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Collateralized dorsal raphe nucleus projections: a mechanism for the integration of diverse functions during stress

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Abstract

The midbrain dorsal raphe nucleus (DR) is the origin of the central serotonin (5-HT) system, a key neurotransmitter system that has been implicated in the expression of normal behaviors and in diverse psychiatric disorders, particularly affective disorders such as depression and anxiety. One link between the DR-5-HT system and affective disorders is exposure to stressors. Stress is a major risk factor for affective disorders, and stressors alter activity of DR neurons in an anatomically specific manner. Stress-induced changes in DR neuronal activity are transmitted to targets of the DR via ascending serotonergic projections, many of which collateralize to innervate multiple brain regions. Indeed, the collateralization of DR efferents allows for the coordination of diverse components of the stress response. This review will summarize our current understanding of the organization of the ascending DR system and its collateral projections. Using the neuropeptide corticotropin-releasing factor (CRF) system as an example of a stress-related initiator of DR activity, we will discuss how topographic specificity of afferent regulation of ascending DR circuits serves to coordinate activity in functionally diverse target regions under appropriate conditions.

Keywords

serotonin; dorsal raphe nucleus; stress; collateral; tract tracing; corticotropin-releasing factor

1. Introduction

The dorsal raphe nucleus (DR) is a bilateral, neurochemically heterogeneous nucleus located in the ventral periaqueductal gray (PAG). Largely characterized as a brainstem region providing widespread serotonergic innervation to forebrain structures, approximately 50% of the serotonin (5-HT) neurons in the rat brain are localized to the DR (50–60% of the 5-

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HT neurons in the human CNS) (Azmitia and Segal, 1978; Jacobs and Azmitia, 1992; Molliver, 1987). Although the DR is the largest of the 5-HT-containing brainstem nuclei (Descarries et al., 1982; Steinbusch and Nieuwenhuys, 1983; Wiklund et al., 1981), less than 50% of the neurons within this region are serotonergic, with clusters of 5-HT neurons interdigitated with non-5-HT neurons (Molliver, 1987; Moore, 1981). Thus, in addition to 5-HT, other neurotransmitter/neuropeptide systems contribute to the afferent and/or efferent connectivity of the DR as well as the modulation of DR-5-HT neurons (Descarries et al., 1982; Jacobs and Azmitia, 1992; Moore, 1981; Steinbusch et al., 1980; Tork, 1990; Wiklund et al., 1981).

Extending approximately 2 mm along its rostrocaudal extent, the DR is bordered ventrally by the well-defined medial longitudinal fasciculus (mlf) fiber bundles and dorsally by the cerebral aqueduct and rostral portion of the fourth ventricle (at rostral/middle and caudal levels, respectively). The DR extends rostrally to the caudal end of the Edinger-Westphal nucleus at the level of the caudal oculomotor nucleus (Paxinos and Watson, 2005) and extends caudally to the caudal level of the dorsal tegmental nucleus (Taber et al., 1960). Both rostrocaudal as well as dorsoventral topography exist in the DR, thus, efforts have been made to more precisely describe DR subregions (Abrams et al., 2004; Clark et al., 2006). Steinbusch et al., divided the DR into 5 major subregions, including the most caudal aspect in the rhombencephalon, the dorsolateral (i.e. lateral wings or bilateral wings), dorsomedial, ventromedial, and interfascicular regions (Steinbusch et al., 1981). The majority of studies describing the location within the DR divide the nucleus into rostral, middle and caudal thirds, as did Abrams et al., using the distribution of tryptophan hydroxylase (*tph*) immunoreactivity as a guide (Abrams et al., 2004), providing corresponding bregma levels in accordance with a commonly used rat brain atlas (Paxinos and Watson, 1997). Clark et al., (Clark et al., 2006) have provided a more extensive analysis of the various DR subregions by describing the mRNA distribution of multiple 5-HT-related transcripts, further dividing the DR into six anterior-posterior subdivisions based on the distribution of tryptophan hydroxylase 2 (*tph2*), the brain-specific enzyme involved in 5-HT synthesis (Clark et al., 2006).

In addition to 5-HT, a multitude of other neurotransmitters and neuropeptides have been identified within the DR. The topography of neurons expressing gamma-aminobutyric acid (GABA) (Belin et al., 1979; Belin et al., 1983; Gamrani et al., 1979; Mugnaini and Oertel, 1985; Nagai et al., 1983; Nanopoulos et al., 1982; Pfister et al., 1981), glutamate (Kaneko et al., 1990), dopamine (Nagatsu et al., 1979; Ochi and Shimizu, 1978), norepinephrine (Grzanna and Molliver, 1980; Grzanna et al., 1978; Steinbusch et al., 1981), and nitric oxide synthase (Nakamura et al., 1991; Pasqualotto et al., 1991; Wang et al., 1995; Wotherspoon et al., 1994) have all been well characterized throughout the DR. The distribution of neuropeptidergic neurons has also been described, including enkephalin (Glazer et al., 1981; Hokfelt et al., 1977a; Hokfelt et al., 1977b; Moss and Basbaum, 1983; Uhl et al., 1979), vasoactive intestinal peptide (Loren et al., 1979; Sims et al., 1980), Substance P (Chan-Palay et al., 1978; Hokfelt et al., 1978; Ljungdahl et al., 1978), cholecystokinin (Bhatnagar et al., 2000; Otake, 2005; Vanderhaeghen et al., 1980), neuropeptide Y (de Quidt and Emson, 1986), galanin (Cortes et al., 1990; Melander et al., 1986; Skofitsch and Jacobowitz, 1985) and corticotropin-releasing factor (CRF) (Commons et al., 2003). These neuropeptide-containing DR neurons also have a topographic distribution and often colocalize 5-HT. The complexity of the 5-HT projections originating in the DR, as well as the vast numbers of other neurotransmitter/neuropeptide systems that extend both local and distant connections, presents multiple layers for communication/regulation of circuits at the level of the DR.

Regions innervated by the DR respond in a specific manner following exposure to a stressor and these responses are often diverse, supporting the heterogeneous connectivity of this

region (Molliver, 1987; Peyron et al., 1998; Tork, 1990). Specific stressors may increase, decrease or have no effect on 5-HT extracellular levels in forebrain regions (Kirby et al., 1995; Kirby and Lucki, 1998) and furthermore, distinct patterns of 5-HT release occur in response to different stressors (Adell et al., 1997; Kirby et al., 1997). The degree of 5-HT input from the DR varies across forebrain regions, with some areas receiving 5-HT innervation solely from the DR and others receiving mixed innervation from the DR and median raphe nucleus (MnR), or MnR alone. Although critical to the understanding of DR functionality, the 5-HT component of efferent connections is beyond the scope of this review and has been extensively discussed elsewhere (Beaudet and Descarries, 1981; Kohler et al., 1982; Kohler and Steinbusch, 1982; Lidov and Molliver, 1982; Moore et al., 1978; O'Hearn and Molliver, 1984; Parent et al., 1981; Steinbusch and Nieuwenhuys, 1981; Steinbusch et al., 1981; Steinbusch et al., 1980; Steinbusch, 1984; Tao and Auerbach, 2002; van der Kooy et al., 1981).

