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THOUGHT LEADERS INVITED REVIEW

Translating stress systems: corticotropin releasing factor, its receptors, and the dopamine system in nonhuman primate models

Julie L. Fudge[1], Emily A. Kelly[1], and Iman Mahoui[1]

Stress is a fact of life, affecting organisms from the smallest invertebrates to humans. Mediating the stress system is the ancient neuropeptide, corticotropin releasing factor (CRF), which works as a neuromodulator to alter brain systems and homeostatic responses to stress. In humans, many stress-related psychiatric illnesses are linked to dysregulation of monoamine systems, which have cross-talk with CRF-enriched circuits. In this review, we focus on the CRF and the midbrain dopamine (DA) system, particularly as it relates to the nonhuman primate. While tremendous knowledge of CRF-DA mechanisms has been gleaned from rodent models, treatments for stress-related diseases have been elusive, raising the question of whether higher animal models might be required. Subtle shifts in CRF peptide or CRF receptor localization, and the expansion and complexity of DA neuron populations, may hold some of the keys to understanding long-standing stress effects on the DA system in humans. Our laboratory has especially been interested in laying out the neural architecture of the CRF-DA system interface in the nonhuman primate, as a close anatomic model for human. Using rodent models as a starting point, we describe aspects of this complex system that inform our understanding of CRF-DA interactions, and focus on results that have been, and those that still need to be, translated to nonhuman primate models.

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Anatomy of the CRF Stress System and DA Neurons

As a key driver of the hypothalamic pituitary axis (HPA) in modulating behavioral stress responses, corticotropin-releasing factor (CRF) [also known as corticotropin-releasing hormone (CRH)] is a 41-amino acid peptide that was first discovered in the rat hypothalamus over 40 years ago (1). This peptide, like all neuropeptides, is packaged with classic "fast" neurotransmitters and acts as a neuromodulator. Working in concert with its primary transmitters, CRF has unique modes of action that can shape stress circuitry throughout the brain. Although there is a vast literature in rodent models, CRF mRNA and its peptide were subsequently identified throughout the brain in human and nonhuman primates, as well as in rodent models, Review (2). In human, there is also considerable interest in CRF receptors that are found in other organ systems, including the immune (3), reproductive (4), metabolic (5), and cardiovascular systems (6, 7) due to known stress effects on these systems and stress-related disease. Because stress, broadly defined, is implicated in the onset and recurrence of numerous neuropsychiatric illnesses (8–11), CRF brain targets continue to be a subject of inquiry.

Cross-species studies indicate that CRF mRNA and protein cellular distribution in the brain shares similarities but also important difference between rodents and nonhuman primates (2). One purpose of this review is to highlight some of the known brain differences between the species. Areas of dense distribution of CRF neurons in both nonhuman primates and rodents include the "central extended amygdala" and ventral pallidum (12–15). Species differences include a generally more diffuse distribution of CRF-containing cells in primate hypothalamus and extended amygdala, and more discrete clusters within these regions in rodents, see Review for details (Figure 1) (2, 14, 16). CRF-containing neurons are found in many areas outside the extended amygdala, which have also been implicated in anxiety-like behavior and/or sensory responses (particularly in the auditory system). These regions include the periaqueductal gray, peripeduncular nucleus, pedunculopontine tegmental nucleus, parabrachial nucleus, locus coeruleus (LC), lateral dorsal tegmental nucleus (LDTg), median raphe nucleus, and pontine reticular nuclei.

This review will also focus on the way CRF neurons influence a broadened and more elaborate dopamine (DA) system in the primate. Stress effects leading to excess DA release are well-documented across species, but there is little known about the cellular and circuit basis of this, especially in higher primates. The "central extended amygdala," a structural continuity of the central amygdala nucleus through cell columns in the forebrain with the lateral bed nucleus of the stria terminalis, that is enriched in CRF-containing neurons, has been one focus (17, 18). Central "extended amygdala" CRF pathways are well known to project to the DA system (19–21), and considered a means by which stress alters DA release, and affects motivated and higher cognitive behaviors (22–27). Outside of the well-studied extended amygdala, however, there are few studies documenting other CRF-modulated paths to the DA system [although see Soden (28) and Chang (29)], which can be assumed to have specific targets and affect specific systems. The mechanisms of how CRF release in general influences DA firing also remains elusive (30, 31). As noted, knowledge of the array of cell type–specific CRF inputs, and the complexity of postsynaptic cellular targets found in the ventral midbrain are missing pieces of the puzzle. This is particularly true when it comes to understanding higher species.

One clear piece of evidence is that there are high concentrations of CRF-positive axons in the midbrain DA system in both rodents and primates (12, 13, 32–34). In both species, CRF-containing fibers overlap the midline ventral tegmental area (VTA), and extend into the lateral VTA (pigmented parabrachial nucleus, PBP) and into the retrorubral field (RRF, or A8 group). These latter, non-midline, regions are especially well-developed in primates (35, 36) and receive an abundance of CRF terminals (Figure 2).

CRF is Packaged as a Neuromodulator

A key level of complexity for understanding CRF actions is that CRF is considered a "neuromodulator" instead of a "neurotransmitter." Like most peptides, it does not directly elicit an action potential (37). Instead, CRF amplifies or attenuates the excitatory/inhibitory function of transmitters

[1]Del Monte Institute for Neuroscience, Departments of Neuroscience and Psychiatry, University of Rochester Medical Center, Rochester, NY 14642, USA
Corresponding Author: Julie L. Fudge. E-mail: julie_fudge@urmc.rochester.edu

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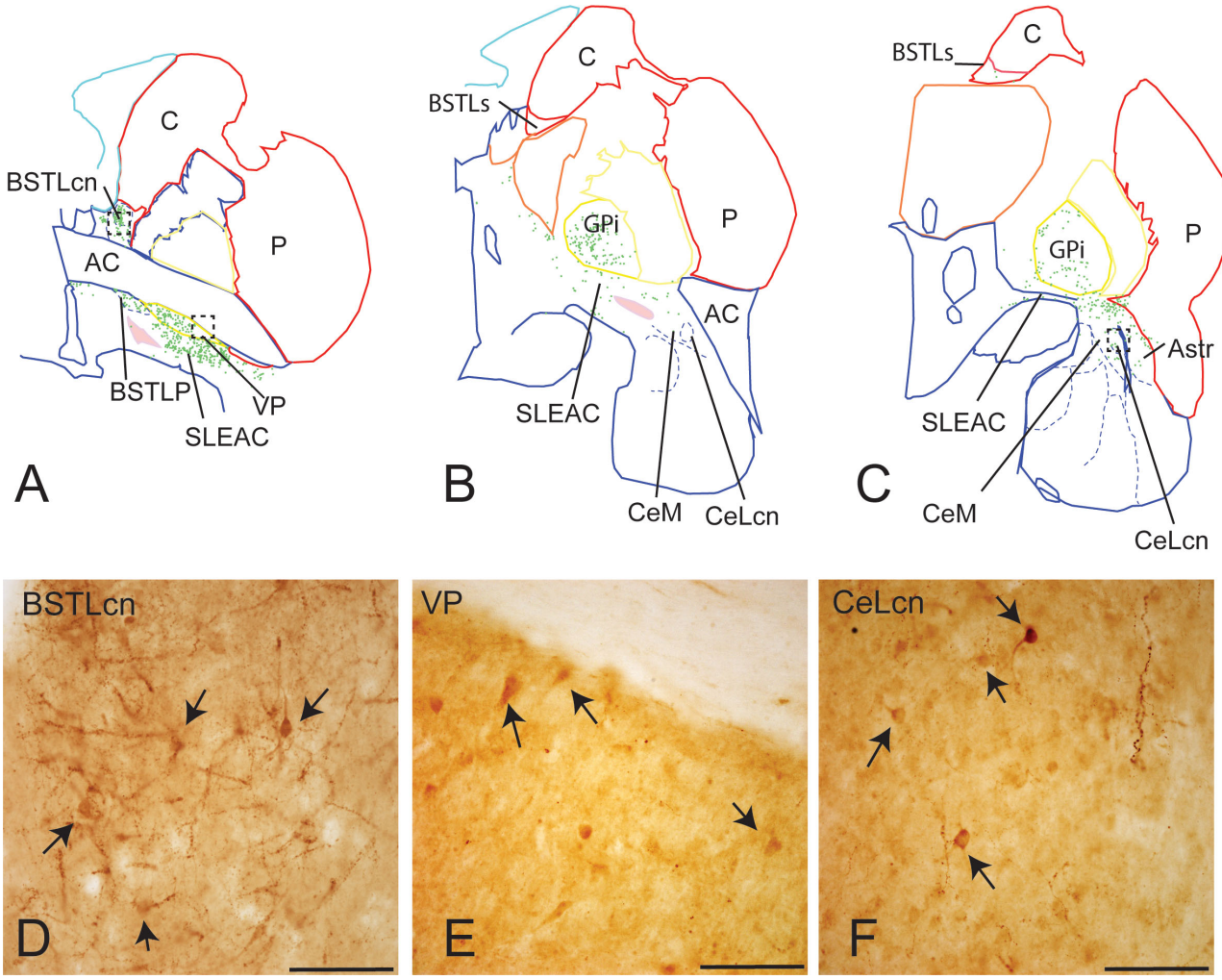

