Summer 8-13-2010

Probing the Neurophysiology of Anxiety: Social Stress Alters the Modulation of Serotonin Neurons

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Probing the Neurophysiology of Anxiety: Social Stress Alters the Modulation of Serotonin Neurons

Abstract
Anxiety disorders are prevalent in human and veterinary medicine yet the underlying mechanism is poorly understood. Because serotonin (5-HT) neurons of the dorsal raphe (DR) are thought to play a prominent role, my goal was to understand the changes in DR 5-HT neurons that underlie anxiety and other stress-related disorders. Two DR subdivisions were studied in a series of experiments: the ventromedial DR (vmDR), a well characterized subfield with a high density of 5-HT neurons, and the lateral wing DR (lwDR), a largely uncharacterized subfield with a more sparse distribution of 5-HT neurons. Many stress paradigms activate 5-HT neurons of the lwDR more so than 5-HT neurons of the vmDR, suggesting a unique role for lwDR 5-HT cells in stress circuits. However, it is not known if lwDR 5-HT neurons possess physiological characteristics that contribute to their increased propensity to be activated by a stressor. I found that lwDR 5-HT neurons demonstrated increased intrinsic excitability, increased glutamatergic input, and similar GABAergic input when compared to vmDR 5-HT neurons. Using the chronic social defeat model of anxiety, the distinctions between lwDR and vmDR neurons were explored further. Social defeat induced anxious behavior and stress-associated pathological changes in the peripheral organs of intruder mice. For the first time, investigation into the neural mechanisms of social defeat has focused on 5-HT neuron physiology, revealing subregion-specific effects within the DR. Increased excitability was seen in the vmDR neurons of the most anxious mice. This was accompanied by a decrease in GABAergic input to vmDR 5-HT neurons potentially mediated by both presynaptic and postsynaptic changes. The lwDR 5-HT neurons demonstrated distinct stress-induced changes limited to the slower kinetics of postsynaptic GABAAR. The differential effect of social stress on inhibitory input to vmDR or lwDR neurons suggest that the 5-HT output in brain regions targeted by each subfield is differentially affected in anxiety disorders. Collectively these findings help fill the gap in our understanding of local DR circuitry, the heterogeneity of 5-HT neurons, and the distinct regulation of vmDR and lwDR neurons in the circuits that mediate stress and contribute to the pathophysiology of anxiety.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Neuroscience

First Advisor
Sheryl G. Beck

Keywords
dorsal raphe, lateral wing, electrophysiology, GABA, glutamate, 5-HT1A receptor

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Behavioral Neurobiology | Mental Disorders | Systems Neuroscience

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PROBING THE NEUROPHYSIOLOGY OF ANXIETY:
SOCIAL STRESS ALTERS THE MODULATION OF SEROTONIN NEURONS

LaTasha K. Crawford

A DISSERTAION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2010

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Probing the Neurophysiology of Anxiety:

Social Stress Alters the Modulation of Serotonin Neurons

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LaTasha K. Crawford
DEDICATION

To the family, friends, and mentors that had nothing but the highest of hopes for me:

Thank you for never lowering your expectations...
ABSTRACT

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LaTasha K. Crawford
Sheryl G. Beck

Anxiety disorders are prevalent in human and veterinary medicine yet the underlying mechanism is poorly understood. Because serotonin (5-HT) neurons of the dorsal raphe (DR) are thought to play a prominent role, my goal was to understand the changes in DR 5-HT neurons that underlie anxiety and other stress-related disorders. Two DR subdivisions were studied in a series of experiments: the ventromedial DR (vmDR), a well characterized subfield with a high density of 5-HT neurons, and the lateral wing DR (lwDR), a largely uncharacterized subfield with a more sparse distribution of 5-HT neurons. Many stress paradigms activate 5-HT neurons of the lwDR more so than 5-HT neurons of the vmDR, suggesting a unique role for lwDR 5-HT cells in stress circuits. However, it is not known if lwDR 5-HT neurons possess physiological characteristics that contribute to their increased propensity to be activated by a stressor. I found that lwDR 5-HT neurons demonstrated increased intrinsic excitability, increased glutamatergic input, and similar GABAergic input when compared to vmDR 5-HT neurons. Using the chronic social defeat model of anxiety, the distinctions between lwDR and vmDR neurons were explored further. Social defeat induced anxious behavior and stress-associated pathological changes in the peripheral organs of intruder mice. For the first time, investigation
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# PROBING THE NEUROPHYSIOLOGY OF ANXIETY:
## SOCIAL STRESS ALTERS THE MODULATION OF SEROTONIN NEURONS

## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter 1:</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Introduction</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2:</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased intrinsic excitability of lateral wing serotonin neurons of the dorsal raphe: a mechanism for selective activation in stress circuits</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 3:</th>
<th>66</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamatergic input is increased in lateral wing 5-HT neurons: the role of morphology and innervation</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 4:</th>
<th>107</th>
</tr>
</thead>
<tbody>
<tr>
<td>Towards an understanding of the pathophysiology of anxiety: social stress alters the inhibitory input to serotonin neurons.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 5:</th>
<th>151</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Conclusions</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Appendix 1:</th>
<th>174</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviations</td>
<td></td>
</tr>
</tbody>
</table>
**LIST OF TABLES**

Table 2.1 Membrane characteristics of 5-HT neurons of the vmDR and lwDR.

Table 2.2 Differences in lwDR membrane characteristics remain despite blockade of synaptic input.

Table 3.1 Results of sEPSC recordings in DR 5-HT neurons

Table 3.2 Results of morphology analysis of 5-HT neurons

Table 4.1 Results of baseline sIPSC recordings in vmDR and lwDR 5-HT neurons

Table 4.2a Membrane properties of vmDR 5-HT neurons after social defeat

Table 4.2b Membrane properties of lwDR 5-HT neurons after social defeat

Table 4.3a Excitatory events: Results of sEPSC recordings in vmDR 5-HT neurons after social defeat

Table 4.3b Excitatory events: Results of sEPSC recordings in lwDR 5-HT neurons after social defeat

Table 4.4a Inhibitory events: Results of sIPSC recordings in vmDR 5-HT neurons after social defeat

Table 4.4b Inhibitory events: Results of sIPSC recordings in lwDR 5-HT neurons after social defeat
LIST OF ILLUSTRATIONS

Figure 1.1 Immunohistochemical detection of markers of 5-HT and GABA neurons in the DR.
Figure 1.2 Diagram of local DR circuitry.
Figure 2.1 5-HT neurons extend across the rostral-caudal extent of the dorsal raphe.
Figure 2.2 Intrinsic membrane properties differentiate lwDR 5-HT neurons from vmDR 5-HT neurons.
Figure 2.3 The firing rate of lwDR 5-HT neurons in vitro is faster than that of vmDR 5-HT neurons.
Figure 2.4 5-HT1A receptor mediated responses in 5-HT neurons.
Figure 2.5 Rostral position was correlated to several intrinsic membrane properties of vmDR 5-HT neurons.
Figure 2.6 Unlike mid-caudal vmDR 5-HT neurons, rostral vmDR 5-HT neurons were comparable to lwDR 5-HT neurons according to several parameters.
Supplemental Figure 2.S1 Subsets of 5-HT neurons demonstrate two unique rectification currents.
Supplemental Figure 2.S2 Ca-channel blockers had a larger effect in lwDR 5-HT neurons.
Figure 3.1 Immunostaining of vGlut proteins in the mouse DR.
Figure 3.2 Confocal images of vGlut proteins and TPH in the vmDR and lwDR subregions.
Figure 3.3 Recordings of sEPSC events revealed increased frequency of glutamatergic synaptic input to lwDR 5-HT neurons.
Figure 3.4 sEPSC events recorded in lwDR 5-HT neurons demonstrate faster decay kinetics than events recorded in vmDR neurons.
Figure 3.5 The 5-HT neurons of the lwDR possess somatic and dendritic morphology that is distinct from vmDR neurons.
Figure 3.6 Correlation analysis reveals several relationships between sEPSC parameters and morphological characteristics.
Figure 3.7 Topographical position of lwDR 5-HT neurons is correlation to increased sEPSC amplitude and longer total dendrite length.
Figure 4.1 There are few differences in sIPSCs between the vmDR and lwDR 5-HT neurons of unmanipulated mice.
Figure 4.2 Chronic social defeat induces anxiety-like behavior in intruder mice.
Figure 4.3 Chronic social defeat induces an array of stress-associated changes in peripheral organs.
Figure 4.4 Increased anxiety-like behavior in the open field test was correlated to increased 5-HT neuron excitability in the vmDR of intruders.
Figure 4.5 Chronic social defeat leads to a decrease in sIPSC frequency and amplitude in the vmDR of intruder mice.
Figure 4.6 Chronic social defeat leads to distinct effects in lwDR 5-HT neurons that were limited to slower in sIPSC kinetics.
Supplemental Figure 4.S1 The vmDR 5-HT neurons of control mice show a trend towards increased sIPSC frequency compared to unmanipulated controls.
Figure 5.1 The summarized effects of stress on the inputs that modulate 5-HT activity.
Anxiety is a state of increased dread or worry that is a normal response to stressful events. It is characterized by cognitive, behavioral, and autonomic signs that likely yield an evolutionary advantage of increased vigilance in the face of potentially threatening stimuli. However, in many individuals the magnitude and duration of excessive, irrational fear and dread is substantial enough to cause severe distress, discomfort in social gatherings, and missed days from work, along with an array of other effects that interfere with normal functioning in their everyday lives. In these individuals, anxiety is not just a normal response to a stressor, but rather a mental disorder that can be debilitating in its most severe forms. The major categories of anxiety disorders include generalized anxiety disorder, panic disorder, obsessive compulsive disorder, post-traumatic stress disorder, social phobia, and specific phobia.

Collectively, anxiety disorders are the most prevalent psychiatric disorders in adults in the United States (Kessler et al., 2005a; NIMH, 2009). It is estimated that anxiety disorders affect 18% of adults each year and over 28% of adults will endure an anxiety disorder over the course of their lifetime (Kessler et al., 2005b; Kessler et al., 2005a). Not only are anxiety disorders more prevalent than other types of mental illness, they also tend to have an earlier...
age of onset (Kessler et al., 2005b). Anxiety disorders are often comorbid with other mental disorders including depression and substance abuse, (Kessler et al., 2005a) and are associated with a wide range of other medical conditions disorders including sleep disorders (Roth et al., 2006), interstitial cystitis (Clemens et al., 2008a; Clemens et al., 2008b), cardiovascular disease, chronic pain, and irritable bowel syndrome (Roy-Byrne et al., 2008). The variety of conditions that are often comorbid with anxiety in human patients underscores the clinical relevance of the effects of stress on both the brain and peripheral organ systems.

Anxiety-related behavioral disorders are also prevalent in veterinary patients including dogs, cats, and horses among other companion animals. In veterinary medicine common anxiety-associated diagnoses include separation anxiety, fear-based aggression, inappropriate elimination, and displacement behavior. Stereotypies and certain types of self-mutilation are behavioral disorders in equine patients for which social stress and social isolation can be contributing factors (Nicol, 1999; McDonnell, 2008). Perhaps the most studied anxiety disorder in veterinary medicine presents as canine acral lick dermatitis resulting from over-grooming, an animal model of obsessive-compulsive disorder with obvious parallels to the grooming behavior often seen in human obsessive-compulsive disorder (Stein et al., 1992; Woods-Kettelberger et al., 1997; Stein et al., 1998). Interestingly, these stress-related disorders are responsive to the same types of drugs that are efficacious in human anxiety disorders including benzodiazepines and selective serotonin reuptake inhibitors. The presence of stress-induced, anxiety-like disorders in a wide range of mammalian species and their responsiveness to anxiolytic drugs suggests a common biology and a mechanism rooted in conserved neural systems.

Much of what is known about the possible mechanisms underlying anxiety stems from the clinical efficacy of anxiolytic drugs. Some of the first drugs to be prescribed for their ability
to quell anxiety disorders were benzodiazepines. Benzodiazepines are a class of drugs that act as agonists of the γ-Aminobutyric acid (GABA) type A receptor (GABA<sub>A</sub>R). Early studies to pinpoint the mechanism responsible for the anxiolytic effects of benzodiazepines highlighted their effect on lowering brain levels of serotonin, also known as 5-hydroxytryptamine (5-HT) (Wise et al., 1972; Stein et al., 1975; Tye et al., 1979; Collinge et al., 1983). The dorsal raphe (DR), the midbrain nucleus containing the majority of 5-HT projections to the forebrain (Azmitia and Segal, 1978; Molliver, 1987), is at the center of these effects. Specifically, these studies showed that manipulations that mimic 5-HT production or increase firing of DR neurons increased anxiety-like behavior and block the effects of benzodiazepines while manipulations that mimic GABA input to DR neurons or otherwise decrease 5-HT production decrease anxiety-like behavior (Wise et al., 1972; Stein et al., 1975; Kohler and Steinbusch, 1982; Higgins et al., 1988). Benzodiazepines applied directly to the DR mimic the systemic anxiolytic effects of benzodiazepines (Soubrie et al., 1981; Thiebot et al., 1982; Maier et al., 1994) and enhance GABAergic inhibitory input to DR 5-HT cells (Gallager, 1978) to enact their anxiolytic effect. Microinjection of a benzodiazepine antagonist into the DR mitigates the anxiolytic effect of systemically administered benzodiazepine (Hindley et al., 1985). Several other studies have likewise demonstrated an anxiolytic effect of other types of GABA agonists and agonists of the inhibitory 5-HT<sub>1A</sub> receptors (5-HT<sub>1A</sub>R) when microinjected directly into the DR (Higgins et al., 1992; Maier et al., 1995a; Graeff et al., 1996; Sena et al., 2003) and anxiogenic effects of drugs that oppose GABA function when microinjected into the DR (Jones et al., 1986; Maier et al., 1995b). Collectively these data suggest that anxiolytic drugs act by decreasing the activity of DR 5-HT neurons and that drugs that increase anxiety-like behaviors act by increasing the activity of DR 5-HT neurons.
While these studies do not elucidate the pathology of the anxious brain, they do underscore the potential role of the DR 5-HT neurons as well as 5-HT\textsubscript{1A} and GABA\textsubscript{A} receptors in the pathogenesis of anxiety itself. The studies of the mechanisms of anxiolytic drug have yielded insightful results but were limited by a few confounds: In early studies, the topography of the DR and heterogeneity of 5-HT neurons found therein was poorly understood; the lateral wings of the DR (lwDR) were never explicitly targeted and it is unclear whether drugs administered by microinjection reach the lwDR and whether such an injection would be considered a “hit” or a “miss”. The ability to probe 5-HT neuron activity and to explore its modulation by local circuits was an impossibility in early studies due to technical limitations that are now easily overcome with the advent of electrophysiology techniques. In addition, by focusing on stress-related circuits in the healthy brain, these studies fail to elucidate the mechanisms of maladaptive stress-related pathology. The advent of well-conceived animal models of anxiety may prove to be useful in supporting the hypotheses generated from findings in the normal, healthy brain.

Despite what is proposed as the mechanism of anxiolytic drugs, very little is known about the neural pathology that underlies anxiety disorders and necessitates the use of anxiolytic drugs. In order to fully understand the pathophysiology of anxiety, the contribution of anxiety to other mental and physical pathologies, and novel methods for treating anxiety disorders, it is crucial to understand the pathological changes which produce the anxious phenotype. As delineated above, the serotonin system is a prime starting point. The 5-HT system is conserved across mammalian species and plays an important role in a wide range of physiological functions including mood, sleep, the limbic stress response, and cardiovascular control (J.M. Monti, 2008; Nalivaiko and Sgoifo, 2009; Monti, 2010). Serotonin efferents from
the DR contribute to a vast neuromodulatory system with targets selectively distributed throughout every major division of the brain (Lowry, 2002; Michelsen et al., 2007). Unlike the norepinephrine and dopamine systems, the serotonin system is not associated with a particular modality of signaling or a single type of stimulus. A recent in vivo study in awake, freely moving rats confirms that DR 5-HT neurons do not encode a unitary signal. Rather, small subsets of DR neurons selectively fire with time-locked precision in response to diverse sensory, motor and reward-related events, which likely enables them to dynamically sculpt information processing in target regions of the brain (Ranade and Mainen, 2009). The complexities of the 5-HT system likely contribute to the breadth and diversity of functions serotonin cells are known to modulate. Characterizing the heterogeneity of DR 5-HT neurons is therefore crucial to our understanding of how the DR operates in stress circuits in the healthy brain and how it contributes to the pathophysiology of anxiety disorders.

The DR nucleus is divided into three major subregions: dorsomedial (dmDR), ventromedial (vmDR), and the lateral wings (lwDR) which is also referred to as dorsolateral DR or ventrolateral DR (Figure 1.1). The midline 5-HT neurons including the vmDR and dmDR are the typical target for microdialysis and electrophysiological studies and are often the only analyzed region in experiments using immunohistochemical or molecular biology techniques. The vmDR is particularly well-characterized because the high density of 5-HT neurons therein makes it an ideal target for electrophysiology recording, microinjection, and even tissue sampling for western blots. On the other hand, the lwDR is poorly understood due to several potential contributing factors including the historical lack of understanding of the heterogeneity of 5-HT neurons, the absence of compelling evidence yet that subregions are differentially regulated in stress-related pathology, and more simply, the relative dearth of 5-HT neurons in the lwDR.
make it more difficult to target than midline subregions where 5-HT neurons are dense and abundant. Though it is often assumed that all DR 5-HT neurons are comparable to the well-characterized vmDR 5-HT cells, emerging evidence suggests otherwise.

Figure 1.1 Immunohistochemical detection of markers of 5-HT and GABA neurons in the DR. A) Tryptophan hydroxylase (TPH, green) can be used to detect 5-HT neurons in the ventromedial (vmDR), dorsomedial (dmDR) and lateral wing (lwDR) subdivisions of the DR. The distribution of 5-HT neurons together with the atlas overlay is used to define subregions. B) The 65kD and 67kD isoforms of glutamate decarboxylase (GAD 65/67, red) is a marker of GABA neurons. GABA neurons are abundant in the lwDR and lateral aspects of the vmDR. mlf – medial longitudinal fasciculus.

Several lines of evidence suggest that the DR subregions contain a heterogeneous population of cells and that each subfield may play a distinct role in mediating stress (O'Hearn and Molliver, 1984; Abrams et al., 2004; Johnson et al., 2004; Lowry et al., 2008). The unique patterns of afferent and efferent input to the lwDR are the first clue that lwDR play a role that is distinct from vmDR neurons. The major limbic forebrain input to the DR extends from the lateral habenula and medial prefrontal cortex, regions that have a higher density of input to the lwDR than to the vmDR (Araki et al. 1988; Peyron et al. 1998; Varga et al. 2003). Inputs from the medial amygdala primarily innervate the vmDR, whereas the lateral portion of the medial
amygdala and the central amygdala innervate the lwDR (Lee et al. 2007). The major difference in efferents is that unlike vmDR and dmDR neurons, lwDR projections are primarily to subcortical targets, especially to regions involved in panic behavior and cardiovascular pressor responses (Johnson et al., 2008; Lowry et al., 2008). In addition, neuronal activation following exposure to stress is more abundant in lwDR 5-HT neurons than in vmDR 5-HT neurons (Commons, 2008; Johnson et al., 2008). Though lwDR neurons likely play a crucial role in stress circuits and stress-related pathology, little is known about their physiology. Because lwDR 5-HT neurons have never been characterized, it is unknown whether they possess distinct membrane characteristics or receptor-mediated responses compared to vmDR 5-HT neurons. Furthermore, it is unknown how stress differentially affects vmDR and lwDR neurons on a cellular level. And finally, an overarching benefit of obtaining a better understanding of the unique physiology of lwDR subregion stems from the fact that the highest abundance of 5-HT neurons in the DR of humans and non-human primates are found not in the vmDR, but rather in the lwDR subregion (Charara and Parent, 1998; Austin and O'Donnell, 1999).