Interest in the anatomical organization of the DR has been driven by its involvement in the regulation of physiological systems and behavioral functions (de Almeida et al., 2005; Jacobs et al., 1978; Laver et al., 1992; Lucki, 1998; Maes and Meltzer, 1995; McGuirk et al., 1992; Simansky, 1996; Vertes, 1988) as well as its dysfunction which has been linked to a number of stress-related psychiatric disorders including depression and anxiety (Carrasco and Van de Kar, 2003; Graeff et al., 1996). In addition to the topographic organization of specific neurotransmitter/neuropeptide systems, the complex, but specific, organization of afferent and efferent DR connections presents multiple locations where DR activity can be modulated under specific circumstances such as stress. Importantly, the potential for different DR targets to be simultaneously affected by individual DR neurons is of interest as this is a means for coordinating distinct responses to a stressor. The collateralization of DR neurons provides a mechanism for this.

The purpose of this review is to provide a detailed description of our current understanding of the major DR-collateralized projections to the forebrain. Although DR-forebrain collateralized projections have been described before (Michelsen et al., 2008), this is the first summary to consider DR-forebrain collaterals in terms of stress-related circuitry, and how stress-related peptides (i.e., CRF) associated with behavioral responses to stress (i.e., forced swim) are poised to modulate DR-forebrain projections.

2. Ascending collateral projections originating in the DR

In the early 1980s following the development of several novel fluorescent tract-tracers the number of studies reporting collateralized neuronal projections significantly increased (see Kobbert et al., 2000 for review). DR projections were assessed using retrograde tract tracers (horseradish peroxidase, fluorescent dyes, plant lectins, latex beads, etc.) that were injected into terminal fields (using pressure or iontophoresis). In the terminal fields, the tracer is incorporated into axons (typically through endocytosis) and transported back to the parent cell bodies located within the DR.

For the most part, efforts were made to describe the distribution of DR efferent projections based on sub-regional distributions (dmDR, vmDR, and dlDR) or rostrocaudal locations (far-rostral, rostral, mid-rostral, mid-caudal, caudal, and far-caudal) (Abrams et al., 2004; Clark et al., 2006). Unfortunately, certain studies lacked specificity and an accurate description of the exact anatomical location of labeled neurons in the expansive DR. Some studies were primarily concerned with the presence of collateralization or limited the analysis to a single rostrocaudal level of the DR. Other studies examined collateral projections using sagittal sections, making it difficult to fully ascertain the distribution of labeled neurons by subregion. When possible, the location of DR efferent collateralized

projections was best described using schematics from universally accepted atlases that could best approximate their location in a consistent manner. When DR neurons were found in the dlDR or lateral areas, neurons were found ipsilateral to the injection site (unless otherwise noted), and a few neurons containing labeling were typically identified in areas contralateral to the injection site. A schematic summarization of the DR collaterals discussed below is included (Fig. 1).

2.1. Anterior-medial and lateral-caudal forebrain

One of the earliest studies using fluorescent retrograde tracers examined the collateral innervation of vast regions of the forebrain by DR neurons (van der Kooy and Kuypers, 1979). Large injections were placed into the anterior-medial forebrain which included large portions of the frontal cortex, olfactory tubercle, as well as rostral portions of the nucleus accumbens and caudate-putamen; injections into the lateral-caudal forebrain covered large parts of the parietal and temporal cortices, amygdala, the caudal caudate-putamen and lateral edges of the internal capsule. At rostral DR levels, neurons projecting to both the anterior-medial and lateral-caudal forebrain were located ipsilateral to the injection sites and found in both dorsomedial (dmDR) and ventromedial (vmDR) locations, including the interfascicular region (ifDR) between the medial longitudinal fasciculus (mlf). In the mid-rostral DR, dual-projecting neurons were only located in the vmDR; no neurons in the caudal DR were found to send collateral projections to the anterior-medial and lateral-caudal forebrain. Given the large areas covered by these injections, it is not surprising that approximately half of the retrogradely labeled neurons in the DR projected to both regions.

2.2. Medial prefrontal and lateral prefrontal areas

Evidence in support of DR collateralized projections to the medial prefrontal cortex (mPFC) and lateral prefrontal cortex (IPFC) is limited. The mPFC, as defined in the work of Sarter and Markowitsch (1984), encompasses the dorsal and ventral portions of the anterior cingulate cortex along with prelimbic and infralimbic cortices, whereas IPFC refers to the dorsal and ventral agranular insular cortex (orbitofrontal cortex). Particular subregions of these prefrontal areas have specific and divergent functions related to cognitive, spatial and motor tasks as well as emotional and motivational behaviors which are beyond the scope of this review (see Perry et al., 2011 and Dalley et al., 2004). Few details are available regarding the dual-projections from the DR to the mPFC and IPFC (Sarter and Markowitsch, 1984). Although DR collateral neurons projecting to the mPFC and IPFC were observed, the only information regarding their topographic distribution was obtained from the schematic depiction of retrogradely labeled neurons. The data suggest that DR-mPFC/IPFC neurons were located in more medial aspects of the middle 1/3 of the DR with no obvious topographical organization. This finding is important because these functionally distinct cortical areas typically receive afferent input from single afferents (Sarter and Markowitsch, 1984). Understanding the distribution of dually-projecting DR neurons to mPFC and IPFC regions is critical, not only because both cortical regions are densely innervated by the DR (Mamounas et al., 1991; Morecraft et al., 1992; Steinbusch, 1981), but also because the IPFC and portions of the mPFC provide reciprocal projections to the DR (Goncalves et al., 2009; Roberts, 2011). This reciprocal connectivity may be particularly important for stress responsivity and motivated behaviors (impulsivity, attention to stimulus, and integrative learning) (Maier et al., 2006; Perry et al., 2011; Roberts, 2011).

2.3. Prefrontal cortex and nucleus accumbens-potential regulation of motivated behavior

The majority of 5-HT innervation to both the mPFC and nucleus accumbens (NAc) originates in the DR (Bjorklund et al., 1971; Descarries et al., 1975; Li et al., 1989; Mantz et al., 1990; North and Uchimura, 1989). Serotonin exerts differential effects in the mPFC and NAc, therefore, determining whether separate populations of DR neurons might be

responsible for this dichotomy is important for understanding the organization of the DR-5-HT projection system (Van Bockstaele et al., 1993). Although DR projections to the mPFC and NAc are largely separate, DR-mPFC/NAc neurons were enriched in the middle 1/3 of the dmDR near the cerebral aqueduct and in the caudal DR along the midline, but not identified in either the vmDR or dlDR. Collateral projections were largely serotonergic when present. While small in number, these DR-mPFC/NAc collaterals allow for potential co-regulation of circuits underlying motivated behavior at an upstream (mPFC) and more downstream (NAc) point. Importantly, 5-HT impacts dopamine release in both the NAc and PFC (see Di Matteo et al., 2008 for review and references therein) and complex interactions between 5-HT and dopamine in these regions have been implicated in mechanisms underlying substance abuse and psychiatric disorders such as major depression. Taken together, these findings implicate 5-HT projections arising at the level of the DR as a practical target in the development of viable pharmacological treatments for addiction and depression (Bubar and Cunningham, 2008; Di Matteo et al., 2008; McBride et al., 2004; Weiss et al., 2001).

2.4. Regions involving nociception and antinociception

The PAG (including DR) has been linked to nociceptive and antinociceptive processes through both physiological studies and anatomical circuitry (Fardin et al., 1984; Li et al., 1993). Numerous ascending and descending 5-HT (and non-5-HT) projections innervate forebrain and brainstem regions which process information related to pain and reduced sensitivity to pain (Azmitia and Segal, 1978; Beitz, 1982; Beitz et al., 1983; Bowker et al., 1981; Conrad et al., 1974; Moore et al., 1978; Morgan et al., 1989). Furthermore, the suggestion that neural substrates underlying pain also overlap with those implicated in processes (i.e., reward and motivation) dysregulated in stress and other psychiatric disorders (Elman et al., 2011) makes a strong case for understanding how the DR-5-HT system interfaces with these areas.

