Relaxin-3 projection on serotonergic centers in the rat brain.

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Resumen

La modulación de los sistemas cognitivos y emocionales depende de las conexiones subcorticales ascendentes en las estructuras telencefálicas, incluidas las proyecciones que surgen de los centros catecolaminérgicos, serotoninérgicos y peptidérgicos. La relaxina-3 (RLN3) es un neuropéptido que se produce principalmente en el núcleo incertus (NI) del tegmento pontino. Aunque los núcleos del rafe, los principales centros que contienen neuronas de serotonina (5HT), reciben fibras procedentes del NI, las interacciones de la RLN3 con el sistema 5HT sólo se han estudiado en el desarrollo postnatal. Nuestra hipótesis es que el sistema 5HT interactúa con el sistema relaxinérgico. Nuestro objetivo es comprender el sustrato anatómico de la proyección de RLN3 sobre los núcleos del rafe en ratas. Para ello, se ha realizado una inmunofluorescencia cuádruple para analizar las relaciones anatómicas de RLN3, del transportador vesicular de glutamato (vGlut2), del transportador vesicular de GABA (vGAT) y de 5HT. En este estudio, hemos cuantificado el número de posibles contactos de RLN3 en estructuras positivas a la 5HT y el número de estos contactos asociados a proyecciones GABAérgicas y glutamatérgicas. Los resultados preliminares apuntan a la ocurrencia efectiva de una alta proporción de fibras RLN3 que contienen marcadores vGlut2 y que contactan con células 5HT-positivas, estando una proporción menor asociada a vGAT. Además, la mayoría de las neuronas 5HT son también vGluT2-positivas. Los datos preliminares indican, por tanto, un papel relevante del sistema RLN3 en la funcionalidad de los sistemas 5HT.

Palabras clave: Relaxina-3, serotonina, núcleos del Rafe, GABA, glutamato.

Resum

La modulació dels sistemes cognitius i emocionals depenen de les connexions subcorticals ascendents a les estructures telencefàliques, incloses les projeccions que sorgeixen dels centres catecolaminèrgics, serotoninèrgics i peptidèrgics. La relaxina-3 (RLN3) és un neuropèptid que es produeix principalment al nucli incertus (NI) del tegment pontí. Tot i que els nuclis del rafe, els principals centres que contenen neurones de

serotonina (5HT), reben fibres procedents del NI, les interaccions de la RLN3 amb el sistema 5HT només s'han estudiat en el desenvolupament postnatal. La nostra hipòtesi és que el sistema 5HT interactua amb el sistema relaxinèrgic. El nostre objectiu és comprendre el substrat anatòmic de la projecció de RLN3 sobre els nuclis del rafe en rates. Per això, s'ha realitzat una immunofluorescència quàdruple per analitzar les relacions anatòmiques de RLN3, del transportador vesicular de glutamat (vGlut2), del transportador vesicular de GABA (vGAT) i de 5HT. En aquest estudi hem quantificat el nombre de possibles contactes de RLN3 en estructures positives a la 5HT i el nombre d'aquests contactes associats a projeccions GABAèrgiques i glutamatèrgiques. Els resultats preliminars apunten a l'ocurrència efectiva d'una alta proporció de fibres RLN3 que contenen marcadors vGlut2 i que contacten amb cèl·lules 5HT-positives, i una proporció menor és associada a vGAT. A més, la majoria de les neurones 5HT són també vGluT2-positives. Les dades preliminars indiquen, per tant, un paper rellevant del sistema RLN3 a la funcionalitat dels sistemes 5HT.

Paraules clau: Relaxina-3, serotonina, nuclis del Rafe, GABA, glutamat.

Abstract

The modulation of cognitive and emotional systems depends on ascending subcortical connections on telencephalic structures including projections arising from catecholaminergic, serotoninergic and peptidergic centers. Relaxin-3 (RLN3) is a neuropeptide that is mainly produced in the nucleus incertus (NI) of the pontine tegmentum. Although the raphe nuclei, the major centers containing serotonin (5HT) neurons, receive fibers arising from the NI, the interactions of RLN3 with the 5HT system have only been studied in postnatal development. We hypothesize that the 5HT system interacts with the relaxinergic system. We aim to understand the anatomical substrate of RLN3 projection on the raphe nuclei in rats. For this purpose, quadruple immunofluorescence has been performed to analyze the anatomical relationships of RLN3, vesicular glutamate transporter (vGlut2), vesicular GABA transporter (vGAT) and 5HT. In this study, we have quantified the number of possible RLN3 contacts on 5HT-positive structures and the number of these contacts associated with GABAergic and glutamatergic projections. Preliminary results point to the effective occurrence of a high proportion of RLN3 fibers containing vGlut2 markers and contacting with 5HT-positive cells, a smaller proportion being associated with vGAT. Moreover, the majority of 5HT neurons are also vGluT2-positive. Preliminary data indicate, therefore, a relevant role of the RLN3 system in the functionality of 5HT systems.

Key Words: Relaxin-3, Serotonin, Raphe nucleus, GABA, glutamate.

1 Introduction

Ascending neuromodulatory systems which includes serotonergic, noradrenergic, cholinergic, and dopaminergic projections arise from subcortical structures and targets forebrain centers involved in emotion and memory processing centers. Specifically, neuromodulators are involved in rewards, risk, effort, arousal, or social cooperation. These neuromodulatory systems adapt the performance of higher cognitive functions such as decision making, goal-directed behaviour, attention, or emotion to the special contextual conditions in which the animal is living, for example, stressful conditions or quiet moments (Avery & Krichmar, 2017). Moreover, drugs affecting the serotonin (5HT) metabolism have been shown to have an effect in modulating control over feeding behaviour, where it has been used in obesity treatment and proved to be effective (Magalhães et al., 2010). Also, serotonin reuptake inhibitors have been widely used as antidepressants (Jakubovski et al., 2016), although the exact mechanism in which this process is being done has been questioned recently (Vashadze, 2006). These projections, especially 5HT which originates in Raphe nuclei, extend to virtually all regions of the forebrain, including the amygdala, hippocampus, ventral striatum, and cortex (Avery & Krichmar, 2017). Although it originates in the raphe nuclei and is usually described as a serotonergic structure, the raphe nuclei also contains large amounts of non-serotonergic cells like neurons of dopamine, GABA, glutamate, nitric oxide, and different types of neuropeptides (Olivier, 2015).

