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REVIEW

Relaxin family peptide systems and the central nervous system

G. E. Callander · R. A. D. Bathgate

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Abstract Since its discovery in the 1920s, relaxin has enjoyed a reputation as a peptide hormone of pregnancy. However, relaxin and other relaxin family peptides are now associated with numerous non-reproductive physiologies and disease states. The new millennium bought with it the sequence of the human genome and subsequently new directions for relaxin research. In 2002, the ancestral relaxin gene *RLN3* was identified from genome databases. The relaxin-3 peptide is highly expressed in a small region of the brain and in species from teleost to primates and has both conserved sequence and sites of expression. Combined with the discovery of the relaxin family peptide receptors, interest in the role of the relaxin family peptides in the central nervous system has been reignited. This review explores the relaxin family peptides that are expressed in or act upon the brain, the receptors that mediate their actions, and what is currently known of their functions.

Keywords Relaxin family peptides · Relaxin · Relaxin-3 · Brain · RXFP3 · RXFP1 · GPCR

Background and historical perspective

Relaxin was one of the first peptide hormones to be discovered. In 1926, Frederick Hisaw observed that injection of virgin guinea pigs with serum from pregnant guinea pigs lengthened the pubic symphysis [1]. It took a further 4 years to extract the agent responsible, relaxin, from sow corpus luteum [2].

The isolation of pure porcine relaxin enabled the amino acid sequence to be determined [3], illuminating many of the features of relaxin we now consider characteristic. James et al. [4] describe a two-chain peptide, derived by proteolytic cleavage from a pro-insulin-like precursor, containing three disulphide bonds, predicted to be identical in arrangement to insulin. Indeed, like insulin, relaxin is synthesized as a pre-prohormone, consisting of a signal sequence and a B–C–A domain configuration. Removal of the C chain and the formation of three disulfide bonds between six highly conserved cysteine residues found on the A and B chains, produces the mature protein.

In the years that followed, relaxin from the ovary, placenta, and plasma of many different species, was isolated, purified, and characterized. Overall, only 30–60% amino-acid sequence identity exists between species. In all species, the conserved residues are largely confined to the cysteine residues that link the A and B domains by two inter-chain disulfide bonds and form an A chain intra-chain disulfide bond and the adjacent glycine residues. Importantly, an amino acid motif in the B-chain (Arg-X-X-X-Arg-X-X-Ile/Val-X) is conserved in all of the relaxin peptides and is crucial for the interaction of these peptides with the relaxin receptor [5].

The emergence of molecular techniques allowed the first human relaxin gene, *RLN1*, to be successfully cloned [6] from a genomic library, which was closely followed by the discovery of a second relaxin gene, *RLN2*, found in a cDNA library from pregnant human corpus luteum (CL) [7]. The *RLN2* gene encodes the relaxin peptide, which had previously been isolated, characterized, and shown to be

G. E. Callander · R. A. D. Bathgate (✉)
Florey Neuroscience Institutes and Department of Biochemistry and Molecular Biology, The University of Melbourne, Melbourne, VIC 3010, Australia
e-mail: bathgate@florey.edu.au

secreted by the CL during pregnancy in humans [8, 9]. The two human relaxin genes are the result of a gene duplication event and non-primates have only the *RLN1* gene. Throughout this review, the human *RLN2* gene and peptide product H2 relaxin will be referred to as relaxin, as it is the functional equivalent of relaxin in non-primates.

The remaining members of the relaxin family of peptides were identified on the basis of sequence similarities to relaxin and the retention of the signal peptide–B–C–A domain structure. In 1993, insulin-like peptide 3 (INSL3) was cloned from a boar testis cDNA library [10] using a differential cDNA screening method to isolate testis-specific transcripts. INSL4 was cloned from a first-trimester human placenta cDNA library [11]. Subsequently, searches of human EST databases for relaxin- or insulin-like prohormone sequences, resulted in the identification of *INSL5* and *INSL6*, respectively [12, 13]. In 2002, the availability of the human genome sequence to the public through the Celera databases, enabled a third relaxin gene, *RLN3,* to be identified [14]. This gene was classified as a "relaxin" as it contained the receptor-binding motif in the B-chain, as outlined above, which is not present in the other members of the family.

The seven genes in the human relaxin family have been mapped to three different chromosomes [15]. On chromosome 9 at 9p24, *RLN1*, *RLN2*, *INSL4* and *INSL6* are present in a tight cluster. *RLN1* and *INSL4* are considered the result of gene duplication, as these genes are only present in primates. *RLN3* and *INSL3* are located on chromosome 19 at 19p13.3 and 19p13.2, respectively. *INSL5*, is not co-located with any family members and can be found on chromosome 1 at 1p31.1.

The increased availability of genomic information and the subsequent discovery of *RLN3* has changed the way we think about the evolution of relaxin family peptides. Firstly, relaxin-3 orthologues are present in the non-vertebrate species *Takifugu rubripes* and *Danio rerio*, indicating relaxin-3 emerged prior to the divergence of fish [16]. Secondly, unlike relaxin, relaxin-3 has high homology between orthologues in the mature peptide region. It is now thought that a single relaxin-3-like ancestor diverged from the insulin peptides and during mammalian evolution two subfamilies arose from this ancestor. Subfamily A consists of INSL3, INSL4, INSL6, H1, and H2 relaxin and subfamily B contains INSL5 and H3 relaxin [16].

This work emphasizes that relaxin-3 is a highly conserved neuropeptide and indeed other family members have been predicted to have important functions in the brain. Hence, this review will focus on the relaxin family peptides that are produced by (or have effects on) the central nervous system and their corresponding receptors (Table 1).

Physiology and endocrine effects of relaxin family peptides and receptors

Relaxin

In most mammals, the main source of relaxin is the CL and/or placenta and uterus of females and the highest tissue and circulating levels are achieved during pregnancy (reviewed in [17]). Relaxin plays a number of roles in reproductive physiology, which vary across species. During pregnancy, relaxin acts on the cervix, pubic symphysis, vagina, uterus, and mammary apparatus. Furthermore, relaxin mediates the cardiovascular changes required to manage the increased blood volume resulting from pregnancy. Relaxin is also produced in the male reproductive tract, specifically the prostate in humans [18], where it has been implicated in prostate growth, sperm motility and penetration into oocytes [19]. In addition to these actions, relaxin is involved with several non-reproductive processes including cardiac protection, wound healing, connective tissue regulation, and allergic responses (reviewed in [17]).

In 2002, Hsu et al. [20] demonstrated the orphan leucine-rich repeat containing G protein-coupled receptor, relaxin family peptide 1 receptor (RXFP1; at the time known as LGR7), was the receptor for relaxin. This important discovery was based on three key findings. Firstly, stimulation of RXFP1 with relaxin results in dose-dependent increases in cAMP. Secondly, the tissue distribution of relaxin-binding sites closely matches that of RXFP1. Finally, a soluble RXFP1-binding domain acts as a functional antagonist by sequestering free relaxin. Subsequently, the phenotype of the RXFP1 knockout (KO) mice was found to be identical to that of the relaxin KO [21, 22].

