Published in final edited form as:

Neuroscience. 2011 June 2; 183: 47–63. doi:10.1016/j.neuroscience.2011.03.047.

Topographical distribution of corticotropin-releasing factor type 2 (CRF₂) receptor-like immunoreactivity in the rat dorsal raphe nucleus: co-localization with tryptophan hydroxylase

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Abstract

Corticotropin-releasing factor (CRF) and CRF-related neuropeptides are involved in the regulation of stress-related physiology and behavior. Members of the CRF family of neuropeptides bind to two known receptors, the CRF type 1 (CRF₁) receptor, and the CRF type 2 (CRF₂) receptor. Although the distribution of CRF₂ receptor mRNA expression has been extensively studied, the distribution of CRF₂ receptor protein has not been characterized. An area of the brain known to contain high levels of CRF₂ receptor mRNA expression and CRF₂ receptor binding is the dorsal raphe nucleus (DR). In the present study we investigated in detail the distribution of CRF₂ receptor immunoreactivity throughout the rostrocaudal extent of the DR. CRF₂ receptor-immunoreactive perikarya were observed throughout the DR, with the highest number and density in the mid-rostrocaudal DR. Dual immunofluorescence revealed that CRF₂ receptor immunoreactivity was frequently co-localized with tryptophan hydroxylase, a marker of serotonergic neurons. This study provides evidence that CRF₂ receptor protein is expressed in the DR, and that CRF₂ receptors are expressed in topographically organized subpopulations of cells in the DR, including serotonergic neurons. Furthermore, these data are consistent with the hypothesis that CRF₂ receptors play an important role in the regulation of stress-related physiology and behavior through actions on serotonergic and non-serotonergic neurons within the DR.

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Keywords

CRF₂ receptor; immunofluorescence; immunohistochemistry; raphe; serotonin; tryptophan hydroxylase

1. Introduction

Corticotropin-releasing factor (CRF) is a 41-amino acid neuropeptide that has been linked to the regulation of stress-related physiology and behavior, including regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Vale et al., 1981; Vale et al., 1983). The CRF family of neuropeptides also includes urocortin (Ucn) 1, Ucn 2, and Ucn 3; the role of these CRF-related neuropeptides in the HPA axis and stress-related physiological and behavioral responses is less clear, although evidence suggests that they also play an important role in modulating these responses (Reul and Holsboer, 2002). CRF and its family of neuropeptides are known to bind to two distinct G protein-coupled receptors, the CRF type 1 (CRF₁) receptor, and the CRF type 2 (CRF₂) receptor (Perrin et al., 1993; Lovenberg et al., 1995b), for which they have different binding affinities. CRF preferentially binds to CRF₁ receptors, Ucn 1 binds with high affinity to both receptors, and Ucn 2 and Ucn 3 preferentially bind to CRF₂ receptors (Lewis et al., 2001; Reyes et al., 2001). Whereas CRF₁ receptors have a widespread distribution in the central nervous system (CNS), including subcortical and cortical regions, CRF₂ receptor distribution is mainly limited to subcortical structures (Chalmers et al., 1995; Radulovic et al., 1998; Sanchez et al., 1999; Van Pett et al., 2000; Chen et al., 2000; Korosi et al., 2006).

Multiple isoforms of the CRF₂ receptor are known to be expressed including the alpha, beta, and gamma (found only in humans) isoforms, a soluble isoform, and a truncated isoform (currently described only in rats). These isoforms have distinct expression patterns and binding affinities for CRF and its family of peptides (Lovenberg et al., 1995a; Kostich et al., 1998; Miyata et al., 1999; Miyata et al., 2001; Chen et al., 2005b; Evans and Seasholtz, 2009).

A subcortical area of the rat CNS that contains a high density of cells expressing CRF₂ receptor mRNA expression is the dorsal raphe nucleus (DR). The DR is a brainstem region that, together with the median raphe nucleus (MnR), contains the majority of serotonergic neurons with ascending projections to forebrain structures (Steinbusch et al., 1978; Steinbusch, 1981). *In situ* hybridization studies have shown that the DR is one of a few brain regions that contains more CRF₂ receptor mRNA expression than CRF₁ receptor mRNA expression in rats (Van Pett et al., 2000; Day et al., 2004; Korosi et al., 2006). Co-localization studies in rat brain have shown that CRF₂ receptor mRNA expression within the DR is expressed predominantly in serotonergic neurons, although, in caudal parts of the DR, CRF₂ receptor mRNA expression is commonly found in non-serotonergic neurons, including γ -aminobutyric acid-synthesizing (GABA)ergic neurons (Day et al., 2004). Consistent with these findings, and consistent with expression of functional receptor protein in the DR, dense CRF₂ receptor binding has been described in the DR of several vole species (Lim et al., 2005). Although the distribution of CRF₂ receptor mRNA expression has been extensively studied, the distribution of CRF₂ receptor protein has not been characterized. This could be due to 1) difficulties generating CRF₂ receptor-specific antibodies, 2) antibodies used in many previous studies, such as the CRF_{1/2} receptor antibody sc-1757 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), recognize both CRF₁ and CRF₂ receptors (Campbell et al., 2003; Chen et al., 2000; Hinkle et al., 2003, also see Figure 4), and 3) some CRF₂ receptor-specific antibodies used in previous studies (Waselus et al., 2009; Wang et al., 2007) are no longer commercially available. Recent studies using

immunoelectron microscopy have demonstrated that CRF₂ receptor immunoreactivity is predominantly intracellular in DR neurons under basal unstressed conditions, and shifts toward a greater expression at the plasma membrane following stress exposure (Waselus et al., 2009). The shift in intracellular versus plasma membrane localization of CRF₂ receptors is associated with a shift from inhibitory to excitatory neuronal firing rate responses to CRF administration (Waselus et al., 2009). The hypothesis that functional CRF₂ receptors are expressed in the DR is supported by studies using immediate-early gene expression (e.g., nuclear c-Fos induction representing increased cellular responses) and electrophysiology to investigate the effects of CRF₂ receptor ligands on responses of serotonergic neurons in the DR. Intracerebroventricular injections of the CRF₂ receptor ligand mouse Ucn 2 (mUcn 2) (Staub et al., 2005) or microinjections of mUcn 2 directly into the DR (Amat et al., 2004) increase c-Fos expression in DR serotonergic neurons and increase serotonin release in DR projection sites, while pretreatment with the CRF₂ receptor antagonist [DPhe11, His12]sauvagine(11–40) (antisauvagine-30; ASV-30) blocks these effects (Amat et al., 2004; Staub et al., 2006). Likewise, electrophysiological studies in anesthetized rats have shown that injections of mUcn 2 directly into the DR can increase the firing rates of serotonergic neurons and these effects can be prevented by pretreatment with ASV-30 (Pernar et al., 2004). Together, these studies are consistent with the finding that activation of CRF₂ receptors in the DR increases extracellular serotonin concentrations within the basolateral amygdaloid nucleus (Amat et al., 2004) and nucleus accumbens (Lukkes et al., 2008), forebrain targets of serotonergic neurons arising from the DR (Abrams et al., 2005; Van Bockstaele et al., 1993).

Understanding the distribution of CRF₂ receptors within the DR is important because there is increasing evidence that the DR may include anatomically and functionally distinct subpopulations of serotonergic neurons (Imai et al., 1986; Abrams et al., 2004; Lowry et al., 2005; Lowry et al., 2008; Lowry and Hale, 2010). Indeed, serotonergic neurons can be divided into several different types, some of which are restricted to specific regions of the DR, based on their physiological properties and behavioral correlates (Rasmussen et al., 1984; Fornal et al., 1996; Jacobs and Fornal, 1999; Sakai and Crochet, 2001; Kocsis et al., 2006).