Figure 1. (A–C) Distribution of CRF-immunoreactive neurons in the nonhuman primate CEA and VP subregions. Labeled neurons are distributed broadly and extend from the ventral striatum through the caudal central nucleus. Colored lines indicate various surrounding structures; red: striatum, cyan: ventricles, yellow: globus pallidus, orange: thalamus, pink: cholinergic cell clusters, green: CRF-positive cells. (D) High-powered micrographs of CRF-labeled cells and processes in BSTLcn, corresponding to boxed area in A. (E) High-powered micrographs of CRF-labeled cells and processes in VP, corresponding to boxed area in A. (F) High-powered micrographs of CRF-labeled cells and processes in CeLcn, corresponding to boxed area in C. Scale bar = 100 μm. Abbreviations: AC, anterior commissure; Astr, amygdalostriatal area; BSTLcn, bed nucleus of the stria terminalis; lateral central subdivision; BSTLP, bed nucleus of the stria terminalis, ventral posterior subdivision; C, caudate nucleus, CeLcn, central nucleus, lateral central subdivision; CEM, central nucleus, medial subdivision; P, putamen; SLEAc, sublenticular extended amygdala, central subdivision; VP, ventral pallidum. *Figure used and modified with permission from (14).*

released with it (38, 39). Therefore, CRF's specific actions at terminals must be considered in the context of the classic fast transmitters including glutamate and/or GABA, with which it is packaged in specific circuits (14, 37, 40). In both primates and rodents, CRF + axon terminals exhibit synaptic profiles in the ventral midbrain based on electron microscopic (EM) studies (33, 34). These terminals are filled with both CRF + dense core vesicles and a more diffuse immunostaining that surrounds clear (fast transmitter) vesicles. The physiologic impact of increased CRF release in response to stress is thought to regulate the excitatory/inhibitory output of its circuit in a duration-dependent manner (41). Complicating the picture, however, is the fact that while CRF is released at classical synapses and binds presynaptic and postsynaptic membrane receptors, it can also be released through exocytosis (also known as "volumetric" or "extrasynaptic" release) directly through the axon membrane into the extracellular space (42). Therefore, while documenting the distribution and characterization of CRF + axon terminals is important for understanding co-modulation of classic transmitters in afferent sources, the location of CRF receptors is also needed to help to clarify which cell types are affected by both synaptic and "extrasynaptic" CRF release.

CRFR1 and CRFR2

The biological actions of CRF at postsynaptic targets are mediated through at least two known receptors that have been identified in rat, primate, and human brains: CRF receptor type 1 (CRFR1) and CRF receptor type 2 (CRFR2) (43). Both receptor subtypes are members of the G protein–coupled receptor family and although they share significant sequence homology, they are pharmacologically and anatomically distinct (44–46). Both the CRFR1 and CRFR2 receptors are coupled with stimulatory G proteins, which undergo specific conformational changes depending on ligand binding (47). CRF bind CRFR1 and CRFR2 with differing affinities, with CRF having a high affinity for CRFR1 and only a moderate affinity for CRFR2. Urocortins, identified after the discovery of CRF, share structural similarities with CRF (48–50), and either bind exclusively to CRFR2 (UCN II, UNC III), or have a higher affinity for CRFR2 (UCN I) (49).

Early electrophysiological and pharmacological studies in rodents demonstrated fundamental differences between CRFR1 and CRFR2 receptors, which have been reviewed extensively (43, 51–53). Generalized CRFR1 antagonism (via intracerebroventricular injection of the CRFR1 antagonist, antalarmin) (54) and CRFR1 knockout (55, 56) suggested

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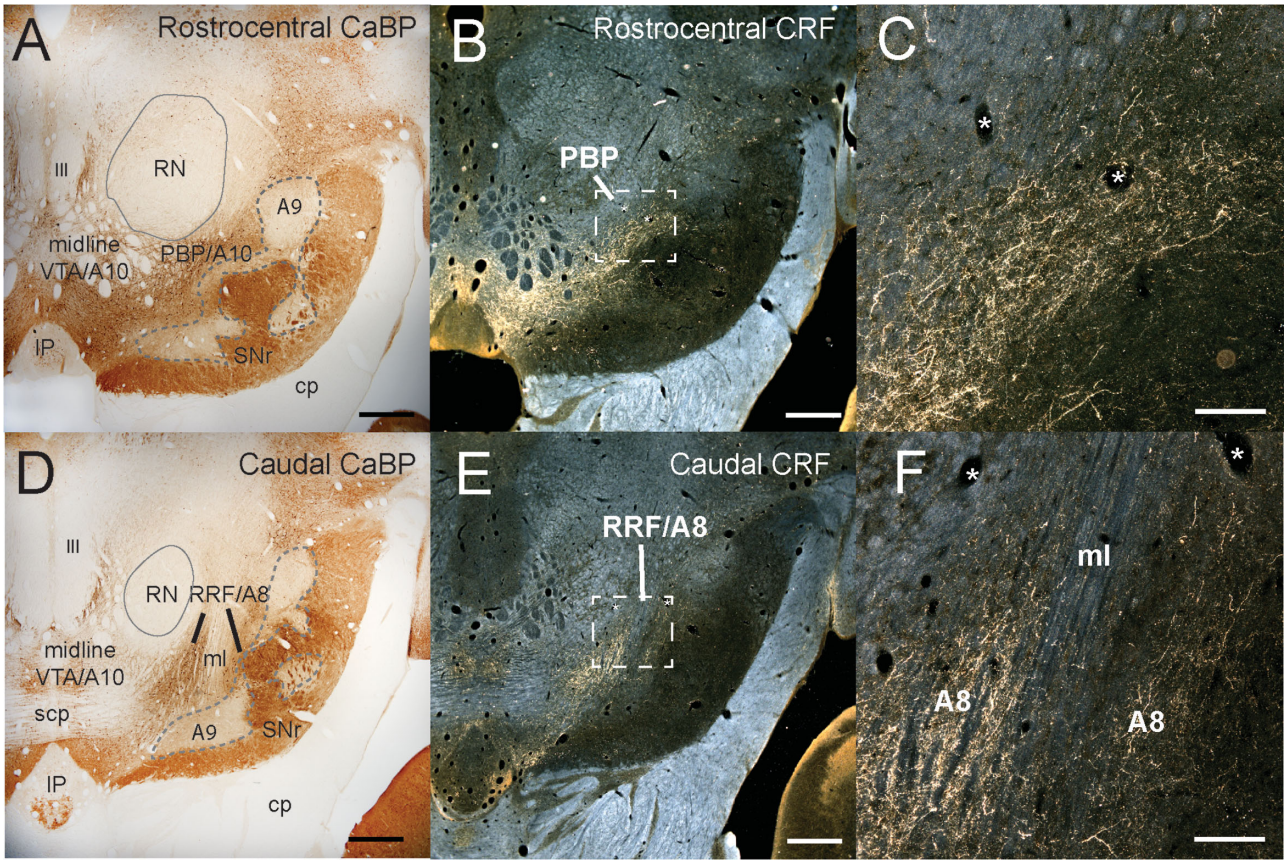

Figure 2. Localization of CRF-immunoreactivity in ventral midbrain. (A) low magnification brightfield micrograph of the ventral midbrain rostrocentral level visualized with CABP-IR, a marker of the A10 and A8 neurons. CABP-positive A10 neurons contrast with CABP-negative SNc/A9 neurons, outlined with dotted lines. (B) CRF-IR in fibers in adjacent sections, visualized with darkfield microscopy. (C) A higher magnification of boxed region in B showing patches of thin beaded CRF-positive fibers in a section of the PBP. (D) Low-magnification brightfield micrograph of CaBP-IR in the caudal ventral midbrain. CaBP-IR neurons are found in the A10 and RRF/A8 and absent in SNc/A9 subregion (dotted line). (E) CRF-IR in neighboring section to D, seen under darkfield magnification. (F) CRF-labeled fibers course through the RRF/A8, which is bisected by the medial lemniscus (ml) seen under higher magnification, boxed region in E. Scale bar = 1 mm (A, B, D, E), 250 μm (C, F). *Abbreviations:* III, third nerve; CRF, corticotropin-releasing factor; CaBP, calbindin; IP, interpeduncular nucleus; cp, cerebral peduncle; scp, superior cerebellar peduncle; ml, medial lemniscus; SNr, substantia nigra reticulata; PBP, parabrachial pigmented nucleus; RN, red nucleus; VTA, ventral tegmental area. *Figure used with permission from (33).*

anxiogenic effects. Early studies also suggested a general opposing role for CRFR1 and CRFR2, but it is now generally acknowledged that this is an overly simplistic view (57). Subsequent studies using more advanced techniques such as conditional and site-specific receptor manipulations indicate a dependence on circuit and cell type–specific characteristics for CRF receptor action. For example, it is now known that low levels of anxiety after enriched housing are associated with low levels of CRFR1 mRNA in the amygdala, and amygdala-specific CRFR1 knockdown reduces anxiety (58). In contrast, CRFR1 knockdown in the globus pallidus (GP) increases anxiety-like behavior, suggesting an anxiolytic role in GP circuitry (59).

