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Corticotropin-Releasing Factor Binding Protein Within the Ventral Tegmental Area Is Expressed in a Subset of Dopaminergic Neurons

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Abstract

Corticotropin-releasing factor (CRF) and related peptides play a role in mediating neuronal effects of stress. These peptides mediate stress responses by their interactions with the CRF receptors and the CRF-binding protein (CRF-BP). Because the CRF-BP is implicated in neurotransmission within the ventral tegmental area (VTA), we investigated whether the CRF-BP is expressed in VTA neurons. By in situ hybridization, we detected cellular expression of CRF-BP mRNA in the VTA; no such expression was seen in neighboring substantia nigra pars compacta (SNC) or substantia nigra pars reticulata. By double in situ hybridization, we determined that VTA neurons with CRF-BP mRNA coexpressed transcripts encoding either tyrosine hydroxylase [TH; a marker for dopamine (DA) neurons] or glutamic acid decarboxylase [GAD; synthesizing enzyme of γ-aminobutyric acid (GABA)]. Neurons with CRF-BP mRNA represented 25% of the total population of TH-expressing neurons and 28% of the total population of GAD-expressing neurons, indicating that discrete subpopulations of dopaminergic and GABAergic neurons are present in the VTA. Within the total population of neurons containing CRF-BP mRNA, 70% coexpressed TH mRNA and only 27% coexpressed GAD mRNA. As far as we are aware, we provide the first anatomical evidence that a molecule, CRF-BP, is encoded by DAergic neurons of the VTA but not by those of the SNC. We propose, based on the observation that the majority of VTA neurons expressing CRF-BP mRNA are DAergic, that in the VTA interactions of CRF-BP with CRF, or with CRF-related peptides, are likely to be mediated predominantly by DAergic neurons.

Indexing terms

GABA; substantia nigra; dopamine; reward and stress

Corticotropin-releasing factor (CRF) is a 41-amino-acid neuropeptide isolated initially from the hypothalamus (Vale et al., 1981) and subsequently shown to be synthesized in numerous cells throughout the brain (Swanson et al., 1983; Sawchenko et al., 1993). CRF plays important roles in hypothalamic and extrahypothalamic responses to stress (Vale et al., 1981; Dunn and Berridge, 1990; Koob and Heinrichs, 1999; Bale and Vale, 2004) by interacting with different neuronal pathways such as the hypothalamic-pituitary-adrenal (HPA) circuit (Vale et al., 1981; Dunn and Berridge, 1990; Bale and Vale, 2004) and the mesocorticolimbic dopamine system (Kalivas et al., 1987; Deutch and Roth, 1990).

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The mesocorticolimbic dopamine (DA) system, which consists of DA-producing neurons concentrated in the ventral tegmental area (VTA) and DA axons targeting, primarily, prefrontal cortex and limbic structures, plays a role in motivation and reward (Wise and Rompre, 1989; Smith, 1995; Wise, 2004). Interactions between CRF and the mesocorticolimbic DAstem are suggested from studies showing an increase in spontaneous motor activity after injections of CRF into the VTA (Kalivas et al., 1987). Moreover, CRF application to midbrain slices potentiates synaptic transmission by N-methyl-D-aspartate (NMDA) receptors in mouse VTA DAergic neurons (Ungless et al., 2003) and excites rat VTA DAergic neurons (Korotkova et al., 2006). These observations indicate that DAergic neurotransmission in the VTA is affected by exogenous CRF. By immunoelectron microscopy, we recently established that CRF in the VTA is concentrated in axon terminals forming synapses mostly with dendrites (Tagliaferro and Morales, 2008). The release of CRF from these axon terminals is likely to be affected by stressors, as in vivo studies demonstrated that mild footshock induces release of CRF in the VTA (Wang et al., 2005).

In addition to CRF, three different genes encoding CRF-related peptides, urocortins (UcnI, -II, and -III), are expressed in mammals (Vaughan et al., 1995; Donaldson et al., 1996; Lewis et al., 2001). CRF and Ucn effects are mediated through two high-affinity membrane receptors, CRF-R1 (Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993) and CRF-R2 (Kishimato et al., 1995; Lovenberg et al., 1995; Perrin et al., 1995; Stenzel et al., 1995). Additionally, activities of CRF and some isoforms of Ucns are affected by their interactions with the glycoprotein CRF-binding protein (CRF-BP; Potter et al., 1991, 1992; Behan et al., 1996). Although a role of peripheral CRF-BP in lowering free circulating CRF levels is well established (Orth and Mount, 1987; Linton et al., 1988; Suda et al., 1988), the effect of CRF-BP in the brain is less clear (for review see Bale and Vale, 2004). CRF-BP appears to play a role in neuronal transmission within the VTA, in that electrophysiological (Ungless et al., 2003) and behavioral (Wang et al, 2007) evidence indicates that CRF and Ucns that bind to CRF-BP affect DAergic and glutamatergic neurotransmission in the VTA. In the present study, we determined, by using anatomical techniques, whether specific resident neurons could be a source of CRF-BP in the VTA.

MATERIALS AND METHODS

Tissue preparation

Ten adult male Sprague-Dawley rats (300–350 g body weight) were used for these studies. Rats were individually housed on a 12-hour light schedule in a temperature-controlled (20°C) animal room and given access to standard rat chow and water ad libitum. All animal procedures were approved by the local Animal Care and Use Committee. Each animal was anesthetized with chloral hydrate (35 mg/100 g) and perfused transcardially with 4% (W/V) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Brains were left in 4% paraformaldehyde for 2 hours at 4°C, rinsed with PB, and transferred sequentially to 12%, 14%, and 18% sucrose solutions in PB. Coronal 20-μm serial cryosections were prepared.

Single in situ hybridization

In situ hybridization was performed as described previously (Yamaguchi et al., 2007). Cryosections were incubated for 10 minutes in PB containing 0.5% Triton X-100, rinsed for 2 × 5 minutes with PB, treated with 0.2 N HCl for 10 minutes, rinsed for 2 × 5 minutes with PB, and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 minutes. Sections were rinsed for 2 × 5 minutes with PB; postfixed with 4% paraformaldehyde for 10 minutes; and, after a final rinse with PB, hybridized for 16 hours at 55°C in hybridization buffer (50% formamide; 10% dextran sulfate; 5× Denhardt's solution; 0.62 M NaCl; 50 mM DTT; 10 mM EDTA; 20 mM PIPES, pH 6.8; 0.2% SDS; 250 μg/ml salmon sperm DNA; 250

μg/ml tRNA) containing ^{35}S- and ^{33}P-labeled single-stranded antisense or sense of rat CRF-BP (nucleotides 615–969, accession No. NM 139183) probes at 10^7 cpm/ml. We found that material hybridized with ^{35}S- and ^{33}P-labeled riboprobes requires exposure times shorter than material hybridized with single ^{35}S- or ^{33}P-labeled riboprobes. Plasmid that contained the CRF-BP was generously provided by Dr. Audrey F. Seasholtz (University of Michigan, Ann Arbor, MI). Sections were treated with 4 μg/ml RNase A at 37°C for 1 hour and washed with 1× SSC, 50% formamide at 55°C for 1 hour and with 0.1× SSC at 68°C for 1 hour. After the last SSC wash, sections were rinsed with TBS buffer (20 mM Tris.HCl, 0.5 M NaCl, pH 8.2). Finally, slides were dipped in Ilford K.5 nuclear tract emulsion (Polysciences, Inc., Warrington, PA; 1:1 dilution in double-distilled water) and exposed in the dark at 4°C for 4 weeks prior to development.

Preparation of digoxigenin-labeled RNA riboprobes for the detection of TH mRNA, GAD_{65} mRNA, and GAD_{67} mRNA

The antisense digoxigenin riboprobes for TH (nucleotides 14 –1165, accession No. NM012740), GAD_{65} (nucleotides 1–1758, accession No. NM012563), and GAD_{67} (nucleotides 1–1782, accession No. NM017007) were obtained by in vitro transcription using digoxigenin-11-UTP labeling mix (Roche, Indianapolis, IN) and T3 RNA polymerase. Plasmids that contained GAD_{65} and GAD_{67} were generously provided by Dr. Allan Tobin (University of California Los Angeles, Los Angeles, CA).

Double in situ hybridization

For double hybridization, cryosections were processed as indicated for single hybridization, but hybridization was performed with hybridization buffer containing ^{35}S- and ^{33}P-labeled single-stranded antisense rat CRF-BP riboprobes at 10^7 cpm/ml, together with the 600 ng TH or mixture of GAD_{65} and GAD_{67} digoxigenin-labeled antisense riboprobes. Sections were treated with 4 μg/ml RNase A at 37°C for 1 hour and washed with 1× SSC, 50% formamide at 55°C for 1 hour and with 0.1× SSC at 68°C for 1 hour. After the last SSC wash, sections were rinsed with TBS buffer (20 mM Tris HCl, 0.5 M NaCl, pH 8.2). Afterward, sections were incubated with an alkaline phosphatase-conjugated antibody against digoxigenin (Roche Applied Science) overnight at 4°C, and the alkaline phosphatase reaction was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science), yielding a purple reaction product. Sections were mounted on slides, air dried, and dipped in Ilford K.5 nuclear tract emulsion (Polysciences, Inc.; 1:1 dilution in double distilled water) and exposed in the dark at 4°C for 4 weeks prior to development.

