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Potential hypothalamic targets of relaxin-3 innervation: a perspective

Craig M. Smith[1,2,3], Berenice E. Chua[1], Andrew W. Walker[1,3], Andrew L. Gundlach[1,2,3,*]

[1]The Florey Institute of Neuroscience and Mental Health, [2]Florey Department of Neuroscience and Mental Health, [3]Department of Anatomy and Neuroscience, The University of Melbourne, Victoria 3010, Australia

Summary

Relaxin-3 is a recently identified neuropeptide transmitter primarily expressed by neurons of the pontine *nucleus incertus*, which binds/activates the $G_{i/o}$-protein coupled receptor, RXFP3. Functional studies have demonstrated that relaxin-3 modulates behavioural arousal in rodents, and although initial anatomical mapping studies have revealed relaxin-3-positive projections within several brain regions containing neurons that control behavioural arousal, further analysis of this topography has been hampered by the unavailability of a suitable specific RXFP3 antibody. In an effort to determine some of the neuron populations that relaxin-3 signalling directly modulates, we examined the distribution of relaxin-3 immunoreactive nerve fibres/terminals within the mouse lateral hypothalamus (LH) and ventrolateral preoptic area (VLPO), relative to elements containing protein markers for arousal-related neurons. Within the LH, relaxin-3 fibres were predominately distributed more laterally than orexin immunoreactive neurons, in the so-called 'parvalbumin-immunoreactive' PV1 region; but direct contacts with these parvalbumin neurons were scarce. Within the VLPO, relaxin-3 fibres were observed in close contact with galanin-immunoreactive elements, but the soma of the galanin/GABA neurons in the area that project to and inhibit arousal-promoting neurons such as orexin/LH cells to promote sleep, were not identified under the conditions employed. Nonetheless, these preliminary studies suggest an interaction between relaxin-3 and VLPO galanin neurons that may contribute to the arousal-promoting action of relaxin-3.

Key words

Relaxin-3, lateral hypothalamus, ventrolateral preoptic area, galanin, parvalbumin, immunohistochemistry

Introduction

Relaxin-3 is a 51 amino acid neuropeptide transmitter that is primarily expressed by GABAergic neurons within the pontine *nucleus incertus* (Ma et al., 2007). The cognate receptor for relaxin-3 is the $G_{i/o}$-protein-coupled receptor, RXFP3 (Bathgate et al., 2006), and several *in vitro* and *in vivo* studies indicate that RXFP3 activation likely inhibits target neurons, which may act synergistically with co-released GABA. Existing neuroanatomical and functional evidence suggests a primary role of relaxin-3/RXFP3 signalling is the modulation of behavioural arousal, which encompasses the control of sleep/wake states and other related modalities (Ma et al., 2009; Smith et al., 2011, 2012).

* Corresponding Author: The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Victoria 3010, Australia; Email: andrew.gundlach@florey.edu.au; Ph: +61 3 9035 6507; Fax: +61 3 9035 0321.

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The neuroanatomical distribution of relaxin-3 immunoreactive fibres has been mapped relative to structural markers within the rat and mouse brain (Ma et al., 2007; Smith et al., 2010). However, due to the current unavailability of a suitable RXFP3 antisera, a need exists to further examine the distribution of relaxin-3 projections relative to neurochemical markers, in order to more clearly define the targets of relaxin-3 action and provide putative neural substrates for the arousal-mediating actions of relaxin-3/RXFP3 signalling in rodents (e.g. Olucha-Bordonau et al., 2012). The present studies aimed to address this goal, by conducting an initial survey of the topography of relaxin-3 fibres relative to neural markers in two arousal-associated brain regions of the mouse, the lateral hypothalamus (LH) and the ventrolateral preoptic area (VLPO). The LH contains orexin expressing neurons which promote behavioural arousal, wakefulness and feeding (Sakurai, 2007); and neurons that express the calcium binding protein, parvalbumin (PV), which notably is expressed by a population of glutamatergic neurons in the 'PV-immunoreactive' (PV1) region (Meszar et al., 2012) that contains strong relaxin-3 staining (Smith et al., 2010). The VLPO contains sleep-active, GABA/galanin-positive neurons which project to and inhibit the major arousal- and wake-promoting neuron populations in the brain (Gaus et al., 2002).