Serotonergic neurons originating in the DR project to the trigeminal sensory complex and spinal cord, regions involved in nociception (Li et al., 1993) as well as nociceptive centers in the forebrain including the ventrolateral orbital cortex (VLO), NAc, and amygdala (Amy). Li and colleagues determined whether these regions implicated in nociception might be coordinately regulated via collateral projections originating in the DR.

DR collateral projections to the caudal spinal trigeminal nucleus (Sp5) and VLO, NAc, and Amy were examined. DR neurons sending collateral ascending and descending projections were found at all rostrocaudal DR levels examined (Li et al., 1993). In the rostral 2/3 of the DR, collateral neurons were predominantly located in the dlDR, with fewer DR neurons sending collaterals to these regions identified at caudal levels. The majority of DR collaterals projecting to the Sp5/VLO and Sp5/Amy were serotonergic at rostral and mid-DR levels, and exclusively serotonergic in the caudal DR. DR-Sp5/NAc neurons were more abundant at mid-DR levels than either the DR-Sp5/VLO or DR-Sp5/Amy collaterals and were serotonergic regardless of rostrocaudal location.

DR projections to the principal sensory trigeminal nucleus (Pr5) and the VLO, NAc, or Amy were also examined. The distribution of DR collaterals was similar to that observed for the Sp5 and VLO/NAc/Amy study. Similarly, most collateral neurons were located in the rostral 2/3 of the DR, with fewer neurons located in caudal DR regions. The majority of collaterals were positive for 5-HT, however, the occasional non-serotonergic collateral was observed for each of the paired injections, even at caudal DR levels (Pr5/NAc and Pr5/Amy). Of note, the DR-Pr5/VLO collaterals had a more dorsal distribution at the level of the trochlear nucleus (4N), lacking the lateral clustering observed for the DR-Sp5/VLO collaterals.

DR collaterals to forebrain and brainstem regions implicated in nociception were suggested by Li et al. to provide concurrent regulation of pain at both primary relay centers (Sp5 and Pr5) as well as higher forebrain centers (VLO/NAc/Amy) which are likely related to the emotional/motivational characteristics of pain (Li et al., 1993).

Antinociception engages descending PAG projections to the raphe magnus (RMg) situated in the rostral ventral medulla (RVM) and ascending projections to multiple forebrain regions including thalamic nuclei (parafascicular and ventroposterior), hypothalamic nuclei (ventrobasal and lateral), the amygdala, frontal cortex (medial and lateral cortices) and parietal and sensorimotor cortices. Reichling and Basbaum determined whether neurons situated in the PAG, including the DR, sent collateral ascending and descending projections (Reichling and Basbaum, 1991).

DR-RVM neurons were most common in the dlDR in contrast to neurons projecting to the forebrain which were more rostral and medially located, however ~8–17% of DR neurons projecting to these regions sent collaterals to the RVM and forebrain. Neurons sending collateral projections to the RVM and either amygdala or cortical regions had a similar topographic distribution throughout the DR. The majority of collaterals were located in the mid-rostral vmDR extending laterally just dorsal to the mlf. Few, if any, collateral neurons were found at rostral DR levels or mid-caudal DR levels (caudal to the decussation of the superior cerebellar peduncle; xscp).

DR collaterals to the RVM and diencephalic regions (thalamic and hypothalamic nuclei) implicated in nociception were identified (Reichling and Basbaum, 1991). The lateral hypothalamus (LH) and RVM receive collateral innervation from DR neurons located in medial and more lateral areas of the DR. Collateral DR-LH/RVM neurons were observed at all DR levels examined, predominantly in the middle 1/3 of the vmDR extending into more lateral regions just dorsal to the mlf. Fewer cells were found at the most rostral and caudal DR levels. Approximately 10% of the DR-LH neurons also extended projections to the RVM. Despite the DR collaterals to the RVM and LH, no neurons were found in the DR that sent collateral projections to the nearby ventrobasal hypothalamus (arcuate and ventromedial nuclei) and RVM at any rostrocaudal level examined, supporting the specific organization of collateral innervation. Further, DR neurons projecting to the medial thalamus and RVM were present at rostral and mid-DR levels, with few neurons present at mid-caudal levels. Collaterals were located mostly in the dmDR, scattered near the midline. Scattered neurons projecting to the ventrobasal thalamus (VB; consisting of VPL and VPM) and RVM were found mostly in the same regions. Additionally, at middle DR levels, dual-projecting neurons extended from the dmDR near the midline into the dlDR area and at mid-caudal levels, collateral neurons were found in the vmDR near the midline.

As discussed above, the finding that neurons involved in opposing functions (nociception vs antinociception) are located in the same brain nucleus (DR), but engage different structures and as a result produce different behavioral outcomes, further supports the complex organization of the DR. Furthermore, many DR collaterals involved in nociceptive and antinociceptive function consist of both ascending and descending projections. While stimulation of regions innervated by the descending limb of DR projections produces nociception or antinociception, regions targeted by the ascending limb of these DR collaterals likely mediate the emotional/motivational responses to stimuli and the general modulation of forebrain activity (Li et al., 1993; Reichling and Basbaum, 1991).

2.5. Collateral projections as a substrate for regulating autonomic functions

Many nuclei with functions related to autonomic modulation are innervated by DR neurons. The simultaneous regulation of ascending and descending autonomic-related nuclei was

assessed by examining collateral DR innervation of the paraventricular nucleus of the hypothalamus (PVN) and lateral parabrachial nucleus (LPB), implicated in visceral and autonomic functions. DR-PVN/LPB neurons were found throughout the rostrocaudal extent of the DR (Petrov et al., 1992) located near the ventricular wall and increased in number from caudal-rostral. Proceeding rostrally through the DR, there was a ventral and midline shift in the neuronal distribution until neuron numbers gradually decreased. A relatively large number of DR neurons sent collaterals to the PVN and LPB, but only a small proportion were serotonergic (8%). Since greater than 2/3 of the DR neurons projecting to the LPB also sent projections to the PVN, the authors suggest that DR neurons projecting to the LPB simultaneously transmit information to higher centers (i.e. PVN) indicating a role for these DR collaterals in the general control of physiological responses (Petrov et al., 1992).

When evaluating neuronal tracing studies in which the injection sites(s) impinge on the ventricular system as was illustrated in this study for the PVN injection, it should be taken into consideration that a population of DR neurons situated just ventral to the aqueduct innervates the ependymal wall and lateral ventricles (Simpson et al., 1998; see 2.6. “Ependymal wall and ventricular system” later in this review). This study indicated that DR-PVN projections were more numerous compared to DR-LPB projections, which may allude to the labeling of additional DR neurons following “PVN” injections. Peyron and colleagues also indicated that a greater number of DR-PVN neurons were observed than described in earlier works, including a well defined cluster ventral to the aqueduct (Levin et al., 1987; Sawchenko et al., 1983) which is consistent with findings reported by Simpson (Simpson et al., 1998).

