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Swim stress excitation of nucleus incertus and rapid induction of relaxin-3 expression via CRF_1 activation

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ABSTRACT

Relaxin-3 (RLX3), a newly identified member of the relaxin peptide family, is distinguished by its enriched expression in GABA projection neurons of the pontine nucleus incertus (NI), which are postulated to participate in forebrain neural circuits involved in behavioural activation and stress responses. In this regard, corticotrophin-releasing factor-1 receptor (CRF_1) is abundantly expressed by NI neurons; central CRF administration activates c-*fos* expression in NI; and various stressors have been reported to increase NI neuron activity. In studies to determine whether a specific neurogenic stressor would activate RLX3 expression, we assessed the effect of a repeated forced swim (RFS) on levels of RLX3 mRNA and heteronuclear (hn) RNA in rat NI by *in situ* hybridization histochemistry of exon- and intron-directed oligonucleotide probes, respectively. Exposure of rats to an RFS (10 min at 23 °C, 24 h apart), markedly increased RLX3 mRNA levels in NI at 30–60 min after the second swim, before a gradual return to basal levels over 2–4 h, while RLX3 hnRNA levels were significantly up-regulated at 60–120 min post-RFS, following a transient decrease at 30 min. Systemic treatment of rats with a CRF_1 antagonist, antalarmin (20 mg/kg, i.p.) 30 min prior to the second swim, blunted the stress-induced effects on RLX3 transcripts. Relative levels of RLX3-immunostaining in NI neurons appeared elevated at 3 h post-swim, but not at earlier time points (30–60 min). These results suggest that acute stress-induced CRF secretion can rapidly alter RLX3 gene transcription by activation of CRF_1 present on NI neurons. More generally, these studies support a role for RLX3 neural networks in the normal neural and physiological response to neurogenic stressors in the rat.

1. Introduction

Relaxin-3 (RLX3) was identified in human, mouse and rat genomes using a homology search strategy (Bathgate et al., 2002; Burazin et al., 2002) and subsequent structural and phylogenetic analyses indicated that RLX3 amino acid sequences are highly homologous across species and that RLX3 is the ancestral peptide of the relaxin family (Wilkinson et al., 2005). Soon after its discovery *in silico*, RLX3 was isolated and purified from porcine brain as the putative ligand for two type I G-protein coupled receptors – GPCR135 and GPCR142 (Liu et al., 2003a,b) (recently reclassified by IUPHAR as RXFP3 and RXFP4; see Bathgate et al., 2006). Extensive molecular and pharmacological studies subsequently revealed that RLX3 is the preferred endogenous ligand for GPCR135/(RXFP3) (Liu et al., 2003b; Sutton et al., 2004), and that another relaxin family peptide member, insulin-like peptide 5 (INSL5), is the native ligand for GPCR142/(RXFP4) (Liu et al., 2005). Notably, RXFP4 is a pseudogene in the rat (Liu et al., 2005) and negligible levels of INSL5 and RXFP4 are expressed in mouse brain (Sutton et al., 2005), highlighting the relative importance of the RLX3/RXFP3 system in rodent brain.

RLX3 mRNA was shown using *in situ* hybridization histochemistry to be primarily expressed in a discrete population of neurons in the brainstem of mouse and rat brain known as the *nucleus incertus* (NI) or *nucleus O* (Bathgate et al., 2002; Burazin et al., 2002; Liu et al., 2003b). Comprehensive, neuroanatomical tracing studies had earlier demonstrated that the NI was strategically positioned to regulate key neural circuits controlling behaviours including attentional state, stress responses and learning and memory via largely ascending projections to an extensive array of forebrain sites, including midbrain, limbic, thalamic and cortical areas,

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several of which return projections to the NI (Goto et al., 2001; Olucha-Bordonau et al., 2003). More recently, many of the distinct brain regions shown to receive NI projections have been shown to contain RLX3-immunoreactive nerve fibres (Tanaka et al., 2005; Ma et al., 2007) and variable densities of neuronal RXFP3 mRNA expression (Sutton et al., 2004; Ma et al., 2007). In fact, ultrastructural examinations revealed that RLX3-immunoreactivity (IR) was present in dense-core vesicles in neuronal soma and in terminals adjacent to synaptic junctions (Tanaka et al., 2005), strongly supporting the idea that RLX3 can be released to act as a neurotransmitter/modulator (see Kizawa et al., 2003) following appropriate activation of the NI.