CRFR1 and CRFR2 mRNA and Protein Distribution: Some Species Differences

Some of the earliest CRF-binding assays were performed in monkey, and revealed intense CRF-binding occurring widely throughout the cortex, amygdala, hippocampus, and cerebellum, in addition to selected thalamic, hypothalamic and brainstem nuclei (60, 61). However, to date, very little is known of the actual distribution of the CRF receptors in the nonhuman primate. Sanchez *et al.* (1999) mapped the neuroanatomic distribution of CRFR1 and CRFR2 receptor subtypes in the adult rhesus monkey utilizing *in situ* hybridization and also receptor autoradiography (62). Noting some differences between the two techniques, CRFR1 mRNA was abundant throughout the cortex (including prefrontal, cingulate, insular, parietal, and temporal neocortical areas), dentate gyrus of the hippocampus, several amygdaloid subnuclei, cerebellar granule cell layer, pituitary, and LC. High densities of CRFR2 were found in neocortical areas (prefrontal, striate, cingulate, and insular cortices), CA1 of the hippocampal formation, the choroid plexus, the paraventricular hypothalamic nucleus, supraoptic nucleus, amygdala, pituitary, and mammillary bodies. Notably, many regions including the cortex, amygdala and hippocampus have strong distributions of both receptors in the primate. However, some brain regions had selective distributions of CRFR1 or CRFR2 mRNA, supporting the hypothesis that each receptor subtype may have distinct but complementary functional roles within the primate central nervous system (CNS). Unfortunately, the midbrain was not included in these results, leaving unaddressed the specific distribution or existence of these receptors in this critical region.

CRFR1 protein studies in nonhuman primate largely converged with the mRNA findings (63). CRFR1 peptide (AA 21-34) was generally localized in cell bodies and dendrites highlighting a strong postsynaptic role. In this broad survey of the monkey brain, CRFR1 immunoreactivity was also present in regions of the cerebral cortex, basal forebrain, basal ganglia, thalamus, and cerebellum converging with prior mRNA results (62). Importantly, CRFR1 was highly expressed in the ventral midbrain, particularly in the lateral substantia nigra and reticulata. No studies have conducted a similarly broad survey of CRFR2 expression in the nonhuman primate brain.

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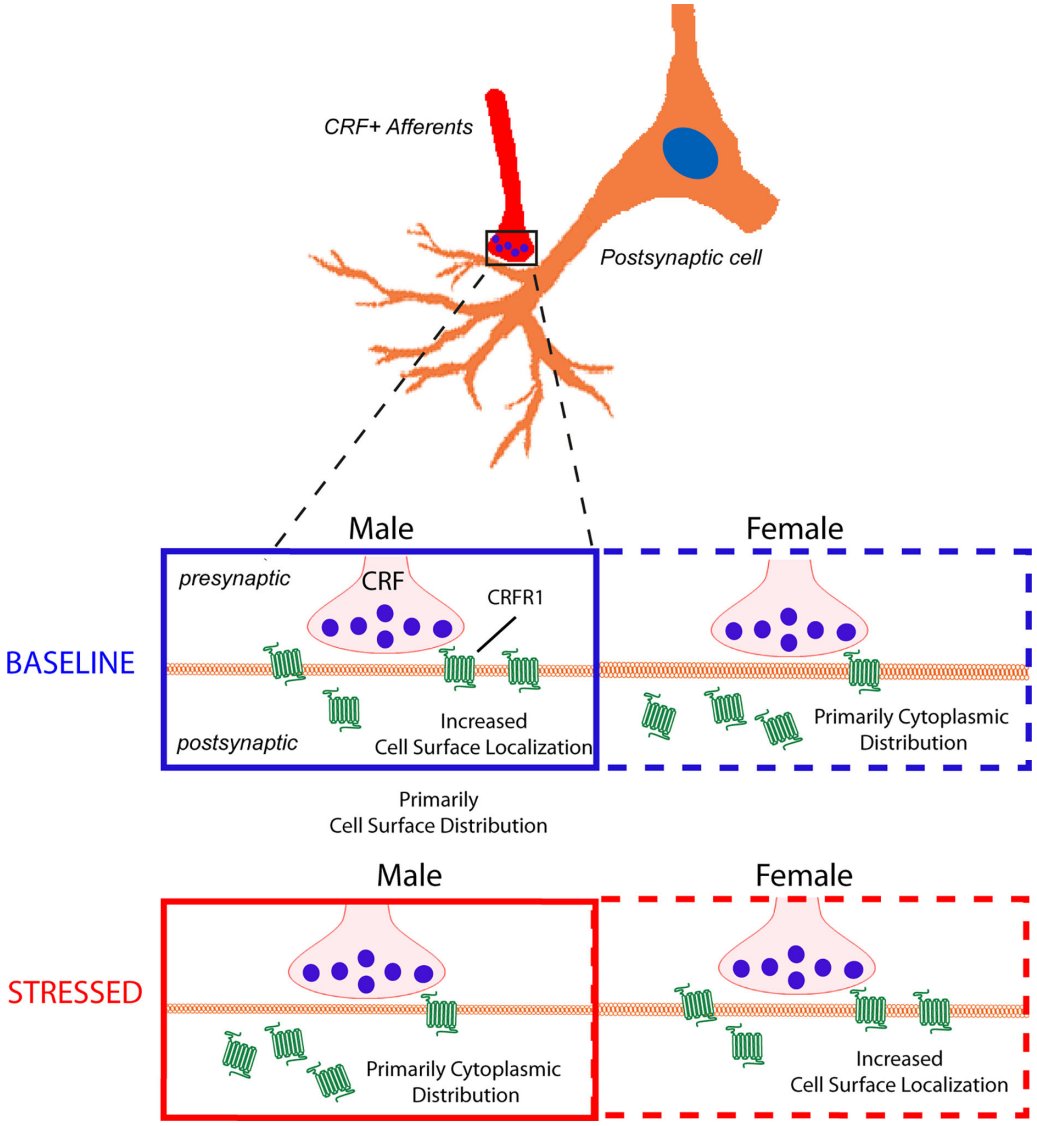

Figure 3. Differential regulation of CRF signaling during baseline and stressed situations in males versus females. At baseline, CRFR is primarily distributed at the cell surface in males versus a primarily cytoplasmic distribution in females. Following stress, CRFR is internalized in males versus relocation to the cell surface in females. *Figure used and modified with permission from* (68).

The general CRF receptor distribution in primates reveals some important differences compared to the rodent. While the human and nonhuman cortex express both CRFR1 and CRFR2 mRNA as noted above, the rodent cortex exclusively expresses CRFR1 mRNA (45, 64). Similarly, in human and monkey, both receptor subtypes are abundant in the pituitary (7, 62), and CRFR1 antagonists alone have relatively minor effects on ACTH or cortisol release in humans (65, 66). In rodent, CRFR1 receptors only are found on adrenotrophs in the anterior pituitary. Other discontinuities in the distributions of the two receptors exist between the species, suggesting possible differences in the balance of CRF receptor subtypes across brain regions, highlighting the importance of nonhuman primate models to improve our understanding of the human CRF system.

Presynaptic and Postsynaptic CRFR1 and CRFR2

Important EM work in rodents reveals that CRFR1R and CRFR2 exist presynaptically and postsynaptically, depending on the brain region assayed (67–71). Thus, CRF effects likely result from a balance of influence on presynaptic versus postsynaptic neural control via each receptor, which vary in a site- and sex-specific manner. CRFR1 is differentially expressed in some brain regions in females and males. Importantly, however, in brain regions without apparent sex differences in CRF receptor expression, differential receptor signaling/sensitivity may be at play (72, 73) Exogenous factors such as stress and pharmacologic manipulations can dynamically regulate CRF signaling with differing behavioral effects in each sex, possibly through differences in CRF receptor sensitivity and engagement with intracellular pathways. Depending on brain region and sex, significant trafficking of postsynaptic CRFR1 and CRFR2 receptors between the membrane and cytosol regulates the availability of the receptor for CRF postsynaptic effects (74, 75) (Figure 3). In brain regions with baseline sex differences in CRFR1 and CRFR2 receptor expression (68, 74), these differences can be exacerbated following stress via intracellular mechanisms that internalize and externalize (recruit) receptors (68). Thus, against a back-drop of sex differences, stress can be homeostatically regulated in each sex by receptor tracking, at least in selected brain regions (74). Knowledge of the basic anatomic locations of CRF receptors, and shifts across development and stress effects in the two sexes, is evolving.