Combination of in situ hybridization and immunocytochemistry

After single or double in situ hybridization, sections were rinsed with PB and incubated for 1 hour in PB supplemented with 4% bovine serum albumin (BSA) and 0.3% Triton X-100. Sections were then incubated with anti-TH mouse monoclonal antibody (Chemicon, Temecula, CA; code MAB318) overnight at 4°C. After rinsing for 3 × 10 minutes in PB, sections were processed with an ABC kit (Vector Laboratories, Burlingame, CA). The sections were incubated for 1 hour at RT in a 1:200 dilution of the biotinylated secondary antibody, rinsed with PB, and incubated with avidin-biotinylated horseradish peroxidase for 1 hour. Sections were rinsed, and the peroxidase reaction was then developed with 0.05% 3,3-diaminobenzidine-4 HCl (DAB) and 0.003% H_2O_2. Sections were mounted on slides, air dried, dipped in nuclear track emulsion, and exposed for 4 weeks prior to development. The mouse monoclonal anti-TH antibody was raised against purified TH from the PC12 pheochromocytoma cell line; this antibody recognizes recombinant TH on Western blot analysis (Wolf and Kapatos, 1989). We previously showed that the anti-TH antibody labels a single protein band in the molecular weight range of 56 – 60 kDa on Western blots prepared

from VTA protein homogenates (Shepard et al., 2006). In addition, we had used this anti-TH antibody to demonstrate that midbrain unilateral lesions with 6-hydroxydopamine (6-OHDA) result in lack of TH immunolabeling in the lesioned site, which is preserved in the contralateral unlesioned midrain (Sarabi et al., 2001).

Data analysis

Sections were viewed, analyzed, and photographed with brightfield or epiluminescence microscopy using a Nikon Eclipse E 800 microscope fitted with ×4 and ×20 objective lenses. Cells expressing CRF-BP mRNA, TH mRNA, or both transcripts were counted in the five sections (500 μm spacing between sections) of five different rats. Double-labeled material was analyzed by using epiluminescence to increase the contrast of silver grains, because neither darkfield nor brightfield optics allows a clear visualization of silver grains when colocalized with immunoproducts. To determine the midbrain cytoarchitecture, adjacent cryosections were either processed for detection of mRNAs or stained with cresyl violet (Nissl stained); midbrain subdivisions were traced according to Phillipson (1979), Halliday and Törk (1986), German and Manaye (1993), and Paxinos and Watson (2007). Nissl images were used to demarcate the SNC and VTA and the boundary between them. A replica of the outlines was superimposed on same-sized images of adjacent sections showing CRF-BP mRNA or TH mRNA. Single- and double-labeled neurons were observed within each traced region at high power (×20 objective lens) and marked electronically. A cell was considered to express CRF-BP mRNA when its soma contained aggregates of silver particles. A neuron was considered to express TH mRNA or GAD mRNA when its soma was clearly labeled as purple. A TH- or GAD-expressing neuron was included in the calculation of total population of TH- or GAD-expressing cells when the stained cell was at least 5 μm in diameter. The cells expressing CRF-BP, TH, GAD, CRF-BP/TH, and CRF-BP/GAD transcripts were counted separately. To determine coexpression of CRF-BP with TH or GAD, 1) silver grains corresponding to CRF-BP expression were focused under epiluminescence microscopy, 2) the path of epiluminescence light was blocked without changing the focus, 3) brightfield light was used to determine whether a purple neuron, a cell expressing either TH or GAD, in focus contained the aggregates of silver grains seen under epiluminescence. Labeled cells were counted three times, each time by a different observer. The background was evaluated from slides hybridized with sense probes. Pictures were adjusted to match contrast and brightness in Adobe Photoshop (Adobe Systems, Seattle, WA).

RESULTS

Neurons expressing CRF-BP mRNA are present in the VTA but absent in neighboring substantia nigra pars compacta and substantia nigra pars reticulata

By using CRF-BP radioactive antisense riboprobes, we found expression of CRF-BP mRNA in the VTA (Figs. 1A,A′, 2B, 3B, 4C). Signal for CRF-BP mRNA was not detected when brain sections were hybridized with the corresponding sense riboprobe (Fig. 1B,B′).

Several areas in the vicinity of the VTA consistently showed expression of CRF-BP mRNA, such as the supra-mammilary nucleus (Fig. 5A′), the interfascicular nucleus (Fig. 6A′), and the interpeduncular nucleus (Fig. 7A′). In contrast, we did not observe expression of CRF-BP mRNA in the substantia nigra pars compacta or substantia nigra pars reticulata (Figs. 2B, 3B, 4C, 5A′, 6A′, 7A′). We did see CRF-BP mRNA in the substantia nigra pars lateralis, a lateral extension of substantia nigra pars compacta that projects to the amygdala (Figs. 5A′, 6A′, 7A′). Notably, the cellular expression of CRF-BP within the PBP appears to correspond to the separation between the VTA and the substantia nigra pars compacta (see white line in Figs. 2B, 3B, 4C).

Subsets of neurons expressing CRF-BP mRNA have different patterns of distribution in the VTA subregions

To visualize better the boundaries of the VTA, brain sections were processed by double in situ hybridization (for identification of cells expressing CRF-BP mRNA or GAD mRNA) and by immunohistochemistry to label cell bodies and dendrites containing TH protein (Figs. 5A, 6A, 7A). We detected expression of CRF-BP mRNA from the rostral to the caudal aspects of the VTA (Figs. 5A′, 6A′, 7A′); the cellular expression of CRF-BP decreased toward the caudal VTA (Fig. 7A′). Within the VTA, there was a medial-lateral gradient of CRF-BP mRNA expression; the highest concentration was found in the medial part of the VTA and the lowest in the lateral portions (Figs. 5A′, 6A′, 7A′, 8A′). Cellular expression of CRF-BP mRNA was found from the rostral to the caudal levels of the PBP. Many cells expressing CRF-BP mRNA were observed at all levels of the rostral linear nucleus (RLi; Fig. 6A′). In contrast, only few neurons expressing CRF-BP mRNA were found at the medial level of the paranigral nucleus (PN; Fig. 6A′) and the rostral level of the parainterfasicular nucleus (PIF). Cells expressing CRF-BP mRNA were rarely found in the caudal level of the PN and the PIF (Fig. 7A′).

In summary, midbrain neurons expressing CRF-BP are distributed mostly within two subregions of the VTA, the parabrachial pigmental area and the rostral linear nucleus. Lower concentrations of cells expressing CRF-BP are present in two other subdivisions of the VTA, the paranigral nucleus and the parainterfasicular nucleus. Although CRF-BP mRNA is expressed in neurons within the substantia nigra pars lateralis, CRF-BP mRNA is not expressed either in the substantia nigra pars compacta or reticulata.

Neurons expressing CRF mRNA coexpress transcripts encoding either TH or GAD

Cellular analysis of double-labeled material clearly showed VTA expression of CRF-BP mRNA in neurons containing TH mRNA (Figs. 8, 9, 11). Neurons coexpressing CRF-BP/TH mRNAs were found in all subdivisions of the VTA: PBP, PN, and PIF (Figs. 8, 9, 11). Cells expressing CRF-BP mRNA but lacking TH mRNA were also found within the same subdivisions of the VTA (Figs. 8, 9, 11) but were located mostly in the medial region of the VTA (Figs. 8, 9, 11). Counting the cells expressing CRF-BP and TH mRNAs, we determined that 70% of all cells expressing CRF-BP mRNA coexpressed TH mRNA (Table 1). However, the population of neurons coexpressing CRF-BP mRNA and TH mRNA corresponds to only 25% of the total population of TH-expressing neurons present in the VTA (Table 1).

Cellular expression of CRF-BP mRNA was also found in neurons containing GAD mRNA (Fig. 10). The CRF-BP/GAD-coexpressing neurons were preferentially distributed in the medial and the dorsal aspects of the VTA. Cellular coexpression of CRF-BP mRNA and GAD mRNA was found in 27% of the total population of neurons expressing CRF-BP mRNA (Table 2). CRF-BP/GAD-coexpressing neurons represent 28% of the total population of neurons expressing GAD mRNA (Table 2).

In summary, although both dopaminergic and GABAergic neurons of the VTA express CRF-BP mRNA, most of the CRF-BP expressing cells are dopaminergic. The CRF-BP/TH neurons represent one-fourth of the total population of VTA dopaminergic neurons.

