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Themed Section: Recent Progress in the Understanding of Relaxin Family Peptides and their Receptors

REVIEW ARTICLE

Relaxin' the brain: a case for targeting the nucleus incertus network and relaxin-3/RXFP3 system in neuropsychiatric disorders

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Relaxin-3 has been proposed to modulate emotional–behavioural functions such as arousal and behavioural activation, appetite regulation, stress responses, anxiety, memory, sleep and circadian rhythm. The nucleus incertus (NI), in the midline tegmentum close to the fourth ventricle, projects widely throughout the brain and is the primary site of relaxin-3 neurons. Over recent years, a number of preclinical studies have explored the function of the NI and relaxin-3 signalling, including reports of mRNA or peptide expression changes in the NI in response to behavioural or pharmacological manipulations, effects of lesions or electrical or pharmacological manipulations of the NI, effects of central microinfusions of relaxin-3 or related agonist or antagonist ligands on physiology and behaviour, and the impact of relaxin-3 gene deletion or knockdown. Although these individual studies reveal facets of the likely functional relevance of the NI and relaxin-3 systems for human physiology and behaviour, the differences observed in responses between species (e.g. rat vs. mouse), the clearly identified heterogeneity of NI neurons and procedural differences between laboratories are some of the factors that have prevented a precise understanding of their function. This review aims to draw attention to the current preclinical evidence available that suggests the relevance of the NI/relaxin-3 system to the pathology and/or symptoms of certain neuropsychiatric disorders and to provide cognizant directions for future research to effectively and efficiently uncover its therapeutic potential.

LINKED ARTICLES

This article is part of a themed section on Recent Progress in the Understanding of Relaxin Family Peptides and their Receptors. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.10/issuetoc

Abbreviations

AD, Alzheimer's disease; CRF, corticotrophin-releasing factor; EPM, elevated plus maze; HC, hippocampal; HP-mPFC, hippocampo-medial prefrontal cortical; MRN, median raphe nucleus; NI, nucleus incertus; PVN, paraventricular nucleus of hypothalamus; RPO, reticularis pontis oralis; SHS, septohippocampal system

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Tables of Links

TARGETS	
GPCRs[a]	PAC_1 receptor
$5\text{-}HT_{1A}$ receptor	RXFP3 receptor
CRF_1 receptor	V_{1A} receptor
CRF_2 receptor	Y_5 receptor
Dopamine D_2 receptor	**Nuclear hormone receptors**[b]
Dopamine D_3 receptor	Glucocorticoid receptor, NR3C1
Neuropeptide S receptor	

LIGANDS	
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Antalarmin	Orexin-A
cAMP	Orexin-B
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β-Endorphin	Relaxin-3
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GABA	Substance P
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Insulin	

These Tables 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 ([a,b]Alexander *et al.,* 2015a,b).

Introduction

The nucleus incertus (NI)/relaxin-3 system, highly conserved across species, has been studied for over a decade (Goto *et al.,* 2001; Bathgate *et al.,* 2002). The NI is heterogeneous, with anatomically distinct compacta and dissipata regions differing in electrophysiological properties (Nunez *et al.,* 2006; Ma *et al.,* 2013; Martinez-Bellver *et al.,* 2015) and expression of neuropeptides, neurotransmitters and receptors (Sutton *et al.,* 2004; Tanaka *et al.,* 2005; Miyamoto *et al.,* 2008; Ryan *et al.,* 2011; Kumar *et al.,* 2015) including the neuropeptide, relaxin-3, which participates in a variety of neurophysiological functions (see Ma *et al.*, (in press)).

The NI/relaxin-3 system in the rat robustly responds to stressors suggesting that it will be up-regulated and potentially dysfunctional in disease states associated with excessive stress. Because neuropeptides are preferentially released under conditions of high neural tone which are more likely to be present in pathological states, neuropeptide receptor antagonists are likely to produce specific and effective actions to correct pathological disequilibrium. In addition, due to the neuromodulatory nature of neuropeptides, interfering with neuropeptide signalling is less likely to have overt, strong side effects than manipulations of classical neurotransmitter systems (Hokfelt *et al.,* 2000). Similarly, we argue that studying a potentially dysregulated relaxin-3 system in preclinical disease models could yield significant insights into its functional roles and simultaneously identify the therapeutic potential of inhibiting or enhancing relaxin-3-related signalling.

There is an exigent need to examine whether the acquired knowledge of this neuropeptide system (Ryan *et al.,* 2011; Smith *et al.,* 2011, 2014; Ma and Gundlach, 2015) can be translated from *bench to bedside*. However, the few human studies on the NI/relaxin-3 system expose a disparity between the animal and human systems and reveal a clear need for more cognizant future studies in this field. Of course, caution will be required when extrapolating preclinical findings to design of human studies, but use of animal models of human diseases and disorders is still indispensable to drug discovery (Markou *et al.,* 2009; Nestler and Hyman, 2010; Baldarelli, 2012). This review will briefly summarize findings linking the NI/relaxin-3 system to neuropsychiatric conditions such as anxiety, depression, disorders related to appetite, stress and cognitive dysfunction (for more detailed reviews see Ryan *et al.,* 2011; Smith *et al.,* 2011; Ma and Gundlach, 2015) and will focus on providing a 'road-map' for future research in preclinical disease models and fundamental clinical studies.

Stress

In humans, dysregulation of adaptive mechanisms with inappropriate and/or exaggerated responses to psychological stressors manifests as a disorder in itself and/or as a cluster of symptoms co-morbid with other psychiatric conditions such as anxiety and depression (American Psychiatric Association, 2013; Gold, 2015). Preclinical studies that have successfully modelled mechanisms, such as plasticity and cognitive dysfunction related to stress, have furthered our understanding of the neurobiology of stress and related disorders and provided screening methods to identify new candidate drugs for treating these disorders (Kormos and Gaszner, 2013; Bock *et al.,* 2015; Chattarji *et al.,* 2015; Constantinof *et al.,* 2015; Musazzi and Marrocco, 2016). Among the multitude of neuropeptides implicated in stress responses, the role of corticotropin-releasing factor (CRF) is central (Aubry, 2013; Kormos and Gaszner, 2013). The conspicuous expression of the CRF_1 receptor in the NI of the rat and its high co-expression with relaxin-3 (Potter *et al.,* 1994; Chalmers *et al.,* 1995; Bittencourt and Sawchenko, 2000; Tanaka *et al.,* 2005; Ma *et al.,* 2013), not only defined the topography of the NI but also inspired early studies to evaluate its role in stress responses (Figure 1) (Rivest *et al.,* 1995; Tanaka *et al.,* 2005).