CRFR1 and CRFR2 Gene Variants in Human Disease

An important issue for translational approaches is splice variation in CRF receptors, which have tissue specific distributions and vary by species (76). Alternative splicing is a known mechanism for regulatory control of signaling that can affect cellular function. Different isoforms of the

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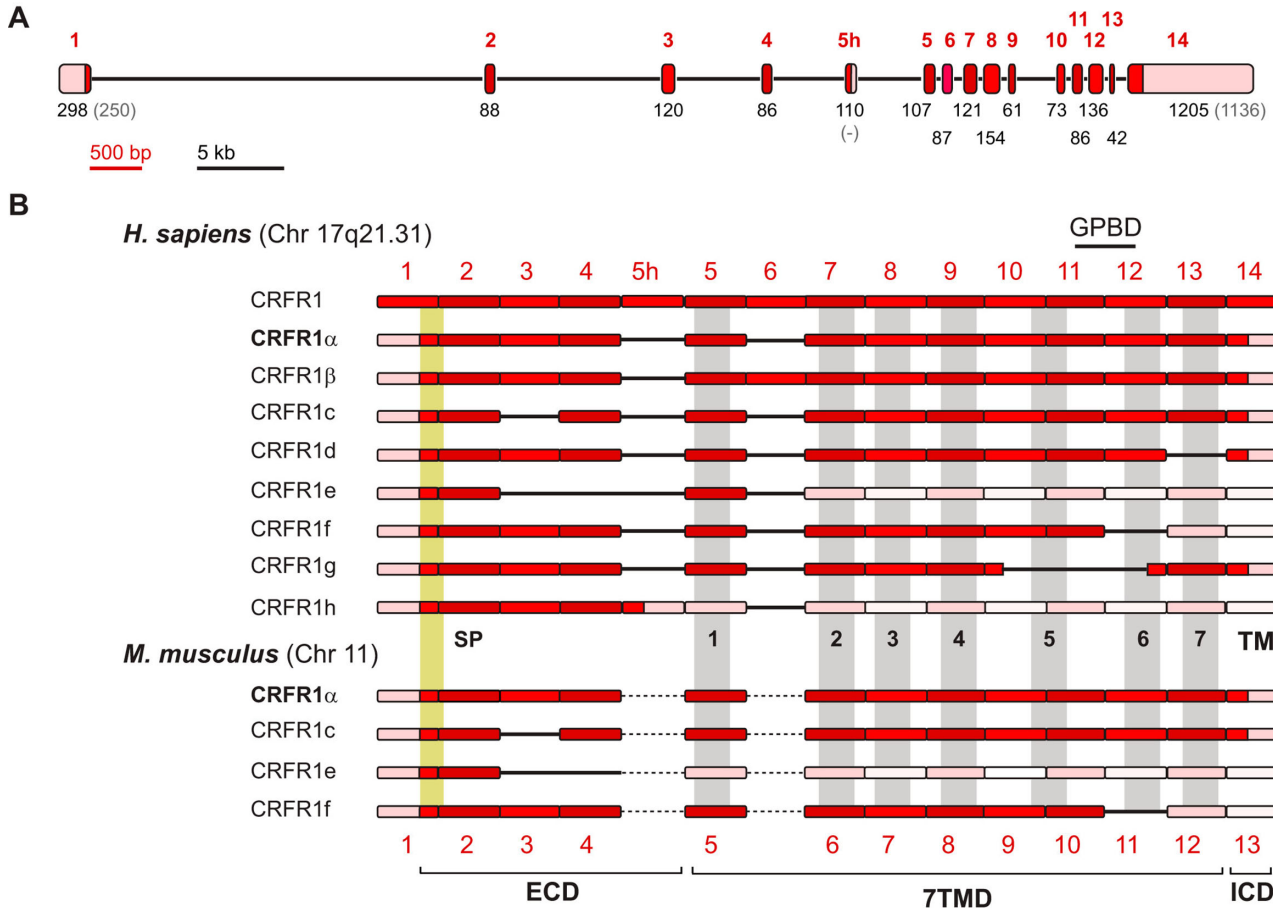

Figure 4. The CRFR1 gene and isoforms following alternative splicing. Note that the CRF1 gene is on different chromosomes in human and mouse. (A) Schematic of the human *CRFR1* gene structure, with base pair (bp) numbers of exons (red) indicated below them. Exons are the coding sequences present in the mature mRNA. Introns (noncoding sequences) are depicted with black lines. Deviations in the size of mouse exons (red) shown in parentheses and gray. (B) Human and murine *CRFR1* splice variants. Red color denotes exons, black color denotes introns, yellow denotes the exon segments encoding the signal peptide (SP). Exon segments coding for transmembrane helices (TMs 1-7) are denoted by gray bars. In both A. and B. pink denotes 5′ or 3′ untranslated regions. ECD = extracellular domain; GPBD = G protein–binding domain; ICD = intracellular domain. *Figure from (76) used with permission.*

receptor can be present in different tissues or cells. In the CRFR1 family, the CRFR1α variant is dominant but its activity is dependent on expression of other isoforms that may compete with or dimerize/oligomerize with the CRFR1α isoform, altering its activity. Human CRFR1 and CRFR2 genes are expressed on chromosomes 17 and 7, respectively, which contrasts with their location on chromosomes 11 and 6 in mouse. Both genes have multiple isoforms, as a result of alternative splicing particularly in human (76) (Figure 4). Gene variants of CRFR1 and CRFR2 are common and, in humans, specific variants are associated with different conditions that are regulated by stress: major depression, type II diabetes, polycystic ovary disease, and irritable bowel syndrome (5, 77–79). The role of CRF signaling in these disorders owes to effects on the HPA, and also to direct CRF effects on external tissues. The tremendous variety in CRFR1 human gene variants, and how they interact with environmental factors such as adverse life experience, has been a focus of psychiatric genetics for several decades (78). Early linkage studies looking for a role for CRF variants in mood and anxiety disorders were not compelling, however, mainly because they were underpowered (80). Nonetheless, the sum of genotyping clinical studies supports a role for CRFR1 in the pathophysiology of depression, anxiety disorders, and alcohol abuse, the latter of which is highly comorbid with anxiety and depression diagnoses. While it is understood that single-gene effects are unlikely in complex disorders, CRFR1 variants likely play a role in conjunction with other genetic risk factors, and also with environmental perturbations (81).

CRF Signaling and Behavior Through the Midbrain DA System

Stressful stimuli lead to dramatic adaptive changes in ongoing behaviors in order to shift the animal into more adaptive responses (82). Stressful manipulations such as footshocks, pinches, or airpuffs, and prolonged anxiogenic events (e.g., restraint), increase DA cell firing (83, 84), and result in DA release in striatum, amygdala, and prefrontal cortex (23–27, 85–87). Similarly, CRF stimulation in the midbrain generally increases DA neuron activity (88) and release at the terminal (89, 90). Thus, stress experience shapes DA signaling and behavior, including motivated behaviors, response to novelty and decision making (91–94). However, the mechanisms behind these effects, or differential effects on specific brain circuits, are far from clear.

In slice preparations, CRF excites VTA DA neurons in a bimodal, dose-dependent manner (88, 95–97). In part, CRF increases excitability by effecting release of calcium from intracellular stores in DA neurons (98). However, CRF promotes excitation of VTA DA neurons via both presynaptic and postsynaptic mechanisms involving both CRFR1 and CRFR2 (88, 96–101). Complicating this picture, CRF release can itself also alter the expression of CRFR1 and CRFR2 expression (102). Stress was recently shown to regulate CRFR1 expression in DA neurons, with consequences for both DA firing and behavior (103).

In vivo, most work has investigated how CRF modulates motivational and decision-making processes, including addictive behaviors. For example, both restraint stress and infusions of CRF into the VTA diminish

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preference for larger rewards with a greater effort cost. Notably, CRF antagonism blocks these effects in stressed animals (104). Similar studies show that stress and CRF administration in the VTA reduce motivation to work for food reward behaviors, but regulate DA firing in a pathway- and stimulus-specific manner (105). This circuit-based approach makes clear that CRF effects on behavior and DA release are critically dependent on gating of specific active afferent/efferent pathways.

