Supplementary materials and methods

2.1 Mice

Male C57BL/6J mice (*Charles Rivers, France)*, 8-10 weeks old, were housed in groups of 4-5, under a standard 12:12 light/dark cycle, with free access to food, water and enrichment items. All experimental procedures followed the ethical guidelines of the International Association for the Study of Pain and were approved by the French Ministry of Agriculture (Agreement #21890).

2.2 Cannula implantation

Mice were anesthetized by inhalation of 4% isoflurane in an induction chamber. The anesthesia was then maintained with 2% isoflurane using a mask. Mice were placed in a stereotaxic frame (*RWD Desktop Digital Stereotaxic Instruments San Diego, CA, USA*) and to alleviate pain, 100 µl of buprenorphine (*0.03 mg/ml*) and 20 µl of 1% lidocaine were injected subcutaneously. The skull was exposed and cleaned with Betadine, and guide cannulae (*Bilaney, Düsseldorf, Germany)* were positioned just above the lateral ventricle (*AP: 0.7 mm, ML: –0.7 mm, DV: –2.85 mm*) and fixed in place by dental cement *(Phymep, Paris, France).*

2.3 Inflammatory pain model

At 4 days post-surgery recovery, 20 µl of Complete Freund's Adjuvant (CFA) (*Sigma-Aldrich, St Louis, MO, USA)* was injected into the hind paw of 'CFA' mice to induce inflammation, whereas 20 µl of NaCl was injected into the hind paw of 'sham' mice.

2.4 Intracerebroventricular infusion of relaxin peptides

Four days after CFA/NaCl injections, mice were anesthetized with isoflurane and relaxin analogs were injected intracerebroventricularly. 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) were delivered via an internal cannula inserted into the guide cannula. The internal cannula was connected by polyethylene tubing to a Hamilton syringe (*ThermoFisher Scientific, Illkirch, France*) mounted on an infusion pump (*Ugo Basile, Gemonio, Italy*). Mice were divided into 10 groups (see Table 1). RXFP1 activation was tested with a single injection of either 2.5 µl of H2-relaxin (H2) at 2 µg/µl or 2.5 µl of B7-33 at 2 µg/µl. Control mice received a single injection of 2.5 µl artificial cerebrospinal fluid (aCSF). The specificity of RXFP1 activation was assessed with a single injection of 5 µl of H2 (2 µg/µl) + RXFP1 antagonist, B-R13/17K-H2 (4 µg/µl) or B7-33 (2 µg/µl) + B-R13/17K-H2 (4 µg/µl).

2.5 Pain assessment

Mechanical response were monitored 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/NaCl (D4). Mice were placed in the testing cage (*Ugo Basile)* one hour before the icv injection for habituation. The withdrawal threshold of the injected and uninjected hind paw/leg was determined with von Frey hairs (0.16 g, 0.4 g, 0.6 g, 1 g, 1.4 g and 2 g) (*Bioseb, Vitrolles, France*) using the ascending method before, and at 30 minutes, 1 hour and 2 hours after icv infusion of relaxin peptide analog(s). Von Frey filaments were applied 5 successive times (with a 30 second delay in between) on the plantar surface of the hind paw of the mouse standing on its four paws. The withdrawal threshold was established when the mouse responded positively (withdrawal of the hind paw) to 3 out of 5 applications.

Thermal sensitivity was evaluated using the Hargreaves test. Mice were placed in Hargreaves cages (*IITC Inc. Life Science, Tem Sega, Pessac, France*) and thermal sensitivity was tested at D–1, and D4 before and at 30 minutes, 1 hour and 2 hours after icv infusion of relaxin peptides. An infrared laser beam (IR 40) was applied 3 successive times (with at least 2 minutes delay in between) on the plantar surface of the hind paw of the mouse standing on its four paws, until the mouse withdrew its hind paw. The average time to withdrawal of the 3 tests was recorded as the paw withdrawal latency.

