

**Relaxin-3 mRNA levels in *nucleus incertus* correlate with alcohol
and sucrose intake in rats**

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ABSTRACT

Background. Chronic alcohol intake produces multiple neuroadaptive changes, including up- and down-regulation of neuropeptides and receptors. There are widespread projections of relaxin-3 containing neurons to, and abundant relaxin family peptide 3 receptor (RXFP3) expression within, brain regions involved in modulating alcohol intake. Recently we demonstrated the involvement of relaxin-3/RXFP3 signalling in alcohol-seeking in rats, therefore in this study we examined whether relaxin-3 and/or RXFP3 expression were altered by chronic alcohol intake in alcohol-preferring iP rats. **Methods.** Expression of relaxin-3 mRNA in the hindbrain *nucleus incertus* and RXFP3 radioligand binding levels in discrete forebrain regions were investigated following voluntary intake of alcohol or sucrose for 12 weeks, with a 2 day washout, using quantitative *in situ* hybridisation histochemistry and *in vitro* receptor autoradiography, respectively, in cohorts of adult, male iP rats. **Results.** Levels of relaxin-3 mRNA in the hindbrain *nucleus incertus* were positively correlated with the level of intake of both alcohol ($r(12) = 0.59, p = 0.03$) and sucrose ($r(7) = 0.70, p = 0.04$) in iP rats. Dense binding of the RXFP3-selective radioligand, [¹²⁵I]-R3/I5, was detected in hypothalamic and extrahypothalamic sites, but no significant changes in the density of RXFP3 were observed in the brain regions quantified following chronic sucrose or ethanol intake. **Conclusions.** Our findings suggest high endogenous relaxin-3 expression may be associated with higher intake of rewarding substances, rather than its expression being regulated in response to their intake; consistent with an active role for the relaxin-3/RXFP3 system in modulating ingestive and alcohol-related behaviours.

KEYWORDS: Relaxin-3, RXFP3, nucleus incertus, neuropeptides, alcohol, mRNA

1. INTRODUCTION

Chronic exposure to alcohol and other drugs of abuse evokes many neuroadaptive changes in the brain, including up- and down-regulation of neuropeptides and their receptors, particularly those which modulate stress-related functions, including corticotropin-releasing factor (CRF), the urocortins (1-3) and their cognate receptors (CRF₁ and CRF₂; Ubaldi et al., 2013). For example, chronic exposure to alcohol is associated with upregulation of CRF mRNA in the paraventricular nucleus of the hypothalamus and central amygdala (Rivier et al., 1990; Sommer et al., 2008), upregulation of CRF₁ mRNA expression within the central amygdala and down-regulation of CRF₂ mRNA expression in the basolateral amygdala (Heilig and Koob, 2007; Sommer et al., 2008), as well as upregulation of CRF₂ binding sites in the dorsal raphe nucleus and lateral septum (Weitemier and Ryabinin, 2005). Chronic alcohol consumption is also associated with up-regulation of the mRNA encoding orexins, in the lateral hypothalamus (Lawrence et al., 2006). By contrast, the daily amount of alcohol consumed correlates with baseline expression levels of certain neuropeptides and their receptors. For example, daily alcohol consumption positively correlates with CRF₁, CRF₂ and vasopressin mRNA expression levels in the hypothalamus and urocortin-1 expression in the midbrain Edinger-Westphal nucleus; and shows a trend to a positive correlation with orexin receptor 1, melanocortin-concentrating hormone (MCH) and α_{1B} adrenoceptor mRNA expression in the hypothalamus, but no up- or down-regulation of these neuropeptides/receptors in these regions (Bachtell et al., 2003; Pickering et al., 2007; Ryabinin and Weitemier, 2006). Understanding the role of neuropeptides/receptors and neuroadaptive changes that occur during chronic alcohol intake can assist the development of new more targeted therapeutic agents for alcohol-related clinical conditions (e.g., clinical trials are currently proceeding for CRF₁ receptor antagonists in alcohol and drug dependence; Zorrilla et al., 2013); and, therefore, it is important to examine the involvement of other relevant neuropeptide/receptor systems in these responses.

In this regard, recent studies have demonstrated that central antagonism of the relaxin family peptide 3 receptor (RXFP3), the cognate receptor for the highly conserved neuropeptide relaxin-3, decreases alcohol self-administration, as well as cue- and stress-induced reinstatement in alcohol-preferring (iP) rats (Ryan et al., 2013). In the rat and other species, relaxin-3 is highly expressed in GABAergic projection neurons in the hindbrain *nucleus incertus*, and in smaller, more diffuse populations of neurons in the pontine raphé nucleus, the anterior and medial periaqueductal grey, and a region dorsal to the substantia nigra (Burazin et al., 2002; Ma et al., 2007, 2009; Smith et al., 2010). Notably, relaxin-3 expressing neurons are strongly regulated by neurogenic stressors (Banerjee et al., 2010; Lenglos et al., 2013; Ryan et al., 2011), arousal-related stimuli (Smith et al., 2012) and associated transmitters, including CRF (Blasiak et al., 2013; Ma et al., 2013) and orexin (Blasiak et al., 2010).

Furthermore, relaxin-3 neurons project widely throughout the rat forebrain (Ma et al., 2007; Tanaka et al., 2005), with relaxin-3 immunoreactive axons/terminals in several regions known to mediate and/or modulate the central effects of alcohol and other drugs of abuse in this species, including the amygdala, bed nucleus of the stria terminalis, thalamic and hypothalamic paraventricular nuclei and lateral hypothalamus (Koob, 2008; Lawrence, 2010; Martin-Fardon and Boutrel, 2012; Nestler, 2005; Smith et al., 2011), which largely coincides with the distribution of RXFP3 expressing neurons (Ma et al., 2007; Sutton et al., 2004). Together these data suggest that examining the response of the relaxin-3/RXFP3 system to chronic alcohol exposure will increase our understanding of the complex neurochemical changes that occur in stress and arousal networks in substance abuse disorders.

In the present study, expression of relaxin-3 mRNA in the hindbrain and the density of RXFP3 binding in selected forebrain areas were investigated using quantitative *in situ* hybridisation histochemistry (Banerjee et al., 2010) and *in vitro* radioligand autoradiography (Ma et al., 2007), respectively, in alcohol-preferring iP rats, using the two-bottle choice paradigm, an established

method for studying voluntary chronic alcohol use (Lodge and Lawrence, 2001; Sanchis-Segura and Spanagel, 2006).

2. METHODS

2.1 *Animals and ethics*

Male, alcohol-preferring (iP) rats were obtained from the breeding colony at The Florey Institute of Neuroscience and Mental Health (University of Melbourne, Victoria, Australia). Parental stock was originally obtained from Professor T.K. Li (currently at Duke University School of Medicine, Durham, NC). All experimental procedures were approved by The Florey Animal Ethics Committee.

2.2 *Two bottle choice paradigm for alcohol and sucrose*

Male iP rats were divided into three age-matched groups (~3 months of age at commencement) and were single-housed under ambient conditions (21°C) and maintained on a 12h light:dark cycle (lights on 0700-1900) with access to food (laboratory chow) and water *ad libitum*. Rats in each group were spread randomly throughout the holding room.

One group of iP rats ($n = 14$) was allowed to consume ethanol in a two bottle free-choice paradigm for 12 weeks. For the first 5 weeks, rats were given a choice of ethanol (5% v/v) or water, after which they had a choice of ethanol (10% v/v) or water. The position of the drink containers was randomly altered to prevent any side preference. Volumes of fluid consumed and preference for ethanol were calculated daily. Two days prior to decapitation, rats were denied further access to ethanol. The average consumption of ethanol was calculated for the last two weeks of consumption.

A second group of iP rats ($n = 9$) was allowed to consume sucrose (a 'natural' reward) in a two bottle free-choice paradigm for 12 weeks. Rats were initially given a choice of sucrose (7.625% w/v; isocaloric to 5% ethanol) or water. The sucrose concentration was then adjusted to ensure a similar caloric intake to ethanol-drinking rats. The position of drink containers was randomly altered

to prevent any side preference. Volumes of fluid consumed and preference for sucrose were calculated daily. Two days prior to decapitation, all rats were denied further access to sucrose. The average consumption of sucrose was calculated for the last two weeks of consumption.

A control group of age-matched iP rats ($n = 9$) was also housed in the same room as the other groups for 12 weeks, with access to rodent chow and tap water. At the end of this period, all rats were injected with ~0.7 mL 'Lethabarb' (325 mg/mL sodium pentobarbitone; Virbac (Australia) Pty Ltd, Milperra, NSW, Australia) and decapitated. Brains were rapidly removed, cooled in ice and blocked in the coronal plane. Tissues were then frozen over liquid nitrogen and stored at -80°C .

