Analgesic effect of central relaxin receptor activation on persistent inflammatory pain in mice: behavioral and neurochemical data

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Abstract

Introduction: The relaxin peptide signaling system is involved in diverse physiological processes, but its possible roles in the brain, including nociception, are largely unexplored.

Objective: In light of abundant expression of relaxin receptor (RXFP1) mRNA/protein in brain regions involved in pain processing, we investigated the effects of central RXFP1 activation on nociceptive behavior in a mouse model of inflammatory pain and examined the neurochemical phenotype and connectivity of relaxin and RXFP1 mRNA-positive neurons.

Methods: Mice were injected with Complete Freund Adjuvant (CFA) into a hind paw. After 4 days, the RXFP1 agonist peptides, H2-relaxin or B7-33, ± the RXFP1 antagonist, B-R13/17K-H2, were injected into the lateral cerebral ventricle, and mechanical and thermal sensitivity were assessed at 30 to 120 minutes. Relaxin and RXFP1 mRNA in excitatory and inhibitory neurons were examined using multiplex, fluorescent in situ hybridization. Relaxin-containing neurons were detected using immunohistochemistry and their projections assessed using fluorogold retrograde tract-tracing.

Results: Both H2-relaxin and B7-33 produced a strong, but transient, reduction in mechanical and thermal sensitivity of the CFA-injected hind paw alone, at 30 minutes postinjection. Notably, coinjection of B-R13/17K-H2 blocked mechanical, but not thermal, analgesia. In the claustrum, cingulate cortex, and subiculum, RXFP1 mRNA was expressed in excitatory neurons. Relaxin immunoreactivity was detected in neurons in forebrain and midbrain areas involved in pain processing and sending projections to the RXFP1-rich, claustrum and cingulate cortex. No changes were detected in CFA mice.

Conclusion: Our study identified a previously unexplored peptidergic system that can control pain processing in the brain and produce analgesia.

Keywords: Chronic pain, G-protein-coupled receptor, Relaxin, RXFP1

1. Introduction

Twenty percent of the world population suffers from chronic pain, which is often associated with emotional comorbidities, such as anxiety and depression.[3,40,67] Unfortunately, chronic pain and its comorbidities are poorly managed clinically, with 40% of patients not receiving adequate treatment.[7,8,70,71] In the brain, changes in the excitation–inhibition balance alter how neurons and microcircuits integrate and respond to chronic nociceptive information.[39] This maladaptive plasticity is controlled by synaptic mechanisms[42] and intrinsic properties of neurons.[24] Neuronal

plasticity is also shaped by regulatory molecules, among which neuropeptides act by binding to G-protein-coupled receptors. Various neuropeptide and receptor systems are highly versatile in function and play key roles in pathophysiological conditions,[21,32] especially chronic pain.[22,75]

How neuropeptides control pain largely depends on their site of release and target cells. In the spinal cord, for instance, neuropeptide Y[16] and substance P[75] modulate pain at primary afferent fiber terminals. Neuropeptides and their receptors are widely distributed in the brain, where they modulate the sensory discriminative and emotional components of pain (eg, in the primary somatosensory cortex and amygdala, respectively).[68,74] Neuropeptides are also involved in the control of descending pain pathways (eg, periaqueductal gray matter).[41,59] We recently demonstrated the ability of the cholecystokinin (CCK)-CCK2 receptor system within the amygdala to inhibit spinal neuron excitability and produce analgesia in an inflammatory pain condition.[56] The role of neuropeptides as pain modulators has attracted increasing interest because their actions can be quite specific and with fewer side effects than targeting classical transmitter systems.[32–34]

Human relaxin-2 (H2-relaxin) is a 6 kDa heterodimeric peptide of 53 amino acids consisting of 2 chains (A and B) linked by 2 disulphide bonds.[4] RXFP1 is the cognate G-protein-coupled receptor for relaxin.[28,37] RXFP1 is characterized by a leucine-rich repeat domain and a low-density lipoprotein A module,[28,37] which are required for activation. Relaxin/RXFP1 signaling is involved in collagen metabolism[5] and in antifibrotic and vaso-dilatory actions in various animal models of disease.[57] The therapeutic potential of relaxin has expanded in recent years to include regulation of cardiovascular and renal function[51] and the treatment of heart failure.[5]

The role of the relaxin-RXFP1 system in the brain has been the subject of very few studies, although it has been implicated in the regulation of thirst and osmotic homeostasis[50,60] and in emotional memory.[44] Anatomical mapping studies[43–45,54] have revealed that RXFP1 is expressed in brain regions involved in pain processing, such as claustrum (CLA), anterior cingulate cortex (ACC), and subiculum (SUB), but to the best of our knowledge, the role of relaxin-RXFP1 signaling has not been investigated in a pain context.

Therefore, this study aimed to characterize the possible effects of the relaxin-RXFP1 signaling system on pain processing in the brain. We used a mouse model of inflammatory persistent pain[1] to investigate any analgesic action of centrally administered relaxin peptides that selectively activate RXFP1. We also conducted a neuroanatomical characterization of the relaxin-RXFP1 system in brain areas involved in pain processing.

2. Materials and methods

Detailed materials and methods are available as supplementary material (available at http://links.lww.com/PR9/A109).

2.1. Mice and surgery

Male C57BL/6J mice (Charles Rivers, France), 8 to 10 weeks old, were used, in agreement with the ethical guidelines of the International Association for the Study of Pain and were approved by the French Ministry of Agriculture (Agreement #21890). Mice were anesthetized with isoflurane and placed in a stereotaxic frame (RWD Desktop Digital Stereotaxic Instruments, San Diego, CA). Guide cannulae were implanted for the injection of relaxin analogs into the lateral ventricle.

At 4 days postsurgery recovery, Complete Freund Adjuvant (CFA) (Sigma-Aldrich, St Louis, MO) or NaCl was injected into the hind paw of inflamed "CFA" or "sham" mice, respectively.

Four days after CFA or NaCl injections, mice were anesthetized with isoflurane and received intracerebroventricular (icv) infusions of relaxin analogs—RXFP1 agonists (H2-relaxin[44] and B7-33[35]) and antagonist (B-R13/17K-H2,[36] referred to as B-R/K in the figures). Ten groups of mice were used (refer to Table S1, supplementary material, available at http://links.lww.com/PR9/A109). RXFP1 activation was tested with a single injection of either H2-relaxin-2 (H2) or B7-33. Control mice received a single injection of artificial cerebrospinal fluid (aCSF). The specificity of RXFP1 activation was assessed with a single injection of H2 + RXFP1 antagonist, B-R13/17K-H2 (or B7-33 + B-R13/17K-H2).