2.6 Multiplex in situ *hybridization histochemistry (RNAscope™)*

Multiplex *in situ* hybridization *(RNAscope™, Advanced Cell Diagnostics, Hayward, CA, USA)* was conducted according to the manufacturer's instructions with slight modifications. Fresh frozen mouse brains were cut on a cryostat *(Leica, Wetzlar, Germany)*. Sixteen (16) µm coronal sections were mounted on Super Frost Plus slides (*ThermoFisher Scientific*), fixed in 4% paraformaldehyde (PFA) for 16 minutes, then dehydrated in increasing concentrations of ethanol (50, 70 and 100%) and left overnight in 100% ethanol at –20°C.

The next day, sections were dried for 10 minutes at room temperature, and a hydrophobic barrier was drawn around sections using a hydrophobic pen (*Advanced Cell Diagnostics*). Sections were incubated with protease pretreatment for 20 minutes at room temperature, then washed twice in wash buffer diluted in PBS (wash buffer/PBS). Sections were then incubated with probe mix 1 [complementary to RXFP1 (Catalog#: 458001-C1), 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 in wash buffer/PBS and signal amplification was conducted by serial incubations in solutions provided by the manufacturer, AMP1 (30 minutes at 40°C), AMP2 (15 minutes at 40°C), AMP 3 (30 minutes at 40°C) and finally AMP4 (AltA for mix 1 and AltB for mix 2; 15 minutes at 40°C). Sections were then incubated in DAPI for 20 minutes at room temperature, covered in fluorescent mounting medium, coverslipped and left in the dark for 30 minutes at room temperature before being stored at 4°C.

2.7 Neuronal tract-tracing

In studies to map some of the projections of brain relaxin neurons, three mice were injected, following the aforementioned surgical procedure, with 200 nl of fluorogold into the

basolateral amygdala (BLA) at two positions (*AP: –1.8 mm, ML: –3.4 mm, DV: –3.4mm and –3.8 mm*). A separate group of 3 mice were injected with 200 nl of fluorogold in the anterior cingulate cortex (ACC) at 2 positions (*AP: 0.8 mm, ML: –0.35 mm, DV: –1.8 mm and –2.2 mm*). Mice were kept single-housed for 2 weeks before brain fixation by intra-cardiac perfusion with 4% PFA in 0.1 M PB),[11] and immunohistochemical staining for relaxin-immunoreactivity, as described below.

2.8 Immunohistochemistry

Perfusion-fixed mouse brains were cut on a cryostat. Coronal sections (25 µm) were incubated in 1× PBS, and 1% bovine serum albumin (BSA) (*Sigma-Aldrich*) for 30 minutes at room temperature. Rabbit anti-rat relaxin primary antibody (1:200) *(Biovision, Milpitas, CA, USA*) was diluted in PBS + BSA (1%) + Triton (0.3%) (*Sigma-Aldrich*) and sections were incubated in this solution for 24 hours at 4°C. Sections were then washed in 1× PBS and incubated for 2 hours at room temperature with Alexa Fluor 568, anti-rabbit secondary antibody (1:500 in 1× PBS + 1% BSA) *(ThermoFisher Scientific*). The specificity of the anti-relaxin antibody has been validated by western blot analysis of Jurkat cells and rat kidney tissue lysates (see supplier website at https://www.biovision.com). Control studies involving pre-adsorption with 10^{-6}M of the H2 antigen, or omission of the primary antibody resulted in a loss of specific staining (Fig. S2). The distribution of relaxin-immunoreactivity observed in mouse brain matched that reported for rat relaxin peptide and mRNA.[27,43-45] For quantification, double-labeling was performed with chicken anti-NeuN (1:500, Millipore, ABN91), visualized with Alexa Fluor 488, anti-chicken secondary antibody (1:500 in 1 × PBS + 1% BSA) *(ThermoFisher Scientific*).

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

Semi-quantitative analysis of relaxin immunostaining was performed in brain areas listed in Tables S2 and S3 (4 sections for each area, in 3 sham mice and in 3 mice injected with fluorogold). Results were expressed as the number and percentage of relaxin-immunopositive cells ± SEM, compared to the total number of NeuN-immunopositive neurons (Table S2), or of relaxin and fluorogold double-labeled cells, compared to the total number of relaxin immunopositive cells (Table S3).