Several studies suggest that interactions between CRF or CRF-related ligands and serotonergic systems may play an important role in the regulation of anxiety-related behaviors in rats (Stout et al., 2001; Maier and Watkins, 2005; Lowry et al., 2005) and anxiety and affective disorders in humans (Arborelius et al., 1999; Austin et al., 2003). These data, together with 1) the potential for mismatch between CRF₂ receptor mRNA and protein expression in neurons within the DR, 2) the potential for expression of multiple isoforms of the CRF₂ receptor, and 3) the evidence for anatomical and functional heterogeneity within the DR, led us to characterize the detailed topographical organization of CRF₂ receptor protein expression within this brainstem region and the extent of its co-localization with cytoplasmic tryptophan hydroxylase (TrpOH), a marker of serotonergic neurons. These studies used an antibody directed against the N-terminus of the CRF₂ receptor, including an epitope that is highly conserved among CRF_{2α} and CRF_{2β} receptor isoforms, and therefore is likely to detect all known CRF₂ receptor isoforms in rat brain, including truncated isoforms of the receptor.

2. Experimental procedures

2.1. DR immunohistochemistry

2.1.1. Animals—Adult male Sprague Dawley rats (n = 8; 250–300 g; Harlan Laboratories, Indianapolis, IN, USA) were used. The rats were group housed, 2 per cage, in wire cages (17 cm × 35 cm × 45 cm; Alternative Design, Siloam Springs, AR, USA) and were maintained

with free access to food and water under a 12 h light/dark cycle with lights on at 0700 h in a room with standard temperature (21 °C) and humidity (22%). Rats were acclimated for at least 1 week before experimental manipulation. All procedures were approved by the University of Colorado at Boulder Institutional Animal Care and Use Committee. In addition, all studies were consistent with the NIH *Guide for the Care and Use of Laboratory Animals* (N.I.H. Publication No. 85-23). All possible efforts were made to minimize the number of animals used and their suffering.

Mice lacking functional CRF₁ receptors (n = 3) and wild type littermates (n = 3) (Smith et al., 1998) were maintained with free access to food and water under a 12 h light/dark cycle. All experimental protocols were approved by The Weizmann Institute of Science Institutional Animal Care and Use Committee. Mice lacking functional CRF₂ receptors were not used as methods used to develop the existing CRF₂ mutants did not delete the 5' region of the gene encoding the N-terminus of the CRF₂ receptor, the region that is targeted by the anti-CRF₂ receptor antibody used in this study.

2.1.2. Tissue processing—Rats or mice were anesthetized with 0.5 ml sodium pentobarbital (200 mg/ml; Vortech Pharmaceuticals, Dearborn, MI, USA). Animals were transcardially perfused with 150 ml of 0.1 M phosphate buffered saline (PBS; pH 7.4) at room temperature (RT), followed by 200 ml of cold (4 °C) 4% paraformaldehyde (prepared using 40 g paraformaldehyde, 404 ml 0.2 M Na₂HPO₄, 96 ml 0.2 M NaH₂PO₄, and 500 ml dH₂O, brought to pH 7.4 with sodium hydroxide pellets). Brains were removed from the cranium, post-fixed for 1 h in the same fixative at 4 °C, then immersed in 0.1 M PBS with 25% sucrose and stored for 2 days at 4 °C. Following cryopreservation with sucrose, brains were then blocked in a coronal plane using a rat (RBM-4000C, ASI Instruments, Warren, MI, USA) or mouse (RBM-2000C, ASI Instruments) brain matrix; brains were cut in the coronal plane at the caudal border of the mammillary bodies (approximately –5.30 mm bregma in rat brains or –2.80 mm bregma in mouse brains) and rapidly frozen in liquid isopentane chilled on dry ice. The brains were stored at –80 °C until sectioning. Brain sections (30 µm) were made using a cryostat and stored as 6 alternate sets of sections at –20 °C in cryoprotectant (prepared using 270 ml ethylene glycol, 160 ml glycerol, 202 ml 0.2 M Na₂HPO₄•7H₂O, 48 ml 0.2 M NaH₂PO₄•H₂O, and 320 ml dH₂O) until immunostaining was performed.

2.1.3. Immunohistochemistry—For immunostaining, every sixth section of the midbrain containing the DR (from approximately –5.8 to –10.3 mm bregma in rat brains; approximately –3.5 to –5.7 mm bregma in mouse brains) was removed from the cryoprotectant and washed in 0.01 M PBS for 15 min. The tissue was then placed in 0.01 M PBS containing 1% H₂O₂ for 15 min and then sections were washed twice in 0.01 M PBS for 15 min each. Sections were then pre-incubated in blocking buffer (0.01 M PBS, pH 7.4, containing 1% normal rabbit serum, Cat. No.011-000-120, Jackson ImmunoResearch, West Grove, PA, USA) at RT for 2 h to reduce non-specific staining. Sections were then incubated in goat anti-CRF₂ receptor-selective polyclonal antibody (1:300; Cat. No. sc-1826; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.01 M PBS containing 0.04% normal rabbit serum and 0.2 % Triton X-100 for 20 hours at 4 °C with gentle agitation (Lukkes et al., 2009). Sections were washed in 0.01 M PBS containing 0.04% normal rabbit serum 2 times for 15 min, and incubated for 2 h at RT in biotin-conjugated donkey anti-goat secondary antibody (1:200; Cat. No.705-066-147, Jackson ImmunoResearch) in 0.01 M PBS containing 0.04% normal rabbit serum and 0.03% Triton X-100 to visualize CRF₂ receptor-like immunoreactivity. After incubation with secondary antibody, sections were washed in 0.01 M PBS containing 0.04% normal rabbit serum 2 times for 15 min, and then placed in an avidin-biotin-peroxidase complex (ABC) reagent (Cat. No. PK-6106; Vector Laboratories, Burlingame, CA, USA) at 1:200 in 0.01 M PBS for

90 min. The tissue was then rinsed in 0.01 M PBS two times for 15 min each. The tissue was then placed in a peroxidase-based substrate reaction (Vector SG chromogen kit; Cat. No. SK-4700; Vector Laboratories) for 6 min. The reaction was stopped by rinsing the tissue in 0.01 M PBS for 15 min. After this the tissue was briefly rinsed in 0.1 M sodium phosphate buffer (PB) and mounted onto clean glass microscope slides. Once mounted the sections were dehydrated, cleared with xylene and mounted with cover slips.

2.1.4. Immunocytochemistry—Human embryonic kidney (HEK-293) cells that stably express the mouse CRF_{2α} receptor and enhanced green fluorescent protein (EGFP) and wild type (WT) HEK-293 cells were used to determine the specificity of the anti-CRF₂ receptor antibody (Chen et al., 2005a). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Cat. No. 10938025; Invitrogen, Paisley, UK) solution supplemented with 10% fetal calf serum. For immunocytochemistry cells were grown on glass cover slips in a 90 cm² petri dish. After reaching the desired confluency the cover slips were placed in 0.05 M PBS, and then placed in 4% paraformaldehyde in 0.05 M PBS for 30 min. The cover slips were then rinsed in 0.05 M PBS and then placed in 0.05 M PBS containing 0.3% Triton X-100 (PBST) for 5 min. Cover slips were then placed in 1% bovine serum albumin (BSA) in 0.05 M PBS for 30 min. Some cover slips were then placed in either goat anti-CRF₂ receptor antibody (1:100; Cat. No. sc-1826; Santa Cruz Biotechnology) in 1% BSA, goat anti-CRF_{1/2} receptor antibody (1:100; Cat. No. sc-1757; Santa Cruz Biotechnology) in 1% BSA, or in 1% BSA overnight at 4 °C. Cover slips were then placed in 0.05 M PBS and then incubated in either a rabbit anti-goat antibody conjugated to Alexa Fluor 555 (1:500; Cat. No. A21431; Invitrogen, Paisley, UK) in 1% BSA, or 1% BSA for 2 h. The cover slips were then placed in 0.05 M PBS and mounted onto slides using a fluorescent mounting medium (Cat. No. S3023; DAKO, Ely, UK).