The local circuitry that regulates 5-HT activity is becoming more clearly understood. The tonic firing of DR 5-HT neurons are driven by noradrenergic input from the locus coerulus (Baraban and Aghajanian, 1981; Vandermaelen and Aghajanian, 1983; Aghajanian, 1985), 5-HT neuron activity is positively modulated by local glutamatergic input mediated by AMPA/kainate receptors (AMPA R) and negatively modulated by local GABAergic input mediated by GABA\(_A\)R as well as feedback regulation whereby 5-HT released within the DR acts at 5-HT\(_{1A}\) autoreceptors and 5-HT\(_{2A}\) receptors on GABA neurons (Figure 1.2). Local glutamatergic input to 5-HT neurons and glutamate released from terminals of severed afferents can be isolated and analyzed in the in vitro slice preparation (Pan and Williams, 1989; Haj-Dahmane and Shen, 2005; Lemos et al.,
However, it remains unclear if glutamatergic input to 5-HT neurons differs across DR subregions or if it is altered in the anxious brain.

Figure 1.2 Diagram of local DR circuitry. 5-HT neurons receive local GABAergic and glutamatergic synaptic inputs that modulate their activity. These inputs are mediated by GABAA and AMPA receptors expressed on 5-HT neurons. Dorsal raphe GABA neurons are modulated by 5-HT receptors, including the 5-HT2A/C receptor (2A/C) and are also modulated by glutamatergic projections originating from the medial prefrontal cortex (mPFC) and lateral habenula (LHb). An additional source of inhibitory feedback to DR neurons includes the 5-HT1A autoreceptor (1A).

As suggested by the negative effects of anxiolytic drugs on 5-HT neuron firing and 5-HT release described above, altered inhibitory feedback regulation of 5-HT neurons may be a crucial component of the pathophysiology of anxiety. In the DR inhibitory feedback is mediated in part by somatodendritic 5-HT1AR, which are implicated in anxiety (Figure 1.2). Human
patients with social anxiety disorder have reduced 5-HT$_{1A}$R binding in several brain regions including the DR (Lanzenberger et al., 2006) and mice lacking the expression of the 5-HT$_{1A}$R show an increase in anxiety-like behavior (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998; Gross et al., 2002). Therefore it would be important to know whether vmDR and lwDR 5-HT neurons differ in their responses mediated by the 5-HT$_{1A}$R or whether these responses are differentially altered in anxiety.

Another key component of inhibitory regulation of 5-HT neurons involves GABA neurons. In the DR, GABA neurons are primarily present in the lateral regions including the lateral vmDR and lwDR (Stamp and Semba, 1995; Day et al., 2004) as indicated by immunohistochemical detection of the 65kD and 67kD isoforms of glutamic acid decarboxylase (GAD65/67, Figure 1.1). Several forms of stress ranging from mild handling to social stress activate non-5-HT, presumably GABA neurons of the lwDR (Martinez et al., 1998; Chung et al., 2000; Roche et al., 2003; Johnson et al., 2004; Abrams et al., 2005; Gardner et al., 2005; Berton et al., 2007; Hale et al., 2008; Johnson et al., 2008). Local GABAergic input to 5-HT neurons mediated by GABA$_A$R can be isolated and analyzed in the in vitro slice preparation (Pan et al., 1989; Pan and Williams, 1989; Liu et al., 2000; Lemos et al., 2006). However, as no one has ever recorded inhibitory current input to 5-HT neurons outside of the midline DR, it is unknown whether GABAergic input to lwDR 5-HT neurons differs from input to vmDR neurons. GABAergic input to 5-HT neurons is itself modulated by several factors, including 5-HT$_{2A/C}$ receptors (5-HT$_{2A/C}$R) expressed by GABA neurons. This serves as another mechanism of inhibitory feedback: 5-HT and 5-HT$_{2A/C}$R agonists increase GABAergic input to vmDR 5-HT cells in a manner that is blocked by selective 5-HT$_{2A}$R and 5-HT$_{2C}$R antagonists (Liu et al., 2000). In fact, the 5-HT$_{2A}$R is also implicated in anxiety, as knock-out mice lacking 5-HT$_{2A}$R show decreased anxiety, though
the mechanism underlying this behavioral phenotype is thought to involve cortical 5-HT$_{2A}$R more so than receptors found in the DR (Weisstaub et al., 2006).

Inhibitory input to DR 5-HT neurons is further modulated by afferents originating in the medial prefrontal cortex (mPFC). Tracing studies have demonstrated that mPFC afferents project to the DR (Sesack et al., 1989; Peyron et al., 1998; Jankowski and Sesack, 2004; Vertes, 2004) where 88% of the synapses formed are asymmetric synapses with GABA cells (Varga et al., 2001). *In vivo* electrophysiological studies show that excitation of mPFC pyramidal cells leads to excitation of GABA cells and indirect inhibition of 5-HT cells within the DR (Celada et al., 2001; Allers and Sharp, 2003). The mPFC projection to the DR is a crucial component of the circuit that mediates the effects of uncontrollable stress, a necessary element of animal models of anxiety and depression (Amat et al., 2005; Maier and Watkins, 2005). The controllable nature of a stressor is sensed by the mPFC which is activated and sends projections to DR GABA neurons that then inhibit 5-HT neurons (Amat et al., 2005; Maier and Watkins, 2005; Maier et al., 2006). With repeated exposure to uncontrollable stress, an animal exhibits learned helplessness, a state that resembles human anxiety and depression (Maier and Watkins, 1998, 2005). In response to uncontrollable stress, there is a lack of mPFC input to GABA cell in the DR whereby this lack of inhibitory feedback results in sensitized DR 5-HT neurons (Maier, 1984; Amat et al., 2005; Maier and Watkins, 2005; Maier et al., 2006). Thus the GABA neurons of the DR are crucial mediators of the mPFC-DR feedback needed to transmit the effects of controllable stress and may contribute to the pathology induced by exposure to uncontrollable stress. Because no one has ever looked at changes in GABAergic input to 5-HT neurons in a model of anxiety, it is unknown whether GABAergic input to DR 5-HT neurons is altered in the anxious brain and whether those alterations differentially affect the vmDR and lwDR subregions.
To delve into cellular mechanisms that underlie anxiety, I explored the local circuitry of the DR in mice following chronic exposure to uncontrollable stress, using a model known as chronic social defeat. The causative agent used to induce anxiety in this model is social stress, a stressor that is ethologically salient for the rodent and also parallels the role of psychosocial stress in the etiology of human anxiety disorders. The chronic social defeat paradigm exposes a male experimental mouse, often called the “intruder”, to social stress by placing it into the cage of an older, larger, territorial male mouse, often called the “resident”. A dominance hierarchy is established almost immediately whereby the intruder is subordinate and is consequently bullied and “defeated” by the dominant resident. Though a single defeat produces an array of neural changes, repeated exposure to social defeat elicits a distinct pattern of neural changes (Matsuda et al., 1996; Martinez et al., 1998; Miczek et al., 2004; Gardner et al., 2005), the behavior of the intruder changes beyond the defeat environment, and an anxious, depressive phenotype ensues. Chronic social defeat reproduces elements of clinical anxiety and depression including increased anxiety-like behavior (Avgustinovich et al., 1997; Keeney and Hogg, 1999), increased anhedonia (Rygula et al., 2005), increased social avoidance (Keeney and Hogg, 1999; Berton et al., 2006; Krishnan et al., 2007), and in some studies increased alcohol consumption (Croft et al., 2005). The validity of the model is further underscored by the successful use of anxiolytic and antidepressant drugs to attenuate each of these behavioral phenotypes (Keeney and Hogg, 1999; Lumley et al., 2000; Von Frijtag et al., 2002; Avgustinovich et al., 2003; Rygula et al., 2006; Rygula et al., 2008).

While chronic social defeat is becoming a prevalent model to study anxiety, depression, and other stress-related disorders, only a few studies have examined how social defeat changes the dorsal raphe of the intruder mouse. Chronic exposure to social defeat induces an increase
in c-Fos expression in the DR, PFC, and several other brain regions in intruder mice as compared to control mice (Matsuda et al., 1996; Martinez et al., 1998; Martinez et al., 2002). Additional studies have examined, often with conflicting results, the social defeat-induced changes in the expression of genes related to 5-HT neurotransmission e.g. serotonin transporter, tryptophan hydroxylase, and the 5-HT1AR (Amstislavskaya and Kudryavtseva, 1997; Filipenko et al., 2002; Abumaria et al., 2006). However, each of these studies analyzed gross tissue samples and thus could not detect changes in specific cell types within the brain regions they examine. Three recent studies examine cellular effects of a single defeat throughout the DR subregions by examining c-Fos protein and the expression of serotoninergic markers (Gardner et al., 2005; Gardner et al., 2009). However these studies only examine the effects of a single defeat and thus have limited insight as to the changes that occur after chronic exposure to social stress. In one study (Cornelisse et al., 2007) in vitro recordings measured vmDR 5-HT1A-R-mediated responses in the rat after chronic social defeat, revealing no significant effect of chronic defeat. However these recordings did not examine the effects of chronic defeat on 5-HT membrane properties or synaptic input to 5-HT cells. Thus, despite the growing use of chronic social defeat model of anxiety, it is still unclear how chronic social defeat alters 5-HT neurons or how the effects of defeat vary across DR subregions. Furthermore, no one has used electrophysiology to assess defeat-induced changes in 5-HT neuron physiology or in the synaptic activity that modulates the 5-HT system.

In summary, before the role of 5-HT neurons in anxiety can be determined, critical issues related to the anatomical distribution of 5-HT neurons need to be addressed. It is unclear whether the intrinsic membrane properties are homogenous throughout DR 5-HT neurons, as no one has ever characterized lwDR neurons or reported on the rostral-caudal position of
recorded neurons. Though it has been demonstrated that glutamate and GABA modulate 5-HT neurons, it is unclear how these inputs vary across subregions. Finally, it is unclear how these components of local DR circuitry are altered in anxiety. Though chronic social defeat is a well established model of anxiety and depression, few investigators have used it to examine specific changes in the serotonin system on a cellular level. Dorsal raphe 5-HT neurons stand at the crux of anxiolytic pharmacotherapy and are thereby likely to be crucial components of pathology underlying anxiety. However, no one has ever examined stress-induced changes in DR 5-HT neuron physiology or modulatory input to DR 5-HT neurons. The experiments described herein will address these deficits in our knowledge of the neurophysiology of anxiety disorders.

I hypothesized that lwDR neurons were distinct from vmDR in terms of membrane characteristics, the 5-HT$_{1A}$R-mediated response, spontaneous glutamatergic synaptic input and spontaneous GABAergic synaptic input. I also hypothesized that the chronic stress induced by a social defeat paradigm would differentially alter the membrane characteristics of lwDR and vmDR neurons and decrease the negative feedback onto both vmDR and lwDR 5-HT cells, mediated by a decrease in baseline GABAergic input and a decrease the 5-HT$_{1A}$R-mediated response.

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CHAPTER 2

Increased intrinsic excitability of lateral wing serotonin neurons of the dorsal raphe: A mechanism for selective activation in stress circuits

(Published Manuscript)


doi:10.1152/jn.01132.2009

ABSTRACT

The primary center of serotonin (5-HT) projections to the forebrain is the dorsal raphe nucleus (DR), a region known for its role in the limbic stress response. The ventromedial subregion of the DR (vmDR) has the highest density of 5-HT neurons and is the major target in experiments that involve the DR. However, studies have demonstrated that a variety of stressors induce activation of neurons that is highest in the lateral wing subregion (lwDR) and includes activation of lwDR 5-HT neurons. Despite the functional role that the lwDR is known to play in stress circuits, little is known about lwDR 5-HT neuron physiology. Whole-cell patch clamp electrophysiology in mice revealed that lwDR 5-HT cells have active and passive intrinsic membrane properties that make them more excitable than vmDR 5-HT neurons. In addition, lwDR
5-HT neurons demonstrated faster in vitro firing rates. Finally, within the vmDR there was a positive correlation between rostral position and increased excitability, among several other membrane parameters. These results are consistent with stressor induced patterns of activation of 5-HT neurons that includes, in addition to lwDR neurons, a small subset of rostral vmDR neurons. Thus, increased intrinsic excitability likely forms a major part of the mechanism underlying the propensity to be activated by a stressor. The membrane properties identified in lwDR recordings may thereby contribute to a unique role of lwDR 5-HT neurons in adaptive responses to stress and in the pathobiology of stress-related mood disorders.

INTRODUCTION

As the primary center of serotonin (5-HT) projections to the forebrain, the dorsal raphe nucleus (DR) is known for modulating the limbic system in response to stressors and for its putative role in stress-related mood disorders such as anxiety and depression. There is a topographical organization within the dorsomedial, ventromedial (vmDR), and lateral “wing” (lwDR) subregions of the DR such that subpopulations of neurons innervate distinct targets, receive disparate afferent input, and are likely to be differentially regulated in stress-related pathology (Abrams et al., 2004; Johnson et al., 2004; Michelsen et al., 2007; Lowry et al., 2008). A subregion that is poised to have a crucial role in the neural circuits that mediate the response to stressors is the lwDR, a subregion whose 5-HT neurons overlap with the ventrolateral periaqueductal gray (Fig. 2.1) (Paxinos and Watson, 1997; Paxinos and Franklin, 2001).
Several types of stressors, including exposure to an open field arena, social stress, swim stress, and interoceptive stressors along with several anxiogenic drugs activate topographically organized subpopulations of DR neurons, particularly within the IwDR subregion, (Martinez et al., 1998; Chung et al., 2000; Roche et al., 2003; Johnson et al., 2004; Abrams et al., 2005; Gardner et al., 2005; Berton et al., 2007; Hale et al., 2008; Johnson et al., 2008). To our knowledge, there are few stressors that do not activate the IwDR. Gene markers of neuronal activation implicate the non-5-HT neurons of the IwDR in swim stress (Roche et al., 2003); however recent studies revealed that 5-
HT neurons are an important part of the swim stress-activated circuitry whose activation may be masked by 5-HT$_{1A}$ receptor mediated inhibitory feedback mechanisms (Commons, 2008). Interestingly, the majority of 5-HT neurons activated by swim stress are not located in the 5-HT-dense vmDR, but rather are found in the lwDR. Intravenous lactate induces panic-like responses in a rat model of panic disorder but has little effect on control animals (Johnson and Shekhar, 2006; Shekhar et al., 2006). This mild stressor activates lwDR 5-HT cells in control animals but fails to activate lwDR 5-HT cells in the model of panic, implicating lwDR activation in the normative response to innocuous stressors (Johnson et al., 2008).

Thus, lwDR 5-HT neurons likely play a distinctive, crucial role in the response to stressors. However, because the vast majority of electrophysiological studies of 5-HT neurons target the midline DR and especially the vmDR, surprisingly little is known about the physiology of lwDR 5-HT neurons. It is unclear whether the distinct role of lwDR 5-HT cells in stress-related circuits might be due to fundamental differences in neuron physiology. I hypothesized that there are intrinsic membrane properties of lwDR 5-HT neurons that distinguish them from vmDR neurons and that contribute to their activation by stressors. To the best of our knowledge, these experiments also represent the first characterization of membrane properties and 5-HT$_{1A}$ autoreceptor-mediated responses of immunohistochemically identified 5-HT cells in the mouse DR. Part of this work was previously presented as an abstract (Crawford and Beck, 2008).

MATERIALS AND METHODS

Animals
Adult male 5-HT-YFP mice or their wild-type littermates were used at 2-4 months of age. These mice contain 5-HT neurons that express yellow fluorescent protein (YFP) under the control of the 5-HT specific Pet-1 promoter (Scott et al., 2005). The mice are on a background containing predominantly C-57/Black6. Mice were housed in a standard animal facility with lights on 06:00 to 18:00. Animal protocols were approved by the Institutional Animal Care and Use Committee and were conducted in accordance to the NIH Guide for the Care and Use of Laboratory Animals.

**Whole-Cell Electrophysiology**

Electrophysiology recordings were conducted as previously described (Beck et al., 2004). In brief, mice were sacrificed using decapitation. While submerged in a cold aCSF solution where NaCl is replaced with sucrose (248 mM), the midbrain was rapidly dissected, blocked, and cut with a Leica VT1000s vibratome (Leica Microsystems, Bannockburn, IL) to generate 200µm thick brain slices. Slices were maintained in aCSF bubbled with 95%O₂/5%CO₂ at 36°C for 1 hour and then at room temperature until used. Individual slices were then placed in a recording chamber and continuously perfused with 32-34°C aCSF solution bubbled with 95%O₂/5%CO₂ with a solution flow rate of 1.5-2ml/min. Neurons were visualized using Nikon E600 (Optical Apparatus, Ardmore, PA) upright microscope fitted with a 40x water-immersion objective, DIC, and infrared filter. Ventromedial and lateral wing DR 5-HT neurons were targeted based on expression of the YFP transgene and later confirmed based on the expression of the synthetic enzyme marker, tryptophan hydroxylase. To visualize YFP-positive 5-HT cells for recording, a fluorescent lamp and yellow-fluorescence filter was used. The image was generated on a computer monitor using a CCD camera and Nikon Elements.
software (Optical Apparatus). Whole-cell recording pipettes fabricated on a Sutter Instruments pipette puller (P-97, Sutter Instrument, Novato, CA) had a resistance of 6-10 MΩ when filled with an intracellular solution of 130mM Kgluconate, 5mM NaCl, 10mM Na-phosphocreatinine, 1mM MgCl₂, 0.02mM EGTA, 10mM HEPES, 2mM MgATP, 0.5mM Na₂GTP, and 0.1% biocytin (pH 7.3). Recordings were collected online with a Multiclamp 700B amplifier, Digidata 1320 A/D converter, and Clampex 9.0 software (Molecular Devices, Union City, CA). Membrane properties of the cell were monitored as was the access resistance of the patch pipette during recordings that lasted a minimum of 20 minutes. Any cell that showed physiological signs of instability or depolarizing resting membrane potential above -50mV was excluded from data analysis. Reported values do not incorporate a junction potential of approximately +15mV, as calculated using Clampex software. The composition of the aCSF was (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2.0 MgSO₄, 2.5 CaCl₂, 10 dextrose, and 26 NaHCO₃. Following the experiment, DR slices were fixed for 2-3 hours with 4% paraformaldehyde and processed for immunohistochemical detection of tryptophan hydroxylase (TPH) and the biocytin-filled, recorded cell.