2.9 Statistical analysis

All statistical analyses were done using Graph Pad Prism. Behavioral analysis was conducted using a 2-way ANOVA, followed by a Tukey test. *In situ* hybridization (RNAscope) data analysis was conducted using a 2-way ANOVA, followed by a Sidak test. Data are presented as mean ± SEM, and differences were considered significant when $p < 0.05$.

Supplementary Data

Table S1. *Groups of mice receiving intracerebroventricular infusion of relaxin peptides*

Relaxin analogs	Paw	Concentration (µmol/L)	Test	CFA (n)	NaCl (n)
B7-33	Injected	0.66	Von Frey	9	3
			Hargreaves	5	5
B7-33	Uninjected	0.66	Von Frey	9	5
			Hargreaves	5	5
B7-33 + B-R13/17K-H2	Injected	0.66+0.664	Von Frey	5	5
			Hargreaves	5	5
B7-33 + B-R13/17K-H2	Uninjected	0.66+0.664	Von Frey	5	5
			Hargreaves	5	5
aCSF	Injected	-	Von Frey	9	5
			Hargreaves	5	5
aCSF	Uninjected	-	Von Frey	9	5
			Hargreaves	5	5
Relaxin analogs	**Paw**	**Concentration (µmol/L)**	**Test**	**CFA (n)**	**NaCl (n)**
H2	Injected	0.33	Von Frey	6	5
			Hargreaves	5	5
H2	Uninjected	0.33	Von Frey	6	5
			Hargreaves	5	5
H2 + B-R13/17K-H2	Injected	0.33+0.664	Von Frey	5	5
			Hargreaves	5	5
H2 + B-R13/17K-H2	Uninjected	0.33+0.664	Von Frey	5	5
			Hargreaves	5	5
aCSF	Injected	-	Von Frey	6	5
			Hargreaves	5	5
aCSF	Uninjected	-	Von Frey	6	5
			Hargreaves	5	5

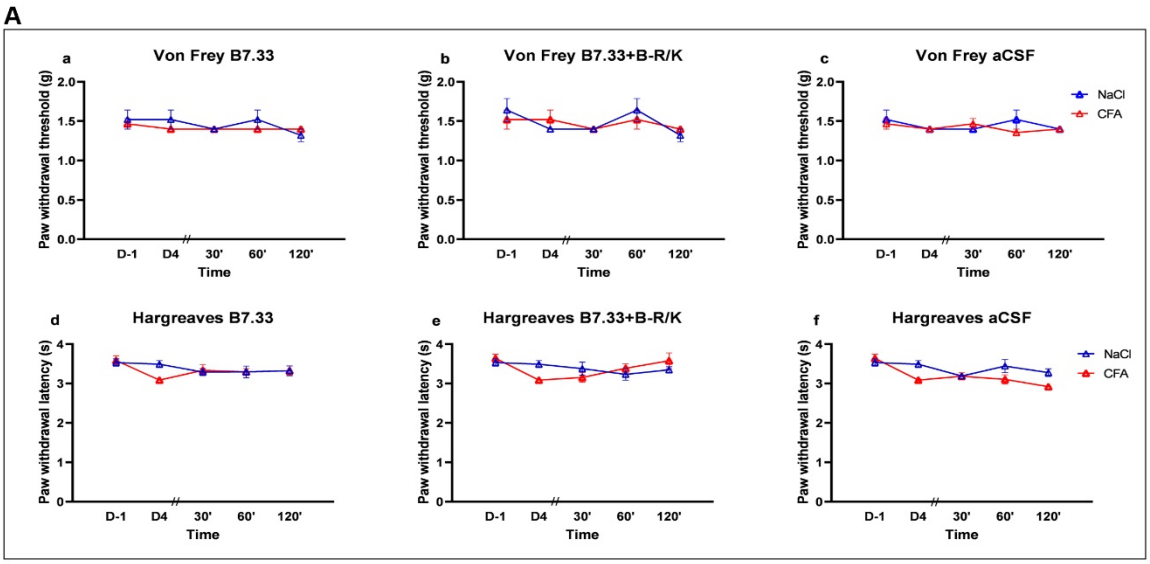
Table S2. *Quantification of relaxin immunopositive cells in different brain areas*