DISCUSSION

CRF-BP expression in the VTA was found in dopaminergic neurons and GABAergic neurons. A role for CRF-BP in signaling actions in the VTA is supported by observations from electrophysiological (Ungless et al., 2003) and behavioral (Wang et al., 2007) studies showing that peptides that bind to the CRF-BP [rat/human CRF (r/hUcnI), mouse UcnI [mUcnI] affect neurotransmission in the VTA. Because CRF-BP within the VTA is expressed in dopaminergic

and GABAergic neurons, we propose that CRF-BP—produced by resident VTA neurons—through its interactions with CRF and CRF-related peptides is likely to affect neurotransmission of VTA dopaminergic and GABAergic neurons.

The CRF-BP is a 37-kDa glycoprotein highly conserved across species (Potter et al., 1991, 1992; Seasholtz et al., 2002). CRF-BP was first isolated from the plasma of pregnant women (Orth and Mount, 1987; Linton et al., 1988; Suda et al., 1988) and was shown to bind CRF with high affinity to lower CRF peripheral circulating levels in the latest stages of pregnancy (Behan et al., 1995; Kemp et al., 1998). Despite the high degree of homology of CRF-BP across species, in humans CRF-BP mRNA is expressed in the liver, the placenta, the pituitary gland, and the brain, whereas, in several other species, it is confined to the brain and pituitary gland (Potter et al., 1991, 1992). In agreement with a previous study (Behan et al., 1995), we found CRF-BP mRNA in the VTA and determined that the majority of CRF-BP-expressing neurons are DAergic.

The role of CRF-BP in brain function is not clear. It has been hypothesized that interactions between CRF and membrane-associated CRF-BP in the brain are important in maintaining synaptic CRF concentrations (Turnbull and Rivier, 1997). An alternative hypothesis proposes that CRF-BP has a role in CRF signaling actions. This alternative hypothesis is based on in vivo observations showing that intraventricular injection of the synthetic r/hCRF$_{6-33}$ fragment that binds to the CRF-BP, but not to CRF receptors, induces c-Fos immunoreactivity in CFR-BP-producing neurons in several brain areas (Chan et al., 2000). A role for CRF-BP in signaling actions in the VTA is also supported by observations from electrophysiological (Ungless et al., 2003) and behavioral (Wang et al., 2007) studies. When added to midbrain slice preparations, the r/hCRF or r/hUcnI induces potentiation of synaptic transmission mediated by NMDA receptors in VTA DAergic neurons (Ungless et al., 2003). Infusions into the VTA of CRF, r/hUcnI, or mUcnII reinstate cocaine seeking and induce local release of dopamine and glutamate in drug-experienced animals (Wang et al., 2007). Based on our results showing that in the VTA the CRF-BP transcripts are encoded mostly by DAergic neurons, we propose that this intrinsic CRF-BP plays a role in the described CRF and CRF-related peptides' induction of potentiation of synaptic transmission mediated by NMDA receptors and that it could have a role in VTA mechanisms involved in stress-induced reinstatement of cocaine seeking in drug-experienced animals.

Cellular expression of CRF-BP mRNA and boundaries between the substantia nigra and the VTA

The mesencephalic DAergic neurons are topographically divided into the SNC, the VTA, and the retrorubral field (Dahlström and Fuxe, 1964; for recent review see Björklund and Dunnett, 2007). It has long been recognized that these topographically divided mesencephalic DAergic neurons are different in their morphology (Dahlström and Fuxe, 1964; Hanaway et al., 1970; Grace and Onn, 1989), biochemistry (Gerfen et al., 1985; Blanchard et al., 1994; Alfahel-Kakunda and Silverman, 1997), vulnerability to neurotoxins (Scheneider et al., 1987; Liang et al., 1996), and neuronal connectivity (Fallon and Moore, 1978; Graybiel and Ragsdale, 1978; Beckstead et al., 1979; Gerfen et al., 1987). However, the cytoarchitectonic boundaries between the midbrain DA cell groups are not well delineated. In the present study, we found that, whereas VTA dopaminergic neurons express high levels of CRF-BP mRNA, neurons of the neighboring SNC do not express these transcripts. This selective expression of CRF-BP in DAergic neurons in the VTA proved to be useful in demarcating the boundaries between the substantia nigra pars compacta and the VTA, especially at the rostral and the medial levels of the VTA. Expression of CRF-BP in the substantia nigra pars lateralis underscores the fact that the pars lateralis is part of the mesocorticolimbic system, innervating the amygdala (Meibach

and Katzman, 1981; Loughlin and Fallon, 1983; Fallon and Loughlin, 1985; Moriizumi et al., 1992).

CRF-BP/DAergic neurons and heterogeneity among dopaminergic neurons in the VTA

Our results showing that a subset of VTA DAergic neurons encode CRF-BP mRNA provide evidence for the notion that discrete subpopulations of DAergic neurons are present in the VTA. The heterogeneity among VTA DAergic neurons is further supported by previous studies showing that fewer than 10% of VTA dopaminergic neurons contain the calcium-binding protein calbindin (Alfahel-Kakunda and Silverman, 1997) and that less than half of the VTA DAergic neurons coexpress brain-derived neurotrophic factor or neurotrophin-3 mRNA (Seroogy et al., 1994). The existence of subpopulations of VTA DAergic neurons is also supported by tract-tracing studies showing a selective organization of the efferent and afferent connectivity among the different VTA DAergic neurons (Swanson, 1982; Carr and Sesack, 2000; Omelchenko and Sesack, 2006). In addition, whole-cell recording of retrogradely labeled VTA DA neurons indicates that opioids differentially inhibit mesolimbic DA neurons depending on their projection targets (Ford et al., 2006; Margolis et al., 2006). Moreover, subsets of DAergic neurons in the VTA have different morphological features (Grace and Onn, 1989) and functional properties (Cameron et al., 1997; Neuhoff et al., 2002; Margolis et al., 2003; Ungless et al., 2003). Thus, our findings demonstrating that CRF-BP is expressed in a subset of VTA DAergic neurons are in line with the view that VTA DAergic neurons are complex in their biochemical composition, morphological features, functional properties, axonal projections, and synaptic connectivity.

CRF-BP/GABAergic neurons and heterogeneity among GABAergic neurons in the VTA

We found that CRF-BP mRNA is expressed mostly in neurons within the PBP and RLi. The concentration of DAergic neurons expressing CRF-BP mRNA in the PBP decreases toward the RLi nucleus. Interestingly, neurons expressing CRF-BP mRNA in the medial dorsal aspect of the PBP are mostly the GABAergic. The differential regional distribution of neurons expressing CRF-BP within the VTA supports the idea of a cellular compartmentalization within the VTA. Similarly to the DAergic/CRF-BP neurons, the GABAergic/CRF-BP neurons constitute a subpopulation of VTA GABAergic neurons, further supporting the notion that there are subpopulations of GABAergic and dopaminergic neurons in the VTA. We recently demonstrated that glutamatergic neurons are present in the VTA (Yamaguchi et al., 2007). These VTA glutamatergic neurons are distributed differently within the VTA and, like the DAergic and GABAergic neurons, are phenotypically diverse (Morales et al., unpublished results). Thus, the VTA has a high degree of complexity in its cellular composition and organization; the efferent and afferent connections of the different phenotypes of VTA DAergic, GABAergic, and glutamatergic neurons remain to be explored.

In summary, we found that CRF-BP mRNA is expressed in the VTA but not the in neighboring SNC or in the SNR. The selective expression of CRF-BP mRNA proved to be useful in demarcating boundaries between the SNC and the VTA. Thus, cellular expression of CRF-BP appears to be a suitable cellular marker to help in delimiting the boundaries between the VTA and the SNC. The detection of CRF-BP mRNA in subsets of either DAergic or GABAergic neurons in the VTA provides support to the notion that the VTA is composed of discrete subpopulations of DAergic and GABAergic neurons. Based on the observation that most VTA neurons expressing CRF-BP mRNA are DAergic, we propose that, in the VTA, interactions between the CRF-BP and CRF or CRF-related peptides are mediated predominantly by DAergic neurons.