2.2. Western blot, microdissected brain regions

Adult male Sprague Dawley rats (n = 2; 250–300 g; Harlan Laboratories) were maintained as described above for immunohistochemical procedures and used for western blots. Western blots were performed to determine the relative molecular weight of the protein(s) recognized by the CRF₂ receptor antibody used, and to determine the relative amounts of CRF₂ receptor protein in different brain regions. Rats were decapitated, brains were then removed, frozen and stored at –80 °C, and then sectioned frozen (300 μm) using a cryostat (Leica CM 1900; North Central Instruments, Plymouth, MN, USA) at –12 °C. The motor cortex (M1), entorhinal cortex (En), lateral septum (LS), ventromedial hypothalamus (VMH), median raphe nucleus (MnR), and DR were dissected at –10 °C using the Palkovits punch technique (Palkovits and Brownstein, 1988) and a 22 gauge cannula (Cat. No. 18036-22; 0.41 mm i.d.; Fine Science Tools, Foster City, CA, USA). Tissue was then homogenized in 60 μl of HEPES buffer (Cat. No. H3375; Sigma-Aldrich, St. Louis, MO, USA) containing 2.4 μl protease inhibitor stock “complete” (1:25 dilution; Roche Diagnostics Ltd, Indianapolis, IN, USA). Total protein concentrations were determined within 5 μl sample duplicates using a Pierce protein assay (Cat. No. 1856210; Thermoscientific, Rockford, IL, USA) and a microplate reader (MultiSkan EX 355; Thermo Electron Corporation, Waltham, MA, USA). Samples (30 μg total protein) were processed for western blotting, and CRF₂ receptor and actin levels were detected using methods described previously (Lukkes et al., 2009). Briefly, protein was mixed in 1 x sodium dodecyl sulfate (SDS)/β-mercaptoethanol loading buffer, vortexed and boiled for 3 min prior to separation by 8% SDS-polyacrylamide gel electrophoresis. Following electrophoresis (Mini-PROTEAN 3 cell, Bio-Rad Laboratories, Hercules, CA, USA), proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immuno-Blot; 0.2 μm, Bio-Rad Laboratories). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 20 min at RT and incubated with goat anti-

CRF₂ receptor polyclonal antibody (1:100; Cat. No. sc-1826; Santa Cruz Biotechnology) in 5% non-fat dry milk in TBS-T overnight at 4 °C. The membranes were rinsed 3 times for 10 min each time at RT in TBS-T. After the rinsing procedure, the membranes were incubated for 2 h at RT in affinity-purified rabbit anti-goat IgG (whole molecule)-peroxidase conjugated secondary antibody (1:30,000; Cat. No. A5420; Sigma-Aldrich) in 5% non-fat dry milk in TBS-T. Control for protein loading was achieved by using mouse anti-actin primary antibody (1:2000; Cat. No. MAB1501R; Chemicon International, Billerica, MA, USA) and goat anti-mouse IgG (Fab specific)-peroxidase conjugated secondary antibody (1:5000; Cat. No. A3682, Sigma-Aldrich) in 5% non-fat dry milk in TBS-T. Proteins were detected using a ChemiDoc-IT imaging system with cooled CCD camera (UVP Bioimaging Systems, Upland, CA, USA).

2.3. Antibodies

The goat anti-CRF₂ receptor antibody used (N-20, Cat. No. sc-1826, Santa Cruz Biotechnology) is an affinity-purified polyclonal antibody raised against a peptide corresponding to amino acid sequence 47–66 mapping at the N-terminus of the CRF_{2β} receptor of mouse origin (RTTIGNFSGPYTYCNTLDQ) (Figure 1). There is no similarity with the amino acid sequence of the CRF₁ receptor (only 4 of 20 amino acids are conserved; Figure 1). The sequence used to generate the antibody is unique based on a search of the BLAST database; this does not preclude the possibility that there are peptides that have not been characterized that have similar sequences.

The goat anti-CRF_{1/2} receptor antibody used (C-20, Cat. No. sc-1757, Santa Cruz Biotechnology) is a polyclonal antibody raised against a peptide corresponding to amino acid sequence 396–415 mapping at the C-terminus of the CRF₁ receptor of human origin (SIPTSPTRVSFHSIKQSTAV). There is a 3 amino acid difference between the rat CRF₁ and rat CRF₂ receptor at the region that corresponds to this sequence. Western blot analysis indicates that this antibody recognizes a band corresponding to the predicted molecular weight of the CRF₁ receptor in mice 77–80 kDa (Radulovic et al., 1998; Chen et al., 2000).

2.4. Analysis of the distribution of CRF₂ receptor immunoreactivity

For analysis, the DR was divided into rostral (−6.92 to −7.64 mm bregma), mid-rostrocaudal (−7.73 to −8.45 mm bregma), and caudal (−8.54 to −9.26 mm bregma) regions (Abrams et al., 2004). Analysis of CRF₂ receptor immunostaining in the DR was performed at 12 anatomical levels at 180 μm intervals (rostral DR; mid-rostrocaudal DR; and caudal DR; 1 section / level / rat). At each level the DR was further divided into different regions. These regions were the dorsal part of the DR (DRD), the ventral part of the DR (DRV), the ventrolateral part of the DR (DRVl), the caudal part of the DR (DRC; subdivided into dorsal (dDRC) and ventral (vDRC) parts at −9.08 mm bregma), and the interfascicular part of the DR (DRI). The rostrocaudal levels and subdivisions were defined based on a standard rat brain stereotaxic atlas (Paxinos and Watson, 1998).

2.5. CRF₂ receptor/TrpOH double immunofluorescence

Adult male Sprague Dawley rats (n=6; 250–300 g; Harlan Laboratories) were maintained as described above for immunohistochemical procedures and used for CRF₂ receptor/TrpOH double immunofluorescence.

Immunofluorescence was used to identify CRF₂ receptor-expressing and TrpOH-expressing cells because this method provides the cellular resolution required to determine co-localization of CRF₂ and TrpOH immunoreactivity. Perfusion and postfixation methods were as described above for CRF₂ receptor immunohistochemistry.

For immunofluorescence, every sixth section of the midbrain containing the DR (from approximately -7.64 to -8.54 mm bregma) was removed from the cryoprotectant and washed in 0.05 M PBS twice, for 15 min each time. Sections were then incubated in 0.05 M PBS containing 0.3% Triton X-100 (PBST) and 0.01% sodium azide for 60 min at RT and then placed in mouse anti-TrpOH monoclonal antibody (1:2000; Cat. No. T-0678, Sigma-Aldrich) in PBST containing 0.01% sodium azide overnight at RT. Following incubation with primary antibody, sections were rinsed 2 times for 15 min each time in 0.05 M PBS and then placed in Cy5-conjugated donkey anti-mouse IgG secondary antibody (1:200; Cat. No. 115-175-205, peak emission 670 nm, red fluorescence; Jackson ImmunoResearch) in 0.05 M PBS for 1 h at RT. Sections were then washed in 0.05 M PBS 2 times for 15 min each time at RT and then incubated in goat anti-CRF₂ receptor polyclonal antibody (1:300; Cat. No. sc-1826, Santa Cruz Biotechnology) in 0.01 M PBS containing 0.04% normal rabbit serum (Cat. No. 011-000-120, Jackson ImmunoResearch) and 0.2% Triton X-100 for 20 h at 4 °C with gentle agitation (Lukkes et al., 2009). Sections were then washed in 0.01 M PBS containing 0.04% normal rabbit serum 2 times for 15 min each time, and incubated for 2 h at RT in FITC-conjugated donkey anti-goat IgG secondary antibody (1:200; Cat. No. 705-095-147, peak emission 520 nm, green fluorescence; Jackson ImmunoResearch) in 0.01 M PBS containing 0.04% normal rabbit serum to visualize CRF₂ receptor immunofluorescence. After incubation in secondary antibody, sections were washed in 0.01 M PBS containing 0.04% normal rabbit serum 2 times for 15 min each time and mounted onto glass slides, then cover-slipped with 4',6-diamidino-2-phenylindole DAPI Vectashield (Cat. No. H-1200, Vector Labs), which fluorescently stains cell nuclei.

