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Authors

Chen, Yuncai
Brunson, Kristen L
Müller, Marianne B
[et al.](#)

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Immunocytochemical Distribution of Corticotropin-Releasing Hormone Receptor Type-1 (CRF₁)-Like Immunoreactivity in the Mouse Brain: Light Microscopy Analysis Using an Antibody Directed Against the C-Terminus

Yuncaï Chen^{1,2}, Kristen L. Brunson¹, Marianne B. Müller³, Wayna Cariaga¹, and Tallie Z. Baram^{1,2,*}

¹Departments of Anatomy and Neurobiology, University of California at Irvine, Irvine, California 92697-4475

²Department of Pediatrics, University of California at Irvine, Irvine, California 92697-4475

³Max Planck Institute of Psychiatry, 80804 Munich, Germany

Abstract

Corticotropin-releasing hormone (CRH) receptor type 1 (CRF₁) is a member of the receptor family mediating the effects of CRH, a critical neuromediator of stress-related endocrine, autonomic, and behavioral responses. The detailed organization and fine localization of CRF₁-like immunoreactivity (CRF₁-LI) containing neurons in the rodent have not been described, and is important to better define the functions of this receptor. Here we characterize in detail the neuroanatomical distribution of CRF₁-immunoreactive (CRF₁-ir) neurons in the mouse brain, using an antiserum directed against the C-terminus of the receptor. We show that CRF₁-LI is abundantly yet selectively expressed, and its localization generally overlaps the target regions of CRH-expressing projections and the established distribution of CRF₁ mRNA, with several intriguing exceptions. The most intensely CRF₁-LI-labeled neurons are found in discrete neuronal systems, i.e., hypothalamic nuclei (paraventricular, supraoptic, and arcuate), major cholinergic and monoaminergic cell groups, and specific sensory relay and association thalamic nuclei. Pyramidal neurons in neocortex and magnocellular cells in basal amygdaloid nucleus are also intensely CRF₁-ir. Finally, intense CRF₁-LI is evident in brainstem auditory associated nuclei and several cranial nerves nuclei, as well as in cerebellar Purkinje cells. In addition to their regional specificity, CRF₁-LI-labeled neurons are characterized by discrete patterns of the intracellular distribution of the immunoreaction product. While generally membrane associated, CRF₁-LI may be classified as granular, punctate, or homogenous deposits, consistent with differential membrane localization. The selective distribution and morphological diversity of CRF₁-ir neurons suggest that CRF₁ may mediate distinct functions in different regions of the mouse brain.

Indexing terms

CRH; CRF; CRF-R1; rodent; immunocytochemistry; central nervous system

Corticotropin-releasing hormone (CRH), a 41-amino acid neuropeptide, is the primary hypothalamic factor involved in controlling the synthesis and release of adrenocorticotropic

hormone (ACTH) from the anterior pituitary (Vale et al., 1981). A large body of evidence indicates that CRH acts as a physiological mediator of stress-related functions (Aldenhoff et al., 1983; Ehlers et al., 1983; Siggins et al., 1985; Swanson et al., 1986; Conti and Foote, 1995; Sawchenko et al., 1996; Baram and Hatalski, 1998). CRH has also been demonstrated to be widely distributed throughout the brain (Swanson et al., 1983; Sawchenko and Swanson, 1985; Imaki et al., 1991; Sawchenko et al., 1993). In several regions, the peptide has been shown to have many of the physiological characteristics of a neurotransmitter (Young et al., 1986; Powers et al., 1987; Valentino and Wehby, 1988; Sawchenko et al., 1993).

The actions of CRH are mediated via interaction with distinct CRH receptors (Grigoriadis et al., 1996). Two general members of the CRH receptor family are currently known and consist of membrane-spanning G-protein-coupled molecules. The first, CRF₁ (or CRF-R1), has been cloned and functionally characterized in a number of species (Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993; Palchadhuri et al., 1998). This receptor binds CRH with high affinity and is positively linked to adenylate cyclase. Rat CRF₁ is found in the pituitary and the central nervous system (CNS) and is the candidate mediator for many of the neuroendocrine and neuromodulatory effects of CRH (De Souza et al., 1985; Potter et al., 1994; Chalmers et al., 1995; Baram et al., 1997; Radulovic et al., 1998). The amino acid sequence of mouse CRF₁ is highly similar to that of rat CRF₁ (Vita et al., 1993). However, recently, Radulovic et al. (1998) showed that CRF₁ is differently glycosylated in rat and mouse, as indicated by a higher molecular weight of mouse CRF₁ protein. These authors, using Western blotting, also suggested that the mouse CRF₁ may not share the regional brain distribution of the rat receptor. The second receptor, CRF₂, is found in at least three subtypes in human and rat (CRF_{2α}, CRF_{2β}, and CRF_{2γ}), one of which, CRF_{2α} is found primarily in the CNS (Lovenberg et al., 1995; Kostich et al., 1998).

The distributions of messenger RNAs (mRNAs) of the two CRH receptors in rat brain have been demonstrated using in situ hybridization. Thus, Potter et al. (1994) and Chalmers et al. (1995) described, respectively, the anatomical distribution of CRF₁ mRNA and CRF₂ mRNA in adult rat brain, demonstrating distinct patterns for each of these receptors. Also, significant differences in CRH receptor mRNA distribution have been described in different species (Sanchez et al., 1999). Importantly, the detailed organization and the fine localization of CRF₁ in rodent brain have not been delineated. In addition, mRNA analysis does not provide information about the intracellular localization of CRF₁ to somata versus processes, or about clustering and synaptic relationships of receptor molecules. Therefore, the goal of the present study was to utilize immunocytochemical methods to provide a detailed, single-cell-resolution description of the distribution of CRF₁ in the mouse brain.

MATERIALS AND METHODS

Animals

Adult male C57 black mice (2 months old) were used in this study. Because the overall goals of this series of studies included understanding the possible regulation of CRF₁ by its ligand, CRH, care was taken to utilize mice of similar genetic make-up in all stages of this effort. Therefore, mice used here were progeny of breeding pairs heterozygous for a null CRH allele (a generous gift from Dr. J Majzoub). Specifically, transgenic mice in whom an allele of the CRH gene has been replaced with a neomycin resistance gene were crossed back to the C57 background strain for a minimum of 14 generations. Progeny of heterozygous breeding pairs were genotyped by polymerase chain reaction analysis (Muglia et al., 1995), and those found to possess both alleles of the CRH gene (wild type) were used in this study.

To account for potential variation in CRF₁ distribution or abundance deriving from the specific strain or genetic make-up of these mice, brains from two additional mice from a separate strain (129/Ola or CD1 strain) were also studied, with essentially indistinguishable results (not shown). Mice were born and maintained in a federally approved animal facility, and litter size was culled to six. Mice were kept on a 12-hour light/dark cycle and given free access to food and water. All experiments were carried out according to NIH guidelines for the care of experimental animals, with approval by the institutional animal care committee.

Antiserum generation

A polyclonal anti-CRF₁ antiserum was generated in goat, against a 20-amino acid peptide sequence (Santa Cruz Biotechnology, Santa Cruz, CA). The synthetic peptide antigen, comprising the C-terminus of CRF₁ (SIPTSPTRVSFHSIKQSTAL), corresponds to amino acids 425–444 of human CRF₁ and is identical with amino acids 396–415 of mouse and rat CRF₁. This sequence differs by three amino acids from the corresponding portion (412–431) of mouse CRF₂. A search of GenBank and SwissProt data bases revealed no known sequences (aside from CRH receptors of other species) with significant homology to this portion of the CRF₁ receptor. To characterize the antigen interacting with the antiserum further, Western blotting was also carried out. To determine whether cross-reactivity with the CRF₂ receptor occurred at the dilution used in this study (as suggested by the manufacturer), sections of mouse heart, known to express only the CRF₂ receptor, were subjected to immunocytochemistry in parallel with brain sections.

Western blotting

Brains were rapidly dissected from adult mice of two strains that were killed by decapitation. The hypothalamus and cerebellum were then separated carefully and homogenized in 50 mM Tris-HCl (pH 7.4, 4°C) containing 150 mM NaCl, 0.25% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, 1 mM EDTA, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 2 µg/ml pepstatin. The crude extracts were centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant was collected and centrifuged for 10 minutes. The clear supernatant fluid (containing soluble proteins and membrane fraction) was then collected for the following analysis. Protein concentration was determined using BCA Protein Assay (Pierce, Rockford, IL), and 50-µg protein aliquots were size-fractionated on 4–12% Tris-glycine gel (Novex, Carlsbad, CA) at 120 V for 2.5 hours.

After electrophoresis, proteins were transferred to Hybond-ECL membrane (Amersham, Arlington Heights, IL). Nonfat milk (6%) in Tris-buffered saline (25 mM Tris, 0.15 M NaCl, pH 7.2) containing 0.05% Tween-20 (TBS-T) was used for blocking and for dilution of primary and secondary antibodies. The membrane was blocked overnight at 4°C and then incubated for 2 hours at room temperature with the anti-CRF₁ antiserum (1:500). After several washes with TBS-T, the membrane was incubated in horseradish peroxidase-conjugated anti-goat IgG (1:10,000, Jackson ImmunoResearch, West Grove, PA) for 1.5 hours at room temperature. Following rinsing with TBS-T, bound antibody was detected using the ECL detection system (Amersham). For control, the primary antiserum was preadsorbed overnight at 4°C with purified CRF₁ blocking peptide (100 µg/ml; Santa Cruz Biotechnology).

Immunocytochemistry

Care was taken to harvest tissue under relatively stress-free conditions (Yan et al., 1998a,b). Briefly, mice (n = 4) were undisturbed for at least 24 hours prior to experiments and then deeply anesthetized with sodium pentobarbital (100 mg/kg intraperitoneally) within 45 seconds of initial handling. Mice were then removed to the laboratory and perfused via the ascending aorta with 0.9% saline solution followed by freshly prepared 4%

paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4, 4°C). Brains were dissected from the skull, postfixed for 4 hours, and immersed in 15%, followed by 25% sucrose for cryoprotection. Brains were blocked in the coronal or sagittal planes and sectioned at 20- μ m thickness using a cryostat. In each plane, one in eight matched sections (one in four for the hypothalamic region) were subjected to immunocytochemistry, and an adjacent series of sections was stained with cresyl violet.