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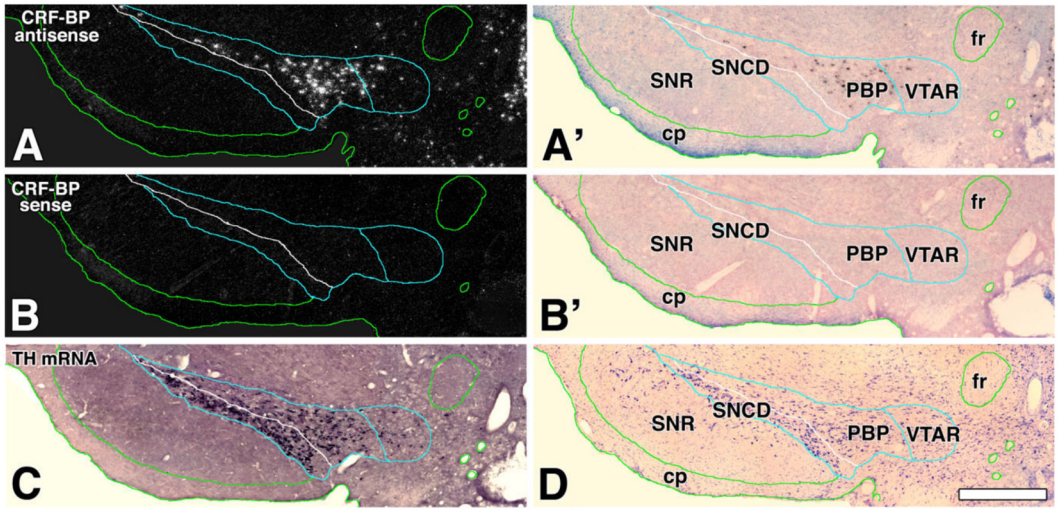

Fig. 1.

Expression of CRF-BP mRNA in the VTA. **A,A′**: Brain section hybridized with the antisense radioactive CRF-BP riboprobe seen under epiluminescence (A) or brightfield (A′) microscopy. Note expression of CRF-BP mRNA (white grain aggregates in A) in the parabrachial pigmental area (PBP) and rostral ventral tegmental area (VTAR). **B,B′**: Brain section hybridized with sense radioactive CRF-BP riboprobe showing lack of signal. **C,D:** Adjacent brain sections were either hybridized with the antisense digoxigenin TH mRNA (C) or Nissl stained (D) to determine subdivisions in the midbrain. cp, Cerebral peduncle; fr, fasciculus retroflexus; SNCD, substantia nigra pars compacta dorsal tier; SNR, substantia nigra pars reticulata. Bregma − 4.92 mm. Scale bar = 890 μm.

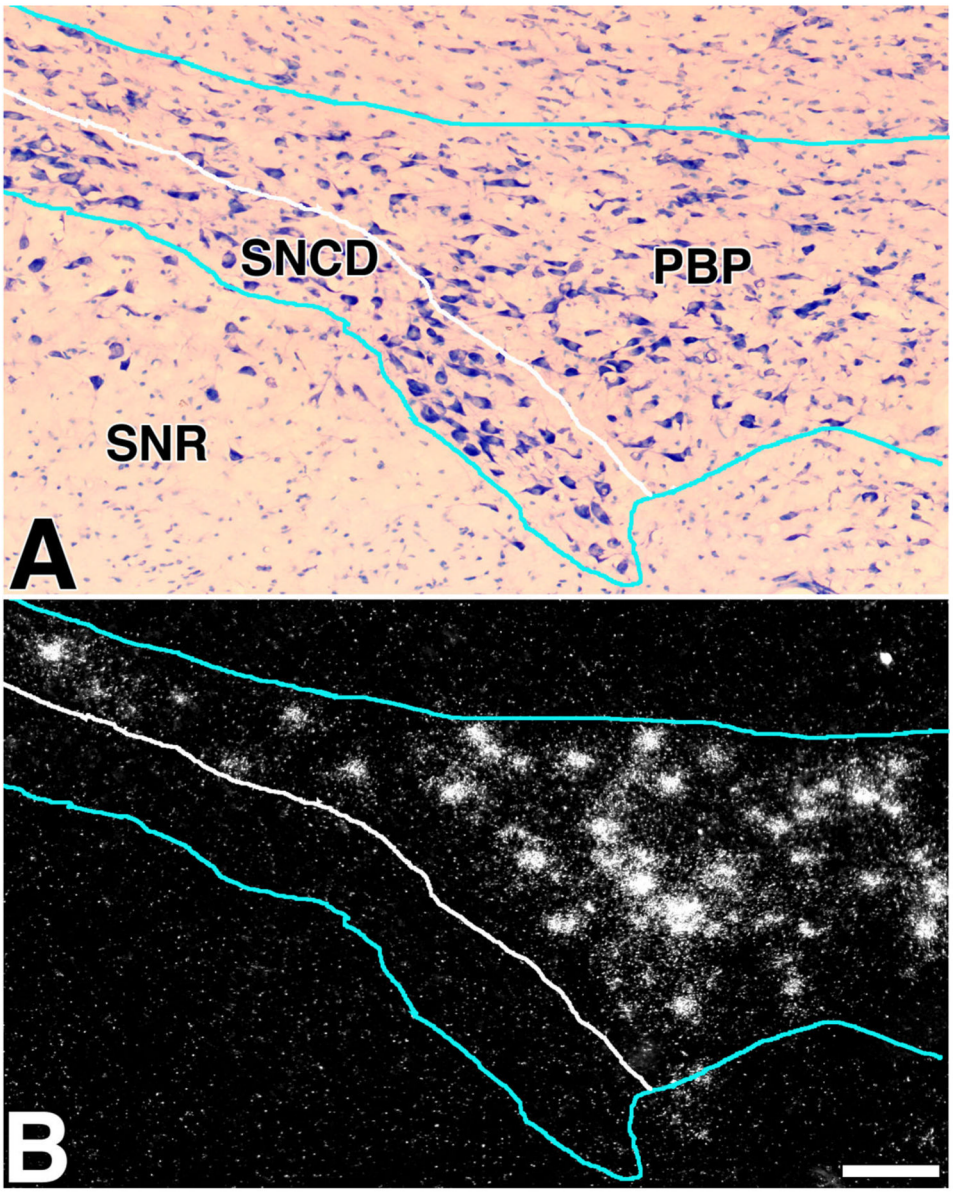

Fig. 2.

Expression of CRF-BP mRNA in the VTA (Bregma −4.92 mm). **A:** Higher magnification of the Nissl-stained section shown at low magnification in Figure 1D. **B:** Higher magnification of the section hybridized with antisense radioactive CRF-BP riboprobe shown at low magnification in Figure 1A. In A, the blue outline demarcates the SNCD and the VTA, and the white line indicates the putative boundary between them. The SNCD encompasses a flat band of compacted, fusiform cells. The VTA has a scattered population of cells lacking a preferential orientation. B shows expression of CRF-BP mRNA within the PBP. Scale bar = 150 μm.

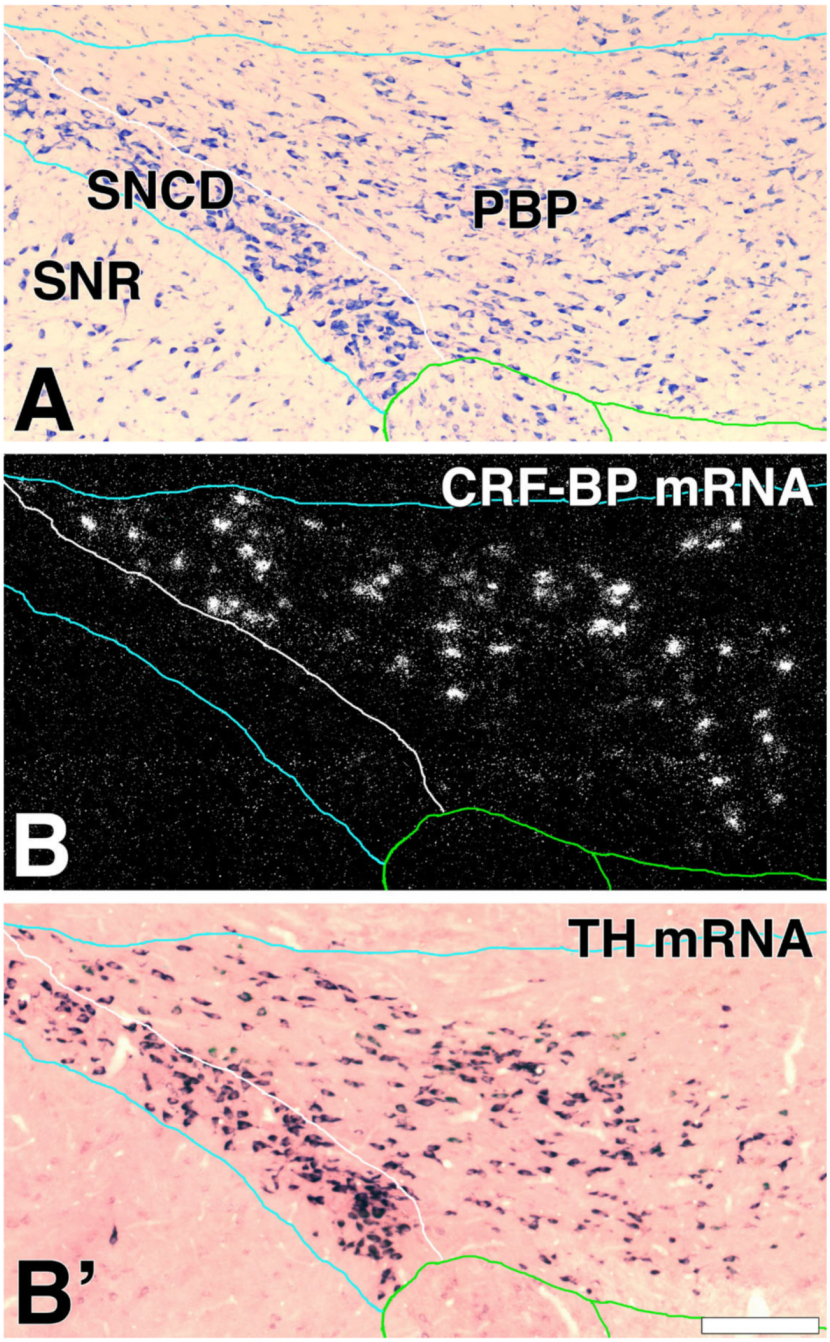

Fig. 3.