2.6. Quantification of CRF₂ receptor/TrpOH double immunofluorescence

For each subject ($n = 6$; Sprague Dawley rats; Harlan Laboratories, Indianapolis, IN, USA), 100X and 200X magnification photomicrographs were generated for regions of interest using a Nikon 90i microscope and a Roper Scientific CoolSNAP ES digital camera linked to a computer with NIS Elements imaging software (A.G. Heinze Inc., Lake Forest, CA, USA). The 100X photomicrographs were used to identify and document the rostrocaudal level of the sample; 200X photomicrographs were used for quantification with each subdivision. For each photomicrograph, we placed the region of interest in the center of the field of view using the 20X objective lens and then took photographs using FITC, Cy5, and DAPI filters. Analysis of fluorescence in the DR and pontine raphe nucleus (PnR) was performed at 6 anatomical levels (-7.46 , -7.64 , -8.00 , -8.18 , -8.54 , and -9.16 mm bregma; 1 section / level / rat; Figure 2), within the dorsal part of the DR (DRD), the ventral part of the DR (DRV), the ventrolateral part of the DR and ventrolateral part of the periaqueductal gray (DRV/VLPAG), the interfascicular part of the DR (DRI), the caudal part of the DR (DRC), and PnR. The anatomical levels and subdivisions were defined based on a rat brain atlas (Abrams et al, 2004; Paxinos and Watson, 1998). Cell counts collected from photomicrographs were probably not biased by differences in cell number or density, as photomicrographs were obtained at a plane of focus in the middle of the tissue sections, which were 30 μ m thick. Separate layers for CRF₂ receptor-immunoreactive (ir) and TrpOH-ir photomicrographs were created using Adobe Photoshpe CS (Adobe Systems Incorporated, San Jose, CA, USA). The numbers of CRF₂ receptor-ir and TrpOH-ir neurons were quantified by placing dots over each CRF₂ receptor-ir and TrpOH-ir profile in additional cell counting layers. The cell counting layers were superimposed and the numbers of single-labeled CRF₂ receptors, single-labeled TrpOH, and double-labeled neurons were counted. Double-labeled (CRF₂ receptor-ir/TrpOH-ir) neurons were confirmed with the slides themselves using 400X magnification.

2.7. Image capture

Brightfield photomicrographs were taken using a Nikon 90i microscope and a Nikon DS-Fi1 digital camera linked to a computer with NIS Elements 3.00 imaging software (A.G. Heinze Inc., Lake Forest, CA, USA). Low magnification images of immunofluorescence in cell culture studies were generated using a Leica DMRB fluorescence microscope, a Leica DC500 camera and image capture software (Leica Microsystems, Heidelberg, Germany). Confocal images of immunofluorescence in cell culture studies were generated using a Leica TCS SP2 AOBS laser scanning confocal microscope using FITC, Cy5, and DAPI filter cubes and Leica Confocal Software (v. 2.00, Leica Microsystems), presented as 8- μ m-thick z-stack projections. Contrast and brightness of the photographs were adjusted using Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA, USA). Photographic plates were prepared in CorelDraw for Windows 12.0 (Viglen Ltd., Wembley, UK).

3. Results

3.1. Immunocytochemistry

In order to determine the specificity of the anti-CRF₂ receptor antibody used in these studies, we conducted immunocytochemical staining of wild type HEK-293 cells that are known to lack CRF₂ receptor expression (Dautzenberg et al., 2000), and HEK-293 cells stably expressing the mouse CRF_{2α} receptor and enhanced green fluorescent protein (EGFP). The anti-CRF₂ receptor antibody did not immunostain wild type HEK-293 cells (Figure 3A,B) but did immunostain HEK-293 cells stably expressing the mouse CRF_{2α} (mCRF_{2α}) receptor (Figure 3D) and EGFP (Figure 3C; for additional controls, see Figure 4). High magnification confocal images revealed that the CRF₂ receptor immunostaining was restricted to the plasma membrane and cytoplasmic compartments of the cell, while EGFP was expressed throughout the cell including the nuclear compartment (Figure 3E–G).

As opposed to CRF₂ receptors, wild type HEK-293 cells are known to express CRF₁ receptors (Dautzenberg et al., 2000). The anti-CRF_{1/2} receptor antibody immunostained both the wild type HEK-293 cells and the HEK-293 cells that stably express the mCRF_{2α} receptor and EGFP (Figure 4). The intensity of staining in the HEK-293 cells stably expressing the mCRF_{2α} receptor was greater than in the wild type HEK-293 cells, suggesting that this antibody detected both endogenous CRF₁ and CRF₂ receptors in cells stably expressing the mCRF_{2α} receptor (Figure 4).

CRF₂ receptor immunostaining in CRF₁ receptor knockout mice—Very few, faintly stained, CRF₂ receptor-ir cells were found in regions of the brain known to contain little to no CRF₂ receptor mRNA expression, including the caudate putamen (CP; Figure 5A,B) and paraventricular nucleus of the thalamus (PV; Figure 4C,D (Van Pett et al., 2000)) in either wild type (Figure 5A,C) or CRF₁ receptor knockout mice (CRF₁ KO; Figure 5B,D). In contrast, large numbers of CRF₂ receptor-ir cells were found in regions known to contain high amounts of CRF₂ receptor mRNA expression, such as the arcuate nucleus (Arc; Figure 5E,F), ventromedial hypothalamus (VMH; Figure 5E,F), and dorsal raphe nucleus (DR; Figure 5G,H; (Van Pett et al., 2000)), in both wild type (Figure 5E,G) and CRF₁ receptor KO mice (Figure 5F,H), indicating that the immunostaining was not due to cross-reactivity with CRF₁ receptors.

3.2. Western blot

The anti-CRF₂ receptor antibody recognized a band between 37 and 50 kDa (the predicted molecular weight of the full-length CRF_{2α} receptor is about 40–45 kDa) in western blot analysis using homogenized microdissected brain regions (Figure 6). All brain regions examined are known to contain CRF₂ receptor mRNA expression (Van Pett et al., 2000),

and the density of the bands in different brain regions corresponded with the density of CRF₂ receptor mRNA expression in the brain (Van Pett et al., 2000). For example, band density was highest in the LS, whereas it was lowest in cortical regions such as the En and M1 (Figure 6). There were no differences in the actin levels in different brain regions (Figure 6). There was no band at the predicted molecular weight for the CRF₁ receptor (76–80 kDa; (Radulovic et al., 1998)) in any region examined (Figure 6).

3.3. CRF₂ receptor immunostaining in the rostral DR

Rostral regions of the rat DR (–6.92 mm to –7.64 mm bregma) contained a low (in the most rostral sections of the rostral DR) to moderate (in the most caudal sections of the rostral DR; Figure 7A–C; Table 1) number of CRF₂ receptor-ir cells. Although some CRF₂ receptor-ir cells were apparent in structures adjacent to the DR, such as the periaqueductal gray region (Figure 7D) both the density and intensity of staining of cells were lower compared to regions in the DR. In the rostral DRD, CRF₂ receptor-ir cells were dense near the cerebral aqueduct (Aq) at the midline, although some were located outside the boundaries of the DRD laterally (Figure 7A,B). In the most rostral part of the rostral DR, the majority of CRF₂ receptor-ir cells were located along the midline within the DR (with some cells scattered outside the DR, in the adjacent oculomotor complex and periaqueductal gray; Figure 7A,B). Large, densely stained CRF₂ receptor-ir cells were observed within the paratrochlear nucleus (Pa4; Figure 7A,C).

3.4. CRF₂ receptor immunostaining in the mid-rostrocaudal DR

Mid-rostrocaudal regions of the DR (–7.73 mm to –8.45 mm bregma) contained the greatest density and numbers of CRF₂ receptor-ir cells in the DR (Figure 8; Table 1). The largest numbers of CRF₂ receptor-ir cells were located in the DRV, particularly at and around –8.00 mm bregma (104.2 ± 3.9 cells). At this level, although there were also many CRF₂ receptor-ir cells scattered around the midline of the DRV, there were bilateral clusters of intensely stained CRF₂ receptor-ir cells above the medial longitudinal fasciculus (mlf) on either side of the midline (Figure 8A,C). The majority of CRF₂ receptor-ir cells were dorsal to the mlf, but some were located between the fiber bundles of the mlf with a few cells in the region lateral to the superior cerebellar peduncle (Figure 8A, C). A dense central cluster of CRF₂ receptor-ir cells in the midline was located in the DRD throughout the mid-rostrocaudal DR (Figure 8A, B), a region referred to as the DRD core region by Abrams et al. (2005). There also were some CRF₂ receptor-ir cells scattered outside this dense cluster (Figure 8A,B), within a region referred to as the DRD shell region by Abrams et al. (2005). Scattered CRF₂ receptor-ir cells were present in the DRVl region (Figure 8A,D).

