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REVIEW

Distribution, physiology and pharmacology of relaxin-3/RXFP3 systems in brain

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Running title: Relaxin-3/RXFP3 systems in brain

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Abstract

Relaxin-3 is a member of a superfamily of structurally-related peptides that includes relaxin and insulin-like peptide hormones. Soon after the discovery of the relaxin-3 gene, relaxin-3 was identified as an abundant neuropeptide in brain with a distinctive topographical distribution within a small number of GABA neuron populations that is well conserved across species. Relaxin-3 is thought to exert its biological actions through a single class-A GPCR – relaxin family peptide receptor 3 (RXFP3). Class-A comprises GPCRs for relaxin-3 and insulin-like peptide-5 *and* other peptides such as orexin and the monoamine transmitters. RXFP3 is selectively activated by relaxin-3, whereas insulin-like peptide-5 is the cognate ligand for the related RXFP4 (see Ang SY *et al*. and Patil NA *et al*. this issue). Anatomical and pharmacological evidence obtained over the last decade supports a function of relaxin-3/RXFP3 systems in modulating responses to stress, anxiety-like and motivated behaviours, circadian rhythms, and learning and memory. Electrophysiological studies have identified the ability of RXFP3 agonists to directly hyperpolarise thalamic neurons *in vitro*, but there are no reports of *direct* cell signalling effects *in vivo*. This article provides an overview of earlier studies and highlights more recent research that implicates relaxin-3/RXFP3 neural network signalling in the *integration* of arousal, motivation, emotion and related cognition, and has begun to identify the associated neural substrates and mechanisms. Future research directions to better elucidate the connectivity and function of different relaxin-3 neuron populations and their RXFP3-positive target neurons in major experimental species and humans are also identified.

Abbreviations: ACTH, adrenocorticotropic hormone; AgRP, agouti-related peptide; BNST, bed nucleus of the stria terminalis; CRF, corticotropin-releasing factor; DREADD, designer receptors exclusively activated by designer drugs; HCN, hyperpolarisation-activated cyclic nucleotide-gated channels; icv, intracerebroventricular; IGL, intergeniculate leaflet; INSL3/5, insulin-like peptide 3/5; MCH, melanin-concentrating hormone; NPY, neuropeptide-Y; POMC, pro-opiomelanocortin; PV, parvalbumin; PVN, paraventricular nucleus of hypothalamus; RXFP1-4, relaxin-family peptide receptor 1-4; SALPR, somatostatin- and angiotensin-like peptide receptor.

Table of Links

TARGETS	**Enzymes**[b]
GPCRs[a]	ChAT
CRF_1 receptor	ERK1
CRF_2 receptor	ERK2
Dopamine D2 receptor	GAD
Orexin OX_2 receptor	PKA
RXFP1-4 receptors	tryptophan hydroxylase
Serotonin-1A receptor	

LIGANDS	Melanin-concentrating hormone
Acetylcholine	Neuropeptide-Y
Agouti-related peptide	Orexin-A
Calcitonin gene-related peptide	Oxytocin
cAMP	Pro-opiomelanocortin
Chlorpromazine	R3/I5
Clozapine	Relaxin
Corticotropin-releasing factor	Relaxin-3
Fluphenazine	Serotonin
Dopamine	Thyroid-stimulating hormone
GABA	Vasoactive intestinal peptide
Insulin-like peptide 5	Vasopressin

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are

permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015a,b).

Introduction

Discovery of relaxin-3 and RXFP3

Relaxin-3 was discovered in 2001 by searching for homologues of the relaxin gene in the Celera Discovery System and Celera Genomics databases (Bathgate *et al.*, 2002) and due to its predominant expression in brain, was subsequently classified as a neuropeptide. Relaxin-3, like other relaxin and insulin-like peptide family members, is a 5 kDa peptide that consists of two chains with three disulphide bonds (Bathgate *et al.*, 2002; Liu *et al.*, 2003b; Hossain *et al.*, 2013). All family members contain the characteristic sequence 'RXXXRXX(I/V)' within their B-chain, which is essential for binding the different cognate receptors (Bullesbach and Schwabe, 2000; Bathgate *et al.*, 2002; 2006b).

The cognate receptor for relaxin-3 is relaxin-family peptide 3 receptor (RXFP3) (Bathgate *et al.*, 2006a). Also known as G-protein-coupled receptor 135 (GPCR135) (Liu *et al.*, 2003b), it was first discovered in 2000 and named somatostatin- and angiotensin-like peptide receptor (SALPR), due to its high amino acid similarity with somatostatin receptor-5 and the angiotensin AT1 receptor (Matsumoto *et al.*, 2000). RXFP3 is a $G_{i/o}$-protein-coupled receptor, and its activation produces inhibition of intracellular cAMP accumulation and activation of extracellular-regulated kinase 1/2 (ERK1/2) (Liu *et al.*, 2003b; van der Westhuizen *et al.*, 2007). Although in cell-based studies, relaxin-3 can bind and activate three related G-protein coupled receptors – RXFP3, RXFP1 (originally named LGR7; Sudo *et al.*, 2003), and RXFP4 (originally named GPCR142; Liu *et al.*, 2003a), there is considerable evidence that RXFP3 is the native receptor for relaxin-3. Firstly, RXFP3 displays the highest affinity for relaxin-3 (Bathgate *et al.*, 2006b), and the genes encoding the peptide and protein have phylogenetically co-evolved (Hsu *et al.*, 2005; Wilkinson *et al.*, 2005). Furthermore, there is a strong overlap between the distribution of relaxin-3-positive nerve fibres and RXFP3 mRNA/binding sites throughout the rat (Sutton *et al.*, 2004; Ma *et al.*, 2007), mouse (Smith *et al.*, 2010) and macaque brain (Ma *et al.*, 2009b, 2009c). Moreover, RXFP4 is primarily expressed within the gastrointestinal tract and is largely absent from brain (Sutton *et al.*, 2006) and is, in fact, a pseudogene in rat (Chen *et al.*, 2005). Also, although RXFP1 is expressed widely throughout the rodent brain (Ma *et al.*, 2006), its distribution pattern does

not correspond with that of the relaxin-3 innervation, and relaxin is expressed by specific neuron populations (Ma *et al.*, 2006). Finally, relaxin-3 is the only relaxin-peptide family member that can activate RXFP3 (Liu *et al.*, 2003b), whereas relaxin is the preferred ligand for RXFP1 and also binds to RXFP2 (Sudo *et al.*, 2003). The related insulin-like peptide 5 (INSL5) is uniquely expressed in enteroendocrine L-cells of the colon and it is the cognate ligand for RXFP4 (Liu *et al.*, 2005b; Sutton *et al.*, 2006; Grosse *et al*., 2014).

Distribution of relaxin-3 and RXFP3 in the brain – a road map to function

Relaxin-3/RXFP3 systems conserved across various mammalian species

The brain is the main site of relaxin-3 mRNA synthesis, with high levels of expression observed in various species including zebrafish (Donizetti *et al.*, 2009), mouse (Bathgate *et al.*, 2002; Smith *et al.*, 2010), rat (Burazin *et al.*, 2002; Tanaka *et al.*, 2005), macaque (Ma *et al.*, 2009b), and human (Liu *et al.*, 2003b). The presence and anatomical distribution of relaxin-3 producing neurons has been best studied in rat and mouse brain, with the largest population observed in the brainstem *nucleus incertus* (**Figure 1**; Bathgate *et al.*, 2002; Burazin *et al.*, 2002; Tanaka *et al.*, 2005; Ma *et al.*, 2007; Smith *et al.*, 2010; Ryan *et al.*, 2011). Relaxin-3 neurons, which use GABA as their primary transmitter, are also present in smaller populations in the pontine raphé nucleus (~350 neurons) medial and ventrolateral periaqueductal grey (~550 neurons), and in an area dorsal to the substantia nigra (~350 neurons), relative to the ~2000 relaxin-3-positive neurons in the rat nucleus incertus (Tanaka *et al.*, 2005; Ma *et al.*, 2007; Smith *et al.*, 2010).