Methods

Brains were harvested from adult, male C57BL/6J mice (n = 3) after transcardial perfusion fixation (4% PFA) and post-fixation for 1 h. Following cryoprotection in 20% sucrose overnight, brains were frozen, sectioned (40 um) and subjected to standard fluorescence immunohistochemistry (see Ma et al., 2007). *Primary antibodies:* Mouse monoclonal α-relaxin-3 (raw culture media from a cell line provided by the International Patent Organism Depositary (IPOD), Japan, 1:5 dilution) (see Tanaka et al., 2005); goat α-orexin-A (Santa Cruz Biotechnology, CA, USA, 1:500 dilution); goat α-parvalbumin (Swant, Switzerland, 1:2,000 dilution); and rabbit α-galanin (Peninsula Laboratories, CA, USA, 1:2,000 dilution). *Secondary antibodies*: Donkey α-mouse DyLight594 and donkey α-rabbit DyLight649 (Jackson Immunoresearch, PA, USA); and donkey α-goat Alexa488 (Invitrogen, CA, USA) (all 1:500 dilution). Images were obtained by conventional confocal microscopy.

Results

Within the LH, relaxin-3-positive fibres were especially abundant within lateral areas – particularly throughout the PV1 region (Fig. 1A), but the identity of the neurons targeted by these fibres remains unknown, as only very limited overlap was observed between relaxin-3 fibres and PV-positive elements. Relaxin-3 fibres were also distributed laterally to orexin-positive neurons/fibres (data not shown), again suggesting very limited, if any, direct interactions under basal physiological conditions.

A high density of relaxin-3 immunoreactive fibres was observed within the lateral preoptic area (LPO; Fig. 1B). At the outer ventral, medial and lateral borders of this main plexus of relaxin-3 fibres, an intermingling of galanin-positive fibres was observed. Within the VLPO, where a large number of relaxin-3 fibres were observed,