3.5. CRF₂ receptor immunostaining in the caudal DR

Caudal regions of the DR (–8.54 mm to –9.26 mm bregma) contained fewer numbers of CRF₂ receptor-ir cells (Figure 9A–C; Table 1), relative to the mid-rostrocaudal DR. CRF₂ receptor-ir cells within the caudal DR were observed in the DRI where a small number of cells, oriented in a vertical plane between the mlf, were arranged in bilateral columns (Figure 9A, C). There were, however, a few scattered, intensely stained CRF₂ receptor-ir cells dorsal to the DRI, in the DRC (Figure 9A, B). These were located in the midline ventrally, extending laterally in clusters of cells located just below the cerebral aqueduct in the dorsal part of the DRC (Figure 9A, B). Lateral to the DRC, scattered CRF₂ receptor-ir cells were observed within the lateral and pericentral part of the dorsal tegmental nucleus (LTDg, DTgP; Figure 9A, D).

3.6. CRF₂ receptor/TrpOH double immunofluorescence

CRF₂ receptor and TrpOH immunofluorescence were found throughout the rostrocaudal extent of the DR (Figure 10 and 11). CRF₂ receptor immunofluorescence was found outside of the DR, but it was most prominent in the DR (Figure 10D–F). In addition, a significant proportion of TrpOH-positive neurons also expressed CRF₂ receptors, (32.5–58.1%, depending on the subdivision) and caudal parts of the DR (Figure 10G–I; Table 1), which is consistent with previous studies describing co-localization of CRF₂ receptor and serotonin transporter mRNA expression (Day et al., 2004).

4. Discussion

CRF₂ receptor-ir cells were distributed throughout the rostrocaudal extent of the DR, with clear regional differences in the number and density of cells. There were low to moderate numbers of CRF₂ receptor-ir cells within the rostral and caudal DR. The highest densities and the greatest numbers of CRF₂ receptor-ir cells were observed in the mid-rostrocaudal DR. At this anatomical level, CRF₂ receptor-ir cells were observed in the dorsal, ventral, and, to a lesser extent, the ventrolateral parts of the DR. The highest densities of CRF₂ receptor-ir cells within the DRD, DRV, and DRI corresponded to the location of the highest densities of serotonergic neurons in these regions (Steinbusch, 1981). In agreement with previous studies of CRF₂ and serotonin transporter mRNA expression (Day et al., 2004), CRF₂ receptor immunofluorescence was co-localized with TrpOH-ir neurons. These data show that CRF₂ receptor-ir cells are expressed throughout the DR, but the highest concentrations seem to be restricted to specific subdivisions of the mid-rostrocaudal DR and in some cases specific parts of these subdivisions. These data indicate that CRF₂ receptor mRNA expression previously described in the DR is associated with CRF₂ receptor immunostaining of perikarya, and that the topographical distribution of the CRF₂ receptor mRNA and protein expression are similar, including expression in serotonergic neurons.

4.1. Technical considerations

Immunostaining resulted in clear CRF₂ receptor immunostaining of perikarya within the DR and other brain regions with high levels of CRF₂ receptor mRNA expression. Recent studies by Waselus et al (2009) using electron microscopy and immunogold labeling have demonstrated that CRF₂ receptor immunoreactivity is predominantly cytoplasmic in DR neurons under basal unstressed conditions (with a ratio of cytoplasmic to total immunogold particles of 0.85 ± 0.01), and shifts toward a greater expression at the plasma membrane following stress exposure (with a ratio of cytoplasmic to total immunogold particles of 0.56 ± 0.03). Although we cannot distinguish plasma membrane-associated from cytoplasmic CRF₂ receptor in our studies, our data are suggestive of expression of CRF₂ receptor in both cellular compartments in unstressed rats, consistent with findings by Waselus et al (2009). Interestingly, the anti-CRF₂ receptor antibodies used in the studies by Waselus et al (2009) and in our studies were both directed at the N-terminus of the CRF₂ receptor (human CRF₂ receptor; Novus Biologicals, personal communication), and both identified a single major band between 37–50 kDa, as determined using western blot (Wang et al., 2007).

4.2. CRF₂ receptor isoforms

It is possible that the CRF₂ receptor immunostaining described here represents immunostaining of CRF_{2α}, CRF_{2β}, CRF_{2α-tr}, and/or soluble (s)CRF_{2α} receptor isoforms in the rat DR. The antibody that was used is likely to recognize all known isoforms of the CRF₂ receptor because the antibody recognizes a 12-amino acid sequence of the N-terminus that is nearly identical in all CRF₂ receptor isoforms that have been characterized (Figure 1). A recent study (Evans and Seasholtz, 2009) has shown that the sCRF_{2α} receptor is found in the rat brain, and that the mRNA is efficiently translated. In addition Evans and Seasholtz

(2009) showed that the sCRF_{2α} receptor is not trafficked to the membrane, which may account in part for the apparent abundance of cytoplasmic staining seen in this study and previous studies (Waselus et al., 2009). Consistent with these findings, recent studies by Schulz and colleagues (2010) showed that CRF_{2α} receptor contains an N-terminal pseudo signal peptide that is unable to target the peptide to the endoplasmic reticulum membrane, resulting in very low cell surface expression (Schulz et al., 2010). Furthermore, Tian and colleagues (Tian et al., 2006) showed that the sc-1826 antibody recognizes the CRF_{2α-tr} receptor isoform using western blot analysis (band at 16–32 kDa) in the forebrain, olfactory bulb, and cerebellum of rats and mice. The sc-1826 antibody was also shown to recognize the CRF_{2α-tr} receptor in HEK-293 cells expressing the CRF_{2α-tr} receptor isoform, using both immunocytochemistry and western blot analysis (Tian et al., 2006). Based on the immunohistochemical and immunofluorescence results in our study, together with *in situ* hybridization studies, receptor autoradiography studies, and functional studies using direct microinjections of selective CRF₂ receptor agonists and antagonists within the DR, it seems likely that at least one high-affinity receptor for CRF₂ receptor ligands is present in the DR, and perhaps more. Future work using specific antibodies or probes will be required to determine which isoform(s) of the CRF₂ receptor are expressed in the DR, their relative abundance, and functional properties.

4.3. Topography of the distribution of CRF₂ receptor-ir cells in the DR

The dorsal and ventral portions of the mid-rostrocaudal DR, where we observed the greatest concentrations of CRF₂ receptor-ir cells, have unique patterns of anatomical projections to and from forebrain limbic structures. The mid-rostrocaudal DRD (relative to other subdivisions of the DR) is known to receive strong projections from specific forebrain structures including the prelimbic cortex, central amygdaloid nucleus, bed nucleus of the stria terminalis, medial and lateral preoptic area, paraventricular nucleus of the hypothalamus, and dorsal hypothalamic area (Peyron et al., 1998; Vertes, 2004). The mid-rostrocaudal DRD is known to project to a number of forebrain regions involved in regulation of emotional behavior, including the medial prefrontal cortex, basolateral amygdaloid nucleus, central amygdaloid nucleus, nucleus accumbens, dorsal hypothalamic nucleus, and bed nucleus of the stria terminalis (Imai et al., 1986; Van Bockstaele et al., 1993; Petrov et al., 1994; Petit et al., 1995; Commons et al., 2003; Abrams et al., 2005). This pattern of projections suggests that this region provides a major contribution to the dorsal raphe forebrain tract, one of six major serotonergic tracts innervating the forebrain (Azmitia and Segal, 1978; Azmitia, 1981; Lowry et al., 2008). The mid-rostrocaudal DRV also contains large numbers of neurons projecting to the basolateral amygdaloid nucleus (Abrams et al., 2005) as well as large numbers of neurons projecting to the caudate putamen and horizontal limb of the diagonal band of Broca (Steinbusch et al., 1980; Steinbusch, 1981; Semba et al., 1988). The mid-rostrocaudal DRV (relative to other subdivisions of the DR) receives strong projections from the lateral orbital cortex and medial and lateral preoptic areas (Peyron et al., 1998). Some of the forebrain regions that project to the mid-rostrocaudal DR contain neurons that express Ucn 2 and Ucn 3, both of which have high affinity for CRF₂ receptors and therefore could be sources of endogenous ligand(s) acting on full-length CRF₂ receptors within the DR (reviewed by (Reul and Holsboer, 2002)). For example, neurons within the paraventricular nucleus of the hypothalamus express Ucn 2 mRNA while neurons within the bed nucleus of the stria terminalis and medial and lateral preoptic areas express Ucn 3 mRNA (Peyron et al., 1998; Lewis et al., 2001; Reyes et al., 2001; Li et al., 2002). Consistent with the hypothesis that urocortins play an important role in regulation of serotonergic systems and behavior, Ucn 1/Ucn 2 knockout mice (Neufeld-Cohen et al., 2009) as well as Ucn 1/Ucn 2/Ucn 3 triple knockout mice have altered baseline serotonergic activity in neural systems regulating anxiety states and altered anxiety-like behavior (Neufeld-Cohen et al., 2010). The pattern of CRF₂ receptor immunostaining in the