RXFP1 is a member of the glycoprotein cluster of the δ group of GPCRs, which also includes the receptors for luteinizing hormone, thyroid-stimulating hormone and follicle-stimulating hormone [23]. RXFP1 has a large ectodomain, consisting of ten leucine-rich repeats, which contain the primary binding site of relaxin [24] and a low-density lipoprotein class A (LDLa) module. Found at the extreme NH2 terminus, the LDLa module is involved in receptor activation but not primary ligand binding [25, 26]. Intracellular signaling produced by RXFP1 activation is remarkably complex and tissue-specific. Signaling from relaxin family peptide receptors is reviewed comprehensively elsewhere [27, 28].

INSL3

First identified in 1993, INSL3 was initially named Leydig cell insulin-like gene, on the basis of its expression profile [10]. However, close structural similarity to relaxin led to the alternative name of relaxin-like factor. In males, INSL3

Table 1 Relaxin family peptides, receptors, and their expression in the brain

Peptide				Receptor			
Gene	Protein	Alternate names	Expression in brain	Gene	Protein	Alternate names	Expression in brain
RLN1[a]	Relaxin-1	–	No	RXFP1	RXFP1	LGR7	Yes
RLN2[b]	Relaxin-2	Relaxin	Yes	RXFP1	RXFP1	LGR7	Yes
RLN3	Relaxin-3	Insulin-like peptide 7	Yes	RXFP3	RXFP3	SALPR, GPCR135, RLN3R1	Yes
INSL3	Insulin-like peptide-3	Leydig insulin-like peptide, relaxin-like factor	Undecided	RXFP2	RXFP2	LGR8, GREAT	Yes
INSL4[a]	Insulin-like peptide-4	Early placental insulin-like factor	No	–	–	–	–
INSL5	Insulin-like peptide-5	Relaxin insulin-like factor 2	Undecided	RXFP4[c]	RXFP4	GPCR142, GPR100, RLN3R2	Yes
INSL6	Insulin-like peptide-6	Relaxin insulin like factor 1	No	–	–	–	–

[a] Only present in higher primates

[b] In rodent, the equivalent gene is known as *RLN1*

[c] Is a pseudogene in the rat

is essential for testicular descent via actions on the gubernaculum [29, 30]. In females, INSL3 is produced in the theca and luteal cells of the adult ovary, where it has potential roles in follicular development [31] and oocyte maturation [32].

The identification of the INSL3 receptor was due in part to the characterization of the INSL3 KO mouse. These mice exhibit bilateral cryptorchidism due to failed gubernaculum development during embryogenesis, which is similar to the phenotype displayed by mice with disruptions in the G protein-coupled receptor affecting testis descent (GREAT) gene [33]. Subsequent phylogenetic analysis proved relaxin family peptide 2 receptor (RXFP2, previously known as LGR8) to be the human orthologue of the mouse GREAT [20]. On the basis of these common phenotypes, INSL3 was tested as a ligand for RXFP2. In HEK293T cells expressing RXFP2, treatment with INSL3 results in dose-dependent increases in cAMP [34], thus demonstrating that RXFP2 is the cognate receptor for INSL3. Stimulation of either RXFP1 or RXFP2 with relaxin results in dose-dependent increases in cAMP, whereas INSL3 only activates RXFP2 [34]. In rodents, relaxin does not activate RXFP2 [35, 36].

In all species tested, INSL3 is a major secretory product of both prenatal and postnatal Leydig cells of the testis [10]. Despite early reports of INSL3 expression being restricted to the testis, in 1995 INSL3 mRNA was also detected in human cyclic corpus lutea and trophoblast [37]. Thereafter INSL3 was detected in tissues of several species including human mammary gland [38], marmoset [39], mouse [40], and ovine and bovine ovary [41, 42].

Relaxin-3

A novel relaxin homologue was identified in the human genome in 2002. It was named human relaxin-3 (H3 relaxin), as it is the third relaxin gene and the mouse equivalent was named relaxin-3 for consistency, despite being only the second relaxin gene in rodent [14]. Both human and mouse relaxin-3 amino-acid sequences contain the residues essential for relaxin receptor binding. Chemical synthesis of H3 relaxin provided proof that relaxin-3 could bind and activate relaxin receptors in THP-1 cells, albeit with lower affinity and activity than relaxin itself [14]. Consequently, relaxin-3 established its place as the final member of the family.

Early attempts to screen for sites of relaxin-3 expression used Southern blotting of RT-PCR products from mouse tissue [14]. Relaxin-3 was found in high abundance in the brain, in moderate abundance in the thymus, kidney, and spleen, and low abundance in the heart and liver, with no noticeable differences between male and female samples [14]. Northern-blot analysis and in situ hybridization confirmed that relaxin-3 is localized to cells of the 'pars ventromedialis of the dorsal tegmental nucleus' and the 'dorsal reticular tegmental nucleus' [14]. Rat relaxin-3 was cloned in the same year and its expression mirrored that of its mouse homologue [43]. The detection of relaxin-3 peptides in the rodent brain was propitious—rodents are the workhorse of neuroscience research—thereby laying the foundations for research into relaxin-3 function.

The cognate receptor for relaxin-3 is RXFP3 but it can also bind and activate RXFP1 [44] and RXFP4 [45]. As

mentioned above, RXFP1 is the receptor for relaxin while RXFP4 is the receptor for INSL5 [46] (Table 1). Both RXFP3 and RXFP4 are members of the γ-group of the rhodopsin family of GPCRs [23] and lack the large extracellular domains characterizing RXFP1 and RXFP2. RXFP3 was previously known as somatostatin and angiotensin-like peptide receptor and GPCR135, this receptor was deorphanized in 2003 [45]. Relaxin-3 binds to RXFP3 with high affinity (0.31 nM) and receptor activation results in inhibition of cAMP accumulation [45]. In situ hybridization and autoradiography using an RXFP3 selective chimeric peptide, human relaxin-3 B-chain/INSL5 A-chain (R3/I5), has been used to map the expression of RXFP3 in the rat brain [47–49]. In the rat, RXFP3 is widely expressed and is strongly expressed in the olfactory bulb, sensory cortex, amygdala, midline thalamus, paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, and the superior colliculus [47].

Functions of central relaxin family peptide and receptor systems

The role of RXFP2 in the apparent absence of INSL3

It is tempting to consider the possibility of an INSL3 system in the brain given the expression of its cognate receptor RXFP2. When RXFP2 was first linked to INSL3, RT-PCR was used to broadly determine the tissue localization of the receptor. In human, RXFP2 is present in brain, kidney, muscle, testis, thyroid, uterus, peripheral blood cells, and bone marrow [20]. In situ hybridization histochemistry in rats found that RXFP2 mRNA is located in the neuronal soma of numerous thalamic nuclei [50] including the parafascicular nucleus, the dorsolateral, ventrolateral and posterior thalamic nuclei and the medial habenula [51]. The distribution of RXFP2 has been determined by mapping [^{125}I]-INSL3-binding studies [51] (Fig. 1).