Corticotrophin-releasing factor (CRF) is expressed in medial parvocellular neurons of the paraventricular hypothalamic nucleus (PVN) and elsewhere in brain, and plays a critical role in initiating hypothalamic- and extra-hypothalamic responses to stress via CRF-1 and -2 receptors ($CRF_{1/2}$) (see e.g. Herman and Cullinan, 1997; Bale and Vale, 2004; Korosi and Baram, 2008 for review). The abundant expression of CRF_1 mRNA by NI neurons (Potter et al., 1994) and evidence that central infusion of CRF produced strong activation of NI cells, reflected by increased Fos protein (Bittencourt and Sawchenko, 2000), raised interest in the anatomy and unknown function of the NI. It was reported that experimental situations producing acute 'psychological or neurogenic stress' and increased locomotor and behavioural activity activated NI neurons (e.g. rats swimming in a water tank searching for a platform, had high levels of Fos-positive cells in the NI, Goto et al., 2001). In line with these data, in preliminary studies we observed that c-*fos* mRNA was increased in NI neurons in rats subjected to the acute neurogenic stressor, swim stress (Cullinan et al., 1995; Dayas et al., 2001), with a stronger response after two swim sessions 24 h apart (Banerjee et al., 2005, unpublished data). Subsequent double-label immunofluorescence studies have revealed that the majority of RLX3-immunoreactive NI neurons co-express CRF_1-IR, confirming the potential for endogenous CRF-like peptides acting via CRF_1 (Bittencourt and Sawchenko, 2000; Bale and Vale, 2004) to activate NI neurons and alter RLX3 production and its utilization in rat brain (see Tanaka et al., 2005).

In this study, we examined the effect of a repeated forced swim (RFS) on RLX3 mRNA levels in NI. In view of the abundance of RLX3 mRNA, and as a potentially more sensitive indicator of rapid and/or long-lasting changes in RLX3 transcription in response to physiological stimuli, we examined heteronuclear RNA (hnRNA) levels, using [^{35}S]-oligonucleotide probes directed against the RLX3 intronic sequence (see Herman et al., 1991; Hofmann and Lyo, 2002). The potential involvement of CRF in the altered activity of RLX3-NI neurons observed was assessed by blocking CRF_1 with the antagonist, antalarmin (Lodge and Lawrence, 2003) prior to exposure of rats to a second FS. Finally, we qualitatively assessed the levels of RLX3-like-immunoreactivity (LI) in the NI at different times after RFS. Preliminary reports of these findings have been published in abstract form (Banerjee et al., 2005; Banerjee et al., 2007).

2. Materials and methods

2.1. Animals

All experiments were conducted according to ethical guidelines issued by the National Health and Medical Research Council of Australia and with the approval of the Howard Florey Institute Animal Welfare Committee. All efforts were made to minimize the number of animals used in this study. Male Sprague–Dawley rats (Australian Research Centre, Canning Vale, WA, Australia) weighing ~300 g were housed 3 per cage (a low stress condition) at the Howard Florey Institute animal facility and maintained on a 12:12 h light/dark cycle (lights on 7:00 a.m.) with access to food and water *ad libitum*.

2.2. Repeated forced swim

Commencing a week prior to behavioural testing, rats were acclimatized to the holding facility and adapted to handling and transportation from the holding room to the behavioural suite, to reduce non-specific stress. Rats ($n = 5$ per group) undergoing RFS were individually removed from home cages and tested in separate behavioural rooms before being group-housed overnight between swim sessions. RFS was conducted between 09:00 and 11:00 to avoid any possible response variations due to circadian activity cycles. On day 1, rats were subjected to a 10 min FS in a tall glass vessel (20 cm W × 28 cm D × 50 cm H) with curved corners with water added to a height of 40 cm at a temperature of 22–24 °C. On day 2, rats were exposed to the same protocol and killed at various time points (0.5, 1, 2, 4, 8 and 24 h) after the completion of the swim, to allow assessment of the time course of RLX3 neuronal responses and changes in other markers of stress-induced changes in brain activity, using *in situ* hybridization histochemistry. Relative levels of relaxin-3-LI were also assessed at selected post-swim times (see below). Control rats ($n = 5$) were handled and treated identically, but not swum and were killed across the same time course as RFS rats.