Together with other neuromodulatory systems, 5HT becomes a key piece in modulating decision-based functions like reward and cost assessment, anxiety, impulsivity and helps avoiding harm situations (Asher et al., 2013). Moreover, problems with the serotoninergic system have been associated to Parkinson's disease, and with the serotoninergic hypothesis to anxiety and depression (Avery & Krichmar, 2017). In contrast, recent studies observed that 5HT is erroneously associated as the cause of depression (Moncrieff et al., 2022).

Another type of neuromodulators are neuropeptides, which can have a homeostatic function on processes like stress, appetite, feeding and the circadian rhythm of the sleep-wake cycle. Likewise, they have modulatory functions in anxiety disorders or depression (as does 5HT). They have modulatory function of synaptic and neuronal function. Thus, neuropeptides could be a good element to treat disorders displaying different symptoms (Smith et al., 2011). For example, oxytocin is being considered in the treatment for anorexia nervosa and autism (Plessow et al., 2018), BDNF could be used in the treatment for psychiatric disorders (Wang et al., 2022), also corticotropin releasing hormone (CRF) together with vasopressin could coordinate behavioural adaptation to stress making it a possible treatment for mood disorders such as depression or anxiety disorders (Holsboer & Ising, 2021; Kritas et al., 2014). Similarly, CRF, melanocortin and opioid peptide could be used in the development and

maintenance of substance-induced hyperalgesia for the treatment of substance use disorders (Delery & Edwards, 2020). Other neuropeptides such as neurotensin, opioid peptides, tachykinins and bradykinin could be used for psychiatric disorders such as schizophrenia (Rodríguez et al., 2020).

We have focused our work on the relaxin-3 (RLN3) neuropeptide that is co-expressed with GABA in the nucleus incertus (NI) of the pontine tegmentum (Ma et al., 2007). Data on the functionality of the system indicate that it exerts an action on stress response, neuroendocrine function, learning and memory processes, social behaviour, and food intake. (García-Díaz et al., 2021; Ryan et al., 2011; Smith et al., 2011). To carry out these roles, the NI displays a wide variety of projections to relevant areas controlling these functions including the prefrontal cortex, the raphe nuclei, the periaqueductal grey, the supramammillary area, diverse nuclei in the hypothalamus, the medial septum, hippocampus and the amygdala (Goto et al., 2001; Olucha-Bordonau et al., 2003). This pattern of connections mimics the distribution of the RLN3 positive fibers (Ma et al., 2007; Tanaka et al., 2005). Additionally, it was found that the natural receptor for RLN3 is the G protein coupled receptor RXFP3 which acts as a Gio (Bathgate et al., 2002; Burazin et al., 2002). The distribution of the receptor is quite similar although no identical to that of RLN3 fibers (Ma et al., 2007; Smith et al., 2009). The activation of this receptor induces the phosphorylation of erk (Albert-Gascó et al., 2017; Albert-Gasco et al., 2019) and the inhibition of adenylate cyclase (Bathgate et al., 2005).

RLN3 is also present in smaller populations of neurons in the pontine raphe nuclei (17.5% relative to the NI), the medial and ventrolateral periaqueductal gray matter (27.5% relative to the NI), and the substantia nigra in its lateral division (17.5% relative to the NI) (Ma et al., 2017). Additionally, the main inputs to the NI come from the midline of the periaqueductal gray matter, the prefrontal cortex, the lateral habenula, the interpeduncular nucleus, the median raphe nuclei, and the lateral hypothalamus (Goto et al., 2001).

Neuroanatomical data (Goto et al., 2001; Olucha-Bordonau et al., 2003) indicate that the NI is the origin of a system of ascending projections that successively contacts to nuclei involved in the generation and/or modulation of the hippocampal theta rhythm, including the raphe nuclei, the supramammillary nucleus and the medial septum (Vertes & Kocsis, 1997). Theta rhythms are part of brain oscillatory waves that appear as a result of synchronic activity in a great proportion of neurons of a given area. Specifically, the medial septum is considered as the pacemaker of the hippocampal theta rhythm since a lesion of that area results in impairment of the hippocampal theta. Additionally, the MS itself contains neurons that fire at a theta frequency and projects to hippocampus and entorhinal cortex spreading the synchronized wave (Petsche et al., 1962). Theta rhythm is associated with behavioural performances requiring active

voluntary (nonstereotyped) movement. This wave also appears during stimulus intake, at the same time as saccadic eye movements in humans, and also occurs during REM sleep (Colgin, 2013). Additionally, theta rhythms favour rhinal transfer from the hippocampus to the neocortex, which could lead to the strengthening of the links between neurons that represent different components of an experience. This makes easier to be remembered. Thus, theta rhythm would influence learning (Paz et al., 2008).

It was shown that the NI is able to induce hippocampal theta as electrical stimulation of the nucleus decreased delta waves and increased theta in urethane anesthetized animals (Nuñez et al., 2006). Moreover, infusion of a RLN3 agonist into the medial septum had the same effect as the electrical stimulation of the NI, and conversely, infusion of a RLN3 antagonist prevented the increase of theta obtained by electrical stimulation of the reticularis pontis oralis (Ma et al., 2009). Thus, the NI projection system via RLN3 in the medial septum may modulate or induce the theta rhythm, and this activity is associated with spatial working memory, a function of theta rhythms (Ma et al., 2009; Nuñez et al., 2006).

