Relaxin-3/RXFP3 system regulates alcohol-seeking

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Relapse and hazardous drinking represent the most difficult clinical problems in treating patients with alcohol use disorders. Using a rat model of alcohol use and alcohol-seeking, we demonstrated that central administration of peptide antagonists for relaxin family peptide 3 receptor (RXFP3), the cognate receptor for the highly conserved neuropeptide, relaxin-3, decreased self-administration of alcohol in a dose-related manner and attenuated cue- and stress-induced reinstatement following extinction. By comparison, RXFP3 antagonist treatment did not significantly attenuate self-administration or reinstatement of sucrose-seeking, suggesting a selective effect for alcohol. RXFP3 is densely expressed in the stress-responsive bed nucleus of the stria terminalis, and bilateral injections of RXFP3 antagonist into the bed nucleus of the stria terminalis significantly decreased self-administration and stress-induced reinstatement of alcohol, suggesting that this brain region may, at least in part, mediate the effects of RXFP3 antagonism. RXFP3 antagonist treatment had no effect on general ingestive behavior, activity, or procedural memory for lever pressing in the paradigms assessed. These data suggest that relaxin-3/RXFP3 signaling regulates alcohol intake and relapse-like behavior, adding to current knowledge of the brain chemistry of reward-seeking.

addiction | dependence

Alcohol abuse is a major cause of morbidity and mortality worldwide, accounting for an estimated 3.8% of all global deaths and 4.6% of the global burden of disease and injury (1). Excessive alcohol use may also lead to alcohol dependence (also termed "alcohol addiction") (2, 3), which has a lifetime prevalence of ∼12.5% (4). Economic costs due to alcohol abuse were in the order of $235 billion in the United States in 2007, or ∼2.7% of GDP (1, 5). Despite the huge impact of alcohol use disorders on society, current first-line therapeutic agents, such as naltrexone and acamprosate, are far from adequate, with high relapse rates during treatment and problems with compliance (6–8). New therapeutic agents are clearly required, particularly for the reduction of hazardous drinking and prevention of relapse (9). To this end, a major goal in addiction neuroscience is to understand the neurobiology and neurocircuitry affected by alcohol use disorders and to identify factors implicated in these conditions, which may lead to improved and more targeted therapies (7–10). Here we investigate the neuropeptide relaxin-3 for its involvement in rodent models of alcohol-seeking and consumption.

Relaxin-3 is the highly conserved, ancestral neuropeptide of the relaxin/insulin superfamily, and its cognate G-protein–coupled receptor is relaxin family peptide 3 receptor (RXFP3) (11–16). Relaxin-3 is predominantly expressed in gamma-aminobutyric acid (GABA) neurons in the hindbrain nucleus incertus, which projects widely to forebrain areas, including the amygdala, bed nucleus of the stria terminalis (BNST), hippocampus, and lateral hypothalamus, which also express high levels of RXFP3 (11, 15, 17–22). This pattern of innervation, along with findings that relaxin-3 can modulate (*i*) food intake (23–25), (*ii*) responses to stress (20, 26, 27), (*iii*) arousal (28, 29), and (*iv*) interactions with the corticotropin-releasing factor (CRF) systems (20, 26), led us to hypothesize that relaxin-3 may modulate aspects of behavior related to substance use and abuse. Such a role would parallel that of other neuropeptides, such as orexin/hypocretin (30, 31), galanin (32), and melanin-concentrating hormone (33).

The relaxin-3/RXFP3 system was investigated using rat models of alcohol self-administration followed by cue- and stress-induced reinstatement, which are considered robust models for the human experience of relapse (34, 35). Because native relaxin-3 displays some pharmacological cross-reactivity with other relaxin family receptors, peptide ligands selective for RXFP3 have been developed and characterized (36–38). Central injection of a RXFP3-selective agonist increases food intake in rats, which is prevented by prior injection of a RXFP3-selective antagonist (37, 38). Here, we demonstrate that the RXFP3-selective antagonists R3(B1-22)R and R3(BΔ23–27)R/I5 (37, 38) decrease alcohol intake and reinstatement behavior in rats.

Results

RXFP3 Antagonism Decreases Alcohol but Not Sucrose Self-Administration. We investigated the effect of the RXFP3 antagonist R3(B1-22)R on male inbred alcohol-preferring (iP) rats that were trained to self-administer ethanol (10% vol/vol) over 6–8 wk in daily operant sessions. Rats were tested over 2 consecutive days. On the first day, rats were injected i.c.v. with vehicle 10 min before their operant chamber session; on the second day, they were injected i.c.v. with R3(B1-22)R (3, 10, or 30 μg). Using a multilevel random-effects generalized least squares (GLS) regression analysis, R3(B1-22)R reduced ethanol self-administration in a dose-related manner. There was a significant difference in

Significance

Relapse and hazardous drinking represent the most difficult clinical problems in treating patients with alcohol use disorders. Increasing our understanding of the brain circuits and chemicals that regulate alcohol intake and relapse offers the potential for more targeted therapeutic approaches to assist in relapse prevention. Using a rat model of alcohol use and alcohol-seeking, we provide the first evidence that a neuropeptide, namely relaxin-3, acts upon specific receptors (relaxin family peptide 3) within the brain to regulate alcohol self-administration and relapse-like behavior. In the case of relapse-like alcohol-seeking, this system appears particularly involved in stress-mediated relapse via actions within a brain region called the bed nucleus of the stria terminalis.