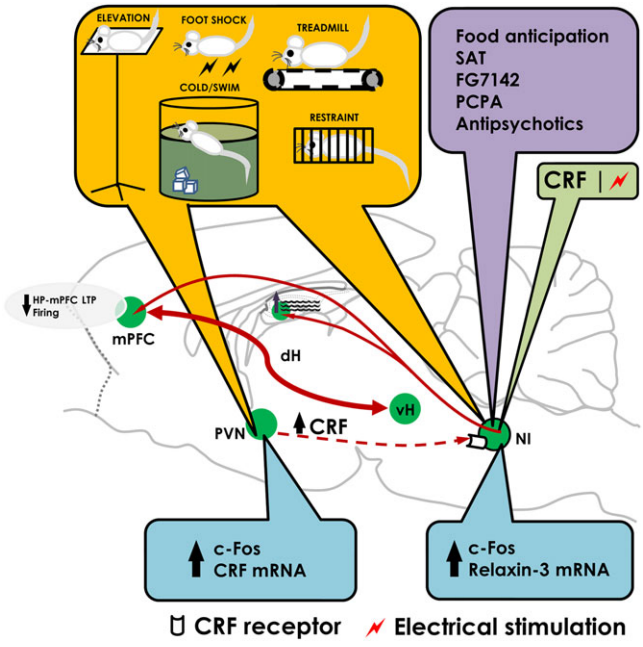

Figure 1

The nucleus incertus responds to stress and contributes to stress responses. The diagram summarises recent findings on the role of the nucleus incertus (NI) in stress. Stressors (yellow box) may directly or indirectly (activating the PVN and increasing CRF released) activate the NI as indicated by c-Fos induction or enhanced expression of relaxin-3 mRNA (blue box). Micro-infusion of CRF or electrical stimulation of NI (green box) suppressed firing of mPFC neurons and LTP in the HP-mPFC pathway. Stimulation of NI is also known to increase theta activity in the dorsal hippocampus. In addition, behavioural or pharmacological manipulations (purple box) such as food anticipation, exposure to spontaneous alternation tasks (SAT), pharmacological treatments (FG-7142, PCPA and antipsychotics) have been shown to induce c-Fos in the NI. dH: dorsal hippocampus; vH: ventral hippocampus; PCPA: para-chlorophenylalanine.

Stress induced by exposure of rodents to different behavioural stressors, exogenous CRF in the brain (presumed to simulate a stressful situation) or treatment with anxiogenic agents such as the inverse benzodiazepine agonist, FG-7142 (Lawther *et al.,* 2015), resulted in up-regulation of c-Fos in the NI as well as other areas (Senba *et al.,* 1993; Cullinan *et al.,* 1995; Bittencourt and Sawchenko, 2000; Passerin *et al.,* 2000; Dayas *et al.,* 2001; Timofeeva *et al.*, 2003; Tanaka *et al.,* 2005; Cano *et al.,* 2008; Rajkumar *et al.,* 2016), and in some cases, increased relaxin-3 expression (Banerjee *et al.,* 2010; Lenglos *et al.,* 2013; Lenglos *et al.,* 2014), which was reversible by pretreatment with a CRF_1 antagonist (Banerjee *et al.,* 2010) (Figure 1). Human relaxin-3 could have a direct stimulatory effect on CRF neurons as suggested by *in vivo* findings, in which i.c.v. infusion of human relaxin-3-augmented plasma corticosterone levels, increased plasma ACTH and prolactin and induced c-Fos and CRF mRNA in the paraventricular nucleus of the hypothalamus (PVN) – a structure with a high density of the relaxin family peptide receptor RXFP3 (Figure 2) (Ma *et al.,* 2007; Watanabe *et al.,* 2011a; McGowan *et al.,* 2014).

We showed that CRF infusion into, or electrical stimulation of, the NI, to simulate a stressful situation or a condition with high neural activity tone, affected plasticity as measured by LTP in the hippocampo-medial prefrontal cortical (HP-mPFC) pathway (Farooq *et al.,* 2013). Infusion of the CRF_1 receptor antagonist antalarmin into the NI, prior to or after exposure of rats to elevation stress, reversed the elevation stress-induced suppression of LTP in the HP-mPFC pathway (Rajkumar *et al.,* 2016). Together, these results demonstrate that the NI-HP-mPFC is a stress-responsive circuit and the NI, especially via CRF_1 receptor activation, contributes to stress-induced impairment in neuronal plasticity that is likely to have implications in stress-related cognitive dysfunction.

Behavioural activation

The NI innervates several areas that are involved in regulating levels of behavioural activity such as the reticularis pontis oralis (RPO), median raphe nucleus (MRN), interpeduncular nuclei and the lateral preoptic area (Goto *et al.,* 2001). During stress and food anticipatory activity, NI activation is positively correlated with high behavioural activity leading to the hypothesis that the NI, together with the interpeduncular and median raphe nuclei, may function to control behavioural activation. Indeed, this circuit may be part of the behavioural inhibition system (McNaughton and Gray, 2000) centred on the septohippocampal system (SHS). This theory is based on the findings that all anxiolytics (typical and novel) increase the threshold of septal driving of hippocampal (HC) theta waves, reduce RPO-induced theta waves and produce similar behavioural responses to lesions of the SHS. According to this theory, the hippocampus functions as a comparator, determining when expected outcomes do not meet actual outcomes and produces an output affecting attention, arousal and behavioural inhibition essentially giving rise to anxiety (McNaughton and Gray, 2000;