Stress likely also influences non-DA neuronal cells in the midbrain DA system, including GABAergic interneurons (106–110). These neurons are proposed to exert tight inhibitory control on DA neurons (108, 111, 112), but the effects of CRF on these neurons have not been studied in detail. CRF increases firing of inhibitory GABA neurons in the VTA, although it is unclear if this a causative or compensatory effect related to CRF-induced DA activation (95, 97). CRFR1 mRNA and CRF-binding protein (associated with CRF receptors) is expressed in some VTA GABAergic neurons per studies in rodent (103, 113). Therefore, to better understand the ways in which CRF differentially impacts the molecularly defined DA neuron subtypes as well as non-DA neurons in the midbrain DA system, anatomic localization of these subpopulations and their relative expression of CRF receptors is a missing piece of the puzzle.

The Midbrain DA Target is Ruled by Anatomic Complexity

The midbrain DA neurons are no longer considered a homogeneous system. DA neurons are physiologically heterogeneous with respect to both intrinsic firing and coding properties. For example, DA neuron pacemaking and spiking depend on a variety of ion channels, which vary across the DA subregions, giving rise to heterogeneity in spontaneous and induced-spiking activity (114, 115). Many recent papers show that physiologic activity, as well as molecular/transmitter content and circuit connections, can be predicted from mediolateral and rostrocaudal anatomy of ventral midbrain in both rodents and primates (116–122). This basic anatomy (based on developmental trajectories) provides an important organizational principle for predicting cell types and connections between the species. Although the primate ventral midbrain system is larger and more elaborate, the mediolateral and rostrocaudal axes provide important anchors for comparison.

The nonhuman primate ventral midbrain is an important, albeit understudied, bridge to understand human disorders because of its similarity to the human (123–125). Because DA neurons serve the individual over the lifespan, the nonhuman primate is also a closer model for understanding specific populations that are particularly plastic or that are vulnerable over long time periods, that is, in aging or chronic environmental insult. Fortunately, identification of the basic DA subregions (A10, A9, and A8) across species can be done with specific histochemical markers, permitting regional comparisons across species (126–128) (Figure 5).

The concept of DA neurons generating "reward prediction errors" in learning was first discovered in nonhuman primate, and became a dominant model of DA function (129, 130). As this concept has been debated and expanded, location-dependent roles of different DA subregions have been raised, with DA neurons in different midbrain regions involved in other functions. In monkey, laterally displaced DA neurons appear to have a separate role, signaling the biologic relevance (salience) of both reward and nonreward predicting stimuli (salience coding) (131, 132). These DA neurons may play a different role in complex behaviors such as orienting, or preparing strategies to avoid potentially aversive cues (84, 131, 133–135). This general medial-lateral trend has been noted as well in rodent studies, with different functional properties distributed along this axis (27, 136).

DA Subregions are a Translational Anchor for Understanding Heterogeneity

Although the vast majority of work on DA heterogeneity is in mouse and rat, the conserved organization of the DA subregions serve as important landmarks to approach higher species (137) (Figure 5). The nonhuman primate system is expanded in both mediolateral and rostrocaudal directions (Figure 5A and B, rostral; C and D caudal). Across rodents and primates, the A10 subregion, referred to as the VTA, contains a number of subnuclei. In addition to multiple midline subnuclei (mVTA), the A10 includes the lateral-most VTA subnucleus, the PBP. The PBP is disproportionately enlarged in higher primates (35). While previously referred to as the "dorsal A9," it is now clear that the primate lateral VTA (PBP) stretches dorsolaterally over the A9, comprising a large expanse of the midbrain. The entire A10 is closely related to the A8 subregion, and is continuous with it in macaque and human (see below) (Figure 5C and D). Both regions, in both rodent and primates (including human), have DA neurons that express calbindin-D28K, a calcium-binding protein (CaBP, Figure 5A, D, and E), which is absent in the A9. The A8 (also known as the RRF), is enlarged volumetrically in nonhuman primates (36, 138), and is also an important component of the mesolimbic path. The A9 is conspicuously lacking in CaBP, but is enriched in other protein markers as noted below.

Major Transmitters in the DA Subregions

All DA subregions contain dopaminergic, GABAergic, and glutamatergic neurons, with variable relative densities of transmitter-specific neurons in each region. This has been shown across mice, rats, and primates (139–143). In addition, "multiplexed" neurons that coexpress at least two of the aforementioned neurotransmitters are well described in rodents (142, 144–147), and are beginning to be identified in higher species. (Although not reviewed here, some DA neurons also coexpress coregulatory neuropeptides: neurotensin (NT), cholecystokinin (CCK) (148–150), and vasoactive intestinal protein (151). The DA subregions also contain glial cells, including astrocytes, oligodendrocytes and microglia, which interact with neurotransmitter release (152–154). Astrocytes in particular are D2 receptor responsive, and are critical for controlling extracellular glutamate levels, which in turn alters the excitability of DA neurons (153, 155, 156).

GABA

GABAergic neurons comprise the largest nondopaminergic subpopulation among the DA subregions (157–159). While the surrounding pars reticulata is comprised entirely of GABAergic cells, inhibitory neurons are also interspersed among DA cells in all subregions (143, 160). All DA neurons appear to be regulated by GABA receptors (158). GABAergic neuron activity in the ventral midbrain can be influenced by astrocytic activity (161), in addition to afferent control by neuronal systems. GABAergic neurons establish local inhibitory connections on DAergic neurons, and also project outside the VTA to the ventral striatum, basal forebrain, the prefrontal cortex, the lateral habenula, lateral hypothalamus, and amygdala (162–167). GABAergic neurons are themselves diverse, based on morphology and immunostaining for neuropeptides, calcium-binding proteins, and nitric oxide synthase (126, 168–170).

While GABAergic neurons make up about one-third of neurons in the midbrain, the ratio of GABA-to-DA cells varies among DA subdivisions (141, 171). In nonhuman primate animals of the same age and sex (pubertal males), we found that although GABAergic neurons comprise 30% neurons, there are marked differences in the DA-to-GABAergic ratios across subpopulations, with the greatest DA-GABA ratio (5:1) in the parabrachial pigmented nucleus of the VTA, and the lowest DA-GABA ratio (1:1) in the A8 neurons. These different ratios are largely accounted for by the changes in DA neurons in each population, with GABAergic neuron numbers remaining similar across regions (140).

Colocalization of traditional markers for DA and GABA occurs in the rodent midbrain but apparently comprise a relatively small subpopulation of DA neurons (10%–15%) (172, 173). Nonetheless, GABA/DA coexpression in selective projections is recognized as an important local regulator of striatal modulation as there is an alternative GABA synthesis pathway involving aldehyde dehydroxygenase-1 (173) found in many DA neurons that may also play a role (see below, *New ways of understanding DA heterogeneity*). As will be described below, a simple "dichotomy" of DA-GABA ratios can be misleading, since many putative DA neurons that co-contain glutamate also contain glutamate aldehyde decaroxylase, GAD, an enzyme in the biosynthesis of GABA, as well as aldehyde dehydroxygenase-1. Although GAD is not detected in some studies in