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ethanol lever presses between rats injected with 3 μg R3(B1-22)R vs. vehicle ($P = 0.003$), 10 μg R3(B1-22)R vs. vehicle ($P < 0.001$), and 30 μg R3(B1-22)R vs. vehicle ($P < 0.001$) (Fig. 1*A* and Tables S1–S3). There was also a significant difference in lever presses for ethanol vs. water lever ($P = 0.005$) (Fig. 1*A* and Tables S1–S3). In addition, there was a significant difference in water lever presses between rats injected with 3 μg R3(B1-22)R vs. vehicle, although the effect size was small [fewer than three lever presses; 95% confidence interval (C.I.) (−5, −0.3); $P = 0.03$]; there was no significant difference at any other dose (Fig. 1*A* and Tables S1–S3).

A separate cohort of rats was injected i.c.v. with 10 μg of a structurally different RXFP3 antagonist, R3(BΔ23–27)R/I5, (37) which also reduced self-administration of 10% (vol/vol) ethanol (repeated measures one-way ANOVA, effect of treatment on lever pressing for ethanol: $F_{(2,32)} = 18.73$, $P < 0.0001$), but demonstrated no significant difference between groups in water lever responding (Fig. S1).

For a comparative assessment of actions on intake of a natural reward, a separate cohort of rats was trained to self-administer sucrose (0.7–2% wt/vol) until the number of lever presses was matched to that in ethanol-trained rats. These rats were injected i.c.v. with vehicle or R3(B1-22)R (10 or 30 μg) 10 min before the operant session. R3(B1-22)R did not significantly alter overall self-administration in this paradigm; however, there was a small but significant increase in sucrose lever presses between rats injected with 30 μg R3(B1-22)R vs. vehicle [31 lever presses; 95% C.I. (1, 60); $P = 0.039$] (Fig. 1*B* and Tables S4–S6). There was no difference in water lever presses at any dose vs. vehicle (Fig. 1*B* and Tables S4–S6).

For comparison with an outbred strain of nonalcohol preferring rats, we investigated the effect of R3(B1-22)R on male Wistar rats that were trained to self-administer ethanol (10% vol/vol). R3(B1-22)R reduced self-administration of 10% (vol/vol) ethanol only at the highest dose (30 μg; $P < 0.001$), suggesting a rightward shift in the dose–response curve (Fig. S2 and Tables S7–S9).

RXFP3 Antagonism Does Not Impair Procedural Memory, General Ingestive Behavior, or Activity. Rats (iP) injected i.c.v. with R3(B1-22)R demonstrated no significant differences in latency to first ethanol reward, suggesting that the effect of R3(B1-22)R was not due to overall sedation or a deficit in procedural memory for lever pressing (Table S10). In a separate cohort of rats, there was no effect of R3(B1-22)R (10 μg, i.c.v.) on food deprivation-stimulated feeding (Fig. S3), suggesting that R3(B1-22)R does not impair general ingestive/consummatory behavior. Notably, this dose of R3(B1-22)R can prevent feeding stimulated by exogenous RXFP3 agonist treatment (38). A group of rats tested in locomotor cells displayed no difference in floor plane distance or rearing activity over a 60-min period following injection of R3(B1-22)R (10 μg; Fig. 2), suggesting that R3(B1-22)B does not affect general activity at doses that regulate alcohol self-administration and seeking.

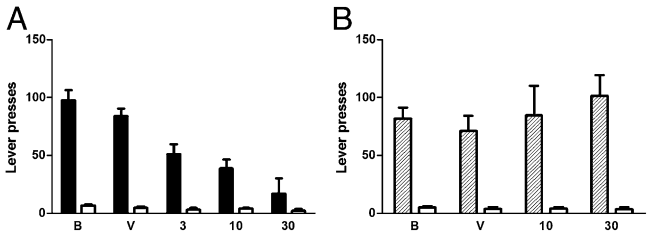

Fig. 1. Self-administration of 10% (vol/vol) ethanol was reduced in a dose-related manner by R3(B1-22)R in male iP rats. (*A*) Lever presses for ethanol (black) and water (white): noninjected baseline (B), vehicle (V), and rats treated with 3 μg (3), 10 μg (10), and 30 μg (30) R3(B1-22)R. Data are mean ± SEM; $n = 5$–11 per dose for R3(B1-22)R. (*B*) Lever presses for sucrose (0.7–2% wt/vol) (hatched) and water (white); $n = 6$ per group.