Major inputs to the nucleus incertus, which lies in the midline periventricular central grey, arise from the prefrontal cortex, lateral habenula, interpeduncular nucleus, median raphe and lateral hypothalamus (see Ma and Gundlach, 2015 for review), but only limited data is available regarding the specific inputs to the relaxin-3 and non-relaxin-3 neurons in the area. Additionally, the proximity of the nucleus incertus to the fourth ventricle in rodent, primate and human (Ma and Gundlach, 2015) makes it a potential target for neurohumoral signals, as described for similarly located structures like the dorsal raphe nucleus (Torterolo *et al.*, 2008). Nonetheless, the identified neural inputs point to a likely role for nucleus incertus/relaxin-

3/RXFP3 networks in the integration of multiple physiological functions, including energy and endocrine homeostasis, circadian rhythmicity, reward, and emotional processing (**Figure 2**). Moreover, nucleus incertus neurons broadly innervate cortical and subcortical structures, such as prefrontal and cingulate cortex, septum, hippocampus, thalamus, hypothalamus, and innervate the brainstem (Goto *et al.*, 2001; Olucha-Bordonau *et al.*, 2003; Ma and Gundlach, 2015), suggesting that nucleus incertus relaxin-3 neurons integrate behavioural and physiological responses to internal and external stimuli.

In the rat, the distribution of relaxin-3-containing fibres throughout the brain largely parallels that of nucleus incertus efferent projections assessed by anterograde neural tract-tracing (Goto *et al.*, 2001; Olucha-Bordonau *et al.*, 2003), suggesting that a substantial component of axonally-transported relaxin-3 originates from nucleus incertus. However, there is evidence for distinct relaxin-3 pathways arising from the smaller populations outside nucleus incertus. For example, the thalamic intergeniculate leaflet (IGL) receives dense relaxin-3 projections (Tanaka *et al.*, 2005; Ma *et al.*, 2007; Smith *et al.*, 2010), that arise from neurons in the periaqueductal grey, *not* nucleus incertus. Indeed, RXFP3 agonist peptides depolarise neuropeptide-Y (NPY) neurons in the intergeniculate leaflet (**Figure 3**; Blasiak *et al.*, 2013), which are known to modulate suprachiasmatic nucleus function and associated circadian rhythms. Therefore, further studies are required to establish the detailed projection patterns of the different relaxin-3 neuron populations, a task that may eventually be facilitated by genetic and/or viral based methods (see e.g. Schwarz *et al.*, 2015).

The distribution of relaxin-3-containing nerve fibres is similar in rat, mouse and macaque brain (Ma *et al.*, 2009b), and 'matches' the distribution of RXFP3, as reflected by the distribution of RXFP3 mRNA, and binding sites for a relaxin-3 agonist analogue, [^{125}I]-R3/I5 (Sutton *et al.*, 2004; Ma *et al.*, 2007; Smith *et al.*, 2010). The relaxin-3/RXFP3 system can be generally viewed as being closely associated with functional circuits involving the septum and hippocampus (septohippocampal system) and hippocampal-modulating regions, the hypothalamus, limbic areas, and the thalamus/cortex. For further details, see Ma and Gundlach (2007) and Smith *et al.* (2011).

Notably, however, the presence of a strong relaxin-3 innervation to the infralimbic, prelimbic and anterior cingulate and posterior retrosplenial areas of the cortex in rat and mouse was not observed in the macaque brain (Ma *et al.*, 2009b). Otherwise, the distribution of the relaxin-3 innervation largely parallels that of nucleus incertus projections, which have been described to be positioned to modulate various higher-cognitive brain circuits, related to behavioural planning and state, motivation, emotion, and learning and memory (Goto *et al.*, 2001; Olucha-Bordonau *et al.*, 2003). With respect to learning and memory, the dense relaxin-3 innervation of septum (Olucha-Bordonau *et al.*, 2012) and hippocampus further suggests the relaxin-3/RXFP3 system modulates cognition via the septohippocampal system and associated effects on hippocampal function (Ma *et al.*, 2009a). To date, however, anatomical and functional studies in human are limited, although in a preliminary study, relaxin-3-like immunoreactivity was reported to be present in neurons in the dorsal raphe and pontine reticular nuclei, and regions of the dorsal and ventral tegmental nucleus, with immunoreactive fibres in the ventrolateral tegmental area, basis pontis, pontine nucleus, and pontocerebellar tracts (Silvertown *et al.*, 2010), and confirmatory studies are now required.

In other studies, human neocortex lysates from Alzheimer's disease patients were reported to contain a moderately higher level of RXFP3 protein detected by immunoblotting, which correlated with longitudinal scores of depression (Lee *et al.*, 2016), and the RXFP3 antiserum was shown to recognise an appropriate sized protein, although tissues from *Rxfp3* gene knockout mice were not tested. Also, in a cohort of patients treated with antipsychotics, two RXFP3 polymorphisms and a relaxin-3 gene polymorphism displayed significant associations with hypercholesterolemia, suggesting a role for relaxin-3/RXFP3 signalling in metabolic disturbances linked to antipsychotic treatment (Munro *et al.*, 2012). In a cohort of female patients, a moderate increase in serum relaxin-3 levels was correlated with component traits of metabolic syndrome (Ghattas *et al.*, 2013), although in this study, the specificity of the assay for relaxin-3 detection was not fully demonstrated, so further confirmation of such links is required. A further issue, given growing pre-clinical evidence for a role of relaxin-3/RXFP3 signalling in modulating central processes underlying cognition and behaviour, is a need for

more comprehensive studies of the system in human brain and its potential involvement in, or therapeutic impact on, dementia, neurodegeneration, and neuropsychiatric disorders (see Kumar *et al*. 2016).

Physiology of relaxin-3 and RXFP3 in the brain

Responsiveness to stress

Substantial anatomical and functional data (e.g. Potter *et al.*, 1994; Bittencourt and Sawchenko, 2000; Banerjee *et al.*, 2010) suggest the nucleus incertus and its relaxin-3 neuron population are highly 'stress-reactive' (see Ryan *et al.*, 2011 for review). Notably, while dorsal raphe serotonergic neurons express corticotrophin-releasing factor (CRF) receptor-1 and 2 ($CRF_{1/2}$) (Kirby *et al.*, 2008), the nucleus incertus expresses higher levels of CRF_1 than CRF_2 (Bittencourt and Sawchenko, 2000; Van Pett *et al.*, 2000; Justice *et al.*, 2008). Neurogenic stress in rats resulting from forced-swim, increased relaxin-3 heteronuclear RNA and mRNA levels in the nucleus incertus, via a CRF_1-dependent action (Banerjee *et al.*, 2010). A major nucleus incertus neuron population expressing CRF_1 (including relaxin-3-containing neurons) exhibited a long-lasting and non-desensitising depolarisation response to CRF (Ma *et al.*, 2013). These responses differ from those within the neighbouring dorsal raphe nucleus, where serotonergic and non-serotonergic display differential, dose-dependent responses to CRF that are rapidly desensitized (Kirby *et al.*, 2008). Similarly, relaxin-3 neurons exhibited increased firing frequency following intracerebroventricular (icv) infusion of CRF (1-3 µg), whereas decreased firing was only observed in relaxin-3 negative neurons (**Figure 4**; Ma *et al.*, 2013). These findings suggest distinct neural populations in the nucleus incertus respond differentially to the stress hormone, but relaxin-3 neurons are robustly stimulated by CRF. The stress reactivity of other relaxin-3 neuron populations has yet to be investigated.

Furthermore, the activity of hypothalamic CRF neurons has been reported to be influenced by central administration of relaxin-3, although the nature of these actions are currently unclear. Intracerebroventricular infusion of relaxin-3 has been shown to increase c-*fos* (a marker of neuronal activation) and CRF mRNA expression in CRF neurons in the rat PVN (Watanabe *et*

al., 2010), and to elevate plasma adrenocorticotropic hormone (ACTH) levels (Watanabe *et al.*, 2010; McGowan *et al.*, 2014). Thus, there appears to exist a reciprocal interaction between relaxin-3 and CRF systems, but further studies are required to determine the nature of any direct or indirect effects of relaxin-3 inputs on the activity of CRF neurons and related physiological/behavioural measures of hypothalamic CRF neural activity. Studies are also required to catalogue the location and identity of the CRF neurons that innervate nucleus incertus relaxin-3 neurons as there are many candidate extrahypothalamic CRF neuron populations that may do so (Lenglos *et al.*, 2013; Ma *et al.*, 2013; Walker *et al.*, 2016). More generally, there is a need to identify and characterise other neurochemical/neural inputs to relaxin-3 neurons that are altered by acute or chronic stressors, such as the hypothalamic orexin neurons (Blasiak *et al.*, 2015; Kastman *et al.*, 2016).