2.3 Tissue processing

Rat brains were coronally sectioned ($14\ \mu\text{m}$) at -18°C using a cryostat (Reichert-Jung Cryocut 1800, Leica Microsystems (Schweia) AG, Heerbrugg, Switzerland). For *in situ* hybridisation experiments, brains were sectioned through the nucleus incertus and pontine raphé nucleus (50 sections per brain; bregma $-9.84\ \text{mm}$ to bregma $-9.12\ \text{mm}$; (Paxinos and Watson, 2007). Sections were thaw-mounted onto slides pre-coated with poly-L-lysine solution ($0.1\ \text{mg/mL}$ in diethylpyrocarbonate (DEPC) treated deionised water). During sectioning, coordinates were identified by microscopic examination of tissue sections counter-stained with 0.01% thionin and reference to a stereotaxic atlas (Paxinos and Watson, 2007). Slides were stored at -80°C until use.

For *in vitro* autoradiography experiments, rat brain sections were collected in four series on slides pre-coated with 0.5% gelatin (Sigma-Aldrich, St Louis, MO, USA) in milli-Q water: series 1 (48 sections per brain; through bregma $3.20\ \text{mm}$ to $2.20\ \text{mm}$); series 2 (48 sections per brain; through bregma $-0.26\ \text{mm}$ to $-0.80\ \text{mm}$); series 3 (48 sections per brain; though bregma $-0.92\ \text{mm}$ to $-1.40\ \text{mm}$); and series 4 (48 sections per brain; through bregma $-1.80\ \text{mm}$ to $-2.56\ \text{mm}$). Two sections per rat brain were mounted per slide.

2.4 *In situ* hybridisation histochemistry

We have chosen this method of investigation because it allows precise regional localization of gene expression in particular tissue, allowing us to not only determine changes in the density of expression, but also changes in the area of expression (Gundlach and O'Shea, 2002). Indeed we have previously used this strategy to demonstrate region-specific effects of chronic alcohol consumption on expression of the mRNA encoding orexin in the rat hypothalamus (Lawrence et al., 2006). For relaxin-3 *in situ* hybridisation, four antisense oligonucleotide probes specific for rat relaxin-3 mRNA were used (Table 1; GeneWorks, Hindmarsh, SA, Australia; Banerjee et al., 2010). A solution containing each probe (10 ng/ μ L solution) was 3'-end labelled with α -[35 S]-dATP (1250 Ci/mmol; PerkinElmer, Waltham, MA, USA) by incubation with terminal deoxynucleotidyl transferase (TdT; Roche Diagnostics, Mannheim, Germany) for 1 h at 37°C (Banerjee et al., 2010; Lawrence et al., 1996). The reaction was then stopped and probes were applied to a Sephadex G25 column pre-equilibrated in TNES buffer (5 M NaCl, 1 M Tris, 0.5 M EDTA, 10% SDS in DEPC-treated water) and centrifuged (2000 rpm, 1 min) to separate unincorporated nucleotides from the labelled probes. Concentration and specific activity of collected α -[35 S]-labelled oligonucleotides were checked in a Tri-Carb 2900-TR liquid scintillation analyser (PerkinElmer); specific activity was $\geq 1-2 \times 10^9$ dpm/ μ g.

Prior to hybridisation, fresh-frozen sections were equilibrated to room temperature and subsequently dehydrated in ethanol (70% for 2 min; 100% for 5 min), delipidated in chloroform for 15 min, rinsed in 100% ethanol, and allowed to air-dry. Sections were hybridised overnight at 42°C with labelled probes (2 pg/ μ L; 70 μ L) in hybridisation buffer (50% formamide, 4 \times SSC, 10% dextran sulphate and 0.2 M DTT). Specificity was confirmed by blocking the signal with a 100-fold molar excess of unlabelled probes (Banerjee et al., 2010; Burazin et al., 2002).

After overnight hybridisation, slides were immediately rinsed in 1 \times SSC, then washed in 1 \times SSC at 55°C for 1 h to remove excess unbound probe. This was followed by rinses in fresh 1 \times SSC and 0.1 \times SSC at room temperature. Sections were then dehydrated in 70% and 100% ethanol and

allowed to air-dry. Slides were apposed to film (Kodak® BioMax® MR film, Rochester, New York, USA) in the presence of brain paste standards overnight (Banerjee et al., 2010). The following day, the film was processed using AGFA CR1000 film processor (AGFA, Mortsels, Belgium).

2.5 *In vitro* receptor autoradiography

Autoradiography with the selective RXFP3 agonist, [¹²⁵I]-R3/I5 (Liu et al., 2005) was based on previous studies (Ma et al., 2007; Sutton et al., 2004). Slide-mounted tissue sections were allowed to warm to room temperature prior to pre-incubation for 15 min at room temperature in HEPES buffer (20 mM HEPES, 120 mM NaCl, 0.22 mM KH₂PO₄, 1.3 mM CaCl₂·2H₂O and 0.8 mM MgSO₄·7H₂O, adjusted to pH 7.4). Sections were then incubated for 60 min at room temperature with 10 pM [¹²⁵I]-R3/I5 (specific activity, 2200 Ci/mmol) in HEPES buffer with 0.017% Pefabloc SC protease inhibitor (Roche Diagnostics) and 0.5% bovine serum albumin (BSA, 5 mg/mL). Non-specific binding was determined in the presence of 100 nM human relaxin-3 (Bathgate et al., 2006). After incubation, excess radioligand was removed by immersing slides in HEPES buffer (briefly at room temperature; then 3 x 5 min at 4°C) and rinsing with distilled water (4°C). Slides were air-dried overnight, and then apposed to film (20 cm × 25 cm Kodak® BioMax® MR) in the presence of ¹⁴C-microscales for 7 weeks, prior to developing using standard methods.

2.6 *Densitometry and data analysis*

Digital images of the *in situ* hybridisation film were acquired using MCID Elite™ 6.0 software (Imaging Research Inc., St Catharines, ON, Canada) as described (Chen et al., 1998; Gundlach and O'Shea, 2002). For analysis of film images, a Sony DXC-930P CCD video camera (Sony Co, Minato, Tokyo, Japan) was used, which was attached to a Nikon micro Nikkor 55mm f 2.8 lens (Nikon Co, Shinjuku, Tokyo, Japan). A 36 mm Vivitar extension tube (Nikon) was used for additional image magnification. The camera was mounted on a Kaiser Copy Stand RS1 (Kaiser Fototechnik GmbH & Co.KG, Buchen, Germany) above a light-box (Northern Lights Precision Illumination Model 890;

Imaging Research Inc., St. Catharine's, ON, Canada; Gundlach and O'Shea, 2002). Slides used for *in situ* hybridisation were stained with 0.01% thionin and examined under a Leica DM LB2 stereology microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) to allow identification and correlation of coronal brain levels (Paxinos and Watson, 2007).

All data are represented as mean \pm SEM unless otherwise specified. An average of three adjacent sections was used for each data point. Statistical analysis was performed using GraphPad Prism V5.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). Assessment of differences in mRNA levels between groups were performed on raw data (hybridisation density in dpm/mg; or area of expression in mm²; or product of density \times area (dpm/mg \times mm²); Gundlach and O'Shea, 2002) by one-way ANOVA with Tukey's *post hoc* Multiple Comparison Test using GraphPad Prism V5.00.

3. RESULTS

3.1 Preference and total fluid intake during chronic alcohol and sucrose intake

Male adult iP rats displayed a range of alcohol/sucrose intake levels and differing levels of preference. A significant positive correlation was observed between alcohol intake (g/kg/day) and alcohol preference (%) ($r(12) = 0.94$, $p < 0.0001$); but no correlation was noted between alcohol intake (g/kg/day) and total fluid intake (g) ($r(12) = 0.22$, $p = 0.45$) (Figure 1A, B). In contrast to alcohol-ingesting rats, no significant correlation was observed between sucrose intake (g/kg/day) and sucrose preference (%) ($r(7) = 0.34$, $p = 0.37$), possibly due to a ceiling effect on sucrose preference. There was however, a significant positive correlation between sucrose intake (g/kg/day) and total fluid intake (g) ($r(7) = 0.99$, $p < 0.0001$) (Figure 1C, D).