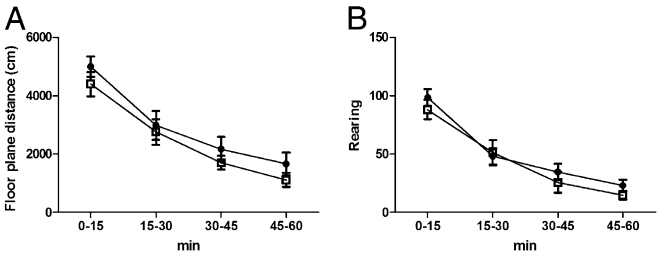

Fig. 2. R3(B1-22)R treatment had no effect on locomotor activity. Rats were injected with vehicle (black circles) or 10 μg R3(B1-22)R (white squares) i.c.v. and then placed into the locomotor cell and recorded over the subsequent 60 min. Two-way repeated measures ANOVA demonstrated no significant main effect of treatment or interaction between time and treatment in (*A*) floor plane distance or (*B*) vertical plane entries (rearing). Data are mean ± SEM; $n = 6$–7 per group.

RXFP3 Antagonism Attenuates Cue- and Stress-Induced Reinstatement of Alcohol- but Not Sucrose-Seeking. Next, we investigated the effect of R3(B1-22)R on cue-induced reinstatement of alcohol-seeking in another cohort of rats. Following operant self-administration of ethanol (10% vol/vol), rats underwent 11 consecutive days of extinction training, where the olfactory cue (vanilla) (S+; discriminative stimulus that signals drug availability) and light stimulus (CS+; conditioned stimulus) were removed, and lever pressing resulted in no programmed response. On day 12, the discrete cue was replaced (S+) and the CS+ was illuminated upon a fixed ratio of 3 (FR3) response, but there was no delivery of fluid contingent with lever pressing. Ten min before the reinstatement session, rats were injected with vehicle or R3(B1-22)R (10 μg), after which they underwent cue-induced reinstatement (39). Rats were subsequently re-extinguished and underwent a second reinstatement session with the opposite treatment during the following week.

R3(B1-22)R significantly attenuated cue-induced reinstatement of alcohol-seeking (repeated measures one-way ANOVA, effect of treatment on lever pressing: $F_{(2,42)} = 11.15$, $P = 0.0001$) (Fig. 3*A*). There was no effect on responding on the "inactive" (formerly water) lever (Fig. 3*B*). We obtained confirmation of the effect of RXFP3 antagonism by injecting a separate cohort of alcohol-trained rats with 10 μg of a structurally different RXFP3-selective antagonist, R3(BΔ23–27)R/I5 (37) or vehicle before cue-induced reinstatement. This treatment also attenuated reinstatement of responding on the "active" (formerly alcohol) lever (repeated measures one-way ANOVA, effect of treatment on lever pressing: $F_{(2,14)} = 6.43$, $P = 0.011$) (Fig. S4).

Next, we investigated the effect of R3(B1-22)R on stress-induced reinstatement for alcohol-seeking, using a similar protocol, except that 30 min before a reinstatement session, rats were injected with the α_2-adrenoceptor antagonist, yohimbine (1 mg/kg i.p.), to precipitate stress-induced reinstatement (40). R3(B1-22)R prevented stress-induced reinstatement of alcohol-seeking (repeated measures one-way ANOVA, effect of treatment on lever pressing: $F_{(2,18)} = 15.00$, $P = 0.0001$) (Fig. 3*C*). There was no effect on responding on the inactive (formerly water) lever (Fig. 3*D*).

For comparison with a natural reward, we tested separate groups of sucrose-trained iP rats for cue- or stress-induced reinstatement. Treatment with 10 μg R3(B1-22)R i.c.v. did not significantly attenuate cue- or stress-induced reinstatement of responding on the active (formerly sucrose) lever (Fig. 4 *A* and *C*), suggesting that the effect was selective for ethanol. There was also no effect on the inactive lever (Fig. 4 *B* and *D*).