However, despite detection of bovine INSL3 in the brain using RT-PCR [41] and Northern blotting [52], to date, INSL3 has not been detected in the rodent brain using in situ hybridization [50]. One explanation for this is that INSL3 is not expressed in the brain. Based on evolutionary analysis, Wilkinson et al. [16] proposed that RXFP1 and RXFP2 were acquired as receptors for relaxin family peptides during mammalian evolution. Thus it is plausible that prior to the emergence of INSL3, RXFP2 existed as a receptor for another neuropeptide and that RXFP2 in the brain is all that remains of a defunct ligand-receptor pairing.

Yet circulating INSL3 secreted from testicular or ovarian cells may be transported across the blood–brain barrier to activate RXFP2. Recently several studies have described immunoassays capable of measuring circulating INSL3 levels [53, 54]. In human males, the circulating levels of INSL3 are relatively high; about 1% of testosterone levels, but much greater than that of the sertoli cell hormone, inhibin-B [54]. If it were possible for peripheral INSL3 to activate central RXFP2, one would expect the central actions of RXFP2 to reflect the fertility-related functions of the receptor observed in the gonads. Currently, studies in the rat brain indicate that RXFP2 is associated with sensorimotor function [51]. Nevertheless, it remains to be assessed whether INSL3 can cross the blood–brain barrier in quantities sufficient to activate RXFP2, or if there is indeed a discrete source of INSL3 in the brain.

Relaxin and RXFP1 system: functions reflect peptide origin and receptor site

The majority of circulating relaxin is the product of reproductive tissues. However, in some species, RT-PCR and in situ hybridization studies have led to the identification of tissues that express secondary sources of relaxin (reviewed in [17]). Rodents are the only species tested thus far to express relaxin in the brain. In the rat, relaxin mRNA is expressed in the anterior olfactory nucleus, lateral orbital cortex, tenia tecta, piriform cortex, neocortex, dentate gyrus, and hippocampus [55–57]. Developmentally, relaxin mRNA is not evident at embryonic day 15, but is observed from post-natal day 1 [55]. Immunohistochemistry consistently detects relaxin in the cytoplasm and proximal processes of cell bodies in the arcuate nucleus, with lower levels also observed in the anterior olfactory nucleus, tenia tecta, and piriform cortex [56, 57]. Preliminary studies in the mouse brain indicate an expression pattern similar to that of the rat [58].

Mapping the topography of relaxin-binding sites was paramount to determining the sites of relaxin action in the brain. Prior to the discovery of RXFP1, Osheroff et al. [59] identified relaxin-binding sites in rat brain using [^{32}P]-labeled relaxin. A more comprehensive study that followed found that in both male and female rats, displaceable relaxin-binding sites were identified in discrete regions of the olfactory system, neocortex, hippocampus, thalamus, amygdala, midbrain and medulla [60]. A subsequent study using [^{33}P]-labeled relaxin together with in situ hybridization for RXFP1 mRNA demonstrated a similar pattern of relaxin-binding sites to that reported by Osheroff, which overlapped with the sites of RXFP1 mRNA expression [61] (Fig. 2).

It is important to recognize that studies investigating the role of relaxin in the central nervous system encompass two distinct categories. Earlier studies focused on the effects of circulating relaxin on the brain during pregnancy