For CRF₁-LI immunocytochemistry, free-floating sections were collected into tissue-culture wells in 0.1 M PB and subjected to standard avidin-biotin complex (ABC) methods (Chen et al., 1998a; Yan et al., 1998a,b). Briefly, after several washes with 0.01 M phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (pH 7.4, PBS-T), sections were treated for 30 minutes in 0.3% H₂O₂ in PBS, followed by blockade of nonspecific sites with 2% normal rabbit serum in PBS for 30 minutes. After a 10-minute rinse in PBS, sections were incubated for 48 hours at 4°C with anti-CRF₁ (1:5,000) in PBS containing 1% bovine serum albumin and 2% normal rabbit serum and washed 3 times in PBS-T, 5 minutes each. Subsequently, sections were incubated in biotinylated rabbit-anti-goat IgG (1:200, Vector, Burlingame, CA) in PBS for 1 hour at room temperature. After washing in PBS-T (3 \times 5 minutes), sections were incubated in ABC solution (1:100; Vector) for 2 hours at room temperature. Sections were then rinsed again in PBS-T (3 \times 5 minutes). The reaction product was visualized by incubating sections for 8–10 minutes in 0.04% 3,3'-diaminobenzidine (DAB) containing 0.01% H₂O₂ with or without 0.5% nickel chloride.

Several approaches were used to address the specificity of the immunostaining obtained with anti-CRF₁. In addition to the Western blotting described above, the specificity of the primary antiserum was also tested by substituting normal goat IgG (1:1,000–5,000; Santa Cruz Biotechnology) for the primary antiserum and by preadsorbing the antiserum overnight with purified CRF₁ blocking peptide (100 μ g/ml). The specificity of the second antibody was tested by omitting the primary antiserum during the first incubation. As indicated above, cross-reactivity with CRF₂ was tested by using the antiserum for staining mouse heart tissue, known to express CRF₂ only.

Regional and laminar distribution patterns of CRF₁-LI were determined by comparison of immunocytochemical sections with adjacent cresyl-violet stained sections and by evaluation of methyl green-counterstained sections. Published nomenclature was used to identify cytoarchitectonic areas in the cortex (Dolorfo and Amaral, 1998), amygdala (Pitkänen et al., 1997), hypothalamic paraventricular nucleus (Swanson and Kuypers, 1980; Swanson et al., 1983), and other regions (Sidman et al., 1971; Paxinos and Watson, 1982).

Density and intensity of CRF₁-LI-labeled neurons

The relative density of CRF₁-ir neurons was assessed with a square lattice system (Chen et al., 1998b) over the entire area examined, using light microscopy at \times 400 magnification. Density was expressed as the percentage of positive cells related to total cell number in the region of interest or in a 0.01-mm² real area, based on the average value from three fields per section. Generally, three to five sections were counted in every mouse for evaluating CRF₁-ir neuronal density in each brain structure or nucleus, and the percentage of CRF₁-ir neurons was calculated by dividing the total number of CRF₁-ir neurons by the sum of Nissl-stained cells. The intensity of CRF₁-LI labeling was scored as weak (+), moderate (+ +), intense (+ + +), or strongly intense (+ + + +).

RESULTS

The specificity of the anti-CRF₁ antiserum was assessed by immunoblotting mouse hypothalamus and cerebellum homogenates (Fig. 1). As shown in lanes 1 and 2, the

antiserum recognized an approximately 80-kDa protein, in good agreement with the molecular weight of mouse brain CRF₁ protein (Radulovic et al., 1998). Preadsorption of the primary antiserum with a blocking peptide consisting of the immunogenic epitope (100 µg/ml) completely abolished the CRF₁ band (Fig. 1, lanes 3 and 4). The specificity of CRF₁ immunoreactivity was also indicated by the absence of labeled neurons or fibers in brain sections that were stained using antiserum pretreated with the antigenic peptide (Fig. 2D). In addition, no staining was observed on the sections when normal goat IgG was substituted for the CRF₁-directed serum or in the absence of the primary antiserum (not shown). Finally, cross-reactivity with the CRF₂ receptor was excluded by the absence of any signal in mouse heart specimens run in parallel to immunopositive brain sections (not shown).

CRF₁-LI neurons were widely but selectively distributed throughout the mouse brain. In addition, the intensity of CRF₁ reaction product in positive neurons differed in a neuron- and region-specific manner. In general (but not always; see Fig. 2C), CRF₁-LI in most involved brain regions showed a pattern typical of membrane localization and was further characterized as either granular (associated with intensely labeled neurons) or finely punctate (generally present in moderately or weakly labeled cells). A third pattern, a homogenous deposit associated with the plasma membrane, was found in several regions (Fig. 2A–C). Notably, intensity of the CRF₁ immunoreaction product was maximal in neocortical pyramidal cells, Purkinje cells, and most positive neurons in the thalamus, hypothalamus, and brainstem nuclei.

The distribution of CRF₁-LI neurons in the four mouse brains studied in detail was essentially identical, with relatively modest variation in labeling intensity among animals. The following is a description of the distribution of CRF₁-LI neurons by region.

Telencephalon

Table 1 outlines the distribution of CRF₁-LI in the telencephalon.

Olfactory bulb and tubercle—In the olfactory bulb (Fig. 3A), many periglomerular cells were intensely CRF₁-LI labeled and thus outlined the glomeruli that were unstained. In addition, a few large, multipolar, intensely labeled neurons were observed just beneath the glomerular layer. Mitral cells were moderately or weakly immunoreactive, and some weakly labeled neurons were also present in the external and internal plexiform layers. In addition, some immunoreactive puncta were observed in the internal plexiform layer and, rarely, in the external plexiform layer. The granular layer contained numerous labeled puncta and scattered CRF₁-LI neurons. The distribution pattern of CRF₁-LI in the accessory olfactory bulb resembled that found in the main bulb; conversely, most neurons in the anterior olfactory nucleus were CRF₁-ir. Within the olfactory tubercle (Fig. 3B), moderately CRF₁-LI-labeled neurons were prominent in the pyramidal layer. Scattered large, multipolar, intensely labeled neurons and some medium-size, weakly labeled cells were seen in the polymorphic layer, but none in the plexiform layer. The islands of Calleja contained moderately labeled CRF₁ granular reaction products, whereas neurons in the taenia tecta were moderately immunoreactive.

Cerebral cortex—Neocortical areas contained a large number of CRF₁-LI neurons, the intensity of which differed substantially across regions. The most intensely labeled neurons occurred in the cingulate cortex and in the somatosensory area of the frontoparietal cortex, followed by the motor area of frontoparietal cortex. CRF₁-LI neurons in the association areas of the striate and temporal cortices were characterized by moderately intense reaction product. The laminar distribution of CRF₁-LI-labeled neurons was striking: in the cingulate, frontoparietal, and most other neocortical regions, whereas immunopositive neurons were

distributed throughout layers II–VI, maximal intensity of the reaction product was found in layer V, most notably in the frontoparietal cortex motor area (Fig. 3C). In addition, rare CRF₁-LI neurons were observed in layer I and in subcortical white matter. Within piriform cortex, moderately labeled CRF₁-ir neurons were common in both the pyramidal (II) and polymorphic (III) layers, intermingled with a smaller population of intensely labeled cells (Fig. 3D). In the entorhinal cortex, many moderately labeled neurons were distributed in layers II and III, accompanied by a scattered population of large, multipolar, intensely labeled neurons. CRF₁-LI neurons in layers V–VI were moderately labeled (Fig. 3E). Perirhinal cortex contained CRF₁-LI-labeled neurons uniformly throughout layers II and III.

Hippocampal formation—Within the hippocampus, CRF₁-LI-containing neurons were most prominent in the pyramidal cell layer: the CA1 pyramidal cell layer was rich in moderately labeled puncta and contained several intensely labeled large neurons. CA2 was distinguished by intense or moderate labeling of virtually all pyramidal neurons. In CA3, maximal intensity of CRF₁-ir was found in CA3a, with a gradual decline over CA3b and a fainter signal over CA3c. Outside the pyramidal layer, only occasional medium-sized, multipolar or fusiform CRF₁-ir neurons were scattered in the strata oriens, lacunosum-moleculare, and radiatum (Fig. 4A,B). Within the dentate gyrus, granule cell somata were outlined by a fine mesh of weakly stained puncta, and occasional strongly labeled basket-like neurons were visible. Large, polymorphic, intensely labeled neurons were present in the hilus, whereas little CRF₁-LI was observed in the molecular layer (Fig. 4A,C). Within the subiculum, many pyramidal neurons were intensely labeled (Fig. 4D).

Amygdala—Large, intensely labeled neurons were widely distributed throughout the basal nucleus (Fig. 5A,B). The staining pattern of the accessory basal nucleus consisted of weakly labeled medium-sized neurons, and this pattern was shared by the medial nucleus: in both nuclei, only a portion of the cell surface typically showed CRF₁-LI (Fig. 5C). Most neurons were immunoreactive in the anterior cortical nucleus, with a similar pattern but lower intensity than in the medial nucleus. In the central nucleus (ACe), weak to moderate CRF₁-LI labeling was found in both lateral and medial divisions, but with a different staining pattern: whereas immunoreactivity in the lateral division consisted of punctate deposits, the medial division pattern consisted of crescent-shaped contours (similar to the pattern found in medial and accessory basal nuclei; Fig. 5D). Finally, weakly labeled neurons were present in the lateral nucleus. Within the bed nucleus of stria terminalis (considered a part of the “extended amygdala”), moderately labeled puncta were abundant in both medial and lateral areas (not shown).

Basal forebrain—Most lateral septal nucleus neurons showed intense CRF₁-LI. Interestingly, each subnucleus demonstrated a distinct immunoreactivity pattern (Fig. 6A–C): the dorsal region contained many medium-size neurons with granular CRF₁-LI deposits (Fig. 6B), whereas the intermediate region also contained (medially) cells with a fine punctate CRF₁-LI pattern and short processes. A third pattern, consisting of large granular deposits without a distinct neuronal contour, was observed in the ventral region of this septal nucleus (Fig. 6C). The medial septum contained many large, intensely or moderately labeled neurons, whereas multipolar, intense CRF₁-LI-labeled magocellular neurons were abundant in both the horizontal and vertical limbs of the diagonal band of Broca (Fig. 6D).

Basal ganglia—Most CRF₁-LI neurons in the striatum (caudate-putamen) were weakly labeled and were accompanied by scattered medium-sized, intensely labeled cells (Fig. 6E). A similar but more intense labeling pattern was evident in the nucleus accumbens (Fig. 6F). The fundus striati and ventral endopiriform nucleus were characterized by abundant, moderately CRF₁-LI neurons. A population of larger, multipolar or fusiform process-

possessing neurons was encountered in the globus pallidus and the ventral pallidum (Fig. 6F), whereas the entopeduncular nucleus contained moderate numbers of intensely CRF₁-LI cells.

Diencephalon

Table 2 outlines the distribution of CRF₁-LI neurons in the diencephalon.