2.2. Pain assessment

Mechanical response thresholds were monitored using von Frey hairs (Bioseb, Vitrolles, France) in "CFA" and "sham" mice, one day before their hind paw injections (D–1; reference value for each mouse). Changes in mechanical sensitivity were evaluated on day 4 after CFA or NaCl (D4) after relaxin analog(s) infusion. Thermal sensitivity was evaluated using the Hargreaves test at the same time points.

2.3 Multiplex in situ hybridization histochemistry (RNAscope)

Multiplex in situ hybridization (Advanced Cell Diagnostics, Hayward, CA) was conducted according to the manufacturer's instructions with slight modifications (see supplementary material, available at http://links.lww.com/PR9/A109). Sections were incubated with probe mix 1 [complementary to RXFP1 (Catalog#: 458001-C1), calmodulin-dependent kinase II (CaMKII) (Catalog#: 445231-C3), and GAD65 (Catalog#: 415071-C2) mRNA] or probe mix 2 [complementary to relaxin (Catalog#: 539521-C2), CaMKII (Catalog#: 445231-C3), and GAD65 (Catalog#: 439371-C1) mRNA] for 2 hours at 40°C. Sections were washed, and signal amplification was performed. Sections were then incubated in DAPI and coverslipped in fluorescent mounting medium.

2.4. Neuronal tract-tracing

In studies to map some of the projections of brain relaxin neurons, 3 mice were injected with fluorogold into the basolateral amygdala (BLA) or the ACC. Mice were kept single-housed for 2 weeks before brain fixation by intracardiac perfusion with 4% paraformaldehyde [PFA] in 0.1 M phosphate buffer.[11]

2.5. Immunohistochemistry

Perfusion-fixed mouse brains were cut on a cryostat, and sections were incubated with rabbit anti-rat relaxin primary antibody (1:200) (Biovision, Milpitas, CA) for 24 hours at 4°C. After washing, sections were incubated with Alexa Fluor 568, anti-rabbit secondary antibody (1:500 in 1x phosphate-buffered saline +1% bovine serum albumin) (Thermo Fisher Scientific, Illkirch, France). The specificity of the anti-relaxin antibody has been validated by western blot (refer to supplier website at https://www.biovision.com). Control studies were performed by preadsorption of the primary antibody with the antigen and omission of the primary antibody. The distribution of relaxin immunoreactivity observed in mouse brain matched that reported for rat relaxin peptide and mRNA.[27,43–45] Quantification of the abundance of relaxin-immunopositive cells is provided in the supplementary material (Tables S2 and S3, available at http://links.lww.com/PR9/A109).

Sections processed for immunohistochemistry or multiplex in situ hybridization were examined in a NanoZoomer 2.0-HT (Hamamatsu, Hamamatsu City, Japan) and under a SPE confocal microscope (Leica, Wetzlar, Germany).

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism. Behavioral analysis was conducted using a 2-way analysis of variance, followed by a Tukey test. In situ hybridization (RNAscope) data analysis was conducted using a 2-way analysis of variance, followed by a Sidak test. Data are presented as mean $\pm$ SEM, and differences were considered significant when $P < 0.05$.

3. Results

3.1. Analgesic effects of RXFP1 activation

We analysed nociceptive behavioral responses to mechanical and heat stimuli using the von Frey test and Hargreaves test in mice (Fig. 1). We studied the effects of RXFP1 activation in control mice (NaCl injection in the hind paw) and in mice with persistent inflammatory pain produced by CFA injection in the hind paw.

Injection of CFA produced a subsequent significant decrease in paw withdrawal threshold (PWT) in mice (Fig. 1A-a; $F_{(1,12)} = 270.63$; $P < 0.0001$). Mechanical sensitization was obtained in the CFA-injected paw on day 4 postinjection (PWT = 1.467 $\pm$ 0.067 g at D–1 vs 0.42 $\pm$ 0.02 g at D4, $P < 0.0001$). No changes in PWT were detected in NaCl-injected mice (Fig. 1A-a; $P > 0.05$) or in the uninjected paw of CFA mice (Fig. S1A-a; $F_{(1,12)} = 1.503$; $P > 0.05$). Intracerebroventricular injection of B7-33 induced a significant PWT increase 30 minutes after the injection (Fig. 1A-a; PWT = 1.467 $\pm$ 0.067 g at 30 minutes, $P < 0.0001$ vs D4, $P > 0.05$ vs D–1) that was absent 1 hour after injection. Similar results were obtained after the injection of H2-relaxin (H2) (Fig. 1B-a; $F_{(1,10)} = 164.3$; $P < 0.0001$), which also increased PWT at 30 minutes after icv injection (Fig. 1B-a; PWT = 1.200 $\pm$ 0.089 g at 30 minutes, $P < 0.0001$ vs D4, $P > 0.05$ vs D-1). The PWT increase was prevented by coinjection of the RXFP1 antagonist (B-R13/17K-H2) with the respective RXFP1 agonists (B7-33 or H2; Fig. 1A-b; PWT = 0.40 g $\pm$ 0.00 at 30 minutes, Fig. 1B-b; PWT = 0.44 $\pm$ 0.04 g at 30 minutes, $P > 0.05$ vs D4, $P < 0.0001$ vs D–1). No effect of the agonists or the antagonist was detected in sham, NaCl-injected mice (Figs. 1A-a, b and 1B-a, b), or after von Frey stimulation of the uninjected paw in CFA mice (Figs. S1A-a, b and S1B-a, b). Intracerebroventricular injection of aCSF did not significantly alter PWT under control or inflammatory pain conditions (Figs. 1A-c, B-c and S1A, B-c).

Paw withdrawal latency (PWL) was decreased after thermal stimulation of the CFA-injected paw (Fig. 1A-d; $F_{(1,8)} = 136.9$; $P < 0.0001$; PWL = 3.34 $\pm$ 0.152 seconds at D–1 vs 1.37 $\pm$ 0.089 seconds at D4, $P < 0.0001$), but not in NaCl-injected mice (Fig. 1A-d; $F_{(1,8)} = 136.9$; $P > 0.05$) or after stimulation of the uninjected paw (Fig. S1A-d; $F_{(1,8)} = 1.062$; $P > 0.05$). Intracerebroventricular injection of B7-33 (Fig. 1A-d) and H2-relaxin (Fig. 1B-d) increased PWL 30 minutes after the injection (Fig. 1A-d; PWL = 2.614 $\pm$ 0.074 seconds at 30 minutes, $P < 0.001$ vs D4, $P > 0.05$ vs D–1) (Fig. 1B-d; PWL = 3.838 $\pm$ 0.398 seconds at 30 minutes, $P < 0.01$ vs D4, $P > 0.05$ vs D–1). This effect was absent 1 hour after injection of B7-33 and 2 hours after H2-relaxin injection (Fig. 1B-d; PWL = 2.214 $\pm$ 0.120 seconds at 60 minutes, $P < 0.0001$ vs D4, $P > 0.05$ vs D–1). Notably, the B7-33-induced effect was not prevented by coinjection of the RXFP1