**In vitro Extracellular Electrophysiology**

Raphe slices from 5-HT-YFP mice were prepared and visualized as described above. YFP-labeled neurons were targeted with extracellular pipettes fabricated on Sutter Instruments pipette puller and filled with 150mM NaCl. The electrode was visually placed on top of the YFP neuron. Firing activity was restored to 5-HT neurons using bath application of 1μM phenylephrine HCl. Any neurons with unstable firing rates over the course of the experiment were deemed unhealthy and excluded.
**Data Analysis**

Current clamp recordings using visualized whole cell patch clamp techniques were analyzed using Clampfit 9.0 (Molecular Devices). Resting membrane potential (RMP), action potential (AP) threshold, AP duration, after-hyperpolarization (AHP) amplitude, and the time it takes for the AHP to depolarize to one-half its peak amplitude (AHP $t_{1/2}$) were measured directly from traces as previously described (Beck et al., 2004). A voltage-current graph was generated using current pulses ranging from -100 to 0pA. Membrane input resistance (IR) was determined from the slope of the linear portion of the plot of the peak voltage induced by each current step. Frequency-intensity plots were obtained by measuring the number of action potentials generated by depolarizing current steps ranging from 0 to +80pA in 20pA increments. Average firing rate (Hz) was determined by the number of APs generated over the 630ms current pulse. Gain was determined from the slope of the frequency-intensity plot. The time constant tau was obtained from an exponential fit of the membrane potential during the first 300ms of a -20pA hyperpolarizing current pulse. AP and AHP characteristics were determined from action potentials generated by injecting just enough current through the recording electrode to elicit a single action potential. AP amplitude, AP duration, and AHP amplitude were measured in relation to the AP threshold. AHP $t_{1/2}$ is the duration of the AHP measured from its peak to half-amplitude. In experiments using CdCl$_2$, AP duration was determined at half-amplitude. 5-HT$_{1A}$R-mediated responses were measured with current clamp recordings using 400ms, -30pA current pulses at 10-second intervals to monitor resistance along with changes in membrane potential. Data generated by extracellular recordings were analyzed using Mini Analysis Program (Synaptosoft,
Decatur, GA). Reported values are mean ± standard error of the mean (s.e.m.) unless otherwise noted; \( p \) values were generated using Students t-test unless otherwise noted and \( p<0.05 \) was deemed significant. Outlying values were included for the purpose of understanding the heterogeneity of 5-HT neurons. Any data that showed non-Gaussian distribution were compared using non-parametric statistical tests. Additional statistical analysis was performed using Prism (GraphPad Software, La Jolla, CA).

**Immunohistochemistry**

Immunohistochemical identification of each neuron recorded in patch clamp configuration was completed as previously described (Beck et al., 2004; Lemos et al., 2006; Kirby et al., 2008). In brief, a standard immunohistochemistry protocol was used on 200\( \mu \)m-thick slices using mouse anti-TPH (1:200, Sigma) along with secondary donkey anti-mouse Alexa Fluor 488 (1:200, Invitrogen) and streptavidin-conjugated Pacific Blue (1:100, Invitrogen). Images were captured using a Leica DMR fluorescent microscope (Leica Microsystems, Bannockburn, IL) and OpenLab 3.0.9 software (Improvision, Lexington, MA) and then confirmed on a Leica DMIRE2 confocal microscope (Leica Microsystems) using Leica confocal software (version 2.5, Leica Microsystems).

**Drugs**

All chemicals for making the sucrose aCSF, aCSF, and extracellular electrolyte solution were purchased from Fisher Scientific (Pittsburgh, PA). All chemicals for the intracellular electrolyte solution, 5-Carboxamidotryptamine (5-CT), cadmium chloride, phenylephrine HCl, and WAY 100635 were purchased from Sigma-Aldrich (St. Louis,
MO). Apamin, bicuculline, CGP 55845, DL-AP5, DNQX, and ZD 7288 were purchased from Tocris (Ellisville, MO).

RESULTS

YFP-labeled 5-HT neurons of the vmDR and lwDR subregions were targeted throughout the rostral-caudal extent of the DR. A total of 85 neurons were recorded from 41 mice; the 5-HT identity of all neurons was confirmed by immunohistochemical detection of tryptophan hydroxylase. Data recorded from YFP-labeled 5-HT neurons and from unlabeled 5-HT neurons from wild-type offspring of the same mouse line did not differ (data not shown). Subregions were defined according to the distribution of 5-HT neurons as shown in Fig. 2.1.

Under current clamp, 5HT cells in the mouse showed passive and active electrophysiological properties resembling those reported in rat including large input resistance, wide action potential (AP), large slow after hyperpolarizations (AHP), and lack of spontaneous firing in brain slice preparations (Vandermaelen and Aghajanian, 1983; Kirby et al., 2003; Beck et al., 2004; Marinelli et al., 2004). In vmDR, as expected, there were a few distinctions between rat and mouse 5-HT neurons. Compared to the values reported in immunohistochemically identified vmDR 5-HT neurons in our previously published rat studies (n=33, Beck et al., 2004), vmDR 5-HT neurons of the mouse (n=19) differed in input resistance (637 ± 32MΩ in rat vs 419.5 ± 34.3 MΩ in mouse, p<0.0001), tau (51 ± 3.0ms in rat vs 23.4 ± 1.9ms in mouse, p<0.0001), AP amplitude (69 ± 1.4mV in rat vs 56.6 ± 1.6mV in mouse, p<0.0001), and AHP amplitude (16 ± 0.8mV in rat vs 29.3 ± 1.6mV in mouse, p<0.0001). Current clamp recordings also
revealed a small subset of confirmed 5-HT cells (5 of 19 vmDR and 3 of 17 lwDR cells) that departed from the classical description for 5-HT neurons and demonstrated an inward rectification current, visible in the non-linear voltage-current plot in response to hyperpolarizing current steps (Supplemental Figs. 2.S1A, 2.S1B). Another small subset

Supplemental Figure 2.S1. Subsets of 5-HT neurons demonstrate two unique rectification currents. (A) Raw data trace of two neurons with comparable input resistance; the trace on the right demonstrates an inward rectification current. Note that larger hyperpolarizing current steps elicited progressively smaller changes in membrane potential. (B) The corresponding voltage-current plots of the cells featured in A; left trace in black, right trace in gray. The non-linear voltage-current plot (gray trace) confirms the presence of the inward rectification current. Only 5 of 19 vmDR and 3 of 17 lwDR cells demonstrated inward rectification currents (scale bar 50mV, 50ms). (C) Raw data traces demonstrating the delayed onset inward rectification $I_h$ current in a vmDR 5-HT neuron before and after bath application of the $I_h$ current blocker ZD7288 (10μM). Only 2 of 19 vmDR cells demonstrated $I_h$ currents (scale bar 20mV, 50ms).

of 5-HT neurons (2 of 19 vmDR cells) featured a delayed-onset inward rectification $I_h$ current (Supplemental Fig. 2.S1C). The cell used to generate traces for Supplemental Fig. 2.S1C was not included in other experiments. Previously, inward rectification currents and $I_h$ currents were reported in the rat vmDR, but only in non-5-HT neurons (Kirby et al., 2003; Beck et al., 2004). The other passive and active membrane
properties of these few 5-HT cells did not differ from other 5-HT neurons (data not shown); these neurons were therefore included in further analysis.

The electrophysiological characteristics of lwDR 5-HT neurons were compared to those of vmDR 5-HT neurons (Table 2.1). All measured parameters demonstrated a single Gaussian distribution with the exception of lwDR resistance, vmDR tau, vmDR AP duration, and vmDR gain, each of which contained 1-2 outliers. Thus comparisons of resistance, tau, AP duration, and gain were conducted using non-parametric tests.

Lateral wing 5-HT neurons had a trend towards a larger membrane resistance compared to vmDR neurons \((p=0.087,\) Mann-Whitney test) (Table 2.1, Fig. 2.2B). This was consistent with the larger time constant (tau) observed in lwDR neurons \((p=0.013,\) Mann-Whitney test) (Table 2.1).

There were also differences in active properties of the neurons, as measured from action potentials generated by current injection through the recording electrode. 5-HT cells of the lwDR showed the broad AP and large amplitude AHP that is characteristic of 5-HT neurons \((\text{Kirby et al., 2003; Beck et al., 2004})\) (Table 2.1). Notably, the APs of lwDR 5-HT neurons had a more hyperpolarized threshold of activation \((p=0.011),\) larger amplitude \((p=0.022)\) and longer duration \((p=0.002,\) Mann-Whitney test) (Table 2.1, Fig. 2.2A). While the average resting membrane potential was
Figure 2.2. Intrinsic membrane properties differentiate lwDR 5-HT neurons from vmDR 5-HT neurons. (A) Representative data traces from vmDR and lwDR neurons demonstrate changes in membrane potential in response to various input current steps (i, iv, scale bar 50mV, 50ms) while enlarged traces demonstrate the AHP (ii, scale bar 20mV, 20ms) and AP (iii, scale bar 20mV, 2ms). Graphs in B-E depict average characteristics (mean ± SEM) for all recorded vmDR neurons (black, n=19) and lwDR neurons (gray with diagonal lines, n=17). (B) There was a trend towards a larger membrane resistance in lwDR neurons. (C) Summary bar graph depicting RMP, AP threshold (AP Thr), and activation gap (RMP - AP Thr). AP threshold was lower in the lwDR than in the vmDR, resulting in a smaller activation gap. (D) Frequency-intensity plots of vmDR and lwDR neurons demonstrate the increased excitability of lwDR neurons given 40pA, 60pA, or 80pA of input current. *p<0.05, **p<0.01 two-way ANOVA, Bonferroni post-test. Inset shows the input current steps and current clamp traces used to generate frequency-intensity plots (scale bar 50mV, 50ms). (E) Bar graph showing that the gain, i.e. slope of the frequency-intensity plot, was significantly greater in lwDR neurons. (F) Bar graph summarizing the effects of CdCl₂ and apamin on AHP amplitude (Amp) in vmDR (n=5) and lwDR (n=5) neurons. Two-way ANOVA revealed that the effect of the Ca-channel blockers on AHP amplitude was significantly larger in lwDR neurons than in vmDR neurons. Also, the effect of CdCl₂ was significantly larger than the effect of apamin. *p < 0.05 Bonferroni post test.
comparable between the two subregions \( p=0.301 \), the lower AP threshold in lwDR neurons resulted in a significantly lower difference between the resting membrane potential and the AP threshold (RMP-AP Thr), a measure I term “activation gap” \( p=0.033 \), (Fig. 2.2C). The AHP of lwDR 5-HT cells had a larger amplitude \( (p=0.034) \) and a longer duration, measured at half amplitude (AHP \( t_{1/2} \), \( p=0.001 \), Table 2.1).

In 5-HT neurons, influx of Ca\(^{2+}\) during the AP repolarizing phase results in a characteristic shoulder which increases AP duration (Aghajanian and Vandermaelen, 1982; Vandermaelen and Aghajanian, 1983). In addition, increased intracellular Ca\(^{2+}\) activates Ca-dependent K-channels that shape the AHP of 5-HT neurons (Aghajanian, 1985; Freedman and Aghajanian, 1987); the amplitude and duration of the late component of the AHP are enhanced by calcium-induced calcium release (CICR) from intracellular stores (Pan et al., 1994). However, because electrophysiological characterization of 5-HT neurons has historically focused on midline raphe neurons in the rat, it is unknown whether Ca\(^{2+}\) contributes to the AP and AHP in lwDR 5-HT neurons and whether the differences in the shape of lwDR AP and AHP from the vmDR neurons could be explained by a difference in calcium influx.

To test this, I assessed active membrane properties in vmDR and lwDR 5-HT neurons before and after bath application of the Ca-channel blocker CdCl\(_2\) or a selective blocker of Ca-dependent small conductance K-channels (SK channels), apamin. Occasional neurons demonstrated an obvious shoulder during the repolarization phase of the AP and did show a reduction in AP duration in the presence of 100\( \mu \)M CdCl\(_2\) (data not shown). However, there was no significant effect of CdCl\(_2\) on average AP duration in
either the vmDR (n=5, p=0.799) or the lwDR (n=5, p=0.184, Fig. 2.2F or Supplemental Fig. 2.S2). Furthermore, the average AP duration of lwDR 5-HT neurons was still longer than vmDR neurons in the presence of CdCl$_2$ (1.8 ± 0.1, n=5 vmDR vs 2.5 ± 0.2, n=5, lwDR, p= 0.004). There was no effect of CdCl$_2$ on AP threshold or AP amplitude (data not shown). Likewise, 100nM apamin had no significant effect on AP duration, AP threshold, or AP amplitude (data not shown).

Supplemental Figure 2.S2. Ca-channel blockers had a larger effect in lwDR 5-HT neurons. (A) Raw data traces demonstrate that CdCl$_2$ (100µM) reduced the AHP amplitude of both vmDR and lwDR 5-HT neurons. In the vmDR a small early component of the AHP persisted while the early component of the AHP was completely attenuated in the lwDR (scale bar 20mV, 50ms on left; 20mV, 0.5ms on right). (B) Raw data traces show that apamin (100nM) also reduced the AHP amplitude of 5-HT neurons but had a smaller effect, as summarized in Fig. 2.2F (scale bar 20mV, 50ms on left; 20mV, 0.5ms on right).

The shape of AHPs was indeed modulated by Ca$^{2+}$, as the presence of a either CdCl$_2$ or apamin decreased the AHP amplitude in both subregions, with significantly larger effects in the lwDR (Fig. 2.2F and Supplemental Fig. 2.S2, two way ANOVA p = 0.0082, main effect of subregion). Irrespective of the subregion, the effect of apamin on
AHP amplitude was significantly smaller than the effect of CdCl₂ (Fig. 2.2F, two way ANOVA p = 0.0013, main effect of drug type). The effect of CdCl₂ was significantly larger in lwDR neurons than in vmDR 5-HT cells (Fig. 2.2F, Bonferroni post test p < 0.05) such that the average AHP amplitudes of the two subregions were nearly equalized in the presence of CdCl₂ (18.3 ± 1.6mV in vmDR vs 15.1 ± 1.0mV in lwDR, t-test p = 0.120).

More specifically, the late component of the AHP mediated by CICR (Pan et al., 1994) was absent in both vmDR and lwDR neurons in the presence of the Ca-channel blockers (Supplemental Fig. 2.S2). In the vmDR a small amplitude early component of the AHP persisted, which was shorter in duration with a faster rise toward resting membrane potential than was observed in the control condition (Supplemental Fig. 2.S2). This early component of the AHP was completely attenuated by CdCl₂ in the lwDR but not by apamin (Supplemental Fig. 2.S2). Even a supramaximal concentration of apamin (300nM) failed to recapitulate the effect of CdCl₂ on AHP amplitude (data not shown).

Collectively, these data suggest that there are apamin-insensitive Ca-channels that contribute to the AHP of 5-HT neurons and these channels may be more abundant in the lwDR.

These data confirm that, in the mouse, Ca²⁺ influx contributes to the shape of the AHP in lwDR 5-HT neurons as it does in vmDR 5-HT neurons. The larger reduction seen in lwDR AHP amplitude produced by Ca-channel blockers parallels the larger AHP amplitude seen under baseline conditions and suggests an enhanced contribution of Ca²⁺ to the shape of AHPs of lwDR neurons compared to vmDR neurons.

Frequency-intensity plots, constructed from the mean firing frequency in response to square current pulses of increasing amplitude, showed that lwDR 5-HT cells
had steeper slopes (i.e. increased gain) than vmDR 5-HT cells \( (p=0.042, \text{Mann-Whitney test, Fig. 2.2D, 2.2E}) \). In addition, lwDR neurons demonstrated increased excitability compared to vmDR neurons in that they reached higher firing rates given the same magnitude of input (Fig. 2.2D). This difference was significant at current steps ranging from 40 to 80pA (Fig. 2.2D).

**Pharmacologic blockade of synaptic input supports the intrinsic nature of distinctive membrane characteristics**

The increase in excitability of lwDR 5-HT neurons is likely due to an array of active and passive membrane properties that are presumed to be intrinsic to the recorded neurons. However, in the slice preparation, 5-HT neurons receive both glutamatergic and GABAergic input (Pan and Williams, 1989; Liu et al., 2000; Lemos et al., 2006). It is unknown whether spontaneous synaptic input to 5-HT neurons may affect membrane characteristics. Thus, I conducted an additional experiment to verify that differences between vmDR and lwDR membrane properties were indeed independent of synaptic input. I measured passive and active membrane properties after bath application of 10\( \mu \)M DL-AP-5, 20\( \mu \)M bicuculline, 10\( \mu \)M CGP 55845, and 10\( \mu \)M DNQX to block synaptic input mediated by NMDA, GABA\( _A \), GABA\( _B \), and AMPA receptors respectively. After blockade of synaptic input, lwDR 5-HT cells maintained their differences in active and passive cell characteristics compared to vmDR neurons (Table 2.2) as well as their higher excitability in F-I plots (20pA: 0.0 ± 0.0 vs 1.6 ± 0.5 Hz, \( p=0.007 \); 40pA: 0.0 ± 0.0 vs 3.5 ± 1.1 Hz, \( p=0.005 \); 60pA: 0.8 ± 0.5 vs 5.4 ± 1.2 Hz, \( p=0.007 \); 80pA: 2.12 ± 0.8 vs 6.7 ± 1.7 Hz, \( p=0.007 \), vmDR versus lwDR respectively).

Furthermore, there were no significant differences in passive membrane properties
between synaptic blockade and the control condition in vmDR neurons (n=4) or lwDR neurons (n=5, data not shown). This suggests that the synaptic input that is present in the in vitro slice preparation cannot account for the increased excitability of lwDR 5-HT neurons and that differences in excitability are due primarily to the differences in their intrinsic membrane properties.

**5-HT neurons of the lwDR demonstrate faster in vitro firing rates**

The increased excitability of lwDR 5-HT neurons suggests that lwDR cells would have faster baseline firing rates in the intact brain. Although the noradrenergic input that drives DR 5-HT firing activity in vivo is severed in the slice preparation, we can examine 5-HT neuron firing rates in vitro using \( \alpha_1 \)-adrenergic agonists (Vandermaelen and Aghajanian, 1983). Extracellular recordings of YFP-labeled 5-HT neuron AP firing rates were obtained in raphe slices following bath application of 1.0µM phenylephrine HCl (Fig. 2.3). In the vmDR, 5-HT neurons fired at a rate of 1.1 ± 0.3 Hz (n=7, Fig.2.3), which is consistent with several reports of in vivo firing rates of vmDR 5-HT neurons (Aghajanian and Haigler, 1974; Allers and Sharp, 2003). However, lwDR 5-HT neurons demonstrated significantly faster firing rates (2.2 ± 0.4 Hz, n=6, \( p=0.042 \), Fig. 2.3). This suggests that the differences in intrinsic excitability of DR 5-HT neurons directly affects the in vitro firing rate and likely contributes to differences in 5-HT cell firing rate and 5-HT output in vivo.
Figure 2.3. The firing rate of lwDR 5-HT neurons in vitro is faster than that of vmDR 5-HT neurons. (A) Extracellular single unit recordings after bath application of 1.0μM phenylephrine HCl show the firing rate of a vmDR 5-HT neuron (scale bar 0.2mV, 2.56s; inset scale bar 0.2mV, 2.6ms). Insets show the characteristic features of the action potential waveform. Differences in amplitude were attributed to electrode position. (B) Summary graph depicting the average firing rate (mean ± SEM) of recorded vmDR and lwDR neurons. The firing rate of lwDR neurons (n=6) was significantly higher than that of vmDR neurons (n=7).

Auto-receptor mediated responses of vmDR and lwDR 5-HT neurons

The 5-HT₁A autoreceptor-mediated response is a defining characteristic of vmDR 5-HT neurons but has yet to be characterized in lwDR 5-HT neurons. It is unknown whether there are differences in autoreceptor responses that may contribute to the increased excitability of lwDR 5-HT neurons. During current clamp recordings at the neuron’s resting membrane potential, bath application of the 5-HT₁₇R agonist 5-CT (100nM) was applied following assessment of membrane characteristics. 5-CT elicited a measurable membrane hyperpolarization and decrease in membrane resistance in all recorded 5-HT neurons, except 1 vmDR 5-HT neuron (Fig. 2.4). Each hyperpolarization was within the linear range of the V-I plot generated for each neuron (data not shown). The magnitude of the hyperpolarization in vmDR and lwDR neurons is shown in Fig. 2.4B. Because the lwDR responses were not normally distributed, the non-parametric
Mann-Whitney U test was used to compare the two groups, revealing no significant difference in 5-HT$_{1A}$R-mediated hyperpolarization ($p=0.182$). Membrane resistance, measured by the change in membrane potential elicited by a periodic 30pA current pulse, was used to quantify change in the resistance imparted by the G-protein coupled K-channels that mediate the 5-HT$_{1A}$R response (Fig. 2.4A, C). The average change in resistance in vmDR 5-HT neurons was -192.1 ± 18.2 MΩ (baseline resistance 415.2 ± 28.4 MΩ, n=13) while the average change in lwDR 5-HT cells was -340.3 ± 65.1 MΩ (baseline resistance 544.1 ± 58.5 MΩ, n=12); there was no significant difference (Mann-Whitney Test, $p=0.383$). However, the variance of lwDR 5-HT$_{1A}$R-mediated responses was significantly larger than that of vmDR responses (F-test, $p<0.0001$, Fig. 2.4C).