Habenula—Small neurons in medial habenula were intensely CRF₁-LI labeled, whereas lateral habenula showed numerous moderately CRF₁-LI neurons and scattered intensely labeled cells (Fig. 7A).

Thalamus—The anterior nuclear group demonstrated numerous CRF₁-LI strongly labeled neurons (in the anterodorsal and anteromedial nuclei) and moderately labeled ones (anteroventral nucleus) (Fig. 7A). Many neurons with intensely labeled somata and short processes were found in the mediodorsal nucleus. In the lateral nuclear group, the dorsal and posterior nuclei contained multipolar or triangular intensely CRF₁-LI neurons possessing short processes. In the ventral group, intensely labeled neurons were abundant in the lateral, posterolateral, and posteromedial nuclei, as well as in the midline nuclear group (paratenial, paraventricular, and reuniens nuclei) and in the intralaminar nuclear group. In the thalamic reticular nucleus, only weak or moderate CRF₁-LI was found (Fig. 7B).

Hypothalamus—Within hypothalamus, most CRF₁-LI labeling of neurons was intense, especially in the supraoptic, paraventricular (PVN), ventromedial (VMH), and arcuate nuclei (Fig. 8A–F). The medial preoptic area contained numerous highly CRF₁-LI neurons, reminiscent of those of the amygdaloid medial nucleus, whereas the lateral preoptic nucleus contained triangular or fusiform, moderately labeled cells bearing several short processes. The suprachiasmatic nucleus was characterized by densely immunoreactive neuropil, whereas the retrochiasmatic area contained a few weakly CRF₁-LI-positive neurons in conjunction with scattered intensely CRF₁-LI-positive puncta. Neurons in the supraoptic nucleus were more strongly immunolabeled than those in the suprachiasmatic nucleus (Fig. 8D). Parvocellular neurons of the PVN demonstrated exceptionally strong CRF₁-LI; in contrast, few positive neurons were present in the magnocellular divisions (Fig. 8A–C). In the VMH, neurons were intensely labeled ventrolaterally and moderately labeled in the dorsomedial part, in a granular pattern. Intensity of CRF₁-ir in the arcuate nucleus was higher than that observed in the VMH (Fig. 8E,F). Staining intensity of CRF₁-LI in neurons of the posterior lateral hypothalamic area was mostly weak or moderate. Interestingly, several subependymal neurons in the median eminence showed CRF₁-LI (Fig. 8E).

Subthalamus and geniculate bodies—Subthalamic neurons showed CRF₁-LI, as did most zona incerta cells (Fig. 7C). In the lateral geniculate body, neurons were generally intensely labeled dorsally and moderately or weakly labeled in the ventral parts, whereas both parts of the medial geniculate body contained intensely immunoreactive neurons.

Mesencephalon

Table 3 outlines the distribution of CRF₁-LI-expressing neurons in the mesencephalon. A large number of CRF₁-LI-labeled neurons was observed in the tectum. Within layers II–VII of the superior colliculus, these neurons were moderately or weakly immunoreactive, accompanied by a few large, multipolar, deeply labeled cells in the lateral part of layer VI and occasionally in layer VII (Fig. 9A). The most striking feature of the inferior colliculus was large, multipolar or triangular, strongly CRF₁-LI neurons in both the cortical and central nuclei, where most medium-sized neurons were moderately stained (Fig. 9B). Comparison

of the dorsal and lateral inferior colliculus cortex revealed a few CRF₁-LI neurons in the latter.

Within tegmental nuclei, labeling intensity was maximal in the ventral (Fig. 9C), moderate in the laterodorsal, and weak in the dorsal nucleus (Fig. 9C,G). Neurons in the pedunculo-pontine-tegmental and reticulotegmental nuclei were strongly labeled. In red nucleus, most large neurons with short processes were intensely labeled (Fig. 9D). The substantia nigra pars reticulata was characterized by large, multipolar, neurons with intense CRF₁-LI, whereas the pars compacta contained medium, moderately to intensely labeled cells (Fig. 9E). Similarly, process-bearing neurons in the interpeduncular nucleus were moderately or intensely labeled. The dorsal raphe nucleus was rich in highly labeled neurons, whereas only modest numbers of intensely stained neurons were observed in the median raphe (Fig. 9G).

Both the accessory (Edinger-Westphal) and principal oculomotor nuclei contained moderate or intense CRF₁-LI (Fig. 9F). Neurons with short processes in the Darkschewitsch nucleus were moderately labeled. The mesencephalic nucleus of the trigeminal nerve showed intense CRF-LI in a granular pattern (see Fig. 11A). In addition, many medium-sized, moderately labeled neurons were present in the central gray, particularly dorsally.

Pons and medulla

Pontine and medullary raphe nuclei shared the staining pattern of their mesencephalic counterparts. Within the reticular formation, large, multipolar, intense CRF₁-LI neurons were abundant in the pontine and gigantocellular reticular nuclei, whereas only scattered positive neurons resided in the intermediate and lateral nuclei. The locus coeruleus was rich in multipolar, intensely CRF₁-LI-labeled neurons (see Fig. 11A). Dorsal parabrachial nuclei were more intensely stained than ventral ones (see Fig. 11B). Large, multipolar neurons in superior olive and nuclei of lateral lemniscus were also intensely stained.

Within trigeminal nerve nuclei, large, multipolar neurons in the motor, principal sensory, and spinal subnuclei showed intense CRF₁-LI. In addition, numerous moderately labeled small and medium cells were found in both principal sensory and spinal nuclei (Fig. 10A). The facial nucleus was rich in large, multipolar CRF₁-LI neurons (Fig. 10B), whereas the vestibular nuclei (medial, lateral, and superior) contained numerous moderately or in-tensely labeled cells (Fig. 10C). In the cochlear complex, the ventral nucleus had more intense CRF₁-LI than the dorsal nucleus, the latter containing rare highly labeled neurons and some moderately immunoreactive neurons (Fig. 11C). Some neurons in vagal, ambiguous, and hypoglossal nuclei showed strong CRF₁-LI (Fig. 10D,E). Weak-to-moderate CRF₁-LI labeling occurred in medial and lateral divisions of the solitary tract nucleus (Fig. 10F).

Medium-sized, highly labeled CRF₁-LI neurons were observed in the medial nucleus of the trapezoid body, in contrast to the occasional, intensely labeled cells of the lateral nucleus (Fig. 11D,E). In the inferior olive, medium-sized CRF₁-LI-stained neurons were found (Fig. 11F).

Cerebellum

Purkinje cells showed intense CRF₁-LI, in a granular pattern (Figs. 2A, 11G), whereas the granular layer contained only scattered intensely labeled neurons accompanied by abundant weakly labeled puncta. Large, multipolar neurons in the deep cerebellar nuclei were intensely stained, whereas the cerebellar white matter was devoid of CRF₁-LI.

DISCUSSION

The current immunocytochemical study describes the distribution of CRF₁-LI throughout the mouse brain. The results indicate that 1) CRF₁-LI is generally localized to the cell membrane and is further characterized as granular, punctate, or homogenous deposits; 2) CRF₁-LI-labeled neurons are morphologically heterogeneous both within and among brain structures; 3) CRF₁-LI is widely and selectively distributed in discrete regions of the mouse brain, in a pattern that—with notable exceptions—generally conforms to that of CRF₁ mRNA; and 4) a gradient of CRF₁-LI labeling intensity exists, with the highest found in certain hypothalamic nuclei, neocortex, septum, basal amygdala, thalamus, select brain stem regions, and cerebellar Purkinje cells.

CRF₁ immunoreaction product was generally localized to the cell membrane. This result is consistent with evidence indicating that this receptor is a G-protein-coupled, membrane-bound protein (Chang et al., 1993; Perrin et al., 1993; Castro et al., 1996). Of the several patterns of CRF₁-LI observed in this study (Fig. 2A–C), some may signify receptor clustering (granular and punctate deposits). In addition, the spectrum of cellular distribution patterns of CRF₁-LI was remarkable, both within a given nucleus as well as among regions. Most commonly, punctate or granular CRF₁-LI outlined a neuronal profile consisting of the soma with or without dendritic processes. In some cases, CRF₁-LI outlined only part of the cell surface, suggesting somatic innervation, perhaps by a spatially discrete bundle of axons (e.g., the climbing fiber innervation of cerebellar molecular layer neurons). Interestingly, numerous CRF₁-LI-labeled puncta were observed in certain regions such as the granular layers of the olfactory bulb and cerebellum, suggesting a presynaptic location of CRF₁. Thus, the findings of this study are consistent with a potential for not only the post- but also presynaptic location of CRF₁. However, electron microscopic (EM) studies are required to demonstrate the presence of presynaptic CRF₁ definitely.

In general, neurons with CRF₁-LI were observed in target regions of CRH-expressing local circuit and projection neurons. An eloquent example of this notion was evident in the amygdala. In the ACE, CRH-containing terminals are abundant and have been shown to arise from cell bodies located in the lateral hypothalamus and dorsal raphe, as well as from intrinsic CRH neurons in the ACE (Swanson et al., 1983; Uryu et al., 1992). In the present study, we observed that essentially all ACE neurons were CRF₁-LI positive. This indicates that in the ACE, CRF₁-ir neurons may be innervated by CRH-expressing cells originating both within and outside the amygdala. Interestingly, the density and intensity of CRF₁-immunoreaction product in the ACE were high. In contrast, CRF₁ mRNA expression in the ACE has been found to be modest (Potter et al., 1994; Chalmers et al., 1995; Avishai-Eliner et al., 1996). Thus, it may be suggested that the CRF₁-LI in the ACE may originate in the somata of other nuclei (e.g., lateral amygdala; Pitkänen et al., 1997) and transported to ACE. An additional, intriguing possibility is that the ACE may contain novel, as yet uncharacterized, ligand for this receptor (e.g., Weninger et al., 1999). Whereas levels of CRF₁-LI and mRNA are somewhat discordant in the ACE, the results of the current study—showing intense labeling of magnocellular neurons in basal nucleus—are in accordance with levels of CRF₁ mRNA expression and CRH receptor binding in this nucleus (De Souza, 1987; Potter et al., 1994; Chalmers et al., 1995; Avishai-Eliner et al., 1996).