antagonist, B-R13/17K-H2 (Fig. 1A-e; PWL = 2.428 $\pm$ 0.253 seconds at 30 minutes, $P < 0.0001$ vs D4, $P > 0.05$ vs D–1). Similarly, the H2-induced PWL increase was not affected by the coinjection of the RXFP1 antagonist (Fig. 1B-e; PWL = 3.342 $\pm$ 0.189 seconds at 30 minutes, $P < 0.0001$ vs D4, $P > 0.05$ vs D–1). Intracerebroventricular injection of RXFP1 agonists or antagonists did not modify PWL in sham, NaCl-injected mice (Figs. 1A-d, e and B-d, e), or after thermal stimulation of the uninjected paw in CFA mice (Figs. S1A-d/e and S1B-d/e). The CFA-induced PWL decrease was not altered by icv aCSF injection under control or inflammatory pain conditions (Figs. 1A-f, B-f, and S1A/B-f).

These data indicate that RXFP1 activation produced both mechanical and thermal analgesia under inflammatory conditions.

3.2. Distribution of RXFP1 mRNA in mouse brain

The mouse ACC, CLA, and SUB, regions strongly implicated in processing pain and emotions,[26,48,49,58,66] contain a high density of RXFP1 mRNA (see Ref 54; Allen Brain Atlas (http://mouse.brain-map.org/experiment/show/70562124). Thus, multiplex fluorescence in situ hybridization (ISH) identified intense labeling in these areas, particularly in the ACC and CLA (Fig. 2A), while the labeling intensity was much weaker in the surrounding areas. Multiplex fluorescent ISH also identified key aspects of the neurochemical phenotype of RXFP1 mRNA-expressing neurons. Presumed excitatory and inhibitory neurons were identified by their expression of CaMKII or GAD65 mRNA, respectively. CaMKII mRNA-positive and GAD65 mRNA-positive neurons were identified as clearly separate populations, with only a very small number of neurons exhibiting colocalization of CaMKII and GAD65 mRNA (Fig. 2B). In the brain areas investigated, virtually all RXFP1 mRNA-positive neurons contained CaMKII mRNA (Fig. 2C), indicating the receptor is almost exclusively expressed by excitatory neurons. No significant differences in the relative colocalization or the levels of RXFP1 mRNA in these areas were observed between sham and CFA mice (Figs. 2B, C).

3.3. Distribution of relaxin immunoreactivity in mouse brain

In studies aimed at identifying the possible sources of endogenous relaxin that would activate RXFP1 in these receptor-rich areas, we first performed immunohistochemical staining for relaxin peptide immunoreactivity throughout the normal mouse brain (Fig. 3). Experiments to examine the specificity of the immunoreaction, including preadsorption with the antigen peptide, resulted in a loss of specific staining (Fig. S2). We identified relaxin-containing areas widely distributed throughout the forebrain (Figs. 3Aa-e, Ba-h) and to a lesser extent in the hindbrain (Fig. 3Af, Bi-l). Notably, the midbrain did not contain prominent populations of relaxin-immunoreactive neurons. The highest density of relaxin-positive cells was found in the cortex (eg, cingulate, claustrum, piriform, and somatosensory) and the hypothalamus (medial preoptic area, basal, and lateral hypothalamus). In the hindbrain, all cerebellar Purkinje cells exhibited clear and homogenous labeling. All positive cells were characterized by diffuse cytosolic labelling, restricted to the cell body or proximal processes, with little immunoreactivity in distal elements, consistent with earlier reports on the rat brain from our laboratory.[44]

We then used fluorogold retrograde tracing to identify the relaxin neuron populations that send projections to the RXFP1-rich ACC and CLA. We focused on these pain processing brain areas because they exhibited the highest density of RXFP1 transcripts, but other areas warrant future investigation. Fluorogold was detected in neurons in
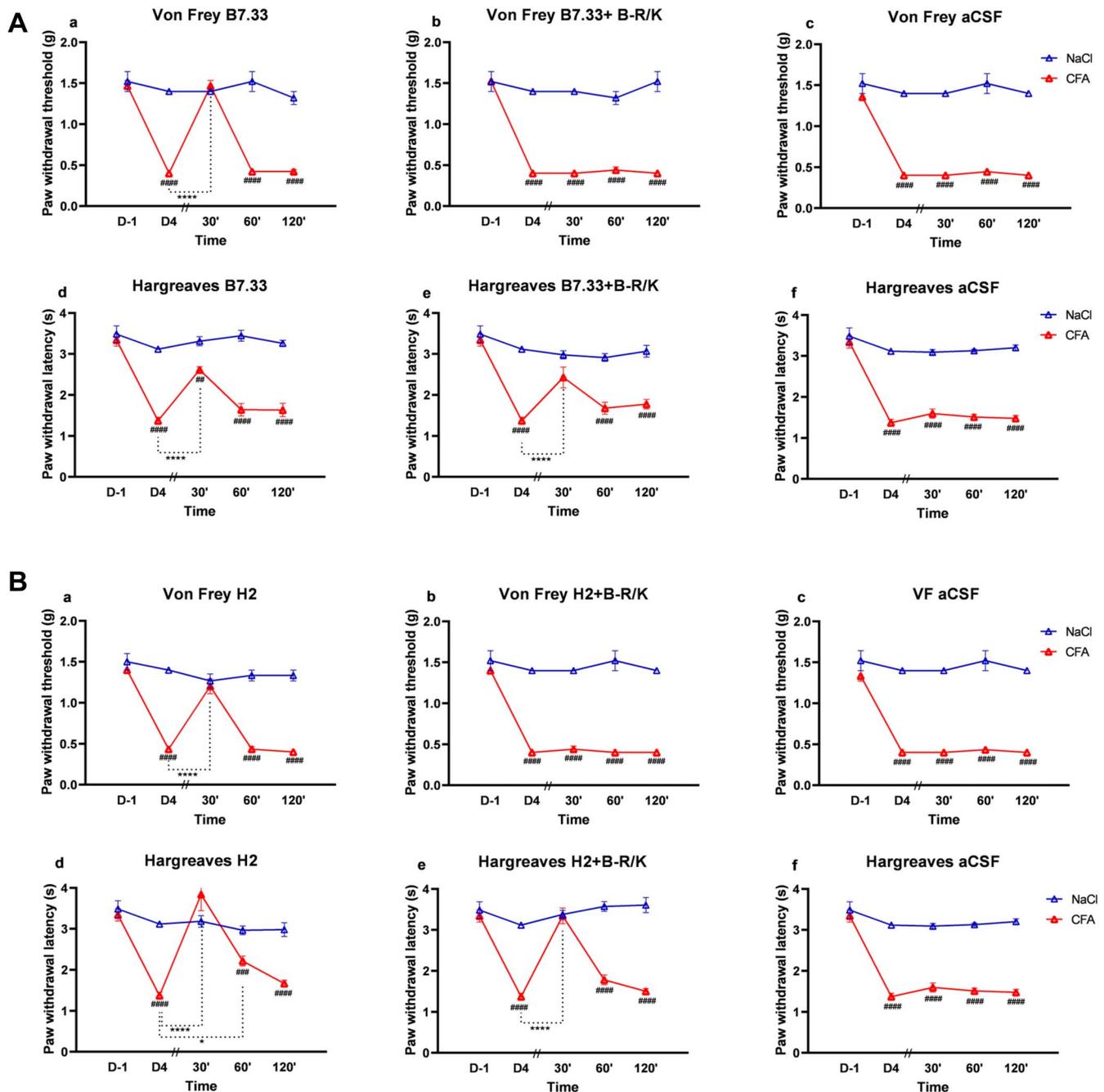
Figure 1. Assessment of mechanical sensitivity (paw withdrawal threshold, von Frey test, VF) and thermal sensitivity (paw withdrawal latency, Hargreaves test) of the CFA-injected hind paw after icv injection of the RXFP1 agonists, (A) B7-33 or (B) H2-relaxin (left panels); coinjection of B7-33 or H2-relaxin with the RXFP1 antagonist, B-R13/17K-H2 (B-R/K), (central panels); or control aCSF injection (right panels). (A) n = 9 von Frey for CFA conditions, n = 5 Hargreaves for CFA conditions, n = 3 von Frey, B7-33 injection for NaCl conditions, n = 5 von Frey and Hargreaves for NaCl conditions. (B) n = 6 von Frey for CFA conditions, n = 5 Hargreaves for CFA conditions, n = 5 von Frey and Hargreaves for NaCl conditions. aCSF, artificial cerebrospinal fluid; CFA, Complete Freund Adjuvant; icv, intracerebroventricular.