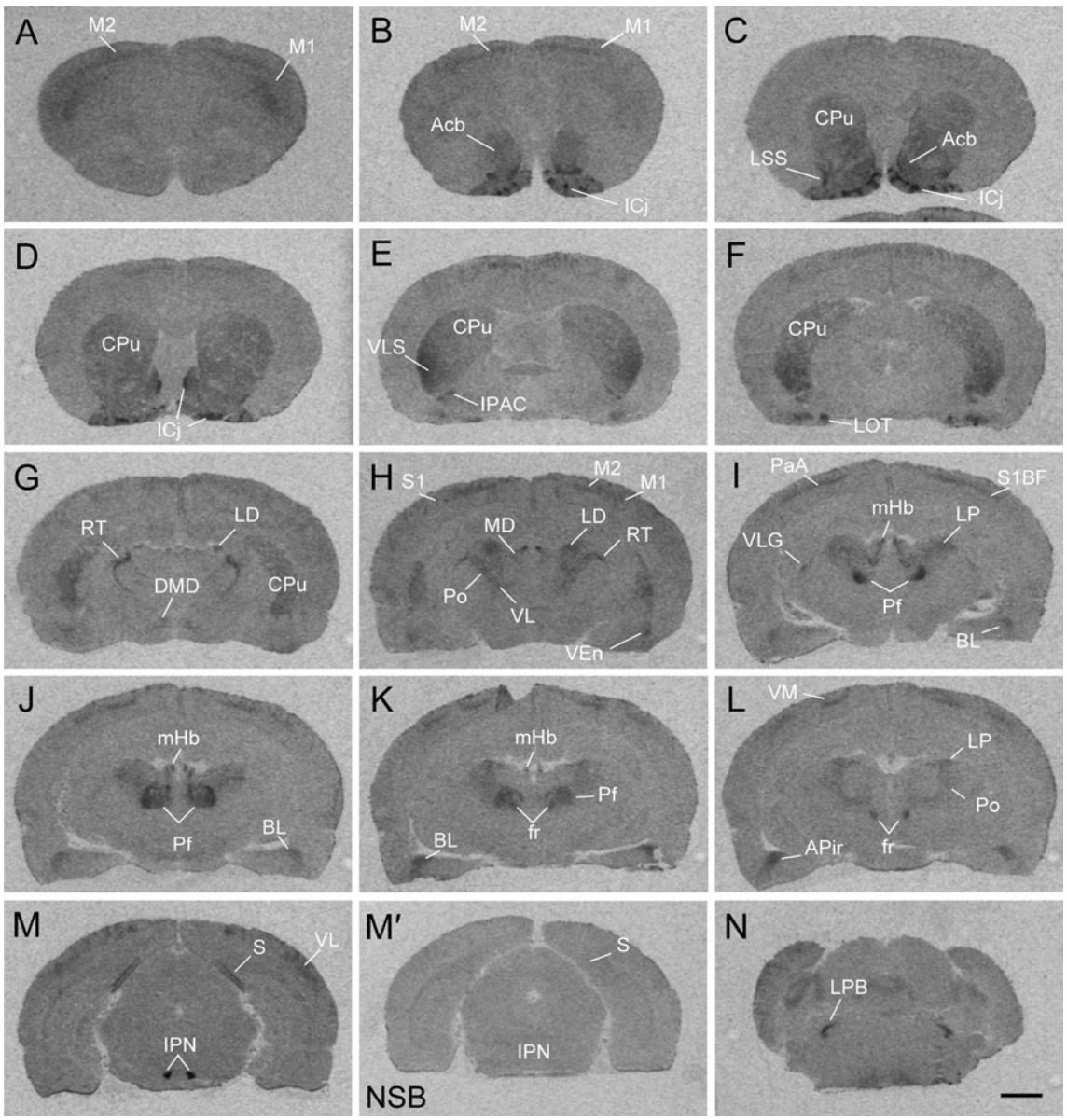

Fig. 1 Distribution of RXFP2, as reflected by [^{125}I]-INSL3-binding sites, in coronal sections of adult rat forebrain. **a**–**n** Representative autoradiographs illustrating the pattern of specific [^{125}I]-INSL3-binding sites throughout the rostrocaudal extent of the rat forebrain. **m′** 100 nM unlabeled INSL3 was added to the slides to demonstrate the specificity of the [^{125}I]-INSL3-binding (NSB). *Scale bar* 1 mm. *Acb* accumbens nucleus (n), *APir* amygdalopiriform transition area, *BL* basolateral amygdaloid n, *CPu* caudate putamen, *fr* fasciculus retroflexus, *DMD* dorsomedial hypothalamic n, *ICj* islands of Calleja, *IPAC* interstitial n of posterior limb of anterior commissure, *IPN* interpeduncular n, *LD* laterodorsal thalamic n, *LOT* n of lateral olfactory tract, *LP* lateral posterior thalamic n, *LPB* lateral parabrachial n, *LSS* lateral stripe of striatum, *M1* primary motor cortex, *M2* secondary motor cortex, *MD* mediodorsal thalamic n, *mHb* medial habenular n, *PaA* parietal cortex, anterior area, *Pf* parafascicular thalamic n, *Po* posterior thalamic nuclear group, *Rt* reticular thalamic n, *S* subiculum, *S1* primary somatosensory cortex, *S1BF* primary somatosensory cortex, barrel field, *VEn* ventral endopiriform n, *VL* ventrolateral thalamic n, *VLG* ventrolateral geniculate n, *VLS* ventrolateral striatum, *VM* secondary visual cortex, medial (adapted from [51] with permission, copyright Elsevier 2009)

and the peripheral consequences, especially in relation to the important cardiovascular effects of relaxin during pregnancy. It was subsequently shown that the circumventricular organs, which have direct contact with the peripheral blood, express RXFP1 [61] and are likely to mediate many of the actions of circulating relaxin on the brain. More recently, research has focused on the potential neuronal functions of relaxin, whereby relaxin produced in the brain acts locally on RXFP1. In an effort to distinguish between the two, this review will address them separately.

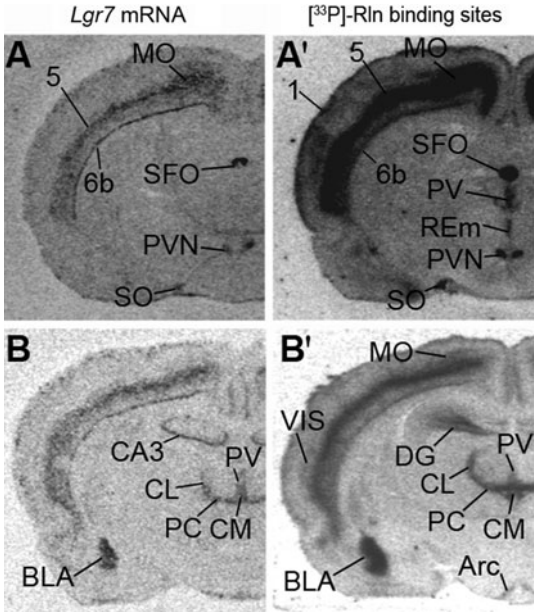

Fig. 2 Comparative distribution of (**a**–**b**) RXFP1 (LGR7) mRNA and (**a′**–**b′**) [^{33}P]-labeled human relaxin-binding sites in coronal sections of adult rat brain. *Arc* arcuate hypothalamic n, *BLA* basolateral amygdala, *CA3* field of hippocampus, *CL* centrolateral thalamic n, *CM* centromedial thalamic n, *DG* dentate gyrus of hippocampus, *MO* motor cortex, *1, 5, 6b* layers of cerebral cortex, *PC* paracentral thalamic n, *PV* paraventricular thalamic n, *PVN* paraventricular nucleus (n), *REm* reuniens thalamic n medial, *SFO* subfornical organ, *SO* supraoptic nucleus, *VIS* visual cortex (from [61] with permission, copyright Elsevier 2009)

Central effects of peripheral relaxin mediated by the circumventricular organs

Before publication of relaxin-binding sites in the rat brain, several groups proposed circumventricular sites of action for relaxin [60, 62–64]. Given sites of relaxin production in the brain had yet to be identified, the hypothesis was that peptides of the periphery could exert their effects on the brain by two mechanisms; by acting on circumventricular organs, which lack a blood–brain barrier, or via actions in highly vascularized regions, such as the hypothalamus, which are more accessible to blood-borne peptides [65].

Effects on milk ejection

The first study on the central actions of relaxin attempted to link the quiescence of the uterus observed in some mammals prior to parturition, with relaxin inhibition of oxytocin release. Summerlee et al. [66] addressed this by studying the effects of porcine relaxin on milk ejection in lactating rats. Intravenous relaxin was found to inhibit milk ejection in a dose-dependent manner and intracerebroventricular (ICV) injection of relaxin disturbed the pattern of the ejection reflex without affecting the response of the mammary gland to oxytocin. Thus, a novel role in the central nervous system was suggested for relaxin.

Subsequent studies aimed to determine the location and mechanism of action of relaxin on the milk ejection reflex. Injections of relaxin into the third ventricle produced the spontaneous and prolonged inhibition of ejection, whereas the response to injection in the lateral ventricles was delayed and injection into the fourth ventricle and cisterna magna had no effect [63]. The authors suggested that relaxin was acting at the level of the diencephalon or mesencephalon. Dayanithi et al.'s [67] in vitro approach aimed to further clarify where relaxin exerts its effects by determining if relaxin acts on the hypothalamus or neurohypophysis to release hormones. Using isolated neural lobes of the male rat pituitary, it was demonstrated that, under basal conditions, relaxin inhibits vasopressin and oxytocin release and when nerve endings are depolarized, vasopressin and oxytocin secretion is potentiated [67]. This suggested that receptors for relaxin are present on magnocellular nerve efferents, and although this has not yet been proven biochemically in the rat, there are high levels of RXFP1 mRNA in the paraventricular and supraoptic nuclei [57]. Using β-galactosidase staining in the pregnant RXFP1 KO/LacZ knock-in mouse, RXFP1 was shown to be expressed in the murine anterior pituitary [22].

Although actions on the pituitary may have a role in mediating the actions of relaxin on oxytocin release, this may not be the site of action in milk ejection. Summerlee et al. [62] demonstrated that lesions of the SFO abolished the inhibitory actions of relaxin on the milk-ejection reflex. Relaxin was still able to inhibit milk ejection in control animals with lesions in adjacent areas of the cerebrum and lesion of the SFO had no effect on normal milk ejection. Hence, it is likely that the effects of relaxin on milk ejection are mediated at the level of the SFO.