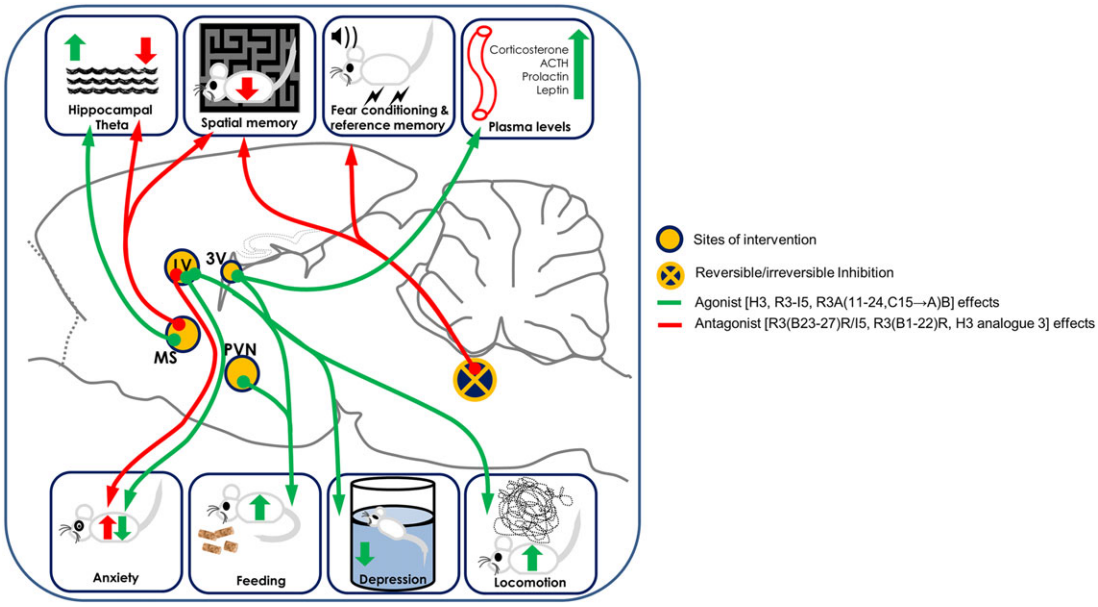

Figure 2

Pharmacological effects of the central administration of RXFP3 receptor ligands and inhibition of the NI. Agonist effects are shown in green and antagonist effects are shown in red. Infusion of relaxin-3 or RXFP3 receptor agonists in the lateral ventricle (LV) causes increased feeding and locomotion, and decreased anxiety and depressive-like behaviour. Infusion of relaxin-3 into hypothalamic centres, especially the PVN, increased feeding behaviour. Infusion of relaxin-3 into the third ventricle (3V) increased both feeding behaviour and plasma levels of corticosterone, ACTH, prolactin and leptin. Infusion of RXFP3 receptor antagonist in the medial septum (MS) impaired spatial memory and decreased hippocampal theta activity, whereas infusion of agonist in the MS increased hippocampal theta activity. Finally, reversible inhibition (by lidocaine) of the NI causes impairment in spatial and reference memory and irreversible inhibition (by CRF-saporin or electrolytic lesions) of the NI causes derangements in fear conditioning.

McNaughton and Corr, 2004). Studies to date indicate that the main inputs to the hippocampus are the septal pacemaker neurons, pedunculopontine tegmental nucleus, amygdala, superior colliculus and substantia nigra, which provide input about signals of non-reward, punishment, unconditioned or conditioned fear stimuli. As direct connections between the RPO and SHS are scarce (Vertes and Martin, 1988; Nunez *et al.*, 1991), the RPO is thought to provide ascending control via the supramammillary nucleus during tasks such as the fixed interval schedule (McNaughton and Gray, 2000); and it is thought that the NI mediates the RPO influence on the SHS during tasks such as exploration, as inhibition of the NI abolished RPO stimulation-induced HC theta waves (Nunez *et al.*, 2006). The NI perhaps modulates anxiety by altering the input to the SHS and contributes to the stress-responsive nature of this circuit (see Ma *et al.*, (in press)).

Studies from our laboratories revealed that the NI/relaxin-3 system may subserve behavioural activation. Infusion of a dopamine D_2/D_3 receptor agonist, quinpirole, into the NI consistently reduced locomotion in different behavioural paradigms (Kumar *et al.*, 2015) demonstrating that activation of D_2 receptors in the NI, that are highly co-expressed with relaxin-3 and CRF_1 receptors, could modulate behavioural activity. Additionally, the role of D_2 receptors may be modulated by stress as activation in a high, but not a low stress environment reduced feeding (Kumar *et al.*, 2015). Furthermore, we observed a causal role for the NI in locomotion and behavioural activation in rats by employing high frequency electrical microstimulation of the NI via chronically implanted microelectrodes (Farooq *et al.*, 2016). Microstimulation of the NI was sufficient to induce robust forward locomotion, rotational behaviour and behavioural activity. The latency of this effect indicated that the NI most likely induced locomotion via modulation of premotor areas rather than directly affecting motor cortices (Farooq *et al.*, 2016). HC theta is strongly linked to locomotion in behaving animals and to processing of spatially-related sensory input (Vanderwolf, 1969; Whishaw and Vanderwolf, 1973). Structures involved in modulating HC theta such as the RPO, supramammillary nucleus and raphe nuclei similarly, upon stimulation, evoke or inhibit locomotion accompanied by increased or decreased HC theta activity. It will therefore be important to concurrently measure HC theta activity and behavioural activation while stimulating the NI. A recent study using designer receptors exclusively activated by designer drugs (DREADDs) to selectively activate NI neurons caused cortical desynchronization, heightened arousal and increased locomotion as well as elevated vigilance behaviours in fear conditioning (Ma *et al.*, 2016). These complementary results indicate that the NI/relaxin-3 system is involved in stress-related modulation of behavioural activity levels, a key feature underlying several functions dysregulated in various neuropsychiatric disorders and manifesting as 'lethargy' (Czeh *et al.*, 2016).

Anxiety

Anxiety is a sustained state of vigilance marked by increased arousal and behavioural inhibition (Davis *et al.*, 2010).