DR lends support to the proposal that the antibody used in the current study recognizes one or more protein products of the CRF₂ receptor gene in the rat brain. It is known that Ucn 1-ir neurons from the Edinger-Westphal nucleus also project to the DR (Bittencourt et al., 1999), but it is currently unclear if the DR is also innervated by Ucn 2-, or Ucn 3-containing fibers. As the DR is one of the few brain structures where CRF₂ receptor mRNA expression is higher than CRF₁ receptor mRNA expression, future studies should examine the innervation of the DR by high-affinity CRF₂ receptor ligands including Ucn 2 and Ucn 3 and the sources of fibers containing these CRF-related neuropeptides.

4.5. Electrophysiological responses to CRF₂ receptor ligands in the DR

The current study, describing high numbers of CRF₂ receptor-ir cells in the mid-rostrocaudal DR, is in agreement with a number of electrophysiological studies investigating the effects of CRF and CRF-related neuropeptides on serotonergic neuronal firing rates.

Electrophysiological studies provide strong evidence that functional CRF₂ receptors are expressed within the mid-rostrocaudal DR. Low concentrations of ovine CRF, concentrations that are likely to bind to CRF₁ receptors (Primus et al., 1997), microinjected into the DR decrease the *in vivo* neuronal firing rates of DR serotonergic neurons (Kirby et al., 2000), while high concentrations of ovine CRF, doses that are likely to bind to CRF₁ and CRF₂ receptors (Primus et al., 1997), increase both *in vivo* and *in vitro* firing rates of DR serotonergic neurons (Lowry et al., 2000; Kirby et al., 2000). *In vitro* extracellular recording studies by Lowry and colleagues (Lowry et al., 2000) revealed that the majority of presumed serotonergic neurons activated by CRF were located in the mid-rostrocaudal DRV relative to the mid-rostrocaudal DRD. These *in vitro* extracellular recordings are consistent with our current findings, as the highest density of CRF₂ receptor-ir cells were found in the mid-rostrocaudal DRV region. Microinjections of low doses of Ucn 2 (mUcn 2; Valentino, personal communication) into the mid-rostrocaudal DR of anesthetized rats inhibits the firing rates of serotonergic neurons, while injections of high doses of mUcn 2 into the same region increase the firing rates of serotonergic neurons (Pernar et al., 2004). Thus, the concentrations of both CRF and mUcn 2 appear to be critical for their effects on the neuronal activity of serotonergic neurons in the DR, with a possibility of both inhibitory and excitatory responses depending on the local concentration of ligand. Future studies should take into account the rostrocaudal and dorsoventral differences in the distribution of CRF₂ receptors as well as the possibility of the presence of multiple isoforms of CRF₂ receptors in the DR.

4.6. Functional implications—anxiety-related physiological and behavioral effects of CRF₂ receptor ligands in the DR

Our current findings of high numbers of CRF₂ receptor-ir cells in the mid-rostrocaudal DR are in agreement with a number of studies investigating neurochemical and behavioral responses to intra-DR injections of CRF₂ receptor ligands. Direct microinjections of mUcn 2 into the DR induce potentiation of fear conditioning and escape deficits measured 24 hours later in a model of learned helplessness (Hammack et al., 2003a). This potentiation of fear conditioning and escape deficits is prevented by prior injections of ASV-30 but not the CRF₁ receptor antagonist 2-methyl-4-(*N*-propyl-*N*-cyclopropanemethylamino)-5-chloro-6-(2,4,6-trichloranilino)pyrimidine (NBI27914) (Hammack et al., 2003b). Furthermore, the potentiation of fear conditioning and escape deficits was observed following injections of high doses of CRF (doses likely to bind to CRF₂ receptors (Primus et al., 1997)) into the caudal, but not the rostral, DR (Hammack et al., 2002). These functional studies suggest that activation of CRF₂ receptors within the DR, particularly within the mid-rostrocaudal and caudal DR may play an important role in the regulation of emotional behavior.

Intra-DR microinjections of Ucn 1, which binds with high affinity to both CRF₁ and CRF₂ receptors, have also been shown to induce physiological and behavioral responses. Intra-DR injections of Ucn 1 induce hypothermia and decrease food and fluid consumption in mice (Turek and Ryabinin, 2005; Weitemier and Ryabinin, 2006). Furthermore, intra-DR injections of the CRF₂ receptor antagonist ASV-30 prevent ethanol-induced hyperthermia (Turek and Ryabinin, 2005). Together, these functional studies support the hypothesis that CRF₂ receptors within the DR are involved in the regulation of multiple physiological and behavioral responses, consistent with their widespread distribution in the DR.

In conclusion, this study describes for the first time the distribution of CRF₂ receptor-ir cells throughout the DR. The highest numbers and the highest densities of CRF₂ receptor-ir cells were found in the mid-rostrocaudal DR, particularly in the DRD and DRV subdivisions. Together with previous studies, these findings are consistent with the hypothesis that CRF₂ receptor activation within the mid-rostrocaudal and caudal DR is an important component of neural mechanisms regulating anxiety- or stress-related physiological and behavioral responses. Based on the widespread distribution of CRF₂ receptors in the DR and the functional heterogeneity of the DR, CRF₂ receptors in this region are likely to modulate diverse physiological and behavioral responses.

Functionally integrated CRF and mesolimbocortical serotonergic systems arising from the mid-rostrocaudal and caudal DR are likely to play an important role in stress-related psychopathology including anxiety and mood disorders, as well as stress-induced relapse to drug abuse (Arborelius et al., 1999; Sarnyai et al., 2001; Heinrichs and Koob, 2004). CRF innervation of the human DR is greatest in the mid-to caudal levels (Austin et al., 1997), and depressed patients have increased CRF immunoreactivity in the caudal subnucleus of the DR (Austin et al., 2003). In addition, recent studies of human depressed suicide patients have revealed that the widely reported increases in tryptophan hydroxylase 2 (*tph2*) mRNA and protein expression in the DR (Underwood et al., 1999; Bonkale et al., 2006; Bach-Mizrachi et al., 2006) may be restricted to the caudal subnucleus (Bach-Mizrachi et al., 2008) and its projection sites (Perroud et al., 2010). Consequently, dysregulation of CRF₂ receptor signaling within the caudal DR may have important implications for affective disorder and other stress-related neuropsychiatric disorders.