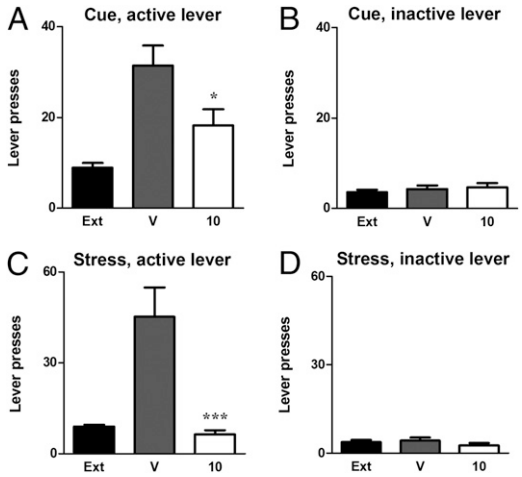

Fig. 3. R3(B1-22)R markedly attenuated cue- and stress-induced reinstatement for alcohol-seeking. Data were analyzed by repeated measures one-way ANOVA with post hoc Tukey's Multiple Comparison Test and expressed as mean ± SEM. Extinguished rats (Ext), vehicle-treated (V), and 10 μg R3(B1-22)R-treated (10) rats. $*P < 0.05$, $***P < 0.001$. (Only P values for vehicle vs. 10 μg R3 (B1-22)R are depicted on the graphs.) (*A*) There was a significant effect of treatment on active lever presses for cue-induced reinstatement in ethanol-trained rats ($F_{(2,42)} = 11.15$, $P = 0.0001$), with post hoc tests indicating that the lever pressing for vehicle-injected rats was significantly different from lever pressing of R3(B1-22)R-treated rats ($P < 0.05$), as well as from lever pressing during extinction ($P < 0.001$); $n = 22$ rats. (*B*) There was no significant effect on inactive lever presses for cue-induced reinstatement in ethanol-trained rats ($F_{(2,42)} = 0.58$, $P = 0.57$). (*C*) There was a significant effect of treatment on active lever presses for stress-induced reinstatement in ethanol-trained rats ($F_{(2,18)} = 15.00$, $P = 0.0001$), with post hoc tests indicating that the lever pressing for vehicle-injected rats was significantly different from lever pressing of R3(B1-22)R-treated rats ($P < 0.001$), as well as from lever pressing during extinction ($P < 0.001$); $n = 10$ rats. (*D*) There was no effect on inactive lever presses for stress-induced reinstatement in ethanol-trained rats ($F_{(2,18)} = 1.30$, $P = 0.30$).

RXFP3 Antagonism in the BNST Decreases Self-Administration and Stress-Induced Reinstatement of Alcohol. Although R3(B1-22)R was active in both cue- and stress-induced reinstatement paradigms of alcohol-seeking, the effect was more robust in the stress paradigm, and therefore we studied this aspect in further detail. Although there is considerable evidence that drugs of abuse interact with the mesocorticolimbic pathway, which comprises dopaminergic neurons in the ventral tegmental area and their projection targets in the nucleus accumbens (18), we considered it unlikely that the effects of RXFP3 antagonism were mediated directly via this pathway because RXFP3 expression is relatively low in these regions (19–21). In contrast, recent studies have suggested a role for the stress-responsive BNST in reinstatement of drug- and alcohol-seeking (41, 42) via a CRF-signaling mechanism (43); and in light of the dense innervation of BNST by relaxin-3 fibers (19), expression of RXFP3 in BNST (21, 42), and the documented interactions between relaxin-3 and CRF systems (20, 26), we targeted this nucleus for discrete microinjections of the RXFP3 antagonist.

Bilateral intra-BNST injections of R3(B1-22)R [1 μg R3(B1-22)R in 0.5 μL artificial cerebrospinal fluid (aCSF), 0.25 μL/min, 4 min before operant session] significantly reduced self-administration (repeated measures one-way ANOVA, effect of treatment on lever pressing: $F_{(2,32)} = 10.77$, $P = 0.0003$) (Fig. 5*A*). The injection sites were validated histologically [17 correct targeting ("hits"); 2 incorrect targeting ("misses")] (Fig. S5 *A–C*).

Intra-BNST R3(B1-22)R also significantly attenuated stress-induced reinstatement of alcohol-seeking (repeated measures one-way ANOVA, effect of treatment on lever pressing: $F_{(2,22)} = 16.77$, $P < 0.0001$) (Fig. 5*B*). There was no effect on responding on the inactive (formerly water) lever (Fig. S6*A*). The injection sites were validated histologically (Fig. S5 *D–F*). R3(B1-22)R injections immediately adjacent to, but outside, the BNST ($n = 8$) were also analyzed and demonstrated no significant effect on active lever pressing, indicating that the effect was anatomically specific to the BNST (Fig. S6*B*).

Discussion

Our data provide evidence that pharmacological antagonism of RXFP3 decreases alcohol intake in a dose-related manner and attenuates both stress- and cue-induced reinstatement of alcohol-seeking. Moreover, we provide support for a role of relaxin-3 signaling in reward processing, which demonstrates a degree of specificity between alcohol and natural rewards, with RXFP3 antagonism reducing intake and reinstatement of alcohol-seeking, but not sucrose-seeking at equivalent doses. Indeed, at the highest dose of R3(B1-22)R used in the self-administration paradigm (30 μg), several rats did not press the alcohol lever enough times to receive even one alcohol reward (i.e., fewer than three lever presses); by comparison, in the sucrose self-administration paradigm, there was a slight increase in sucrose responding at the same dose in the same strain of rats. R3(B1-22)R also decreased alcohol intake in outbred Wistar rats, although only at the highest dose, suggesting reduced efficacy to decrease alcohol intake in nonpreferring rats. A structurally different RXFP3-selective antagonist, R3(BΔ23–27)R/I5, also decreased alcohol self-administration and attenuated cue-induced alcohol-