Preclinical research on the mechanisms underlying anxiety places most emphasis on the role of the amygdala, bed nucleus of stria terminalis (BNST), ventral hippocampus (vHC) and prefrontal cortex (PFC) – all of which have moderate to strong connections with the NI (Goto *et al.,* 2001; Olucha-Bordonau *et al.,* 2003; Santos *et al.,* 2016).

Central infusions of RXFP3 receptor ligands suggest that relaxin-3 signalling has an anxiolytic function physiologically (Figure 2) (Nakazawa *et al.,* 2013; Ryan *et al.,* 2013a) (see Ma *et al.*, (in press)). Relaxin-3 signalling has been shown to alter oxytocin levels in the hypothalamus (Ganella *et al.,* 2013; Nakazawa *et al.,* 2013). Oxytocin is a likely effector of relaxin-3/RXFP3 receptor signalling based on the robust expression of these receptors in the hypothalamic nuclei synthesizing oxytocin, the PVN and supraoptic nucleus and the well-researched role of oxytocin in modulating behavioural responses, such as anxiety, fear, social behaviour and stress responses (McCarthy *et al.,* 1996; Carter, 2003; Slattery and Neumann, 2010; Viero *et al.,* 2010) and neuropsychiatric diseases like autism and obsessive–compulsive disorder (Leckman *et al.,* 1994; Hollander *et al.,* 2003; Hollander *et al.,* 2007).

Selective ablation of NI cells by CRF-saporin administration (Lee *et al.,* 2014) and electrolytic lesions caused deficits in fear conditioning, particularly in the extinction of conditioned fear, with no effect on fear acquisition or retrieval of extinction memory (Pereira *et al.,* 2013). Extinction of learned fear features in post-traumatic stress disorder, panic disorder and phobias and is impaired in rats bred for high anxiety (Anderson and Insel, 2006; Pull, 2007).

Studies of neuropeptides such as CRF, neuropeptide Y (NPY), arginine vasopressin (AVP), oxytocin and substance P highlight that standard behavioural tests are designed with neurotransmitters in mind and may not be the most suited to detect neuropeptide-mediated changes in anxiety (Hokfelt *et al.,* 2000; Rotzinger *et al.,* 2010). In many cases, it is necessary to introduce stress as an external stimulus to observe neuropeptide-induced behavioural effects (Heinrichs *et al.,* 1994). This phenomenon has been demonstrated with regard to relaxin-3/RXFP3 receptor signalling, whereby i.c.v. administration of RXFP3 agonist did not alter basal anxiety but attenuated FG7142-induced elevated anxiety (Zhang *et al.,* 2015); suggesting the relaxin-3/RXFP3 receptor system may be activated in states of elevated anxiety. This is of therapeutic interest because, if the system is dysregulated in diseased states, modulating it may not affect baseline functioning, thus reducing side effects. Despite reports of apparent inconsistent phenotypes of relaxin-3 knockout mice in tests of innate anxiety-like behaviour (Smith *et al.,* 2009; Watanabe *et al.,* 2011a), studying how these knockout mice respond to treatments that are designed to induce stress-induced anxiety may better reveal the importance of relaxin-3 signalling.

Depression

Several neuropeptides have strong regulatory roles in the function of the hypothalamic–pituitary–adrenal (HPA) axis, which is dysfunctional in depressed patients (Rybakowski and Twardowska, 1999; Maletic *et al.,* 2007; Pariante and Lightman, 2008). CRF, substance P, NPY, AVP and oxytocin all offer novel therapeutic possibilities for treatment of depression (Catena-Dell'Osso *et al.,* 2013), and several studies suggest relaxin-3 may influence the HPA axis (McGowan *et al.,* 2008; McGowan *et al.,* 2014). Further, reduced neurogenesis in the dentate gyrus has also been implicated in depression, as patients have smaller hippocampus volumes, and neurogenesis is required for effective antidepressant action in preclinical models (Santarelli *et al.,* 2003; Small *et al.,* 2011). Additionally, maturation of new neurons may explain the time lag in antidepressant action (Malberg *et al.,* 2000). Interestingly, relaxin-3 fibres are present in the dentate gyrus, and our unpublished data indicate that neurogenesis and neuronal maturation is perturbed in an age-, sex- and septotemporal axis-dependent manner in relaxin-3 knockout mice (Dawe and Gundlach laboratories), indicating a possible involvement of relaxin-3 in regulation of adult neurogenesis in hippocampus and depression. In the forced swim test, central infusion of a RXFP3 receptor agonist reduced immobility (Ryan *et al.,* 2013a), while one strain of relaxin-3 knockout mice displayed increased immobility (Smith *et al.,* 2009), indicating that relaxin-3 has an antidepressant effect. However, in another relaxin-3 knockout strain, forced swim test behaviour was unaltered (Watanabe *et al.,* 2011b). These results highlight the pressing need for detailed behavioural phenotyping of knockout strains in a series of antidepressant assays including the tail suspension, social interaction and sucrose preference tests, as well as sleep EEG studies.

PET studies conducted in patients of major depressive disorder consistently reveal widespread reductions in binding to 5-HT_{1A} receptors in the raphe, limbic and cortical regions (Savitz and Drevets, 2013; Kohler *et al.,* 2016). In this regard, depletion of 5-HT in rats, by p-chlorophenylalanine (fenclonine) administration, nearly doubled relaxin-3 mRNA in the NI and these neurons express 5-HT_{1A} receptor-like immunoreactivity (Miyamoto *et al.,* 2008). Thus, dysregulation of 5-HT in depression may be accompanied by altered relaxin-3 tone, which could contribute to changes in sleep, appetite, mood or stress responses. Infusion of an RXFP3 receptor antagonist, both i.c.v. and intra-BNST, prevented stress-induced alcohol relapse in alcohol-preferring rats (Ryan *et al.,* 2013a) (see Ma *et al.*, (in press)), linking relaxin-3 signalling to stress and reward, perhaps similar to that in depression where uncontrollable stress usually acts as a trigger and anhedonia is a symptom. Depression is also characterized by disturbances in appetite, as discussed below.