On the other hand, it has been found that the raphe nuclei had an antagonistic action, compared to the NI, on theta activity. Stimulation of the median raphe inhibited theta activity while its inactivation increased it (di Prisco et al., 2002; Kocsis & Vertes, 1996). Thus, the raphe nuclei had a direct control over the hippocampus EEG, making this structure a modulator of the hippocampal electroencephalogram (EEG) at the level of synchronization and desynchronization of these theta rhythms during learning and spatial working memory (di Prisco et al., 2002).

In spite of a putative common developmental origin and overlapping connections, there are few studies on the relationships between the relaxinergic and serotonergic systems. Nonetheless, in a paper on the development of the NI, it was observed that 5HT depletion by chlorophenylalanine led to increased expression of the RLN3 gene in the NI in the young adult (Miyamoto et al., 2008). These data suggest that the function of RLN3 neurons in the brain is influenced by serotonergic activity, which would limit the degree of development of the NI. The fact that the two systems, serotonergic and relaxinergic, may control similar aspects of behaviour, especially emotional aspects, leads to the conclusion that the interaction between them would allow a series of interadjustments and compensatory mechanisms. The existence of a serotonergic projection on the NI through H1A receptors has already been observed (Miyamoto et al., 2008), However, the reciprocal circuit from the NI to the raphe nuclei has not been analysed in detail.

We hypothesized a strong projection of the NI over the raphe nuclei that could subserve a complementary regulation of the hippocampal theta activity Therefore, in the present work we aim to analyse the anatomical relationships

between the RLN3 and the serotonergic systems in the dorsal, median, and pontine raphe nuclei and to study their GABAergic and/or glutamatergic nature.

2 Methods

2.1 Animals

Eight adult female Wistar rats of a weight between 350-450g have been used in this study. All protocols were approved by the Animal Ethics Committee of the Universitat Jaume I of Castellón (Spain). All procedures were in accordance with the European Community Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

2.2 Brain fixation and sectioning

For immunofluorescence experiments, untreated female wistar rats were euthanized by a lethal dose of pentobarbital (Dolethal, 200 mg/kg i.p; Vetoquinol S.A., Madrid, Spain). After losing all signs of consciousness and sensory reflexes, animals were transcardially perfused with saline (250 ml) followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4) for 30 min (~500 ml). The brains were then removed from the skull and immersed in the same fixative for 4 h at 4°C. Brains were subsequently immersed in 30% sucrose in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 48 h at 4°C, and coronal sections (40 µm) were obtained using a freezing slide microtome (Leica SM2000R, Leica Microsystems, Heidelberg, Germany). For each brain, 6 sets of sections were collected and stored in cryoprotective solution (30% glycerol, 30% ethylene glycol and 40% 0.1 M PBS, pH 7.4).

2.3 Immunofluorescent detection of neuronal markers (5HT-RLN3-vGAT-vGlut2)

For detection of neuronal marker proteins 6 sections per animal (n=5) were rinsed 3 times for 10 minutes in PBS and immersed in PBST blocking medium containing 4% NDS (Normal Donkey Serum), 4% NGS (Normal Goat Serum) and 0.2% Triton X-100 for 1 hour at room temperature. The sections were incubated in a primary antibody solution containing 1:5 mouse anti-RLN3 and 1:8000 rabbit anti-Serotonin (5HT), 1:500 Guinea pig anti-VGAT (Vesicular GABA transporter), 1:250 chicken IgY anti-VGlut2 (Vesicular glutamate transporter), in PBST with 4% NDS, 4% NGS and 0.2% Triton X100 for 48 h at 4ºC. The sections were then rinsed 3 times for 10 min in PBS and for quadruple labelling were incubated in 1:200 donkey anti-rabbit – Alexa 488 IgG (cat. no. 711-545-152, Jackson ImmunoResearch), 1:200 donkey anti-mouse – Alexa 647 IgG (cat. no. 715-605-150, Jackson ImmunoResearch), in 1:200 goat anti-chicken - 405 IgG (Cat. No. 20375-1mg, Biotium) and in 1:200 goat anti-guinea pig – Cy3 IgG (Cat. No. 106-

165-003, Jackson ImmunoResearch) in PBST with 4% NDS, 4% NGS and 0.2% Triton X100 for 2 hours at room temperature in the dark. Sections were briefly rinsed in 0.01 M PBS and mounted on gelatin-coated slides, air-dried, and cover slipped with Mowiol.

2.4 Immunofluorescent detection of neuronal markers (5HT-RLN3-Syn)

For immunofluorescence with synaptophysin, 6 sections per animal (n=3) were rinsed 3 times for 10 minutes in PBS 0.01M ph7.4 and immersed in PBST blocking medium containing 2% NDS (Normal Donkey Serum), 2% NGS (Normal Goat Serum) and 0.2% Triton X-100 for 1 hour at room temperature. Sections were incubated in a primary antibody solution containing 1:5 mouse anti-RLN3,1:8000 rabbit anti-Serotonin (5HT) and 1:500 Guinea pig anti-synaptophysin (Syn), in PBST with 2% NDS, 2% NGS and 0.2% Triton X100 for 48 h at 4°C. The sections were then rinsed 3 times for 10 min in PBS and for quadruple labelling were incubated in 1:200 donkey anti-rabbit – Alexa 488 IgG (cat. no. 711-545-152, Jackson ImmunoResearch), 1:200 donkey anti-mouse – Alexa 647 IgG (cat. no. 715-605-150, Jackson ImmunoResearch) and in 1:200 goat anti-guinea pig-Cy3 IgG (Cat. No. 106-165-003, Jackson Immuno-Research) in PBST with 2% NDS, 2% NGS and 0.2% Triton X100 for 2 hours at room temperature in the dark. Sections were briefly rinsed in 0.01 M PBS and mounted on gelatin-coated slides, air-dried, and cover slipped with Mowiol.