3.2 *In situ* hybridisation of relaxin-3 mRNA

Relaxin-3 mRNA expression in the hindbrain was investigated in three groups of rats: (i) control (water intake only), (ii) alcohol intake and (iii) sucrose intake. Coronal sections from two

different brain levels were analysed: (i) caudal nucleus incertus (bregma -9.7 mm) and (ii) rostral nucleus incertus and the adjacent 'pontine raphé nucleus' which extends ventrally from the nucleus incertus (bregma -9.4 mm; Paxinos and Watson, 2007), to determine if the level of relaxin-3 mRNA reflects a possible difference in function and consequent response to activation between these two rostrocaudal levels. A one-way ANOVA revealed no significant differences in density, area, or 'density × area' for any groups of rats at bregma -9.7 mm nor at bregma -9.4 mm (Figure 2), suggesting that chronic alcohol or sucrose intake did not markedly up- or down-regulate regional relaxin-3 mRNA expression.

3.2.1 Correlation between alcohol intake and relaxin-3 mRNA levels. The relationship between alcohol intake (g/kg/day) and relaxin-3 mRNA expression (density, area, density × area) was assessed by calculating Pearson product-moment correlation coefficients. At bregma -9.7 mm, there was a significant positive correlation between alcohol intake and area ($r(12) = 0.65, p = 0.01$) and between alcohol intake and 'density × area' ($r(12) = 0.59, p = 0.03$), with an approximate doubling in relaxin-3 mRNA 'density × area' values across the lowest to the highest levels of daily alcohol intake; whereas there was no significant correlation between alcohol intake and density ($r(12) = 0.34, p = 0.23$; Figure 3A-C and 4). In addition, relaxin-3 mRNA expression was compared for rats that drank larger daily volumes of ethanol (>5g/kg/day) versus smaller volumes, and there was a significant difference in terms of area of relaxin-3 mRNA expression ($t(12) = 2.74; p = 0.02$); and area × density ($t(12) = 2.38; p = 0.04$).

At bregma -9.4 mm, there were no significant correlations between alcohol intake and density ($r(11) = 0.23, p = 0.45$), between alcohol intake and area ($r(11) = 0.19, p = 0.54$), or between alcohol intake and 'density × area' ($r(11) = 0.24, p = 0.43$) (Figure 3D-F), suggesting that the correlation of relaxin-3 mRNA to alcohol intake is restricted to the caudal nucleus incertus.

3.2.2 Correlation between sucrose intake and relaxin-3 mRNA levels. The relationship between sucrose intake (g/kg/day) and relaxin-3 mRNA levels was also assessed by calculating Pearson product-moment correlation coefficients. At bregma -9.7 mm, there was a significant positive correlation between sucrose intake and density ($r(7) = 0.68, p = 0.04$), between sucrose intake and area ($r(7) = 0.69, p = 0.04$), and between sucrose intake and 'density \times area' ($r(7) = 0.70, p = 0.04$), with an approximate three-fold increase in relaxin-3 mRNA 'density \times area' values across the lowest to the highest levels of daily sucrose intake (Figure 5A-C).

At bregma -9.4 mm, there was a significant positive correlation between sucrose intake and area ($r(7) = 0.69, p = 0.04$), suggesting that the correlation of relaxin-3 mRNA to sucrose intake is more widespread than for alcohol intake. However, there was no significant correlation between sucrose intake and density ($r(7) = 0.49, p = 0.18$), or between sucrose intake and 'density \times area' ($r(7) = 0.64, p = 0.07$; Figure 5D-F).

3.3 Receptor autoradiography

Specific [125 I]-R3/I5 binding was observed in the supraoptic nucleus (SON) and the magnocellular part of the paraventricular nucleus of the hypothalamus (mPVN), the central amygdala (CeA), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the thalamus (PVT) and central medial thalamic nucleus (CM), as described (Ma et al., 2007). A one-way ANOVA revealed no significant differences in density, area, or 'density \times area' of specific [125 I]-R3/I5 binding in any of these regions between the different groups of rats, suggesting that chronic alcohol or sucrose intake did not markedly alter the binding of this selective radioligand to RXFP3 in these areas (Table 2). In addition, there were no significant correlations between alcohol or sucrose intake and radioligand binding in these regions (data not shown). Notably however, there was a significant correlation between total area of the PVN and alcohol intake in the alcohol-ingesting rats ($p = 0.03$; data not shown), in accordance with a previous study (Silva et al., 2002).

4. DISCUSSION

Here we identify a positive correlation between the level of relaxin-3 mRNA detected in the nucleus incertus and the daily intake of ethanol or sucrose by adult, male iP rats in a two-bottle choice paradigm. This supports the hypothesis that relaxin-3 levels in the nucleus incertus and associated signalling may regulate the voluntary intake of 'rewarding' substances. Relative relaxin-3 mRNA levels detected in the control (water) group displayed a similar spread to levels in the chronic alcohol and sucrose groups, supporting the interpretation that brain levels of relaxin-3 mRNA were not markedly up- or down-regulated by chronic alcohol or sucrose intake, but rather predisposed rats to consume alcohol or sucrose.

Similarly, previous reports demonstrated a positive correlation between alcohol (but not sucrose) consumption and urocortin-1 mRNA levels in the Edinger-Westphal nucleus (Bachtell et al., 2003; Ryabinin and Weitemier, 2006). Also a strong positive correlation exists between alcohol/saccharin consumption and hypothalamic expression of CRF₁, CRF₂ and vasopressin (in a paradigm involving 9 days self-administration of alcohol and saccharin, followed by 20 days abstinence), but no up- or down-regulation, suggesting that these systems also predispose rats to consume alcohol (Pickering et al., 2007). By contrast, several neuropeptides and their receptors are modulated following alcohol consumption, including orexin mRNA levels in the lateral hypothalamus which are upregulated (reflected by an ~3-fold increase in hybridisation area (mm²)) following chronic alcohol intake in a similar paradigm to this study (Lawrence et al., 2006); and increased CRF mRNA in PVN and CeA following chronic exposure to alcohol (Rivier et al., 1990; Sommer et al., 2008).

Several studies have demonstrated a strong relationship between increased consumption of 'sweets' and alcohol intake, in animals and humans, which may be at least partially genetically based, as alcohol preferring rats often consume sucrose and saccharin well in excess of their normal fluid intake (Kampov-Polevoy et al., 1999). This may explain the positive correlation of relaxin-3 mRNA

with consumption of both alcohol *and* sucrose. An alternative explanation of our data, albeit less likely given the similar spread for sucrose consumption, is that decreased relaxin-3 mRNA levels are associated with increased sensitivity to the effects of alcohol, thus requiring reduced intake. This could be investigated by assessing the sensitivity to the acute intoxicating (e.g., sedative/hypnotic) effects of ethanol in rats with high vs low relaxin-3 mRNA levels in nucleus incertus. Such an experiment is difficult to conduct given that mRNA expression levels are only known post-mortem, which would mean behavioural assessment of large numbers of rats followed by retrospective correlation of brain neurochemistry.

High-alcohol ingesting rats increased their alcohol intake in a different manner from high sucrose-ingesting rats. Higher alcohol intake correlated with a higher percentage preference for alcohol, but similar total fluid intake to the lower alcohol-ingesting rats, while higher sucrose intake correlated with higher total fluid intake. One explanation is that the ethanol solution contains higher calories per gram (7.1 Cal/g or 29.7 kJ/g) than the sucrose solution (4.2 Cal/g or 17.6 kJ/g), and there was a ceiling effect of sucrose preference, requiring higher total fluid intake of the sucrose solution to achieve a higher caloric intake. Despite the correlation between intake of 'rewarding' substances and relaxin-3 mRNA, there was no correlation between body weight and relaxin-3 mRNA expression (data not shown). An additional analysis was performed comparing the amount of weight gained during the 12 week experiment versus relaxin-3 mRNA levels at the end of this period, which also demonstrated no significant correlation (data not shown), suggesting endogenous relaxin-3 (levels/activity) does not strongly influence body weight, but does influence the amount of 'rewarding' substances ingested. It also indicates that the total caloric intake of rats remains similar; suggesting that rats with higher intake of alcohol or sucrose may have eaten less chow than their counterparts. Alternatively, rats with a higher intake of alcohol or sucrose may have had increased activity and/or a higher baseline metabolic rate.