Appetite regulation

Appetite, which is regulated by central and peripheral mechanisms (Gao and Horvath, 2007; Lenard and Berthoud, 2008; Keen-Rhinehart *et al.,* 2013), is dysregulated in various disorders such as depression, schizophrenia and anxiety, which are associated with derangements in levels of neurotransmitters (dopamine and 5-HT) and neuropeptides, including insulin, ghrelin, agouti-related protein, β-endorphin (proopiomelanocortin), orexins and NPY.

There is good evidence that relaxin-3 is an orexigenic peptide in the rat. Acute and chronic central administration of relaxin-3 or RXFP3 receptor agonists consistently induced hyperphagia, accompanied by weight gain and metabolic changes (Liu *et al.,* 2005; McGowan *et al.,* 2005; Hida *et al.,* 2006; Kuei *et al.,* 2007; Liu *et al.,* 2009; Sutton *et al.,* 2009; Shabanpoor *et al.,* 2012; Ganella *et al.,* 2013; Hossain *et al.,*

2013). This is likely to be due to actions in hypothalamic areas such as the paraventricular, supraoptic and arcuate nuclei and the anterior preoptic area, as direct peptide injections into these areas elicits a robust orexigenic effect (McGowan *et al.,* 2006; McGowan *et al.,* 2007).

Weight gain is a common side effect of antipsychotic drug treatments, and acute treatment of rats with antipsychotics produced a distinct pattern of c-Fos expression in appetite-related neuronal structures namely, arcuate nucleus, PVN and paraventricular thalamic nucleus, and in the NI, suggesting it may play a role in acute antipsychotic-induced hyperphagia (Rajkumar *et al.,* 2013). An orexigenic action of relaxin-3 in the hypothalamus thus presents a paradigm in which to explore effects of selective RXPF3 antagonists for treatment of appetite disorders, including management of antipsychotic drug-induced weight gain and hyperphagia associated with depression.

Cognition

HC theta activity is strongly associated with memory consolidation, arousal, behavioural inhibition, anxiety, sleep states, exploration and movement (Bland, 1986; Bland and Bland, 1986; Vinogradova, 1995; McNaughton and Gray, 2000; Vertes *et al.,* 2004; Vertes, 2005; Buzsaki and Moser, 2013; McNaughton *et al.,* 2013). Anatomical connections suggest that the NI can influence rat HC theta rhythm via several pathways, including bidirectional connections to (i) medial septum/vertical limb of the diagonal band [later identified to be both GABAergic (inhibitory) and glutamatergic (excitatory) in nature] (Ma *et al*., 2007; Cervera-Ferri *et al*., 2012) and hippocampus; (ii) RPO, a brainstem 'generator' of HC theta waves; (iii) habenula and interpeduncular nucleus, known to control HC theta rhythm (Valjakka *et al.,* 1998); (iv) MRN, involved in desynchronizing theta rhythm (Kinney *et al.,* 1995; Vertes and Kocsis, 1997; Viana Di Prisco *et al.,* 2002); and (v) posterior hypothalamus (Bland *et al.,* 1994; Oddie *et al.,* 1994).

The NI/relaxin-3 system has been shown to induce (Nunez *et al.,* 2006), display coherence with (Cervera-Ferri *et al.,* 2011) and fire in a phase-locked manner with (Ma *et al*., 2013) HC theta oscillations and modulate relevant behavioural functions such as spatial memory (Ma *et al.,* 2009; Albert-Gasco *et al*., 2016) (see Ma *et al*., (in press)). Interestingly, tetanic stimulation of the NI in freely-moving rats evoked robust forward locomotion at latencies consistent with its modulation of premotor areas such as the SHS and, probably, HC theta activity (Farooq *et al.,* 2016).

We have explored the contribution of NI to the plasticity in the HP-mPFC pathway. Firstly, it was demonstrated that $CRF_{1/2}$ receptor-like immunoreactivity was present in neurons of the NI that project to the mPFC (Hoover and Vertes, 2007; Farooq *et al*., 2013). Secondly, electrical stimulation of the NI suppressed spontaneous firing of mPFC neurons, as reflected by a decreased firing rate, and infusion of CRF to the NI suppressed the firing rate and burst firing observed (Farooq *et al.,* 2013). Thirdly, high frequency stimulation of NI or exogenous CRF administration into the NI attenuated LTP in the HP-mPFC pathway (Farooq *et al.,* 2013). Furthermore, inactivation of NI with lidocaine, prior to high frequency stimulation of the perforant path, affected LTP induction in the dentate gyrus (Nategh *et al.,* 2016). These reports, along with the finding that direct infusion of antalarmin into the NI reverses elevation stress-induced suppression of LTP in the HP-mPFC pathway (Rajkumar *et al.,* 2016), reveal that CRF_1 receptor-expressing neurons in the NI contribute to synaptic plasticity in the HP-mPFC pathway and via connections to the mPFC may affect cognitive processes such as spatial, working and fear memory, under stress-related conditions (Granon and Poucet, 1995; Seamans *et al.,* 1995; Delatour and Gisquet-Verrier, 1996; Laroche *et al.,* 2000; Burgos-Robles *et al.,* 2009).

Future directions

Better targeting of RXFP3 receptors and novel peptide and drug development. The lack of a broad range of molecular tools to target RXFP3 receptors, including non-peptide agonists and antagonists that penetrate the blood–brain barrier seriously impedes behavioural neuroscience research on the relaxin-3/RXFP3 receptor system. As discussed, the NI/relaxin-3 system is a prospective target for pharmaceutical intervention in a range of neuropsychiatric disorders, and thus, suitable ligands active at RXFP3 receptors are crucial. Currently available RXFP3 receptor agonists are human relaxin-3, R3/I5 (Haugaard-Jonsson *et al.,* 2008) and R3A(11–24,C15 → A)B (human relaxin-3 analogue 2); RXFP3-A2 (Shabanpoor *et al.,* 2012), while the available antagonists are R3(BΔ23–27)R/I5 (Kuei *et al.,* 2007), R3(B1–22)R (Haugaard-Kedstrom *et al.,* 2011) and human relaxin-3 analogue 3; RXFP3-R3 (Shabanpoor *et al.,* 2012) (Table 1) (see Patil *et al*., (in press)). Stapling of peptides, whereby peptides are chemically stabilized by crosslinking with small molecules, has been proposed as a highly effective method of improving the "druggability" of peptides (Verdine and Walensky, 2007; Jubb *et al.,* 2012; Higueruelo *et al.,* 2013; Walensky and Bird, 2014). Recent advances in stapling relaxin-3 B chain analogues (Hojo *et al.,* 2016; Jayakody *et al.,* 2016) suggest that stapling may hold promise for development of more 'druggable' peptide ligands for the RXFP3 receptor (see Patil *et al*., (in press)).