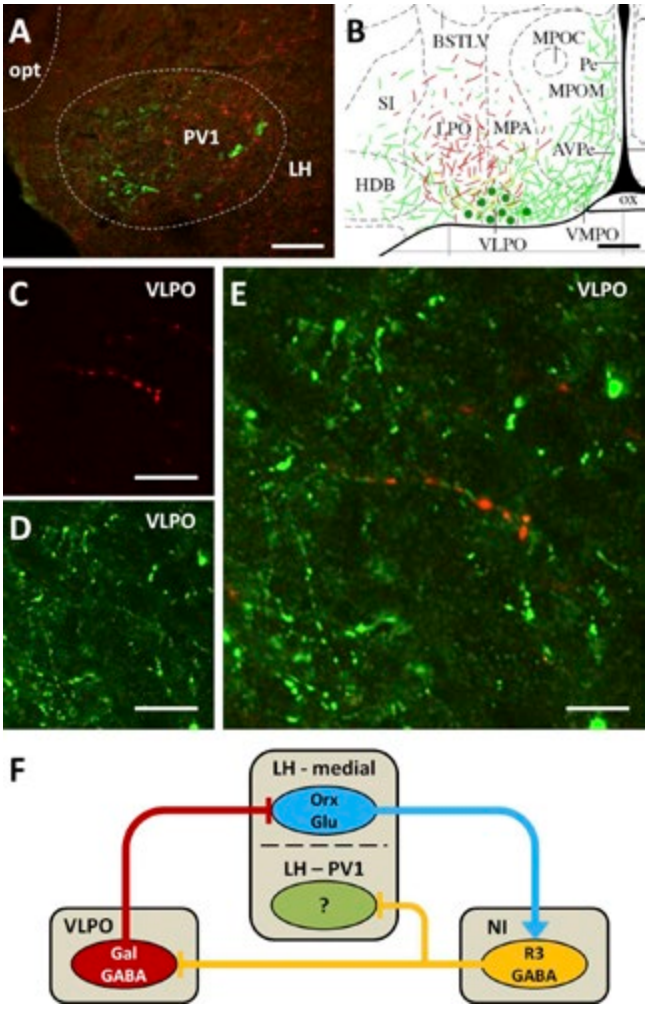

Figure 1. A. Low power micrograph of the distribution of relaxin-3 immunoreactive fibres (red) within the parvalbumin neuron-positive PV1 region of mouse lateral hypothalamus (LH) (green cells). B. Schematic representation of the distribution of relaxin-3 immunoreactive fibres (red lines), galanin immunoreactive fibres (green lines), and the approximate location of close-adjacent galanin and relaxin-3 immunoreactivity (yellow lines) within the preoptic region of the mouse brain. The approximate location of sleep-active, galanin-positive neurons within the VLPO, based on literature reports (Gaus et al., 2002), is also shown (green dots). High-power confocal micrographs illustrating relaxin-3 (red, C), and galanin (green, D) immunoreactivity within the mouse ventrolateral preoptic area (VLPO), with a larger merged image shown in (E). F. Summary of putative circuit whereby relaxin-3 (R3)/GABA neurons within the nucleus incertus (NI) project to galanin (Gal)/GABA neurons within the VLPO, and to an undefined neuronal population within the PV1 region of the LH. Direct innervation of orexin (Orx) neurons in the more medial portion of the LH by relaxin-3 fibres may be minimal, but VLPO galanin/GABA neurons are known to directly inhibit LH orexin/glutamate (Glu) neurons, which can directly activate relaxin-3 neurons in the nucleus incertus. Abbreviations: AVPe, anteroventral periventricular nucleus; BSTLV, bed nucleus of the stria terminalis, lateral division, ventral part; HDB, horizontal limb of the diagonal band; LPO, lateral preoptic area; MPA, medial preoptic area; MPOC/M, medial preoptic nucleus, central/medial part; opt, optic tract; ox, optic chiasm; SI, substantia innominata; VMPO, ventromedial preoptic nucleus. A. Bregma -2.06 mm; B-E. Bregma 0.02 mm. Scale bars (μm): A 100; B 250; C, D 20; E 10.

these were often adjacent to galanin-positive fibres (Fig. 1C-E). However, there was no evidence of galanin-positive cell bodies in the material examined.

Discussion

These preliminary studies suggest that relaxin-3 neurons innervate galanin-positive elements associated with galanin neurons within the VLPO. However, in line with similar studies that have examined galanin immunoreactivity in non-colchicine-treated mice, only galanin fibres, and not cell bodies, were observed within the VLPO (Perez et al., 2001). Colchicine treatment prior to brain perfusion may improve the detection of these galanin neurons, but may also reduce the efficiency of detection of relaxin-3 positive fibres. Alternatively, staining brains from mice following disruption of sleep patterns to facilitate increased galanin neuron activity may assist their detection. Thereafter confocal and/or electron microscopic analysis of combined relaxin-3, synaptic protein and galanin staining should allow confirmation of whether these neurons receive a relaxin-3 innervation (see e.g. Olucha-Bordonau et al., 2012).

If proven, this represents a potentially powerful mechanism through which relaxin-3 may promote arousal, as a major target of these inhibitory galanin/GABA neurons is the orexin neurons in the LH (Yoshida et al., 2006); and it is possible that relaxin-3 promotes arousal by disinhibiting orexin neurons via direct inhibition of galanin/GABA VLPO neurons. Preliminary evidence that LH orexin neurons directly innervate and activate relaxin-3 neurons (Blasiak et al., 2010), provides further evidence for this putative neural circuit (Fig. 1F).