The correlation between alcohol intake and relaxin-3 mRNA expression is in line with recent studies from our laboratory, which demonstrated that central injections of an RXFP3 antagonist decreased alcohol self-administration and attenuated cue- and stress-induced reinstatement following extinction in adult rats (Ryan et al., 2013). Interestingly, there was no significant effect on self-administration or reinstatement of sucrose-seeking in that study, despite the current correlation between sucrose consumption and relaxin-3 mRNA expression. There are several possible explanations: perhaps higher doses of the RXFP3 antagonist are required in the sucrose paradigm compared to alcohol, or alternatively other neural circuits and neuropeptides may regulate sucrose intake more powerfully. In addition, the amount of sucrose consumed in a 20 minute operant session may not represent a 'ceiling' level for satiation in the rats (in the two bottle choice paradigm, rats drank much larger daily volumes of sucrose solution than ethanol).

Both stress and CRF regulate expression of relaxin-3 mRNA in the nucleus incertus (Banerjee et al., 2010; Tanaka et al., 2005). A recent study also demonstrated a significant increase in relaxin-3 mRNA in the nucleus incertus of female but not male Sprague-Dawley rats following repeated food restriction and weekly restraint stress (Lenglos et al., 2013). Interestingly, these female rats considerably overate chow during recovery from stress and food restriction and significantly increased their body weight, while male Sprague-Dawley rats lost considerable weight following a similar paradigm, suggesting that increased relaxin-3 mRNA levels may be associated with stress-induced overeating (Lenglos et al., 2013). Further studies are warranted to establish the relationship between stress-induced increases in relaxin-3 expression and their impact on food *and* alcohol intake. Such studies could also investigate changes in expression of other stress- and arousal-related peptides, some of which are known to influence relaxin-3 neuron activity (Ma et al., 2013). Further studies are also warranted to determine whether differences in relaxin-3 are associated with the differences between male and female rats regarding alcohol intake, particularly given the difference in relaxin-3 expression between male and female rats observed in relation to stress-induced overeating (Lenglos et al., 2013).

A recent genetics study described a possible association between relaxin-3 polymorphisms and hypercholesterolemia, obesity and diabetes in patients treated with antipsychotic agents (Munro et al., 2012). While we did not find a correlation of relaxin-3 mRNA and increased body weight, it is possible that administering antipsychotic medication to rats with different expression levels of relaxin-3 mRNA may influence body weight, and this could be addressed in future investigations. In support of this possibility, it has been reported that antipsychotic treatments, such as clozapine and chlorpromazine increased c-Fos immunoreactivity in the nucleus incertus (Rajkumar et al., 2013). Further studies are also warranted to investigate relaxin-3 polymorphisms in relation to alcohol related disorders.

Recent reviews have highlighted the overlap in the neurocircuitry involved in drug addiction and in the ingestion of palatable food intake leading to obesity (Volkow et al., 2013a, b). Drugs of abuse tend to exert their major effects directly on brain reward pathways, while food tends to affect multiple peripheral and central pathways, conveying information to brain reward pathways both directly and indirectly. This difference may explain our observation that high alcohol intake correlated with relaxin-3 mRNA expression in a smaller, more distinct, region (caudal nucleus incertus) than high sucrose intake (which correlated with both the rostral and caudal nucleus incertus, *and* the pontine raphé nucleus). Future studies could potentially identify the anatomical and functional differences between brain regions activated by alcohol and sucrose, in particular details of the neural and humoral regulation of relaxin-3 neuron populations.

The hypothalamic SON and magnocellular PVN expressed robust [¹²⁵I]R3/I5 binding densities (Ma et al., 2007; Sutton et al., 2004; Tanaka et al., 2005), but no correlation between binding to RXFP3 and alcohol or sucrose intake. Markedly decreased neuronal numbers occur in these regions following prolonged alcohol intake, which was attributed to cell death; but surviving neurons were hypertrophied and the total volume of the SON and magnocellular PVN increased (Madeira et al., 1993; Silva et al., 2002). We found that total PVN (but not SON) size correlated with alcohol intake.

RXFP3 levels were also quantified in the CeA, BNST, PVT and CM, although no significant up- or down-regulation was detected. The BNST was of particular interest, as we have recently identified it as an anatomic locus where RXFP3 signalling modulates stress-induced alcohol-seeking (Ryan et al., 2013), although our current data would suggest that chronic alcohol intake does not appear to markedly regulate RXFP3 expression. It is possible, however, that downstream receptor signalling mechanisms, rather than receptor density, may be impacted by alcohol intake; or that RXFP3 expression/signalling is susceptible to adaptation during protracted withdrawal from alcohol use. Such possibilities warrant further examination in future studies. In conclusion, our results reveal a correlation between the expression of relaxin-3 mRNA in the nucleus incertus and the amount of alcohol and sucrose ingested in iP rats, suggesting endogenous relaxin-3/RXFP3 signalling may play a role in setting the intake of 'rewarding' substances.

REFERENCES

- Bachtell, R.K., Weitemier, A.Z., Galvan-Rosas, A., Tsivkovskaia, N.O., Risinger, F.O., Phillips, T.J., Grahame, N.J., Ryabinin, A.E., 2003. The Edinger-Westphal-lateral septum urocortin pathway and its relationship to alcohol consumption. *J. Neurosci.* 23, 2477-2487.
- Banerjee, A., Shen, P.J., Ma, S., Bathgate, R.A.D., Gundlach, A.L., 2010. Swim stress excitation of nucleus incertus and rapid induction of relaxin-3 expression via CRF1 activation. *Neuropharmacology* 58, 145-155.
- Bathgate, R.A.D., Lin, F., Hanson, N.F., Otvos, L., Jr., Guidolin, A., Giannakis, C., Bastiras, S., Layfield, S.L., Ferraro, T., Ma, S., Zhao, C., Gundlach, A.L., Samuel, C.S., Tregear, G.W., Wade, J.D., 2006. Relaxin-3: improved synthesis strategy and demonstration of its high-affinity interaction with the relaxin receptor LGR7 both in vitro and in vivo. *Biochemistry* 45, 1043-1053.
- Blasiak, A., Blasiak, T., Lewandowski, M.H., Hossain, M.A., Wade, J.D., Gundlach, A.L., 2013. Relaxin-3 innervation of the intergeniculate leaflet of the rat thalamus - neuronal tract-tracing and in vitro electrophysiological studies. *Eur. J. Neurosci.* 37, 1284-1294.
- Blasiak, A., Gundlach, A.L., Lewandowski, M.H., 2010. Orexins increase nucleus incertus neuronal activity: implications for a possibly reinforcing a arousal drive. *FENS Abstracts* 5, 104.102.
- Burazin, T.C.D., Bathgate, R.A.D., Macris, M., Layfield, S., Gundlach, A.L., Tregear, G.W., 2002. Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. *J. Neurochem.* 82, 1553-1557.
- Chen, F., Rezvani, A., Jarrott, B., Lawrence, A.J., 1998. Distribution of GABAA receptors in the limbic system of alcohol-preferring and non-preferring rats: in situ hybridisation histochemistry and receptor autoradiography. *Neurochem. Int.* 32, 143-151.
- Gundlach, A.L., O'Shea, R.D., 2002. Quantitative analysis of in situ hybridization histochemistry. *Int. Rev. Neurobiol.* 47, 135-170.

- Heilig, M., Koob, G.F., 2007. A key role for corticotropin-releasing factor in alcohol dependence. *Trends Neurosci.* 30, 399-406.
- Kampov-Polevoy, A.B., Garbutt, J.C., Janowsky, D.S., 1999. Association between preference for sweets and excessive alcohol intake: a review of animal and human studies. *Alcohol Alcohol.* 34, 386-395.
- Koob, G.F., 2008. A role for brain stress systems in addiction. *Neuron* 59, 11-34.
- Lawrence, A.J., 2010. Regulation of alcohol-seeking by orexin (hypocretin) neurons. *Brain Res.* 1314, 124-129.
- Lawrence, A.J., Cowen, M.S., Yang, H.J., Chen, F., Oldfield, B., 2006. The orexin system regulates alcohol-seeking in rats. *Br. J. Pharmacol.* 148, 752-759.
- Lawrence, A.J., Krstew, E., Jarrott, B., 1996. Actions of nitric oxide and expression of the mRNA encoding nitric oxide synthase in rat vagal afferent neurons. *Eur. J. Pharmacol.* 315, 127-133.
- Lenglos, C., Mitra, A., Guevremont, G., Timofeeva, E., 2013. Sex differences in the effects of chronic stress and food restriction on body weight gain and brain expression of CRF and relaxin-3 in rats. *Genes Brain Behav.* 12, 370-387.
- Liu, C., Chen, J., Kuei, C., Sutton, S., Nepomuceno, D., Bonaventure, P., Lovenberg, T.W., 2005. 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.* 67, 231-240.
- Lodge, D.J., Lawrence, A.J., 2001. Comparative analysis of the central CCK system in Fawn Hooded and Wistar Kyoto rats: extended localisation of CCK-A receptors throughout the rat brain using a novel radioligand. *Regul. Pept.* 99, 191-201.