Preclinical translational studies. The key avenues of preclinical research in this area are to identify (1) regulation of other neurotransmitter systems by relaxin-3; (2) interplay of second messenger systems due to interactive effects of relaxin-3, GABA and CRF in the NI neurons; (3) real-time patterns of NI relaxin-3 neuron firing during home cage activity and on exposure to stressors; (4) effects of selective optogenetic stimulation/suppression of NI relaxin-3 neurons in behaving rodents; (5) effects of stimulation/suppression of NI neurons (including specific populations) on cortical EEG and theta wave rhythms; (6) use of DREADDs (Roth, 2016) to target relaxin-3 and/or CRF_1 receptor positive neurons to understand the effects of acute and chronic activation/suppression of the NI neurons (Ma *et al.,* 2016); and (7) measuring expression levels of target proteins or high resolution MRI of the NI during basal conditions and in response to pharmacological intervention especially, with drugs and ligands implicated in neuropsychiatric conditions.

Importantly, research so far has been largely limited to the investigation of NI neurons and relaxin-3/RXFP3 receptor signalling in normal rodents. Thus, further research on the

Table 1

Effects of intra-cerebral microinfusion of RXFP3 receptor ligands

Ligand	Infusion site	Infusion type	Species	Effects observed	References
Agonists					
Human relaxin-3	i.c.v. (lateral ventricle)	Acute	Rat	Sex-specific increase in feeding with higher food intake induction in females.	(Calvez *et al.*, 2015)
Human relaxin-3	i.c.v. (lateral ventricle)	Acute	Rat	Increased food intake in female but not male rats. Increased plasma corticosterone in male but not female rats. Increased CRF and c-fos mRNA in the parvocellular PVN of male but not female rats. Increased CRF mRNA in the BNST of female but not male rats.	(Lenglos *et al.*, 2015)
Human relaxin-3	i.c.v. (lateral ventricle)	Acute	Rat	Decreased anxiety behaviour in elevated plus maze and shock probe-burying test. Increased locomotion in novel environment indicating reduced stress response.	(Nakazawa *et al.*, 2013)
Human relaxin-3	i.c.v. (lateral ventricle)	Acute	Rat	Increased plasma ACTH. Increased c-fos and CRF mRNA in PVN.	(Watanabe *et al.*, 2011a)
Human relaxin-3	i.c.v. (lateral ventricle)	Acute	Rat	Increased food intake 1 h after infusion.	(McGowan *et al.*, 2007)
Human relaxin-3	i.c.v. (lateral ventricle)	Chronic (osmotic minipump for 14 days)	Rat	Increased food consumption and weight gain. Plasma concentrations of leptin and insulin increased.	(Hida *et al.*, 2006)
Human relaxin-3	i.c.v. (third ventricle)	Acute	Rat	Increased plasma corticosterone.	(McGowan *et al.*, 2014)
Human relaxin-3	i.c.v. (third ventricle)	Acute	Rat	Increased food intake 1 h after infusion in satiated rats in early light/dark phase.	(McGowan *et al.*, 2005)
Human relaxin-3	PVN	Acute	Rat	Increased plasma ACTH, corticosterone and prolactin.	(McGowan *et al.*, 2014)
Human relaxin-3	PVN	Subchronic (twice daily for 7 days)	Rat	Increased cumulative food intake. Plasma leptin increased. Plasma thyroid stimulating hormone was decreased.	(McGowan *et al.*, 2007)
Human relaxin-3	PVN	Acute	Rat	Increased food intake 1 h after infusion. Plasma thyroid stimulating hormone was decreased.	(McGowan *et al.*, 2006)
Human relaxin-3	PVN	Acute	Rat	Increased food intake 1 h after infusion in satiated rats in early light/dark phase.	(McGowan *et al.*, 2006)
Human relaxin-3 analogue 2	i.c.v. (lateral ventricle)	Acute	Rat	Increased food intake 1 h after infusion in satiated rats in early light phase.	(Shabanpoor *et al.*, 2012)

(continues)

Table 1 (Continued)

Ligand	Infusion site	Infusion type	Species	Effects observed	References
R3/I5	i.c.v. (lateral ventricle)	Acute	Rat	Dose-dependent increase in locomotion.	(Sutton *et al.*, 2009)
R3/I5	i.c.v. (lateral ventricle)	Chronic (osmotic minipump for 14 days)	Rat	Increased food intake and body weight. Increased epididymal fat, plasma insulin, leptin, adiponectin, plasma testosterone and angiotensinogen. Decreased growth hormone.	(Sutton *et al.*, 2009)
R3/I5	PVN	Chronic (rAAV expression of R3/I5 for 8 weeks)	Rat	Increase in daily food intake and body weight gain.	(Ganella *et al.*, 2013)
R3/I5	Medial septum	Acute	Rat	Increase HC theta power.	(Ma *et al.*, 2009)
R3A(11–24,C15 → A)B	i.c.v. (lateral ventricle)	Acute	Mouse	Reduced elevated anxiety induced by FG7142 in light dark box and social interaction test.	(Zhang *et al.*, 2015)
R3A(11–24,C15 → A)B	i.c.v. (lateral ventricle)	Acute	Rat	Decreased anxiety behaviour in EPM and light/dark box. Decreased depressive-like behaviour in forced swim test in pretested rats but not experimentally naïve rats.	(Ryan *et al.*, 2013a)
Antagonists					
R3(B1–22)R	i.c.v. (lateral ventricle)	Acute	Mouse	Increased anxiety behaviour in EPM.	(Zhang *et al.*, 2015)
Human relaxin-3 analogue 3	i.c.v.(lateral ventricle)	Acute	Rat	Blocked increase in food intake by H3 analogue 2.	(Shabanpoor *et al.*, 2012)
R3(B23–27)R/I5	i.c.v. (lateral ventricle)	Chronic (osmotic minipump for 14 days)	Rat	Plasma growth hormone decreased.	(Sutton *et al.*, 2009)
R3(B23–27)R/I5	Medial septum	Acute	Rat	Decreased HC theta power. Impairs spatial working memory performance in spontaneous alternation task.	(Ma *et al.*, 2009)
R3(B23–27)R/I5	i.c.v. (lateral ventricle)	Acute	Rat	Blocks increase in food intake induced by agonist R3/I5.	(Kuei *et al.*, 2007)

outcomes of disease pathologies in relevant preclinical disease models on NI neurons and the relaxin-3 system will be crucial to understand its full physiological role more clearly and its therapeutic potential as mapped out below.