Hemodynamic actions

In light of the effect of relaxin on vasopressin release, the action of relaxin on blood pressure has been extensively studied. The first study to investigate the central effect of relaxin on blood pressure demonstrated that injection of relaxin into the third ventricle caused increased arterial blood pressure and vasopressin release, and this effect was partially negated by lesion of the SFO [64]. Consequent studies revealed that the brain angiotensin system plays a role in partially mediating the pressor effects of relaxin [68]. However, like the studies on reflex milk ejection, progress in determining a mechanism for these effects was impaired by a lack of knowledge about where relaxin exerts its effects.

In rodents, changes in the central set points of cardiovascular control occur during the second half of pregnancy.

Increases in plasma volume and glomerular filtration rate and decreases in plasma osmolality and blood pressure are accommodated by substantial increases in drinking [69]. In rats, ICV infusion of relaxin caused dose-dependent dipsogenesis, but the effect was influenced by photoperiod, with the largest response during the dark (active) period [70]. Conversely, ICV administration of MCA1, a purified monoclonal neutralizing antibody to rat relaxin, ablated the dipsogenic effect of endogenous relaxin during the dark period in the second half of pregnancy [70]. This effect also appears to occur in mice, as relaxin KO mice exhibit higher plasma osmolality compared to wild-type mice over the last few days of pregnancy, indicating that relaxin may play a similar role in rats and mice [21].

Levels of relaxin-binding sites are high in regions known to be involved in central hemodynamic control, including the organum vasculosum (OVLT), SFO, SON, and PVN [60]. In 2001, Sunn et al. [71] investigated the effects of circulating relaxin on the laminar terminalis, a region of the brain involved in the regulation of body fluid and electrolyte homeostasis. Relaxin was found to activate neurons, as indicated by increases in Fos expression, in the outer region of the SFO, the OVLT, SON, and PVN. As a result, the role of these regions in mediating the dipsogenic effects of exogenous relaxin was investigated. Relaxin was found to directly stimulate neurons of the SFO to elicit the water drinking response, as ablation of the SFO and not the OVLT abolished relaxin-induced drinking [72] (Fig. 3). Additionally, ablation of the OVLT was found to decrease Fos expression in the SON and PVN in response to relaxin, suggesting that the OVLT mediates the relaxin-induced release of vasopressin from these regions [72].

Angiotensin has been implicated in the dipsogenic response to relaxin, as in 1999 Sinnayah et al. [73], demonstrated that the angiotensin 1 receptor antagonist, losartan, reduces the response to systemic exogenous relaxin and ablates the response to central exogenous relaxin. Additionally, angiotensin II when co-administered at a non-dipsogenic dose could potentiate the effects of relaxin [73]. The effects of relaxin on oxytocin and vasopressin secretion can also be partially blocked by an angiotensin II receptor antagonist, suggesting that this effect is also mediated by angiotensinergic pathways [74]. Hence, it is likely that angiotensinergic pathways originating in RXFP1-expressing neurons of the circumventricular organs mediate the effects of relaxin on both dipsogenesis and oxytocin and vasopressin secretion.

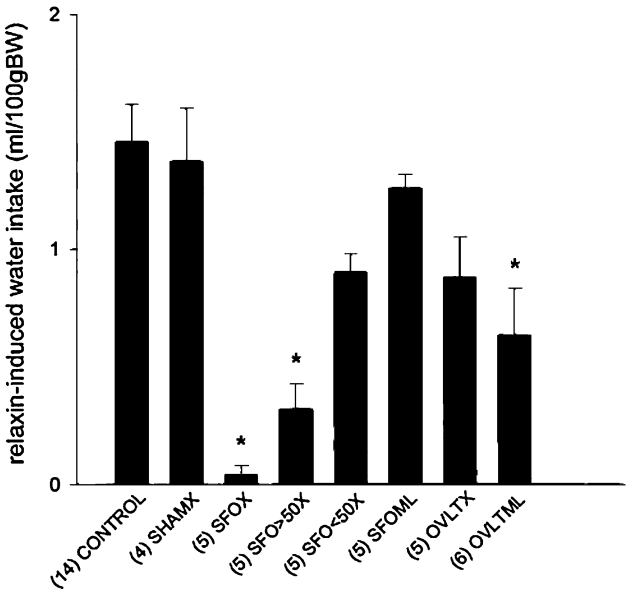

Fig. 3 Circulating relaxin acts on subfornical organ (SFO) neurons to stimulate water drinking in rats. Water intake during 90 min following i.v. infusion of human relaxin for 1 h in normal rats or rats with ablation of the SFO, OVLT, parts of the SFO, or tissue adjacent to these regions, or sham lesions. Mean and SEM are shown, and number of rats in each group are given in *parentheses*. Control, normal rats; SHAMX, sham lesions; SFOX, >90% of SFO ablated; SFO >50×, 50–90% of SFO ablated; SFO <50×, <50% of SFO ablated; SFOML, lesion missed SFO and damaged adjacent tissue; OVLTX, >90% of OVLT ablated; OVLTMX, lesion missed OVLT and ablated tissue in the adjacent regions. *$p < 0.05$ versus control group (from [72] with permission)

Central versus peripheral effects during pregnancy

As many of the hemodynamic effects of relaxin mediated through the CVOs are related to the cardiovascular changes necessary to maintain pregnancy, it is reasonable that circulating relaxin would be responsible for such effects. However, there are two key sets of experiments that potentially demonstrate the functional differentiation between the central and peripheral effects of relaxin during pregnancy. Passive immunization by peripheral administration of MCA1 caused significant disruption of the birthing process [75], whereas passive immunization by chronic ICV infusion of MCA1 resulted in a significant decrease in the length of gestation, in the absence of any effect on duration of straining or parturition, delivery interval, live birth rate, or newborn body weight [76]. Hence it is possible that central relaxin has pregnancy-specific actions that are not related to its peripheral actions. Furthermore, relaxin is also expressed in the rodent brain in areas unrelated to reproductive physiology, suggesting that relaxin produced in the central nervous system may act locally on receptors to exert other functions.

Neuronal effects

The brain relaxin system was first realized in the 1990s with the identification of relaxin mRNA, immunoreactivity, and binding sites in the brain. However, the

investigation of function was triggered by the localization of RXFP1 mRNA in the rat brain. In 2006, Ma et al. comprehensively mapped the distribution of RXFP1 expression in the male rat brain [61], finding that the distribution was closely correlated with the relaxin-binding sites described by Osheroff and Phillips (Fig. 2). The particularly high levels of RXFP1 mRNA identified in the basolateral complex of the amygdala (BLA), an area involved in regulating memory consolidation of emotionally arousing experiences [77], led to the investigation of whether relaxin could play a role in modulating memory consolidation. Relaxin was found to impair memory consolidation of an aversive-context in rats following intra-BLA injection [57]. This effect was proposed to be mediated by RXFP1 activation, but no specific agonist or antagonist studies have been conducted in order to confirm this possibility or to establish the endogenous source of relaxin for these receptors. The extensive expression of relaxin and RXFP1 in the brain suggest that there are many other potential functions for relaxin as a neuropeptide.