CRF₁-LI was localized to regions receiving CRH-containing projections from the amygdala. Indeed, the amygdala is considered an origin of major CRH-containing pathways, emanating from the ACE, the major output nucleus for amygdaloid projections to the brainstem and hypothalamus (Swanson et al., 1983; Sakanaka et al., 1986; Gray and Bingaman, 1996; Pitkänen et al., 1997). CRH neurons in ACE have been shown to project to the bed nucleus of the stria terminalis (BNST), lateral hypothalamus, midbrain central gray, parabrachial

nucleus, mesencephalic nucleus of the trigeminal nerve, mesencephalic reticular formation, solitary tract nucleus, and vagal nucleus (Veening et al., 1984; Sakanaka et al., 1986; Moga et al., 1989). In view of the widespread CRH projections from the ACe, it is not surprising that abundant CRF₁-LI-expressing neurons are observed in the current study. Indeed, the distribution of CRF₁-LI-labeled neurons demonstrated in this study highly overlaps that of target regions of CRH-expressing projections from the ACe. An additional CRH-expressing group of projection neurons originates in the amygdaloid corticomедial nucleus, innervating the VMH (Sakanaka et al., 1986). Accordingly, the current study demonstrated numerous CRF₁-LI-labeled neurons in VMH.

CRF₁-LI-positive neurons were abundant in most cholinergic nuclei of the basal forebrain, including the septum and the diagonal band of Broca. Interestingly, we found high levels of CRF₁-LI in both the lateral and medial septum, whereas reports on the distribution of CRF₁ mRNA have suggested a preferential localization to the medial septum (Chalmers et al., 1995). In addition, within the lateral septum, regional differences were evident in the cellular pattern of CRF₁-ir, implying regional specificity in the localization and perhaps physiological functions of this receptor. Thus, the demonstration of CRF₁-LI-positive neurons in cholinergic nuclei afferent to the hippocampal formation may suggest that the receptor participates in modulation of cholinergic input to the hippocampus.

The current study demonstrated a rich array of CRF₁-LI-expressing neurons in the hippocampal formation. CRH has been shown to influence directly synaptic function in hippocampal CA1 (Aldenhoff et al., 1983; Smith and Dudek, 1994) and CA3 (Hollrigel et al., 1998) and to excite hippocampal neurons both in vivo (Baram et al., 1997) and in vitro (Aldenhoff et al., 1983; Smith and Dudek, 1994; Hollrigel et al., 1998). These effects may be important in modulating learning and memory processes (Liang and Lee, 1988; Lee et al., 1993; Behan et al., 1995). Furthermore, recent evidence indicates that at least some of these direct effects of CRH on hippocampal neurons are mediated by CRF₁ (Baram et al., 1997).

In the absence of CRH-containing afferent projections to the hippocampus, the endogenous ligand for CRF₁—shown here to reside primarily on pyramidal layer neurons—is likely to be CRH found in local hippocampal interneurons, as described by Swanson et al. (1983) and Sakanaka et al. (1987). Recently most of these neurons have been found to be basket and chandelier cells, synapsing on soma and axon initial segment of pyramidal neurons, respectively (Yan et al., 1998b). Thus, local CRH-expressing interneurons are the likely source of ligand activating the abundant CRF₁-LI on hippocampal pyramidal layer neurons. This study documented a particularly striking density and intensity of CRF₁ immunoreactivity in CA3 pyramidal neurons. In this context, it is interesting to note that the excitatory and particularly the excitotoxic effects of CRH are maximal on CA3 hippocampal neurons (Baram and Ribak, 1995; Ribak and Baram, 1996).

In the hypothalamus, the current study demonstrated an abundant population of generally intense CRF₁-LI labeling of neurons in the PVN, VMH, and supraoptic and arcuate nuclei. While a small proportion (about 24%; 96 of 398) of PVN magnocellular neurons showed faint CRF₁-LI, parvocellular neurons were very intensely labeled. Of these, most were concentrated in the anterior, medial, and lateral parts of the parvocellular division (Swanson and Kuypers, 1980; Swanson et al., 1983). Previous studies have demonstrated that CRH-expressing parvocellular neurons of the dorsomedial division of PVN give rise to the pathway terminating in the external zone of the median eminence (Swanson et al., 1987; reviewed in Sawchenko and Swanson, 1990) that controls the release of ACTH from the pituitary. These CRH neurons thus participate in the control of ACTH release from the anterior pituitary, a key neuroendocrine component of the response to stress. In addition to this stress axis-related role, subpopulations of PVN CRH neurons, such as those residing in

the medial and lateral parvocellular regions, project to autonomic cell groups in the brainstem and spinal cord (Sawchenko, 1987). The current study documents the presence of CRF₁-LI in PVN regions containing both neuroendocrine and autonomic-projecting cells. These receptors may thus be positioned to modulate both the neuroendocrine and autonomic functions of CRH.

The current study demonstrates a striking abundance of CRF₁-LI in the PVN. This may be contrasted with previous *in situ* hybridization studies, showing unexpectedly little CRF₁ mRNA expression in the PVN of unstressed rats (Potter et al., 1994; Chalmers et al., 1995; Avishai-Eliner et al., 1996). However, CRF₁ mRNA levels were highly increased by stressful manipulations (Luo et al., 1994; Rivest et al., 1995; Imaki et al., 1996; Hatalski et al., 1998), as well as by intracerebroventricular administration of CRH. The current study demonstrated CRF₁-LI in parvocellular neurons from hypothalami of animals obtained under relatively stress-free circumstances (see Materials and Methods). This indicates that high CRF₁ protein levels may exist concurrent with modest steady-state CRF₁ mRNA levels. This is consistent both with a long half-life of CRF₁ and with regulation of receptor levels at the protein level. Because receptor binding studies have generally demonstrated modest levels of CRH binding in the PVN (De Souza and Kuhar, 1986), our data may suggest increased sensitivity of the immunocytochemical approach. In agreement with our results, Radulovic et al. (1998) recently reported the presence of CRF₁-ir neurons in PVN of both rat and mouse.

It should be noted that it is unlikely that the CRF₁-LI observed in this study was a result of cross-reactivity with CRF₂. Substantial homology exists between CRF₁ and CRF₂ proteins, and the immunogenic epitope used to generate the antiserum used here shares 17 of 20 amino acids with the C-terminus of CRF₂. However, Western blotting at high antiserum concentration of mouse brain of several strains revealed a single band. In addition, the overall distribution of CRF₁-ir in the current study matched that reported for CRF₁ binding and mRNA, and not that of CRF₂. Finally, using the antiserum on mouse heart, known to express CRF₂ but not CRF₁, no immunoreactivity was evident, again strongly suggesting that the antiserum does not recognize CRF₂.

In the present study, neurons strongly labeled for CRF₁-LI were also evident in the supraoptic nucleus. In view of the association of such neurons with CRH-expressing cells in PVN (*vide supra*), it is notable that a subset of supraoptic nucleus neurons have been shown to synthesize CRH (Kawata, 1983). CRF₁-LI was abundant in both the dorsomedial and ventromedial VMH. At the mRNA level, CRF₁ expression in the VMH has been found to be low compared with that of CRF₂ in both adult and immature animals (Chalmers et al., 1995; Eghbal-Ahmadi et al., 1998). The VMH functions to regulate food intake and energy balance. In addition, it is involved with integrating inputs from the HPA-axis, circadian rhythm, and sensory relay centers (Lovenberg et al., 1995; Eghbal-Ahmadi et al., 1999). However, the relative roles of CRF₁ and CRF₂ in mediating the effects of CRH and similar ligands in the VMH are not fully understood. The relatively high levels of VMH CRF₁-LI found in this study suggest that this receptor may participate in mediating some of the effects of CRH (and related ligands) in this region.

CRF₁-LI was abundant in monoaminergic cell groups such as the locus coeruleus, raphe, ventral tegmentum, and substantia nigra. Of these, the locus coeruleus has been shown to contain CRH-expressing neurons and fibers. A number of studies have suggested that CRH may function as a neurotransmitter in this region, involved with central integration of autonomic, behavioral, and anxiogenic effects of stressful stimuli (Valentino et al., 1992; Lehnert et al., 1998). For example, CRH injected into the rodent locus coeruleus produces an anxiogenic response (Owens and Nemeroff, 1993). In this regard, the present study

provides an anatomical basis for the involvement of the locus ceruleus CRF₁ in mediating anxiety-related effects of CRH.

CRF₁-LI was abundant in cerebellar circuits. CRH has been localized to cerebellar mossy fiber projections originating in vestibular-related brainstem nuclei (Cha and Foote, 1988; Cummings et al., 1994) and to the climbing fibers and their origin, the inferior olive (Young et al., 1986; Cummings et al., 1994; Chang et al., 1996). In accordance with the functional role of CRH as a cerebellar neurotransmitter, previous studies have demonstrated the expression of CRF₁ and CRF₂ mRNAs in the molecular and granular layers, sites of termination of climbing and mossy fibers, respectively (Potter et al., 1994; Chalmers et al., 1995). In the present study, almost all Purkinje cells showed intense CRF₁-LI, whereas these levels were generally low in both molecular and granular layers. Thus, whereas the current immunocytochemical data correlate with mRNA distribution in Purkinje cells (Chalmers et al., 1995; King et al., 1997), the current study shows low CRF₁-LI levels in the granular layer, considered rich in CRF₁ mRNA. This apparent discrepancy may be due to the synthesis of CRF₁ within cell bodies located in the granular layer and transport of the protein away from the soma, located in the granular layer, resulting in low signal intensity in this layer (Table 3).

The current study focused on CRF₁ detection in the mouse brain; the distribution may differ from that in rat. Thus, Radulovic et al. (1998) reported significant, although mainly quantitative, species differences in the distribution of the receptor between the rat and mouse. Compared with the rat, the mouse was found to express less CRF₁ protein in the neocortex and basal amygdala and significantly larger amounts of CRF₁ protein in several subcortical regions including the substantia nigra, central gray, red and Darkschewitsch nuclei, and pontine reticular nuclei.

In summary, the present immunocytochemical study reveals that the CRF₁-LI is widely yet selectively expressed in the mouse brain and that its localization generally overlaps target regions of CRH projections. In addition, CRF₁-LI distribution is generally congruent with previously published data on CRF₁ mRNA expression. However, discrepancies do exist in several regions including the central amygdaloid nucleus, septum, locus coeruleus, certain hypothalamic nuclei, and the cerebellar granular layer. These, as discussed above, may be due to differential regulation of receptor transcription and protein, to protein transport, to the cellular detail afforded by the current study, or to the presence of as yet undiscovered or cross-reacting ligands and receptors, respectively.