Ma, S., Blasiak, A., Olucha-Bordonau, F.E., Verberne, A.J., Gundlach, A.L., 2013. Heterogeneous responses of nucleus incertus neurons to corticotrophin-releasing factor and coherent activity with hippocampal theta rhythm in the rat. *J. Physiol.* 591, 3981-4001.

Ma, S., Bonaventure, P., Ferraro, T., Shen, P.J., Burazin, T.C., Bathgate, R.A.D., Liu, C., Tregear, G.W., Sutton, S.W., Gundlach, A.L., 2007. 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* 144, 165-190.

Ma, S., Sang, Q., Lanciego, J.L., Gundlach, A.L., 2009. Localization of relaxin-3 in brain of *Macaca fascicularis*: identification of a nucleus incertus in primate. *J. Comp. Neurol.* 517, 856-872.

Madeira, M.D., Sousa, N., Lieberman, A.R., Paula-Barbosa, M.M., 1993. Effects of chronic alcohol consumption and of dehydration on the supraoptic nucleus of adult male and female rats. *Neuroscience* 56, 657-672.

Martin-Fardon, R., Boutrel, B., 2012. Orexin/hypocretin (Orx/Hcrt) transmission and drug-seeking behavior: is the paraventricular nucleus of the thalamus (PVT) part of the drug seeking circuitry? *Front. Behav. Neurosci.* 6, 75.

Munro, J., Skrobot, O., Sanyoura, M., Kay, V., Susce, M.T., Glaser, P.E., de Leon, J., Blakemore, A.I., Arranz, M.J., 2012. Relaxin polymorphisms associated with metabolic disturbance in patients treated with antipsychotics. *J. Psychopharmacol.* 26, 374-379.

Nestler, E.J., 2005. Is there a common molecular pathway for addiction? *Nat. Neurosci.* 8, 1445-1449.

Paxinos, G., Watson, C., 1997. *The Rat Brain in Stereotaxic Coordinates: Compact Third Edition.* Academic Press, London.

Paxinos, G., Watson, C., 2007. *The Rat Brain in Stereotaxic Coordinates.* Academic Press, London.

Pickering, C., Avesson, L., Liljequist, S., Lindblom, J., Schioth, H.B., 2007. The role of hypothalamic peptide gene expression in alcohol self-administration behavior. *Peptides* 28, 2361-2371.

- Rajkumar, R., See, L.K., Dawe, G.S., 2013. Acute antipsychotic treatments induce distinct c-Fos expression patterns in appetite-related neuronal structures of the rat brain. *Brain Res.* 1508, 34-43.
- Rivier, C., Imaki, T., Vale, W., 1990. Prolonged exposure to alcohol: effect on CRF mRNA levels, and CRF- and stress-induced ACTH secretion in the rat. *Brain Res.* 520, 1-5.
- Ryabinin, A.E., Weitemier, A.Z., 2006. The urocortin 1 neurocircuit: ethanol-sensitivity and potential involvement in alcohol consumption. *Brain Res. Rev.* 52, 368-380.
- Ryan, P.J., Kastman, H.E., Krstew, E.V., Rosengren, K.J., Hossain, M.A., Churilov, L., Wade, J.D., Gundlach, A.L., Lawrence, A.J., 2013. Relaxin-3/RXFP3 system regulates alcohol-seeking. *Proc. Natl. Acad. Sci. U S A* 110, 20789-20794.
- Ryan, P.J., Ma, S., Olucha-Bordonau, F.E., Gundlach, A.L., 2011. Nucleus incertus - an emerging modulatory role in arousal, stress and memory. *Neurosci. Biobehav. Rev.* 35, 1326-1341.
- Sanchis-Segura, C., Spanagel, R., 2006. Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. *Addict. Biol.* 11, 2-38.
- Silva, S.M., Madeira, M.D., Ruela, C., Paula-Barbosa, M.M., 2002. Prolonged alcohol intake leads to irreversible loss of vasopressin and oxytocin neurons in the paraventricular nucleus of the hypothalamus. *Brain Res.* 925, 76-88.
- Smith, C.M., Hosken, I.T., Sutton, S.W., Lawrence, A.J., Gundlach, A.L., 2012. Relaxin-3 null mutation mice display a circadian hypoactivity phenotype. *Genes Brain Behav.* 11, 94-104.
- Smith, C.M., Ryan, P.J., Hosken, I.T., Ma, S., Gundlach, A.L., 2011. Relaxin-3 systems in the brain - The first 10 years. *J. Chem. Neuroanat.* 42, 262-275.
- Smith, C.M., Shen, P.J., Banerjee, A., Bonaventure, P., Ma, S., Bathgate, R.A.D., Sutton, S.W., Gundlach, A.L., 2010. Distribution of relaxin-3 and RXFP3 within arousal, stress, affective, and cognitive circuits of mouse brain. *J. Comp. Neurol.* 518, 4016-4045.

Sommer, W.H., Rimondini, R., Hansson, A.C., Hipskind, P.A., Gehlert, D.R., Barr, C.S., Heilig, M.A., 2008. Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. *Biol. Psychiatry* 63, 139-145.

Sutton, S.W., Bonaventure, P., Kuei, C., Roland, B., Chen, J., Nepomuceno, D., Lovenberg, T.W., Liu, C., 2004. 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* 80, 298-307.

Tanaka, M., Iijima, N., Miyamoto, Y., Fukusumi, S., Itoh, Y., Ozawa, H., Ibata, Y., 2005. Neurons expressing relaxin 3/INSL 7 in the nucleus incertus respond to stress. *Eur. J. Neurosci.* 21, 1659-1670.

Ubaldi, M., Bifone, A., Ciccocioppo, R., 2013. Translational approach to develop novel medications on alcohol addiction: focus on neuropeptides. *Curr. Opin. Neurobiol.* 23, 684-691.

Volkow, N.D., Wang, G.J., Tomasi, D., Baler, R.D., 2013a. The addictive dimensionality of obesity. *Biol. Psychiatry* 73, 811-818.

Volkow, N.D., Wang, G.J., Tomasi, D., Baler, R.D., 2013b. Obesity and addiction: neurobiological overlaps. *Obes. Rev.* 14, 2-18.

Weitemier, A.Z., Ryabinin, A.E., 2005. Brain region-specific regulation of urocortin 1 innervation and corticotropin-releasing factor receptor type 2 binding by ethanol exposure. *Alcohol.Clin. Exp. Res.* 29, 1610-1620.

Zorrilla, E.P., Heilig, M., de Wit, H., Shaham, Y., 2013. Behavioral, biological, and chemical perspectives on targeting CRF(1) receptor antagonists to treat alcoholism. *Drug Alcohol Depend.* 128, 175-186.

FIGURE LEGENDS

Figure 1. Linear regression analyses of alcohol/sucrose intake (g/kg/day) vs alcohol/sucrose preference (%) and total fluid intake (g) in adult, male iP rats after chronic alcohol or sucrose intake in a two-bottle choice paradigm

(A) Alcohol intake (g/kg/day) vs alcohol preference (%) demonstrated a positive correlation ($r(12) = 0.94$, $p < 0.0001$). (B) Alcohol intake (g/kg/day) vs total fluid intake demonstrated no significant correlation ($r(12) = 0.22$, $p = 0.45$). (C) Sucrose intake (g/kg/day) vs sucrose preference (%) demonstrated no significant correlation ($r(7) = 0.34$, $p = 0.37$). (D) Sucrose intake (g/kg/day) vs total fluid intake demonstrated a significant positive correlation ($r(7) = 0.99$, $p < 0.0001$). $n = 9-14$ rats per group for the alcohol and sucrose studies; alcohol (closed circles), sucrose (open circles).