Pharmacological effects of RXFP3 activation

Neurophysiological effects

Relaxin-3 activation of its cognate receptor, RXFP3, leads to the inhibition of intracellular cAMP accumulation and activation of the ERK1/2 enzyme in cell-based assays (Liu *et al.*, 2003b; van der Westhuizen *et al.*, 2007). Regulation of the cAMP pathway is a common intracellular signalling cascade target for neuropeptides (e.g. CRF, vasoactive intestinal peptide and calcitonin gene-related peptide (Haug and Storm, 2000) and other transmitters, including catecholamines (Pedarzani and Storm, 1995). cAMP activates the protein kinase A (PKA) enzyme, which phosphorylates target proteins, including ion channels that can mediate suppression of membrane ion currents (e.g. the slow calcium-activated potassium current) (Haug and Storm, 2000; Hu *et al.*, 2011). Moreover, cAMP can exert direct effects on ion channels independent of PKA, such as hyperpolarization-activated cyclic nucleotide–gated (HCN) channels, whereby activation increases non-selective Ih cation currents that lead to membrane depolarisation (Pedarzani and Storm, 1995; Sun *et al.*, 2003). Currently, there are few published reports of the direct impact of RXFP3 activation on the physiological or neurochemical activity of target neurons, but these studies are underway and there are several candidate target areas/neurons for investigation.

For example, the medial septum component of the septohippocampal system is a major innervation target of relaxin-3 neurons and contains a high density of RXFP3 mRNA expressing neurons (Sutton *et al.*, 2004; Ma *et al.*, 2007). Electrical stimulation of the nucleus incertus in anesthetised rats evoked hippocampal theta oscillations and lesions of the nucleus incertus abolished theta rhythm evoked by brainstem stimulation (Nunez *et al.*, 2006). Moreover, selective activation of RXFP3 in the medial septum promoted hippocampal theta rhythm, as well as spatial memory and exploratory activity (Ma *et al.*, 2009a). In this regard, HCN h-currents exist in septal fast-spiking GABAergic and, to a lesser extent, fast-firing glutamatergic neurons (Sotty *et al.*, 2003); and rhythmic firing at theta frequency is characteristic of all HCN-expressing neurons (Varga *et al.*, 2008). Therefore, the role of relaxin-3 in the regulation of septohippocampal activity may rely on RXFP3-dependent modulation of cAMP in GABAergic septal neurons, which play a critical role in synchronising the hippocampal neuron network at theta frequency (Toth *et al.*, 1997). Importantly, inhibition of cAMP accumulation reduces neuronal excitability and produces membrane hyperpolarisation (Molosh *et al.*, 2013) and RXFP3 activation inhibits a population of IGL neurons *in vitro* (**Figure 3B**; Blasiak *et al.*, 2013).

In addition to GABAergic neurons, hippocampal theta rhythm is also regulated by cholinergic pacemaker neurons of the medial septum (Yoder and Pang, 2005). A recent study reported that icv administration of the selective RXFP3 agonist, RXFP3-A2, increased ERK phosphorylation in septal cholinergic neurons (20 and 60 min post-injection) and impaired spatial working memory in a spontaneous alternation test assessed 5 min post-treatment (Albert-Gasco *et al.*, 2016). ERK1/2 activation is capable of increasing neuronal excitability through inhibition of transient potassium (A-type) currents (Fu *et al.*, 2008), but the recent study did not assess the direct or indirect nature of the excitatory/inhibitory effect of RXFP3 activation on different septal neurons, as the site of peptide administration was outside the septum (Albert-Gasco *et al.*, 2016). Moreover, these recent behavioural findings contrast earlier studies, which reported an increase in the power of hippocampal theta activity following infusion of RXFP3 agonist, R3/I5, directly into the medial septum, and an impairment in spatial memory performance in the spontaneous alternation task with intra-

septal infusion of an RXFP3 antagonist, R3(BΔ23-27)R/I5 (Ma *et al.*, 2009a). Thus, additional studies are required to investigate the precise nature of relaxin-3/RXFP3 signalling within the medial septum, which may differ depending on the neural circuits and the neuronal cell types involved when using different 'pharmacological' approaches. Notably however, a key goal is to determine the physiological/behavioural effects of 'global' RXFP3 modulation initiated via a peripheral route of administration, as this is vital in a therapeutic context.

Feeding and other motivated behaviours

The first reported pharmacological effect of relaxin-3 on behaviour in rats, was a potent orexigenic action (McGowan *et al.*, 2005; see also Calvez *et al*. 2016b). In satiated rats, relaxin-3 injected into the lateral cerebral ventricle (180 pmol) or the paraventricular nucleus of the hypothalamus (PVN, 18 pmol) during the early light phase, produced a marked increase in food intake. This orexinergic response did not appear to involve *classical* peptidergic feeding pathways, as no change in NPY, pro-opiomelanocortin (POMC) or agouti-related peptide (AgRP) mRNA levels was produced by the peptide. Later studies indicated that chronic intra-PVN relaxin-3 injections (180 pmol/twice a day for 7 days) also promoted food intake, an effect associated with an increase in plasma leptin levels and decreased thyroid-stimulating hormone levels (McGowan *et al.*, 2006). Similar effects were produced by chronic (14-day) relaxin-3 infusion into the cerebral ventricles via osmotic minipumps (Hida *et al.*, 2006), which in addition to the increase in food intake and body weight, caused severe hyperleptinemia and hyperinsulinemia - symptoms that accompany obesity in humans (Leon-Cabrera *et al.*, 2013). A caveat of these early studies was the possible activation of RXFP3 *and* RXFP1 by exogenously administrated relaxin-3, as both are expressed in the hypothalamus and PVN (Sutton *et al.*, 2004; Bathgate *et al.*, 2006b; Ma *et al.*, 2006; Ganella *et al.*, 2013b).

Studies using the first selective RXFP3 agonist, R3/I5 (Liu *et al.*, 2005a; Sutton *et al.*, 2009) and the 'next generation' minimised agonist, RXFP3-A2 (Shabanpoor *et al.*, 2012) confirmed the involvement of RXFP3 in promoting feeding in rats. Furthermore, the likely involvement of oxytocin and vasopressin signalling in the orexigenic action of relaxin-3 was revealed as

viral-mediated, chronic secretion of R3/I5 in the PVN region (Ganella *et al.*, 2013a), produced a robust reduction in whole hypothalamic oxytocin and vasopressin mRNA levels (50% and 25% decrease relative to control, respectively). Importantly, chronic activation of RXFP3 in this study led to a modest, but significant, increase in body weight and in daily food intake, and so similar studies using an RXFP3 antagonist to determine its ability to attenuate feeding in rats would be of interest. In this regard, RXFP3 antagonist peptides are capable of blocking acute agonist-induced feeding (Kuei *et al.*, 2007; Haugaard-Kedstrom *et al.*, 2011) and stress-induced increase in sucrose intake in binge-like eating prone, but not binge-like eating resistant, female rats (Calvez *et al.*, 2016a,b). Therefore, while these studies suggest a lack of a strong direct influence of RXFP3 activation on hypothalamic NPY, AgRP and POMC neurons, the mechanisms and hypothalamic neural circuits underlying relaxin-3-induced feeding including effects via oxytocin and/or vasopressin, and other feeding-related peptides such as orexins require further investigation. These studies should also examine other experimental species such as mice and non-human primates and investigate the impact of stress and different diet compositions on outcomes.

Other motivation and stress-sensitive behaviours are also influenced by relaxin-3/RXFP3 signalling, including alcohol seeking and self-administration, and stress-induced relapse to alcohol seeking following abstinence in alcohol-preferring (iP) rats (Ryan *et al.*, 2013b). Infusion of the RXFP3-selective antagonist, R3(B1-22)R, into the lateral cerebral ventricle or directly into the bed nucleus of the stria terminalis (BNST) of iP rats significantly attenuated lever pressing for alcohol, and cue- and stress-induced reinstatement of lever pressing (Ryan *et al.*, 2013b). Importantly, these rats display increased stress/CRF responsiveness, and decreased brain CRF levels (Ehlers *et al.*, 1992); and relaxin-3 mRNA levels in the nucleus incertus are positively correlated with their alcohol and sucrose intake (Ryan *et al.*, 2014). Together, these findings suggest relaxin-3/RXFP3 signalling in key hypothalamic and limbic circuits is capable of integrating stress-related external and internal information, by regulating networks responsible for orexigenic and goal-directed (motivated) behaviours.