Index of Abbreviations

4V	4th ventricle
Aq	cerebral aqueduct
Arc	arcuate nucleus
ASV-30	antisauvagine-30
bv	blood vessel
CP	caudate putamen
CNS	central nervous system
CRF	corticotropin-releasing factor
CRF₁ receptor	CRF type 1 receptor
CRF₂ receptor	CRF type 2 receptor
dIPAG	dorsolateral periaqueductal gray
dDRC	dorsal raphe nucleus, caudal part, dorsal part

DR	dorsal raphe nucleus
DRD	dorsal raphe nucleus, dorsal part
DRI	dorsal raphe nucleus, interfascicular part
DRV	dorsal raphe nucleus, ventral part
DRVl	dorsal raphe nucleus, ventrolateral part
DTgP	dorsal tegmental nucleus, pericentral part
En	entorhinal cortex
EGFP	enhanced green fluorescent protein
KO	knockout
LDTg	laterodorsal tegmental nucleus
LV	lateral ventricle
LS	lateral septum
M1	motor cortex
mlf	medial longitudinal fasciculus
MnR	median raphe nucleus
Pa4	paratrochlear nucleus
PV	paraventricular nucleus of the thalamus
TrpOH	tryptophan hydroxylase
Ucn1 2, 3	urocortin 1, 2, 3
mUcn 2	urocortin 2 (mouse)
TrpOH	tryptophan hydroxylase
vDRC	dorsal raphe nucleus, caudal part, ventral part
VMH	ventromedial hypothalamus
WT	wild type

Acknowledgments

C.A. Lowry was supported by a Wellcome Trust Research Career Development Fellowship (RCDF 068558/Z/02/Z), and a 2007 NARSAD Young Investigator Award and is currently supported by an NSF CAREER Award (NSF-IOE #0845550). The project described was supported by Award Numbers R01MH065702 (AS/CAL), R01MH086539 (CAL) and 1F32MH084463 (JLL) from the NIMH. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Mental Health or the National Institutes of Health.

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Fig. 1.

Amino acid sequences of the N-termini, up to the first predicted transmembrane domain, of mouse and rat CRF_{2α} and CRF_{2β} isoforms of the CRF₂ receptor, as well as a soluble form of the mouse and rat CRF_{2α} receptor, a truncated form of the rat CRF_{2α} receptor, and the rat CRF₁ receptor. Black line indicates the amino acid sequence corresponding to the 20 amino acid peptide from the mouse CRF_{2β} receptor isoform that was used as the antigen for development of the polyclonal CRF_{2β} receptor antibody (sc-1826) used in this study. Amino acid sequences were obtained from the following sources: mCRF_{2β} (Perrin et al., 1995) (NCBI Protein database accession no.: Q60748), rCRF_{2β} (NCBI Protein database accession no.: EDL88098), mCRF_{2α} (Chen et al., 2005a) (NCBI Protein database accession no.: AAS07021), mCRF_{2αsol} (Chen et al., 2005b; Evans and Seasholtz, 2009), (NCBI Protein database accession no.: AAU94301), rCRF_{2α}-tr receptor (Miyata et al., 1999), rCRF_{2α} (Lovenberg et al., 1995b) (NCBI Protein database accession no.: NP_073205), rat CRF_{2αsol} (Evans and Seasholtz, 2009), rCRF₁ receptor (Perrin et al., 1993) (NCBI Protein database accession no.: NP_112261). Abbreviations: mCRF_{2α}, mouse CRF_{2α} receptor; mCRF_{2β}, mouse CRF_{2β} receptor; mCRF_{2αsol}, mouse soluble CRF_{2α} receptor; rCRF_{2α}, rat CRF_{2α} receptor; rCRF_{2β}, rat CRF_{2β} receptor; rCRF_{2αsol}, rat CRF_{2αsol} receptor; rCRF_{2αtr}, rat truncated CRF_{2α} receptor; rCRF₁, rat CRF₁ receptor. Black shading indicates conserved amino acids; light gray shading indicates conservative amino acid substitutions. Alignments were obtained using: www.ebi.ac.uk/Tools/clustalw2/index.html.

Fig. 2.

Photomicrographs of tryptophan hydroxylase (TrpOH) immunofluorescence illustrating the subdivisions analyzed in the dorsal raphe nucleus (DR) at 6 different rostrocaudal levels (A–F). White boxes indicate regions photographed at higher magnification (200X) and used for analysis. The entire fields of view of the boxes indicating the regions sampled at 200X magnification for the dorsal raphe nucleus, ventrolateral part/ventrolateral part of the periaqueductal gray (DRV/VLPAG; B–D) and pontine raphe nucleus (PnR; F) are not visible in the figure. Abbreviations: DRD, dorsal raphe nucleus, dorsal part; DRV, dorsal raphe nucleus, ventral part; DRV/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral part of the periaqueductal gray; DRI, dorsal raphe nucleus, interfascicular part; DRC, dorsal raphe nucleus, caudal part; and PnR, pontine raphe nucleus. Scale bar: 50 μ m; inset scale bar: 100 μ m.

Fig. 3.

Photomicrographs illustrate immunocytochemical staining of HEK-293 wild type cells (A and B) or HEK-293 cells stably expressing enhanced green fluorescent protein (EGFP) and mCRF_{2α} receptor (C–G). The green fluorescence (A, C, E, G) results from EGFP expressed in conjunction with the mCRF_{2α} receptor. Note that only light green autofluorescence is present in HEK-293 wild type cells (A). B, D, F and G illustrate results of immunofluorescence using the goat anti-mCRF_{2β} receptor antibody (sc-1826) used in this study. B, D, low magnification photomicrographs illustrating immunofluorescence with the goat anti-mCRF_{2β} receptor antibody (sc-1826) used in this study. (E–G) high magnification confocal photomicrographs of immunofluorescence of HEK-293 cells stably expressing EGFP and mCRF_{2α} receptor: (E) EGFP expression in the HEK-293 cells, (F) mCRF_{2α} receptor immunofluorescence, (G) photomicrograph illustrating the cellular distribution of EGFP and mCRF_{2α} receptor expression in HEK-293 cells. Scale bar = A–D, 60 μm; E–G, 20 μm.

Fig. 4.

Photomicrographs illustrate immunofluorescence of HEK-293 wild type cells (A, B, E, and F) or HEK-293 cells stably expressing enhanced green fluorescent protein (EGFP; C, G) and mCRF_{2α} receptor (D, H). The green fluorescence (C and G) results from EGFP expressed in conjunction with the mCRF_{2α} receptor. Note that only light green autofluorescence is present in HEK-293 wild type cells (A and E). (B, D) photomicrographs illustrating immunofluorescence using the goat anti-CRF_{1/2} receptor antibody (sc-1757). (E–H) photomicrographs illustrating immunofluorescence using the goat anti-CRF₂ receptor antibody (sc-1826) used in this study, replicating results from the experiment illustrated in Figure 3. Scale bar = 50 μm.

Fig. 5. Photomicrographs illustrating the distribution of CRF₂ receptor-immunoreactive (ir) cells in specific brain regions of wild type (A, C, E, G) and CRF₁ receptor knockout (B, D, F, H) mouse brains. (A–B) Very few, faintly immunostained, CRF₂ receptor-ir cells were found in the caudate putamen of either wild type (A) or CRF₁ receptor knockout mice (B). (C–D) Very few, faintly immunostained, CRF₂ receptor-ir cells were found in the paraventricular nucleus of the thalamus of either wild type (C) or CRF₁ receptor knockout mice (D). (E–F) Numerous and densely packed CRF₂ receptor-ir cells were found throughout the arcuate nucleus (Arc) and ventromedial hypothalamus (VMH) of both wild-type (E) and CRF₁ receptor knockout (F) mice. (G–H) CRF₂ receptor-ir cells were found throughout the dorsal

raphe nucleus (DR) of both wild type (G) and CRF₁ receptor knockout (H) mice. Abbreviations: Arc, arcuate nucleus; DR, dorsal raphe nucleus; mlf, medial longitudinal fasciculus; VMH, ventromedial hypothalamus. Scale bar = A–B, E–H, 100 μ m; C–D, 50 μ m.

Fig. 6.

Western blot analysis using the anti-CRF₂ receptor antibody (sc-1826) in microdissected rat brain tissue. In all brain regions analyzed, the anti-CRF₂ receptor antibody recognized one major band at between 37 and 50 kDA, which corresponds to the predicted molecular weight of the full-length CRF₂ receptor. The density of the bands in different brain regions corresponded with the density of CRF₂ receptor mRNA expression previously described in rat brain. Band density was highest in the lateral septum (LS), whereas it was lower in cortical regions such as the entorhinal cortex (En) and motor cortex (M1). There were no differences in actin levels among the different regions. Results are representative of three replicate western blots. Abbreviations: DR, dorsal raphe nucleus; En, entorhinal cortex; LS, ventral part of the lateral septum; M1, motor cortex; MnR, median raphe nucleus; MWM, molecular weight markers; VMH, ventromedial hypothalamus.