Figure 2. Relaxin-3 mRNA expression in adult male iP rat brain (at bregma -9.7 and -9.4 mm) after chronic alcohol or sucrose intake in a two-bottle choice paradigm

Semi-quantitative *in situ* hybridisation histochemistry of relaxin-3 mRNA levels in rats at the level of nucleus incertus (caudal NI; bregma -9.7 mm, Fig. 2A-C) and nucleus incertus and pontine raphe nucleus (rostral NI; bregma -9.4 mm, Fig. 2D-F), demonstrating no significant differences between groups. The density and area of mRNA hybridisation of each rat was the average of values from three coronal sections. Group data were analysed by one-way ANOVA, and are expressed as mean \pm SEM, $n = 9-14$ /group; control (closed squares), alcohol (closed circles), sucrose (open circles). (A) Bregma -9.7 mm: density of relaxin-3 mRNA (dpm/mg). (B) Bregma -9.7 mm: area of relaxin-3 mRNA (mm^2). (C) Bregma -9.7 mm: product of density \times area ($\text{dpm/mg} \times \text{mm}^2$). (D) Bregma -9.4 mm: density of relaxin-3 mRNA (dpm/mg). (E) Bregma -9.4 mm: area of relaxin-3 mRNA (mm^2). (F) Bregma -9.4 mm: product of density \times area ($\text{dpm/mg} \times \text{mm}^2$).

Figure 3. Linear regression analyses of alcohol intake (g/kg/day) and relaxin-3 mRNA levels in adult, male iP rats after chronic alcohol intake in a two-bottle choice paradigm

Linear regression analysis of alcohol intake (g/kg/day) and relaxin-3 mRNA levels at the level of nucleus incertus (caudal NI; bregma -9.7 mm; Fig. 3A-C) and nucleus incertus and pontine raphé nucleus (rostral NI; bregma -9.4 mm; Fig. 3D-F). The density and area of mRNA hybridisation of each rat was the average of values from three coronal sections and all data are expressed as mean \pm SEM, $n = 13-14$ /group. (A) Bregma -9.7 mm: alcohol intake (g/kg/day) vs density of relaxin-3 mRNA (dpm/mg) demonstrated no significant correlation ($r(12) = 0.34$, $p = 0.23$). (B) Bregma -9.7 mm: alcohol intake (g/kg/day) vs area of relaxin-3 mRNA (mm^2) demonstrated a positive correlation ($r(12) = 0.65$, $p = 0.01$). (C) Bregma -9.7 mm: alcohol intake (g/kg/day) vs product of density \times area ($\text{dpm/mg} \times \text{mm}^2$) demonstrated a positive correlation ($r(12) = 0.59$, $p = 0.03$). (D) Bregma -9.4 mm: alcohol intake (g/kg/day) vs density of relaxin-3 mRNA (dpm/mg) demonstrated no significant correlation ($r(11) = 0.23$, $p = 0.45$). (E) Bregma -9.4 mm: alcohol intake (g/kg/day) vs area of relaxin-3 mRNA (mm^2) demonstrated no significant positive correlation ($r(11) = 0.19$, $p = 0.54$). (F) Bregma -9.4 mm: alcohol intake (g/kg/day) vs product of density \times area ($\text{dpm/mg} \times \text{mm}^2$) demonstrated no positive correlation ($r(11) = 0.24$, $p = 0.43$).

Figure 4. Representative images of relaxin-3 mRNA in the nucleus incertus detected by *in situ* hybridisation in rats that consumed alcohol for 12 weeks in a two-bottle choice paradigm

(A) Nissl-stained section illustrating the location of the nucleus incertus (NI) (bregma -9.7 mm; also known as 'nucleus O' and 'central gray, alpha part'); 4V, 4th ventricle; PDTg, posterodorsal tegmental nucleus. Inset displays full coronal section at bregma -9.68 mm (Paxinos and Watson, 1997; Ryan et al., 2011). (B-D) Levels of relaxin-3 mRNA detected in rats which ingested (B) 1.64 g/kg/day alcohol (low alcohol intake), (C) 3.7 g/kg/day alcohol (medium alcohol intake), (D) 5.29 g/kg/day alcohol (high alcohol intake). Scale bar: (B-D) 250 μm .

Figure 5. Linear regression analyses of sucrose intake (g/kg/day) and relaxin-3 mRNA levels in adult, male iP rats after chronic sucrose intake in a two-bottle choice paradigm

Linear regression analysis of sucrose intake (g/kg/day) and relaxin-3 mRNA levels in nucleus incertus (caudal NI; bregma -9.7 mm; Fig. 4A-C) and in the nucleus incertus and pontine raphe nucleus (rostral NI; bregma -9.4 mm; Fig. 4D-F). The density and area of mRNA hybridisation of each rat was the average of values from three coronal sections and all data are expressed as mean \pm SEM, $n = 9$. (A) Bregma -9.7 mm: sucrose intake (g/kg/day) vs density of relaxin-3 mRNA (dpm/mg) demonstrated a significant positive correlation ($r(7) = 0.68$, $p = 0.04$). (B) Bregma -9.7 mm: sucrose intake (g/kg/day) vs area of relaxin-3 mRNA expression (mm^2) demonstrated a positive correlation ($r(7) = 0.69$, $p = 0.04$). (C) Bregma -9.7 mm: sucrose intake (g/kg/day) vs product of density \times area ($\text{dpm/mg} \times \text{mm}^2$) demonstrated a positive correlation ($r(7) = 0.70$, $p = 0.04$). (D) Bregma -9.4 mm: sucrose intake (g/kg/day) vs density of relaxin-3 mRNA (dpm/mg) demonstrated no significant correlation ($r(7) = 0.49$, $p = 0.18$). (E) Bregma -9.4 mm: sucrose intake (g/kg/day) vs area of relaxin-3 mRNA expression (mm^2) demonstrated a positive correlation ($r(7) = 0.69$, $p = 0.04$). (F) Bregma -9.4mm: sucrose intake (g/kg/day) vs product of density \times area ($\text{dpm/mg} \times \text{mm}^2$) demonstrated no significant correlation ($r(7) = 0.64$, $p = 0.07$).

Table 1. Relaxin-3 oligonucleotide probes

Probe	Sequence
H3-1	5' CAC TCG CAA CAG CTG CTG GAA AGG CCA GCC AGC ACA TCT 3'
H3-3	5' CTG TTT GCC TGG ATA GAG GAT GGC CAG CAG CAA AAG TGG 3'
H3-4	5' CAC AGC TTC GTC CAG TTC GCT GGC CAG GTG GTC TGT ATT 3'
H3-5	5' GCC AGT TTG GGT AGA TGG ACA TCT ATC TGG TTG CTG CCG 3'

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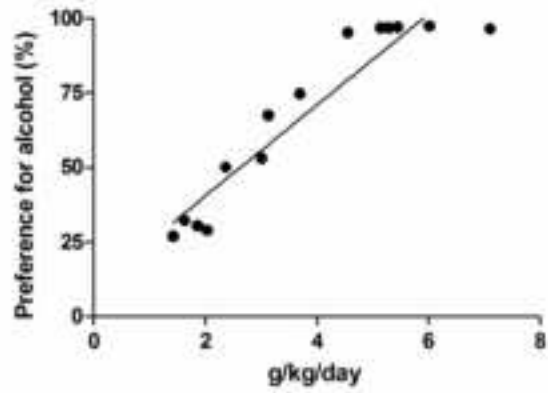
Table 2. Radioligand (¹²⁵I-R3/I5) binding expression.

	Control			Ethanol			Sucrose		
	Density (dpm/mg)	Area (mm ²)	Density x area	Density (dpm/mg)	Area (mm ²)	Density x area	Density (dpm/mg)	Area (mm ²)	Density x area
SON	479.4 ± 34.8	0.10 ± 0.01	49.3 ± 4.7	422.1 ± 24.8	0.10 ± 0.003	42.9 ± 2.8	411.0 ± 35.7	0.10 ± 0.01	42.3 ± 5.6
mPVN	303.3 ± 36.6	0.08 ± 0.02	25.0 ± 5.2	226.5 ± 28.1	0.07 ± 0.01	16.0 ± 3.3	288.7 ± 80.5	0.06 ± 0.01	18.6 ± 5.2
CeA	315.2 ± 36.2	0.08 ± 0.01	28.3 ± 6.1	349.2 ± 23.0	0.09 ± 0.01	31.5 ± 4.1	317.7 ± 40.3	0.08 ± 0.01	25.6 ± 3.8
PVT	287.3 ± 38.5	0.29 ± 0.03	90.0 ± 17.3	327.6 ± 35.1	0.28 ± 0.03	101.3 ± 15.8	393.9 ± 75.0	0.30 ± 0.06	132.4 ± 37.1
CM	308.5 ± 58.3	0.57 ± 0.09	187.8 ± 54.5	285.6 ± 41.2	0.60 ± 0.07	156.2 ± 26.8	384.3 ± 35.2	0.58 ± 0.08	204.2 ± 5.8
BNST	117.3 ± 11.1	0.54 ± 0.06	62.7 ± 8.5	122.1 ± 4.6	0.54 ± 0.04	63.4 ± 5.1	133.3 ± 9.0	0.63 ± 0.04	88.1 ± 4.4