Responses within the lwDR were distributed into two major groups according to the magnitude of the 5-CT-induced change in resistance, where large responses were above the mean and small responses below the mean. The response of a single cell that was near the mean was not included in either group in further analysis. Within the lwDR, small 5-HT$_{1A}$R-mediated responses (109.8 ± 17.0 MΩ, n=6) were significantly smaller than those measured in the vmDR ($p=0.013$) while large responses were significantly larger (572.6 ± 17.6 MΩ, n=6, $p<0.0001$). While those cells with large and small responses did not differ according to any other parameters, there was a significant correlation between larger 5-HT$_{1A}$R-mediated responses and more caudal localization within the lwDR (Fig. 2.4D, Spearman’s $r = -0.617$, $p=0.025$). In addition, larger 5-HT$_{1A}$ autoreceptor responses were also correlated with higher initial membrane resistance (Spearman’s $r = 0.571$, $p=0.041$) and larger AHP amplitude (Spearman’s $r=0.610$, $p=0.027$). Other than the 5-CT-induced change in resistance, no parameter was
Figure 2.4. 5-HT<sub>1A</sub> receptor mediated responses in 5-HT neurons. (A) Graph depicting the membrane resistance before (Ctrl) and after (5-CT) bath administration of 100nM 5-CT in vmDR and lwDR recordings. 5-CT decreased membrane resistance in both vmDR and lwDR 5-HT neurons. Inset: Raw data trace of membrane hyperpolarization and decreased resistance generated by administration of 5-CT (scale bar 20mV, 50ms). (B) Graph depicting the distribution of the change in membrane potential, i.e., membrane hyperpolarization, for all recorded neurons following administration of 5-CT. (C) Graph depicting the distribution of the change in membrane resistance induced by 5-CT in the vmDR and the lwDR. In the lwDR the change in resistance was distributed into two major groups. ### p<0.001, F test for differences in variance. (D) Correlation between the change in resistance and position along the rostral-caudal axis for lwDR neurons. The magnitude of the 5-CT response in lwDR cells was correlated to rostral-caudal position where cells with the largest 5-CT responses were located in the caudal regions of the DR. Rostral-caudal levels indicated in Fig. 2.1. Correlation determined by Spearman’s test.
correlated to rostral-caudal position in the lwDR. No correlation between the magnitude of the 5-HT₁₆R-mediated response and rostral-caudal position or initial membrane resistance was seen in the vmDR (rostral position: \( r = 0.09, p = 0.762 \); initial membrane resistance: \( r = 0.196, p = 0.563 \)).

To ensure that 5-HT₁₆R-mediated responses in the lwDR were mediated by the same underlying inward rectifying potassium current seen in previous studies (Innis et al., 1988; Okuhara and Beck, 1994), voltage ramps were conducted in several cells to measure current elicited before and after 5-CT administration. The 5-CT activated current was determined by subtracting the 5-CT response from the baseline current and the reversal potential for the 5-CT-induced current was determined by the x-intercept of the plot of activated current versus input voltage (data not shown). The reversal potential was comparable in the vmDR \((-78.9 \pm 1.3, n = 4\)) and the lwDR \((-82.8 \pm 5.0, n = 4\) \( p = 0.481 \)). Given the junction potential of approximately +15mV (see Methods), the reversal potentials lie close to the theoretical equilibrium potential for K⁺ \((-99.1mV,\) determined by the Nernst equation). This suggests that the G-protein coupled inward rectifying K-channels responsible for 5-HT₁₆R responses in other brain regions (Innis et al., 1988; Okuhara and Beck, 1994) also underlie the responses measured in both vmDR and lwDR neurons.

Because 100nM 5-CT elicited changes in resistance that ranged from -64.6 to -647.5 MΩ, I wanted to know if this broad range of responses represented differences in the concentration-response curve of individual neurons. First, to determine whether 5-HT₁₆R-mediated responses desensitize in the mouse DR, repeated doses of 5-CT were administered and responses measured by both current clamp and voltage clamp.
recordings in 5-HT neurons. While the 5-HT1aR-mediated response did not desensitize in some cells, the responses of several 5-HT neurons in both the vmDR and lwDR did desensitize. In the vmDR, 2 of 3 5-HT neurons demonstrated a degree of desensitization where the 2nd response was on average 81.4 ± 4.8% of the first response. In the lwDR, 2 of 3 5-HT neurons demonstrated a degree of desensitization where the 2nd response was on average 82.3 ± 8.3% of the first response (data not shown). These results precluded repeated application of 5-CT to obtain data for the construction of concentration-response curves in individual cells.

Distinguishing characteristics are found amongst another stress-activated subpopulation of DR neurons.

I hypothesize that the distinctive intrinsic membrane properties described above are a crucial part of the mechanism that makes lwDR neurons more excitable in slice preparations and preferentially activated by stressors in the intact brain. I therefore expect that other subpopulations of 5-HT neurons activated by stressors would have intrinsic membrane properties that are similar to that of lwDR 5-HT cells. Rostral vmDR 5-HT neurons are activated by several stressors, albeit it to a lesser extent than lwDR 5-HT neurons (Bouwknecht et al., 2007; Commons, 2008; Hale et al., 2008). To understand whether intrinsic membrane properties may also contribute to rostral vmDR activation, I analyzed the correlation between vmDR cellular characteristics and the position of the recorded neuron along the rostral-caudal axis. Indeed, a more rostral position within the vmDR was positively correlated with a smaller activation gap, larger tau, longer AHP duration, and most notably, increased firing rate elicited by 80 pA of current (Fig. 2.5) and larger gain (Spearman’s r = -0.562, p=0.012, data not shown).
There was also a trend towards a correlation between rostral position and larger resistance (Pearson’s \( r = -0.373, p=0.115 \)) as well as lower AP threshold (\( r = 0.396, p=0.116 \)).

![Figure 2.5](image)

**Figure 2.5.** Rostral position was correlated to several intrinsic membrane properties of vmDR 5-HT neurons. Graphs demonstrate the correlation between vmDR membrane properties and rostral-caudal position as defined in Fig. 2.1. Rostral position was correlated to (A) faster firing rates in response to an 80pA input current step, (B) smaller magnitude activation gap, (C) larger tau, and (D) longer AHP t 1/2. See Results for abbreviations. Correlation determined by Pearson’s test in A, B, D and by Spearman’s test in C due to the non-Gaussian distribution of tau values.

I then conducted a direct comparison between lwDR cells, rostral vmDR neurons (levels 1-2 in Fig. 2.1), and the remaining mid-caudal vmDR neurons (levels 3-6 in Fig. 2.1). Two-way ANOVA with Bonferroni post-tests were used to evaluate the frequency intensity plots while one-way ANOVA analyses with Tukey-Kramer post-tests
Figure 2.6. Unlike mid-caudal vmDR 5-HT neurons, rostral vmDR 5-HT neurons were comparable to lwDR 5-HT neurons according to several parameters. Graphs demonstrate the comparison between all lwDR neurons, rostral vmDR neurons (levels 1-2), and mid-caudal vmDR neurons (levels 3-6, see Fig. 2.1). (A) The frequency-intensity plot demonstrates that rostral vmDR cells had increased excitability and gain that were comparable to lwDR cells and were distinct from mid-caudal vmDR cells. *** p < 0.001, lwDR vs mid-caudal vmDR, two-way ANOVA Bonferroni post-test; ## p < 0.01, ### p < 0.001, rostral vmDR vs mid-caudal vmDR, two-way ANOVA Bonferroni post-test. (B) The activation gap and (C) tau values of lwDR and rostral vmDR cells differed significantly from mid-caudal vmDR cells. (D-G) For several parameters, lwDR cells differed significantly from mid-caudal vmDR cells but rostral vmDR cells did not differ from either group. These included (D) resistance, (E) AP threshold, (F) AHP amplitude, and (G) AHP t 1/2. (H) The bimodal distribution of 5-CT responses was unique to the lwDR while rostral vmDR neurons remained comparable to mid-caudal vmDR neurons. (I) Both rostral and mid-caudal vmDR neurons remained distinct from lwDR neurons in AP duration. One-way ANOVA and Tukey-Kramer post-test used for B-I. *p<0.05, **p<0.01, ***p<0.001 obtained from post tests.
were used for all other comparisons. The frequency-intensity plot of rostral vmDR cells closely resembled lwDR cells, indicating both an increased excitability and increased gain when compared to mid-caudal vmDR neurons (Fig. 2.6A). In both rostral vmDR and lwDR neurons, the firing rate during an 80pA input current step was larger than mid-caudal vmDR cells ($p=0.005$, rostral vs mid-caudal $p<0.05$, lwDR vs mid caudal $p<0.01$, Bonferroni post-tests), as was the gain ($p=0.001$, rostral vs mid-caudal $p<0.05$, lwDR vs mid caudal $p<0.01$, Tukey-Kramer post-tests). Both lwDR and rostral vmDR neurons demonstrated a smaller activation gap and larger tau than mid-caudal vmDR cells (Fig. 2.6B, C). For several parameters, rostral vmDR cells did not differ from either group while lwDR cells remained significantly different from mid-caudal vmDR cells; these parameters included resistance, AP threshold, AHP amplitude, and AHP $t_{1/2}$ (Fig. 2.6D-G). The AP duration and 5-HT$_{1A}$R-mediated responses did not differ between rostral and mid-caudal vmDR cells and remained distinguishing factors for lwDR 5-HT neurons (Fig 6H, I). Thus, the rostral vmDR cells share many of the same membrane properties that typified lwDR 5-HT neurons, including increased excitability, larger gain, smaller activation gap, and larger tau than mid-caudal vmDR neurons. The common parameters of lwDR and rostral vmDR neurons suggest a common physiological mechanism for the stress-induced neuronal activation that has been observed in both subpopulations.

**DISCUSSION**

Behavioral studies have previously demonstrated that stressors activate lwDR 5-HT cells more than vmDR 5-HT cells (Commons, 2008; Johnson et al., 2008); however a mechanism underlying this difference has not been identified. In this study, whole-cell
patch clamp electrophysiology experiments demonstrated that lwDR 5-HT neurons possess distinctive membrane properties that make lwDR neurons more excitable than vmDR neurons. The increased excitability is intrinsic to the lwDR 5-HT neuron, i.e. independent of synaptic input, and is accompanied by faster firing rates demonstrated in vitro.

**Active and passive membrane properties likely contribute to excitability**

Among passive membrane properties, the resistance was notably larger in lwDR neurons and likely contributed to the increased excitability seen in frequency-intensity plots. In addition the larger tau seen in lwDR cells would result in greater summation of post-synaptic potentials, such as would occur with the full complement of synaptic connections of the intact brain.

Analysis of active membrane properties revealed that the AP threshold was more hyperpolarized in lwDR neurons; this could also contribute to increased excitability. The lower AP threshold in lwDR 5-HT neurons may be due to differences in the kinetics of the voltage-gated Na-channels or in the subtypes of Na-channels expressed in the lwDR. Because AP amplitude was measured relative to threshold, the lower AP threshold of lwDR neurons contributed to the larger AP amplitude; however it would not account for the larger AHP amplitude. In the presence of Ca-channel blockers, lwDR 5-HT neurons demonstrated a larger decrease in AHP amplitude than vmDR neurons. In addition, the effect of apamin was only a fraction (approx 53%) of that of CdCl₂, which may indicate that apamin-insensitive Ca-channels also contribute to the AHP in 5-HT neurons. Based on these data I propose that the influence of Ca²⁺ influx is greater in the lwDR than in the vmDR, possibly due to an increased Ca²⁺ conductance,
increased membrane expression of Ca-channels, or increased release of calcium-induced calcium release. The AHP of lwDR neurons may therefore be more sensitive to signaling that alters intracellular Ca^{2+} levels, including but not limited to neurotransmitter receptor pathways (Pan et al., 1994).

Blockade of SK channels in midline DR 5-HT neurons results in burst firing patterns in vivo and irregular firing patterns in vitro (Rouchet et al., 2008; Crespi, 2009). The data presented above are the first evidence that apamin-sensitive SK channels are present in lwDR neurons in the mouse where they likely contribute to regular firing patterns as they do in midline 5-HT neurons of the rat. In the presence of apamin a small early component of the AHP persisted in all recorded neurons. The early component also persisted in vmDR recordings following CdCl₂ application but was attenuated by CdCl₂ in the lwDR. This finding is consistent with midline DR studies where apamin only blocks the late component of the AHP (Pan et al., 1994) and blocks only 80-90% of the post-activation outward current that underlies the AHP (Freedman and Aghajanian, 1987). One explanation for the persistent early AHP component is that some of the channels involved in the falling phase of the action potential 1) drive the early component of the AHP, 2) are not Ca-dependent, and 3) are more abundant in vmDR neurons than in lwDR neurons. Another explanation is that an inward tail current, as has been demonstrated in midline DR neurons (Penington and Kelly, 1993), is larger in lwDR neurons and attenuates the early AHP in this subpopulation. Further experiments will help distinguish between these possibilities.

The physiological significance of differences in membrane properties between the DR subregions was demonstrated by in vitro recordings of firing rate. Extracellular
single unit recordings demonstrated that lwDR 5-HT neurons have a faster firing rate than vmDR 5-HT neurons. This suggests that the increased intrinsic excitability of lwDR 5-HT neurons has significant functional consequences, resulting in increased sensitivity to the noradrenergic signals required for the characteristic firing pattern of 5-HT neurons (Baraban and Aghajanian, 1980, 1981; Vandermaelen and Aghajanian, 1983). The increased excitability of lwDR neurons was independent of synaptic input, though additional neurotransmitters may modulate 5-HT activity in vivo. With the full complement of synaptic connectivity in the intact brain, the increased gain of lwDR 5-HT neurons would translate into increased 5-HT output in response to excitatory modulating input from stress-activated afferents.

Correlative analysis of vmDR 5-HT neurons throughout the rostral-caudal axis revealed that vmDR cells with more rostral position tended to be more excitable. In addition, the rostral subset of vmDR neurons was largely comparable to lwDR neurons and significantly more excitable than the mid-caudal subset of vmDR 5-HT neurons. Rostral vmDR 5-HT neurons are a stressor-activated subpopulation (Bouwknecht et al., 2007; Commons, 2008; Hale et al., 2008) thought to be part of the ascending mesostriatal serotonergic system related to behavioral arousal and motor function (Steinbusch et al., 1981; Loughlin and Fallon, 1982; Imai et al., 1986b; Imai et al., 1986a; Lowry, 2002; Waselus et al., 2006). Interestingly, the distinctive membrane properties of rostral vmDR, i.e., firing rate, activation gap, AHP duration and tau, were among the distinguishing properties of lwDR 5-HT neurons. This finding further supports the hypothesis that this constellation of membrane characteristics, which typifies lwDR 5-HT neurons, forms the physiological mechanism underlying increased responsiveness to
stress. Furthermore, increased AP duration and large 5-CT responses were restricted to lwDR cells and absent in rostral vmDR cells, suggesting that prolonged AP duration and enlarged 5-HT$_{1A}$R-mediated responses do not play a crucial role in the increased excitability of 5-HT neurons.

**Heterogeneous subpopulations of DR 5-HT neurons are topographically organized**

The heterogeneity of 5-HT neurons has become more evident over recent years. Extracellular *in vivo* recordings in the DR paired with juxtacellular labeling has revealed small subsets of 5-HT neurons with unique properties, including fast firing rates, bursting activity, and synchronization with theta rhythm (Allers and Sharp, 2003; Kocsis et al., 2006; Hajos et al., 2007). Previously we found differences between median raphe and vmDR 5-HT membrane properties in the rat (Beck et al., 2004). The results reported here are the first to be obtained from immunohistochemically identified 5-HT cells in the mouse and provide additional evidence for the heterogeneity of 5-HT neurons. Subsets of neurons found mostly in the vmDR demonstrated rectification or $I_h$ currents (Supplemental Fig. 2.S1) that are typically seen in non-5-HT neurons (Beck et al., 2004). Thus, there is likely a topographic distribution of the channels that mediate these currents or differential post-translational modification of these channels within 5-HT cells.

Our experiments also highlighted a subset of lwDR neurons with larger 5-HT$_{1A}$R responses than those of vmDR 5-HT neurons. Correlation analysis showed that lwDR neurons demonstrating large membrane resistance and more caudal position were most likely to have large 5-HT$_{1A}$R-mediated responses. Neurons with large 5-HT$_{1A}$R responses may have increased receptor expression, efficacy of coupling to $G_{i/o}$ effector molecules,
and/or affinity to the agonist 5-CT. I could not obtain reliable concentration-response data from individual cells because of desensitization of the 5-HT_{1A}R mediated response. The desensitization is consistent with a previous study that demonstrated that 5-HT_{1A}R in the DR internalize when activated (Riad et al., 2001). Techniques other than those used in the above experiments may yield more useful insight as to the relative abundance of 5-HT_{1A}R in the lwDR as well as the efficacy of other components of the signaling pathway.

**Implications for the neural circuits that mediate stress**

Although a wide range of stressors preferentially activate lwDR neurons (Martinez et al., 1998; Chung et al., 2000; Martinez et al., 2002; Roche et al., 2003; Johnson et al., 2004; Gardner et al., 2005; Berton et al., 2007; Commons, 2008; Johnson et al., 2008), the data presented here fill the void in our understanding of why lwDR 5-HT neurons are more responsive. The intrinsic membrane properties of lwDR neurons contribute to a heightened gain, enabling lwDR 5-HT cells to convert converging input from a stress-activated circuit into greater output in projection regions that are responsible for physiological and behavioral responses to stressors.

A few tracer studies in the rat describe the afferent input to the DR with respect to the lwDR subregion or the rostral-caudal axis. The major limbic forebrain input to the DR extends from the lateral habenula and medial prefrontal cortex, regions that have a higher density of input to the lwDR than to the vmDR (Araki et al., 1988; Peyron et al., 1998; Varga et al., 2003). The locus coeruleus, long thought to be the primary driver of spontaneous firing in the raphe, exhibits input of equal density to the vmDR and lwDR (Kim et al., 2004). Inputs from the medial amygdala primarily innervate the vmDR,
whereas the lateral portion of the medial amygdala and the central amygdala innervate the lwDR (Lee et al., 2007). In addition fibers containing the stress hormone corticotropin releasing factor (CRF) course from the interfascicular region of the rostral vmDR, through the middle DR, towards the dorsolateral edge of the caudal lwDR (Kirby et al., 2000; Valentino et al., 2001; Waselus and Van Bockstaele, 2007). Thus, to complement their unique intrinsic physiology, there are also anatomical factors that make lwDR neurons well suited for integrating input known to be part of the neural circuitry that mediates stress, i.e., input from CRF fibers, the limbic forebrain, the central amygdala, and afferents with diffuse projections throughout the DR.