Although most relaxin-3-related pharmacological research to date has been conducted in rats, studies in mice have contributed to our knowledge of relaxin-3 biology. In agreement with a role in motivated feeding, which is well-established in rats, icv infusion of the RXFP3 antagonist, R3(B1-22)R, in mice reduced the consumption of palatable food, and of regular chow during the early dark phase and following mild food deprivation (Smith *et al.*, 2014a). Furthermore, icv infusion of this same RXFP3 antagonist reduced the consumption of NaCl salt in sodium-depleted mice (Smith *et al.*, 2015), and *Rxfp3* gene knockout mice displayed reduced motivation to consume sucrose compared to wildtype controls (Walker *et al.*, 2015b). Despite a clear ability of relaxin-3/RXFP3 signalling to modulate feeding in both rats and mice, it is interesting that central infusion of RXFP3 agonists (or native relaxin-3 peptide) potently increases food consumption in rats (e.g. Shabanpoor *et al.*, 2012, but not mice (Smith *et al.*, 2013b, 2014a)). The reason for this species discrepancy is not obvious, as, for example, both species display strong and roughly equivalent regional patterns of RXFP3 expression within hypothalamic feeding centres (Ma *et al.*, 2007; Smith *et al.*, 2010). However, the neurochemical identity of RXFP3-positive neurons within each of these regions, and their efferent and afferent connectivity, remains to be determined in each species. For example, differences exist between rat and mouse hypothalamic melanin-concentrating hormone (MCH) neurons as reflected by their gene expression and projection patterns, birthdates and a divergence in their developmental differentiation, which may underlie observed species-specific effects of MCH signalling in the control of feeding behaviour and the sleep/wake cycle (Croizier *et al.*, 2010).

Another consummatory behaviour relaxin-3 signalling is able to modulate in both rats and mice, is alcohol consumption. In line with rat studies, in which icv infusion of the RXFP3 antagonist R3(B1-22)R reduced alcohol seeking (Ryan *et al.*, 2013b), *Rxfp3* gene knockout mice on a C57BL/6J background displayed reduced alcohol preference relative to wildtype controls following chronic stress (Walker *et al.*, 2015a). This study also demonstrated that basal alcohol preferences were equivalent between genotypes; while a recent study reported that male *Rln3* gene knockout mice on a C57BL/6N background displayed *increased* baseline alcohol intake compared to wildtype controls (Shirahase *et al.*, 2016). These differences may

be attributable to genetic differences in the C57BL/6 mice used, as it has been established that substrains of these mice display marked behavioural differences (Kiselycznyk and Holmes, 2011). Again, further studies will be required to explore these possibilities and clarify the true nature and biological importance of the alcohol consumption differences observed.

Circadian rhythm and arousal

An ability of relaxin-3/RXFP3 signalling to promote a range of consummatory behaviours is in line with its likely primary role in driving arousal and motivated behaviour more broadly (Smith *et al.*, 2011; Ma and Gundlach, 2015). For example, male and female relaxin-3 (*Rln3*) (Smith *et al.*, 2012) and *Rxfp3* gene knockout mice (Hosken *et al.*, 2015) display reduced circadian dark phase running wheel activity compared to wildtype controls (**Figure 5**). Furthermore, acute icv injection of the RXFP3 antagonist, R3(B1-22)R, reduced food anticipatory activity displayed by pre-conditioned mice (Smith *et al.*, 2014a), and viral vector-mediated chronic secretion of an RXFP3 agonist within the mouse cerebral ventricular system reduced locomotor habituation to a novel environment (Smith *et al.*, 2013a). Central arousal systems are also strongly involved in mediating the response to stress (Smith *et al.*, 2014b), and similar to rats (Ryan *et al.*, 2013a), icv injection of an RXFP3 agonist reduced (elevated) anxiety-like behaviour in mice (Zhang *et al.*, 2015). Although subtle signs of altered anxiety-like behaviour have been detected in *Rln3* (Watanabe *et al.*, 2011) and *Rxfp3* knockout mice (Hosken *et al.*, 2015), life-long relaxin-3 or RXFP3 deletion did not alter depressive-like behaviours relative to wildtype controls during methamphetamine withdrawal (Haidar *et al.*, 2016). Although the mechanisms underlying the ability of relaxin-3/RXFP3 signalling to promote arousal and modulate stress responses in mice are unknown, based on the similar distribution of ligand and receptor in both species (Ma *et al.*, 2007; Smith *et al.*, 2010), mechanisms identified in rats (such as modulation of the septohippocampal system, amygdala and PVN; see above) are likely to be involved.

Furthermore, in the context of arousal, recent studies have demonstrated that nucleus incertus relaxin-3 neurons receive an excitatory orexinergic innervation from the lateral hypothalamus and perifornical area, and that orexin-A produces depolarisation and action potential firing of

neurons *in vitro* via the OX_2 receptor (Blasiak *et al.*, 2015). Conversely, nucleus incertus relaxin-3 neurons also express inhibitory D_2 dopamine receptors which, when pharmacologically activated, result in decreased locomotor activity in rats (Kumar *et al.*, 2015, 2016).

Among brain sites that might underlie the relaxin-3/RXFP3 signalling modulation of arousal patterns, the IGL, which is a primary regulator of circadian rhythm, is a candidate. The largely GABAergic and NPY-expressing IGL neurons, have strong projections to the suprachiasmatic nucleus, which is considered to be the main circadian 'pacemaker' in the circadian timing system (Morin and Blanchard, 2005; Moore, 2013). The IGL displays dense RXFP3 mRNA levels and relaxin-3-immunoreactive nerve fibres (Tanaka *et al.*, 2005; Ma *et al.*, 2007), but is not a target of nucleus incertus projections (Goto *et al.*, 2001; Olucha-Bordonau *et al.*, 2003). Thus, retrograde neural tract-tracing studies identified that a large population of relaxin-3 neurons in the periaqueductal grey innervate the IGL (Blasiak *et al.*, 2013). Furthermore, *in vitro* electrophysiological studies of these neurons revealed that RXFP3 activation led to excitation *or* inhibition of neurons (**Figure 3**), depending on their neurochemical nature; and suggesting that the actions of relaxin-3/RXFP3 signalling can be bidirectional/opposing within different neural circuits (Blasiak *et al.*, 2013).

Other findings that support a putative involvement of relaxin-3/RXFP3 in arousal arise from studies of the nucleus incertus, which has been described as a 'key GABAergic projection hub for the regulation of cortical arousal' (Brown and McKenna, 2015). Consistent with this hypothesis, our laboratory has recently demonstrated that chemogenetic activation of the nucleus incertus network in rats led to long-lasting wakefulness, and enhanced EEG measures of cortical arousal/desynchronisation that was independent of movement; *and* enhanced vigilance in response to impending threat (Ma *et al.*, 2016). Similarly, unilateral electrical stimulation of the nucleus incertus induced forward locomotion and rotation, accompanied by an increase in movement velocity (Farooq *et al.*, 2016). In both studies, it is suggested that the promotion of arousal and movement may be via the septohippocampal system, as glutamatergic neuron activation in the medial septum controls the initiation and velocity and

locomotion, and associated entrainment of hippocampal theta oscillations (Fuhrmann *et al.*, 2015; Robinson *et al.*, 2016). Furthermore, the septohippocampal system also underlies anxiety-related hippocampal theta rhythm (Wells *et al.*, 2013). Thus, further studies examining the impact of nucleus incertus (and relaxin-3) neurons in modulating stress-associated arousal and related behaviours, will be of immense interest.

Learning, memory and hippocampal theta rhythm

Neural substrates underlying learning and memory chiefly reside in the hippocampus and associated brain regions that regulate its activity, particularly a distinct activity known as hippocampal theta rhythm, which are distinct oscillations at theta frequency (4-12 Hz) that reflects mnemonic processing (Vertes, 2005). Theta rhythm is detectable in the electroencephalogram (EEG) recording of brain activity in many mammals, and the temporal aspects and behavioural correlations of these detected brain rhythms are highly conserved (Buzsaki *et al.*, 2013). In addition to memory, hippocampal theta rhythm has also been associated with arousal states, exploratory behaviour and spatial navigation, rapid eye movement (REM) sleep, and in anxiety-related behaviours (Vertes, 1984; McNaughton and Gray, 2000; Vertes, 2005; Stujenske *et al.*, 2014).