2.5 Microscopic Analysis

Immunofluorescence analysis was performed with a confocal microscopy (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany). The images were taken with a 63x objective. A stack of 20 images at 0.5 µm was made for each of the regions, serial sections of 0.5 µm were captured with Leica Confocal software (V 2.61). For vGlut2, the 405 fluorophore was used, excitation was 405 nm for 410-493nm emission. For 5HT, the Alexa 488 fluorophore was used, excitation was 488 nm for an emission of 493-553 nm. For vGAT, the Cy3 fluorophore was used, the excitation was 532 nm for an emission of 566-641 nm. For RLN3, the Alexa 647 fluorophore was used, the excitation was 647 for an emission of 649-776 nm.

In contrast, when analyzing 5HT-RLN3-Syn immunofluorescence we used the following combination. For 5HT, the Alexa 488 was used, and the excitation was 488 nm and emission of 495-549nm. For synaptophysin, the Cy3 fluorophore was used, the excitation was 532 nm for an emission of 566-641 nm. For RLN3, the Alexa 647 fluorophore was used, the excitation was 647 the emission was set at 646-748 nm.

3 Results

3.1.1 Immunofluorescent imaging of 5HT-RLN3-vGAT-vGlut2

5HT immunofluorescence resulted in labelling in dispersed cells of the dorsal raphe nucleus, median raphe nucleus and pontine raphe nucleus. RLN3 labelling resulted in fibers displaying puncta and swelling morphologies that were distributed throughout the afore mentioned raphe nuclei. A high density of these positive fibers was seen in all raphe nuclei. The vGAT labelling was in the form of granulation which left a series of empty spaces corresponding to the cell bodies. Finally, the vGlut2 labelling resulted in two kinds of labelling, one granular, which was similar to that of the vGAT and covered extensive areas of the neuropil and one thin that appeared in the empty areas of the vGAT, which was associated to the cell bodies.

5HT-positive cells appeared as a heterogeneous population of cells with circular, triangular or polygonal somata of different sizes. In the 5HT-labeled neurons, it was possible to observe primary and secondary branches of the dendritic tree in variable number. 5HT cells were more densely packed in the dorsal and pontine raphe than in the median raphe. All raphe nuclei contained a dense plexus of RLN3 fibers.

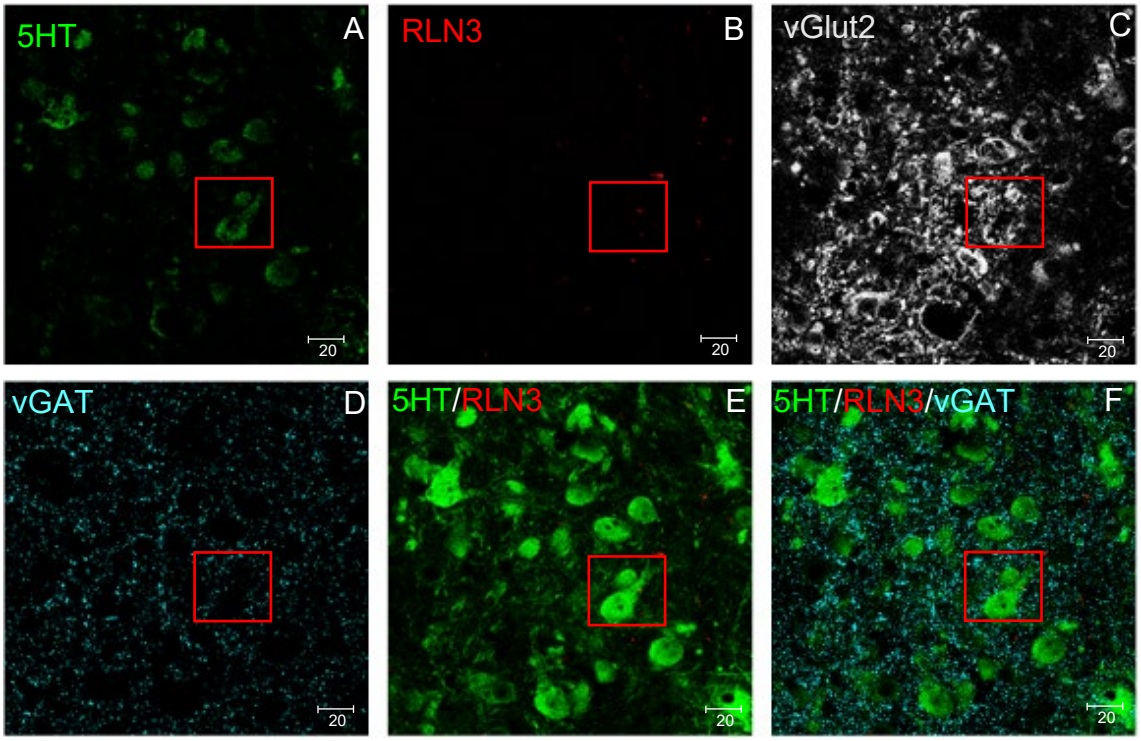

Figure 1 ***Image of a region of the dorsal raphe (N=5).*** *(**A**) 5HT-positive cell labelling (in green) in a 0.5 µm section. (**B**) RLN3 fiber labelling (in red) in maximum projection of 10 µm. (**C**) vGlut2 labelling (in white) in a 0.5 µm section. (**D**) Marking of vGAT (in cyan) in a 0.5 µm section. (**E**) Marking of 5HT-positive cells together with RLN3 fibers at maximum projection of 10 µm (**F**) Triple*

labelling of 5HT-positive cells, RLN3 and vGAT fibers at maximum projection of 10 µm.