Relaxin-3: the neuropeptide

The distribution of relaxin-3 in the rat brain has been characterized in two separate studies utilizing different antibodies against relaxin-3 [48, 78] (Fig. 4). Relaxin-3 is expressed in a distinct group of neurons located in the nucleus incertus (NI), a group of cells also described as the dorsal tegmental nucleus pars ventromedialis [79], nucleus recessus pontismedialis [80] and nucleus "O" [81]. This result was consistent with the finding of relaxin-3 mRNA in the equivalent cells of the mouse brain. A small number of relaxin-3-expressing neurons are also present in the pontine raphe nucleus, the periaqueductal gray, and an area dorsal to the substantia nigra in the midbrain reticular formation [48]. Studies in relaxin-3 KO/LacZ knock-in mice demonstrate high levels of reporter gene expression in the soma and proximal extensions of the neurons of the NI and these other regions, indicating similarities between the relaxin-3 systems in rodents [82].

Recently, a study of relaxin-3 expression in zebrafish highlighted the similarities in expression between rodents and the non-mammalian vertebrate [83]. In post-embryonic zebrafish, relaxin-3 expression is restricted to two groups of neurons, the first near the fourth ventricle, which also express type 1 corticotrophin releasing factor receptor (CRF-R1) and the second in the PAG, as confirmed by co-localization with proenkephalin-like gene 1.

In the interest of progressing to studies of human relaxin-3, a recent paper describes the distribution of relaxin-3 in the macaque brain [84]. Immunohistochemistry and in situ hybridization revealed that relaxin-3 is located in a group of neurons equivalent to the rodent NI. The similarities in distribution between mammalian species suggest not only conservation of function, but implications for relaxin-3 in the human brain. A separate study using a different antibody against relaxin-3 has shown a similar pattern of relaxin-3 expression in the macaque brain [85].

The nucleus incertus

At the time of relaxin-3 discovery, a seminal paper on the NI had just been published. Goto et al. [86] described the NI as a region of the pontine periventricular grey, consisting of two visually distinct groups of cells, the pars compacta (NIc) and the pars dissipata (NId). The NIc lies within the caudoventral pontine grey, extending from the caudal pole of the dorsal raphe nucleus to the caudal end of the pontine periventricular gray [86]. The neurons are dense, medium-sized, multipolar, and deeply staining. NId neurons are also medium-sized, multipolar, and deeply staining, however, unlike NIc neurons, they are sparse and tend to be oriented mediolaterally, rather than dorsoventrally. The NId is located near the ventromedial border of the caudal dorsal tegmental nucleus and spans the same distance rostrocaudal as the NIc, increasing in area caudally [86].

Neurons of the NI have been characterized using immunohistochemical and cytochemical techniques. NI neurons express glutamate decarboxylase, the enzyme required for the synthesis of GABA [87, 88]. Cholecystokinin (CCK) antiserum specifically labels neurons of the NIc, whereas fewer neurons in the NId express CCK [88]. Additionally, the NId contains large acetyl cholinesterase-positive cells [88]. Relaxin-3-containing neurons were demonstrated to be GABAergic [48] and almost all co-express the CRF-R1 [78].

Although Goto et al. were not the first to describe the NI as we currently know it [79], their retrograde tracing studies lead us to conclude that the dissipata and compacta—while histologically distinct—form a functional group. Telencephalic afferents arise from the prelimbic, anterior cingulate, and orbital areas of the cortical mantle and the diagonal band of the septum. Inputs to the NI from the diencephalon arise from the medial region of the lateral habenular nucleus, rostral zona incerta, lateral pre-optic and lateral hypothalamic areas, posterior hypothalamic nucleus, and supramammillary nucleus. From the brainstem, inputs include the supraoculomotor region of the periaqueductal grey, superior central (median raphe) nucleus (CS), interpeduncular nucleus (IPN), and the retrolaterodorsal tegmental nucleus.

Using PHA-L anterograde tracing, Goto et al. revealed the efferent projections from the NIc and NId have near identical targets and can be divided into ascending and descending projections. The ascending group of projections

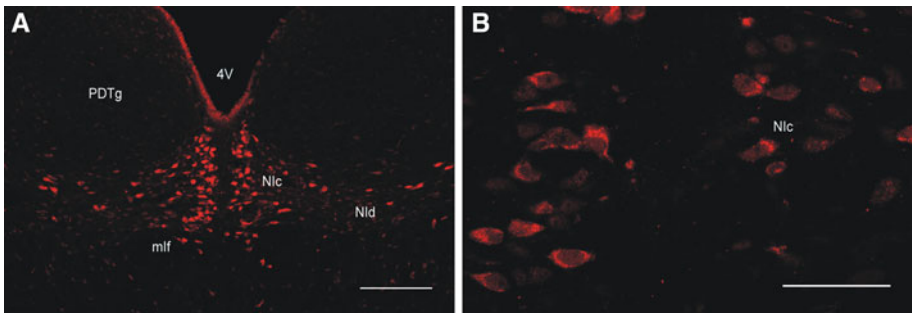

Fig. 4 The distribution of relaxin-3 immunoreactivity in the rat brain. **a**, **b** Representative confocal micrographs in coronal sections through the nucleus incertus (NI). **b** High-resolution image reveals staining in the cytoplasm of cells. *4V* fourth ventricle, *mlf* medial longitudinal fasciculus, *NIc* nucleus incertus pars compacta, *NId* nucleus incertus pars dissiparta, *PDTg* posterodorsal tegmental nucleus. *Scale bars* 200 μm (**a**) and 50 μm (**b**)

innervate targets rostral to the NI in the pons, midbrain, diencephalon, and telencephalon and the descending group innervates regions of the lower brainstem. Overall, the results demonstrate that the NI has strong bi-directional connections with the CS and the IPN. The authors hypothesize that this group of nuclei control the release of behavioral responses: the NI provides inhibitory inputs to both nuclei, thus suppressing the behavioral inhibition of the CS and IPN [86]. The presence of relaxin-3 in many of these NI projections and RXFP3 in target nuclei [48], indicates the relaxin-3/RXFP3 system is involved in this behavioral network and the modulation of locomotor behavior, attentive states, and learning.