Abbreviations

3	principal oculomotor nucleus
3V	third ventricle
7	facial nucleus
10	dorsal motor nucleus of vagus
12	hypoglossal nucleus
AB	accessory basal amygdaloid nucleus
aca	anterior commissure, anterior part
Acb	accumbens nucleus
ACe	central amygdaloid nucleus
AD	anterodorsal thalamic nucleus

AHy	anterior hypothalamic area
Amb	ambiguous nucleus
ap	anterior parvocellular part, paraventricular hypothalamic nucleus
Arc	arcuate hypothalamic nucleus
AV	anteroventral thalamic nucleus
Bmc	basal amygdaloid nucleus, magnocellular division
CA1–3	cornu ammonis, hippocampus
cc	corpus callosum
CC	central canal
CEl	central amygdaloid nucleus, lateral division
CEm	central amygdaloid nucleus, medial division
CL	centrolateral thalamic nucleus
COa	anterior cortical amygdaloid nucleus
d	dorsal nucleus, inferior olive
DCo	dorsal cochlear nucleus
Dk	Darkschewitsch nucleus
DPB	dorsal parabrachial nucleus
DR	dorsal raphe nucleus
DTg	dorsal tegmental nucleus
ec	external capsule
En	endopiriform nucleus
EPl	external plexiform layer of olfactory bulb
EW	accessory oculomotor nucleus
FStr	fundus striate
Gl	glomeruli
Grc	granular layer of cerebellum
GrCo	granular layer of cochlear nucleus
GrDG	granular layer of dentate gyrus
Hi	hilus of dentate gyrus
ICj	islands of Calleja
icp	inferior cerebellar peduncle
Igr	internal granular layer of olfactory bulb
IO	inferior olive
IPl	internal plexiform layer of olfactory bulb
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus

lfp	longitudinal fasciculus of pons
LH	lateral hypothalamic area
LI	lateral amygdaloid nucleus, lateral division
Lm	lateral amygdaloid nucleus, medial division
LMol	stratum lacunosum-moleculare of hippocampus
lp	lateral parvocellular part, paraventricular hypothalamic nucleus
LRt	lateral reticular nucleus
LRtS5	lateral reticular nucleus, subtrigeminal part
LSD	lateral septal nucleus, dorsal part
LSI	lateral septal nucleus, intermediate part
LSV	lateral septal nucleus, ventral part
LTz	lateral nucleus of trapezoid body
LV	lateral ventricle
Lve	lateral vestibular nucleus
Med	medial amygdaloid nucleus, dorsal part of central division
Mcv	medial amygdaloid nucleus, ventral part of central division
MD	mediodorsal thalamic nucleus
ME	median eminence
Me5	mesencephalic trigeminal nucleus
MHb	medial habenular nucleus
Mi	mitral cell layer of olfactory bulb
Mo5	motor trigeminal nucleus
Mol	molecular layer of cerebellum
MolDG	molecular layer of dentate gyrus
mp	medial parvocellular part, paraventricular hypothalamic nucleus
MS	medial septal nucleus
MSO	medial superior olive
MTz	medial nucleus of trapezoid body
MVe	medial vestibular nucleus
opt	optic tract
Or	oriens layer of hippocampus
PAC	periamygdaloid nucleus
PCRt	parvocellular reticular nucleus
PGi	paragigantocellular reticular nucleus
Pir	piriform cortex
pm	posterior magnocellular part, paraventricular hypothalamic nucleus

Pn	pontine nuclei
PnC	pontine reticular nucleus, caudal
PnO	pontine reticular nucleus, oral
Pr5	principal sensory trigeminal nucleus
Pur	Purkinje cell layer
PVN	paraventricular hypothalamic nucleus
py	pyramidal tract
Py	pyramidal layer of hippocampus
PyTu	pyramidal layer of olfactory bulb
Rad	stratum radiatum of hippocampus
Rt	reticular thalamic nucleus
sep	superior cerebellar peduncle
sm	stria medullaris thalamus
SNC	substantia nigra pars compacta
SNR	substantia nigra pars reticulata
SO	supraoptic hypothalamic nucleus
sol	solitary tract
SolL	nucleus solitary tract, lateral part
SolM	nucleus solitary tract, medial part
STh	subthalamic nucleus
Su7	suprafacial nucleus
VCo	ventral cochlear nucleus
VDBV	nucleus of vertical limb of diagonal band, ventral part
VL	ventrolateral thalamic nucleus
VLL	ventral nucleus of lateral lemniscus
VM	ventromedial thalamic nucleus
VMH	ventromedial hypothalamic nucleus
VMHD	ventromedial hypothalamic nucleus, dorsomedial part
M	
VMHV	ventromedial hypothalamic nucleus, ventrolateral part
L	
VP	ventral pallidum
VPB	ventral parabrachial nucleus
VTg	ventral tegmental nucleus
ZI	zona incerta

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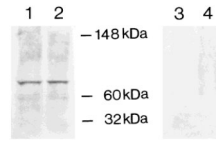


Fig. 1.

The anti-CRF₁ antiserum recognizes a single protein species, as evident from Western blot analysis. Cell lysates were prepared from mouse hypothalamus (**lanes 1 and 3**) and cerebellum (**lanes 2 and 4**). Aliquots of 50 μ g protein were loaded in each lane. Following incubation (see Materials and Methods), a single band of approximately 80 kDa was recognized by CRF₁ antiserum (1:500). Lanes 3 and 4 show that preadsorption of CRF₁ antiserum with the antigenic peptide leads to disappearance of this band.

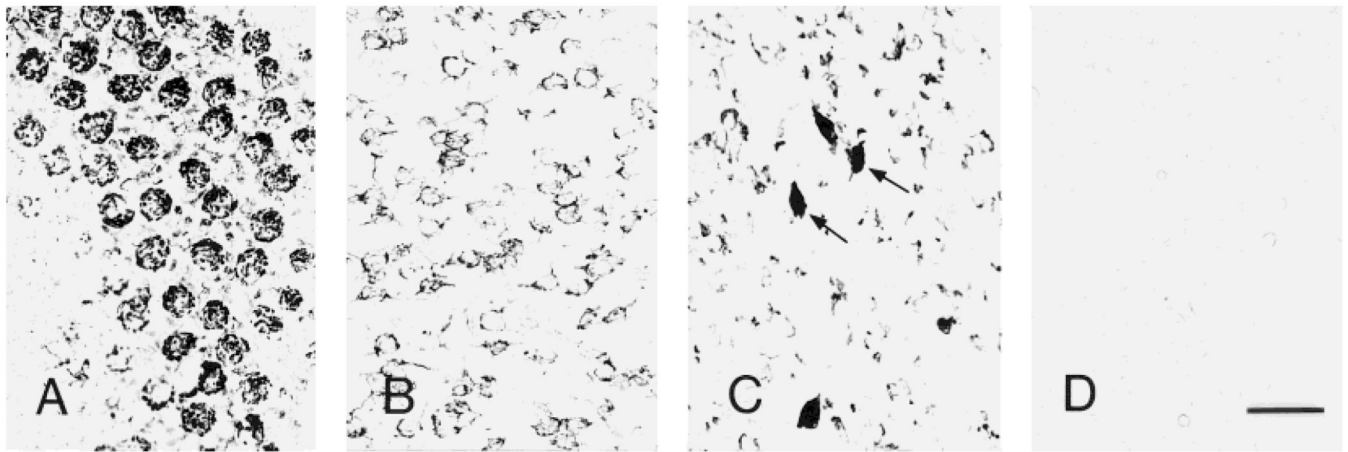


Fig. 2. Illustration of the three principal patterns of neuronal CRF₁-like immunoreactivity (CRF₁-LI) in mouse brain. CRF₁-LI is characterized as granular (**A**, cerebellar Purkinje cells), punctate (**B**, parafascicular thalamic nucleus), or homogenous (arrows in **C**, lateral hypothalamic area). **D**: Preadsorption of the antiserum with the immunogenic epitope eliminates all the reaction signal (section shown is from thalamus). Scale bar = 40 μ m (applies to A–D).

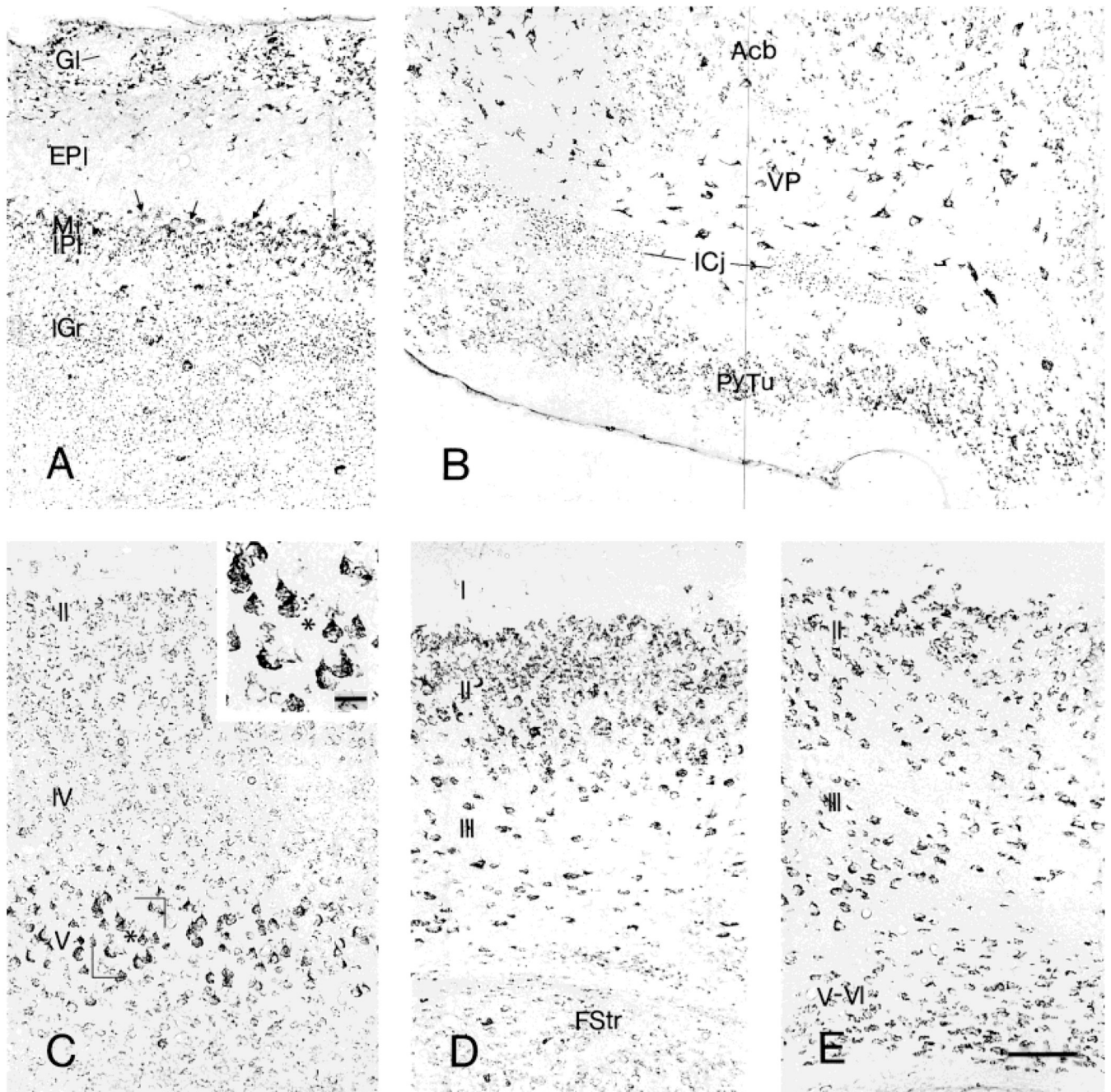


Fig. 3. Distribution of CRF₁-like immunoreactivity in the olfactory and cortical regions: (A) olfactory bulb, (B) olfactory tubercle, (C) neocortex, (D) piriform cortex, and (E) entorhinal cortex. Enlargement in C shows the strongly labeled pyramidal neurons in layer V. Cortical layers are denoted by Roman numerals. Scale bars = 80 μ m in E (applies to A–E); 20 μ m in inset in C.