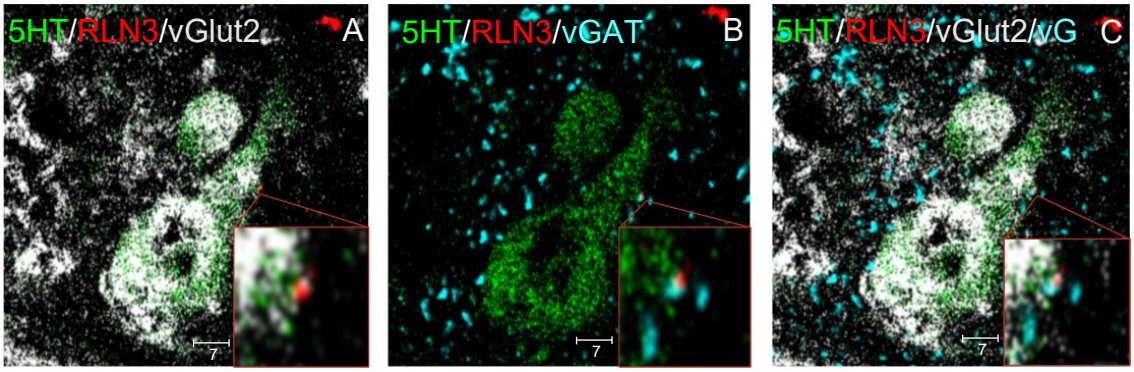

Figure 2 ***Image of the marked cell in images A,B,C, D, E, and F in Figure 1.*** *(**A**) 5HT-positive cell labelling together with RLN3 and vGlut2 in a 0.5 µm section (**B**) 5HT-positive cell labelling together with RLN3 and vGAT in a 0.5 µm section (**C**) Quadruple labelling in the same cell in a 0.5 µm section.*

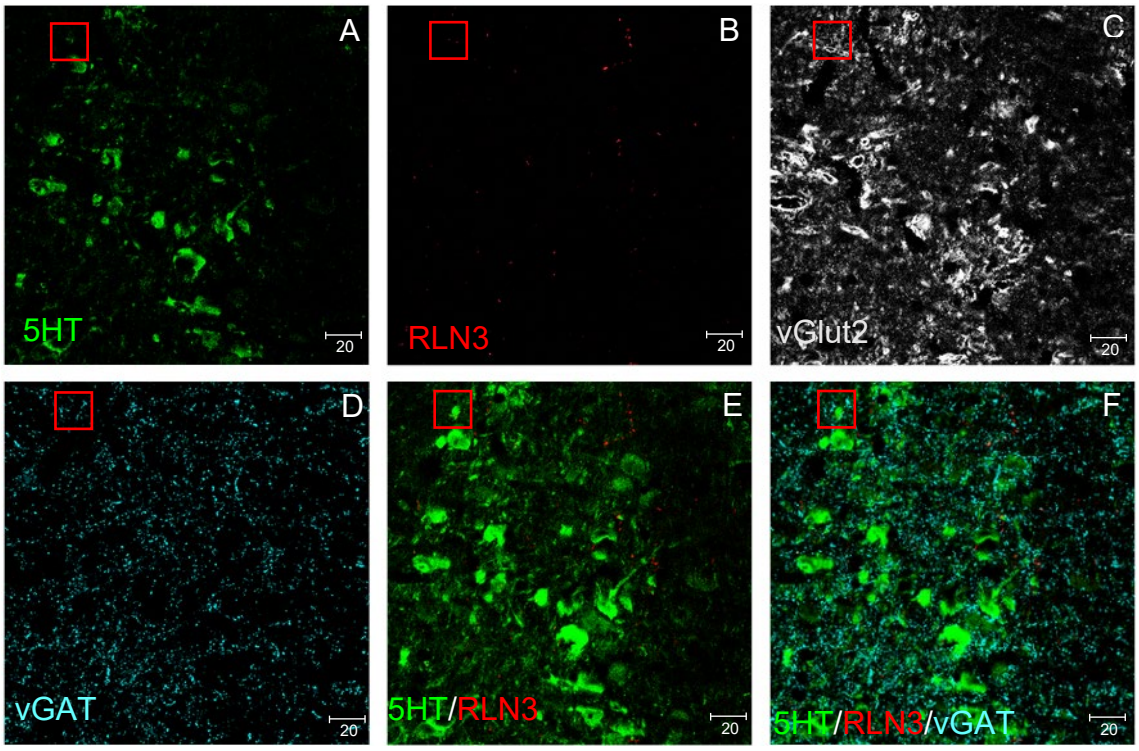

Figure 3 ***Image of a region of the dorsal raphe (N=5).*** *(**A**) 5HT-positive cell labelling (in green) in a 0.5 µm section. (**B**) RLN3 fiber labelling (in red) in maximum projection of 10 µm. (**C**) vGlut2 labelling (in white) in a 0.5 µm section. (**D**) Marking of vGAT (in cyan) in a 0.5 µm section. (**E**) Marking of 5HT-positive cells together with RLN3 fibers at maximum projection of 10 µm (**F**) Triple labelling of 5HT-positive cells, RLN3 and vGAT fibers at maximum projection of 10 µm.*