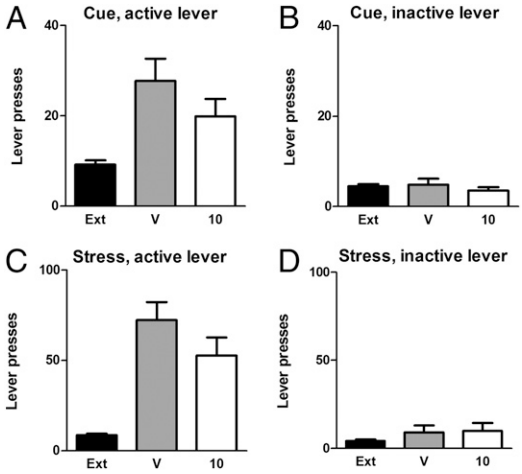

Fig. 4. R3(B1-22)R had no significant effect on cue- and stress-induced reinstatement for sucrose-seeking. Data were analyzed by repeated measures one-way ANOVA with post hoc Tukey's Multiple Comparison Test and expressed as mean ± SEM. Extinguished rats (Ext), vehicle-treated (V), and 10 μg R3(B1-22)R-treated (10) rats. (*A*) Although there was a significant effect of treatment on active lever presses for cue-induced reinstatement in sucrose-trained rats ($F_{(2,44)} = 6.12$, $P = 0.0045$), post hoc tests indicated that the lever pressing for vehicle-injected rats was not significantly different from lever pressing of R3(B1-22)R-treated rats. However, there was a significant difference between vehicle-injected rats and lever pressing during extinction ($P < 0.01$); $n = 23$ rats. (*B*) There was no significant effect on inactive lever presses for cue-induced reinstatement in sucrose-trained rats ($F_{(2,44)} = 0.68$, $P = 0.51$). (*C*) Although there was a significant effect of treatment on active lever presses for stress-induced reinstatement in sucrose-trained rats ($F_{(2,22)} = 16.60$, $P < 0.0001$), post hoc tests indicated that the lever pressing for vehicle-injected rats was not significantly different from lever pressing of R3(B1-22) R-treated rats. However, there was a significant difference between vehicle-injected rats and lever pressing during extinction ($P < 0.001$) and between R3 (B1-22)R-treated rats and lever pressing during extinction ($P < 0.01$); $n = 12$ rats. (*D*) There was no significant effect on inactive lever presses for stress-induced reinstatement in sucrose-trained rats ($F_{(2,22)} = 1.89$, $P = 0.17$).

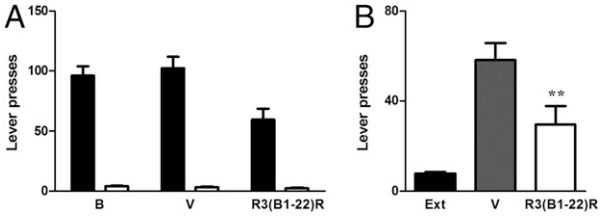

Fig. 5. (*A*) Self-administration of 10% (vol/vol) ethanol was reduced following infusion of 1 μg R3(B1-22)R bilaterally into the BNST. Repeated measures one-way ANOVA demonstrated a significant effect of treatment on lever pressing for the ethanol lever ($F_{(2,32)} = 10.77$, $P = 0.0003$), with post hoc tests indicating a significant difference between baseline and R3(B1-22)R treatment ($P < 0.01$) and vehicle and R3(B1-22)R ($P < 0.001$). There was no significant effect of treatment on lever pressing for the water lever. Lever presses for ethanol (black) and water (white): noninjected baseline (B), vehicle-treated (V), R3(B1-22)R treated with 1 μg bilaterally. Data are mean ± SEM; $n = 17$. (*B*) Stress-induced reinstatement for alcohol was attenuated following infusion of 1 μg R3(B1-22)R bilaterally into the BNST. Active lever presses for ethanol-trained rats, extinguished rats (Ext), vehicle-treated (V), and 1 μg R3(B1-22)R-treated rats. Repeated measures one-way ANOVA demonstrated a significant effect of treatment on lever pressing ($F_{(2,22)} = 16.77$, $P < 0.0001$), with post hoc tests indicating that the lever pressing for vehicle-injected rats was significantly different from lever pressing of R3(B1-22)R-treated rats ($P < 0.01$), as well as from lever pressing during extinction ($P < 0.001$). Data are mean ± SEM; $n = 12$ rats. $^{**}P < 0.01$. [Only the *P* value for vehicle vs. 1 μg R3(B1-22)R is depicted on the graph.]

seeking, providing pharmacological confirmation that the effects observed are mediated via antagonism of RXFP3.

RXFP3 antagonism at equivalent doses did not impact general arousal or activity or procedural memory for lever pressing, which is an important possibility to exclude, given that previous studies have suggested a role for relaxin-3 in arousal, learning, and memory (28, 29, 44). RXFP3 antagonism produced no effect on food intake in rats following overnight restricted food access, suggesting no major impact of the relaxin-3/RXFP3 system on appetitive drive stimulated by the need for food in this paradigm. By comparison, in satiated rats, RXFP3 antagonism can prevent RXFP3 agonist-induced food intake (37, 38), suggesting the relaxin-3/RXFP3 system may modulate the motivational or rewarding properties of food.