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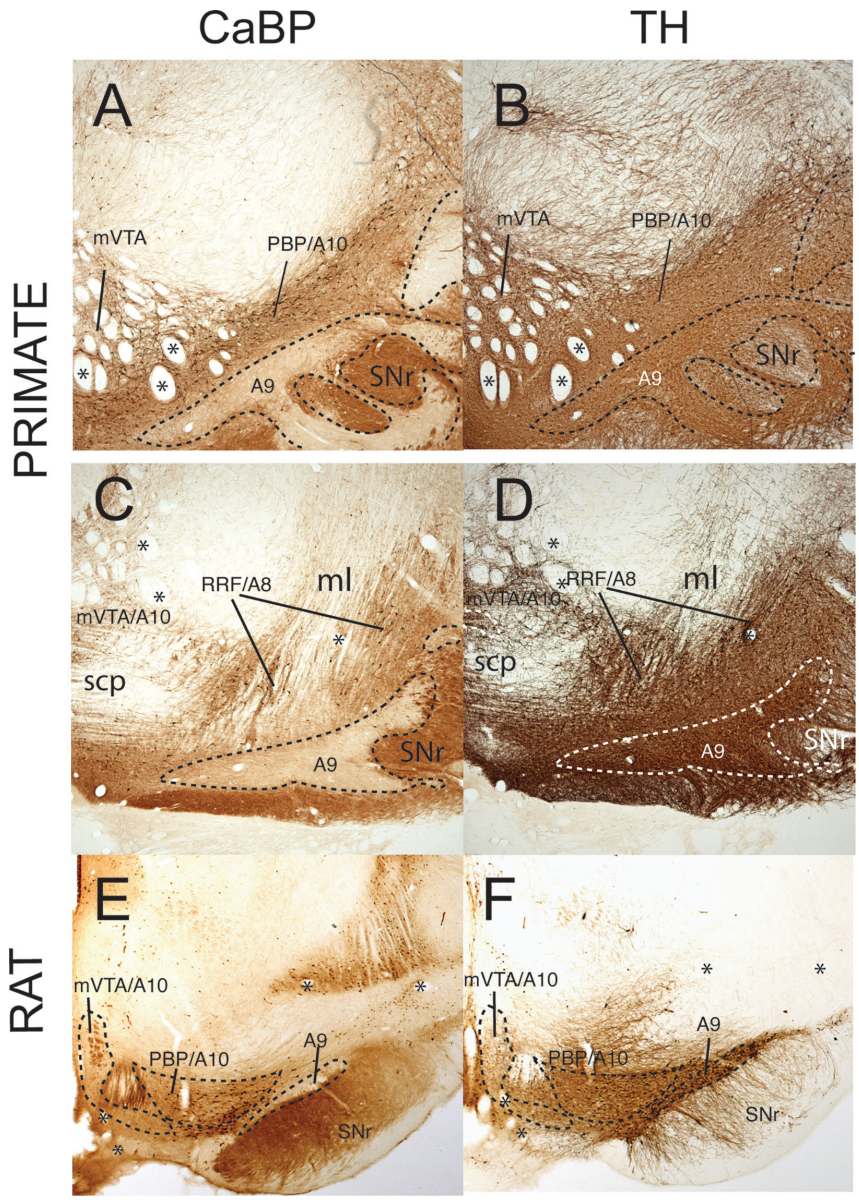

Figure 5. Localization of the A10, A9, and A8 DA subregions following immunohistochemical labeling for CaBP and TH in nonhuman primate and murine ventral midbrain. (A) Rostrocentral level of nonhuman primate ventral midbrain with CaBP-positive cells in mVTA (A10), PBP (A10) in contrast to CABP-negative labeling in A9 (dotted outline). (B) Neighboring TH-IR section to A. (C) Caudal level of nonhuman primate ventral midbrain with CaBP-positive cells in RRF/A8 in contrast to CABP-negative labeling in A9 (dotted outline). (D) Neighboring TH-IR section to C. (E) Murine ventral midbrain demonstrating CaBP-IR in VTA. A9 (substantia nigra pars compacta) is CaBP-negative. (F) Neighboring section to E showing TH-IR. Abbreviations: ml, medial lemniscus; mVTA, medial ventral tegmental area; PBP, parabrachial pigmented nucleus; RRF, retrorubral field; SNr, substantia nigra reticulata; vta, ventral tegmental area; zi, zona inserta.

mouse (174), we detected GAD1 mRNA signal both in interneurons and DA neurons in the primate.

Glutamate

The discovery that some DA neurons coexpress glutamate over twenty years ago created a paradigm shift in conceptualizing DA neuron function (142, 175). DA neurons can selectively express the glutamate vesicular transporter 2 (VGluT2), which is differentially expressed in different DA subregions (144, 176–178). Consistent with this, glutamate release from DA neurons is only detected in some forebrain targets in rodents (179).

Importantly, the corelease of DA and glutamate appears to be developmentally regulated, due to the dynamic expression of tyrosine hydroxylase (TH) (128). In early postnatal development, most postmitotic DA neuron progenitors express VGluT2 (180). These DA progenitors migrate in waves so that the earliest neurons arrive in the lateral midbrain, and the most recently differentiated neurons populate the midline structures (181, 182). During development, TH mRNA gradually increases throughout the midbrain neurons, and VGluT2 expression declines (147, 183, 184). Depending on the animal's age, the complement of VGluT2-only cells, VGluT2-TH, and TH-only neurons can theoretically shift (183) particularly in long-lived species like primates. "Pure" glutamatergic VTA neurons are largely are localized to the midline in adult mice, marmosets, and humans (185, 186), but found throughout the ventral midbrain (178).

Newer Ways of Understanding DA Heterogeneity

The understanding of the heterogeneity of DA subpopulations across the midbrain has been advanced by newer transcriptomic methods in rodents, including single-cell RNA sequencing (scRNA-seq) (187). When gene expression heterogeneity in single cells is mapped and validated spatially,

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the distribution of combinations of mRNA transcripts is evident across the medial-to-lateral and dorsal/ventral extent of the system. These approaches not only confirm DA subregional "markers," also shed light on molecules not previously identified within these regions (128, 188–190). Replicating the results of traditional immunostaining studies, scRNA-seq approaches, combined with in situ hybridization, show that calcium-binding protein D28-K mRNA (CABP) maps onto the "dorsal tier" of A10 and the A8 neurons. Functionally, high CaBP levels are associated with fast buffering of calcium influx, which controls the rate of synaptic vesicle release following an action potential (191, 192). In primates CaBP + DA neurons also lack high levels of autoregulatory molecules such as the dopamine transporter (DAT) and the D2 receptor (193), suggesting a relative dependence on calcium buffering for controlling release at synapses. "High" and "low" expressing levels of these autoregulatory molecules in different DA subregions are now confirmed in scRNA-seq in mouse (194). In contrast, CaBP-negative DA neurons of the ventral substantia nigra (A9), are likely to express aldehyde dehydroxylase-1, which regulates DA production and also serves as an alternate path for GABAergic synthesis (173).

In macaques, CaBP + DA neurons neurons (A10 and A8) have specific projections to the ventral striatum, to the entire prefrontal cortex, and the amygdala (122, 138, 195–197). In mouse studies, it is found that a subset of CaBP + DA neurons further colocalize VGluT2 mRNA (121, 128, 179, 194). For example, CaBP/VGluT2-positive neurons of the midline VTA project to the ventral striatum, while the CaBP/VGluT2 of the lateral VTA(PBP), project specifically to the central nucleus of the amygdala (179) and to the tail of the caudoventral striatum (121). As with VGluT2 expression, developmental shifts in CaBP have not been fully accounted for, so that the picture as viewed from studies in adult animals may not be static through life, or applicable to younger animals (189, 190). This may be also relevant in higher species where development occurs over longer time periods. For example, in the human, CaBP gene expression in DA neurons is not seen prior to P7 (128).

Attempting to control for age and sex effects, we preliminarily examined two young male macaques for evidence of "multiplexed" transmitter content using mRNA expression for TH, VGluT2, and GAD1 (Figure 6A–H). These animals were considered "adolescent" at age 3 (early puberty) and 6 (late puberty) (198). Using RNAScope methodology and a semiautomated cell counting strategy, we found few differences between these animals in either the numbers of single-labeled or "multiplexed" neurons. Our method used RNAScope methods in evenly spaced coronal sections throughout the midbrain (5–6 sections, spaced 1 mm apart). After setting criteria for automated labeled cell detection in individual channels in the triple-labeled sections in preliminary studies, the regions of interest were drawn from adjacent CaBP-IR sections. After automated detection of each cells in individual channels, colocalization was performed (Neurolucida 360, Microbrightfield, Williston, VT) [see also (14)]. An independent investigator randomly chose sections and subregions for manual count validation, to check the fidelity of the semiautomated settings to markers placed by human expert users. We compared each animal in terms of number of cells and the relative proportion of cells counted, which was similar.

In this pilot, we were surprised to find that the majority of labeled neurons contained two or more of these three transmitters, and formed 59% of all cells (Figure 6G). The remaining 41% were single-labeled for either TH, VGluT2, or GAD1 mRNA. As expected, the majority of neurons contained TH mRNA (82%). Somewhat surprisingly, however, the majority (69%) of TH mRNA-positive neurons were multiplexed (Figure 6H), with the predominant type being TH/VGluT2-coexpressing cells (light and dark purple). A sizeable proportion of TH/VGluT2-expressing neurons also cocontained GAD1 mRNA (dark purple). Pure glutamatergic neurons (orange) were relatively rare (7%). Interestingly, GAD1 mRNA + neurons constituted about 30% of all cells, consistent with prior results for single-labeled immunohistochemical studies (140); yet these too contained sizeable "multiplexed" populations (Figure 6G). We found that GAD1 mRNA colocalization in TH-positive neurons almost always occurred against a VGluT2 background (Figure 6H). Since GAD1 protein is a synthetic enzyme responsible for converting glutamate to GABA, this tendency for TH-GAD1-VGluT2 co-localization makes anatomic sense. Past studies in rodent have not appreciated GAD1 mRNA in DA neurons, but do find corelease of GABA at the DA terminals (173, 174).