Functions of the relaxin-3 and RXFP3 system

Stress

Recent interest in the NI was due to evidence that the NI is a periventricular site with the highest levels of CRF-R1 [89]. CRF-R1 is a GPCR that mediates neural responses to CRF, which is released in response to stress [90]. Thus it was no surprise when the first functional studies of relaxin-3 investigated the relationship between CRF, relaxin-3, and stress. Almost all relaxin 3-containing neurons in the NI co-express CRF-R1 and the response of relaxin-3 neurons to ICV administration of CRF has been investigated. In rats, after administration of CRF, 65% of relaxin-3 neurons showed elevated c-*fos* expression and after water-restraint stress, relaxin-3 mRNA in the NI was significantly increased [78]. Additionally, electron microscopy revealed that relaxin-3 is present in pre-synaptic vesicles of NI neurons. These results suggest that the relaxin-3 produced in the NI is released from nerve endings and is involved in the regulation of the stress response. Recently, a role for relaxin-3 in stress has been reinforced by a study demonstrating repeat forced swim stress increases relaxin-3 mRNA and heteronuclear RNA 30–60 min after the second swim, before a return to baseline levels within 2–4 h [91]. These effects could be blunted by pre-treatment with the CRF-R1 antagonist, antalarmin (Fig. 5).

Interestingly, the principal site of CRF production in brain is the PVN, which also expresses RXFP3 [78], which suggests RXFP3 may mediate actions of relaxin-3 on the CRF system at this level, but this remains to be demonstrated experimentally. Future studies will undoubtedly focus on the down-stream effects of elevated relaxin-3 levels in response to stress; particularly whether relaxin-3 release potentiates or inhibits stress pathways and which receptor and signaling pathway mediates such effects.

Feeding

The PVN is part of the hypothalamic-pituitary–adrenal axis and thus is a region crucial in the hypothalamic regulation of appetite. As a result, a number of recent studies have investigated the role of relaxin-3 in appetite. McGowan et al. [92] found that acute ICV and intra-PVN injections of relaxin-3 in male Wistar rats significantly increased feeding in the early light phase and dark phase. This effect was found to be specific to increases in feeding and not the consequence of increased spontaneous activity or arousal. In addition, relaxin administration was found to have no effect, indicating that the orexigenic effects of relaxin-3 are mediated by RXFP3, rather than RXFP1 [92].

Numerous hypothalamic nuclei express RXFP3 and subsequent experiments demonstrated that the relaxin-3 injection into the SON also increased feeding [93]. In the SON, expression of c-*fos*, a neuronal marker of activation, was increased 130% by relaxin-3 [93]. In the PVN, however, there was no difference in c-*fos* expression between rats receiving relaxin-3 and those receiving vehicle, despite the use of a dose tenfold greater than required to elicit a feeding response in the previous study. Therefore, further studies are required to determine the underlying cellular mechanisms associated with these changes in food intake.

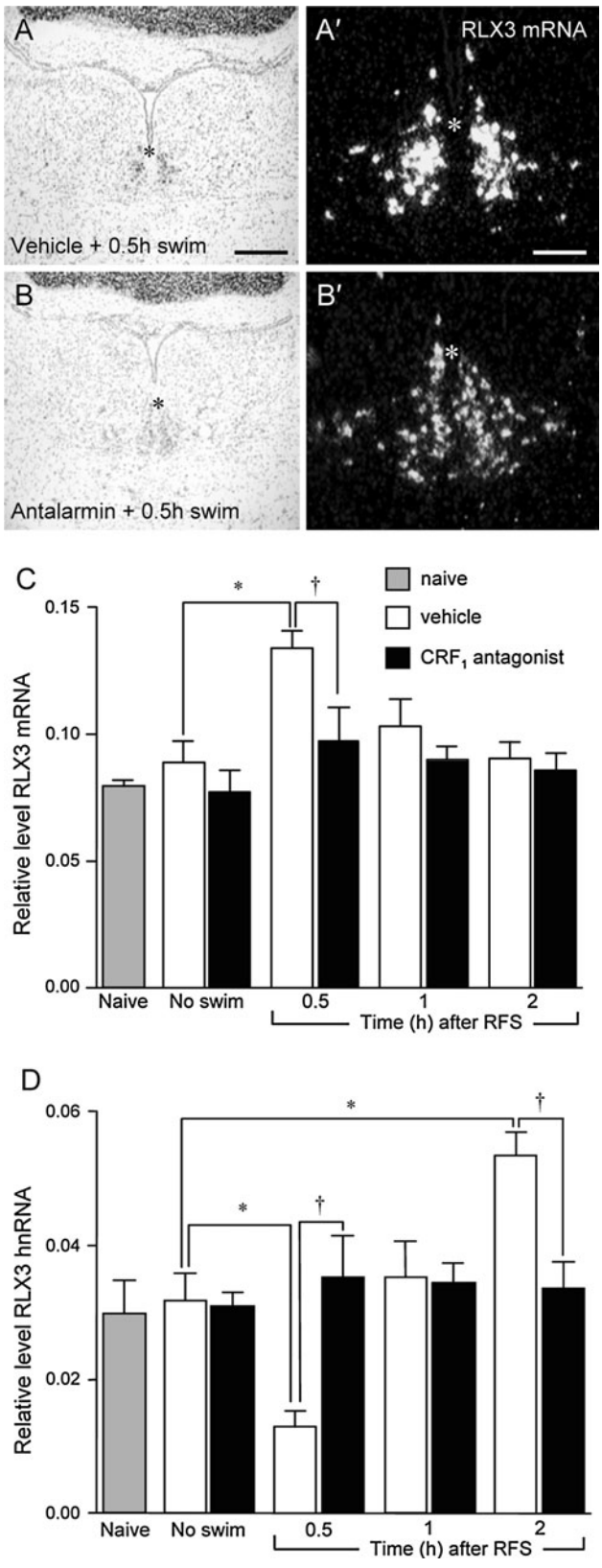

Fig. 5 The effect of repeat forced swim (RFS) on relaxin-3 mRNA and hnRNA in the rat nucleus incertus (NI) in the presence or absence of the CRF-R1 antagonist antalarmin. **a**, **b** Representative thionin-stained sections used to detect RLX3 mRNA in vehicle- and antalarmin-injected rats at 0.5 h after RFS and **a′**, **b′** corresponding high-power, darkfield digital micrographs of the NI in these sections. **c**, **d** Semi-quantitative analysis of the effects of antalarmin on (**c**) RLX3 mRNA and (**d**) hnRNA expression following RFS. Values are mean ± SEM, $n = 4$/group. RLX3 mRNA $^{*}p < 0.05$ versus control, $^{\dagger}p < 0.05$ versus antalarmin-treated; and RLX3 hnRNA $^{*}p < 0.05$ versus control, $^{\dagger}p < 0.05$ versus antalarmin-treated. *Scale bars*, 150 mm (**a**, **b**) 40 mm (**a′**, **b′**) (from [91] with permission, copyright Elsevier 2009)

In early 2006, McGowan et al. [94] extended their studies to examine the effects of chronic, repeated intra-PVN administration of relaxin-3 (Fig. 6). Chronic administration of relaxin-3 increases cumulative food intake (Fig. 6a) and plasma leptin, but does not significantly increase body weight. Over 7 days, relaxin-3 also suppresses plasma TSH, independent of food intake [94] (Fig. 6b). Hida et al. [95] found that 14 days of ICV infusion resulted in significant body weight gain, increases in food intake, and increased levels of plasma leptin and insulin. Both studies suggest that the orexigenic effects of relaxin-3 are independent of energy expenditure; however, comprehensive studies of TSH, BAT, and sympathetic nervous activity are required to substantiate such claims.