various areas of the forebrain and midbrain areas that contained relaxin immunoreactivity. A fluorogold injection into the ACC (**Fig. 4**, left panel) resulted in fluorogold accumulation in relaxin-positive neurons in the CLA, BLA, primary somatosensory cortex, and the ACC itself. Fluorogold injection into the CLA (**Fig. 4**, right panel) resulted in colocalization of fluorogold and relaxin immunoreactivity in the BLA, ACC, and the CLA itself, in addition to the posterior complex of the thalamus. These data indicate that the ACC, CLA, and BLA contain relaxin neurons that project to both ACC and CLA.

3.4. Neurochemical phenotype of relaxin mRNA-containing neurons in mouse brain

In studies aimed at identifying the neurochemical phenotype of relaxin-expressing neurons in the ACC, CLA, and BLA that project to these RXFP1-expressing brain nuclei involved in pain processing, we first attempted double immunohistochemical labeling experiments to colocalize relaxin and phenotypical markers of excitatory and inhibitory neurons. However, unfortunately these experiments were unsuccessful because
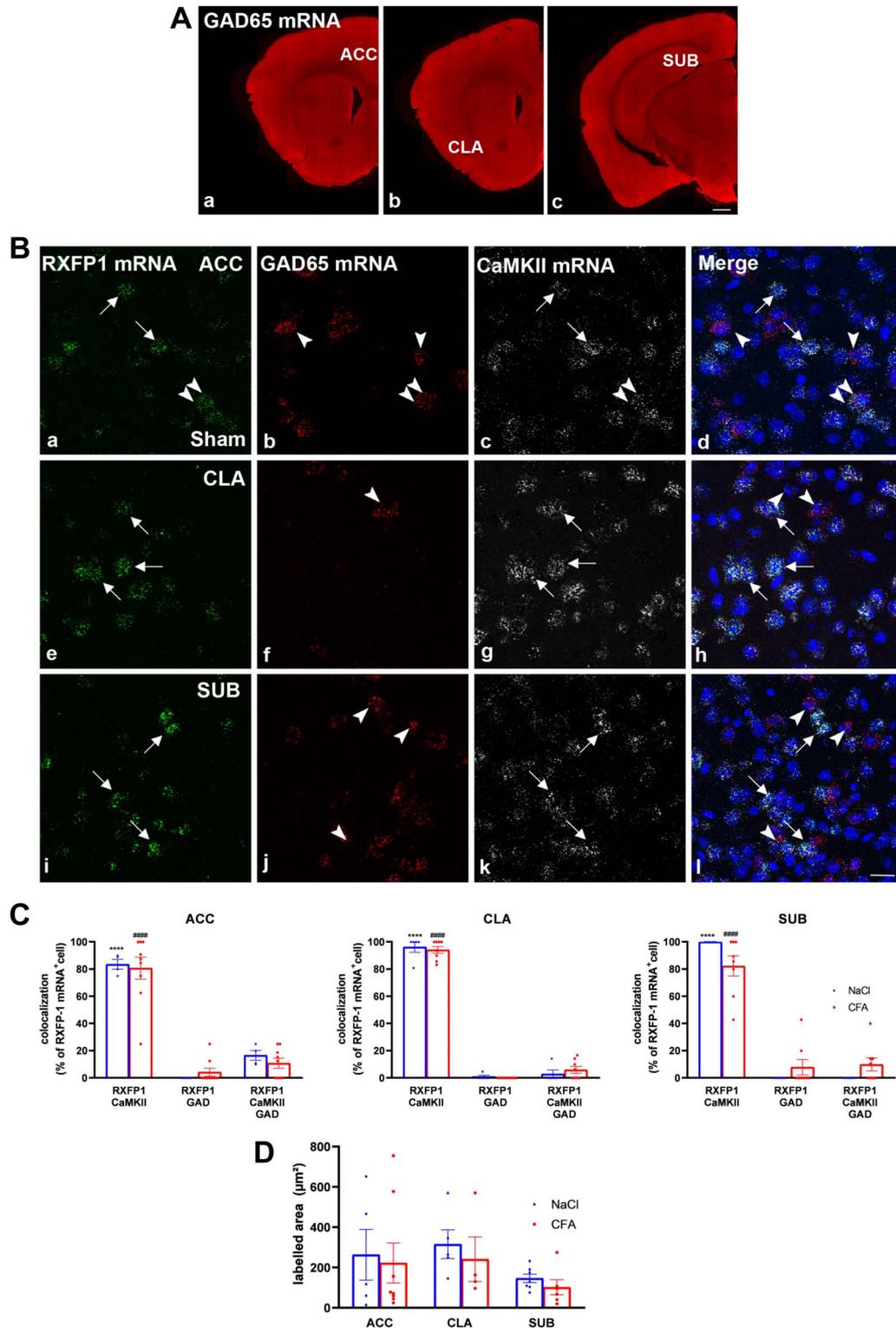
Figure 2. Multiplex fluorescent ISH detection of RXFP1, GAD65, and CaMKII mRNA in the forebrain of sham mice. (A) Low-magnification images of RNAscope experiments for GAD65 mRNA illustrating the areas observed in (B) and quantified in (C, D). (B) Detection of RXFP1 mRNA (green; a, e, and i), GAD65 mRNA (red; b, f, and j), CaMKII mRNA (white; e, g, and k), and DAPI staining (blue; d, h, and l) in the ACC (a–d), CLA (e–h), and SUB (i–l). RXFP1 mRNA was frequently colocalized with CaMKII mRNA (arrows), whereas colocalization with GAD65 mRNA was scarce (arrowheads). Rare cells display colocalization of all 3 transcripts (double arrowheads). Bar (a-l) = 20 μm. (C) Quantification of neurons displaying colocalization of RXFP1 mRNA with GAD65 mRNA or CaMKII mRNA (5 sections in each area, n = 4 mice under sham [blue] and CFA [red] conditions). ****$P < 0.0001$ vs sham RXFP1/GAD65; ####$P < 0.0001$ vs CFA RXFP1/GAD65. (D) Quantification of areas labeled using multiplex fluorescent ISH for RXFP1 mRNA in CLA, ACC, and SUB of sham (blue) and CFA (red) mice (4 sections through each area, n = 4 mice under sham [blue] and CFA [red] conditions). In total, for the colocalization study, 561 (ACC), 366 (CLA), and 385 (SUB) cells were counted. For the assessment of relaxin mRNA-labeled areas, 286 (ACC), 191 (CLA), and 123 (SUB) cells were counted. ACC, anterior cingulate cortex; CFA, Complete Freund Adjuvant; CLA, claustrum; SUB, subiculum.
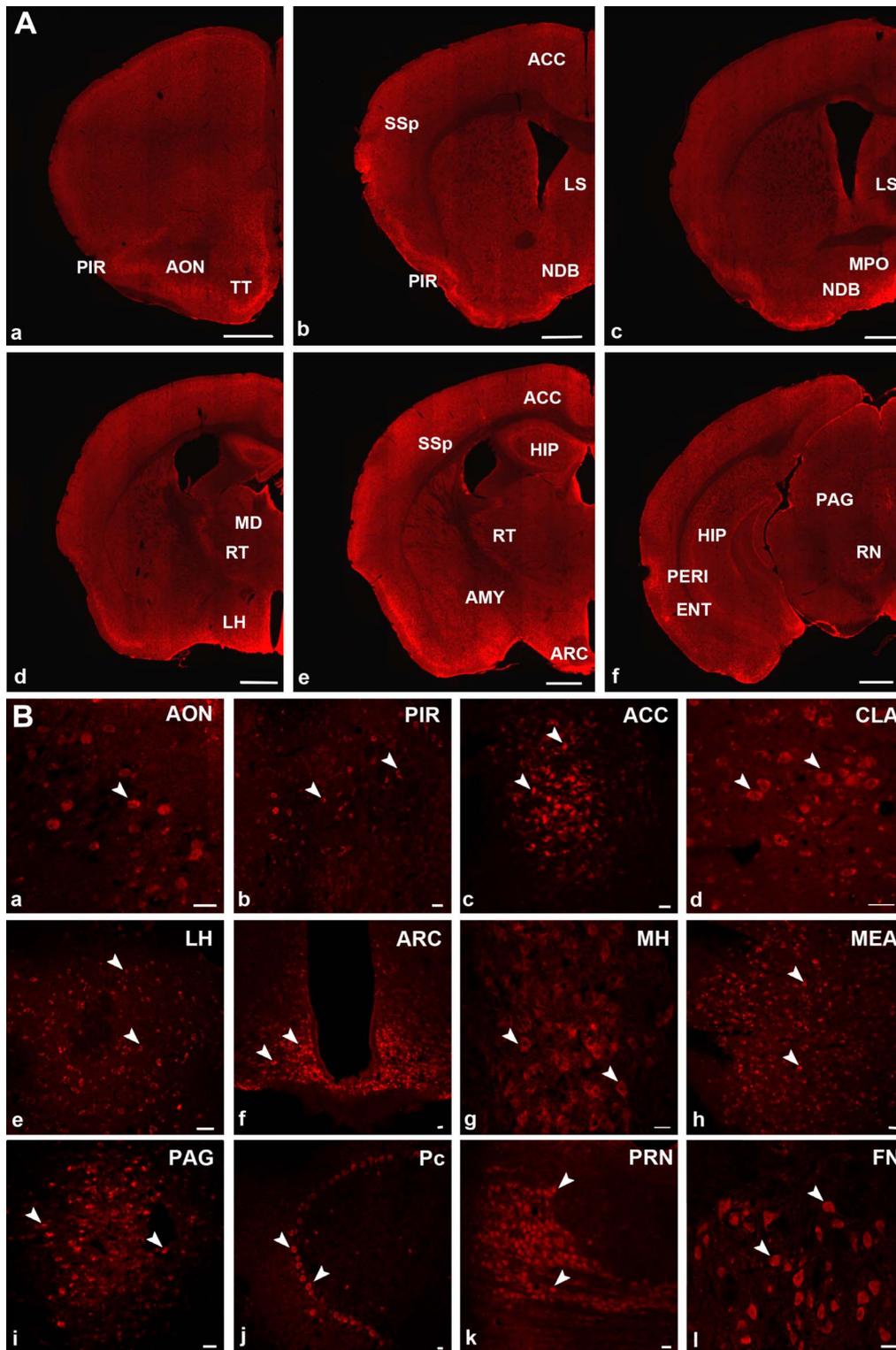
Figure 3. Relaxin immunoreactivity in the forebrain of sham mice. (A) Low-magnification images were acquired using a NanoZoomer. Bars = 1 mm. (B) Confocal micrographs illustrating relaxin immunoreactivity (arrowheads) in the anterior olfactory nucleus (AON), piriform cortex (PIR), anterior cingulate cortex (ACC), claustrum (CLA), lateral hypothalamus (LH), arcuate nucleus (ARC), medial habenula (MH), medial amygdala nucleus (MEA), periaqueductal gray (PAG), Purkinje cells (Pc), pontine reticular nucleus (PRN), and facial nucleus (FN). Bars = 20 μm.

relaxin labeling could not be effectively visualized when antisera were used in combination or sequentially.