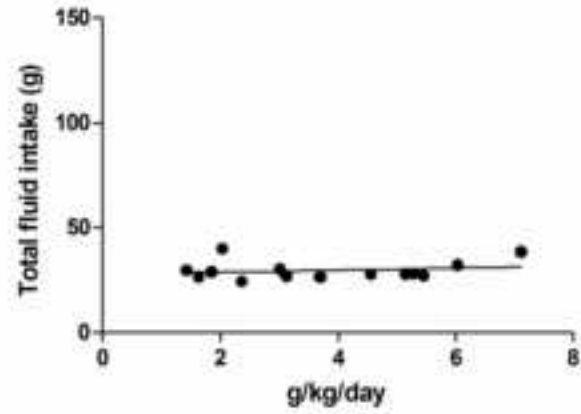
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Alcohol

A

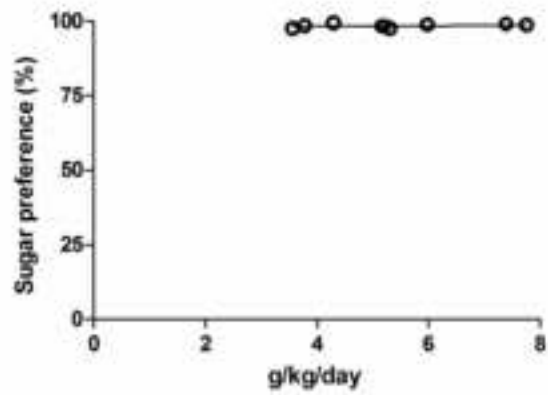


B

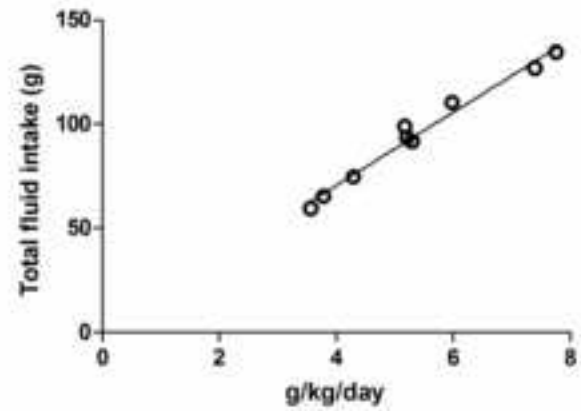


Sucrose

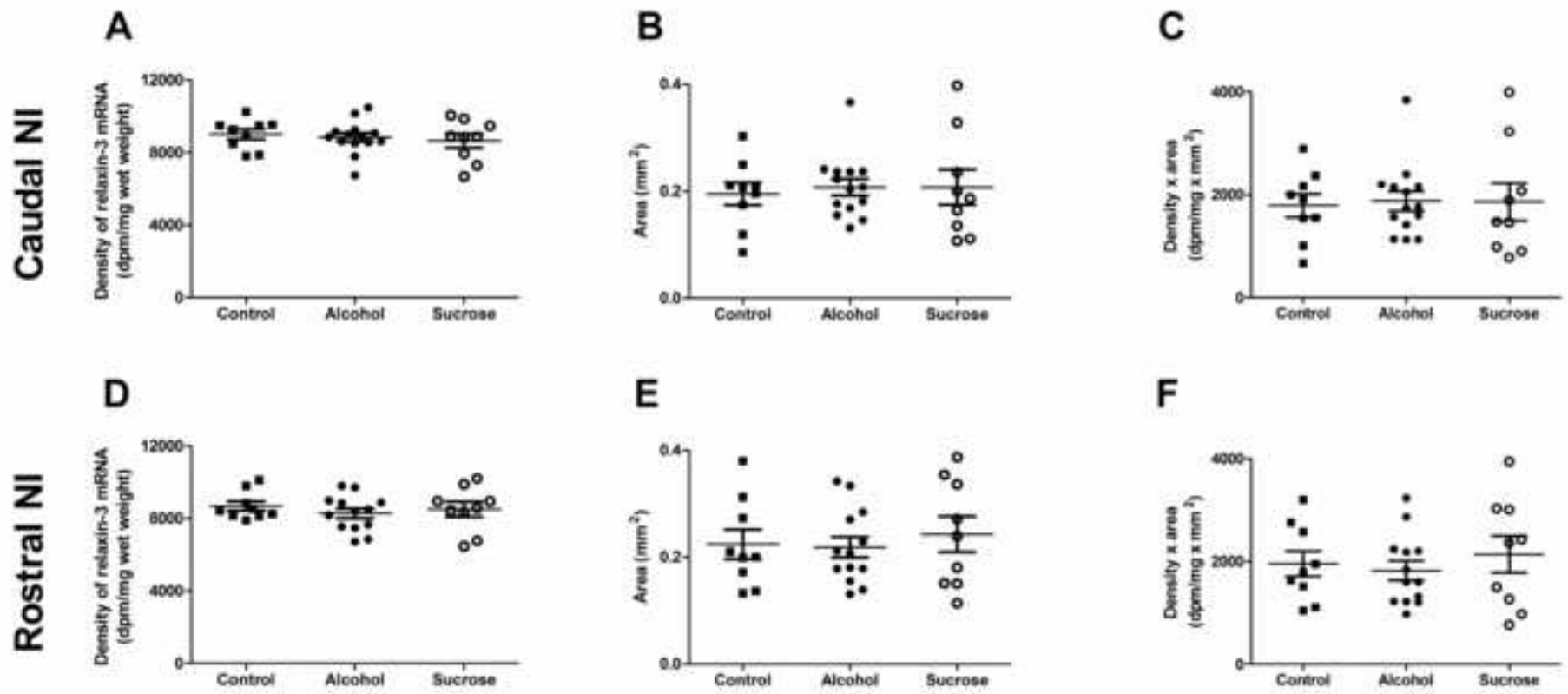
C



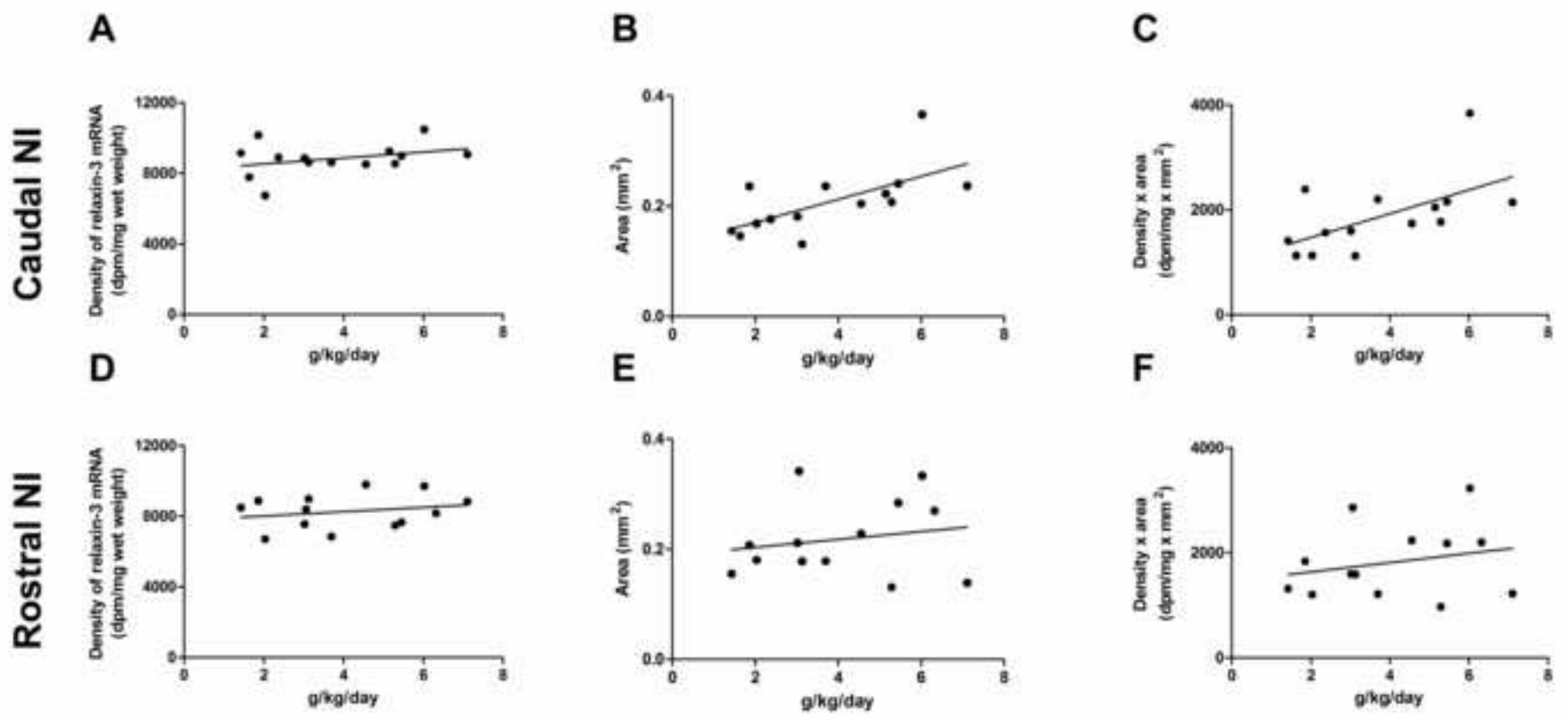
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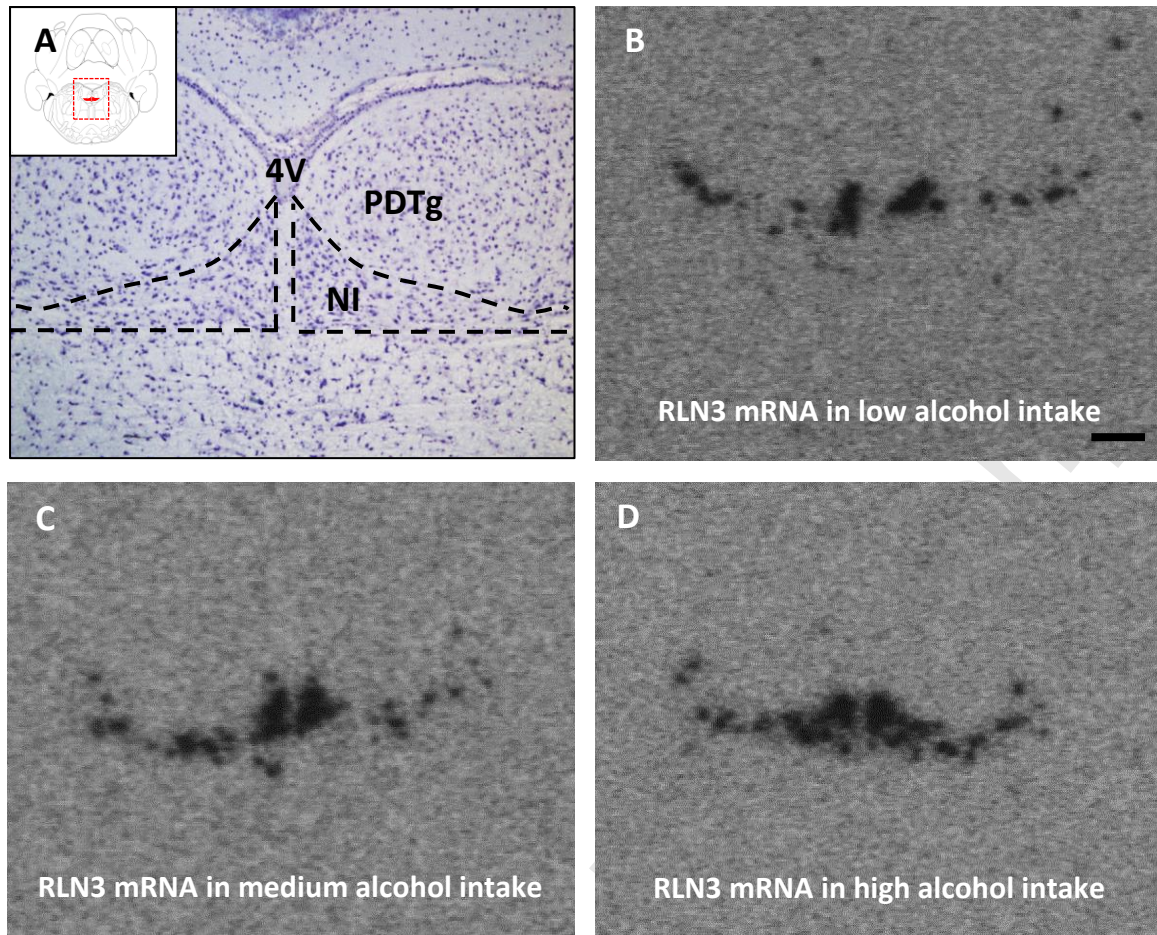