At present, the identity of RXFP3 expressing neurons within the LH is unknown, but resolving this question is of interest, as although little is known about the PV1 region, strong efferent projections to major arousal centres including the periaqueductal grey and dorsal tegmental nucleus have been reported (Meszar et al., 2012), and PV1 neurons express other neuropeptides with putative roles in arousal, such as PACAP (Girard et al., 2011).

In conclusion, further detailed anatomical studies are required to provide insights into the identity of neurons targeted by the broad relaxin-3 innervation of the hypothalamus and other brain areas, in order to reveal the potential mechanisms through which relaxin-3 signalling may modulate arousal and behavioural state control.

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References

Bathgate R.A.D., Ivell R., Sanborn B.M., Sherwood O.D., Summers R.J. (2006) International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. Pharmacol. Rev. 58: 7-31.

Blasiak A., Gundlach A.L., Lewandowski M.H. (2010) Orexins increase nucleus incertus neuronal activity: implications for a possible reinforcing arousal drive. 7th Forum of European Neuroscience (FENS) Abstract. 5: 104.2.

Gaus S.E., Strecker R.E., Tate B.A., Parker R.A., Saper C.B. (2002) Ventrolateral preoptic nucleus contains sleep-active, galaninergic neurons in multiple mammalian species. Neuroscience. 115: 285-294.

Girard F., Meszar Z., Marti C., Davis F.P., Celio M. (2011) Gene expression analysis in the parvalbumin-immunoreactive PV1 nucleus of the mouse lateral hypothalamus. Eur J Neurosci. 34: 1934-1943.

Ma S., Bonaventure P., Ferraro T., Shen P.J., Burazin T.C.D., Bathgate R.A.D., Liu C., Tregear G.W., Sutton S.W., Gundlach A.L. (2007) Relaxin-3 in GABA projection neurons of nucleus incertus suggests widespread influence on forebrain circuits via G-protein-coupled receptor-135 in the rat. Neuroscience. 144: 165-190.

Ma S., Olucha-Bordonau F.E., Hossain M.A., Lin F., Kuei C., Liu C., Wade J.D., Sutton S.W., Nunez A., Gundlach A.L. (2009) Modulation of hippocampal theta oscillations and spatial memory by relaxin-3 neurons of the nucleus incertus. Learn Mem. 16: 730-742.

Meszar Z., Girard F., Saper C.B., Celio M.R. (2012) The lateral hypothalamic parvalbumin-immunoreactive (PV1) nucleus in rodents. J Comp Neurol. 520: 798-815.

Olucha-Bordonau F.E., Otero-Garcia M., Sanchez-Perez A.M., Nunez A., Ma S., Gundlach A.L. (2012) Distribution and targets of the relaxin-3 innervation of the septal area in the rat. J. Comp. Neurol. 520: 1903-1939.

Perez S.E., Wynick D., Steiner R.A., Mufson E.J. (2001) Distribution of galaninergic immunoreactivity in the brain of the mouse. J Comp Neurol. 434: 158-185.

Sakurai T. (2007). The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. Nat Rev Neurosci. 8: 171-181.

Smith C.M., Hosken I.T., Sutton S.W., Lawrence A.J., Gundlach A.L. (2012) Relaxin-3 null mutation mice display a circadian hypoactivity phenotype. Genes Brain Behav. 11: 94-104.

Smith C.M., Ryan P.J., Hosken I.T., Ma S., Gundlach A.L. (2011) Relaxin-3 systems in the brain - the first 10 years. J Chem Neuroanat. 42: 262-275.

Smith C.M., Shen P.J., Banerjee A., Bonaventure P., Ma S., Bathgate R.A.D., Sutton S.W., Gundlach A.L. (2010) Distribution of relaxin-3 and RXFP3 within arousal, stress, affective, and cognitive circuits of mouse brain. J Comp Neurol. 518: 4016-4045.

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

Yoshida K., McCormack S., Espana R.A., Crocker A., Scammell T.E. (2006). Afferents to the orexin neurons of the rat brain. J Comp Neurol. 494: 845-861.