The presence of DR collaterals to the PVN and central nucleus of the amygdala (CeA) were examined to glean information regarding the regulation of homeostatic circuits at the level of the DR (Petrov et al., 1994). DR-PVN/CeA collaterals were identified in the caudal 1/2 of the DR in what appeared to be clusters just below the ventricular wall (overall in DR 7% of cells were double-labeled) in the far-caudal DR. At the level of the caudal DR, neurons projecting to both regions were found primarily along the midline between the aqueduct and mlf, and at mid-caudal levels extending into the dIDR. No DR-PVN/CeA neurons were detected in the rostral 1/2 of the DR. As in the studies examining DR collaterals to the PVN and LPB, the clusters of neurons situated just below the aqueduct in the caudal DR could be the result of the diffusion of retrograde tracer into the ventricular system, although it is impossible to determine the quantity of tracer that may have entered the ventricles and whether or not diffusion played a role in this finding.

The coregulation of autonomic nuclei by DR neurons has important implications for the regulation of physiological responses to stress. For example, activation of the stress response by DR collaterals is possible through mechanisms where the synchronized activation of PVN and LPB neurons elicits neurohormone release and pressor responses in these regions, respectively (Petrov et al., 1992). Therefore, the activation of DR collateral projections to regions involved in autonomic regulation (PVN, LPB, CeA) in response to stress is an important mechanism for regulation of the hypothalamic-pituitary-adrenal (HPA) activity (Bhatnagar and Dallman, 1998).

2.6. Ependymal wall and ventricular system

Previous studies have described a unique population of neurons in the DR that send projections (serotonergic) to the ependymal wall and/or ventricular system (Aghajanian and Gallagher, 1975; Mikkelsen et al., 1997; Richards et al., 1973; van der Kooy and Kuypers, 1979). Elegant studies carried out by Simpson and colleagues (1998) characterized a substantial and well described population of DR neurons projecting to the ventricular system

and ependymal wall. A major finding from these studies showed that DR innervation of the ventricular system is topographically heterogeneous. The lateral ventricle is innervated by a significant, bilateral population of neurons clustered just ventral to the cerebral aqueduct in the middle (intermediate) DR, with fewer cells clustered in the vmDR, dorsal to the mlf (Simpson et al., 1998). DR neurons, retrogradely labeled by tracer injections made into the 4th ventricular space, had a similar distribution to DR neurons containing retrograde tracer from the lateral ventricle; however, the number of DR neurons projecting to the 4th ventricle was significantly less (Simpson et al., 1998). Further, DR neurons projecting to the lateral ventricle ependyma (lining) are clustered in the subependymal dmDR, ipsilateral to the injection site. Due to the similarities in the distribution of DR neurons projecting to the ependyma and ventricular space, Simpson et al., determined whether DR neurons sent collateral projections to these regions and whether they were 5-HT positive. In contrast to most other DR collateral projections which comprise a small percentage of the overall number of projecting neurons, most (50–70%) retrogradely labeled neurons in the DR sent collateral projections to both the ependymal wall and contralateral lateral ventricle, and furthermore, these neurons contained 5-HT (Simpson et al., 1998).

The consideration of DR neuronal innervation of the ventricular system is important when evaluating neuronal tracing studies in which the placement of tracer encroached on the ependymal wall or ventricles themselves. Examples of such encroachment can be visualized along the ependyma subsequent to PVN injections (Petrov et al., 1992, 1994). Because appropriate controls were not carried out in these studies, one cannot be certain that tracer was not released into the ventricular space where it would be available for transport to downstream ventricular regions and taken up, resulting in the labeling of additional neurons, many of which reside in the DR. In fact, studies by Larsen and colleagues addressed this issue by depositing tracer directly into the 3rd ventricle and showed that only neurons located in the DR had taken up the tracer (Larsen et al., 1996). Furthermore, the DR neuronal populations described following these injections were nearly identical to those described following PVN injections (Petrov et al., 1992). In other studies where robust labeling of the ependyma was obvious at the level of the PVN, clusters of DR neurons located ipsilateral to the injection site were observed below the cerebral aqueduct, and also in the vmDR at more rostral DR levels.

Recent studies elucidated the activation of a subset of DR-5-HT neurons which project to the ependyma/ventricular system which are activated following administration of urocortin 2 (Hale et al., 2010), a member of the CRF family of neuropeptides which preferentially binds to and activates corticotropin-releasing factor receptor 2 (CRF₂) (Reul and Holsboer, 2002). Although a role for these DR-5-HT neuronal projections to the ependyma/ventricular system is not yet obvious, the activation of subsets of DR neurons by stress-related peptides implicates a role for these neurons in mechanisms underlying stress-related behaviors and neuropsychiatric disorders that have a stress component (see Hale et al., 2010 and references therein).

2.7. Collateral projections involving the caudate-putamen: modulation of extrapyramidal function

Numerous anatomical tracing studies have examined DR collaterals that involve projections to the caudate-putamen (CPu), a major input center of the basal ganglia. An early study examining DR efferents examined the DR in the sagittal plane to assess the rostrocaudal distribution of neurons projecting to the CPu, Amy, hippocampus (HC), substantia nigra (SN) and locus coeruleus (LC) (Imai et al., 1986). This study topographically delineated the rostrocaudal distribution of these DR projections neurons showing that neurons related to motor function (CP and SN) were located in the rostral DR, whereas neurons related to the limbic function (HC and LC) were located in the caudal DR, with neurons projecting to the

amygdala interdigitated at intermediary levels. Thus, the paucity of DR neurons sending collateral projections to the CPu/HC or CPu/LC was of no surprise due to the primarily rostral distribution of DR-CPu neurons and predominantly caudal distribution of DR-HC and DR-LC neurons (Imai et al., 1986).

DR neurons extending collateral projections to the CPu and SN have been described in the rostral dmDR at the level of the trochlear nucleus (Imai et al., 1986) as well as at slightly more caudal DR locations in the dorsal cluster (van der Kooy and Hattori, 1980). This slight difference in the localization of dual-projecting neurons is likely due to subtle differences in tracer placement within target regions. The ability of DR collateral projections to coregulate motor-related dopamine circuits at a downstream and upstream level is similar to that described above for potential coregulation of dopamine circuits involved in motivation.

DR neurons sending collateral projections to the CPu and Amy were described as part of a larger study that described a considerable number of DR collateral projections to large regions encompassing the anterior-medial and caudal-lateral forebrain (van der Kooy and Kuypers, 1979). The authors briefly stated that even when much smaller injections were made into regions such as the CPu and Amy, DR neurons were identified that projected to both of these regions. Unfortunately, the specific topographic location of these neurons within the DR was not described in any further detail. A later study described DR-CPu/Amy collaterals that were present in the ifDR at far-rostral levels where the oculomotor nucleus is visible (Imai et al., 1986).

The DR-5-HT system is involved in mediating feeding behaviors (Medeiros et al., 2005), although dopamine is also associated with feeding circuits (Volkow et al., 2011). For example, enhanced activation of limbic regions (including the CPu and Amy) in response to food stimuli was expressed by insulin-resistant subjects (Chechacz et al., 2009) and in response to ghrelin, which stimulates food intake (Batterham et al., 2007). Since the CPu and Amy, both part of the reward and motivational circuits, are activated in response to feeding-related stimuli, DR neurons sending collaterals to these regions may play an important role in the regulation of feeding-related behaviors.