Expression of CRF-BP mRNA in the VTA (Bregma −5.16 mm). **A:** Nissl-stained section. **B,B′**: Adjacent section to the one shown in A, displaying expression of CRF-BP mRNA (B) and TH mRNA (B′). In A, the blue outline demarcates the SNCD and the PBP, and the white line indicates the putative boundary between the SNCD and the PBP. B: Cells expressing CRF-BP mRNA are seen in the PBP, but not in the neighboring SNCD. B′: Cells expressing TH mRNA are present in the SNCD and the PBP. Scale bar = 230 μm.

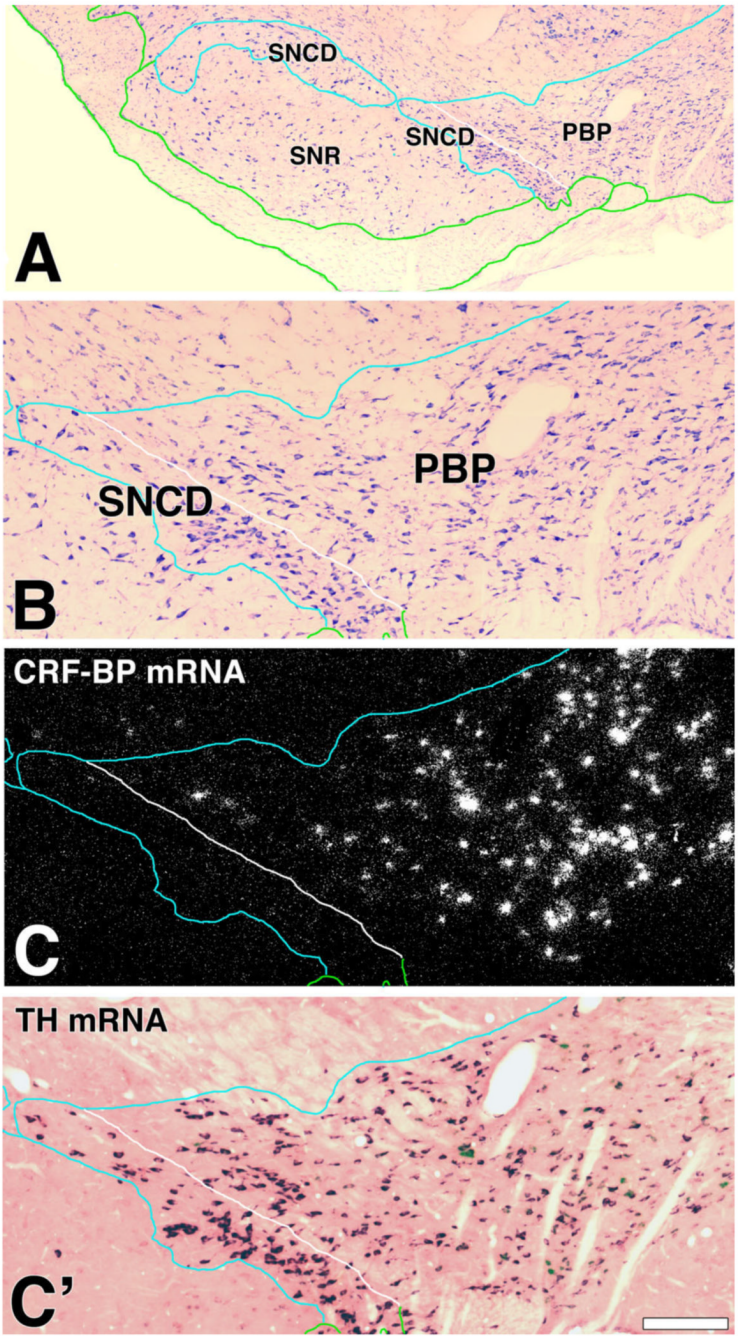

Fig. 4.

Expression of CRF-BP mRNA in the VTA (Bregma −5.52 mm). **A,B:** Nissl-stained section. **C,C′**: Adjacent section to the one shown in B, displaying expression of CRF-BP mRNA (C) and TH mRNA (C′). In A and B, the blue outline demarcates the SNCD and the PBP, and the white line indicates the putative boundary between the SNCD and the PBP. **C:** Cells expressing CRF-BP mRNA are seen in the PBP, but not in the neighboring SNCD. **C′**: Cells expressing TH mRNA are present in the SNCD and the PBP. Scale bar = 254 μm in C′ (applies to B,C,C′); 457 μm for A.

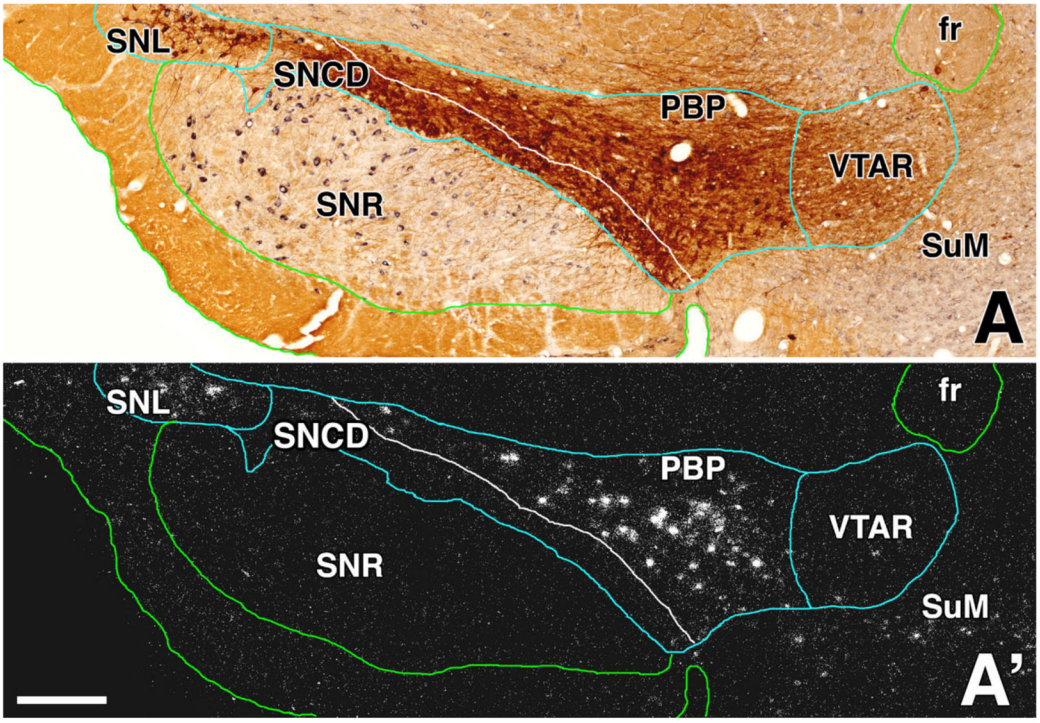

Fig. 5.

Expression of CRF-BP mRNA in the rostral VTA. **A:** Light microscopy showing TH immunoreactivity (dark brown label) and cellular expression of GAD mRNA (purple cells). The blue outline demarcates the VTA (PBP and VTAR), SNCD, and substantia nigra pars lateralis (SNL) as defined by the presence of TH immunoreactivity. The white outline demarcates the separation between the VTA and SNCD. Note numerous neurons expressing GAD mRNA in the SNR. **A′**: Epiluminescence microscopy showing cellular expression of CRF-BP mRNA (white grain aggregates) in the VTA. Low levels of CRF-BP mRNA expression are present in the VTAR and high levels in the PBP. CRF-BP mRNA expression is also present in the SNL and supramammillary nucleus (SuM). Bregma −4.92 mm. Scale bar = 370 μm.