Therefore, we used multiplex fluorescent ISH, as described above, to detect the possible coexpression of relaxin mRNA with GAD65 and CaMKII mRNA. Although some differences were observed in the areas examined, most relaxin mRNA-positive neurons were identified as GAD65 mRNA-containing neurons (**Fig. 5A**). Thus, the proportion of relaxin mRNA-positive cells was 76.6 ± 4.96% in the CLA, 70.8 ± 11.37% in the ACC, and 78.4 ± 4.82% in the BLA (**Fig. 5B**). By contrast,
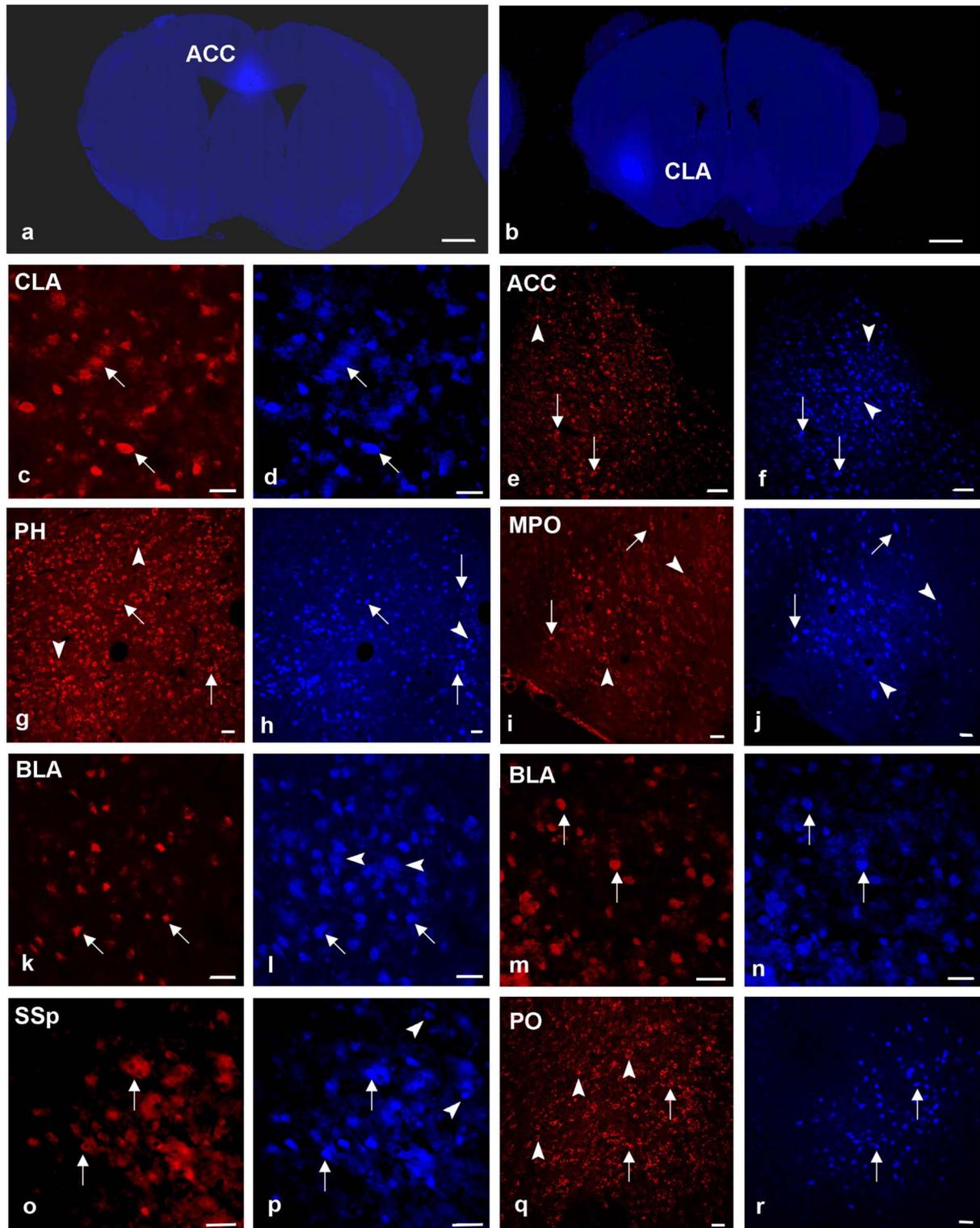
Figure 4. Fluorogold retrograde tracing (blue) of relaxin-immunoreactive neurons (red) in various forebrain areas that project to the ACC (left panel) or CLA (right panel) of sham mice. Examples of injection sites, visualized one day after fluorogold injection, are shown in a and b. Results of retrograde labeling were observed after 2 weeks (c–r). Arrows indicate neurons retrogradely labeled with fluorogold and immunopositive for relaxin. Arrowheads indicate neurons that are single labeled for one marker alone. Bars = 20 μm. ACC, anterior cingulate cortex; CLA, claustrum; BLA, basolateral amygdala; MPO, medial preoptic area; PH, posterior hypothalamus; PO, posterior complex of the thalamus; SSp, primary somatosensory cortex..