A variety of stressors (e.g., forced swim stress, tail pinch, cat exposure, etc.) have been shown to differentially alter 5-HT levels in distinct forebrain regions (Kirby et al., 1997; Rueter and Jacobs, 1996). The effects of forced swim on extracellular forebrain 5-HT levels are some of the best characterized, in terms of examining a stressor that produces robust changes in multiple forebrain areas (Kirby et al., 1997) and in examining multiple forebrain areas which are innervated by at least to some extent by 5-HT projections originating in the DR (Vertes, 1991). Given the finding that forced swim stress had opposite effects on 5-HT release in the lateral septum (LS) and CPu (Kirby et al., 1995), our laboratory investigated DR projections and the potential for collateralization to these regions (Waselus et al., 2006). Consistent with microdialysis findings, few DR neurons were identified which sent projections to both the LS and CPu, due largely in part to little overlap in DR neuronal populations projecting to these regions (Waselus et al., 2006). The few DR-LS/CPu collaterals identified were located in the middle 1/3 of the vmDR and were serotonergic.

The collateralization of ascending and descending DR projections to motor centers was evaluated. DR collaterals to the CPu and region including both the raphe magnus nucleus (RMg) and gigantocellular reticular nucleus alpha part (GiA), implicated in controlling the atonia of rapid eye movement, were determined (Li et al., 2001). Individual DR neurons projecting to both the CP and RMg/GiA occurred ~8% of the time. Only about 10% of the DR-CPu neurons also innervated the RMg/GiA, however a much larger number (28.5%) of the DR-RMg/GiA neurons projected to the CPu. Almost 2/3 of the DR collaterals were 5-

HT-positive, the majority of which were located in the dDR, especially at middle and caudal DR levels. These findings implicate the DR as a site for coordinated regulation of forebrain and brainstem functions related to motor system activity.

2.8. Collateral projections to limbic areas

The early examination of DR collaterals using fluorescent tracing methods described collateral DR projections to the anterior olfactory cortex (AO) and other limbic areas including the mediodorsal thalamus (MD) and septum (S) (De Olmos and Heimer, 1980). Although individual DR-MD neurons are found primarily in the dDR, DR-S and DR-AO neurons are found in more medial locations. DR collaterals to all combinations of these regions were identified. DR-MD/S collaterals were found in the dmDR and vmDR at mid-rostral levels, extending into the dDR in the mid-caudal DR. DR-AO/S collaterals were located in the vmDR in the intermediate 1/3 of the DR. Occasionally, DR-AO/MD or DR-AO/MD/S collaterals were identified in the vmDR at mid-caudal levels.

Studies implicating a functional connection between balance control and anxiety (Balaban and Thayer, 2001; Furman et al., 2005; Furman and Jacob, 2001) prompted the examination of DR collateral projections to centers involved in vestibular processing (the vestibular nuclei; VN) and centers which process the emotional importance of vestibular/visceral stimuli (the central nucleus of the amygdala; CeA) (Balaban and Porter, 1998; Halberstadt and Balaban, 2006). DR-VN/CeA collaterals were detected, and more specifically found at all rostrocaudal levels of the DR examined (mid-caudal through far-rostral; rostral 2/3 of the DR). DR-VN/CeA neurons were most numerous in the middle 1/3 of the DR and in greater proportions along the midline as opposed to lateral regions. More than half of these collateral neurons contained 5-HT suggesting the coordination of these regions by both 5-HT and non-5-HT projections. The authors propose that DR-VN/CeA collaterals may coordinate neural processing to ensure that vestibular system stimulation results in an aversive response when necessary, relating balance and posture information to contextual behaviors related to anxiety and/or fear (Halberstadt and Balaban, 2006).

DR collaterals also project to the HC and Amy (Imai et al., 1986) where schematics indicated injection sites which spanned multiple amygdalar nuclei and ventral portions of the HC. DR-HC/Amy neurons were predominant in the caudal 2/3 of the DR, located in the vmDR at mid-DR levels with fewer cells located in the caudal DR scattered just beneath the aqueduct and between the mlf. Amygdala-hippocampal connections have been implicated in the retrieval of emotionally-related contextual information (Smith et al., 2006), and these collaterals are of great interest given that the Amy and HC are involved in the acquisition and expression of conditioned fear (Davis, 2000; Fanselow, 2000; Fanselow and Poulos, 2005; Kim and Fanselow, 1992; LeDoux, 2000; Maren, 2001). Additionally, specific reciprocal connections exist between the basolateral amygdala and ventral hippocampus (Pitkanen et al., 2000), and both of these regions which receive collateral innervation from the caudal DR are necessary for fear expression and the extinction of memory (Sierra-Mercado et al., 2011). Since DR-5-HT actions at the level of the amygdala and hippocampus are both implicated in fear-related behavior (Graeff, 1993; Merali et al., 2006), the ability of collateral 5-HT projections to simultaneously impact both forebrain targets allows for a potentially powerful regulation of fear behavior. Additional work to elucidate the specific behavioral and physiological contributions of midbrain 5-HT populations (DR vs MRN) to fear-related behaviors in precisely described subnuclei/regions of the amygdala and hippocampus are necessary.

DR collaterals to the entorhinal area (Ent) and septum (S) are of interest due to the intimate connection of both of these regions with the hippocampal formation and their importance in proper hippocampal functioning (Gray, 1978). DR-S/Ent neurons comprised 13.3% of the

cells projecting to these regions (Kohler et al., 1982). The rostral DR contained the fewest collaterals, with numbers increasing at progressively more caudal DR levels. The majority of DR collaterals were detected in the dlDR and vmDR.

2.9. Collateral projections to motor and/or sensory areas

The DR innervation of visual processing centers such as the lateral geniculate (LG) and superior colliculus (SC) has been described (Parent et al., 1981; Pasquier and Tramezzani, 1979; Pasquier and Villar, 1982). DR neurons projecting to both the SC and LG are located primarily in the dlDR (10–15%) with the majority of collaterals exhibiting 5-HT (Villar et al., 1988). No collaterals were observed in the caudal DR although individual DR-LG and DR-SC neurons were detected. DR, and primarily serotonergic, innervation of these primary visual centers suggests a coordinated regulation of these and potentially other centers involved in vision.

Kirifides and colleagues characterized collateral DR efferent projections to multiple, functionally related sites along the rat trigeminal somatosensory pathway, responsible for conveying tactile information from the face and whiskers to the contralateral cerebral cortex (Kirifides et al., 2001). Sites analyzed included the principal nucleus of V (Pr5), ventral posteromedial nucleus of the thalamus (VPM), and barrel field regions of the primary somatosensory cortex (S1BF). Individual DR collaterals to the VPM and S1BF were identified in the dmDR and vmDR at rostral to mid-caudal levels, but most numerous in the middle 1/3 of the DR. DR neurons sending collaterals to the S1BF and contralateral Pr5 were restricted to the middle 1/3 of the DR, with a distribution similar to DR-VPM/S1BF. The VPM and contralateral Pr5 were innervated by collateral innervation from DR neurons located in the dmDR and vmDR at rostral through caudal DR levels. DR-VPM/Pr5 collaterals extended into the dlDR at mid-DR levels and were present in the caudal DR, unlike DR-VPM/S1BF or DR-Pr5/S1BF collaterals. Consistent with other reports, DR neurons extend collaterals to functionally related areas along the trigeminal somatosensory pathway (14%) as opposed to functionally unrelated (5%) areas (i.e., regions conveying trigeminal somatosensory and visual information).