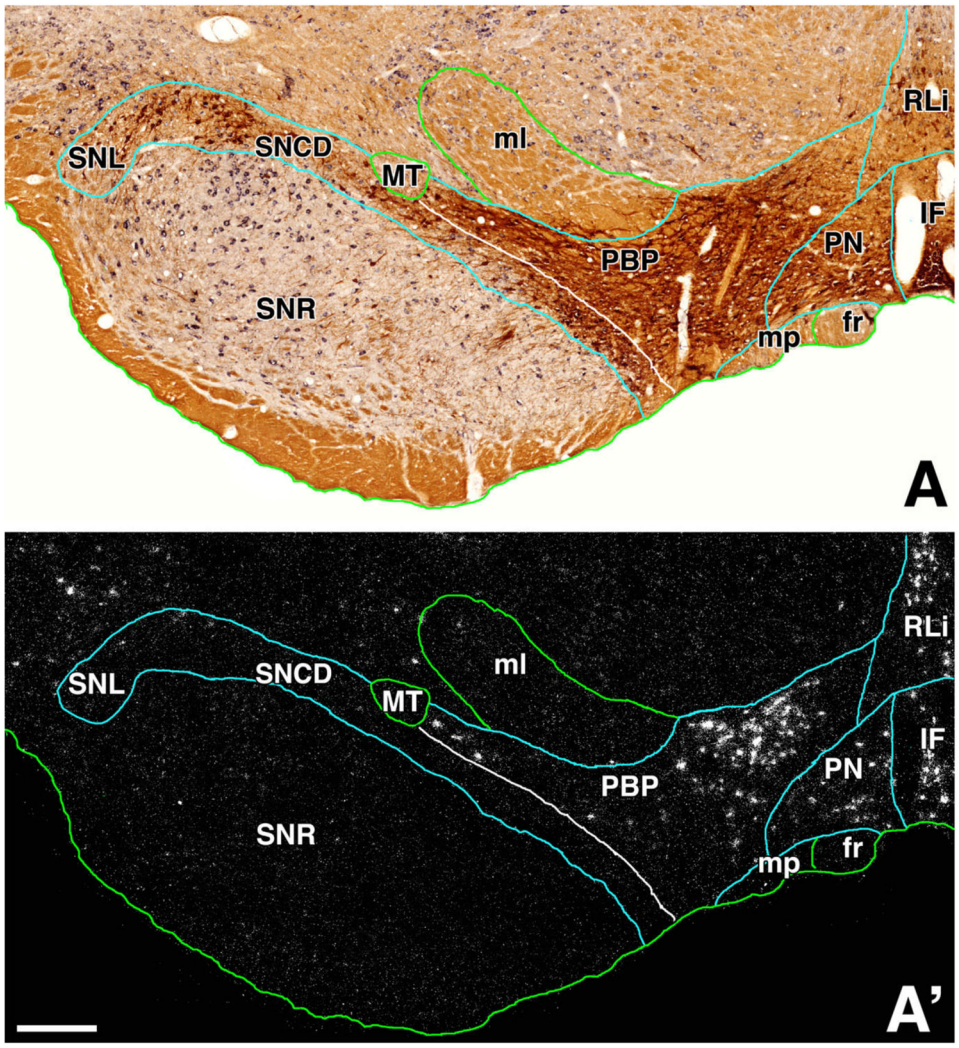

Fig. 6.

Expression of CRF-BP mRNA in the mediocaudal VTA. **A:** Light microscopy showing TH immunoreactivity (dark brown label) and cellular expression of GAD mRNA (purple cells). The blue outline demarcates the VTA, SNCD, and SNL. The white outline demarcates the separation between the VTA and the SNCD. **A′**: Epiluminescence microscopy. Cells expressing CRF-BP mRNA (white grain aggregates) are seen mostly in the medial aspect of the PBP, the rostral linear nucleus (RLi), paranigral nucleus (PN), and interfascicular nucleus (IF). Note the lack of CRF-BP expression in the SNCD and SNR. ml, Medial lemniscus; MT, medial terminal nucleus of the accessory optic tract; mp, mammillary peduncle. Bregma −5.40 mm. Scale bar = 370 μm.

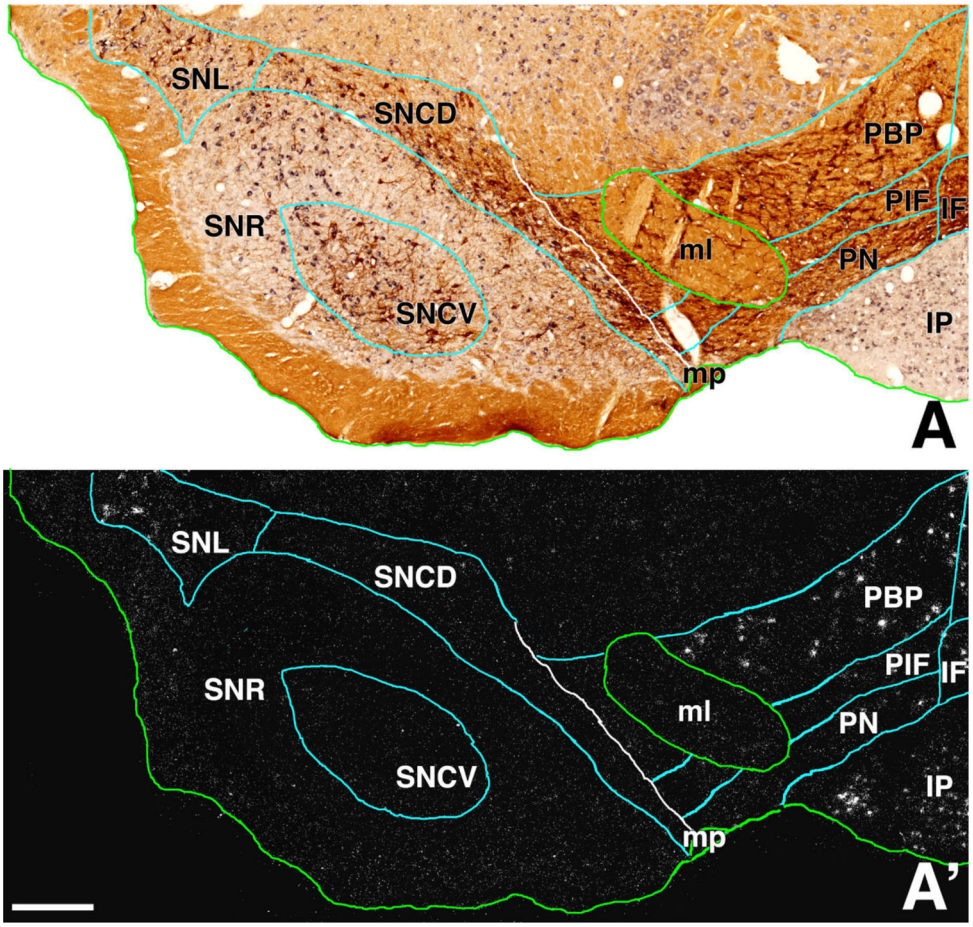

Fig. 7.

Expression of CRF-BP mRNA in the caudal VTA. **A:** Light microscopy showing TH immunoreactivity (dark brown label) and cellular expression of GAD mRNA (purple cells). The blue outline demarcates the VTA, SNCD, and SNL. The white outline demarcates the separation between the VTA and the SNC. **A′**: Epiluminescence microscopy. Within the VTA, cells expressing CRF-BP mRNA (white grain aggregates) are seen mostly in the medial aspect of the PBP, but not in the PN and the parainterfascicular nucleus (PIF). Cells expressing CRF-BP mRNA are present in the SNL, IF, and interpeduncular nucleus (IP). Note the lack of CRF-BP expression in the SNR and the dorsal (SNCD) and ventral (SNCV) aspects of the SNC. Bregma −5.88 mm. Scale bar = 370 μm.