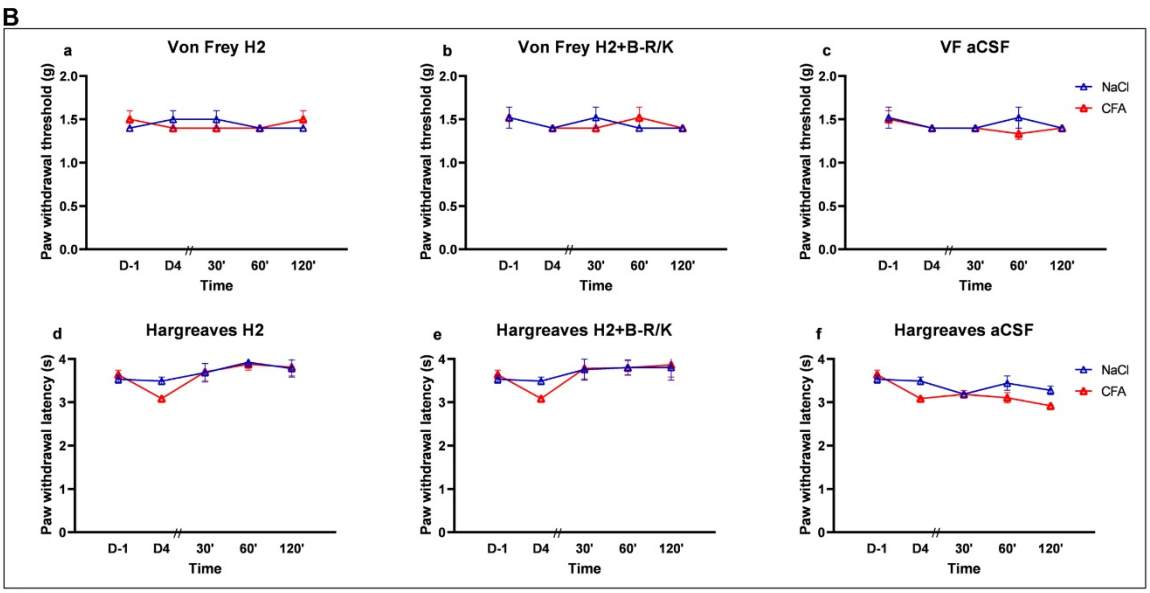
Brain area	No. of relaxin immunopositive neurons/total no. of neurons	Percentage (%) of relaxin immunopositive neurons
Accessory olfactory nucleus	192/806	24 ± 5
Piriform cortex	372/1242	30 ± 7
Anterior cingulate cortex	384/961	39 ± 12
Claustrum	252/687	36 ± 8
Lateral hypothalamus	660/1914	34 ± 11
Arcuate nucleus	756/1259	60 ± 7
Medial habenula	348/730	47 ± 7
Basolateral amygdala	156/484	32 ± 5
Cerebellar Purkinje cells	327/384	85 ± 8

Table S3. *Quantification of relaxin immunopositive cells in different brain areas projecting to the anterior cingulate cortex (upper panel) or to the claustrum (lower panel).*

Fluorogold injection into anterior cingulate cortex		
Brain area	**No. of relaxin + fluorogold positive cells/no. relaxin cells**	**Percentage (%) of double-labeled cells/total no. of relaxin cells**
Claustrum	235/324	72 ± 15
Posterior hypothalamus	621/772	80 ± 14
Basolateral amygdala	156/252	62 ± 13
Somatosensory cortex	396/412	96 ± 9
Fluorogold injection into claustrum		
Brain area	**No. of relaxin + fluorogold positive cells/no. relaxin cells**	**Percentage (%) of double-labeled cells/total no. of relaxin cells**
Anterior cingulate cortex	278/357	69 ± 13
Medial preoptic area	468/665	70 ± 16
Basolateral amygdala	217/289	75 ± 9
Posterior thalamic complex	308/696	44 ± 11