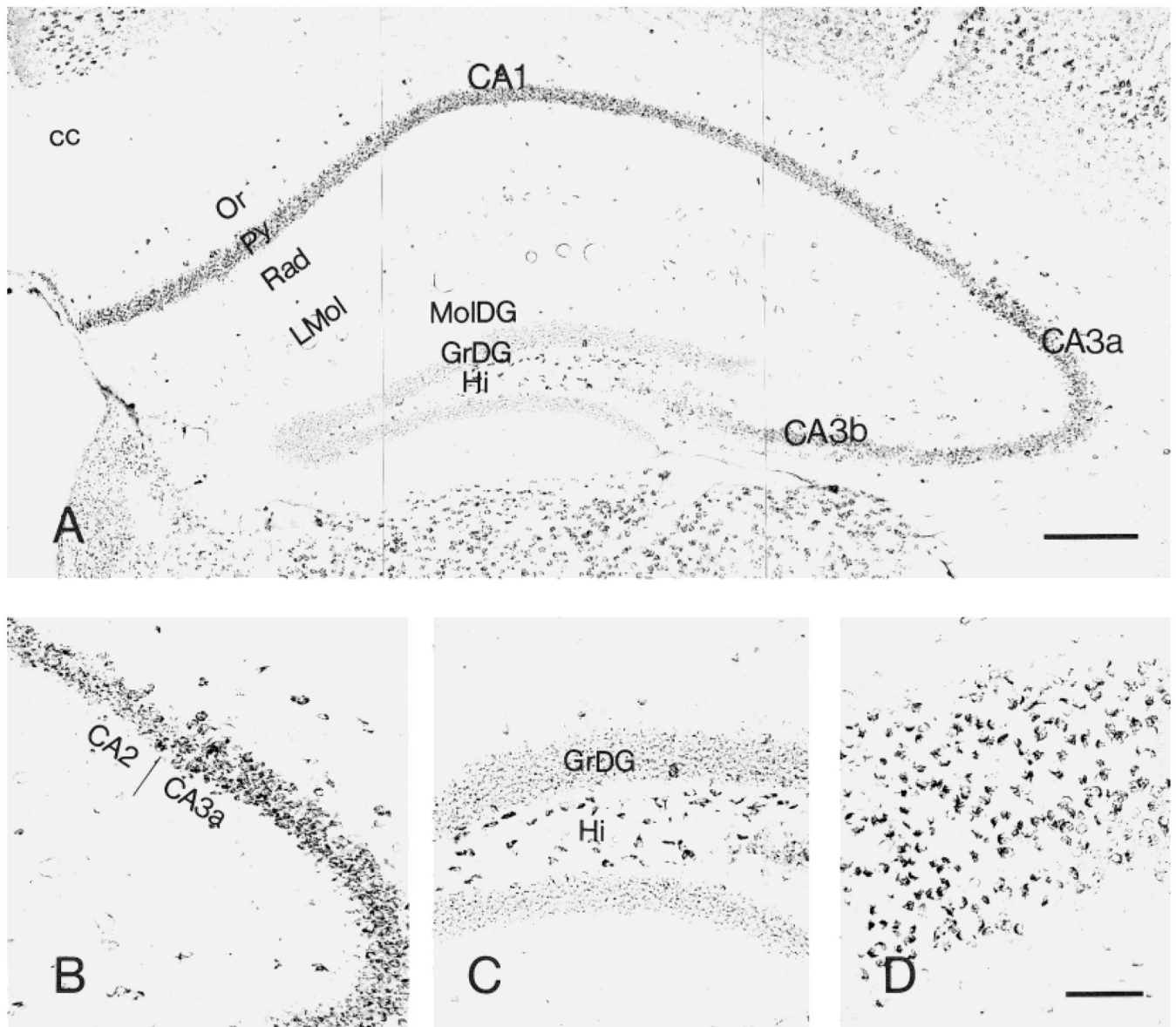


Fig. 4. Distribution of CRF₁-like immunoreactivity in the hippocampal formation (A). **B:** Morphology of CRF₁-LI-expressing neurons in hippocampal CA2–3a area. **C,D:** CRF₁-like immunoreactivity in the dentate gyrus and subiculum, respectively. Within the dentate gyrus, granular cell somata are outlined by a fine mesh of weakly stained puncta, and intensely labeled neurons are present in the hilus. Most pyramidal neurons are intensely labeled in the subiculum. Scale bars = 200 μ m in A; 80 μ m in D (applies to B–D).

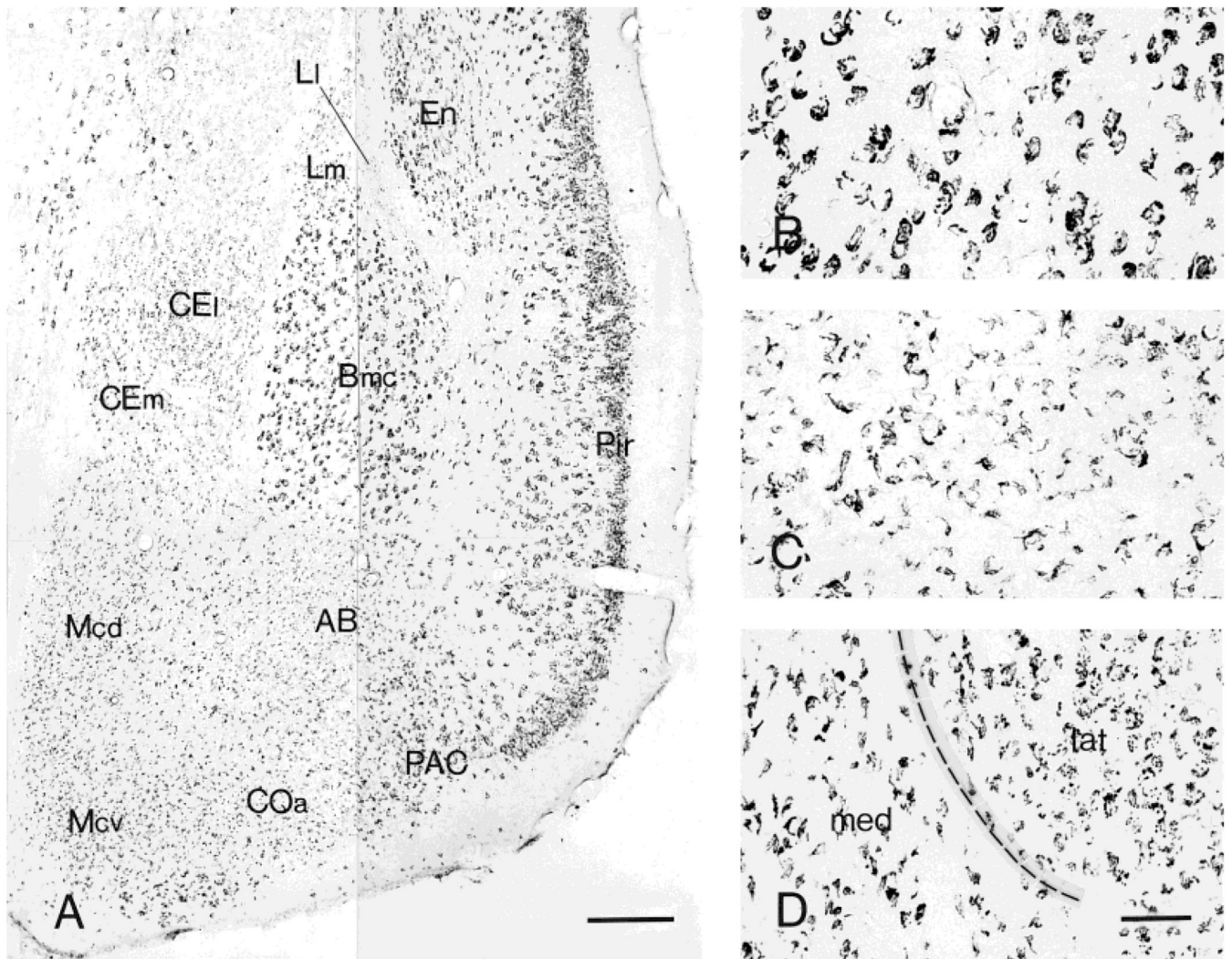


Fig. 5. Distribution of CRF₁-like immunoreactivity in the amygdala (A) and the morphology of neurons showing CRF₁-LI in basal (B), medial (C), and central (D) nuclei. These neurons are intensely labeled throughout the magnocellular division of basal nucleus (Bmc). Within the medial nucleus (Mcd and Mcv), CRF₁ immunoreaction product frequently outlines only a portion of the cell surface. In the central nucleus, the lateral (CEI) and medial (CEm) divisions show a different staining pattern: whereas immunoreactivity in CEI consists of punctate deposits, the CEm consists of an outline of only a portion of the cell surface. Scale bars = 200 μ m in A; 40 μ m in D (applies to B–D).

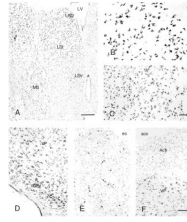


Fig. 6. Distribution of neurons showing CRF₁-LI in septum (**A**). Higher magnification, illustrating characteristic CRF₁-ir neurons in dorsal (**B**) and lateral (**C**) septal nuclei, and in the vertical limb of the nucleus of the diagonal band of Broca (**D**). **E,F**: CRF₁-like immunoreactivity in the basal ganglia, including the accumbens nucleus (Acb) and ventral striatum (VP). Scale bars = 200 μm in A; 40 μm in C (applies to B,C); 80 μm in F (applies to D-F).