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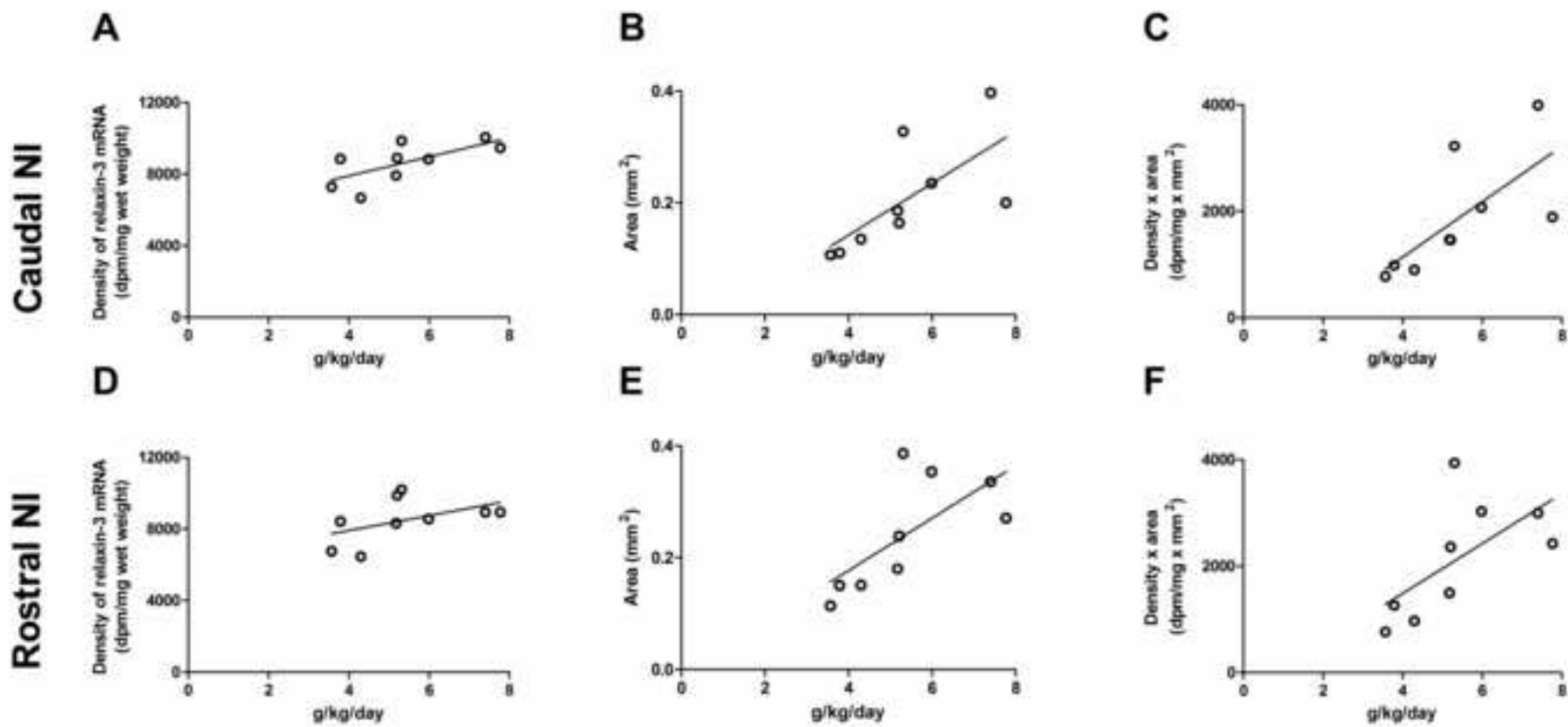


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Contributors. Study conceived and planned by AJL & PJR. Reagents provided by MS.

Study performed by PJR & EVK. Data analysed and interpreted by PJR & AJL.

Manuscript written by PJR, AJL & ALG.

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