RXFP3 antagonism produced robust attenuation of stress-induced reinstatement in alcohol-trained rats, so we examined this action in more detail. The BNST, which contains a high density of RXFP3 (19, 21), has an established role in stress-induced reinstatement via a CRF-signaling pathway (41, 43) and therefore was an obvious candidate region for targeted injections of R3(B1-22)R. Accordingly, intra-BNST injections of R3(B1-22)R produced a significant decrease in both alcohol self-administration and stress (yohimbine)-induced reinstatement of alcohol-seeking. The attenuation of alcohol-seeking, rather than complete prevention, suggests other brain regions may also be involved. Nevertheless, our data clearly support a role for RXFP3 signaling in the BNST in stress-induced reinstatement of alcohol-seeking. Importantly, injections adjacent to the BNST had no effect, suggesting anatomical specificity of the action within the BNST.

The relationship between brain stress systems and addiction has been the focus of considerable research in recent years, particularly because of the central role of CRF in both (17, 45, 46). In the clinical setting, there is high comorbidity between substance abuse and stress-related psychiatric disorders such as depression (47). Our studies suggest a role for the relaxin-3/RXFP3 system, which has established interactions with CRF signaling (20, 26), in mediating interactions between stress and drug-seeking. In particular, RXFP3 signaling in the BNST appears to influence stress-related alcohol-seeking behavior. Studies are now required to "close the loop" and deconstruct reward-seeking neurocircuitry in further detail and to widen the investigation of the role of relaxin-3 signaling to studies with other drugs of abuse.

In summary, the current findings provide important information that identifies a previously unknown role for the relaxin-3/RXFP3 system in the regulation of reward-seeking. Moreover, we demonstrate that RXFP3 signaling in the BNST can modulate both alcohol self-administration and stress-induced reinstatement of alcohol-seeking. These findings highlight the need to further delineate the circuitry of relapse-like behavior.

Methods

Animals. All experimental procedures were approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics Committee. Male iP rats were obtained from the breeding colony at The Florey Institute of Neuroscience and Mental Health (The University of Melbourne) and were used for experimentation at ≥8 wk of age. Parental stock was originally obtained from T. K. Li (Duke University School of Medicine, Durham, NC). Male Wistar rats were supplied by the Animal Resources Centre. Rats were housed under ambient conditions (21 °C) and maintained on a 12 h light:dark cycle (lights on 0700–1900 h), with access to food (laboratory chow) and water ad libitum, unless otherwise stated. Rats were acclimated to the holding room for at least 1 wk before experimentation. Before surgery, rats were generally housed two or three per standard box; postsurgery, rats were single-housed.

RXFP3-Selective Antagonists. The RXFP3 antagonists R3(B1-22)R and R3(BΔ23–27)R/I5 were synthesized using solid-phase peptide synthesis and purified using reverse-phase HPLC (38, 48, 49). The identity and purity of the peptide was confirmed by reverse-phase HPLC and MALDI-TOF mass spectrometry. The amino acid composition was checked, and absolute quantities were measured using amino acid analysis. Receptor-binding affinity was assessed in competition binding assays using a single concentration of Europium-labeled INSL5-A/H3 relaxin-B (0.5 nM), Europium-labeled H2 relaxin (1 nM), or Europium-labeled mouse INSL5 (2.5 nM) in the presence of increasing concentrations of H3 relaxin analogs in comparison with the native receptor ligands. Potency was assessed by measuring the influence on cAMP signaling using a cAMP reporter gene assay (37, 38). The peptide content of batches used for i.c.v. injections was >97% for R3(B1-22)R and 71% for R3(BΔ23–27)R/I5, which was adjusted by dilution with vehicle to equimolar doses of active peptide.

Stereotaxic Implantation of Cannulae into Lateral Ventricle and BNST. Each rat was deeply anesthetized with 4% (vol/vol) isoflurane in room air, 2 L/min (Delvet), and maintained with ~2% (vol/vol) isoflurane in room air (0.2 L/min). The head was positioned in a stereotaxic frame (Stoelting Co.), and the surgical site was shaved and cleaned with riodine [povidone–iodine 10% (wt/vol); Orion Laboratories] and an 80% (vol/vol) ethanol solution. A small incision was made in the skin, and the area was cleaned and dried. Three to four pits were drilled into the skull adjacent to the eventual cannulation site, and screws (1.4 mm diameter × 3 mm length) (Mr Specs) were inserted to anchor the cannula in place. A small hole was drilled in the skull, through which a stainless-steel guide cannula was inserted. For lateral ventricular cannulation, the stainless-steel guide cannulae were 22 gauge, cut 2 mm below the pedestal (PlasticsOne), and were implanted using the following coordinates relative to bregma: anteroposterior, −0.7 mm; mediolateral, −1.4 mm; and dorsoventral, −2 mm (50). For BNST cannulation, the stainless-steel guide cannulae were 26 gauge, cut 6 mm below the pedestal (PlasticsOne), and were implanted bilaterally using the following coordinates relative to bregma: anteroposterior, −0.6 mm; mediolateral, ±4.5 mm; and dorsoventral, −7.2 mm on a 23° angle (50). Cannulae were affixed to the skull and screws using dental cement (Vertex-Dental).