Models of anxiety and depression. High anxiety (HAB) rats, selectively bred based on elevated plus maze (EPM) performance, display hyper-emotionality, hyper-reactivity of the HPA axis, impaired fear extinction, passive coping strategies and stronger responses to anxiolytics and antidepressants (Liebsch *et al.,* 1998; Henniger *et al.,* 2000; Ohl *et al.,* 2001; Wigger *et al.,* 2001; Landgraf and Wigger, 2002; Keck *et al.,* 2003), suggesting they effectively model co-morbid depression and anxiety, commonly observed in the clinic (Landgraf *et al.,* 1999; Keck *et al.,* 2001). AVP expression is elevated in the PVN of HAB rats compared with their counterpart low anxiety rats and is functionally relevant as intra-PVN infusion of a V_{1A} receptor antagonist produced an anxiolytic effect in HAB rats (Wigger *et al.,* 2004). CRF expression in the BNST is reduced, and CRF_2 receptor binding in hypothalamic nuclei and amygdala is increased in HAB rats (Wigger *et al.,* 2004). Though not entirely consistent, previous results indicate that the NI/relaxin-3 system has an anxiolytic effect, and it is possible that there is reduced relaxin-3 expression and concentrations in the NI and/or key target regions like the amygdala and BNST in HAB rats.

Similarly, the Flinders sensitive line (FSL) of rats were developed through selective breeding to exhibit cholinergic supersensitivity, a feature seen in depressed patients (Overstreet, 1993). FSL rats display high levels of immobility in the forced swim test, low social interaction, altered appetite and weight, impaired response to reward, lethargy and particularly strong stress responses, such as increased anhedonia in response to a chronic mild stress and behavioural inhibition after a foot shock (Overstreet *et al.,* 1995). Reduced NPY expression in the hippocampus of FSL rats indicates its involvement in the pathophysiology of depression (Jimenez Vasquez *et al.,* 2000). As relaxin-3 neurons are stress-responsive, FSL rats may display an altered profile of relaxin-3 signalling and provide insights into a possible therapeutic opportunity in treating depression symptoms. Furthermore, because relaxin-3 signalling has been strongly implicated in stress-induced alcohol relapse (Ryan *et al.,* 2013b) (see Ma *et al.*, (in press)), the fawn-hooded rat, which is a model for co-morbid depression and alcoholism displaying high immobility on the forced swim test and high voluntary ethanol intake, is also worth considering for relaxin-3 system studies (Overstreet *et al.,* 2007; Rezvani *et al.,* 2002).

In addition to these life-long models created by selective breeding, more convenient models with greater construct validity may be those induced by environmental behavioural stressors. The chronic mild stress (CMS) model of depression, which is preferred for rats but has also been used in mice, is extensively validated and produces enduring changes in behaviour, neurotransmitter and hormonal concentrations and the immune system (Overstreet, 2012; Abelaira *et al.,* 2013; Czeh *et al.,* 2016). Notably, a similar 8-week chronic stress regime imposed on relaxin-3 knockout mice resulted in a sustained loss of body weight, but no marked changes in the forced swim test (Smith *et al.,* 2009). Further studies in this area are warranted, including further tests of depressive phenotypes such as those revealed by the sucrose/saccharin preference test, because such changes are a primary characteristic of mice undergoing CMS (Katz, 1982; Willner, 2005).

Furthermore, a model of post-traumatic stress disorder (PTSD) symptoms developed by exposing rats to 'chronic plus acute prolonged stress' treatment (Green *et al.,* 2011) was reported to increase basal anxiety and fear responding while impairing extinction of learned fear and altering stress coping styles from active to passive, reducing HPA reactivity to acute stressors and reducing glucocorticoid receptor expression in the mPFC (Roth *et al.,* 2012). Therefore, alterations in expression of relaxin-3 in the NI or RXFP3 receptors in regions such as the amygdala, PFC, BNST and vHC could be examined in this model. If changes were observed, experiments could be designed to examine the ability of RXFP3 receptor agonists and antagonists to improve or worsen behavioural phenotypes and identify their main sites of action.

Need for clinical studies. While the vast majority of the experimental studies of the NI/relaxin-3 system have been preclinical, there have been some efforts to examine links between relaxin-3 signalling and metabolic and psychiatric disorders in patients, and the substantial body of evidence supporting important roles for this neuropeptide demands targeted translational studies. It is perhaps prudent to base any systematic clinical investigations of the relaxin-3/RXFP3 system on successful studies linking other neuropeptide and neuropeptide receptor systems to neuropsychiatric disorders. These have largely been through molecular genetic studies combined with assays of peptide content in CSF, blood serum and/or *post mortem* brain samples from relevant patient groups. Recently, the serum levels of circulating pituitary adenylate cyclase activating polypeptide neuropeptide were positively correlated with PTSD symptoms only in women, not men (Ressler *et al.,* 2011). A single nucleotide polymorphism in the PAC_1 receptor gene could predict PTSD in women but not men and was associated with elevated reactivity of the amygdala and hippocampus to threatening stimuli as well as lower functional connectivity between the two structures studied via functional MRI (Stevens *et al.,* 2014). Similarly, a single nucleotide polymorphism of the neuropeptide S receptor resulting in the mutated T-allele was found to be associated with panic disorder, increased sensitivity to anxiety, greater stress response, a hyperactive HPA axis and increased activity of the basolateral amygdala when exposed to stress (Kumsta *et al.,* 2013). NPY expression has been found to be reduced in the caudate nucleus and frontal cortex of suicide victims and in the CSF of depressed patients (Widerlov *et al.,* 1988; Widdowson *et al.,* 1992) and blood samples from PTSD patients (Sah *et al.,* 2009). Currently, intranasal infusion of NPY is being developed and tested preclinically as a treatment for PTSD, to enable rapid delivery to the brain and avoid the side effects of peripheral administration (Serova *et al.,* 2013; Sabban *et al.,* 2016).