It is hard to compare this work to prior work in humans and marmosets, or rodents (185) not only due to potential species differences, but also the relative age of the individuals [human samples are elderly, when DA neurons decrease normally (183, 199)]. Importantly, a bigger sample size and the inclusion of female animals is essential to fully interpret our results. In general, however, as found previously, there are relatively fewer VGluT2-single labeled neurons in nonhuman primate, compared to rodents. In addition, there are three clear populations of TH-single, TH/VGluT2, and TH/VGluT2/GAD1 containing neurons. In contrast to rodents, and similar to one other study in marmoset and human, TH/VGluT2 containing neurons were not restricted to the midline VTA structures (185).

The relatively young age of our animals may explain the relatively high colocalization of VGluT2 mRNA in TH-positive neurons, since glutamate in dopaminergic cells is developmentally regulated as noted above [see also Fortin (147, 200)]. Furthermore, different methodologies across studies have differing sensitivity for detecting these molecules. Prior work compared immunohistochemistry for TH in conjunction with traditional methods for *in situ* double labeling of VGluT2 mRNA, whereas these results employ RNAScope to detect transcripts for all molecules.

Correlating our mRNA results with protein levels is relatively straightforward for TH mRNA and protein, and the distribution is robust and similar, as expected. The general distribution of GAD1 mRNA comported with our previous experiments for GAD1 protein, as noted above. VGluT2 protein is found at terminals, although its transcript is found in the cell body, leading to a mismatch of protein and transcript localization (201). The presence of VGluT2 mRNA at the cell body, however, has been found to be necessary and sufficient for glutamate exocytosis at the synapse, and is considered a marker of glutamatergic transmisson (202).

Mapping CRF Receptors onto a Heterogeneous DA System

A comprehensive understanding of CRF effects in the DA system requires a thorough understanding of CRF receptors distribution among specific cell types and circuits in the DA system. In older anatomic studies, CRFR1 mRNA is distributed over the VTA and other midbrain structures (64, 203, 204), whereas there is reportedly little CRFR2 mRNA (204). One important issue with lower resolution anatomic studies is that CRFR2 mRNA and protein are found in axon terminals innervating the ventral midbrain (70, 97). Therefore, if CRFR2 mRNA (or protein) is not properly colocalized in presynaptic or postsynaptic structures using appropriate markers, the data can be hard to interpret (67, 203).

More recent studies using mouse genetics indicate that CRFR1 is found with relative abundance in DA versus non-DA cells (100, 103, 205), consistent with patch clamp studies showing the CRFR1 activates DA neurons, increasing spontaneous firing (88). CRFR1-containing GABAergic neurons exist, but do not change their spontaneous firing pattern in response to CRF (103). A recent report in mouse indicates that CRFR1 + neurons in the VTA are > 87% DAergic, and segregate to the lateral VTA (PBP) neurons which project to the lateral shell and core, but not the medial shell, of the ventral striatum (205). While data in monkey are preliminary, we find a very widespread distribution of CRFR1 mRNA in the ventral midbrain, mainly localized to DA neurons. CRFR1 is distributed broadly, not only in the A10 neurons but also in the substantia nigra, pars compacta (SNc) (Figure 6I). As in mouse, the lateral VTA (PBP) cells appear more enriched in CRFR1 mRNA than the midline VTA cells, and CRFR1 mRNA is strongly expressed across the SNc DA neurons as well. In adolescent animals, we found that on average, 72% of CRFR1 mRNA-positive neurons across all ventral midbrain subregions contained TH mRNA. Most of the CRFR1-TH–positive neurons were "multiplexed" with GAD1 and/or VGluT2 (not shown) (Figure 6J).

Less is understood about the anatomy of the CRFR2 distribution in the midbrain. Several reports using real-time PCR found low-level CRFR2

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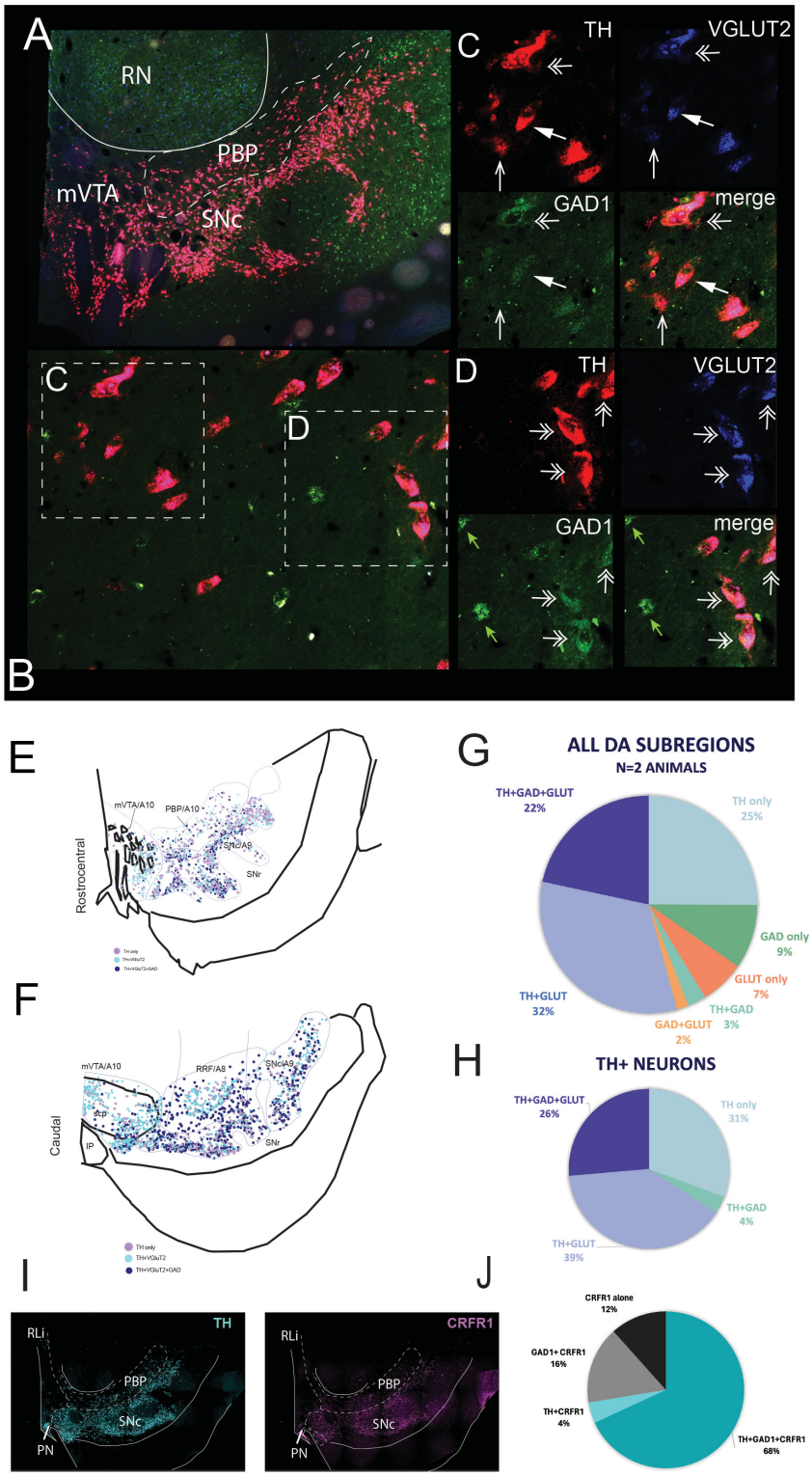

Figure 6. Preliminary characterization of "multiplexed" neurons in A10, A9, and A8 subregions in macaque. (A) Overview of RNAScope processed section at 4X, (B–D) Higher power (20X) images of various neuronal types in separate and merged channels. (C) Insets with TH mRNA + neurons in red, either single labeled, or colabeled with VGluT2 (blue) and/or GAD1 (green) mRNA. Simple arrow = single-labeled TH + neurons, arrowhead = TH/VGluT2 + neuron, double arrowhead = triple-labeled TH/VGluT2/GAD1 neurons (not all cells are labeled for simplicity). (D) Insets with similar neurons types, but depicting many triple-labeled neurons (double arrowhead), and single labeled GAD1 mRNA + neurons (green arrows). (E, F) Maps of the distribution of TH-positive phenotypes at two rostrocaudal levels. Light blue, TH only; light purple, TH/vGLUT2; dark purple, TH/vGLUT2/GAD1. (G) Mulitplexed and nonmultiplexed neurons in the ventral midbrain. (H) Proportions of multiplexed neuronal types by transmitter types. (I) Macroscopic view of CRFR1 mRNA in macaque ventral midbrain. The majority of CRFR1 mRNA is colocalized in TH-positive neurons (n = 3 animals). (J) Triple labeling for TH-GAD1-CRFR1 mRNA shows that the majority of CRFR1 neurons cocontain TH mRNA.