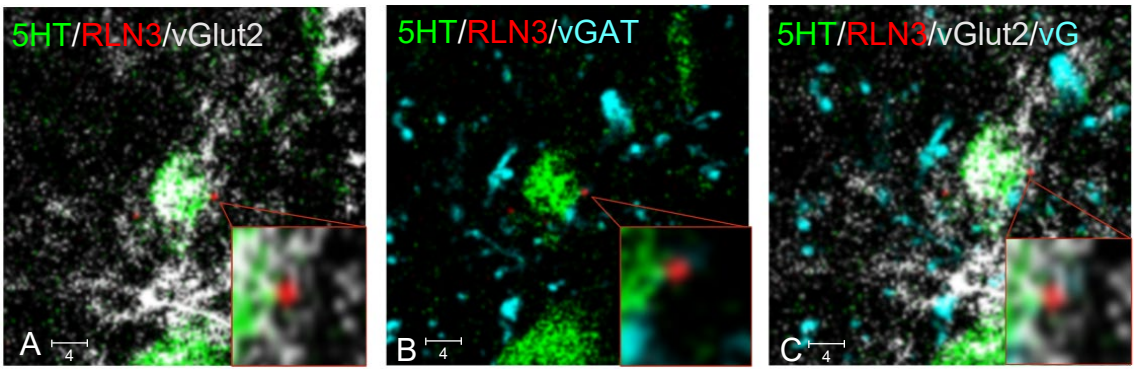

Figure 4 ***Image of the marked cell in images A, B, C, D, E, and F in Figure 3.*** *(**A**) 5HT-positive cell labelling together with RLN3 and vGlut2 in a 0.5 µm section (**B**) 5HT-positive cell labelling together with RLN3 and vGAT in a 0.5 µm section (**C**) Quadruple labelling in the same cell in a 0.5 µm section.*

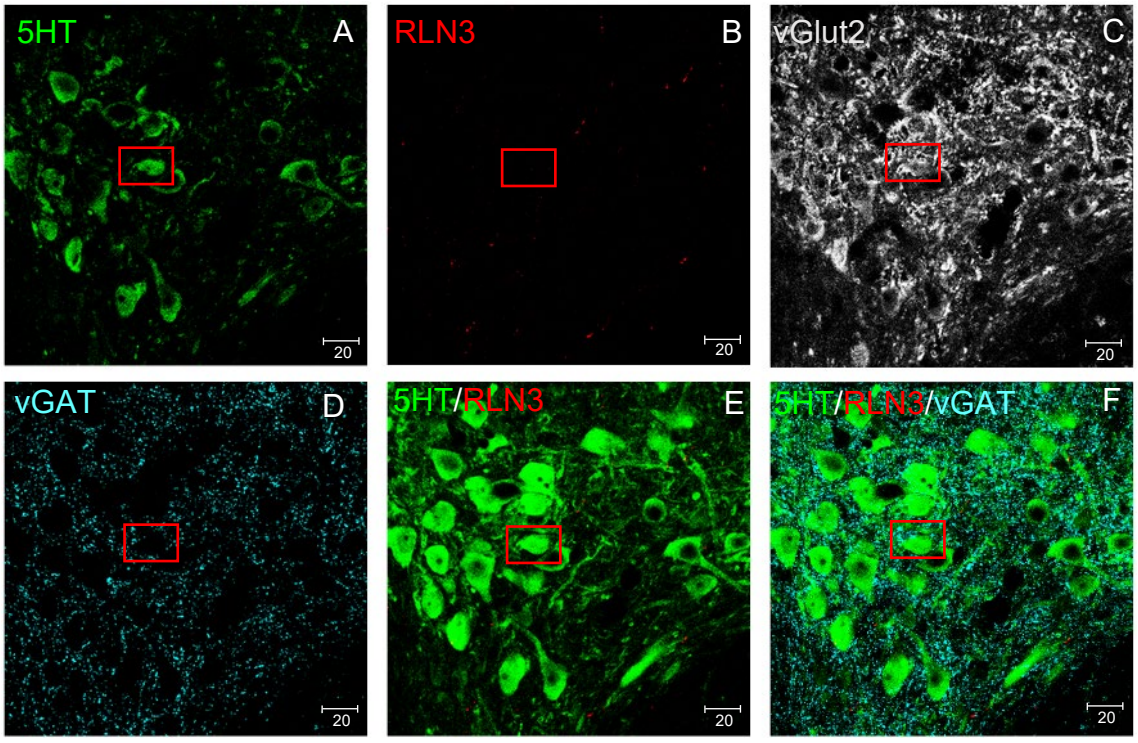

Figure 5 ***Image of a region of the dorsal raphe (N=5).*** *(**A**) 5HT-positive cell labelling (in green) in a 0.5 µm section. (**B**) RLN3 fiber labelling (in red) in maximum projection of 10 µm. (**C**) vGlut2 labelling (in white) in a 0.5 µm section. (**D**) Marking of vGAT (in cyan) in a 0.5 µm section. (**E**) Marking of 5HT-positive cells together with RLN3 fibers at maximum projection of 10 µm (**F**) Triple labelling of 5HT-positive cells, RLN3 and vGAT fibers at maximum projection of 10 µm.*

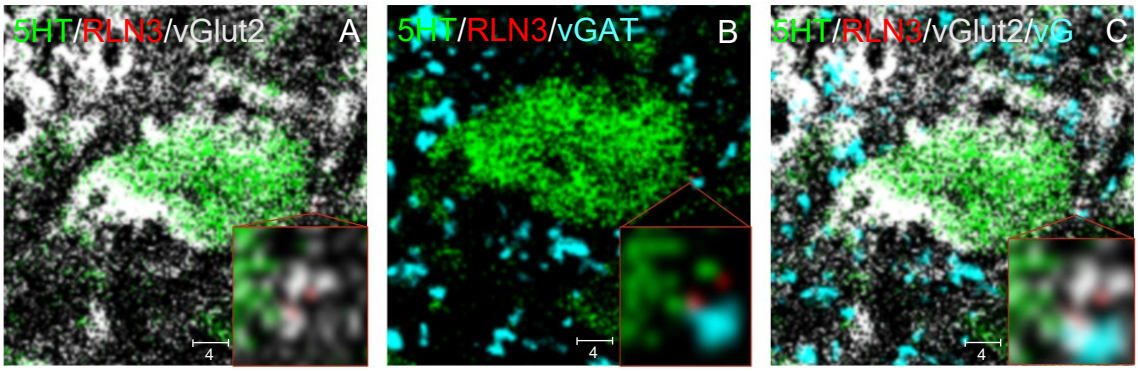

Figure 6 ***Image of the marked cell in images A, B, C, D, E, and F in Figure 5.*** *(**A**) 5HT-positive cell labelling together with RLN3 and vGlut2 in a 0.5 µm section (**B**) 5HT-positive cell labelling together with RLN3 and vGAT in a 0.5 µm section (**C**) Quadruple labelling in the same cell in a 0.5 µm section.*