In the first studies of this type related to the relaxin-3/RXFP3 receptor system, polymorphisms in the relaxin-3 and RXFP3,

RXFP4 receptor genes were reported to be associated with hypercholesterolemia, obesity and diabetes in patients being treated with antipsychotic drugs, corroborating a likely role of the relaxin-3 system in metabolism and regulation of appetite and body weight (Munro *et al.,* 2012). In separate studies, serum relaxin-3 levels were reported to be elevated in female patients with metabolic syndrome (Ghattas *et al.,* 2013) but unaffected in patients with diabetes (Zhang *et al.,* 2013), although these studies did not fully demonstrate that their assays specifically detected the relaxin-3 peptide. However, if these studies are reflective of the existence of detectable levels of relaxin-3 in the bloodstream, it might be prudent to examine the profile of relaxin-3 levels in patients with eating disorders and/or appetite irregularities associated with neuropsychiatric diseases such as schizophrenia and depression (Nestler *et al.,* 2002; Newcomer, 2007; Lungu *et al.,* 2013).

Interestingly, in a gene association study of familial schizophrenia, a mutation in chromosome 5p locus was identified, and the RXFP3 receptor gene is located on this chromosome (Bespalova *et al.,* 2005). Based on preclinical data, the relaxin-3 system could be linked to the cognitive impairment as well as the negative symptoms of schizophrenia. While this is only an anecdotal observation at present, with the rapid increase in genetic information available from different patient groups, due to improvements in and reduced costs of gene sequencing, more detailed information about the profile of RXFP3 receptors in neuropsychiatric disorders should become available.

In a recent study of the neocortex of patients with Alzheimer's disease (AD) and their age-matched controls, we have found that alterations in the levels of RXFP1 and RXFP3 receptors were more closely associated with depressive symptoms than with cognitive decline or Aβ42 levels (Lee *et al.,* 2016). While RXFP3 receptor-like immunoreactivity in the parietal cortex was up-regulated in depressed AD patients and unchanged in non-depressed AD patients, RXFP1 receptor-like immunoreactivity in the parietal cortex was unchanged in depressed AD patients and down-regulated in non-depressed AD patients (Lee *et al.,* 2016). Neuropsychiatric conditions such as depression, anxiety and psychosis in AD patients (also known as 'behavioural and psychological symptoms of dementia') are thought to arise from degeneration of monoaminergic neurons and accompanying perturbations in neurotransmission (Francis *et al.,* 2010; Ramirez *et al.,* 2014). Given the proposed role for relaxin-3 in regulating monoaminergic transmission (Miyamoto *et al.,* 2008), the relaxin-3/RXFP3 receptor system may represent a novel target for treating neuropsychiatric symptoms. Previous reports suggest that dysregulation of the HPA axis in AD could underlie neuropsychiatric behaviours (Notarianni, 2013; Lucassen *et al.,* 2014). Exogenous relaxin-3 altered activity of the HPA axis and it is possible that changes in endogenous relaxin-3/RXFP3 receptor signalling contribute to altered HPA axis functioning and adaptive plasticity in AD (Lee *et al.,* 2016), although further studies are required to understand this relationship and confirm its existence in human brain.

Strong evidence demonstrates an association between the disruption of synchronous brain neural oscillations, which can be studied non-invasively, and neuropsychiatric diseases (Buzsaki and Watson, 2012; Uhlhaas and Singer, 2012). Theta and alpha-band activities evoked during sensorimotor gating are significantly reduced in schizophrenia patients, as well as their first-degree relatives (Hong *et al.,* 2008). A similar association exists between alterations of rhythmic activity and depression, bipolar disorder and autism spectrum disorders (Buzsaki and Watson, 2012). In this regard, the lack of direct connections of the RPO and other brainstem modulating structures to the SHS indicates that the NI relaxin-3 signalling may provide an important relay centre for brainstem-initiated signals driving HC theta activity and may play a causal role in the disruption of theta activity associated with neuropsychiatric diseases.

Brain imaging is a technique that has provided detailed macroscale information about the distribution and magnitude of neurotransmitter systems. For instance, using PET and single-photon emission computed tomography tracers, dopamine transporter density has been found to be significantly reduced in the basal ganglia of depressed patients (Nutt, 2006). Although not tested in humans yet, PET tracers have been developed and tested successfully preclinically for Y_5 receptors, which is linked to the pathophysiology of depression, anxiety and obesity (Hostetler *et al.,* 2011; Kumar *et al.,* 2016). Similarly, a labelled ligand specific for RXFP3 receptors would enable brain imaging of the densities of these receptors in patients.

Thus, it may be informative to conduct studies of relaxin-3 levels in the CSF (blood) and genetic variants of relaxin-3/RXFP3 receptors in both control subjects and patients with neuropsychiatric disorders to build a data set that might allow further rationally designed studies of causative associations. Comprehensive studies of relaxin-3 and RXFP3 receptor concentrations in *post mortem* brain samples from key brain regions important in emotional regulation such as amygdala, hippocampus and BNST from these populations may also provide insights, particularly if the identity of the RXFP3 receptor-expressing neurons in the different regions can be discovered.

In conclusion, it is hoped that the substantial preclinical evidence for the broad neurophysiological actions of relaxin-3/RXFP3 receptor signalling and the growing momentum of research in the area will encourage more basic and clinical researchers to consider this conserved neuromodulatory system in their investigations.

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Conflict of interest

The authors declare no conflicts of interest.

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