Although the DR is known for its forebrain serotonergic projections, only vmDR and dorsomedial neurons project to the cortex while lwDR projections are primarily to subcortical targets (Lowry et al., 2008). The lwDR descending output to the dorsolateral periaqueductal gray (dIPAG) and the rostral ventrolateral medulla (RVLM) is a crucial part of the circuitry that mediates the response to stressors. Serotonergic projections from the lwDR inhibit the dIPAG and RVLM, thereby attenuating sympathoexcitatory behavioral responses and decreasing vasopressor responses to stress (Beckett et al., 1992; Beckett and Marsden, 1997; Underwood et al., 1999; Bago and Dean, 2001; Bago et al., 2002; Johnson et al., 2004). The emerging hypothesis is that the lwDR 5-HT neurons are activated and help inhibit sympathetic excitation as part of the normative response to innocuous stressors. This circuit is dysregulated in a rat model of panic disorder where decreased activation of lwDR neurons accompanies excessive panic-like responses to a mild stressor (Johnson et al., 2008).
In summary, the increased intrinsic responsiveness of lwDR neurons distinguishes them from vmDR neurons and likely underlies both their increased in vitro firing rate and increased propensity to be activated by an array of stressors. Their anatomical location near putative stress related inputs in concert with increased intrinsic gain make lwDR 5-HT neurons particularly suited for generating output to sympathomotor targets and forebrain regions involved in adaptive responses to stress. Because the excitability of lwDR 5-HT neurons is an underpinning of the normative response to stressors, dysregulation of lwDR neurons likely contributes to the pathobiology of anxiety and other stress-related mood disorders.

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CHAPTER 3

Glutamatergic input is increased in lateral wing 5-HT neurons: the role of morphology and innervation

ABSTRACT

Characterizing glutamatergic input to dorsal raphe (DR) 5-HT neurons is crucial for our understanding of how the glutamate and serotonin systems interact in the healthy brain and contribute to psychiatric disorders such as anxiety, depression, and schizophrenia. Three markers of glutamatergic terminals, vGlut1, 2, and 3, are distributed throughout the rostral-caudal extent of the DR and may reflect relative inputs from regions such as the mPFC, lateral habenula, or local inputs from the DR, respectively. I found that in the mouse, punctate staining of vGlut2 was homogenous throughout the DR subregions while vGlut1 and vGlut3 demonstrate a slight decrease in density of puncta in areas where lateral wing (lwDR) 5-HT neurons are found. Whole-cell patch clamp recordings of sEPSCs recording revealed both presynaptic and postsynaptic differences in glutamatergic input to lwDR 5-HT neurons compared to ventromedial (vmDR) neurons. Despite the equal or decreased density of various types of glutamate terminals, lwDR 5-HT neurons had an increased frequency of synaptic glutamatergic input. Because the dendritic morphology of DR 5-HT neurons has never been quantified, it was unknown whether differences in sEPSC frequency might be due to differences in dendritic length. The morphology of lwDR 5-HT neurons was unique from vmDR neurons, including larger cell somas and longer dendrites with more extensive branching. The differences between
vmDR and lwDR dendrite number and dendrite length were specific for 2nd order and 3rd order branches. While increased sEPSC frequency was correlated with increased mean length of 2nd order branches in all recorded cells, this relationship was predominately seen in vmDR neurons and was not present when lwDR neurons were grouped separately. Thus, the increased dendritic complexity cannot fully explain the increased frequency of sEPSCs in lwDR neurons, as there were several lwDR cells for which this relationship did not hold true. This suggests that spontaneous glutamatergic input in the DR is the result of selective innervation of specific subpopulations of 5-HT neurons and is rooted in the activity of those glutamatergic neurons. The differences between vmDR and lwDR neurons in terms of glutamatergic activity and morphology, in concert with previously described differences in membrane characteristics (Crawford et al., 2010), provide a foundation for understanding the 5-HT-limbic circuit, how it regulates stress, and how it contributes to anxiety and other psychiatric disorders.

INTRODUCTION

The serotonin (5-HT) system has been implicated in stress circuits and numerous psychiatric disorders including anxiety, depression, Alzheimer’s, and schizophrenia. Because the majority of 5-HT neurons that project to the forebrain are housed in the dorsal raphe nucleus (DR) (Azmitia and Segal, 1978; Molliver, 1987), understanding the local circuitry of the DR has yielded mechanistic insight into stress circuits of the brain and the pathobiology of stress-related disorders. However the patterns of local excitatory synaptic inputs to DR neurons are still poorly understood. The 5-HT neurons of the DR exhibit a topographical distribution across the rostral-caudal axis and within the ventromedial (vmDR), dorsomedial (dmDR), and lateral wing (lwDR) subregions. The subregions of the DR have distinct patterns of afferent input, efferent target regions and intrinsic properties that likely contribute to unique functions in
stress circuits (Abrams et al., 2004; Johnson et al., 2004; Michelsen et al., 2007; Lowry et al., 2008; Crawford et al., 2010). While the vmDR subregion has been a common target due to the high density of 5-HT neurons found therein, the lwDR is a subregion that has been shown to have a unique role in stress circuits, due in part to the differences in afferent input, efferent projections, and neurochemical content (reviewed in Abrams et al. 2004, Johnson et al. 2004, Michelson et al. 2007, Lowry et al. 2008). In addition, a series of studies have demonstrated that among 5-HT neurons, stress-induced neuronal activation is more common in the lwDR than in the vmDR. While this is due in part to the increased intrinsic excitability of lwDR 5-HT neurons (Crawford et al., 2010), it is unknown whether excitatory synaptic modulation may contribute to the differential role of vmDR and lwDR neurons.

Glutamate is also implicated in the etiology of several psychiatric disorders including anxiety, depression and schizophrenia amongst several others (Cortese and Phan, 2005). While the cortex is a prime brain region where 5-HT influences glutamate output (Aghajanian and Marek, 2000; Hajos et al., 2003; Puig et al., 2005), another major site of interaction between the two neurotransmitter systems occurs within the DR, as glutamate forms an important influence over 5-HT activity (Pan and Williams, 1989; Adell et al., 2002) and can modulate 5-HT output to target brain regions (Tao and Auerbach, 1996; Tao et al., 1997; Tao and Auerbach, 2000; Celada et al., 2001; Tao and Auerbach, 2003). Tracer studies have delineated both cortical and subcortical sources of glutamatergic input to the DR (Lee et al., 2003). The glutamatergic input has been further elucidated upon the discovery of three isoforms of the vesicular glutamate transporter proteins (vGlut) that serve as presynaptic markers of glutamate input throughout the brain. The primary markers of glutamate terminals, vGlut1 and vGlut2, are present in diffuse puncta throughout the rat DR (Commons et al., 2005; Waselus and Van Bockstaele, 2007), though their distribution in the mouse DR is unclear. Previous studies have shown that
vGlut1 is a marker associated with glutamate terminals of axons that tend to originate from the cortex, including the medial prefrontal cortex (mPFC, reviewed in Fremeau et al., 2004 and Takamori, 2006). The marker vGlut2 is a marker of terminals of glutamate projections that tend to originate from subcortical regions, including midbrain regions outside of the DR (Hioki et al., 2010) and the lateral habenula (Barroso-Chinea et al., 2007), a prominent source of glutamate input to the DR that links the DR to the limbic forebrain (Wang and Aghajanian, 1977; Kalen et al., 1985; Araki et al., 1988; Peyron et al., 1998; Lee et al., 2003). The third isoform, vGlut3, is a glutamatergic marker that can be found in the axon terminals and cell soma of neurons that typically release other types of neurotransmitters, including brainstem 5-HT neurons (Fremeau et al., 2002; Gras et al., 2002; Fremeau et al., 2004a; Herzog et al., 2004). Several studies have shown that vGlut3 is found in DR 5-HT neurons of the rat (Gras et al., 2002; Hioki et al., 2004; Shutoh et al., 2008; Commons, 2009; Jackson et al., 2009; Hioki et al., 2010), Syrian hamster (Mintz and Scott, 2006), and mouse (Schafer et al., 2002; Amilhon et al., 2010). These reports suggest that while there are some non-5-HT neurons that express vGlut3, the colocalization of vGlut3 with 5-HT is extensive in the DR. However, one study conducted in rat (Amilhon et al., 2010) found subregional differences in vGlut3 expression whereby 82% of vmDR neurons that express TPH2 also express vGlut3 mRNA while only 4% of TPH2-expressing lwDR neurons express vGlut3 mRNA. Reports of vGlut3 protein in the cell bodies of 5-HT neurons tend to focus on midline DR subregions and especially the vmDR. It is unclear whether the lack of explicit description of vGlut3 protein in lwDR cell bodies is due to a paucity of immunoreactive cell bodies in that subregion, or due to a failure to fully characterize the lwDR subfield. Overall, it is unclear how vGlut protein are distributed throughout the rostral-caudal axis of the mouse DR and whether the distribution of vGlut proteins across DR subregions can predict relative glutamatergic synaptic input to 5-HT neurons.
Electrophysiology studies have helped us begin to understand glutamatergic neurotransmission in the DR on a functional level. Projections from the glutamate-rich mPFC and lateral habenula to both 5-HT and non-5-HT DR neurons have been demonstrated using in vivo electrophysiology techniques (Hajos et al., 1998; Varga et al., 2003). However, these studies did not distinguish between recordings in different DR subregions. Additional studies have revealed evidence of input to vmDR 5-HT neurons mediated by AMPA/kainate glutamate receptors in the in vitro slice preparation (Liu et al., 2002; Haj-Dahmane and Shen, 2005; Lemos et al., 2006; Kirby et al., 2007; Haj-Dahmane and Shen, 2009). However, glutamatergic synaptic input to lwDR neurons has never been characterized.

An understanding of the local circuitry of the DR subregions is crucial to our understanding of the interaction between the glutamate and 5-HT systems. However, as the majority of DR studies focus on midline structures, the relative glutamatergic input to lwDR 5-HT neurons remains poorly understood. Thus, to investigate how glutamatergic input to the lwDR differs from the vmDR, we set out to confirm the distribution of vGlut1, vGlut2, and vGlut3 in the lwDR subregion of the mouse brain in comparison to the vmDR. We then used whole-cell recordings in the slice preparation to investigate whether the patterns of immunostaining of glutamate terminals in the lwDR and vmDR could predict the levels of glutamatergic synaptic input to 5-HT cells in these subregions. Finally we sought to identify possible sources of the differences between lwDR and vmDR glutamatergic input to more fully understand the mechanism by which glutamate modulates the 5-HT system.

METHODS:
**Animals**

Adult male 5-HT-YFP mice or their wild-type littermates were used at 2-4 months of age. These mice contain 5-HT neurons that express yellow fluorescent protein (YFP) under the control of the 5-HT specific Pet-1 promoter (Scott et al., 2005). The membrane characteristics of YFP-labeled 5-HT neurons did not differ from wild type 5-HT neurons (Crawford et al., 2010). The mice were on a background containing predominantly C-57/Black6. Mice were housed in a standard animal facility with lights on 06:00 to 18:00. Animal protocols were approved by the Institutional Animal Care and Use Committee and were conducted in accordance to the *NIH Guide for the Care and Use of Laboratory Animals*.

**Immunohistochemistry**

Adult male mice were euthanized by pentobarbital via intraperitoneal injection, followed by transcardial perfusion of 20mL 0.9% saline then 50-60mL of 4% paraformaldehyde in phosphate buffer (PB), pH 7.4. Brains were postfixed in the same solution for 2 hours. Brains were then sunk for several days in 30% sucrose in phosphate buffer containing 0.1% (w/v) azide, frozen in dry ice and cut into 30μm-thick floating sections on a cryostat. Sections were washed in phosphate buffered saline (PBS), then blocked in a PBS blocking solution containing 0.03% (v/v) Triton-X, 0.04% (w/v) bovine serum albumin (BSA), and 0.1% (w/v) azide. Tissue was then incubated in primary antibody diluted in the blocking solution overnight at room temperature. Primary antibodies used were guinea pig anti-vGlut1 (1:1000, Millipore), guinea pig anti-vGlut2 (1:1000, Millipore), guinea pig anti-vGlut3 (1:1000, Millipore), and mouse anti-tryptophan hydroxylase (TPH, 1:500, Sigma). Tissue was then washed in PBS containing 0.03% (v/v) Triton-X, 0.04% (w/v) bovine serum albumin (BSA), then incubated for 2 hours in fluorescent secondary antibody diluted in the same solution. Secondary antibodies used included goat anti-guinea pig
Alexa Fluor 647 (1:200, Invitrogen) and donkey anti-mouse Alexa Fluor 488 (1:200, Invitrogen). Finally, sections were washed in PBS, mounted in 0.05M PB, air dried in the dark, and coverslipped with Fluormount G (Southern Biotech) or ProLong Antifade (Invitrogen) coverslipping medium. Dual immunohistochemistry was performed sequentially where vGlut staining was followed by TPH staining. Control experiments that omitted primary antibody yielded no staining (data not shown). Images were captured using a Leica DMR fluorescent microscope (Leica Microsystems, Bannockburn, IL) and OpenLab 3.0.9 software (Improvision, Lexington, MA). Confocal images of 30μm slices were obtained on an Olympus Fluoview FV1000 confocal microscope (Olympus, Center Valley, PA) using Fluoview confocal software (FV10-ASW v1.7, Olympus). Images were pseudocolored and optimized by adjusting levels using Adobe Photoshop 6.0 (Adobe Systems Incorporated; San Jose, CA).

Immunohistochemical identification of each neuron recorded in patch clamp configuration was completed as previously described (Beck et al., 2004; Lemos et al., 2006; Kirby et al., 2008). In brief, a standard immunohistochemistry protocol was used on 200μm-thick slices using mouse anti-TPH (1:200, Sigma) along with secondary donkey anti-mouse Alexa Fluor 488 (1:200, Invitrogen) and streptavidin-conjugated Pacific Blue (1:100, Invitrogen). TPH labeling of biocytin filled cells was imaged on a Leica DMR fluorescent microscope (Leica Microsystems) using OpenLab 3.0.9 software (Improvision) and confirmed on a Leica DMIRE2 confocal microscope (Leica Microsystems, Bannockburn, IL) using Leica confocal software (version 2.5, Leica Microsystems). The dendritic morphology of filled neurons was analyzed as described below.

**Whole-Cell Electrophysiology**

Electrophysiology recordings were conducted as previously described (Beck et al., 2004;
Lemos et al., 2006; Crawford et al., 2010). In brief, mice were sacrificed using decapitation. While submerged in a cold artificial cerebral spinal fluid (aCSF) solution where NaCl is replaced with sucrose (248 mM), the midbrain was rapidly dissected, blocked, and cut with a Leica VT1000s vibratome (Leica Microsystems, Bannockburn, IL) to generate 200µm thick brain slices. Slices were maintained in aCSF bubbled with 95%O<sub>2</sub>/5%CO<sub>2</sub> at 36°C for 1 hour and then at room temperature until used. Individual slices were then placed in a recording chamber and continuously perfused with 32-34°C aCSF solution bubbled with 95%O<sub>2</sub>/5%CO<sub>2</sub> with a solution flow rate of 1.5-2.0ml/min. The composition of the aCSF was (in mM): 124 NaCl, 2.5 KCl, 1.25 Na<sub>H</sub>2PO<sub>4</sub>, 2.0 MgSO<sub>4</sub>, 2.5 CaCl<sub>2</sub>, 10 dextrose, and 26 NaHCO<sub>3</sub>. Neurons were visualized using Nikon E600 (Optical Apparatus, Ardmore, PA) upright microscope fitted with a 40x water-immersion objective, DIC, and infrared filter. Ventromedial (vmDR) and lateral wing (lwDR) 5-HT neurons were targeted based on expression of YFP and later confirmed using immunohistochemical detection of the synthetic enzyme marker TPH. To visualize YFP-positive 5-HT cells for recording, a fluorescent lamp and yellow-fluorescence filter were used. The image was generated on a computer monitor using a CCD camera and Nikon Elements software (Optical Apparatus). Whole-cell recording pipettes fabricated on a Sutter Instruments pipette puller (P-97, Sutter Instrument, Novato, CA) had a resistance of 6-10MΩ when filled with an intracellular solution of 130mM Kgluconate, 5mM NaCl, 10mM Na-phosphocreatinine, 1mM MgCl<sub>2</sub>, 0.02mM EGTA, 10mM HEPES, 2mM MgATP, 0.5mM Na<sub>2</sub>GTP, and 0.1% biocytin (pH 7.3). Voltage clamp recordings were conducted at a holding potential of -60mV such that spontaneous excitatory post-synaptic currents (sEPSCs) were downward and spontaneous inhibitory post-synaptic currents (sIPSCs) were upward; only downward events were analyzed. In experiments where the AMPA-kainate receptor blocker DNQX (20μm) was added at the end of the recordings, all downward events were abolished (data not shown). Recordings were
collected online with a Multiclamp 700B amplifier, Digidata 1320 A/D converter, and Clampex 9.0 software (Molecular Devices, Union City, CA). Membrane properties of the cell were monitored as was the access resistance of the patch pipette during recordings that lasted a minimum of 20 minutes. Recordings were discarded if access resistance increased over 5x the initial access resistance noted prior to patching onto the cell. Any cells that showed physiological signs of instability, baseline holding currents below -30pA, or resting membrane potential above -50mV were excluded from data analysis. Reported values do not incorporate a junction potential of approximately +15mV, as calculated using Clampex software. Following the experiment, DR slices were fixed for 2-3 hours with 4% paraformaldehyde and processed for immunohistochemistry.

**Electrophysiology Data Analysis**

Current clamp recordings using visualized whole cell patch clamp techniques were analyzed using Clampfit 9.0 (Molecular Devices). Resting membrane potential (RMP) and membrane resistance were measured as previously described (Beck et al., 2004; Crawford et al., 2010). Membrane input resistance was determined from the slope of voltage-current plot generated using current pulses ranging from -100 to 0pA. The time constant tau was determined for each cell by fitting a single exponential function to the first 200ms of the membrane potential response to a -20pA hyperpolarizing input current step. MiniAnalysis (Synaptosoft; Decatur, GA) was used to analyze sEPSC data as has been reported in the past (Lemos et al., 2006). The parameters for synaptic event analysis were optimized for each cell with the threshold set beyond the maximum values of the all-points noise histogram for a segment of the trace containing no detectable synaptic events. The threshold generally ranged from 5 to 8pA. The MiniAnalysis program provides a summary table for each cell containing
values for mean and median frequency, amplitude, rise time (10-90%), decay time (50%) and event half-width. For each cell, 200 randomly chosen events were manually filtered to exclude multiple peaks and combined to obtain an averaged EPSC for each cell. The decay tau reported was the tau obtained from the single exponential fit of the 10-90% decay of the averaged EPSC. The area-under-curve of the averaged EPSC was the charge per EPSC event; this value was multiplied by the frequency to obtain mean phasic current. For each parameter, group averages were obtained by compiling the mean and/or median values for each cell. Histograms and cumulative probability plots describing all events from all cells in each group were compared using the Kolmogorov-Smirnov test. To determine differences in the populations of events recorded in the 2 subregions, OriginPro 8.1 software (Origin Lab Corporation, Northampton, MA) was used to generate Gaussian fits of event histograms using the Peak Analyzer function. The fit was generated by choosing 1-5 peaks in order to minimize the reduced Chi squared value.