a minority of relaxin mRNA-positive cells were identified as excitatory neurons (ie, the proportion of relaxin mRNA-positive cells was $9.20 \pm 1.07\%$ in the CLA, $29.00 \pm 6.82\%$ in the ACC, and $8.20 \pm 1.77\%$ in the BLA). No significant differences in relaxin mRNA expression were observed between sham and CFA mice in either the level of colocalization (**Fig. 5B**) or the levels of relaxin mRNA in various brain areas (**Fig. 5C**).
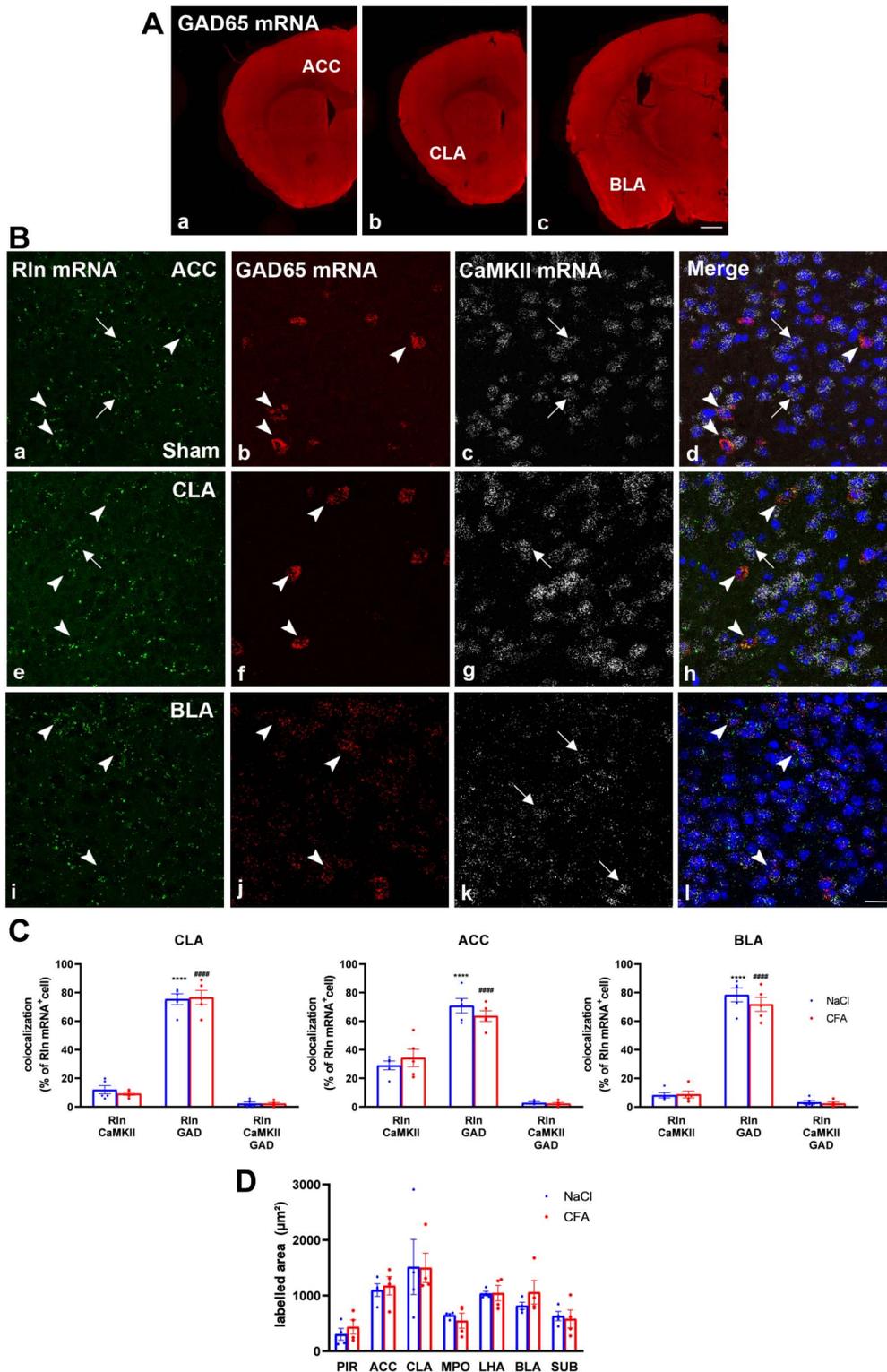
Figure 5. Multiplex fluorescent ISH detection of relaxin (Rln) mRNA, GAD65 mRNA, and CaMKII mRNA in the forebrain of sham mice. (A) Low-magnification images of RNAscope experiments for GAD65 mRNA illustrating the areas observed in B and quantified in C. (B) Multiple detection of Rln mRNA (green; a, e, and i), GAD65 mRNA (red; b, f, and j), CaMKII mRNA (white; e, g, and k), and DAPI staining (blue; d, h, and l) in the ACC (a–d), CLA (e–h), and BLA (i–l). Rln mRNA was frequently colocalized with GAD65 mRNA (arrowheads), whereas colocalization with CaMKII mRNA was scarce (arrows). Bar (a–l) = 20 μm. (C) Quantification of neurons displaying colocalization of Rln mRNA with GAD65 mRNA or CaMKII mRNA (5 sections in each area, n = 4 mice under sham [blue] and CFA [red] conditions). ****$P < 0.0001$ vs sham Rln/CaMKII; ####$P < 0.0001$ vs CFA Rln/CaMKII). (D) Quantification of areas labeled using multiplex fluorescent ISH for Rln mRNA in various forebrain regions of sham (blue) and CFA (red) mice (4 sections through each area, n = 4 mice under sham [blue] and CFA [red] conditions). In total, for the colocalization study, 476 (ACC), 306 (CLA), and 263 (BLA) cells were counted. For the assessment of relaxin mRNA-labeled areas, 258 (PIR), 169 (ACC), 101 (CLA), 154 (MPO), 109 (LHA), 88 (BLA), and 74 (SUB) cells were counted. ACC, anterior cingulate cortex; BLA, basolateral amygdala; CFA, Complete Freund Adjuvant; CLA, claustrum; SUB, subiculum.

4. Discussion

This study represents the first to demonstrate the function of the relaxin-RXFP1 system in pain processing in the brain. Relaxin was originally identified as a hormone of reproduction and pregnancy[31] but was subsequently found not to be mandatory for pregnancy in humans.[28] Relaxin is now well recognized for its vasodilatory action on blood vessels,[14,28] its positive chronotropic and ionotropic effects on the heart,[38,72] and its powerful antifibrotic actions,[57] among others.[5] Despite these pleiotropic effects described with various organs[4] and some early anatomical studies,[43–45] very few studies have been performed in recent years to explore the role of the relaxin-RXFP1 system *within* the central nervous system. Circulating relaxin is known to activate RXFP1 present in the subfornical organ and the organum vasculosum of the lamina terminalis, outside the blood–brain barrier, to cause a reduction in plasma osmolality.[61] Circulating and centrally administered relaxin also increases water consumption in rats, through actions at these sites.[60] In addition to the circumventricular organs and hypothalamic nuclei accessible to circulating peptides such as relaxin, relaxin was also shown to alter the activity of the BLA after local administration in the rat, which impaired fear memory consolidation.[44]

Thus, in light of the presence of RXFP1 mRNA and relaxin-binding sites in areas involved in pain processing,[9,45,54] in the current study we tested the effect of intracerebroventricular administration of relaxin peptide analogs on nociceptive behaviors in mice. Although this strategy lacked anatomical specificity, it allowed testing for a global effect of RXFP1 activation in the brain. Our results demonstrated an analgesic effect of centrally injected RXFP1 agonist peptides on 2 sensory modalities, mechanical and thermal sensitivity. The results obtained after icv injection of H2-relaxin and B7-33 were similar, indicating the analgesic effect is mediated by RXFP1. Indeed, in cell-based systems, H2-relaxin can act as a biased ligand at the relaxin-3 receptor, RXFP3,[4,69] and therefore its effects in vivo cannot be unequivocally attributed to RXFP1 activation.