The 'septohippocampal system' is an important regulator of hippocampal theta rhythm, whereby GABAergic and cholinergic neurons located in the medial septum function as "pacemakers" for the genesis and pacing of hippocampal theta rhythm (Vertes and Kocsis, 1997; Simon *et al.*, 2006; Hangya *et al.*, 2009). Both septum and hippocampus receive a dense relaxin-3 innervation, and relaxin-3-positive nerve fibres make close contacts (putative synapses) with various types of pacemaker cells, including choline acetyltransferase (ChAT-), and inhibitory GAD67-positive neurons, and those containing the calcium-binding proteins parvalbumin, calbindin and calretinin (Olucha-Bordonau *et al.*, 2012). In addition, medial septum calretinin-positive neurons project to the nucleus incertus (Sanchez-Perez *et al.*, 2015), forming a closed-loop neural circuit, although the function of this bidirectional feedback is still unknown. The effects of relaxin-3 on cognitive performance and EEG markers of septohippocampal activity have been investigated in rats, whereby the RXFP3-

selective agonist, R3/I5, or antagonist, R3(BΔ23-27)R/I5, were locally infused into the medial septum. Infusion of RXFP3 agonist significantly enhanced, whereas the antagonist attenuated hippocampal theta power in freely-moving rats, and impaired spatial working memory performance in a spontaneous alternation task (Ma *et al.*, 2009a).

In electrophysiological studies in anesthetized rats, hippocampal theta oscillations were induced by electrical stimulation of the nucleus incertus (Nunez *et al.*, 2006). In contrast, brainstem-induced hippocampal theta rhythm was blocked by electrolytic lesion of, or muscimol injection into, the nucleus incertus (Nunez *et al.*, 2006), suggesting it may act as a key relay node between brainstem and forebrain theta-pacing regions (Brown and McKenna, 2015). Notably in this regard, nucleus incertus relaxin-3 neurons exhibit spontaneous firing activity that is coherent with the early ascending phase of theta oscillations (while other neurons do not), further supporting the proposed functional link (Ma *et al.*, 2013).

Emotional and anxiety-like behaviour

Dysfunction in neural circuits controlling emotional behaviour underlies disorders such as anxiety, depression and related psychiatric illnesses. In addition to broad modulatory effects on cognition and arousal, which have interrelated importance for affective behaviour, RXFP3 is also densely expressed in regions critical for emotional control, such as the amygdala, ventral hippocampus, bed nucleus of the stria terminalis (BNST) and prefrontal cortex (see Smith *et al.*, 2014b for review). A key transmitter that is an established regulator of anxiety states and anxiety-related behaviour is serotonin, and the dorsal raphe nucleus is a major source of this monoamine (Hale *et al.*, 2012). Early studies in rats demonstrated that most relaxin-3 neurons of the nucleus incertus co-express the inhibitory serotonin-1A receptor and depletion of serotonin by pharmacological inhibition of tryptophan hydroxylase, resulted in increased expression of relaxin-3, suggesting that serotonin normally suppresses relaxin-3 expression (Miyamoto *et al.*, 2008). More recent studies revealed that treatment of rats with the anxiogenic benzodiazepine, FG-7142, resulted in enhanced anxiety-like behaviour in the elevated plus maze that was associated with activated populations of relaxin-3 neurons in the nucleus incertus and serotonergic neurons in the dorsal raphe (Lawther *et al.*, 2015). Such co-

activation of serotonergic and relaxin-3 systems suggests a functional association between these signalling systems that warrants further investigation.

Indeed, previous studies demonstrated that icv administration of relaxin-3 (Nakazawa *et al.*, 2013) or the RXFP3-selective agonist, RXFP3-A2 (Ryan *et al.*, 2013a), resulted in anxiolytic and antidepressant-like behavioural effects in rats, although in studies in which relaxin-3 mRNA knockdown was achieved by viral driven expression of relaxin-3 microRNA in nucleus incertus of rats, no overt changes in measures of anxiety-like behaviour were observed in the light-dark box (Callander *et al.*, 2012). However, because relaxin-3 neurons are highly stress-responsive, such a behavioural change may have been better observed if pre-stressed rats were studied. Administration of typical (chlorpromazine and fluphenazine) and atypical (clozapine) antipsychotic drugs to rats activates nucleus incertus neurons, suggesting that nucleus incertus relaxin-3 neurons are directly responsive to antipsychotic drugs of various modes of action (Rajkumar *et al.*, 2013).

Novel technologies to investigate relaxin-3/RXFP3 function *in vivo*

The recent boom in the use of viral vector technology for the dissection of complex neural circuits underlying physiology and behaviour (Schaffer *et al.*, 2008), has revolutionised our understanding of how the brain works. Gene delivery technology, coupled with optogenetic and chemogenetic methods, now allows researchers to investigate and dissect complex neural circuit neuroanatomy and neurophysiology (Wulff and Wisden, 2005; Betley and Sternson, 2011; Deisseroth, 2015; Roth, 2016), and furthermore, gene therapy is currently being assessed for clinical applications related to CNS treatments (Ojala *et al.*, 2015). To date, there have been limited studies using these technologies to investigate the relaxin-3/RXFP3 system, but viral vectors *have* been used to determine physiological effects of relaxin-3 mRNA knockdown in the nucleus incertus (Callander *et al.*, 2012), and effects of chronic local secretion of a selective RXFP3 agonist peptide in hypothalamus (Ganella *et al.*, 2013a) on feeding and body weight regulation. The effect of chemogenetic activation of the nucleus incertus on cortical and behavioural arousal (as reflected by EEG and locomotor activity changes) has also been explored (Ma *et al.*, 2016).

Future applications of optogenetic and chemogenetics methods to study the role of relaxin-3 and RXFP3-regulated neurons should be greatly facilitated by the development of tools such as viral vectors driven by a cell-specific promoter to regulate relaxin-3 neurons and/or a relaxin-3-Cre or RXFP3-Cre transgenic mouse/rat, which would allow discrete functional manipulations of relaxin-3 neurons and their specific target neurons (Madisen *et al.*, 2015). Furthermore, such technology could also address the importance of relaxin-3 *and* GABA co-transmission in brain, in studies similar to those used to evaluate histaminergic and GABA co-transmission in controlling wakefulness (Yu *et al.*, 2015).

In light of growing evidence the nucleus incertus is a heterogeneous population of relaxin-3 positive and negative neurons that co-express a range of inhibitory neuron markers and other neuropeptides (Ma *et al.*, 2013), viral-based methods could be used to map the efferent and afferent connections of relaxin-3 neurons, which would complement and advance current mappings of the 'whole' nucleus incertus (Goto *et al.*, 2001; Olucha-Bordonau *et al.*, 2003). The connectivity of the populations of relaxin-3 neurons in the pontine raphé nucleus, periaqueductal grey, and dorsal substantia nigra could also be characterised.

Relaxin-3/RXFP3 related transgenic mouse strains

Although 'whole-body/whole-of-life' *Rln3* and *Rxfp3* gene knockout mouse strains have been useful tools for exploring relaxin-3/RXFP3 biology (Watanabe *et al.*, 2011; Smith *et al.*, 2012; Hosken *et al.*, 2015), they potentially undergo developmental compensatory adaptations in their behaviour and brain chemistry. For example, differences in the consumption of palatable food (Smith *et al.*, 2014a) and salt appetite (Smith *et al.*, 2015) were detected in wildtype mice following acute injection of the RXFP3 antagonist, R3(B1-22)R, compared to vehicle, but there were no differences in these behaviours between *Rxfp3* gene knockout and wildtype mice. Therefore, anticipated future studies that utilise conditional *Rxfp3* gene knockout mice, which might combine the use of 'floxed *Rxfp3*' mice with viral vector-induced expression of Cre recombinase to produce local receptor deletion, will be important, not only to avoid developmental compensation (i.e. provide temporal control), but also to

allow chronic *Rxfp3* gene depletion within one or more target region(s) of the brain (i.e. spatial control). Transgenic mice that express a fluorophore within RXFP3-positive neurons would be of benefit for histological *and* electrophysiological studies, as a fully-validated RXFP3 antibody is not currently available. Indeed, studies using commercially-available RXFP3 antibodies have been conducted (Meadows and Byrnes, 2015; Albert-Gasco *et al.*, 2016; Lee *et al.*, 2016), although these antibodies have not yet been tested in *Rxfp3* gene knockout mice, which will be an important validation of specificity. Finally, transgenic mice that express Cre recombinase within relaxin-3- or RXFP3-positive neurons would be invaluable for facilitating viral-vector optogenetic or DREADD approaches to selectively activate or inhibit target neuron populations within conscious, freely-behaving mice, as this approach has been widely adopted to study neurons of a particular neurochemical phenotype (see e.g. Krashes *et al.*, 2014; Fuzesi *et al.*, 2016).