Neuron Morphology Analysis

Biocytin-filled neurons filled during whole-cell patch clamp recording were processed for immunohistochemical detection as described above. Tissue slices were imaged on a Leica DMR fluorescent microscope to confirm the subregional location of the neuron and TPH content. Cells were then imaged on a Leica DMIRE2 confocal microscope (Leica Microsystems) using Leica confocal software (version 2.5, Leica Microsystems). For confocal image stacks, slices were imaged using a 20x objective lens and optical z-slices of 0.8µm thickness were taken to capture the entire extent of the dendritic tree. The confocal stacks were then analyzed using Neurolucida (v8 and v9, MBF Bioscience Inc., Williston, VT). The cell soma and dendrite were traced using the autoneuron feature and then manually edited if necessary to ensure accurate
tracing. Branch order was assigned using centrifugal branch order. Neurolucida Explorer was then used to obtain measurements of traced neurons. The neuron soma characteristics included enclosed volume, surface area, mean length, total cross-sectional area, and mean cross-sectional area. The dendrite parameters included the number of nodes, i.e. branch points, the number of ends, the total dendrite length and the mean dendrite length. In addition data was obtained for number of branches, total length, and mean length according to branch order. A dendrogram was generated for each cell to obtain the length of the longest segment and the longest soma-to-tip dendritic length. Sholl analysis was performed using radial shells at 20μm intervals. Polar histograms with 30 degree bins were obtained for each cell to ascertain directional preferences for dendrite orientation in the medial-lateral and dorsal-ventral directions. Because morphology analysis was conducted in 200μm-thick brain slices, some of the axons and dendrites of analyzed cells could have been severed. While this problem applies to all neurons, it could potentially lead to underestimation of true dendritic length, especially of longer dendrites. Despite these caveats, observed differences between vmDR and lwDR 5-HT neuron morphology were an important, relevant proxy for 5-HT neurons in the intact brain. It should be noted that recordings targeted neurons deep within the 200μm-thick slice and obviously severed dendrites were uncommon in confocal images of filled neurons.

**Statistics**

Reported values are mean ± standard error of the mean (s.e.m.); p values were generated using Students t-test unless otherwise noted and p< 0.05 was deemed significant. Outlying values were excluded from group means but are denoted in scatter plots. After exclusion of outliers, any data that showed non-Gaussian distribution according to the D’Agostino and Pearson omnibus normality test were compared using non-parametric statistical
tests. Additional statistical analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA) and OriginPro 8.1 (Origin Lab Corporation).

**Drugs**

All chemicals for making the sucrose aCSF, aCSF, and extracellular electrolyte solution were purchased from Fisher Scientific (Pittsburgh, PA). All chemicals for the intracellular electrolyte solution were purchased from Sigma-Aldrich (St. Louis, MO). Bicuculline and DNQX were purchased from Tocris (Ellisville, MO).

**RESULTS**

*The distribution of vGlut proteins in the mouse DR varies between subregions*

The distribution of vesicular glutamate transporter proteins 1, 2, 3 (vGlut1, 2, and 3) was examined in the mouse DR. Dual immunohistochemistry was conducted to enable the use of the distribution of TPH neurons as an indicator of DR subregions and rostral-caudal level. Immunostaining for vGlut1 revealed punctate staining throughout every subregion of the DR, though an increased density of the punctate staining was present in bilateral regions of the ventral lwDR, just dorsal to the major longitudinal fasciculus (Fig. 3.1A). While this region of increased vGlut1 puncta partially overlapped with TPH immunopositive 5-HT neurons, the majority of 5-HT neurons were just dorsal to this region (Fig. 3.1A). Higher magnification confocal images demonstrated that despite the increased density in the ventral lwDR, the puncta surrounding the cluster of 5-HT cell bodies was actually lower in density than that seen surrounding cell bodies in the vmDR (Fig. 3.2A). Because reports of vGlut1 staining in the rat
Figure 3.1. **Immunostaining of vGlut proteins in the mouse DR.** Fluorescent microscopy was used to generate the top panels showing the vGlut protein and the bottom panels shows the overlay of vGlut (magenta) and TPH (green).

A) Immunohistochemistry revealed the punctate pattern of vGlut1 staining through the rostral-caudal axis of the DR. Note the increased density of vGlut1 staining in the lwDR, ventral to the cluster of 5-HT neurons. B) The staining for vGlut2 puncta was dense and homogenous throughout the DR. C) Staining for vGlut3 was present in both puncta and in cell soma that were immunopositive for TPH. Images taken at 20x. Scale bar 104.0 μm.
Figure 3.2. Confocal images of vGlut proteins and TPH in the vmDR and lwDR subregions. High magnification (60x)

Confocal images were taken to demonstrate the variations in punctate staining across the vmDR and lwDR subregions. In A, B, and C vGlut is shown in magenta, TPH in green. Panels to the left demonstrate staining in the vmDR while panels to the right show the lwDR. A) Confocal imaging confirms the lower density of vGlut1 punctate staining surrounding 5-HT neurons of the lwDR. B) Puncta expressing vGlut2 appeared to have the same density in the vmDR and the lwDR. C) Cell bodies and puncta expressing vGlut3 are shown. Note that several cell bodies also contained punctate staining. Comparison of the images of vGlut3 staining demonstrated that punctate staining of vGlut3 was slightly less dense in the lwDR than in the vmDR, though it is unclear if that was due to fewer terminals in the lwDR or
due to fewer cell bodies containing punctate staining. The majority of vGlut3 immunopositive neurons also expressed TPH, though a few were TPH-negative. Scale bar 42.4 μm.

described homogenous puncta throughout the DR (Commons et al., 2005; Waselus and Van Bockstaele, 2007) this may represent a species difference.

Immunostaining of vGlut2 revealed homogeneous puncta that were much denser than vGlut1 puncta. Throughout all of the DR subregions, staining was present everywhere except the spaces occupied by cell bodies (Fig. 3.1B). Higher magnification confocal images revealed a clearer demonstration of the punctate nature of the staining (Fig. 3.2B). This was consistent with previous reports of vGlut2 staining in the rat (Commons et al., 2005; Waselus and Van Bockstaele, 2007).

Detection of vGlut3 revealed immunopositive cell bodies and punctate staining of presumed axon terminals throughout the neuropil (Fig. 3.1C). Puncta that were less dense than vGlut1 or vGlut2 staining were present throughout the DR and appeared less dense in the lwDR than in the vmDR (Fig. 3.2C). This was consistent with previous reports of punctate vGlut3 staining in the rat (Commons, 2009). While the intensity of vGlut3 and TPH were not equal within every cell, the majority of the cell bodies containing vGlut3 also contained TPH in both the vmDR and lwDR. There were a few neurons that were singly stained for vGlut3 or TPH (Fig. 3.2C and data not shown). Although a colocalization of vGlut3 and 5-HT fibers in the supraependymal plexus just ventral to the aqueduct has been reported in the caudal DR of the rat, (Commons, 2009), this was not seen with our TPH immunostaining of the mouse.

Overall, the patterns of vGlut protein expression in the mouse resemble those seen in the rat. The degree of glutamatergic input to the lwDR may be different from that of the vmDR because vGlut3 terminals were lower in density in the lwDR and vGlut1 terminals were lower in the dorsal lwDR in regions surrounding 5-HT neurons. However, immunohistochemistry alone
cannot determine the relative influence of glutamatergic inputs on vmDR and lwDR 5-HT neurons.

**Lateral wing 5-HT neurons exhibit presynaptic and postsynaptic differences in glutamatergic synaptic input**

Glutamate input to 5-HT neurons mediated by AMPA-kainate receptors is present in the *in vitro* slice preparation (Liu et al., 2002; Haj-Dahmane and Shen, 2005; Lemos et al., 2006; Haj-Dahmane and Shen, 2009). However, as previous studies have only targeted the vmDR, it is unclear how local glutamatergic inputs may differ between the lwDR and vmDR subregions of the DR. Electrophysiology recordings were conducted in a total of 36 cells from 20 male 5-HT-YFP mice. Cells expressing YFP were targeted for electrophysiology recording and later confirmed using immunohistochemical detection of TPH. Outlying values occurring more than 2 standard deviations from the mean were identified and excluded from group means. Neurons of the lwDR had a higher resistance than vmDR neurons (*p*=0.024, Table 3.1, Fig. 3.3D).

Voltage clamp recordings of spontaneous excitatory post-synaptic current (sEPSC) were conducted in vmDR 5-HT neurons (n=19 neurons in 12 mice) and lwDR 5-HT neurons (n=17 neurons in 11 mice), revealing similarities and several striking differences (Table 3.1, Figs. 3.3 and 3.4). Events recorded in vmDR and lwDR neurons were comparable in amplitude (Table 3.1).

**Table 3.1. Results of sEPSC recordings in DR 5-HT neurons**

<table>
<thead>
<tr>
<th>Subregion</th>
<th>Frequency (Hz)</th>
<th>Decay Tau (ms)</th>
<th>Average Amplitude (pA)</th>
<th>Median Amplitude (pA)</th>
<th>Average Rise Time (ms)</th>
<th>Median Rise Time (ms)</th>
<th>Charge per EPSC (pA/ms)</th>
<th>Mean Phasic Current (pA)</th>
<th>Input Resistance (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vmDR (n=19)</td>
<td>13.8</td>
<td>1.7</td>
<td>16.9</td>
<td>13.5</td>
<td>1.3</td>
<td>1.2</td>
<td>47.9</td>
<td>0.6</td>
<td>417.2</td>
</tr>
<tr>
<td>lwDR (n=17)</td>
<td>24.0</td>
<td>1.2</td>
<td>18.1</td>
<td>14.2</td>
<td>1.3</td>
<td>1.1</td>
<td>39.0</td>
<td>1.0</td>
<td>568.5</td>
</tr>
</tbody>
</table>

| t-test P value | 0.009 | 0.0001 | 0.397 | 0.508 | 0.428 | 0.224 | 0.070* | 0.081** | 0.024 |

* *p* value generated by Mann-Whitney test; ** *p* value generated by Welch’s test due to unequal variances.
Figure 3.3. Recordings of sEPSC events revealed increased frequency of glutamatergic synaptic input to lwDR 5-HT neurons. A) Raw data traces of sEPSC activity were obtained from representative vmDR (left trace) and lwDR (right trace) 5-HT neurons. Scale bar 512ms, 20pA. B) The all-events amplitude histogram shows the division between noise (in grey) and analyzed events (black) for the vmDR cell featured in A. The inset demonstrates large amplitude events using an enlarged scaling of the y-axis. C) The all-events amplitude histogram for the lwDR cell featured in A. D) The average amplitude was comparable between vmDR (black) and lwDR (gray) neurons. The graph on the left shows the average amplitude values obtained from each recorded cell. The graph on the right shows the cumulative probability plot of all events from all recorded cells, demonstrating only a slight difference in amplitudes of the vmDR and lwDR event populations (K-S test, \( p = 0.006 \)). E) The average frequency of sEPSC events was higher in lwDR neurons as shown in the graph on the left of averaged frequency for each recorded cell. The graph on the right demonstrates the significant difference in cumulative probability plots of inter-event interval, whereby lwDR events were shifted towards lower inter-event intervals. Outlying values denoted by the symbol “X” were not included in group means but are indicated in scatter plots to demonstrate the heterogeneity of 5-HT neurons.
3.1), although a comparison of the cumulative frequency histogram of all recorded events revealed an increased number of large-amplitude events in the lwDR compared to events in the vmDR (K-S test \( p = <0.006 \), Fig. 3.3E). The rise times of sEPSC events in the vmDR and lwDR were also comparable (Table 3.1). In recordings of both subregions, there was a small, negative correlation in both subregions between rise time and amplitude such that the largest amplitude events had the fastest rise times and smaller amplitude events had slower rise times (vmDR: \( R = -0.057, \ p=2.2E-16 \), lwDR: \( R = -0.043, \ p=7.9E-11 \), Spearman’s correlation test). This indicated that a small degree of electrotonic filtering persisted in our voltage clamp recordings (Ling and Benardo, 1999).

There were also several differences in glutamatergic synaptic events recorded in vmDR and lwDR neurons. A monoexponential fit of the averaged post-synaptic event was generated for each cell, revealing a faster decay tau in lwDR neurons (1.2 \( \pm 0.1 \)ms) compared to vmDR neurons (1.7 \( \pm 0.1 \)ms, \( p=0.0001 \), Fig. 3.4B). This difference in kinetics was explored further by looking at the distribution of decay time-50% of all the events recorded in each subregion. The histogram revealed a population of fast-decaying events centered at a decay time-50% of 0.62ms that was larger in the lwDR (32.3% of all events, Fig. 3.4D) than in the vmDR (14.7% of all events, Fig. 3.4C). This resulted in a significant leftward shift of the lwDR cumulative frequency curve of decay time-50% (K-S test \( p<0.0001 \), Fig. 3.4E). The faster decay in lwDR neurons resulted in narrow events and a trend towards a smaller charge flux per event (47.9 \( \pm 3.6 \)pAms vmDR vs 39.0 \( \pm 2.1 \)pAms lwDR, Mann-Whitney \( p=0.070 \), Fig. 3.4B). However this did not reduce the mean phasic current to lwDR neurons, due to distinctions in the frequency of sEPSC events. The increased frequency of sEPSC events in the lwDR neurons (24.0\( \pm 3.1 \)Hz) compared to that of vmDR neurons (13.8\( \pm 2.0 \)Hz, \( p=0.009 \), Fig. 3.3F) was, perhaps, the most interesting distinction between the two subregions. Amongst the values of sEPSC frequency obtained from vmDR
cells, there were two outlying values (64.4 and 84.5 Hz) that were over 2 and 3 standard deviations from the mean, respectively. In the lwDR there was one outlying value (56.4 Hz) that

Figure 3.4. sEPSC events recorded in lwDR 5-HT neurons demonstrate faster decay kinetics than events recorded in vmDR neurons. A) Enlarged averaged events show differences in decay kinetics between the two cells featured in Fig. 3.3. Scale bar 3.0ms, 4.0pA. B) Summary scatter plots show group means of several sEPSC parameters. The rise time was comparable between vmDR (black) and lwDR (gray) neurons. The decay tau obtained from exponential fit of the averaged event (see Methods) was smaller in the lwDR, indicating faster decay kinetics. The charge per sEPSC event was comparable between subregions ($p = 0.084$). C) The all-events histogram containing decay time-50% from all recorded events in vmDR neurons was analyzed using a Gaussian fit to obtain peaks centers and percent area under the curve for each peak. D) The all-events histogram of decay time-50% of lwDR demonstrated a larger peak centered at 0.62ms and smaller peak centered at 1.78ms compared to the distribution of vmDR decay times. The inset features the vmDR (black) and lwDR (gray) histograms overlaid for easier comparison. E) This resulted in a significant leftward shift of lwDR decay times towards faster decay times. Outlying values denoted by the symbol “X” were not included in group means but are indicated in scatter plots to demonstrate the heterogeneity of 5-HT cells.
was over 2 standard deviations from the mean. Because these cells may make up a unique population of neurons, these cell were excluded from group means but are indicated in Fig. 3.3F. The increased frequency of glutamatergic input to lwDR neurons was surprising in light of our immunohistochemical data whereby lwDR 5-HT neurons were surrounded by a decreased density of glutamatergic puncta in comparison vmDR 5-HT neurons. Thus, we sought to determine whether the recorded lwDR neurons had other properties that might help explain the differences in recorded glutamatergic input.

**Lateral wing 5-HT neurons exhibit unique morphological characteristics including larger cell soma and increased dendritic complexity**

We analyzed the morphology of 5-HT neurons that were filled during whole-cell electrophysiology recordings. The cell bodies of lwDR 5-HT neurons were significantly larger than vmDR neurons in surface area and total cross-sectional area with a trend towards a larger enclosed volume (Table 3.2, Fig. 3.6C). These results were consistent with previous descriptions of cell bodies in the DR (Steinbusch et al., 1981). Because the dendrites of 5-HT neurons of DR subregions have never been described, we also performed analysis of dendritic characteristics of vmDR and lwDR 5-HT cells. On average, lwDR 5-HT cells featured a larger number of dendrites with more nodes and longer mean branch length (Table 3.2, Fig. 3.6D-E). Looking more closely at dendritic branching structure, lwDR neurons have a larger number of secondary and tertiary branches.

Table 3.2. Results of morphology analysis of 5-HT neurons

<table>
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<tr>
<th>Subregion</th>
<th>Enclosed Volume</th>
<th>Surface Area</th>
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<th>Longest Tip to Tip Length</th>
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<th>Number of Trees</th>
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<th>Number of Tails</th>
<th>Total Dendrite Length</th>
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| vmDR (n=26) | 1821.0 | 757.6 | 43.2 | 112.6 | 170.3 | 149.6 | 3.5 | 1.7 | 5.5 | 438.9 | 128.6 | Avg
| lwDR (n=23) | 2473.2 | 954.9 | 46.2 | 133.9 | 287.7 | 242.5 | 3.4 | 4.8 | 8.4 | 901.9 | 293.4 | Avg
| Mean P-value | 0.005 | 0.046 | 0.130 | 0.072 | 4.6E-04 | 0.002 | 0.100 | 0.083 | 2.2E-06 | 5.3E-05 | 1.2E-05 |
Figure 3.5. The 5-HT neurons of the lwDR possess somatic and dendritic morphology that is distinct from vmDR neurons. A representative tracing demonstrates the morphology of a vmDR neuron (A) and a lwDR neuron (B) as generated by Neurolucida. The corresponding polar histogram to the right of the trace demonstrates the dendritic length in each direction, using 30-degree bins. Scale bar 100 µm. C) The polar histograms were averaged across groups to generate an average polar histogram, showing orientation preferences for dendrites of vmDR neurons (black) and lwDR neurons (grey). The longest dendrites of vmDR neurons were oriented in the dorsal-ventral direction while the longest dendrites of lwDR neurons did not show a particular preference. The 5-HT neurons of the lwDR had large cell soma surface area (D), longer mean dendritic length (E), and higher numbers of nodes, i.e. branch points (F). G) The increased number of branches seen in lwDR neurons were limited to 2nd and 3rd order dendrites. H) The increased mean branch length of lwDR neurons was limited to 2nd order branches. I) Dendritic tree complexity was quantified by Sholl analysis, revealing that lwDR neurons had more intersections and longer dendritic length in region ranging from 40 to 160 µm from the cell soma. Outlying values denoted by the symbol “X” were not included in group means but are indicated in scatter plots to demonstrate the heterogeneity of 5-HT neurons.
branches, resulting in longer total branching length that is specific for secondary and tertiary branches (Fig. 3.5G, 3.5H). Sholl analysis revealed that lwDR dendritic trees are significantly more complex than the dendritic tress of vmDR neurons (Fig. 3.6I), as was predicted by the higher number of nodes and ends measured in lwDR dendrites. Polar histograms were constructed for the dendritic tree of each cell and then averaged for each group to look for trends in orientation of dendrites within the coronal section. The polar histograms of all analyzed cells positioned to the left of the midline (vmDR n=5, lwDR n=3) were transformed to provide meaningful comparisons with the remaining cells. Directional analysis revealed that vmDR dendrites tended to be oriented in a dorso-ventral direction while lwDR dendrites demonstrated no preferred orientation (Fig. 3.5C). While most dendrites of vmDR neurons were contained wholly within the vmDR, the longest dendrites of vmDR 5-HT cells putatively pointed towards the MR and dmDR. While most dendrites of lwDR neurons were contained wholly within the lwDR, the longest dendrites of lwDR neurons tended to be oriented in the dorso-lateral, dorsal, medial, and ventromedial directions (Fig 3.5C). These dendrites putatively pointed towards the ventrolateral periaqueductal gray, the aqueduct, the dmDR, and the dorsolateral edge of the vmDR. A few lwDR dendrites ramified in the supraependymal plexus just ventral to the aqueduct.

The dendritic complexity of 5-HT neurons may contribute in part to the increased frequency of glutamatergic synaptic input.