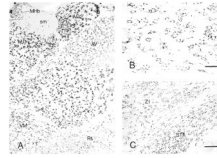


Fig. 7. Distribution of neurons labeled for CRF₁-LI in thalamus (**A**). **B**: Higher magnification showing CRF₁-ir neurons in the nucleus reticularis. **C**: CRF₁-like immunoreactivity in the subthalamus (STh) and zona incerta (ZI). Scale bars = 40 μ m in B; 80 μ m in C (applies to A,C)

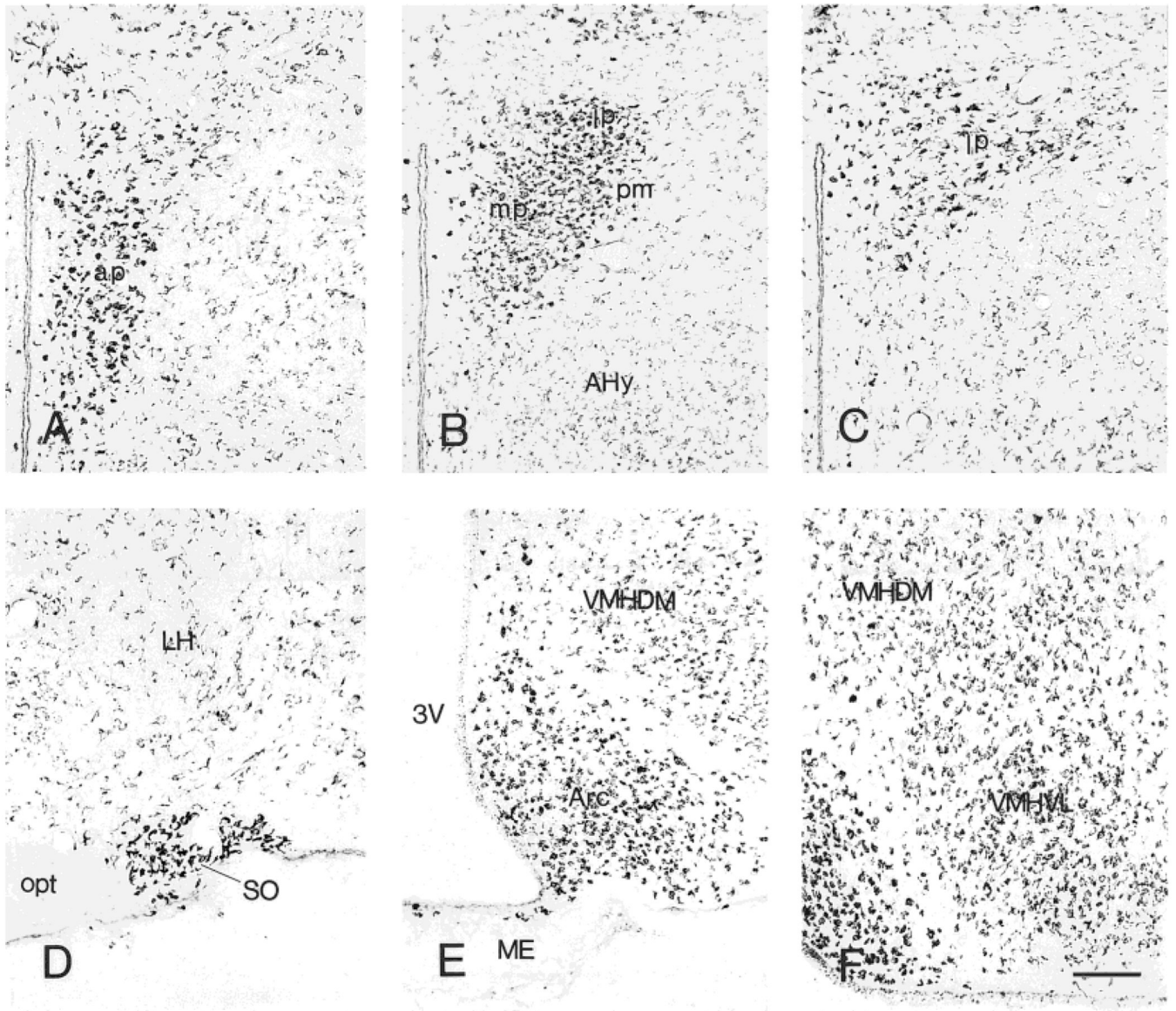


Fig. 8. A series of coronal sections from rostral to caudal of the paraventricular nucleus, showing neurons intensely labeled for CRF₁-LI (A–C). Similar neurons in the supraoptic nucleus (D), arcuate nucleus and median eminence (E), and ventromedial nucleus (F). Scale bar = 80 μ m (applies to A–F).

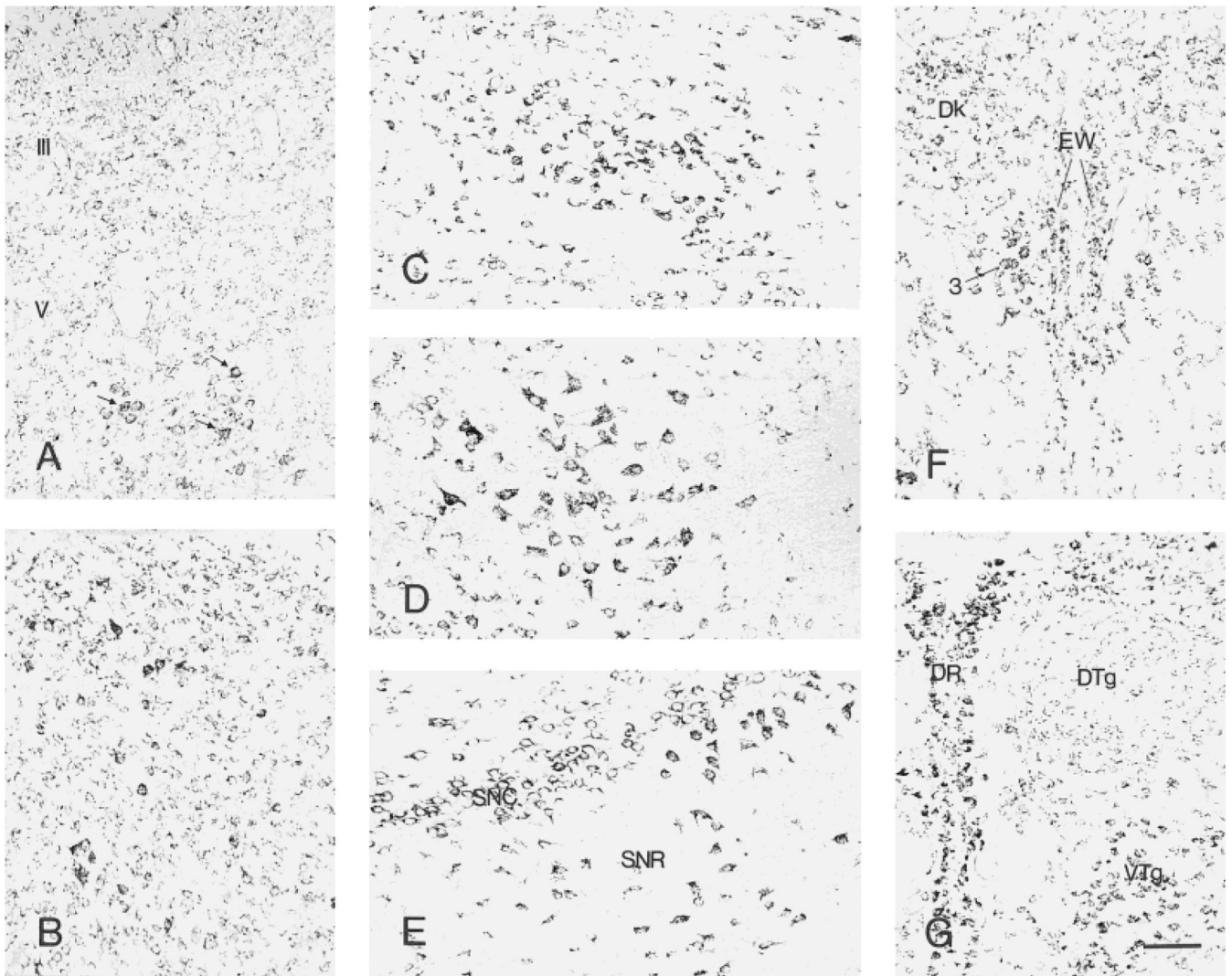


Fig. 9. Distribution of CRF₁-like immunoreactivity in the superior (**A**) and inferior (**B**) colliculus. A few deeply labeled neurons are present in the lateral part of layer VI. In the inferior colliculus, strongly stained neurons are scattered in both the cortical and central nuclei, where most medium-sized neurons are moderately labeled. CRF₁-LI-expressing neurons in the ventral tegmental nuclei (**C**), red nucleus (**D**), and substantia nigra (**E**) are strongly labeled. The accessory oculomotor nucleus (Edinger-Westphal, **F**) is moderately or intensely labeled, whereas the dorsal raphe nucleus (**G**) is rich in highly labeled neurons. Scale bar = 80 μ m (applies to A–G).

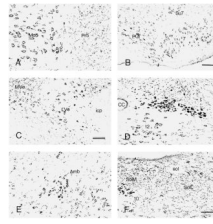


Fig. 10. Neurons showing CRF₁-like immunoreactivity in nuclei of the trigeminal nerve (**A**), facial nucleus (**B**), vestibular nuclei (**C**), vagal dorsal motor nucleus and hypoglossal nucleus (**D**), ambiguous nucleus (**E**), and solitary tract nucleus (**F**). Scale bars = 160 μ m in B,C; 80 μ m in F (applies to A,D-F).