Conclusions and Future Perspectives

In light of demonstrated anatomical and/or functional interactions between relaxin-3 and multiple transmitter and neuropeptide systems (i.e. serotonin, dopamine, CRF and orexin); evidence for a role for relaxin-3/RXFP3 signalling in arousal, motivation and cognition, particularly in response to stress; and a range of additional putative such interactions and functions, research on relaxin-3/RXFP3 neurobiology should flourish in the future, both basic investigations and in relation to human neuropathology and the system's plasticity in animal models of psychiatric illness, metabolic/feeding disorders and neurodegenerative disease. For example, growing evidence for the impact of stress and CRF in the aetiology of neurodegenerative disorders such as Alzheimer's disease (Campbell *et al.*, 2015; Park *et al.*, 2015; Zhang *et al.*, 2016), and the involvement of serotonin, orexin and other arousal networks in normal and abnormal cognitive processing and in the expression of comorbid symptoms of sleep dysregulation, anxiety and depression in multiple disorders (Chen *et al.*, 2015; Kohler *et al.*, 2016), suggest there are exciting opportunities to examine the importance/involvement and/or therapeutic potential of relaxin-3/RXFP3 signalling for the treatment of cognitive, affective and mood deficits and/or neurological disease progression in

a range of clinical conditions or their validated experimental models (Smith *et al.*, 2014b; see Kumar *et al*. 2016).

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Conflict of Interest

The authors have no conflict of interest to declare.

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Figures

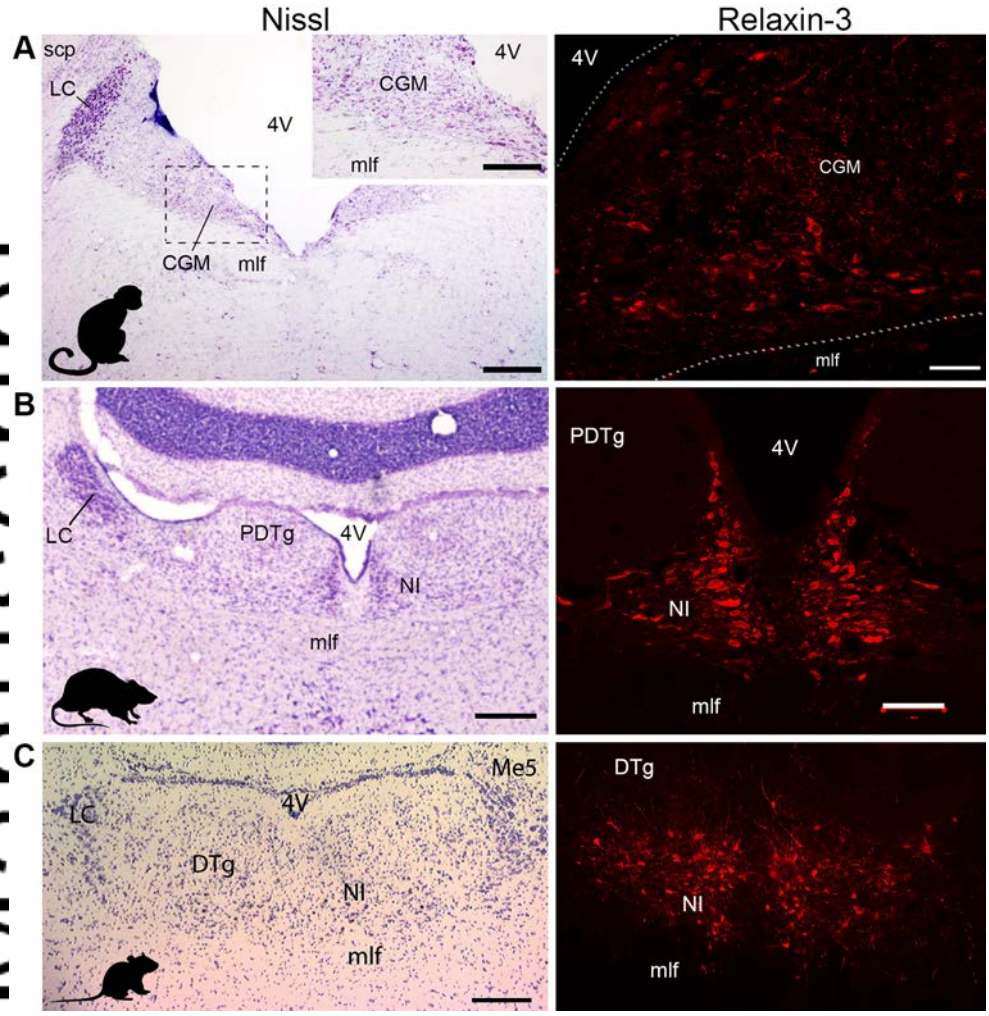


Figure 1. The nucleus incertus and its relaxin-3 neurons are similarly located in the midline periventricular central grey of non-human primate (macaque), rat and mouse brains and conserved across other species (adapted from Ma *et al.*, 2007; 2009b; Smith *et al.*, 2010). Nucleus incertus (NI) is the primary source of neurons expressing relaxin-3 mRNA and abundant relaxin-3 immunoreactivity and are located in the midline periventricular central grey at the base of the fourth ventricle (4V) of (A) macaque, (B) rat and (C) mouse. Abbreviations: CGM, mid central gray; DTg, dorsal tegmental nucleus; LC, locus coeruleus; Me5, mesencephalic trigeminal nucleus; mlf, medial longitudinal fasciculus; PDTg,

posterodorsal tegmental nucleus; scp, superior cerebellar peduncle. Scale bars, (A) Nissl, 0.6 mm, inset, 80 µm, relaxin-3, 0.2 mm; (B) Nissl, 0.3 mm, relaxin-3, 0.1 mm; (C) 0.2 mm.

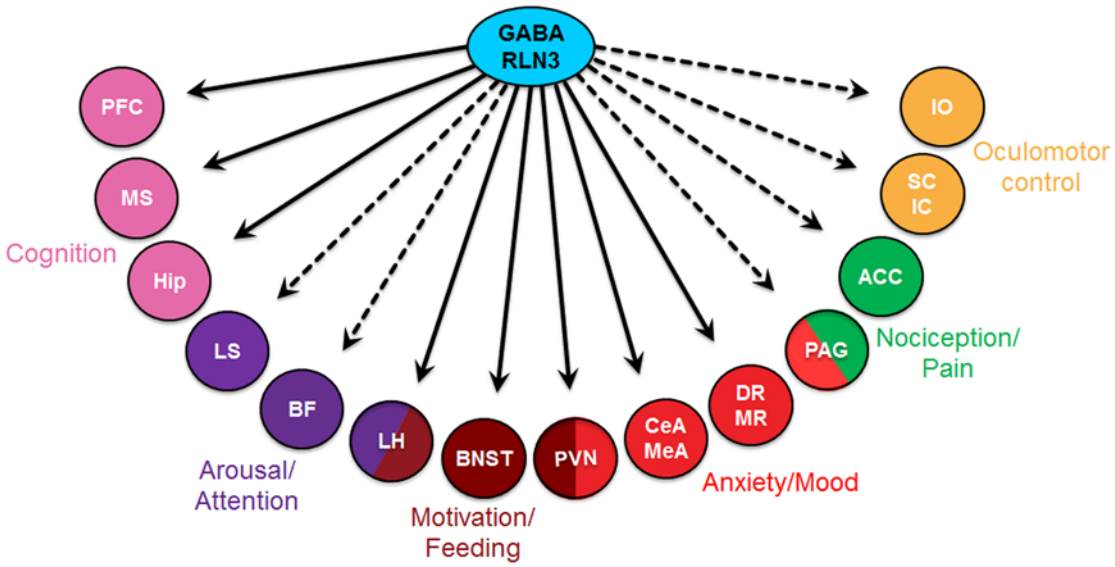


Figure 2. Schematic illustration of some of the major downstream neural targets of relaxin-3 neurons and the likely, 'tested' (solid lines) or putative, untested (dotted lines) functional roles of relaxin-3/RXFP3 signalling in the coordinated regulation of modalities including cognition, arousal, motivation, anxiety, mood, pain and oculomotor control. Abbreviations: ACC, anterior cingulate cortex; BF, basal forebrain; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; DR, dorsal raphe; Hip, hippocampus; IC, inferior colliculus; IO, inferior olive; LH, lateral hypothalamus; LS, lateral septum; MeA, medial amygdala; MR, median raphe; MS, medial septum; PAG, periaqueductal gray; PFC, prefrontal cortex; PVN, paraventricular hypothalamic nucleus; SC, superior colliculus.