By contrast, however, B7-33 is a single-chain analog of the H2-relaxin B-chain, which retains binding to RXFP1.[35,55] B7-33 has lower binding affinity than H2-relaxin at RXFP1 but has similar potency to H2-relaxin in the activation of the phosphorylated extracellular signal-regulated kinase pathway in RXFP1-expressing human embryonic kidney cells and rodent myofibroblasts.[35] Functionally, the small B7-33 peptide agonist efficiently prevented or reversed organ fibrosis and dysfunction in rodent models of heart or lung disease.[35,46,55] B7-33 also conferred cardioprotection and attenuated cardiomyocyte death after cardiac infarction.[15,25] In the current study, the concentration of H2-relaxin used was based on that reported as effective in previous reports.[44] The concentration of B7-33 was twice that of H2-relaxin to take into account the lower receptor affinity of the single-chain agonist.[35]

Relaxin-induced analgesic effects are transitory after a rapid onset but are not likely to be due to RXFP1 desensitization because prolonged exposure of RXFP1 to H2-relaxin does not trigger β-arrestin coupling and receptor internalization and results in sustained signaling (up to 6 hours) in vitro.[4,10,30] The short duration of the analgesic effects observed (between 30 and 60 minutes) more likely indicates that the ligand is degraded quite rapidly or possibly that the intracellular signaling cascade is rapidly terminated in vivo.

In previous studies, the peptide, B-R13/17K-H2, was characterized as an RXFP1 antagonist in cells that endogenously express RXFP1—rat renal myofibroblasts and MCF-7 cancer cells.[36] Notably, in the current study, the thermal analgesia produced by H2-relaxin and B7-33 was not reversed by the antagonist. One interpretation is that any action that is not reversed by a higher molar amount of RXFP1 antagonist is caused by the relaxin agonist analogs interacting with other transducing systems independent of RXFP1.[29] The glucocorticoid receptor is a potential target of H2-relaxin,[17,18] but its involvement in activation of gene expression is not consistent with the rapid effect of H2-relaxin and B7-33 on thermal sensitivity. Ligand-directed signaling bias at the relaxin-3 receptor, RXFP3, is a further possibility because H2-relaxin can activate RXFP3 and has potency and efficacy at the MAP kinase and AP-1 transduction pathways in cell lines.[69] However, such biased signaling has not been observed for B7-33, which is a more selective RXFP1 agonist.[5,55]

Alternatively, it is possible that B-R13/17K-H2 preferentially inhibits RXFP1 transduction in a specific population of those neurons activated by the RXFP1 agonists or at selective signaling pathways, as observed with RXFP3. This hypothesis implies that different cell populations or different signaling pathways modulate mechanical and thermal pain in the brain. In this regard, different circuits convey noxious mechanical[19,47,53] and thermal[6,23] information. However, although the existence of specific thermal and mechanical transduction systems and circuits are well described in the periphery and spinal cord,[20,52] such a distinction remains elusive in the brain. Therefore, further studies are warranted to investigate the complex mechanisms associated with brain RXFP1 signaling and modulation of mechanical and thermal pain.

Because the injection of relaxin analogs into the lateral cerebral ventricle does not provide information on their precise sites of action, in initial efforts to identify these loci, we studied the distribution of RXFP1 mRNA expression in a limited number of brain areas known to play a role in pain transmission. The pain responses tested in our study rely on spinal reflexes, and therefore, we focused on brain areas potentially involved in descending pain pathways whose modulatory effects on spinal circuits could be altered by RXFP1 activation.

RXFP1 mRNA expression has been demonstrated in the brain of several species.[43] It has been well characterized in rat brain[44,45] and some data are available in the mouse (see Ref 46; Allen Brain Atlas). A comparison of previous studies suggests RXFP1 mRNA expression is more restricted in mouse brain than in rat brain. For example, the BLA displays a much higher density of RXFP1 mRNA in the rat than in the mouse. Thus, we chose to investigate 3 forebrain regions involved in pain processing, namely the ACC, CLA, and SUB.[2,66] The relative abundance of RXFP1 mRNA in the ACC and CLA and the ability of RXFP1 activation to produce analgesia are consistent with the prominent role of these areas in modulating descending pain pathways. Indeed, the ACC exerts a facilitating action on pain transmission.[12,58,64] The broad and unique cortical connections of the CLA suggest it serves as a central network hub, coordinating activity within cortical circuitry.[76] The CLA displays a strong connectivity with sensory modalities and a significant preference for peripheral sensory information.[26] In all the brain areas examined, RXFP1 mRNA was almost exclusively expressed in CaMKII mRNA-positive, excitatory neurons and thus identifies a novel signaling system to control modulatory pain outputs from these regions.

The detailed cellular and regional distribution of relaxin, the preferred, cognate ligand for RXFP1, remains largely unexplored in the brain. Therefore, we conducted a broad mapping of relaxin immunoreactivity throughout the mouse

brain using immunohistochemistry. Although not exhaustive, our data are in agreement with published reports[43,44] and identify relaxin-immunoreactive neurons in several brain areas of importance for pain processing. We identified many cortical and subcortical regions, hypothalamic nuclei, and midbrain structures that contain a moderate to high number of relaxin-immunoreactive cells. The presence of relaxin immunoreactivity in the cell body, with only rare positive neural processes, indicates that relaxin may serve as a paracrine neuromodulator. However, RXFP1 mRNA and relaxin-binding sites also exist in rat brain areas devoid of relaxin immunoreactivity.[43] Moreover, our study has determined that several neuronal populations immunoreactive for relaxin project to distant RXFP1-containing brain areas. Thus, relaxin might be transported along the axon and released in the target sites, as proposed earlier.[44] Interestingly, the ACC and CLA have strong ipsilateral reciprocal connections[73] that potentially contain relaxin. The mouse BLA also sends relaxin-immunoreactive neural outputs to the ACC and CLA. In these different regions connected to the ACC and CLA, relaxin neurons are mostly inhibitory, based on their expression of GAD65 mRNA, consistent with a recent analysis of the transcriptome of neurons in the mouse visual cortex.[65]

The effects of RXFP1 activation on PWTs were observed when the mice tested were experiencing persistent pain, but not under control conditions. Our study demonstrated that this restriction of RXFP1 action to CFA-induced persistent pain-like states does not depend on changes in the relative expression of relaxin or RXFP1 transcripts. Instead, it could be due to alterations in RXFP1 signaling under persistent pain conditions, including possible RXFP1 oligomerization[62,63] or heterodimerization.[13] Another possibility is that specific neuronal systems must be physiologically challenged to become sensitive to RXFP1 activation, as observed for other neuropeptides that play a particular role in pathophysiological conditions.[32]

In the current study, we centrally administered exogenous relaxin analogs to demonstrate the analgesic action of RXFP1 activation in adult mice. These data do not preclude a possible role for endogenous relaxin signaling in nociception that warrants further assessment in future studies. Additional studies will be also required to identify the brain areas involved in the analgesic effects and the underlying signaling mechanisms associated with the relaxin-RXFP1 system control of mechanical and thermal pain.

Disclosures

Acknowledgements

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PR9/A109.

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