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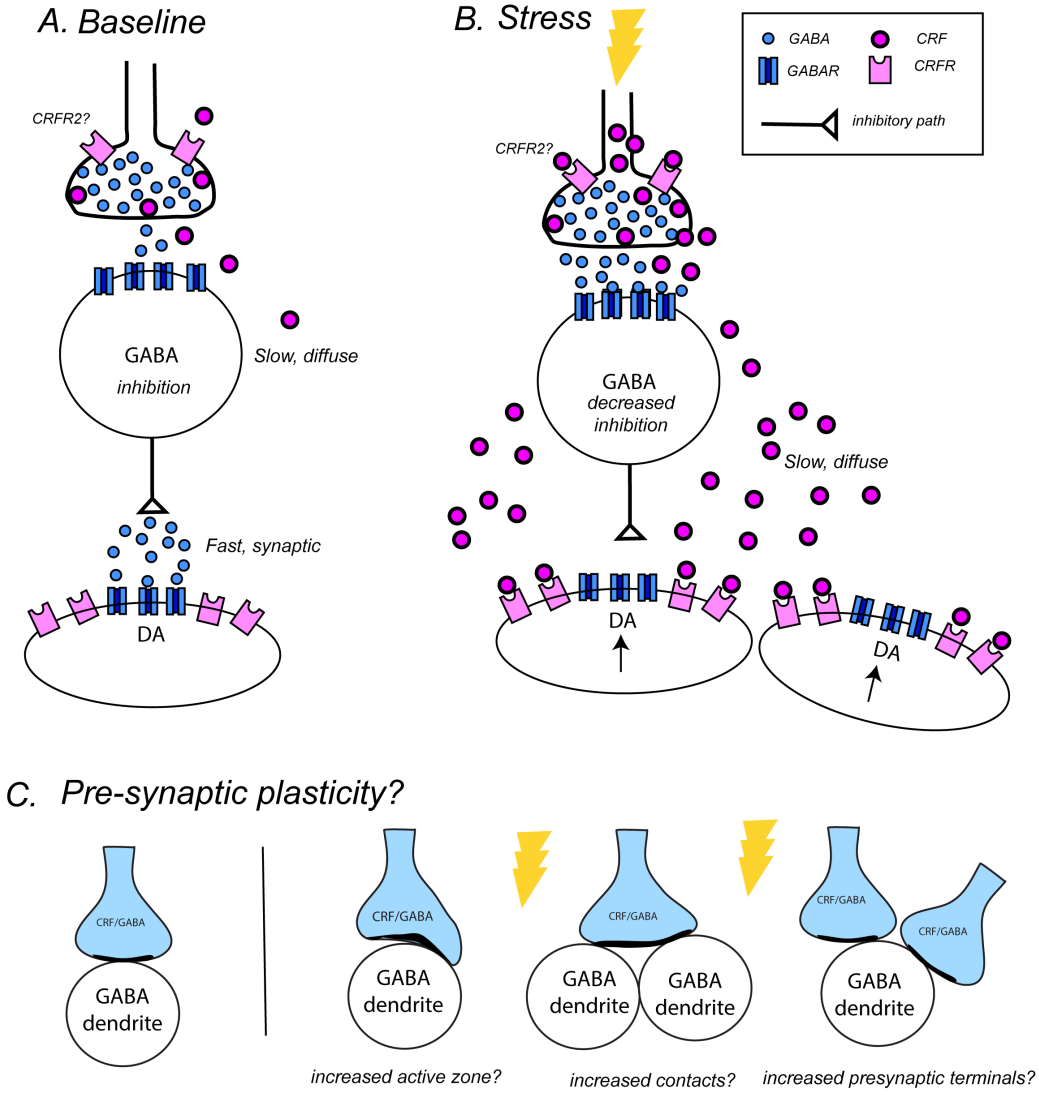

Figure 7. Piecing together CRF-DA anatomic relationships. (A) CRF can act via dual "fast"/short range and "slow"/long range mechanisms. CRF/ GABA afferent terminals mainly contact non-DA neurons (putative interneurons). GABA-CRF terminals may autoregulate GABA release through presynaptic CRF2 receptors, as in other brain regions. In the midbrain, classic GABAergic transmission "inhibits" the inhibitory GABAergic neuron, to modulate DA firing. (B) Under stress, increased activity-dependent CRF production acts via both increased presynaptic transmission (increasing GABAergic fast signaling, blue) and volume (slow, pink) transmission. Volume transmission permits CRF to more completely saturate CRFR1 receptors on DA membranes at some distance from extrasynaptic release sites. In this way, it works in tandem with GABAergic signaling, ensuring that interneuron inhibition is decreased, before direct excitation via CRF1R signaling. (C) Stress and CRF can change synaptic structure to enhance CRF effects (see text), although this possibility has not been explored in the CRF-DA path. Increased active zone length and increased contact sites are potential mechanisms.

expression in the mouse VTA; however, these results have not been reproduced using in situ hybridization until recently.

While one report using real-time PCR found low-level CRFR2 expression in the mouse VTA (101), other researchers were unable to reproduce those results (34, 113). Recently, using modern methods for in situ hybridization, low baseline levels of CRFR2 were found in astrocytes (206). Interestingly, CRFR2 was significantly upregulated by dopaminergic agonists (cocaine and methamphetamine), and was seen in astrocytes and TH-positive neurons after drug treatment. These studies suggest that low baseline levels of CRFR2 in the midbrain are up-regulated by manipulations such as stress or substances (86, 206), similar to effects on CRFR1 in diverse brain regions.

Future Directions

In summary, the mapping of gene expression patterns within distinct DA subpopulations highlights a nuanced spatial distribution of TH, GAD1, and VGluT2 mRNA transcripts that may change dynamically with age (182). We are attempting to add in the distribution of CRF and CRFR1 the macaque. We previously found that the vast majority CRF + axon terminals in this region make contacts onto non-DA (presumptive GABAergic) neurons (33). CRFR1 mRNA is broadly distributed on DA neurons in the lateral VTA and in the substantia nigra (A9), but it is unclear how development, sex differences, and stress factor into this pattern. This first "snapshot" suggests that at least in young male animals, control of GABAergic neurons by CRF in the lateral VTA is through direct synaptic, possibly presynaptic, mechanisms, while strong labeling for CRFR1 mRNA in DA neurons (and relatively few direct CRF + synapses onto these cells), suggests that CRF "volume" transmission is also in play (Figure 7A and B).

"Volume transmission," long described in seminal studies by Agnati, Fuxe, and colleagues (207), explains how peptides confer long-range and enduring effects on signaling, which are complementary to excitatory, "fast" transmission. Peptides such as CRF "packaged" with classic fast

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transmitters in the same neuron allows for cooperativity and flexibility at the target. They are packaged in large dense core vesicles, which require high-frequency action potentials for release, in contrast to classic transmitters. Once released, peptides such as CRF may take seconds to minutes to reach their receptors which are often found some distance from the synapse, in contrast to synaptic fast transmission that occurs within milliseconds through a synaptic cleft of less than 50 nm (208, 209). Neuropeptide clearance is slower, unlike classic transmitter reuptake, and depends on extracellular peptidases. These dual mechanisms provide variations in spatial and temporal control of DA firing (37, 68, 210). In a simple model suggested by our preliminary results, GABA release can quickly release the inhibitory (GABA interneuron) brakes on DA, while more intense firing rates in CRF/GABA terminals activates release of extrasynaptic CRF which diffuses through the extracellular space to directly activate CRFR1 in a concentration dependent manner (Figure 7A and B). On the postsynaptic side, CRFR1 receptors on DA neurons may form oligomeric complexes with other G protein–coupled receptors on the membrane surface, to integrate peptide signaling (211).

Beyond signaling dynamics, another relatively underexplored facet of the field is that CRF release has neuroplastic effects on synapse structure (212–215). This is a potentially important modality for conferring long-lasting synaptic changes in the stress system, including DA release (Figure 7C). Determining neuroplastic effects of CRF-containing projections on the DA system will require exhaustive work using laborious techniques like electron microscopy, but will be important on shedding light on how long-term adaptations in the stress system occur, particularly in higher species.

Authors Contributions

J.L.F. supervised I.M. on literature review, and both wrote the manuscript; J.L.F. and E.A.K. provided editing and selection of figures. I.M. and E.A.K collected and analyzed preliminary, original data presented in the manuscript. E.A.K. contacted authors of adapted figures for permission to present their work in the literature review. All authors take full responsibility for all data, figures, and text and approve the content and submission of the study, supervised by J.L.F. No related work is under onsideration elsewhere. All authors state that all unprocessed data are available, and all authors read and approved the paper.

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Author Disclosures

The authors have no competing interests to declare.

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