Functionally parallel regions of the cerebrum and cerebellum are also innervated by collateral projections originating in the DR (Waterhouse et al., 1986). DR collaterals innervating the visual cortex (area 17) and paraflocculus of the cerebellum (PFI) were scattered between the mlf across the middle 2/3 of the DR, where these dual-labeled neurons comprised roughly 30% of the DR neurons projecting to these ipsilateral paired visual areas. Ipsilateral injections into paired motor areas (forelimb sensorimotor cortex and crus II of the lateral cerebellum (Crus2)) were distributed in the rostral 1/3 of the DR along the midline or displaced slightly laterally in the vmDR, comprising roughly 1/3 of the labeled cell population. Dual injections into the visual cortex and crus II of the cerebellum indicated that the DR lacked collateral projections to these functionally unrelated motor and visual areas (Waterhouse et al., 1986).

Recent studies have determined whether DR neurons play a role in multi-modal regulation by examining collateral projections to motor and sensory centers related to whisker function at the cortical, thalamic and medullary level (Lee et al., 2008). DR collaterals to the primary sensory (S1) and primary motor (M1) cortices were predominantly serotonergic and concentrated in the rostral 2/3 of the vmDR along the midline, with additional collateralizing neurons observed in the dmDR. DR neurons sending collaterals to the ventral posteromedial (VPM) and ventrolateral (VL) thalamic nuclei were found primarily in the dorsolateral DR (dlDR), in contrast to dual-projections to primary centers (S1/M1) which were more medially located. At the medullary level, DR collaterals to the facial motor (Mo7) and principal sensory trigeminal (Pr5) nuclei were localized to the dlDR. In sum, DR collaterals

to whisker-related cortical regions were located in the vmDR along the midline, however DR collaterals to thalamic relay (VPM/VL) and whisker-related medullary nuclei (Mo7/Pr5) were located in the dlDR along the middle 2/3 of the DR. These dual-projections to functionally related areas indicates a role for DR collaterals in the coordination of sensory-guided motor behaviors (Lee et al., 2008).

3. Impact of stress on DR projections

As reviewed above, a relatively unique feature of the DR is its complex network of abundant forebrain projections (Fig. 1). It is noteworthy that targets of DR collateral innervation are implicated in the regulation of mood, appetite, sleep, and attention, to name a few, all of which can be dysregulated in affective disorders such as depression (for review, see Krishnan and Nestler, 2010). The significant numbers of collateral projections and the diverse regions to which collaterals extend suggests that the DR is a common region targeted to exert regulation over multiple brain regions implicated in complex processes.

The specific mechanisms underlying regionally specific 5-HT changes in DR terminal fields following stress are steadily emerging. A number of variables need to be considered when elucidating the cellular basis for stress-induced 5-HT modulation. These include the distribution of receptor subtypes on neurochemically heterogeneous populations of DR neurons, the diverse afferent inputs to select DR neuronal populations and the DR target regions involved. Complex responses of 5-HT neurons have been described following swim stress. Specifically, both direct and indirect effects have been reported in the DR, with distinct effects on 5-HT vs non-5-HT neuronal activity (Kirby et al., 2007). Moreover, stress promotes differential activation of glutamatergic afferents regulating distinct DR neuronal populations, adding to the complexity of the DR response (Kirby et al., 2007). In subsequent sections, we further detail the effects of swim stress, as well as the stress-related neuropeptide CRF, on DR neuronal circuitry.

3.1. Impact of stress on the DR and its collateral projections

The extensive efferent projections of the DR play a crucial role in the modulation of forebrain activity and are impacted by stress. Virtually every forebrain region receives input from the DR and the neurons that innervate these regions are not random, but have a topographical distribution within the DR. For these reasons, clarification of the anatomical circuitry within the DR is essential for understanding how stressors impact the DR-5-HT system and thus, forebrain responses to stress.

Stress alters 5-HT release in projection fields in a regionally and stressor-specific manner (Adell et al., 1997; Kirby et al., 1997). For example, a single acute stressor (i.e., swim stress) increases extracellular 5-HT in the caudate-putamen and at the same time decreases levels in the lateral septum and amygdala, while having no effect in the cortex or hippocampus (Kirby et al., 1995). One explanation for regional differences in forebrain 5-HT release is the finding that 5-HT innervation of these regions arises from distinct neuronal populations in the DR that may be differentially affected by the same stressor. Our previous work indicated that two forebrain regions that exhibit differential regulation of 5-HT release following stress (lateral septum and caudate-putamen) receive serotonergic innervation from distinct populations of DR neurons (Fig. 2A–C and Waselus et al., 2006). In fact, the distribution of CRF immunoreactive fibers in relation to DR-LS and DR-CPu neurons (Fig. 2D–F) indicates that the proximity of CRF-containing fibers to DR-forebrain projections is different at caudal DR levels (Fig. 2D) compared to middle (Fig. 2E) or rostral DR levels (Fig. 2F). This suggests an organization whereby forebrain regions may be differentially engaged via activation at the level of the DR following exposure to a stressor (Waselus et al., 2006). The lateral septum, a limbic structure involved in regulation of emotion as well as

cognitive function, and the caudate-putamen, a region involved in planning, modulation of movement pathways, and other cognitive processes involving executive function, are primarily innervated by separate populations of forebrain projecting DR neurons, and although some DR-LS/CPu neurons were identified in the vmDR, this population was small and restricted (Waselus et al., 2006). Given that DR-LS/CPu collaterals were located in the middle 1/3 of the vmDR (Waselus et al., 2006), a region lacking a preponderance of CRF-immunoreactive fibers (Fig. 2E), it is more likely that CRF regulation of DR-LS (Fig. 2D) and DR-CPu (Fig. 2F) neurons occurs independently and this differential regulation by CRF may contribute to the differences in forebrain 5-HT release observed following stress (Kirby et al., 1995). Our findings, together with reports describing the stress-specific activation of DR neurons in a subregion-specific manner, substantiate a complex stress-specific circuitry in the DR whereby stress differentially impacts populations of 5-HT (and non-5-HT) neurons which in turn target specific forebrain regions. Together, these findings support the precise elucidation of the complex organization and regulation of DR efferent neurons.

3.2. CRF effects on DR circuitry

The effects of stress on the DR-5-HT system are likely mediated by the stress-related peptide corticotropin-releasing factor (CRF), which alters the firing rates of DR neurons, and consequently, 5-HT release in forebrain targets. CRF exerts bimodal effects on DR neuronal activity *in vivo*, with low doses of CRF inhibiting DR discharge rate and higher doses having no effect or increasing discharge rate (Kirby et al., 2000; Price et al., 1998). This is paralleled by changes in extracellular 5-HT in the lateral septum and the caudate-putamen as measured by microdialysis (Price et al., 1998; Price and Lucki, 2001).