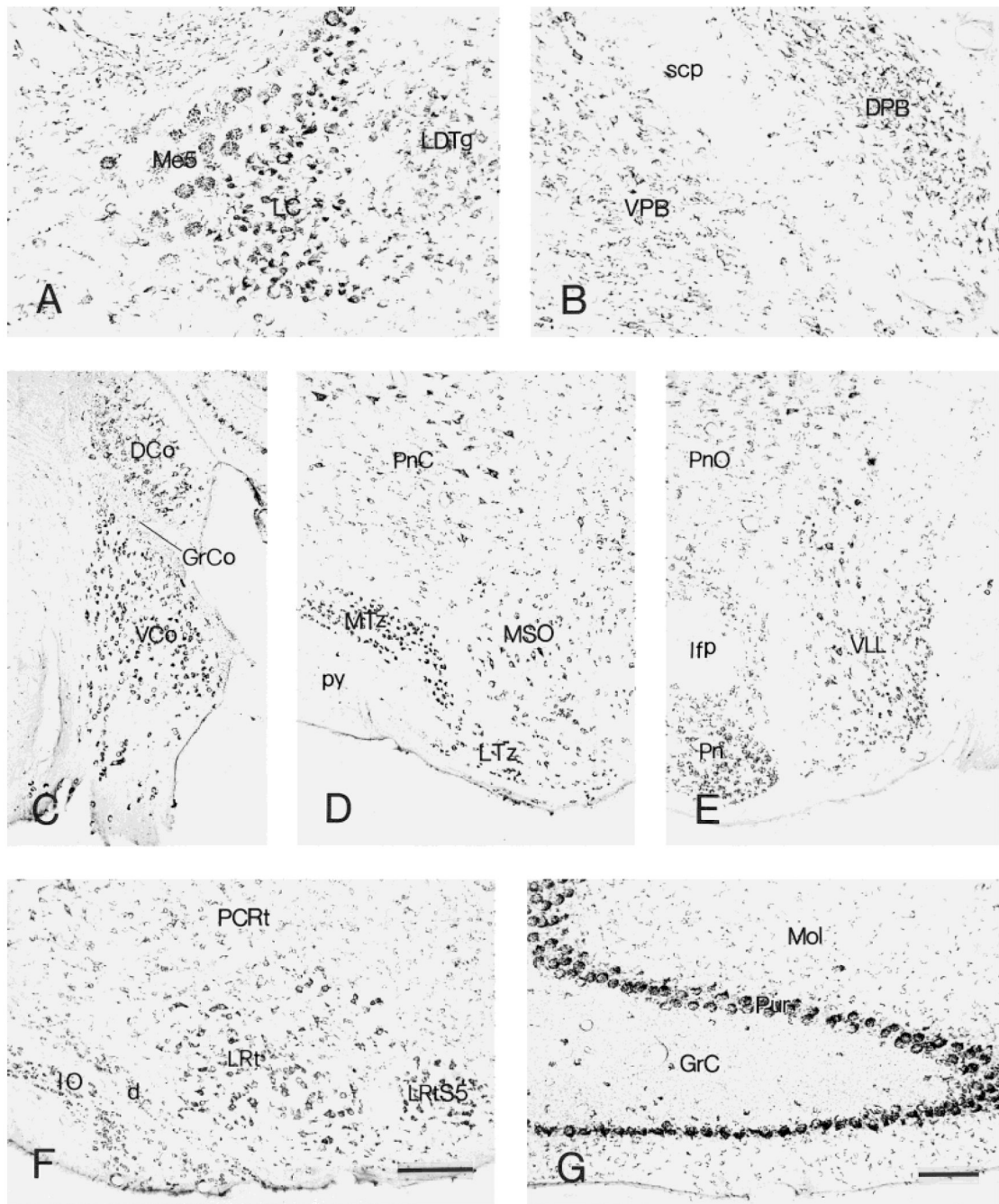


Fig. 11. CRF₁-like immunoreactive neurons in locus coeruleus (A) and parabrachial nuclei (B). C–E: Distribution of labeled neurons in cochlear nuclei (C), trapezoid body (D), and ventral nucleus of lateral lemniscus (E). F,G: Immunoreactive neurons in the inferior olive and cerebellum, respectively. Within the cerebellum, Purkinje cells are intensely labeled. The granular layer contains scattered intensely labeled neurons, as well as numerous weakly stained fine puncta. Scale bars = 200 μ m in F (applies to C–F); 80 μ m in G (applies to A,B,G).

TABLE 1

Distribution of Neurons Expressing CRF₁-LI in the Mouse Telencephalon: Density and Intensity

Region	Density ¹	Intensity ²
Olfactory bulb		
Glomerular layer	++	++/ +++ ³
External plexiform layer	+	+
Mitral cell layer	++++	++
Internal plexiform layer	++	++
Granular cell layer	++++	+/>++
Accessory olfactory bulb		
Mitral cell layer	++++	++
Granular cell layer	++++	+/>++
Olfactory tubercle		
Polymorphic layer	+++	+/>++
Pyramidal layer	++++	+/>++
Island of Calleja	+++	+/>++
Taenia tecta	++++	++
Clastrum	++++	+/>++
Endopiriform nucleus	+++	++
Neocortex		
Layer I	+	+
Layer II	++++	++
Layer III	++++	++
Layer IV	++++	+
Layer V	++++	++++
Layer VI	++++	+/>++
Piriform cortex		
Layer I	+	++
Layer II	++++	++/>+++
Layer III	++++	++/>+++
Entorhinal cortex		
Layer IIa	++++	++/>+++
Layer IIb	+++	++
Layer III	++++	++
Layers V–VI	+++	++/>+++
Perirhinal cortex	++++	++
Hippocampal formation		
Hippocampus		
CA1 pyramidal layer	++++	++
CA3a pyramidal layer	++++	+++
CA3b,c pyramidal layer	++++	+/>++

Region	Density ¹	Intensity ²
Dentate gyrus		
Hilus	++	+++
Granular layer	++++	+
Molecular layer	-/+	++
Subiculum	+++	+++/ ++++
Amygdala		
Medial nucleus	++++	++
Cortical nucleus	++++	+/>++
Central nucleus		
Medial	+++	+/>++
Lateral	++++	+/>++
Basal nucleus	+++	++++
Lateral nucleus	+++	+
Bed nucleus of stria terminalis		
Medical part	+++	++
Lateral part	++++	++
Basal ganglia		
Caudate-putamen	++++	+
Nucleus accumbens	++++	+/>++
Fundus striati	+++	++
Globus pallidus	++	++/>+++
Ventral pallidus	++	+++
Entopeduncular nucleus	++	+++
Septum		
Lateral septal nucleus	++++	+++
Medical septal nucleus	++++	++/>+++
Diagonal band of Broca		
Vertical limb	+++	+++
Horizontal limb	+++	+++

¹Density values are based on the percentage of positive cells related to total cell number. -, no positive cells; -/+, occasional cells, <10%; +, low, 10–25%; ++, moderate, 25–50%; +++, dense, 50–75%; +++++, very dense, >75%.

²Staining intensity: +, weakly positive; ++, moderately positive; +++, intensely positive; +++++, strongly intense.

³Two scores are noted because the region contains cell populations with different labeling intensity.

TABLE 2

Distribution of Neurons Expressing CRF₁-LI in the Mouse Diencephalon: Density and Intensity

Region	Density ¹	Intensity ²
Habenula		
Medial part	++++	+++
Lateral part	++	++
Thalamus		
Anterodorsal nucleus	++	+++ / +++++ ³
Antroventral nucleus	+++	++
Anteromedial nucleus	+++	+++
Mediodorsal nucleus	+++	+++
Lateral dorsal nucleus	++	+++
Lateral posterior nucleus	++	+++
Ventral lateral nucleus	++	+++
Ventral postrolateral nucleus	++	+++
Ventral postromedial nucleus	++	+++
Paraventricular nucleus	+++	+++ / +++++
Midline nuclear group	++	++ / +++
Intralaminar nuclear group	++	++ / +++
Thalamic reticular nucleus	++	+ / ++
Hypothalamus		
Medial preoptic area	+++	+++
Lateral preoptic area	+++	++
Suprachiasmatic nucleus	+++	++
Retrochiasmatic nucleus	++	+
Supraoptic nucleus	++++	++++
Paraventricular nucleus		
Medial parvocellular division	+++	+++ / +++++
Magnocellular division	+	+
Ventromedial nucleus	++++	++ / +++
Arcuate nucleus	++++	+++ / +++++
Anterior hypothalamic area	+++	++
Lateral hypothalamic area	+++	+ / ++
Dorsal hypothalamic area	+++	+
Dorsomedial nucleus	+++	+
Median eminence	+	+++
Subthalamic nucleus	++++	++ / +++
Zona incerta	+++	+ / ++
Medial geniculate body	+++	+++
Lateral geniculate body	+++	++ / +++

¹Density values are based on the percentage of positive cells related to total cell number. -, no positive cells; -/+, occasional cells, <10%; +, low, 10–25%; ++, moderate, 25–50%; +++, dense, 50–75%; +++++, very dense, >75%.

²Staining intensity; +, weakly positive; ++, moderately positive; +++, intensely positive; +++++, strongly intense.

³Two scores are noted because the region contains cell populations with different labeling intensity.

TABLE 3

Distribution of Neurons Expressing CRF₁-LI in the Mouse Hindbrain: Density and Intensity

Region	Density ¹	Intensity ²
Superior colliculus	++	+/ ³
Inferior colliculus	++	+/ ³
Tegmental nuclei	+++	+/+++
Pedunculopontine nucleus	+++	+++
Red nucleus	++++ ⁴	+++
Substantia nigra		
Pars compacta	++	+++
Pars reticulata	++	+/+++
Raphe nuclei		
Dorsal nucleus	++	+++/ ⁴
Median nucleus	+	++
Edinger-Westphal nucleus	+++	+/ ³
Principal oculomotor nucleus	+++	+++
Darkschewitsch nucleus		
Nuclei of the trigeminal nerve		
Mesencephalic nucleus	++++	+++
Motor nucleus	++++ ⁴	+++
Principal sensory nucleus	+++	+/ ³
Spinal nucleus	++	+/ ³
Facial nucleus	++++ ⁴	+++
Vestibular nuclei	+++	+/+++
Cochlear nuclei		
Dorsal nucleus	++	++
Ventral nucleus	++	+++
Vagus nucleus	++++	++++
Hypoglossal nucleus	++++	+++
Solitary nucleus	+++	+/ ³
Ambiguus nucleus	+++	++++
Locus coeruleus	+++	++++
Superior olive	+++	+++
Inferior olive	+++	+/+++
Nuclei of lateral lemniscus	+++	+++
Nucleus of trapezoid body		
Medial nucleus	++++	++++
Lateral nucleus	+++	++
Cerebellum		

Region	Density ¹	Intensity ²
Molecular layer	+++	+
Purkinje cell layer	++++	++++
Granular cell layer	+++	+
Cerebellar nuclei	+++	+++

¹ Density values are based on the percentage of positive cells related to total cell number. -, no positive cells; +/-, occasional cells, <10%; +, low, 10–5%; ++, moderate, 25–50%; +++, dense, 50–75%; +++++, very dense, >75%.

² Staining intensity: +, weakly positive; ++, moderately positive; +++, intensely positive; +++++, strongly intense.

³ Two scores are noted because the region contains cell populations with different labeling intensity.

⁴ All large, multipolar neurons were CRF1 immunoreactive.