2.3. CRF_1 antagonist treatment

Antalarmin, *N*-butyl-*N*-ethyl-[2,5,6,-trimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-d] pyrimidin-4-yl]amine, a selective, centrally-acting CRF_1 antagonist (Tocris Bioscience, Bristol, UK; kindly provided by Prof AJ Lawrence, FNI) was dissolved in 10% ethanol and 10% Cremophor EL in dH_2O at 65 °C and cooled (Lodge and Lawrence, 2003). Antalarmin (20 mg/kg, 1 ml/kg, i.p.) or vehicle was administered to rats ($n = 4$/group), 40 min prior to the second FS, and rats were killed at 0.5, 1, 2 and 4 h thereafter.

2.4. Tissue preparation

For *in situ* hybridization histochemistry studies, rats were deeply anaesthetized with isoflurane and decapitated. Brains were rapidly removed and blocked in the coronal plane using the Watson and Paxinos Brain Blocker (David Kopf Instruments, Tujunga, CA, USA), then frozen over liquid nitrogen and stored at −80 °C. Consecutive coronal sections (10 μm) were cut on a Cryocut 1800 cryostat (Leica Microsystems, Heerbrugg, Switzerland) at −16 °C through the NI [and nearby LC] from −9.60 to −10.10 mm relative to Bregma [and through the PVN (14 μm) at −2.70 to −2.20 mm relative to bregma] (Paxinos and Watson, 1986) and thaw-mounted on microscope slides coated in 0.01% poly-L-lysine. During sectioning, brain regions and anatomical coordinates were identified by microscopic examination of tissue sections counter-stained with 1% thionin and with reference to a stereotaxic atlas (Paxinos and Watson, 1986).

For immunohistochemistry studies, rats ($n = 4$/group) were killed at 0.5, 1 and 3 h after the second swim, along with a group of control, no swim rats by isoflurane inhalation and perfused transcardially with ~100 ml ice-cold PBS solution (PBS: 137 mM NaCl, 2.7 mM KCl, 11.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) followed by ~300 ml ice-cold 4% paraformaldehyde in PBS (pH 7.4) and then decapitated. Brains were dissected and immersed in fixative for 1 h at 4 °C before cryoprotection in 20% sucrose in PBS overnight at 4 °C. Tissues were then frozen over liquid nitrogen and stored at −80 °C. Coronal sections (40 μm) were cut on a cryostat at −16 °C through the NI from −9.60 to −10.10 mm relative to Bregma (Paxinos and Watson, 1986) and collected into PBS immediately before immunohistochemical processing.

2.5. In situ *hybridization histochemistry*

In situ hybridization was performed essentially as described (Shen et al., 1995; Burazin et al., 2002). Briefly, frozen sections were dehydrated, delipidated in chloroform, rinsed in 100% ethanol, and stored in 100% ethanol at 4 °C. RLX3 mRNA was detected with a mixture of three synthetic oligonucleotide probes complementary to different regions of the rat RLX3 cDNA and six oligonucleotide probes were designed against the single intronic sequence of RLX3 cDNA to detect RLX3 hnRNA (Table 1) (Burazin et al., 2002). C-*fos* was detected with a mixture of two oligonucleotides complementary to different regions of the rat c-*fos* cDNA (Shen et al., 1995) and CRF mRNA was detected with a mixture of two synthetic oligonucleotides complementary to different regions of the rat/mouse CRF cDNA (Table 2). Probes were routinely 3′-end labeled with α-[^{35}S]deoxy-adenosine 5′-triphosphate (dATP; 1200 Ci/mmol; Perkin–Elmer, Boston, USA) by incubation with terminal deoxynucleotidyl transferase (TdT; Roche Diagnostics, Sydney, Australia) at 37 °C for 15–30 min to yield an average specific activity of 1–2 × 10^9 dpm/μg.

Sections were hybridized at 42 °C overnight with the different probes (2 pg/μl; 65 μl per slide) in a buffer consisting of 50% formamide; 4 × saline sodium citrate (SSC, 0.6 M sodium chloride, 0.06 M sodium citrate, pH 7.0); 10% dextran sulfate and 0.2 M dithiothreitol (DTT). The authenticity of the hybridization was assessed by demonstration that the signal could be successfully blocked in all areas, except those which correspond to non-specific binding and background, by the addition of a 100-fold excess of unlabeled probes to the hybridization buffer. After hybridization, slides were washed in 1 × SSC at 55 °C for 1 h to remove excess unbound probe, rinsed in fresh 1 × SSC and 0.1 × SSC, dehydrated in 70% and 95% ethanol and