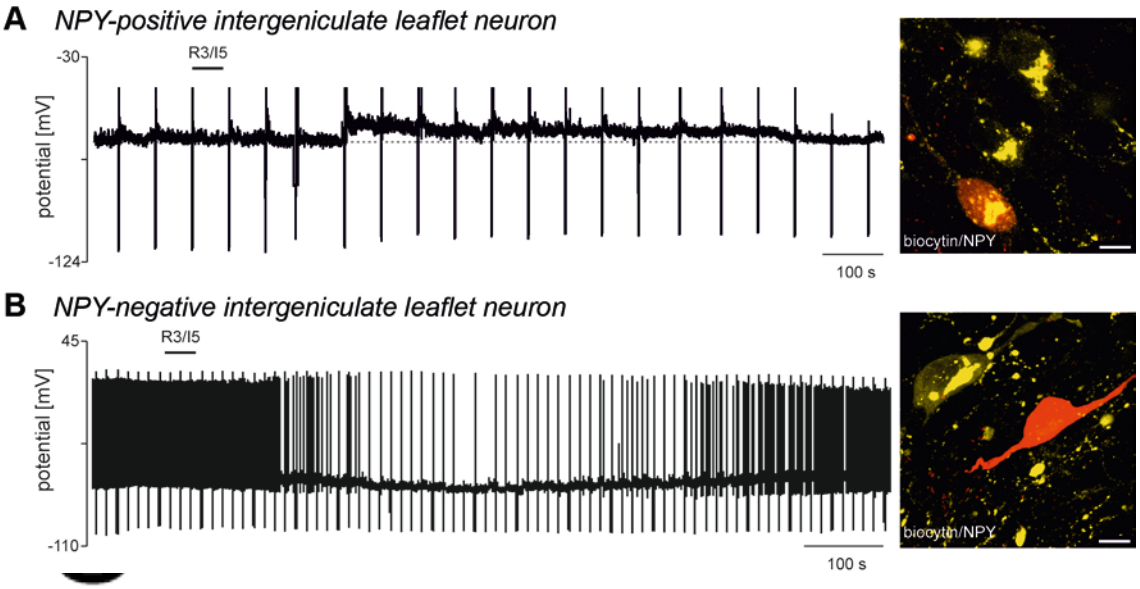


Figure 3. RXFP3 activation excites or inhibits intergeniculate leaflet neurons, depending on their neurochemical nature (adapted from Blasiak *et al.*, 2013). (A) A zero current-clamp recording illustrating the depolarising effect of bath-applied RXFP3 agonist, R3/I5 (100 nM, horizontal bar). Upwards deflections represent truncated action potentials present on top of calcium spikes evoked by membrane potential recovery from hyperpolarisation induced by current injection (downward deflections), and a confocal projection image of the neuron depolarised by R3/I5 stained for biocytin injected into the neuron (red) and neuropeptide Y (NPY) immunoreactivity (yellow) revealing the NPY nature of the recorded neuron. Scale bar, 10 µm. (B) A zero current-clamp recording illustrating the hyperpolarising effect of bath-applied R3/I5 (100 nM, horizontal bar) on the membrane potential and firing properties of another intergeniculate leaflet neuron, and a confocal projection image of the neuron hyperpolarised by R3/I5 stained for biocytin (red) and NPY immunoreactivity (yellow) revealing the NPY-negative nature of the recorded neuron. Scale bar 10 µm.

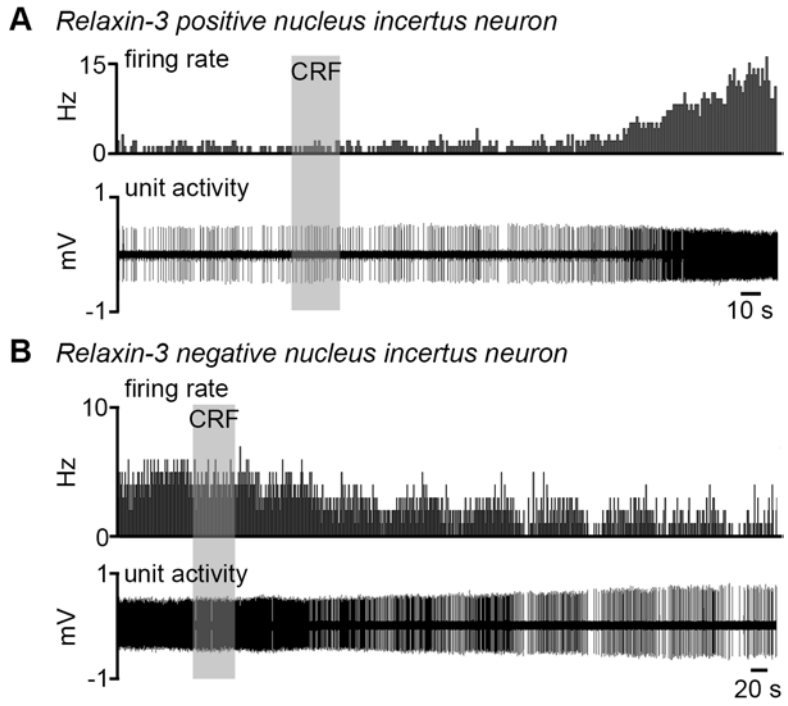


Figure 4. Differential response of different types of nucleus incertus neurons to CRF *in vivo* (adapted from Ma *et al.*, 2013). Extracellular recording and juxtacellular-filling of nucleus incertus neurons in rat revealed that (A) relaxin-3 neurons increased firing in response to icv administration of CRF, whereas (B) some non-relaxin-3 neurons exhibited decreased firing in response to CRF, suggesting specific but complex responses to the stress hormone.

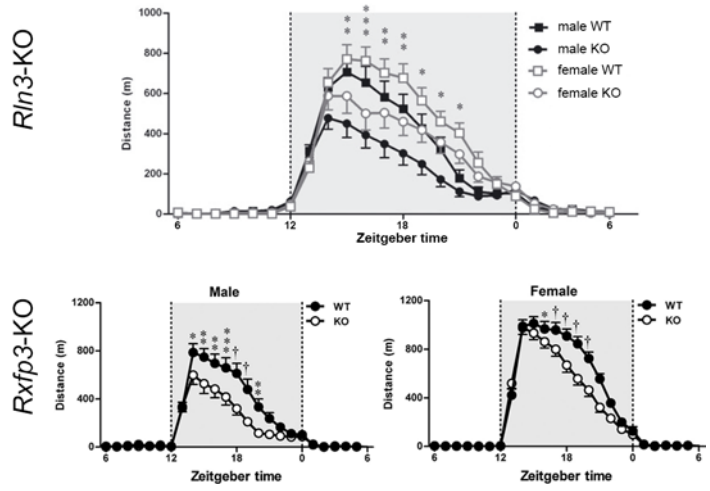
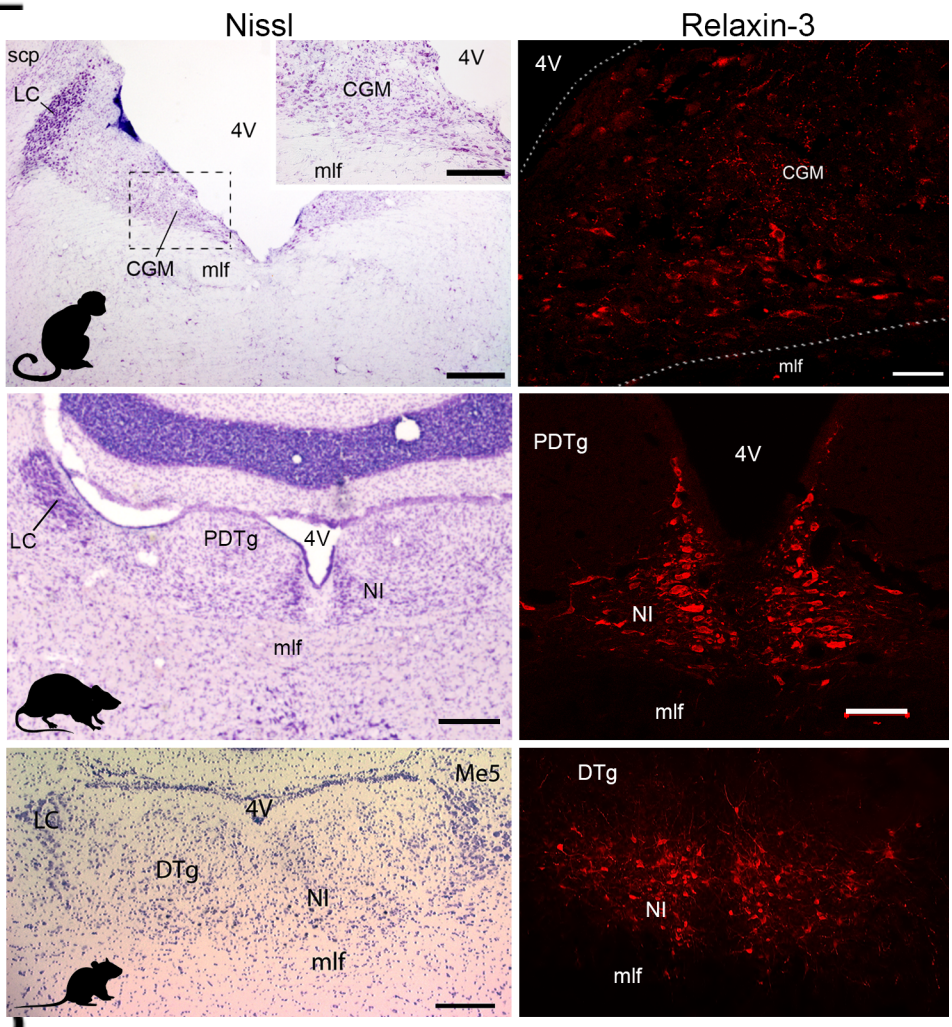