Fig. 7. Photomicrographs illustrating the distribution of CRF₂ receptor-ir cells in the rostral DR. (A) Low magnification image of CRF₂ receptor immunostaining in the rostral DR (−7.10 mm bregma) and surrounding regions. The black boxes indicate regions photographed at higher magnification and shown in panels B and C. (B) Scattered CRF₂ receptor-ir cells were found throughout the rostral dorsal part of the dorsal raphe nucleus (DRD). (C) Scattered CRF₂ receptor-ir cells were found within the midline ventral part of the dorsal raphe nucleus (DRV) and the adjacent paratrochlear nucleus (Pa4). (D) Few, faintly-stained CRF₂ receptor-ir cells were found within the dorsolateral periaqueductal gray (dIPAG) dorsal to the DR. Abbreviations: Aq, cerebral aqueduct; bv, blood vessel; DR, dorsal raphe nucleus; DRD, dorsal raphe nucleus, dorsal part; DRV, dorsal raphe nucleus, ventral part; mlf, medial longitudinal fasciculus; Pa4, paratrochlear nucleus; dIPAG, dorsolateral periaqueductal gray. Scale bar = A, 500 μm; B–D, 100 μm.

Fig. 8.

Photomicrographs illustrating the distribution of CRF₂ receptor-ir cells in the mid-rostrocaudal DR. (A) Low magnification image of CRF₂ receptor immunostaining in the mid-rostrocaudal DR (-8.00 mm bregma) and surrounding regions. The black boxes indicate regions photographed at higher magnification and shown in panels B–D. (B) CRF₂ receptor-ir cells were differentially distributed in the dorsal part of the dorsal raphe nucleus (DRD) core (DRDc) and DRD shell (DRDsh) regions, with dense clusters of CRF₂ receptor-ir cells in the DRDc and scattered CRF₂ receptor-ir cells in the DRDsh. (C) Numerous and densely packed CRF₂ receptor-ir cells were found throughout the mid-rostrocaudal dorsal raphe nucleus, ventral part (DRV). (D) Numerous CRF₂ receptor-ir cells were found in the dorsal raphe nucleus, ventrolateral part (DRVl). Abbreviations: Aq, cerebral aqueduct; bv, blood vessel; DR, dorsal raphe nucleus; DRD, dorsal raphe nucleus, dorsal part; DRDc, dorsal raphe nucleus, dorsal part, core region; DRDsh, dorsal raphe nucleus, dorsal part, shell region; DRV, dorsal raphe nucleus, ventral part; DRVl, dorsal raphe nucleus, ventrolateral part; mlf, medial longitudinal fasciculus. Scale bar = A, 500 μm; B–D, 100 μm.

Fig. 9.

Photomicrographs illustrating the distribution of CRF₂ receptor-ir cells in the caudal dorsal raphe nucleus (DR). (A) Low magnification image of CRF₂ receptor immunostaining in the caudal DR (-8.90 mm bregma) and surrounding regions. The black boxes indicate regions photographed at higher magnification and shown in panels B–D. (B) Densely immunostained CRF₂ receptor-ir cells were evident in the midline of the dorsal raphe nucleus, caudal part, dorsal part (dDRC) and in bilateral clusters ventral to the cerebral aqueduct. (C) CRF₂ receptor-ir cells were found within the interfascicular part of the dorsal raphe nucleus (DRI), showing a vertical orientation and bipolar, fusiform shape, features that are consistent with the phenotype of serotonergic neurons in the region. (D) Scattered CRF₂ receptor-ir cells were found within the adjacent dorsal tegmental nucleus, both the lateral (LDTg) and pericentral parts (DTgP). Abbreviations: Aq, cerebral aqueduct; bv, blood vessel; DR, dorsal raphe nucleus; dDRC, dorsal raphe nucleus, caudal part, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DTgP, dorsal tegmental nucleus, pericentral part; LDTg, dorsal tegmental nucleus, lateral part; mlf, medial longitudinal fasciculus. Scale bar = A, 500 μ m; B–D, 100 μ m.

Fig. 10. Dual CRF₂ receptor and tryptophan hydroxylase (TrpOH) immunofluorescence in the dorsal raphe nucleus (DR). Photomicrographs of TrpOH (Panels A–C), CRF₂ receptor (Panels D–F), and CRF₂ receptor/TrpOH double immunofluorescence (G–I) at the rostral (A, D, G), mid-rostrocaudal (B, E, H), and caudal (C, F, I) levels of the DR. Arrows indicate double-labeled cells shown in insets at the lower right corner of each photomicrograph. Scale bar, 100 μ m, insets, 15 μ m.

Fig. 11.

Photomicrographs of CRF₂ receptor immunofluorescence and tryptophan hydroxylase (TrpOH) immunofluorescence in the rat DR that were used for quantification.

Photomicrographs of TrpOH immunofluorescence (Panels A–F), CRF₂ receptor immunofluorescence (Panels B', D', F'), DAPI (B'', D'', F''), and CRF₂ receptor/TrpOH/DAPI triple immunofluorescence (B''', D''', F''') at a rostral anatomical level (–7.64). Row A–B''' represents the dorsal raphe nucleus, dorsal part (DRD), row C–D''' represents the dorsal raphe nucleus, ventral part (DRV) and row E–F''' represents the dorsal raphe nucleus, ventrolateral part (DRVl). Scale bar for Panels A, C, E: 50 μm, scale bar for rest of panels: 100 μm).

Table 1

Expression of CRF₂ receptors in serotonergic cells of the rat DR, determined by dual immunofluorescence

Region	Rostrocaudal level (mm bregma)	# CRF ₂ R+ neurons	# TrpOH+ neurons	# double-labeled neurons	% TrpOH+ neurons that are CRF ₂ R+	% CRF ₂ R+ neurons that are 5-HT+
DRD	-7.46	63.3 ± 8.2	42.7 ± 5.4	13.8 ± 1.8	35.2 ± 6.7	23.1 ± 3.6
DRV	-7.46	51.8 ± 8.4	40.8 ± 5.9	16.3 ± 2.9	40.0 ± 6.6	31.7 ± 4.8
DRD	-7.64	75.0 ± 9.6	41.3 ± 3.0	13.8 ± 2.5	32.6 ± 5.4	20.3 ± 3.8
DRV	-7.64	98.4 ± 4.8	61.0 ± 7.5	32.5 ± 6.7	50.5 ± 7.9	38.0 ± 3.2
DRV/VLPAG	-7.64	83.9 ± 2.9	29.8 ± 4.6	10.8 ± 3.2	32.9 ± 5.2	14.5 ± 4.2
DRD	-8.00	91.8 ± 4.6	64.2 ± 7.7	29.5 ± 5.0	44.6 ± 3.1	31.6 ± 4.8
DRV	-8.00	104.2 ± 3.9	87.4 ± 1.8	44.3 ± 3.2	47.9 ± 2.5	42.0 ± 3.3
DRV/VLPAG	-8.00	78.8 ± 7.7	59.4 ± 5.7	18.8 ± 3.5	32.5 ± 6.7	26.7 ± 6.4
DRD	-8.18	89.5 ± 10.8	64.0 ± 3.0	34.8 ± 0.9	54.4 ± 2.3	36.5 ± 2.4
DRV	-8.18	86.2 ± 7.6	62.0 ± 6.9	36.0 ± 6.2	58.1 ± 8.3	41.2 ± 5.0
DRV/VLPAG	-8.18	68.8 ± 7.5	39.3 ± 3.7	17.8 ± 2.4	46.3 ± 6.3	26.0 ± 2.1
DRC	-8.54	78.3 ± 8.5	54.7 ± 7.2	23.3 ± 4.4	43.3 ± 1.6	30.4 ± 6.0
DRI	-8.54	28.2 ± 4.9	29.4 ± 3.8	12.4 ± 3.0	39.7 ± 7.6	44.9 ± 9.1
DRC	-9.16	42.4 ± 5.5	40.5 ± 4.7	15.0 ± 1.8	38.5 ± 4.9+	35.6 ± 5.4
PnR	-9.16	29.2 ± 5.7	24.8 ± 0.8	10.7 ± 1.7	39.3 ± 6.3	38.0 ± 4.1