Multiple lines of evidence support a neurotransmitter role for CRF actions in the DR during stress. For example, swim stress-induced decreases in 5-HT extracellular levels in the lateral septum are dependent on endogenous CRF (Price et al., 2002). Functional anatomical studies have suggested a circuit whereby CRF released during swim stress can inhibit DR activity (Fig. 3A). These studies demonstrated that swim stress increases expression of the immediate early gene, *c-fos*, in GABA-immunolabeled neurons in the dlDR that also express CRF receptor immunolabeling (Roche et al., 2003). The findings implied that CRF indirectly inhibits DR-5-HT cells by activating GABA neurons (Fig. 3B). Notably, excitatory effects of CRF have been reported using *in vitro* slice preparations containing DR neurons (Fig. 3C), although these were restricted to the caudal DR and were not consistent, occurring in 30% of recorded neurons (Lowry et al., 2000). High doses of CRF comparable to those that increase DR discharge facilitate learned helplessness, a behavior thought to be mediated via DR-5-HT system activation (Hammack et al., 2002). This same group demonstrated that low doses of CRF, such as those that inhibit the DR-5-HT system, attenuate learned helplessness, consistent with DR neuronal inhibition produced by low doses of CRF (Hammack et al., 2003). These findings have led to the hypothesis that the dose-dependent effects of CRF on DR activity promote alternative coping strategies (active vs passive) (Valentino and Commons, 2005). Moreover, the organization of the DR is such that stimulus specific control of 5-HT release is possible in regionally discrete terminal fields that are likely to be differentially regulated by CRF.

3.3. Direct vs indirect CRF effects on the DR-5-HT system

The DR is a complex nucleus not only in terms of afferent and efferent organization, but also with respect to the multitude of neurotransmitter/neuropeptide systems distributed throughout this region and the presence of multiple members of the CRF family of peptides and CRF receptor subtypes. The effects of CRF and related ligands are mediated by two receptors, CRF₁ and CRF₂, in the mammalian central nervous system. Neurophysiological and neurochemical findings suggest that activation of CRF₁ and CRF₂ in the DR has

opposing effects that may facilitate distinct behaviors. Activation of CRF₁ is thought to inhibit DR neurons and increase 5-HT release in many forebrain regions, and promote active coping responses such as escape behavior in response to swim stress (Kirby et al., 2000; Lukkes et al., 2007). In contrast, CRF₂ activation in the DR increases both discharge rates of 5-HT neurons and 5-HT release in certain forebrain targets (Amat et al., 2004; Forster et al., 2008; Lukkes et al., 2007; Pernar et al., 2004). CRF₂ activation in the DR is thought to be integral to the passive behavior that characterizes learned helplessness (Hammack et al., 2002; Hammack et al., 2003). Consistent with this, CRF₂ antagonists in the DR decrease immobility in response to swim stress (Valentino et al., 2010). Studies dissecting the phenotype of DR neurons impacted by CRF₂ activation indicated that CRF₂ excites DR-5-HT neurons via an inhibition of non-5-HT-DR neurons (Fig. 3D), presumed to be GABA (Pernar et al., 2004).

The finding that activating CRF₁ vs. CRF₂ in the DR has opposing physiological and behavioral consequences, taken with the different potency of CRF for its receptor subtypes, leads to a compelling model that relates stress magnitude with an appropriate behavioral response. The model suggests that during mild stress or the initial stages of a more severe stress, relatively low levels of CRF are released to engage CRF₁ receptors in the DR primarily and this will initiate an active behavioral response. In contrast, higher levels of CRF that may be released with more severe stress or stress of a longer duration can engage CRF₂ in the DR and promote passive behavior. This distinction may help to determine an alternate passive strategy, if the active strategy is not successful. Interestingly, in the initial characterization of CRF₂, Chalmers and colleagues suggested that the low affinity of CRF for CRF₂ may be physiologically relevant in that it may allow activity at CRF₂ only under conditions of prolonged CRF release, such as during episodes of stress (Chalmers et al., 1995). This distinction may help to determine an alternate passive strategy, if the active strategy is not successful. Thus, the net influence of the DR on behavior will be determined by whether CRF₁-mediated inhibition or CRF₂-mediated excitation predominates (Valentino and Commons, 2005).

We recently identified differential trafficking of CRF receptor subtypes as a novel cellular mechanism by which prior stress could change the predominant CRF receptor subtype in the DR, the physiological response of DR neurons and the behavioral response to stress (Waselus et al., 2009). These studies demonstrated that a single swim stress, which inhibits the DR-5-HT system, changes the cellular distribution of CRF₁ and CRF₂ such that CRF₁ internalizes and CRF₂ is recruited to the plasma membrane. This is associated with a shift in the physiological response of DR neurons to CRF from inhibition to excitation and a loss of inhibition of 5-HT release in target regions (Kirby and Lucki, 1998; Waselus et al., 2009). At a behavioral level the coping strategy shifts towards passive behavior with a subsequent exposure to swim stress. This mechanism is consistent with the requirement for CRF₂ activation to produce passive coping, such as described for learned helplessness (Hammack et al., 2002; Hammack et al., 2003).

A hypothetical circuit can be proposed whereby neuronal excitation and enhanced release of 5-HT in response to stress are brought about by the activation of CRF₂ on DR-5-HT neurons (Fig. 3E). We recently demonstrated that the majority of CRF₂-containing somatodendritic profiles colocalize 5-HT in the DR (Waselus et al., 2009). This finding, coupled with the increased ratio of CRF₂ at the plasma membrane, suggests a mechanism whereby the elevated concentrations of CRF observed following stress activate the now-enriched population of CRF₂ located at the plasma membrane on DR-5-HT neurons and as a result leading to neuronal excitation and subsequently increased release of 5-HT (Fig. 3E). Further studies are needed to examine the precise distribution of CRF_r on both 5-HT and non-5-HT

neurons in the DR, and further, to characterize the effects of stress on these subpopulations of DR neurons.

The findings that 1) GABA neurons activated by swim stress express CRF receptors (Roche et al., 2003), 2) GABAergic axon terminals synapse with 5-HT neurons in the DR (Lee et al., 1987; Tao et al., 1996; Wang et al., 1992) and 3) CRF-containing axon terminals were frequently in synaptic contact with GABAergic profiles (compared with 5-HT profiles) in the dlDR and vmDR (Waselus et al., 2005) all implicate GABA as a mediator of CRF effects on DR-5-HT circuits (Fig. 3B, D). DR-LS neurons were identified in DR regions caudal and ventral to the population of activated GABA neurons in the dlDR, which are thought to be necessary for the swim stress-induced decreases in lateral septal 5-HT release suggesting that GABA neurons in the dlDR are more than interneurons and project to other DR subnuclei (unpublished observations and Roche et al., (2003). Current studies are examining the differential targeting of DR-forebrain projections by CRF fibers that topographically innervate the DR to better understand afferent regulation of the DR system during stress (Fig. 3F).

Additional studies contributing to the understanding of DR circuitry include the identification of CRF cotransmitters in DR axon terminals to ascertain how CRF actions at the synapse may be modulated. We previously identified glutamate as a cotransmitter in a subpopulation of DR axon terminals containing CRF, however, the postsynaptic targets of CRF-glutamate terminals have not been elucidated (Waselus and Van Bockstaele, 2007) and further studies identifying such targets would contribute to the understanding of activity-specific circuits in this region. Further, CRF receptors have been observed on some DR axon terminals at the ultrastructural level (Waselus et al., 2009) suggesting that CRF_r may be modulating the presynaptic release of CRF or other neurotransmitters in the DR. Prior studies focused only on 5-HT and GABA as postsynaptic targets of CRF axon terminals in the DR (Waselus et al., 2005), however these are likely not the only systems regulated by CRF making the neurochemical identification of other postsynaptic targets of CRF necessary in piecing together if and how other DR neuropeptide/neurotransmitter systems are modulated by CRF. In summary, determining whether DR-forebrain projections to functionally related regions are targeted directly by CRF-containing axon terminals (Fig. 3F) or are indirectly regulated by CRF via non-5-HT mechanisms (Fig. 3F) will aid in the elucidation of the substrates underlying the stress-related modulation of DR circuits.