Differences in synaptic input to a neuron can sometimes be attributed to differences in the size of the dendritic tree (Hajos and Mody, 1997). Thus, the increased frequency of sEPSC observed in many lwDR neurons could be due to a longer, more complex dendritic tree. To examine this relationship more closely we looked for correlations in morphological characteristics and sEPSC parameters obtained from the same neurons.
Neurons were analyzed both as a single group of DR neurons and as separate DR subregions to assess correlations between measures of dendritic length and sEPSC frequency. There was no correlation between sEPSC frequency and total dendrite length or mean dendrite length. However, a significant correlation was found between the mean length of 2nd order branches and sEPSC frequency. This correlation between frequency and mean length of 2nd order branches was strong among vmDR neurons but was not significant in lwDR neurons, suggesting that the increased branch length of lwDR neuron, which is specific for 2nd order branches, cannot account for the increased sEPSC frequency seen in all lwDR neurons. There was no correlation between sEPSC rise time and the number of nodes, i.e. dendritic branch points, when all DR neurons were grouped together, or when vmDR neurons were separated. However, there was a significant correlation between slow rise times and a larger number of nodes that was limited to lwDR neurons. This has implications for differential levels of electrotonic filtering in lwDR neurons (see Discussion).
Because morphological analysis revealed that the largest distinction in the dendritic length of lwDR neurons was seen in 2nd order branches, I decided to analyze secondary branch length in particular. In fact there was a significant correlation between the mean length of secondary branches of all recorded neurons and sEPSC frequency ($R = 0.540, p = 0.009$, Fig. 3.6A). This correlation was strong in the vmDR subpopulation ($R = 0.809, p = 0.008$, Fig. 3.6B) but was not significant in the lwDR subpopulation ($R = 0.422, p = 0.151$, Fig. 3.6B). As shown in the 2D scatter plot comparing sEPSC frequency and secondary branch length (Fig. 3.6A), there were several cells, including outliers that did not fit the correlation. This suggests that while the longer length of secondary dendritic branches may contribute to the increased frequency seen in many neurons, it cannot completely explain the increased sEPSC frequency seen the lwDR.

Further analysis of sEPSC measurements and morphology revealed an additional relationship, present only in lwDR neurons, between the average rise time of recorded events and measures of increased dendritic branching. Slower rise times of lwDR sEPSC events were correlated to an increased number of nodes ($R = 0.598, p = 0.031$, Fig. 3.6D) along with increased number of ends ($R = 0.623, p = 0.023$), and increased number and total length of 3rd order branches (data not shown). This relationship between rise time and number of dendritic nodes was not present in the vmDR ($R = 0.039, p = 0.893$, Fig. 3.6D) or when all cells were grouped together ($R = 0.066, p = 0.744$, Fig. 3.6C). There were no correlations with number of ends, number of 3rd order branches, or total length of 3rd order branches in the vmDR alone or when all cells were grouped together (data not shown). Nevertheless the relationship between the number of nodes and rise time provides evidence for electrotonic filtering in DR 5-HT neurons and suggests that filtering may preferentially affect those lwDR neurons with the longest dendrites and most branch points.
Topographical position within the lwDR subregion is correlated to particular features of sEPSCs and morphology

Because immunohistochemistry revealed specific topographical distributions of putative synaptic inputs, I then examined the relationship between topographical position within the DR and the array of sEPSC measurements and morphology characteristics obtained from each cell. An atlas of recorded neurons was then used to assign a pair of coordinates to every cell with the origin of the x-y coordinate system positioned at the base of the aqueduct. The x, y coordinate of any cell positioned on the left half of the brain slice was transformed to allow comparison with the majority of cells on the right side of the brain slice. Because neither sEPSC parameters nor morphology measurements demonstrated a relationship with rostral-caudal position (data not shown), cell position was collapsed across the rostral-caudal axis. Pearson’s correlation analysis was used to examine the relationship between y-values and various sEPSC and morphology parameters. There was no correlation found between the x-coordinate, i.e. medial-lateral position, and any parameter. In the vmDR there was a significant correlation between a more negative y-coordinate, i.e. more ventral position, and the time constant tau (R=-0.722, p = 0.004) as measured in current clamp configuration (see Methods). In the lwDR a significant correlation was found between ventral position and several parameters including increased amplitude (R = -0.731, p = 0.003, Fig. 3.7A,D), increased charge per event (R = -0.534, p = 0.049), and longer total dendrite length (R = -0.458, p = 0.049, Fig. 3.7B,D). These parameters of lwDR neurons are represented in a bubble map in Figure 3.7 where the total dendrite length of each recorded cell is the radius of each data point. The color of each data point corresponds to the average sEPSC amplitude from that cell. The cell was positioned according to its x, y coordinate mapped over an atlas image of the mid-rostral-caudal DR (Paxinos and Watson, 1997), as shown in Fig. 3.7C. The same graph was mapped over an image of the mouse DR near the same rostral-
caudal level where TPH immunostaining more accurately demonstrates the distribution of 5-HT neurons (Fig. 3.7D). Though there are a few cells that do not fit the pattern, these data demonstrate that the dorso-ventral position of lwDR neurons is associated with both the amplitude of sEPSC events and the dendritic length of the postsynaptic neuron. This suggests a

Figure 3.7. Topographical position of lwDR 5-HT neurons is correlation to increased sEPSC amplitude and longer total dendrite length. A) After assigning x,y coordinates to the positions of recorded neurons, a negative correlation demonstrated the relationship between negative y-coordinate, i.e. ventral position within the lwDR, and increased sEPSC amplitude. B) A similar correlation was found between more ventral position within the lwDR and increased total dendrite length. C) This relationship was visualized in a bubble map overlaying an atlas image of the mid-rostral-caudal DR. Each point is positioned according to its x, y coordinate; the size of the point represents the total dendrite length of the cell and the color represents the average sEPSC amplitude recorded in that cell. Note that in the lwDR, larger points contain the darkest colors and are situated more ventral than the other cells. D) The same graph is mapped over an image of mouse DR tissue where TPH staining more accurately demonstrates the distribution of 5-HT neurons in the mid-rostral-caudal DR.
functional consequence of the topographical organization of the lwDR that facilitates communication between a subset of lwDR neurons and putative synaptic partners in the ventral lwDR and neighboring regions.

**DISCUSSION**

To understand how the glutamate and serotonin systems interact at the level of the DR, immunohistochemical detection of glutamatergic terminals was confirmed in the mouse DR, revealing subtle differences in punctate staining the vmDR and lwDR subregions. Though vGlut2 puncta were homogenous through the DR, vGlut1 and vGlut3 puncta were less dense in the region surrounding lwDR 5-HT neurons. I then compared spontaneous glutamatergic input in the coronal midbrain slice. Surprisingly, the relative density of vGlut punctate staining was not predictive of sEPSC frequency, as lwDR neurons had increased sEPSC frequency compared to vmDR neurons, in addition to faster decay kinetics of sEPSC events. Morphological assessment revealed the lwDR neurons had longer, more complex dendrites that may contribute, in part, to the increase sEPSC frequency. However, correlations between sEPSC frequency and dendrite morphology were not significant in the lwDR, indicating that for several lwDR 5-HT neurons, mechanisms other than increased dendrite length contribute to the increased excitatory synaptic input. Within the lwDR, ventral position was associated with increased sEPSC amplitude and longer dendrite length, suggesting that topographical location of 5-HT neurons likely contributes excitatory input of lwDR neurons as well.

*Methodological considerations for electrophysiology recordings of lwDR neurons.*

Voltage clamp recordings of sEPSC activity are optimal under conditions that enable clamping of the entire cell, including distal neuronal processes; however some degree of filtering due to incomplete space clamp is inherent in even the best of voltage clamp conditions.
(Spruston and Johnston, 2008). Our data revealed a negative correlation between event rise time and event amplitude, which suggests a degree of electrotonic filtering of events occurring in distal dendrites. This could be due to the cable properties of the cell and complex dendritic branching, as branch points can increase filtering (Rall et al., 1967; Ling and Benardo, 1999).

Correlations between longer, more extended trees and slow rise times were present in the lwDR and not the vmDR, suggesting that filtering of distant events may be a more relevant phenomenon for signal processing in lwDR neurons, which is not surprising given their larger cell bodies and longer, more complex dendritic trees. Events arising from distal sites along the lwDR dendrite arrive at the soma (and my recording electrode) with smaller amplitudes and slow rise times, though passive properties, synaptic clustering, the size of dendritic spines, and the presence of voltage-dependent elements may also influence the degree of filtering and the summation of coincident post-synaptic potentials (Jaslove, 1992; Koch and Segev, 2000). My data suggest that a greater degree of filtering of distal events occurs in lwDR 5-HT neurons than in vmDR neurons, which may have led to an underestimation of the amplitude of lwDR events originating at distal dendrites. This also suggests that the extended dendritic tree and increased number of nodes of lwDR neurons contributes to their ability to filter input and may provide increased resolution of the array of synaptic input that the lwDR neuron receives in the intact brain.

In addition, the degree of filtering seen in lwDR neurons with the longest, most branched dendrites underscores the importance of proximity to putative synaptic inputs. A correlation was found between ventral position within the lwDR, increased dendrite length, and increased sEPSC amplitude, data that support the hypothesis that topographical position within the DR has a functional consequence. The dorsal-ventral position of lwDR neurons is related to their dendrite length and the amplitude of the sEPSC events they receive, which has implications
for putative synaptic partners and the postsynaptic receptors that modulate the strength of that synaptic input. It may be that ventrally located lwDR neurons may express more AMPA receptors which enable cells to increase the strength of glutamatergic input, despite the potential filtering of distal events bestowed by the increased length of their dendrites. Alternatively, the more ventral position situates the lwDR neuron in closer proximity to major glutamatergic inputs, resulting in events whose amplitudes are not diminished due to filtering. Glutamatergic synapses that are situated closer to the ventral lwDR cell soma would then be more effective at making the neuron fire. Interestingly, immunohistochemical detection of vGlut protein revealed topographic evidence for such inputs in the ventral lwDR.

**Functional significance of vGlut protein in the mouse DR**

With a few subtle differences, the patterns of immunostaining of vGlut proteins throughout the rostral-caudal extent of the DR is consistent with what has been reported previously in the rat (Commons et al., 2005; Waselus and Van Bockstaele, 2007; Commons, 2009). The marker vGlut1 is associated with glutamate terminals of axons that tend to originate from the cortex, including the mPFC (Fremeau et al., 2004a; Takamori, 2006). In addition, the distribution of vGlut1 throughout the brain is correlated to regions with low probability of glutamate release, and thus may mark terminals possessing an increased potential for synaptic plasticity (Fremeau et al., 2004b; Fremeau et al., 2004a). Punctate labeling of vGlut1 terminals was present throughout the DR but the density was highest in the lwDR, just ventral to the cluster of 5-HT neurons found in the lwDR (Fig. 3.1). This density of vGlut1 terminals may be a putative source of synaptic input for the more ventral lwDR neurons that demonstrated large amplitude events (Fig. 3.7). The location of the increased density of vGlut1 puncta was also consistent with the finding that projections from the mPFC tend to synapse on GABA DR
neurons (Jankowski and Sesack, 2004) which are preferentially located in the lateral regions of the DR (Stamp and Semba, 1995; Day et al., 2004). The increased density of vGlut1 puncta may represent projections from mPFC to GABA neurons of the DR. Given the role of mPFC input to the DR in stress-related mood disorders (Maier et al., 2006), the potential for increased synaptic plasticity at vGlut1 synapses in the DR is worthy of further investigation.

Because the lwDR is not a common target of DR studies, the nature of mPFC input to lwDR 5-HT neurons remains unclear. Although studies underscore the mPFC projection to GABA neurons of the DR, they identify a small minority of mPFC projections synapse with 5-HT neurons (Jankowski and Sesack, 2004) and can excite 5-HT neurons (Varga et al., 2001; Varga et al., 2003). Unfortunately, because these studies fail to account for the subregional organization of the DR, it is unknown whether any of those 5-HT neurons excited by mPFC projections may lie in the lwDR. Further investigation as to the forebrain inputs to lwDR 5-HT neurons is needed for a comprehensive understanding of how the glutamate and serotonin systems interact in stress circuits and stress-related disorders.

The dense and homogenous punctate staining of vGlut2 demonstrated input that could potentially originate from the lateral habenula (Barroso-Chinea et al., 2007), a prominent source of glutamate afferents to the DR that link the DR to the limbic forebrain (Wang and Aghajanian, 1977; Kalen et al., 1985; Araki et al., 1988; Peyron et al., 1998; Lee et al., 2003). Because vGlut2 it is also found in midbrain regions outside of the DR such as the periaqueductal grey (Hioki et al., 2010), it presents a good candidate for inputs that may be active within the slice.

Detection of vGlut3 revealed staining throughout the DR of cell bodies along with puncta that were slightly less dense in the lwDR. The majority of vGlut3 was found in TPH immunopositive neurons, although a few cells were singly labeled for either TPH or vGlut3. These patterns were consistent with what has been previously reported (Commons et al., 2005;
vGlut3 can play a role in glutamate neurotransmission, as has been demonstrated in auditory cortex (Ruel et al., 2008; Seal et al., 2008). In addition, a recent study of the vGlut3 knockout mouse (Amilhon et al., 2010) suggests that vGlut3 also increases vesicular filling in subsets of 5-HT terminals. However the contribution of neurons expressing vGlut3 to glutamatergic neurotransmission in the DR and its projection regions remains unclear.

**Contributing factors and functional consequences of lwDR glutamatergic input.**

Glutamatergic synaptic input to DR 5-HT neurons may originate from any of the three types of glutamate terminals identified by immunohistochemistry. While it is not known the degree to which subtypes of glutamatergic terminals contribute to excitatory input to DR neurons, based on the density of vGlut puncta alone, one might expect lwDR 5-HT cells to receive equal or less glutamatergic input than vmDR 5-HT cells. Surprisingly, lwDR neurons received a higher frequency of glutamatergic synaptic input than vmDR neurons. Identifying factors that contribute to this difference in glutamate input was the subject of further experiments that included analysis of the morphology of recorded cells. Compared to vmDR neurons, lwDR 5-HT neurons had a large cell soma with longer, more complex dendritic trees. The increased dendrite number and length of lwDR neurons was especially apparent in 2nd and 3rd order branches. While the total dendrite length of all branches was not correlated to sEPSC frequency, there was a significant correlation amongst all recorded cells between the mean length of 2nd order branches and sEPSC frequency. This correlation was strong in vmDR subpopulation but failed to reach significance in the lwDR subpopulation. This suggests that while the length of 2nd order branches may contribute to increased synaptic input in some neurons, especially those of the vmDR, it cannot account for the increased input seen in many lwDR neurons. Rather, glutamatergic input to lwDR neurons is predominantly governed by
selective innervation of lwDR cells and the spontaneous activity of the afferent neurons and axon terminals.

The increased glutamate input to lwDR neurons has several functional ramifications. Compared to vmDR neurons, lwDR 5-HT demonstrate an increased propensity to be activated by stressors due in part to their increased intrinsic excitability (Crawford et al., 2010). In our previous studies I have found that lwDR 5-HT neurons demonstrate a higher excitability and gain that is independent of synaptic input and thereby attributable to intrinsic membrane properties. The increased glutamatergic input to lwDR neurons reported here likely synergizes with increased gain of this subpopulation, enabling an increased responsivity to stress-activated afferents and increased output in target regions that are crucial for the behavioral and physiological responses to those stressors. The recorded lwDR neurons showed sEPSCs with faster decay times than those of vmDR neurons, possibly due to a population of AMPA receptors made up of subunits with faster closing kinetics. However the narrow events and trend towards a decreased charge flux did not decrease the mean phasic current in lwDR neurons, suggesting that the increased sEPSC frequency ensures that lwDR receive more glutamatergic input than vmDR neurons. It is likely no coincidence that the highest glutamatergic input is going to those 5-HT cells whose intrinsic properties make them especially suited to integrate input. The extended dendritic tree of lwDR 5-HT neurons provides a likely milieu for integration of a variety of inputs from the intact brain, e.g., glutamate input as characterized above, inhibitory input from DR GABA neurons, as well as noradrenergic input, CRF input, and other modulators of 5-HT activity. Subregion-specific differences in excitatory input and glutamate receptor modulation translate to differential regulation of 5-HT output in brain regions targeted by the vmDR and lwDR. There is a presumed degree of overlap in projection regions of the two subregions but studies that explicitly include the lwDR are helping us to understand the distinctions. While
vmDR 5-HT neurons project to regions such as the mPFC, ventral hippocampus, and amygdala, lwDR projections tend to be limited to subcortical regions and include projection to midbrain and hindbrain regions responsible for panic and escape behaviors as well as vasoconstriction responses mediated by the sympathetic system (Abrams et al., 2004; Johnson et al., 2004; Michelsen et al., 2007; Lowry et al., 2008). Thus, subregional differences in glutamate modulation of 5-HT neuron activity likely results in specific, diverse effects on 5-HT output to limbic brain regions and brainstem centers responsible for behavioral and physiological components of a range of psychiatric disorders. Continued investigation into the distinct circuitry that governs lwDR activity is needed to understand how a heterogeneous 5-HT system modulates neurotransmission in regions ranging from sympathetic response centers to higher order processing regions of the brain.

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102


Brain Res 1174:53-65.
CHAPTER 4

Towards an understanding of the pathophysiology of anxiety: social stress alters the inhibitory input to serotonin neurons.

ABSTRACT

Anxiety disorders are prominent in human and veterinary medicine yet the underlying mechanism is poorly understood. Because serotonin (5-HT) is thought to play a prominent role, my goal was to understand the changes in the 5-HT system that underlie anxiety and other stress-related disorders. Five days of social defeat induced a multifaceted anxiety-like phenotype in intruder mice which included anxiety-like behavior in the elevated plus maze and open field tests, increased stressor-induced grooming, an increase in bladder weight, and an increase in heart weight. To begin to understand the changes in the serotonin system induced by this model of anxiety, electrophysiology recordings were conducted in dorsal raphe (DR) brain slices from intruder mice and control mice after five days of social defeat. Assessment of the intrinsic physiology of 5-HT neurons of the ventromedial (vmDR) and lateral wing (lwDR) subregions revealed no significant effect of social defeat. However, DR 5-HT neurons are regulated by a wide array of inputs, including excitatory glutamatergic input and inhibitory GABAergic input. Comparisons between intruders and controls suggest that while there were few differences in input mediated by glutamate AMPA receptors, GABAergic input was altered in a topographically specific way. The frequency and amplitude of baseline sIPSCs is decreased in the vmDR of intruders while the kinetics of sIPSC events were altered in the lwDR of intruders. These data suggest that chronic social stress induces changes in GABA release and altered
expression of GABA receptor subunits in subpopulations of 5-HT neurons. Overall, social defeat decreased GABAergic current input to vmDR neurons but had little effect or may even increase the GABAergic current input to lwDR neurons. The distinct stress-induced changes in inhibitory input to vmDR or lwDR neurons suggest that the 5-HT output in brain regions targeted by either subfield is differentially affected in anxiety disorders.

INTRODUCTION

Anxiety disorders are among the most prevalent of psychiatric disorders, yet the underlying mechanisms are poorly understood. Because serotonin (5-HT) is known for its role in the etiology of anxiety and in anxiolytic medical treatment, our goal is to understand the changes in the 5-HT system that underlie this and other stress-related mood disorders. The dorsal raphe (DR) is likely to play a crucial role in anxiety, as the center of the majority of the 5-HT projections to the forebrain. In addition, DR 5-HT activity is a sufficient and necessary component of the effects of uncontrollable stress a common element of many models of anxiety and the related disorder depression (Maier and Watkins, 2005). An understanding of how DR 5-HT neurons are modulated in the face of chronic uncontrollable stress is central to understanding the mechanisms underlying stress-related disorders like anxiety.