Figure 3. Whole-of-life' deletion (knockout) of the relaxin-3 or the Rxfp3 gene results in circadian hypoactivity in adult mice (adapted from Smith *et al.*, 2012; Hosken *et al.*, 2015). The distance travelled on home-cage voluntary running wheels by male and female *Rln3* (Smith *et al.*, 2012) and *Rxfp3* (Hosken *et al.*, 2015) gene knockout mice is markedly less than their wildtype littermates, possibly reflecting a reduced level of sustained attention or motivation. Distance is shown as cm/hour during an average 24 hour day. Gray shading indicates the dark phase.



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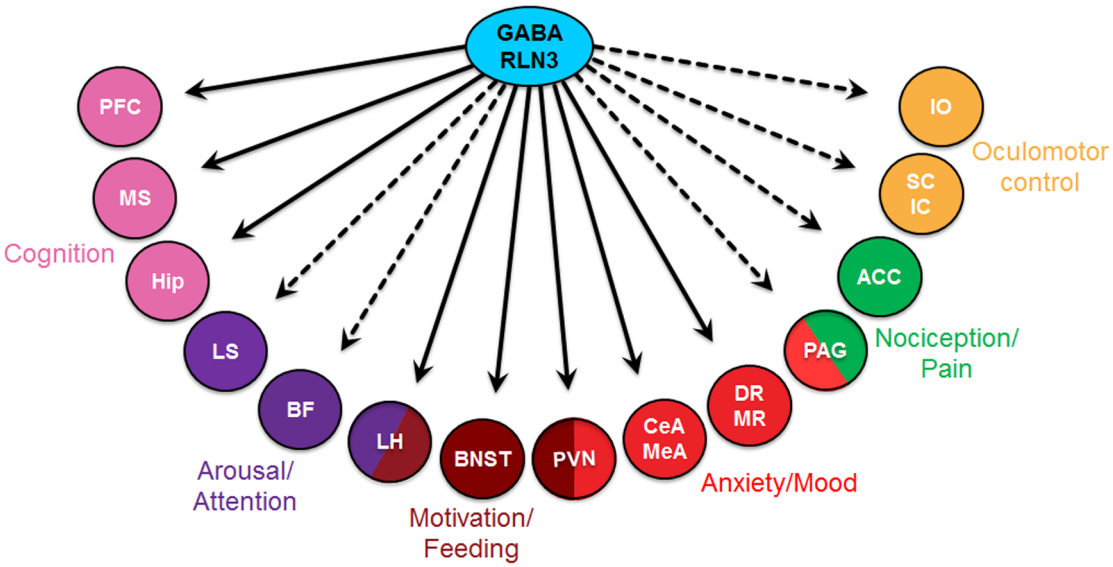

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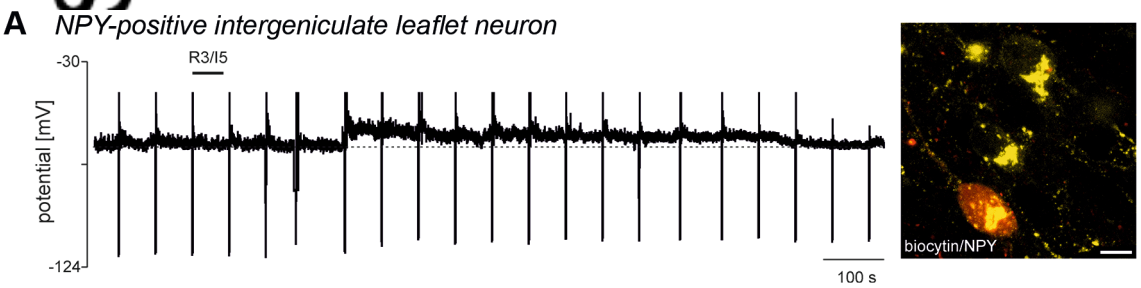

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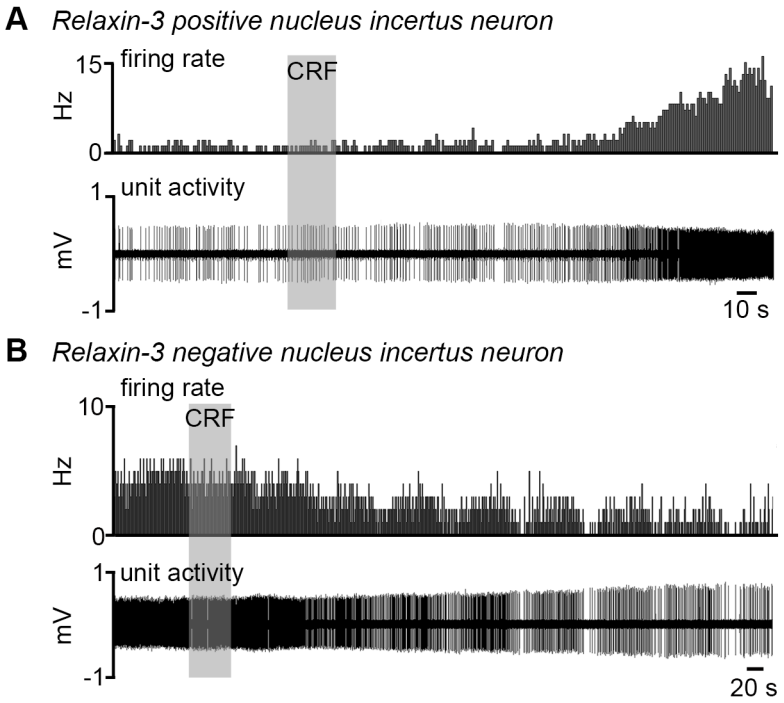

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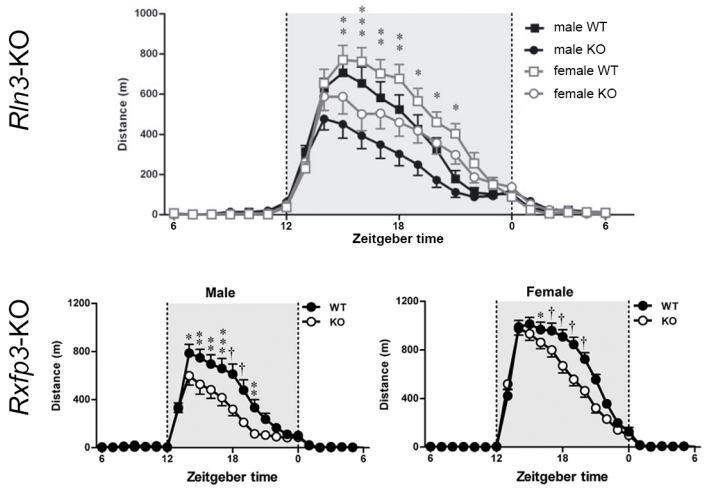

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