3.1.2 Immunofluorescent data of 5HT-RLN3-vGAT-vGlut2

The confocal images showed an average of 24.42 RLN3 fibers displaying contact with 5HT positive cells. We found that at least half of these RLN3 fibers, presented contact contained vGlut2 (as occurred in the pontine raphe nucleus with 43.54%), on the other hand, vGAT occurred in a rank from 27.27% of RLN3 fibers in the dorsal raphe nucleus to a maximum of 53.26% in the median raphe nucleus. Therefore, a higher number of RLN3 contacts containing vGlut2 was visualized in respect to RLN3 fibers containing vGAT.

Table 1. Percentage of contact between vGlut2 or vGAT with RLN3 contacting 5HT cells.

Region	RLN3	VGlut2	VGAT
Dorsal raphe nucleus	19,25	9,75 (50,65%)	5,25 (27,27%)
Median raphe nucleus	23	21,5 (93,48%)	12,25 (53,26%)
Pontine raphe nucleus	31	30,5 (98,38%)	13,5 (43,54%)

Note: mean no. of RLN3 connections with 5HT-positive cells, in addition to the mean no. of RLN3 fibers that have contact with VGlut2 and VGAT.

3.2.1 Immunofluorescence imaging of 5HT-RLN3-Syn

As previously observed, 5HT immunofluorescence resulted in labelling in dispersed cells that were in the dorsal raphe nucleus, median raphe nucleus and pontine raphe nucleus. Additionally, RLN3 labelling resulted in fibers with a trajectory that was distributed throughout the afore mentioned raphe nuclei. Whereas synaptophysin labelling displayed granular morphology, which was

representative of the presumptive synaptic connection. In fact, black holes among the granular synaptophysin labelling represented areas occupied by neuronal somata.

In addition to 5HT cells, RLN3 was observed to exhibit synaptic contacts in the raphe nuclei in the black holes free of synaptophysin labelling what we interpret as no-5HT raphe cells.

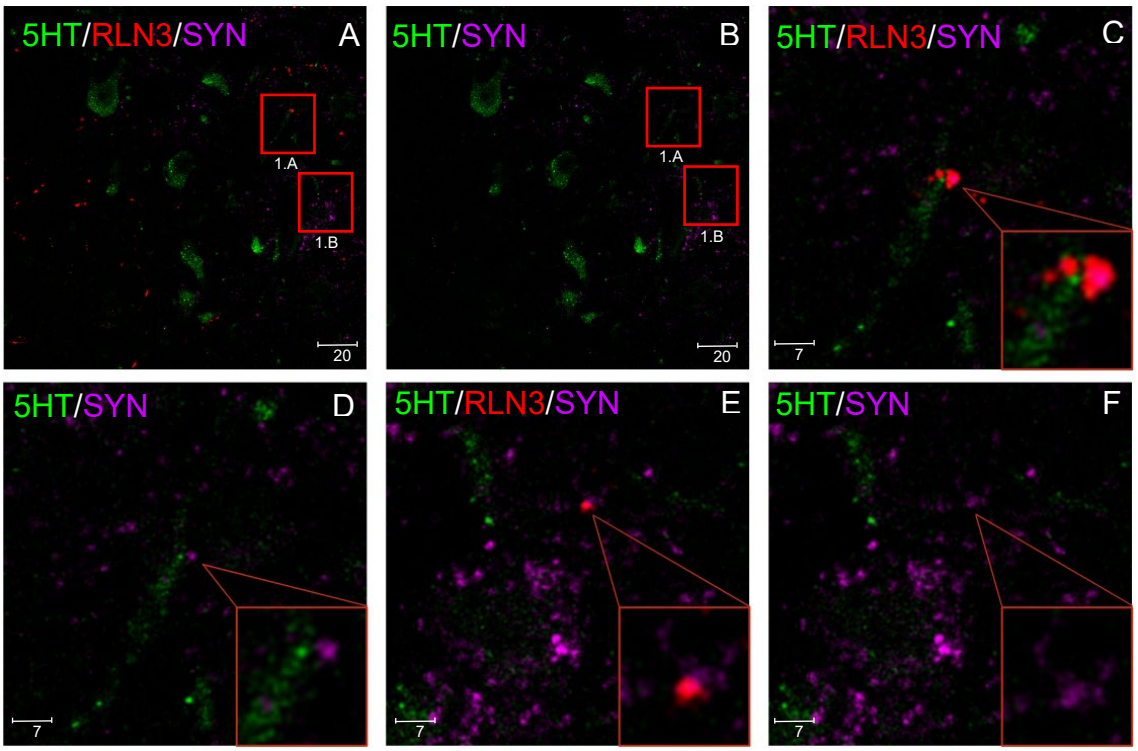

Figure 7 ***Image of a region of the dorsal raphe (N=3).*** *(**A**) Labelling of 5HT-positive cells (in green), with RLN3 (in red) and synaptophysin (in magenta) in a 0.5 µm section. (**B**) Labelling of 5HT-positive cells (in green) and synaptophysin (in magenta) in a 0.5 µm section. (**C**) Image of the marked cell in A and B (1.A), with labelling of 5HT-positive cells (in green), with RLN3 (in red) and synaptophysin (in magenta) in a 0.5 µm section. (**D**) Image of the marked cell in A and B (1.A), with labelling of 5HT-positive cells (in green) and synaptophysin (in magenta) in a 0.5 µm section. (**E**) Image of the marked cell in A and B (1.B), with labelling of 5HT-positive cells (in green), with RLN3 (in red) and synaptophysin (in magenta) in a 0.5 µm section. (**F**) Image of the marked cell in A and B (1.B), with labelling of 5HT-positive cells (in green) and synaptophysin (in magenta) in a 0.5 µm section.*

3.2.2 Immunofluorescence data of 5HT-RLN3-Syn

Confocal images showed an average of 13.52 of RLN3 fibers closely apposed to 5HT-positive cells. Of these RLN3 fibers, a rank between 11.36% to 26.93% of RLN3 fibers also contained synaptophysin thus indicating a putative synaptic relationship between fiber and cell.