Such functional studies of the role of relaxin-3 in feeding currently lack the supporting mechanisms required to make conclusions about the role of the endogenous peptide in feeding behavior. More detailed studies focused on the neural, signaling, and gene-transcription changes that lead to the relaxin-3-induced feeding response will enable the differentiation between any confounding effects on reward, motivation, and arousal pathways and a true feeding response.

Behavioral activation and arousal

Currently, much of the evidence for a role for relaxin-3 in behavioral activation and arousal arises from the neuroanatomy of relaxin-3 projections and sites of RXFP3 expression. Serotonin (5-HT) has long been implicated in numerous cognitive, emotional, and behavioral control processes (reviewed in [96]). The proximity of the NI to the dorsal raphe, a region enriched in 5-HT neurons, led Miyamoto et al. [97] to investigate the effects of 5-HT on relaxin-3 expression. Immunohistochemistry revealed that most relaxin-3 neurons of the NI co-express the 5-HT type 1A receptor. Depleting 5-HT, using an inhibitor of 5-HT synthesis for 3 days, caused an increase in relaxin-3 mRNA in the NI. The events occurring between changes in serotonin levels and relaxin-3 expression remain to be explored.

Goto et al. demonstrated that the NI has strong reciprocal connections with the median raphe and the interpeduncular nuclei [86] and consequently proposed that

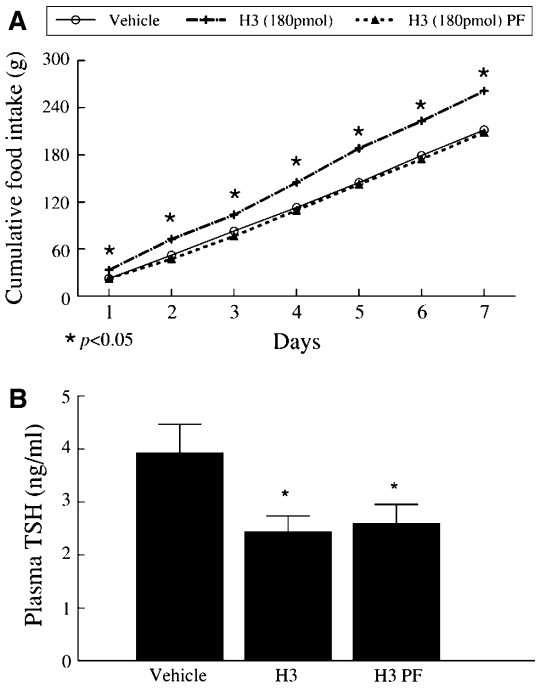

Fig. 6 The effect of chronic relaxin-3 administration on food intake and plasma TSH in rats. **a** Cumulative food intake after repeated intra-paraventricular nucleus (iPVN) administration of vehicle (*white circles*), human relaxin-3 (H3) in ad libitum fed rats (*plus sign*) or H3 in pair-fed (PF; *black triangle*) rats for 7 days. **b** Effect of repeated iPVN administration of vehicle, H3 in ad libitum-fed rats or H3 in pair-fed (H3PF) for 7 days on plasma TSH. *$p < 0.05$ versus vehicle, $n = 8$–11 (from [94] with permission, copyright Elsevier 2009)

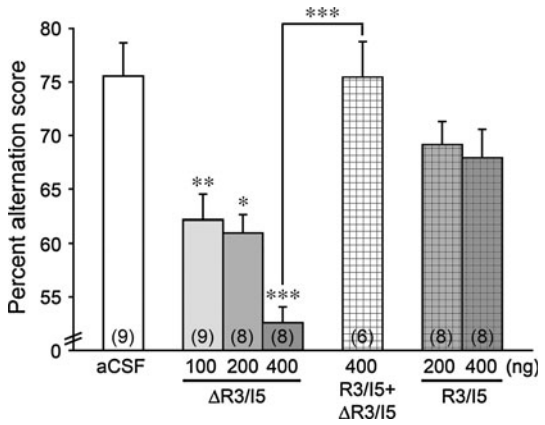

Fig. 7 Effects of RXFP3-specific agonist (R3/I5) and/or antagonist (R3(BΔ23–27)R/I5; ΔR3/I5) infusion on spatial working memory performance assessed in the spontaneous alternation task (SAT). Artificial cerebrospinal fluid (aCSF) vehicle or different concentrations of peptides (ng) were infused into the medial septum of rats 10 min prior to the SAT. Numbers of rats in each group ($n = 6$–9) are indicated in *brackets within the bars* on the figures (from [99] with permission, copyright 2009)

the NI forms a behavioral network that utilizes the neurotransmitter GABA and neuropeptides. The presence of relaxin-3 in the NI and the distribution of RXFP3 suggest they may be involved in this system and behavior such as exploration, orientation, navigation, locomotor control, and working memory.

Hippocampal theta rhythm, oscillations of 4–12 Hz, has been implicated in several of these behaviors and is modulated by "pacemaker" neurons in the medial septum (MS). The NI functions as a relay station in this process, as electrical stimulation of the NI induces theta rhythm in the hippocampus and lesion of the NI disrupts theta initiated by stimulation of the reticularis pontine oralis [98]. Consequently, the contribution of relaxin-3 to MS activity on theta rhythm and an associated learning behavior was investigated. In anaesthetized rats, infusions of the RXFP3 specific agonist, R3/I5, into the MS enhanced evoked field potentials and significantly increased spontaneous theta activity [99]. In conscious rats, MS infusions of R3/I5 and the RXFP3 antagonist R3(BΔ23–27)R/I5 [100], differentially modulated hippocampal theta activity. In a behavioral paradigm that investigates theta rhythm-dependent spatial working memory, infusions of R3(BΔ23–27)R/I5 impaired rats performance of the task in a dose-dependent manner [53] (Fig. 7).

Conclusions

The recent discoveries of the distribution of relaxin family peptides and their receptors in the central nervous system indicate that these peptides will more than likely have important functions in the brain. This is particularly relevant for relaxin-3, which has a highly conserved peptide structure and pattern of expression in the brain from fish to mammals. Current evidence based on the projections of the NI and the distribution of RXFP3 suggests that relaxin-3 is a neurotransmitter and is likely to have important roles in behaviors such as exploration, orientation, navigation, locomotor control, and working memory. The recent studies outlined already indicate relaxin-3 plays a role in stress regulation and spatial working memory. The availability of relaxin-3 and RXFP3 KO mice will aid in the determination of the precise roles of relaxin-3. Furthermore, recently developed viral systems to modulate relaxin-3 expression in adult rats [101] may prove useful in determining phenotypes that may otherwise be masked in the KO mice due to compensation by other genes during development. Behavioral studies with these animal models together with central injections of the specific RXFP3 agonists and antagonists will enable the determination of the specific roles of relaxin-3 in the central nervous system.

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