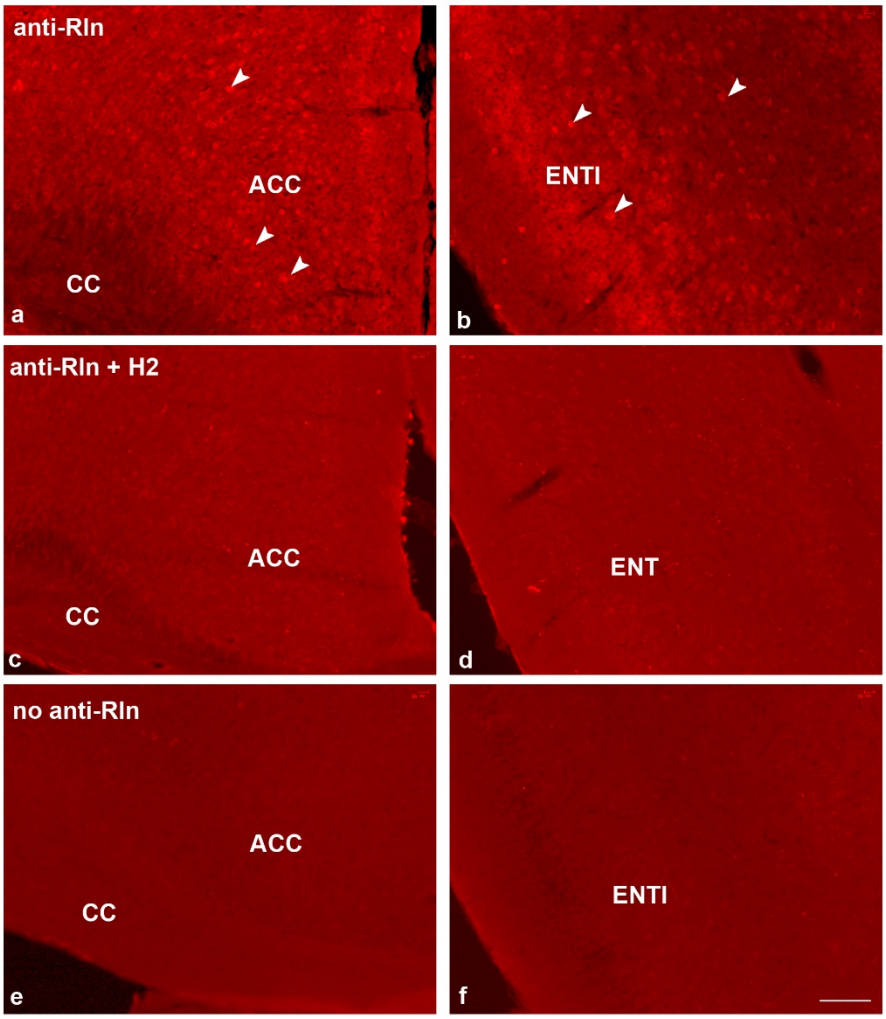
Supplementary Figures





(Fig. S1, Abboud et al.,)

Figure S1. Assessment of mechanical sensitivity (paw withdrawal threshold, von Frey test, VF) and thermal sensitivity (paw withdrawal latency, Hargreaves test) of the uninjected hind paw after icv injection of the RXFP1 agonists (**A**) B7-33 or (**B**) H2-relaxin (left panels), co-injection of B7-33 and H2-relaxin with the RXFP1 antagonist, B-R13/17K-H2 (B-R/K) (centre 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.



(Fig. S2, Abboud et al.)

Figure S2. Assessment of the specificity of the anti-relaxin antibody. Relaxin immunoreactivity was detected in the anterior cingulate (ACC, left panel) and entorhinal (ENT, right panel) cortex. Sections were incubated with anti-relaxin (Rln) antibody (a, b), anti-relaxin antibody pre-adsorbed with 10^{-6}M of H2-relaxin antigen (c, d), or no primary antibody (e, f). Immunopositive profiles were observed only when incubating sections with the anti-relaxin antibody without pre-adsorption (arrowheads in a and b). Bars = 100 μm.