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) was administered to provide acute postoperative analgesia, along with empirical antibiotic treatment (enrofloxacin, 5 mg i.p., Bayer Australia Ltd.). Rats were single-housed and allowed to recover for 7 d, during which they were handled and weighed daily to habituate them to the experimenter. Patency was maintained by inserting an obturator fashioned from stainless steel wire (30 gauge) into the lateral ventricular cannulae and by inserting dummies bilaterally into the BNST cannulae (which projected 2.25 mm beyond the tip; PlasticsOne).

Intracerebroventricular and BNST Infusions and Verification of Cannula Location and Patency. Intracerebroventricular infusions were made using 29-gauge hypodermic tubing (Small Parts Inc.) connected to a 10-μL microsyringe (Hamilton Instruments) by polyethylene tubing (0.80 mm outer and 0.40 mm internal diameter; Microtube Extrusions). Rats were gently held, and the injector was inserted into the guide cannula. Infusions of 5 μL were delivered over the course of ∼20 s with care to ensure that all of the solution was delivered. The injector was left in place for ∼10 s after infusion. Correct cannula positioning for lateral ventricular cannulation was verified in each rat by testing the acute dipsogenic response to an injection of angiotensin II (5 μL of a 0.2 ng/μL solution; Auspep) in artificial cerebrospinal fluid (147 mM NaCl; 4 mM KCl; 0.85 mM $MgCl_2$; 2.3 mM $CaCl_2$). Dipsogenesis was defined as repeated drinking episodes that commenced promptly after angiotensin II administration. Correct cannula placement was tested pre- and post-experimentation, and rats were excluded if they failed to exhibit a positive dipsogenic response.

Bilateral BNST infusions were made using 40-cm polyethylene connectors (PlasticsOne) attached to 1-μL microsyringes (SGE Analytical Science). A 0.5-μL volume was simultaneously infused into each side at a rate of 0.25 μL/min by an automated syringe pump (Harvard Apparatus). The injectors were left in place for ∼2 min after infusion. Rats were well habituated to the equipment before experimentation. Correct cannula positioning for BNST cannulation was verified in each rat by infusing methylene blue (0.5 μL/side). Rat brains were sectioned in the coronal plane (40 μm) using a cryostat microtome at −18 °C (Reichert-Jung Cryocut 1800, Leica Microsystems) and mounted on SuperFrost Plus slides (Menzel-Gläser microscope slides). Slides were placed for 2 min in Neutral Red solution (Sigma-Aldrich), dehydrated, and cleared through a series of ethanol and X-3B (Oilchem Pty Ltd.) solutions, coated with DPX (distyrene, plasticizer, and xylene mixture) mountant (Sigma-Aldrich) and coverslipped (Menzel-Gläser). Slides were viewed under an Olympus BH-2 microscope to verify injection sites by an examiner who was blinded to the associated behavioral data.

Alcohol Self-Administration. Male iP rats [for i.c.v., n = 5–11 for each dose of R3(B1-22)R; for BNST, n = 19] and male Wistar rats [n = 4–5 for each dose of R3(B1-22)R] were tested for operant responding to alcohol in operant chambers supplied by Med Associates Inc. Each chamber was housed individually in a sound-attenuation cubicle, featuring a fan to provide airflow and mask external noise, and the chambers were connected to a computer running Med-PC IV software (Med Associates Inc.) to record activity. Within the chambers, a small light provided soft illumination during operant sessions.

On either side of the operant chambers was a retractable lever that emerged during the operant session and was centrally placed below a stimulus light and adjacent to a fluid receptacle. Each receptacle was fed by a solenoid-controlled liquid dispenser with a 20-mL reservoir. In addition, availability of alcohol was conditioned by the presence of an olfactory cue (S+: two drops of vanilla essence, placed on the bedding of the operant chamber directly under the active lever); in addition, a 1-s light stimulus occurred when the solution was delivered (CS+). The correct instrumental response resulted in delivery of 100 μL of solution.

Over a 10-d period, rats were trained to self-administer ethanol (10% vol/vol) with an FR3 (rats had to press a lever three times to obtain 100 μL of 10% vol/vol alcohol) as previously described (39). For each session, the total number of lever presses was recorded for both the alcohol and water solutions.

After stabilization of alcohol self-administration (∼5 wk of 10% vol/vol ethanol), each rat was anesthetized and underwent stereotaxic implantation of a cannula into its lateral cerebral ventricle or BNST. After recovery and restabilization of daily alcohol responding, rats were habituated to the treatment protocol over 2 consecutive days by injecting aCSF before the operant chamber session.