Table 2. Percentage presence of synaptophysin in RLN3 fibers contacting serotonin cells.

Region	5HT	RLN3	Synaptophysin
Dorsal raphe nucleus	18,8	11,33	2,2 (19,41%)
Median raphe nucleus	20	18,25	4,75 (26,03%)
Pontine raphe nucleus	15,75	11	1,25 (11,36%)

Note: mean no. of RLN3 connections with serotonin-positive cells in addition to the mean no. of RLN3 fibers presenting synaptophysin.

4 Discussion and conclusions

We have obtained preliminary data from a small number of specimens that strongly suggest synaptic contacts between RLN3 fibers and 5HT-positive neurons. This statement is supported by the co-occurrence of synaptophysin and RLN3 and the close apposition of these complexes over 5HT-positive cells. Thus, anatomical data support the idea of an interaction of the relaxinergic system with the serotonergic system. The data obtained also point out that these interactions may be mediated by GABA or glutamate, thus could be excitatory or inhibitory.

As for the observed results, it can be seen how the cells of the raphe nuclei receive projections mediated by RLN3. This projection is located especially in dendrites or in the soma of serotonin-positive cells and would be associated with the vesicular glutamate transporter. However, the labelling obtained with vGlut2 is relatively dispersed and although sometimes it seems to be effectively associated with neuronal structures, it is also possible to observe it not associated with them and therefore it could contain unspecific elements. In contrast, VGAT labelling displays in all cases a granular appearance that leaves unlabelled spaces corresponding to the cell bodies of 5HT neurons and other cell types and is, therefore, considered a specific labelling associated with GABAergic transmission. Further analyses are necessary to discriminate the specificity of these detection systems.

The GABAergic character of the projections was already pointed out in the early work on connections (Olucha-Bordonau et al., 2003) and has also been proven the association with the RLN3 peptide (Ma et al., 2007). However, some other data also point out the possibility that part of the RLN3 transmission could be also mediated by glutamate (Szlaga et al., 2022).

Thus, the modulatory effects of the NI projections to the raphe nuclei could be a combination of GABA+RLN3 or Glu+RLN3 and these combinations could also include other neuropeptides like neuromedin B which correlates with arousal level, locomotor speed and hippocampal theta power in the NI (Lu et al., 2020). Given that glutamate and GABA produce excitation and inhibition respectively, the co-release with RLN3 could provide additional features to this transmission.

It is also followed that, as that the main effect of RXFP3 activation by RLN3 agonist is inhibition (Kania et al., 2017), this co-release could increase the inhibitory effect of GABA or attenuate the excitatory effect of glutamate. In any case, the inhibition of the raphe nuclei fits well with the hypothesis that NI-RLN3 effect on theta rhythm could be done through an inhibition of the raphe nuclei which have been proved to desynchronize the hippocampal theta (di Prisco et al., 2002).

Given the demonstrated role of RLN3 involvement as a theta rhythm activator, this action could be the result of inhibition on raphe nuclei whose action is antagonistic to NI. The antagonism between RLN3 and 5HT opens the possibility of RLN3-mediated therapeutic use. Serotonergic transmission enhancer systems have been widely used as antidepressants (di Giovanni et al., 2016; Mogha et al., 2012). But in recent studies, it has been shown that the therapeutic action of serotonin-based antidepressants could not be a direct action on 5HT transmission but a long-term indirect effect on the role of 5HT in the configuration of the emotional managing system (Moncrieff et al., 2022). Data on an anxiolytic and antidepressant effect of RLN3-mediated transmission have also been obtained (Ryan et al., 2013). With RLN3 being used as a modulator as with 5HT, depression could be addressed with different types of drugs, with new antidepressant options such as RLN3 itself.

It is necessary in successive steps to test the pattern of collateralization over the medial septum and hippocampus. Recent data indicate that indeed the raphe nuclei constitute a heterogeneous population of neurons organized in discrete arrays each of which performing specific actions (Paquelet et al., 2022). The NI and its projections onto the raphe nuclei could contribute to this subdivision of functional compartments.

These preliminary work stands that the connection between the NI and raphe nuclei actually exists and opens new paths of research on the function implications of this step. It is needed to know if the connection is associated to arousal, memory, or emotional processes. Thus, manipulation of RLN3 transmission on the raphe nuclei could report new vistas on the role of 5HT in these functions and the way to control this through RLN3-RXFP3 system. On the other hand, it is relevant to answer the question on the effect of rln3 transmission over 5HT cells on hippocampal theta.

Another of the future steps to be carried out in order to verify the hypothesis is the increase in 5HT caused by the administration of prozac and the observation of the effects on the expression of the RLN3 gene, since, as has been observed in the studies of (Miyamoto et al., 2008) 5HT depletion caused increased RLN3 gene expression and it would be interesting to see if it causes the opposite effect, a decrease in RLN3 gene expression. In the case of having an effect, a final step would be the administration of RLN3 agonists to observe whether the increase in

RLN3 has an effect on the serotonergic system in the raphe nuclei, to confirm that the serotonergic system and the relaxinergic system present a mutual interaction.

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