Another example of how DR circuitry and CRF might interact to impact behavior relates to the intersection of stress and vulnerability to drug abuse. Stress can influence different aspects of substance abuse and addiction through effects on multiple neuronal systems. The DR-5-HT system plays a complex role in addiction through its ability to regulate discharge activity of dopaminergic neurons and dopamine release in numerous targets (for reviews, see Bubar and Cunningham, 2008; Di Matteo et al., 2008; Weiss et al., 2001). The multiplicity of 5-HT receptors and their presence on different neurons within reward circuitry suggests a complex regulation of the dopamine release that ultimately drives drug-seeking behavior. Collateralized projections from the DR to functionally linked circuits in the reward pathway would be an effective way to co-regulate several targets simultaneously. Future studies designed to elucidate behavioral functions of distinct DR subnuclei and how CRF affects cells in these subnuclei are necessary to understand the precise role of CRF-5-HT interactions in psychiatric disease and substance abuse.

4. Conclusions

The DR is a complex brain region, implicated in responses to stress, whose dysregulation contributes to the etiology of affective disorders, including depression. Numerous studies

have characterized efferent projections from the DR (both 5-HT and non-5-HT) and, although many of these project to a single brain area, there are significant divergent projections to two or more target regions. The overview of DR collaterals, provided here, suggests that the mid-region of the DR is enriched with neurons that send projections to multiple forebrain regions. However, in general, collateralized projections to functionally similar or distinct areas are not restricted to any specific DR subregion. This results in challenges to the development of a cohesive model of efferent organization. However, the complexity of DR efferent pathways stands in contrast to the topographic specificity of afferent regulation, such as that from the stress-related peptide, CRF. It is by continuing systematic functional neuroanatomical studies that knowledge will be advanced regarding how different transmitter systems and neural circuits impact the DR and its efferent projections, whether singular or collateral. These advances will certainly lead to the development of more specific pharmacological treatments for stress-related psychiatric disorders.

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List of Abbreviations

17	visual cortex, area 17
4N	trochlear nucleus
5-HT	5-hydroxytryptamine
Amy	amygdala
A-Mfor	anterior-medial forebrain
AO	anterior olfactory cortex
CeA	central nucleus of the amygdala
CNS	central nervous system
CPu	caudate-putamen
CRF	corticotropin-releasing factor
CRF₁	corticotropin-releasing factor receptor, type 1
CRF₂	corticotropin-releasing factor receptor, type 2
CRF_r	corticotropin-releasing factor receptor
Crus2	crus 2 of the lateral cerebellum
dIDR	dorsolateral dorsal raphe nucleus
dmDR	dorsomedial dorsal raphe nucleus
DR	dorsal raphe nucleus
Ent	entorhinal cortex
GABA	gamma-aminobutyric acid
GiA	gigantocellular reticular nucleus, alpha part

HC	hippocampus
ifDR	interfascicular dorsal raphe nucleus
L-Cfor	lateral-caudal forebrain
LC	locus coeruleus
LG	lateral geniculate
LH	lateral hypothalamus
LPB	lateral parabrachial nucleus
IPFC	lateral prefrontal cortex
LS	lateral septum
M1	primary motor cortex
MD	mediodorsal thalamus
mlf	medial longitudinal fasciculus
MnR	median raphe nucleus
Mo7	facial motor nucleus
mPFC	medial prefrontal cortex
MThal	medial thalamus
NAc	nucleus accumbens
PAG	periaqueductal gray
PFI	paraflocculus of the cerebellum
Pr5	principal sensory trigeminal nucleus
PVN	paraventricular nucleus of the hypothalamus
RMg	raphe magnus
RVM	rostral ventral medulla
S	septum
S1	primary sensory cortex
S1BF	primary somatosensory cortex, barrel field
SC	superior colliculus
scp	superior cerebellar peduncle
SN	substantia nigra
Sp5	spinal trigeminal nucleus
VB	thalamic ventrobasal complex
VL	ventrolateral nucleus of the thalamus
VLO	ventrolateral orbital cortex
vmDR	ventromedial dorsal raphe nucleus
VN	vestibular nuclei
VPM	ventral posteromedial nucleus of the thalamus

xscp decussation of the superior cerebellar peduncle

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Figure 1. Ascending collateral projections of DR neurons

DR schematics through representative caudal (−8.72mm bregma), middle (−8.00mm bregma), and rostral (−7.30mm bregma) levels. Collateral projections occurring along the midline (in pink) are represented in the schematics on the left. On the right are depicted collaterals known to originate in specific DR subregions (dmDR in blue, vmDR in green, dlDR in orange, or lateral in purple) at the three rostrocaudal levels.

Figure 2. Differential distribution of CRF-immunoreactive fibers in the DR with respect to DR efferent projections

A–C) DR neurons projecting to the lateral septum (green) or caudate-putamen (red) are topographically distributed at different rostrocaudal DR levels (adapted from Waselus et al., 2006). DR-LS neurons are primarily located at caudal DR levels (A). At mid-DR levels (B), both DR-LS and DR-CPu neurons are present, with DR-LS neurons located in the vmDR and DR-CPu neurons mostly in the dmDR. DR-CPu neurons are enriched at rostral levels (C) in both the dmDR and vmDR, with some neurons located laterally, ipsilateral to the injection site. Shaded area in gray indicates the location of the medial longitudinal fasciculus (mlf). **D–F)** The overlap of CRF-immunohistochemistry (black fibers/puncta) with DR-LS (magenta) or DR-CPu (cyan) neurons indicates a distinct distribution of CRF immunoreactivity related to DR neurons projecting to each of these regions, dependent on the rostrocaudal DR level examined. At caudal levels (D), CRF-containing fibers are enriched just below the aqueduct, and also in regions dorsal and lateral to the primarily midline distributed DR-LS neurons. At mid-DR levels, CRF fibers are enriched in the dlDR, where few (if any) DR-LS or DR-CPu neurons are detected (E). At rostral DR levels (F), CRF is enriched in more medial locations, especially in the vmDR where there are a large number of DR-CPu neurons. (*) 4th ventricle or cerebral aqueduct; (mlf) medial longitudinal

fasciculus. Scale bars: D–F=100µm. Schematics in A–C adapted from Paxinos and Watson (Paxinos and Watson, 1997) and (Waselus et al., 2006).

Figure 3. Functional models for complex effects in the DR mediated by a stress-related peptide Direct and indirect regulation of DR-5-HT neurons can produce opposing behavioral outcomes (active vs passive coping) and differential effects in forebrain regions based on the specific underlying biological substrates. Evidence exists for the inhibition of DR-5-HT circuits (A,B) mediating active coping behaviors (i.e., swimming on Day 1 of forced swim test), as well as the excitation of DR-5-HT circuits (C,D) which mediate passive coping behaviors (i.e., learned helplessness). CRF effects on DR-5-HT neurons can occur by both direct (A,C) or indirect mechanisms involving non-5-HT neurons (B,D). A hypothetical circuit for CRF actions on DR-5-HT neurons in subjects with prior stress exposure is illustrated (E). Elevated CRF levels that occur subsequent to stress act on CRF₂ receptors that have been recruited to the plasma membrane of DR-5-HT neurons, resulting in neuronal activation and increased forebrain release of 5-HT. Elucidating the mechanisms (F), either direct or indirect, by which DR projection neurons (possibly collateral projections) are regulated by CRF_r will contribute to the understanding of stress-related DR activity.