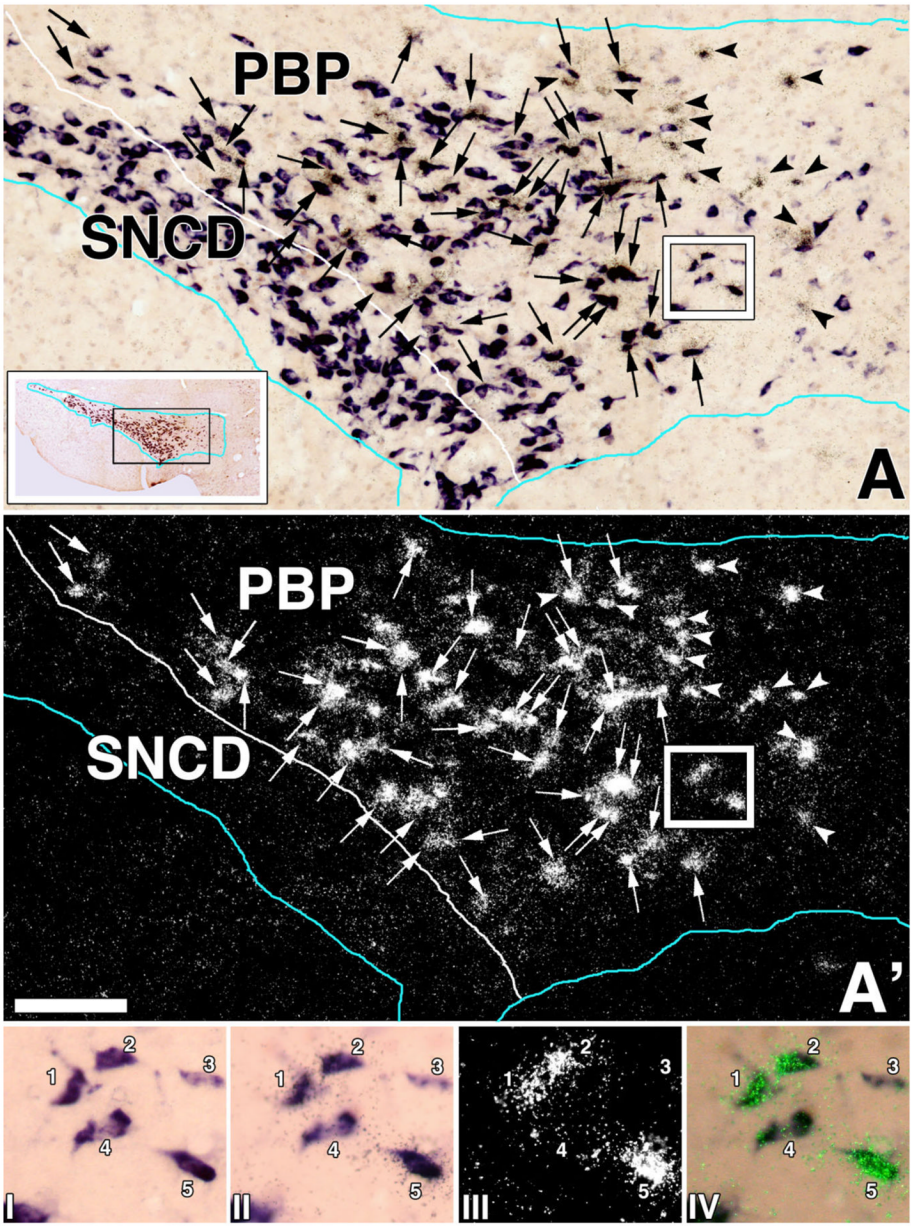

Fig. 8.

High degree of cellular coexpression of CRF-BP mRNA and TH mRNA in the rostral VTA. **A,A′**: Brain section simultaneously hybridized with Dig-TH (A) and radioactive CRF-BP (A ′) antisense riboprobes. The blue outline demarcates the VTA and SNC dopamine groups as defined by the extent of the cellular expression of TH mRNA (purple cells in A). The white outline demarcates the separation between the VTA and the SNC based on the expression of CRF-BP mRNA. A,A′: Pair of micrographs showing two types of neurons in the PBP: neurons coexpressing CRF-BP and TH mRNAs (arrows) and neurons expressing CRF-BP mRNA but lacking TH mRNA (arrow-heads). Note that neurons coexpressing CRF-BP and TH mRNAs (arrows) are distributed throughout the PBP, but neurons expressing CRF-BP and lacking TH mRNA (arrowheads) are concentrated in the medial portion of the PBP. Despite the high concentration of neurons expressing TH mRNA in the SNC, none of them expresses CRF-BP mRNA. **I–IV:** High magnification of single-labeled cells expressing TH mRNA (cells 3 and

4), and double-labeled cells coexpressing TH and CRF-BP transcripts (cells 1, 2, and 5). Bregma – 4.92 mm. Scale bar = 200 μm in A′ (applies to A,A′); 1.8 mm for inset; 55 μm for I–IV.

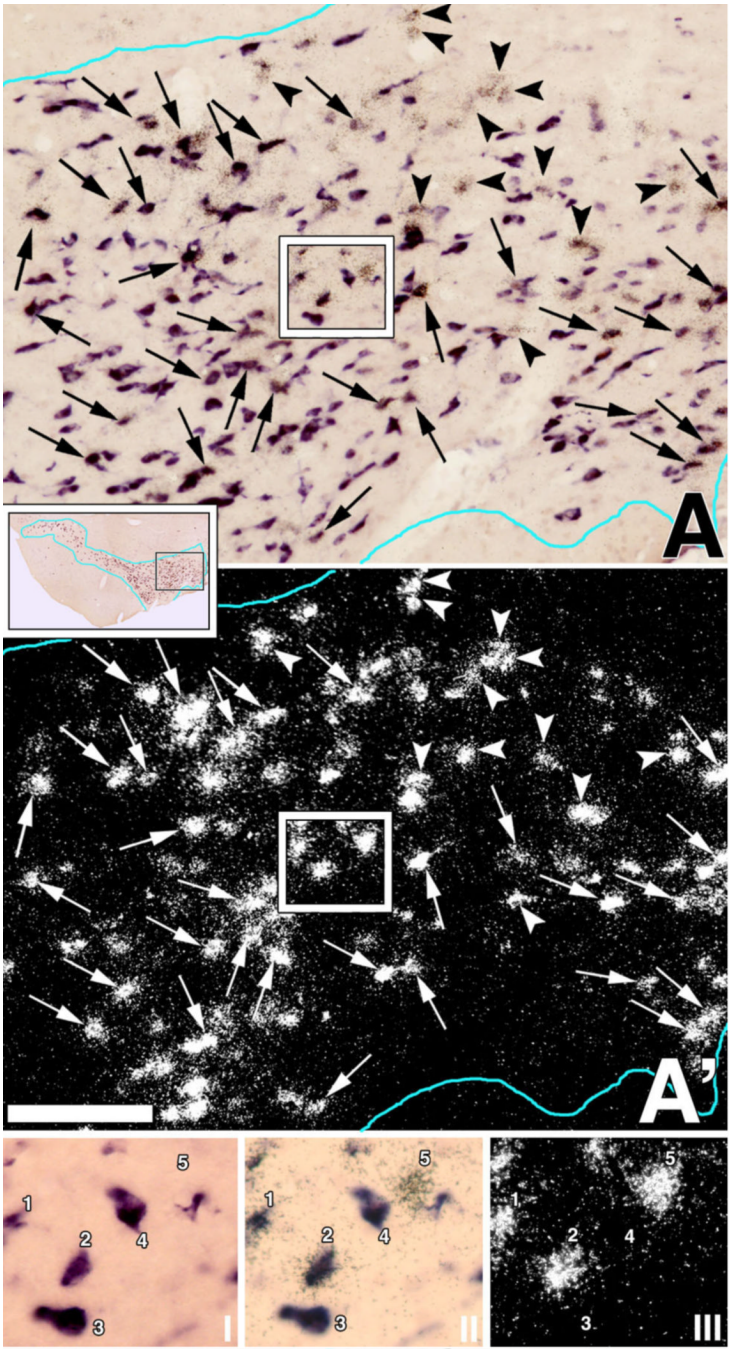

Fig. 9.

High degree of cellular coexpression of CRF-BP mRNA and TH mRNA in the mediocaudal VTA. **A,A′**: Brain section simultaneously hybridized with Dig-TH (A) and radioactive CRF-BP (A′) antisense riboprobes. The blue outline delimits the VTA and SNCD dopamine groups as defined by the extent of the cellular expression of TH mRNA (purple cells in A). A,A′: Pair of micrographs showing two types of neurons in the PBP; neurons coexpressing CRF-BP and TH mRNAs (arrows) and neurons expressing CRF-BP mRNA but lacking TH mRNA (arrowheads). Note that neurons coexpressing CRF-BP and TH mRNAs (arrows) are distributed throughout the PBP, but neurons expressing CRF-BP and lacking TH mRNA (arrowheads) are concentrated in the medial dorsal portion of the PBP. **I–III:** High

magnification of single-labeled cells expressing either TH mRNA (cells 3 and 4) or CRF-BP mRNA (cell 5) and double-labeled cells coexpressing TH and CRF-BP transcripts (cells 1 and 2). Bregma −5.40 mm. Scale bar = 200 μm in A′ (applies to A,A′); 2.3 mm for inset; 100 μm for I–III.