Each treatment session occurred over 2 consecutive days. On the first day, rats were injected with vehicle (aCSF) before their operant chamber session (for i.c.v., 5 μL aCSF injected 10 min before session; for BNST, 0.5 μL aCSF injected bilaterally 4 min before session); on the second day, rats were injected with R3(B1-22)R [for i.c.v., 3, 10, or 30 μg R3(B1-22)R in 5 μL aCSF 10 min prior; for BNST, 1 μg R3(B1-22)R in 0.5 μL aCSF bilaterally 4 min prior]. A separate cohort of rats was injected i.c.v. with another RXFP3 antagonist, 10 μg R3(BΔ23–27)R/I5 in 5 μL solution (n = 17). Measures recorded included the number of lever presses for both alcohol and water and the latency to obtain the first reinforced response.

Sucrose Self-Administration. Rats (n = 6) were trained to self-administer sucrose (0.7–2% wt/vol) with an FR3 in a similar manner to alcohol, such that the number of lever presses was matched to alcohol-trained rats (51). Following training, rats underwent surgery for i.c.v. cannula implantation. Following recovery and restabilization in operant training sessions, rats underwent injection of vehicle (aCSF) or R3(B1-22)R (10 and 30 μg).

Alcohol Cue- and Stress-Induced Reinstatement. For cue-induced reinstatement, a separate cohort of rats (n = 22) underwent operant training sessions for ethanol (10% vol/vol) on an FR3 schedule. Following acquisition of self-administration, each rat was implanted with a cannula into the lateral ventricle. Following recovery and restabilization of alcohol self-administration, rats underwent 11 consecutive days of extinction training where lever pressing resulted in no consequence. On day 12, rats underwent cue-induced reinstatement, in which the cues were replaced (S+ and CS+), but not the alcohol or water. Ten min before the reinstatement session, rats were injected i.c.v. with aCSF or 10 μg R3(B1-22)R. The rats were subsequently re-extinguished over four sessions, followed by a second reinstatement test with the opposite treatment, such that each rat was exposed to both treatments. A separate cohort of rats (n = 8) underwent cue-induced reinstatement for alcohol following injection with aCSF or another RXFP3 antagonist, 10 μg R3(BΔ23–27)R/I5 in 5 μL.

For stress-induced reinstatement, two separate cohorts of rats (for i.c.v., n = 10; for BNST, n = 20) were trained using a similar protocol, with an additional i.p. injection of the α_2-adrenoceptor antagonist yohimbine (1 mg/kg dissolved in distilled water; Tocris Bioscience) 30 min before the reinstatement session.

Sucrose Cue- and Stress-Induced Reinstatement. As a natural reward comparison, a separate cohort of rats underwent similar protocols for sucrose (0.7–5% wt/vol) (for cue-induced, n = 23; for stress-induced, n = 12).

Deprivation-Stimulated Feeding. Rats (n = 6 per group) were restricted to two pellets (∼3 g) of food each overnight. The next morning, rats were injected with aCSF or 10 μg R3(B1-22)R and then given access to a preweighed amount of rat chow (∼16–18 g) in the cage food compartment and a preweighed water bottle. Food and water intake were measured 1 h later.

Locomotor Cell. Rats (n = 6–7 per group) 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) for 60 min with photobeam detectors to track the horizontal and vertical activity. Testing was performed under low light conditions (∼15–20 lx). Rats were injected with vehicle (V) or 10 μg R3(B1-22)R (10) i.c.v. and then placed directly into the locomotor cell and recorded over the subsequent 60 min. Parameters measured included total floor plane distance moved and rearing (vertical plane entries).

Data Collection and Statistical Analysis. Data analysis and generation of histograms were performed using GraphPad Prism Version 5.00 for Windows (GraphPad Software). Results are expressed as mean ± SEM.

For alcohol and sucrose self-administration data where multiple doses of R3(B1-22)R were administered, the data were analyzed using a multilevel random-effects GLS regression model using Stata Data Analysis and Statistical Software Version 12 (StataCorp LP). This analysis allowed for the fact that certain rats were given several doses of R3(B1-22)R, with an individual animal being treated as a "level" within the model. This model is highly appropriate for treating "missing" data in a nonrandom manner.

For latency to first ethanol reward data, the latency was measured in a non–time-to-event manner, using the multilevel random-effects GLS regression model. There were two observations where the FR3 event did not occur [for the 30 μg R3(B1-22)R dose], which were subsequently excluded from this analysis.

For self-administration data where one dose of R3(B1-22)R or R3(BΔ23-27)R/I5 was administered, the data were analyzed using a repeated measures one-way ANOVA with post hoc Tukey's Multiple Comparison Tests (GraphPad Prism). The cue- and stress-induced reinstatement of alcohol and sucrose data were analyzed for the effect of treatment on lever pressing using repeated measures one-way ANOVA with post hoc Tukey's Multiple Comparison Tests (GraphPad Prism).

The locomotor cell data were analyzed in 15-min time bins using a two-way repeated measures ANOVA on SPSS (IBM SPSS Statistics 20.0). The food deprivation-stimulated feeding data were analyzed using Student t test (GraphPad Prism).

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