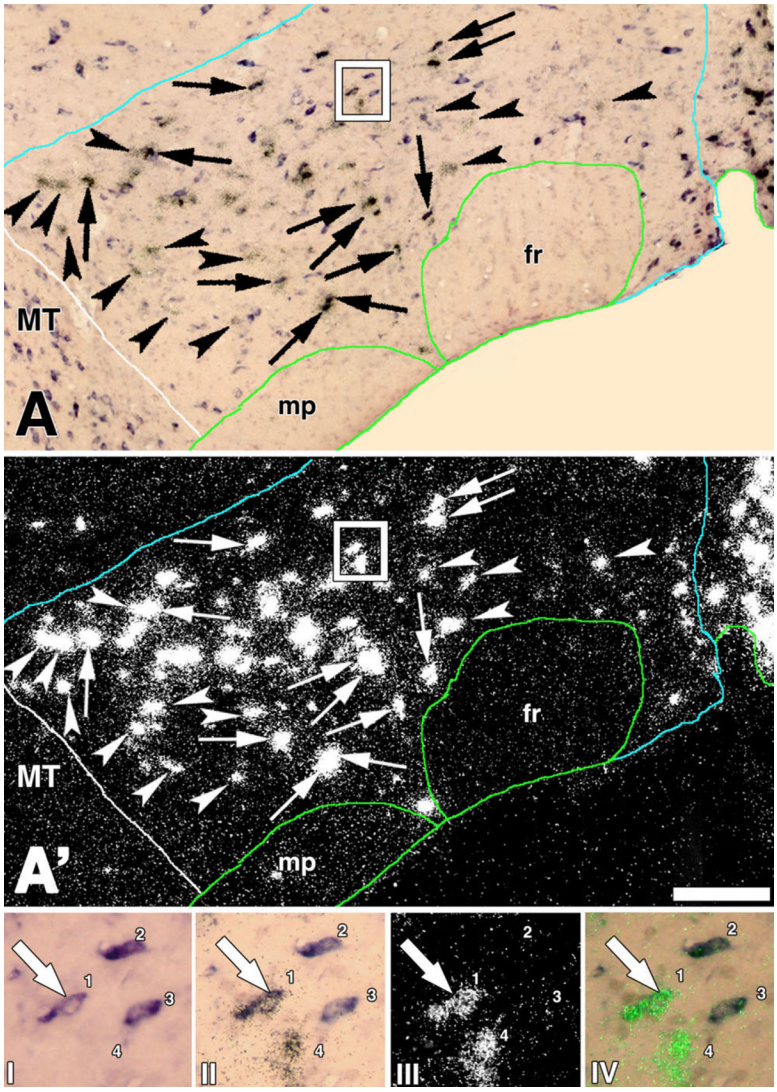

Fig. 10.

Cellular coexpression of CRF-BP mRNA and GAD mRNA in the VTA. **A,A′**: Brain section simultaneously hybridized with Dig-GAD (A) and radioactive CRF-BP (A′) antisense riboprobes. The VTA is demarcated by the blue outline. The white line demarcates the separation between the VTA and the MT. A,A′: Pair of micrographs showing two types of neurons in the PBP: neurons coexpressing CRF-BP and GAD mRNAs (arrows) and neurons expressing CRF-BP mRNA but lacking GAD mRNA (some of them are indicated by arrowheads). **I–III:** High magnification of single-labeled cells expressing either GAD mRNA (cells 2 and 3) or CRF-BP mRNA (cell 4) and a double-labeled cell coexpressing GAD and CRF-BP transcripts (cell 1, indicated by arrow). In **IV**, note silver grains seen as green aggregates. Bregma −5.40 mm. Scale bar = 180 μm in A′ (applies to A,A′); 55 μm for I–IV.

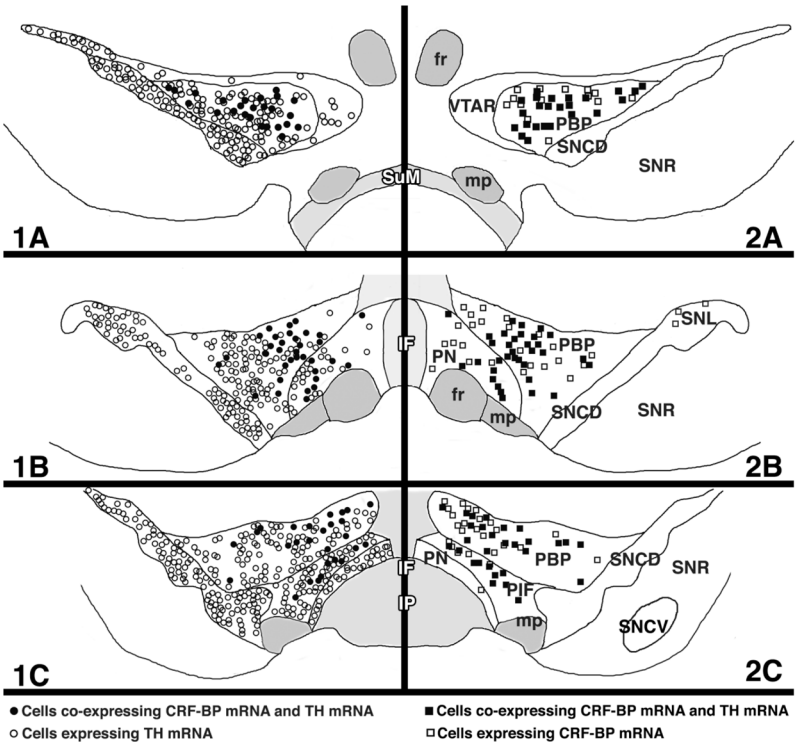

Fig. 11.

Summary diagram of VTA rostrocaudal distribution of cells coexpressing CRF-BP and TH mRNAs (solid circles in **1A–C** and solid squares in **2A–C**), cells expressing only TH mRNA (open circles), and cells expressing CRF-BP without TH (open squares). 1A–C show the distribution of neurons coexpressing CRF-BP and TH mRNAs in relation to the total population of neurons expressing TH mRNA. 2A–C show the distribution of neurons coexpressing CRF-BP and TH mRNAs in relation to the total population of neurons expressing CRF-BP mRNA. Note that most of the cells coexpressing CRF-BP and TH mRNAs represent a subset of the total population of TH-containing neurons (1A–C). The vast majority of neurons expressing CRF-BP mRNA have TH (2A–C). Subregions of the VTA are named according to Paxinos and Watson (2007). Bregma −4.80 mm for 1A,2A; −5.40 mm for 1B,2B; −5.76 mm for 1C, 2C.

TABLE 1

Frequency of Cellular Coexpression of CRF-BP mRNA and TH mRNA in the Ventral Tegmental Area

Mean percentage of neurons coexpressing CRF-BP mRNA and TH mRNA in the total population of neurons expressing CRF-BP mRNA[1]		Mean percentage of neurons coexpressing CRF-BP mRNA and TH mRNA in the total population of neurons expressing TH mRNA[2]	
Subject	**Mean ± SEM**	**Subject**	**Mean ± SEM**
1 (n = 600 cells)	69 ± 2	1 (n = 1,944 cells)	22 ± 1
2 (n = 1,025 cells)	72 ± 4	2 (n = 2,747 cells)	26 ± 2
3 (n = 674 cells)	70 ± 3	3 (n = 1,854 cells)	26 ± 1
4 (n = 770 cells)	68 ± 4	4 (n = 2,186 cells)	25 ± 2
5 (n = 807 cells)	69 ± 3	5 (n = 2,230 cells)	25 ± 3
Total (n = 3,876 cells)	Average 70 ± 1	Total (n = 10,961 cells)	Average 25 ± 1

[1]Total number of cells expressing CRF-BP mRNA, TH mRNA, or both transcripts were counted in five sections from five rats. n, Total number of cells expressing CRF-BP mRNA.

[2]Total number of cells expressing CRF-BP mRNA, TH mRNA, or both transcripts were counted in five sections from five rats. n, Total number of cells expressing TH mRNA.

TABLE 2

Frequency of Cellular Coexpression of CRF-BP mRNA and GAD mRNA in the Ventral Tegmental Area

Mean percentage of neurons coexpressing CRF-BP mRNA and GAD mRNA in the total population of neurons expressing CRF-BP mRNA[1]		Mean percentage of neurons coexpressing CRF-BP mRNA and GAD mRNA in the total population of neurons expressing GAD mRNA[2]	
Subject	**Mean ± SEM**	**Subject**	**Mean ± SEM**
1 (n = 624 cells)	25 ± 1	1 (n = 496 cells)	29 ± 1
2 (n = 816 cells)	24 ± 3	2 (n = 672 cells)	29 ± 4
3 (n = 759 cells)	31 ± 1	3 (n = 1,068 cells)	25 ± 1
4 (n = 727 cells)	25 ± 1	4 (n = 701 cells)	26 ± 2
5 (n = 717 cells)	30 ± 2	5 (n = 766 cells)	29 3 2
Total (n = 3,643 cells)	Average 27 ± 2	Total (n = 3,703 cells)	Average 28 ± 1

[1] Total number of cells expressing CRF-BP mRNA, GAD mRNA, or both transcripts were counted in five sections from five rats. n, Total number of cells expressing CRF-BP mRNA.

[2] Total number of cells expressing CRF-BP mRNA, GAD mRNA, or both transcripts were counted in five sections from five rats. n, Total number of cells expressing GAD mRNA.