The DR is made up of dorsomedial (dmDR), ventromedial (vmDR), and lateral wing (lwDR) subregions. Though the vmDR is a well-characterized, common target of DR studies, the lwDR contains 5-HT neurons with distinct patterns of connectivity to other brain regions (Abrams et al., 2004; Johnson et al., 2004; Lowry et al., 2008). In addition, recent studies have demonstrated that lwDR 5-HT neurons have more complex dendritic morphology, are intrinsically more excitable, and receive more excitatory synaptic input than vmDR neurons (Crawford et al., 2010; Crawford et al., in preparation). The relative inhibitory input to vmDR
and lwDR 5-HT neurons remains uncharacterized. Because GABA neurons of the DR are activated by a wide array of stressors it is likely that GABA input to vmDR and lwDR neurons plays an important role in stress and anxiety. In addition, input from the limbic forebrain regions, including the medial prefrontal cortex (mPFC) and lateral habenula is primarily to GABA neurons that inhibit 5-HT neurons of the DR (Hajos et al., 1998; Peyron et al., 1998; Varga et al., 2001; Varga et al., 2003; Jankowski and Sesack, 2004). GABAergic synaptic input to vmDR 5-HT neurons has been characterized in the healthy brain (Pan et al., 1989; Pan and Williams, 1989; Jolas and Aghajanian, 1997; Liu et al., 2000; Lemos et al., 2006; Kirby et al., 2008), but studies of inhibitory inputs have never included lwDR 5-HT neurons, and have never used a model of anxiety. Despite findings that suggest a unique role for lwDR 5-HT neurons in stress circuits and stress-related pathology, the changes in vmDR and lwDR neuron physiology induced by chronic stress or other models of anxiety are not known.

Chronic social defeat is a stress paradigm that produces many of the behavioral and physiological attributes seen in human anxiety disorders. The chronic social defeat paradigm exposes a male experimental mouse, the “intruder”, to social stress by placing it into the cage of an older, larger, territorial male mouse, the “resident” where it is bullied and “defeated” by the dominant resident. Though an array of neural changes occur after a single defeat (Matsuda et al., 1996; Martinez et al., 1998; Gardner et al., 2005), with repeated exposure to social defeat, the behavior of the intruder changes beyond the defeat environment and an anxious, depressive phenotype ensues. Although many variations of the paradigm exist in the literature, the general outcome of chronic social defeat increased anxiety-like behavior (Avgustinovich et al., 1997; Keeney and Hogg, 1999; Haller and Bakos, 2002), anhedonia (Rygula et al., 2005), increased social avoidance (Keeney and Hogg, 1999; Berton et al., 2006), and increased alcohol consumption (Croft et al., 2005). The validity of the model is further underscored by the
successful use of anxiolytic and antidepressant drugs to attenuate each of these behavioral phenotypes (Keeney and Hogg, 1999; Lumley et al., 2000; Von Frijtag et al., 2002; Avgustinovich et al., 2003; Rygula et al., 2006).

While the behavioral effects of chronic social defeat are well-described in the literature, only a few studies have examined how social defeat changes the activity of the DR of the intruder mouse. Chronic exposure to social defeat induces an increase in c-Fos expression in the DR, PFC, and several other brain regions in intruder mice as compared to control mice (Matsuda et al., 1996; Martinez et al., 1998; Martinez et al., 2002; Berton et al., 2007). Additional studies have examined, often with conflicting results, the social defeat-induced changes in the expression of genes related to 5-HT neurotransmission e.g. serotonin transporter, tryptophan hydroxylase, and the 5-HT$_{1A}$ receptor (5-HT$_{1A}$R) (Amstislavskaya and Kudryavtseva, 1997; Filipenko et al., 2002; Abumaria et al., 2006). However, each of these studies analyzed gross tissue samples and thus could not detect changes in specific cell types or at the cellular level within the brain regions they examined. Three recent studies examined cellular effects of a single defeat through the DR subregions on c-Fos and the expression of serotonergic markers (Gardner et al., 2005; Gardner et al., 2009a; Gardner et al., 2009b). However these studies only examined the effects of a single defeat and thus have limited insight as to the changes that occur after chronic exposure to social stress. Despite the growing use of chronic social defeat model of anxiety, it is still unknown how chronic social defeat alters 5-HT neurons of the DR or how the effects of defeat vary across DR subregions. Furthermore, no one has used electrophysiology to assess defeat-induced changes in 5-HT neuron physiology or in the receptor-mediated activity that modulates the 5-HT system.

To begin to fill the gaps in our understanding of the mechanisms underlying anxiety, we first characterized the GABAergic synaptic input to vmDR and lwDR neurons in healthy,
unmanipulated mice. We then characterized a 5-day chronic social defeat paradigm and compared intruders to behavioral controls. Electrophysiology was used to examine the social defeat-induced changes in DR 5-HT neuron physiology as well as changes in the inhibitory and excitatory synaptic inputs to 5-HT cells. Some of these data has been presented previously in abstract form (Crawford and Beck, 2009).

METHODS

Animals

Adult male 5-HT-YFP mice or their wild-type littermates were used at 2–4 months of age. These mice contain 5-HT neurons that express yellow fluorescent protein (YFP) under the control of the 5-HT specific Pet-1 promoter (Scott et al., 2005). The mice are on a background containing predominantly C-57/Black6. The membrane properties of YFP-labeled 5-HT neurons are comparable to those of 5-HT neurons from wild-type littermates (Crawford et al., 2010). For pilot studies of elevated plus maze behavior, Adult male C-57/Black6 mice from Taconic (place) were used. All mice were housed in a standard animal facility with lights on 06:00 to 18:00. Animal protocols were approved by the Institutional Animal Care and Use Committee and were conducted in accordance to the *NIH Guide for the Care and Use of Laboratory Animals*.

Whole-Cell Electrophysiology

Electrophysiology recordings were conducted as previously described (Beck et al., 2004; Lemos et al., 2006; Crawford et al., 2010). In brief, mice were sacrificed using decapitation. While submerged in a cold aCSF solution where NaCl is replaced with sucrose (248 mM), the midbrain was rapidly dissected, blocked, and cut with a Leica VT1000s vibratome (Leica Microsystems, Bannockburn, IL) to generate 200µm thick brain slices. Slices were maintained in
aCSF bubbled with 95%\textsubscript{O2} /5%\textsubscript{CO2} at 36°C for 1 hour and then at room temperature until used. Individual slices were then placed in a recording chamber and continuously perfused with 32-34°C aCSF solution bubbled with 95%\textsubscript{O2} /5%\textsubscript{CO2} with a solution flow rate of 1.5-2ml/min. The composition of the aCSF was (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 2.0 Mg\textsubscript{SO}\textsubscript{4}, 2.5 CaCl\textsubscript{2}, 10 dextrose, and 26 NaHCO\textsubscript{3}. Neurons were visualized using Nikon E600 (Optical Apparatus, Ardmore, PA) upright microscope fitted with a 40x water-immersion objective, DIC, and infrared filter. Ventromedial (vmDR) and lateral wing (lwDR) 5-HT neurons were targeted based on expression of YFP and later confirmed based on the expression of the synthetic enzyme marker, tryptophan hydroxylase (TPH). To visualize YFP-positive 5-HT cells for recording, a fluorescent lamp and yellow-fluorescence filter were used. The image was generated on a computer monitor using a CCD camera and Nikon Elements software (Optical Apparatus). For membrane characteristics and spontaneous excitatory post synaptic currents (sEPSCs), whole-cell recording pipettes fabricated on a Sutter Instruments pipette puller (P-97, Sutter Instrument, Novato, CA) had a resistance of 6-10MΩ when filled with an intracellular solution of 130mM Kgluconate, 5mM NaCl, 10mM Na-phosphocreatinine, 1mM MgCl\textsubscript{2}, 0.02mM EGTA, 10mM HEPES, 2mM MgATP, 0.5mM Na\textsubscript{2}GTP, and 0.1% biocytin (pH 7.3). For sEPSCs, voltage clamp recordings were conducted at a holding potential of -60mV such that EPSCs were downward and spontaneous inhibitory post-synaptic currents (sIPSCs) were upward; only downward events were analyzed. In experiments where the AMPA-kainate receptor blocker DNQX (20µm) was added at the end of the recordings, all downward events were abolished, (n=5, data not shown). For sIPSCs, pipettes had a resistance of 3-7MΩ when filled with an intracellular solution of 70mM Kgluconate, 70mM KCl, 2mM NaCl, 10mM Na phosphocreatine, 4mM EGTA, 10mM HEPES, 2mM MgATP, 0.3mM Na\textsubscript{2}GTP, and 0.1% Biocytin (pH 7.3). Because of the high concentration of Cl\textsuperscript{-} in the sIPSC electrolyte solution, sIPSCs were downward and were isolated by blocking EPSCs with
20µm DNQX. Recordings were collected online with a Multiclamp 700B amplifier, Digidata 1320 A/D converter, and Clampex 9.0 software (Molecular Devices, Union City, CA). Membrane properties of the cell were monitored as was the access resistance of the patch pipette during recordings that lasted a minimum of 20 minutes. Recordings were discarded if access resistance increased to 5x the initial access resistance that was noted prior to patching onto the cell. Any cells that showed physiological signs of instability, baseline holding currents below -30pA, or resting membrane potential above -50mV were excluded from data analysis. Reported values do not incorporate a junction potential of approximately +15mV, as calculated using Clampex software. Following the experiment, DR slices were fixed for 2-3 hours with 4% paraformaldehyde and processed for immunohistochemistry.

**Electrophysiology Data Analysis**

Current clamp recordings using visualized whole cell patch clamp techniques were analyzed using Clampfit 9.0 (Molecular Devices). Membrane properties were measured as previously described (Beck et al., 2004; Crawford et al., 2010). Resting membrane potential (RMP), action potential (AP) threshold, AP duration, after-hyperpolarization (AHP) amplitude, and the time it takes for the AHP to depolarize to one-half its peak amplitude (AHP t\(_{1/2}\)) were measured directly from traces as previously described (Beck et al., 2004). A voltage-current graph was generated using current pulses ranging from -100 to 0pA. Membrane input resistance was determined from the slope of the linear portion of the plot of the peak voltage induced by each current step. Frequency-intensity plots were obtained by measuring the number of action potentials generated by depolarizing current steps ranging from 0 to +80pA in 20pA increments. Average firing rate (Hz) was determined by the number of APs generated over the 630ms current pulse. Gain was determined from the slope of the frequency-intensity
plot. The time constant tau was obtained from an exponential fit of the membrane potential during the first 300ms of a -20pA hyperpolarizing current pulse. AP and AHP characteristics were determined from action potentials generated by injecting just enough current through the recording electrode to elicit a single action potential. AP amplitude, AP duration, and AHP amplitude were measured in relation to the AP threshold. AHP t_{1/2} is the duration of the AHP measured from its peak to half-amplitude. 5-HT_{1A}R-mediated responses were measured with current clamp recordings using 400ms, -30pA current pulses at 10-second intervals to monitor resistance along with changes in membrane potential.

Voltage clamp data were analyzed using MiniAnalysis (Synaptosoft; Decatur, GA) as has been reported in the past (Lemos et al., 2006). The parameters for synaptic event analysis were optimized for each cell with the threshold set beyond the maximum values of the all-points noise histogram for a segment of the trace containing no detectable synaptic events. The threshold generally ranged from 5 to 8pA. The MiniAnalysis program provides a summary table for each cell containing values for mean and median frequency, amplitude, rise time (10-90%), decay time (50%) and event half-width. For each cell, 200 randomly chosen events were manually filtered to exclude multiple peaks and used to obtain an average EPSC for each cell. The reported decay tau value is the tau obtained from an exponential fit of the 10-90% decay of the averaged PSC. A single exponential fit was used for sEPSCs and a double exponential fit for sIPSCs. Using the equation for the double exponential fit $y=(A_1)*\exp(-x/T_1)+(A_2)*\exp(-x/T_2)$, the weighted tau was calculated for the averaged sIPSC using the following formula: $T_w = \frac{A_1 T_1 + A_2 T_2}{A_1 + A_2}$. The area under curve of the averaged PSC was the charge per PSC. Charge per PSC was multiplied by the frequency to obtain mean phasic current. Gaussian fits of event histograms were obtained using the peak analyzer function in OriginPro 8.1 software (Origin Lab Corporation, Northampton, MA); 1-5 peaks were chosen in order to minimize the reduced Chi
For each PSC parameter, group averages were obtained by compiling the mean and/or median values for each cell. Histograms and cumulative probability plots describing all events from all cells in each group were compared using the Kolmogorov-Smirnov test. Reported values are mean ± standard error of the mean (s.e.m.) unless otherwise noted; p values were generated using Students t-test unless otherwise noted and p< 0.05 was deemed significant. Outlying values were excluded from group means but are indicated in scatter plots. Any data that showed non-Gaussian distribution according to the D’Agostino and Pearson omnibus normality test were compared using non-parametric statistical tests. Additional statistical analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA) and OriginPro 8.1.

**Immunohistochemistry**

Immunohistochemical identification of each neuron recorded in patch clamp configuration was completed as previously described (Beck et al., 2004; Lemos et al., 2006; Kirby et al., 2008). In brief, a standard immunohistochemistry protocol was used on 200μm-thick slices using mouse anti-TPH (1:200, Sigma) along with secondary donkey anti-mouse Alexa Fluor 488 (1:200, Invitrogen) and streptavidin-conjugated Pacific Blue (1:100, Invitrogen). Images were captured using a Leica DMR fluorescent microscope (Leica Microsystems, Bannockburn, IL) and OpenLab 3.0.9 software (Improvision, Lexington, MA) and then confirmed on a Leica DMIRE2 confocal microscope (Leica Microsystems) using Leica confocal software (version 2.5, Leica Microsystems).

**Chronic Social Defeat**

The 5-day episodic social defeat paradigm used was based a paradigm used in the rat (Bhatnagar and Vining, 2003; Bhatnagar et al., 2006) and mouse (Yap et al., 2005; Yap and
Miczek, 2007; Nikulina et al., 2008). Adult male mice were singly housed one day prior to the start of the episodic chronic social defeat protocol. Mice were transported to an experimental room and allowed to adjust for 15-30 minutes. Intruders were then placed into the home cage of a larger, older, male “resident” mouse and allowed to interact until the resident initiated an attack or a threat leading to a defeat posture that is typical of mice (Miczek et al., 1982). The intruder was then placed behind a wire partition for the remainder of the 30-minute session with continued exposure to visual, auditory, and olfactory cues from the aggressive resident mouse. Mice were returned to their home cage and housed in their home room. Defeat sessions were repeated daily for 5 days and intruders encountered a different resident on each day. Control mice were placed into a clean, empty cage for 2 minutes and then behind a partition for the remainder of the 30-minute session. Pilot experiments demonstrated that the behavior of control mice were significantly more variable when processed in the same room at the same time as intruder mice (Crawford and Beck, 2007). Thus, control mice were processed and removed from the experimental room just prior to the start of the intruder defeats to minimize their exposure to auditory and olfactory stress cues present during the intruder defeat session.

“Resident” mice were singly housed wild type males of a various backgrounds, were over 4 months old, and were screened for aggressive behavior by introducing an intruder into the cage several times during the week preceding the first defeat. Any resident that did not defeat the intruder within 2 minutes during screening trials was not used in experiments. The intruders used to screen residents were not used in any other experiments.

Groups of 6 to 16 mice were used for initial pilot experiments to establish ideal conditions needed to consistently generate intruders with an anxious phenotype as well as
controls with low variability in anxiety-like behavior. Thereafter, the first day of social defeat was staggered to generate 1-2 mice per day for electrophysiology experiments.

**Behavioral tests of anxiety-like behavior**

Following the 5-day defeat paradigm, mice were tested on day 6 and day 7 to assess anxiety-like behavior. Mice were transported to the experimental room and allowed to acclimate for 20 min. Behavioral tests were conducted in the dark under dim, red light. For the elevated plus maze test, mice were placed in the center of the maze, facing an open arm and behavior was videotaped for 5 minutes. Video was hand-scored to assess the number of four-paw entries into the open and closed arms as well as the time spent in each. The scores obtained from two observers blinded to the treatment groups were averaged for each mouse.

For the open field test mice were placed facing the corner of a 16” by 16” box and behavior videotaped for 5 minutes. The floor of the open field was divided into 16 (4 x 4) squares of equal size with an inner zone consisting of the inner 4 squares, and the outer zone consisting of the outermost 12 squares around the perimeter. Videotaped behavior was assessed for four-paw entries and duration of time spent in the inner zone vs outer zone. The scores obtained from two observers blinded to the treatment groups were averaged for each mouse.

In pilot experiments, mice were subjected to the 5-day social defeat paradigm and then tested on day 6 and day 7 for behavior in the elevated plus maze. In a separate set of experiments, mice were subjected to the 5-day social defeat paradigm followed by an open field test on day 6, and were then sacrificed on the morning of day 7 for electrophysiology recordings and to assess organ weights. Social defeat was conducted in the late afternoon between 16:00 and 19:00 hours. Open field tests were conducted as described above at the beginning of the dark phase, between 18:00 and 20:00 hours. Behavior was analyzed using Ethnovision software to assess distance traveled, velocity, duration of time spent, and number of entries within the
inner zone and outer zone. Distance-traveled and velocity were also assessed for the entire open field arena. Because Ethnovision tracks the center of gravity, the boundaries of the inner zone were defined in order to be consistent with the zone definition used when hand-scoring four-paw entries.

**Organ weights**

Body weight was measured on day 1 following the first defeat session, on day 6 following the behavioral test of anxiety, and on day 7 just before mice were sacrificed. The change in body weight reported is the difference between weight taken on day 1 and day 6. On day 7, after brains were dissected for slice preparation, the bladder and heart were dissected and weighed.

**RESULTS**

*Inhibitory input to vmDR and lwDR 5-HT neurons is comparable in mice*

The GABAergic input to DR 5-HT neurons is crucial to understanding stress circuits and stress-related pathology, but the inhibitory synaptic input to lwDR neurons has never been characterized. We performed whole-cell patch clamp recordings of spontaneous inhibitory post-synaptic currents (sIPSCs) in 26 neurons from a total of 10 grouped housed, untreated mice. Several of the parameters of sIPSCs recorded in vmDR and lwDR neurons were comparable between groups, including the average frequency, rise time, and weighted decay tau (Table 1). Though the average amplitude was also comparable (12.6 ± 1.2pA vmDR vs 11.7 ± 0.9pA lwDR, p=0.586), analysis of histograms of all events showed a different distribution of
Figure 4.1. There are few differences in sIPSCs between the vmDR and lwDR 5-HT neurons of unmanipulated mice.

L) Scatter plots summarize the comparable average sIPSC frequency values obtained from recorded cells in the vmDR and lwDR.

B) The average amplitude of sIPSC events was also comparable between vmDR and lwDR neurons, although the cumulative probability plot shown in the right panel suggests a shift toward larger amplitudes in the population of events recorded in the lwDR (K-S test, p<0.0001).

The population of sIPSC events were shifted towards larger amplitudes in lwDR 5-HT neurons (K-S test p<0.001, Fig. 4.1). This difference was suggestive of subtle differences in post-synaptic GABA_R between vmDR and lwDR 5-HT neurons.

Though the lwDR 5-HT neurons have increased intrinsic excitability and increased excitatory synaptic input in relation to vmDR neurons (Crawford and Beck, 2008; Crawford et al., 2010; Crawford et al., in preparation), the inhibitory input is largely comparable. These distinctions between DR subregions in the healthy brain have several implications for the
putative role of the vmDR and lwDR in stress-related pathology. Chronic stress may differentially affect the vmDR and lwDR 5-HT neurons, contributing to distinct role in anxiety and other stress-related mood disorders. With the aid of a chronic stress model of anxiety, we directly tested this hypothesis.

*Chronic social stress induces an anxiety-like syndrome consisting of an array of physiological and behavioral changes*

A 5-day chronic social defeat paradigm was tested for its ability to produce anxiety-like behavior in adult male mice and to quell concerns that arose due to the discordance we observed in the literature as to the effects of episodic defeat paradigms. Pilot studies were conducted using a total of 67 mice to ascertain appropriate conditions for control animals and determine the age range of mice that demonstrate the most consistent effects of defeat (data not shown). In these pilot studies, intruders and behavioral controls were tested on day 6 and day 7 for anxiety-like behavior in the elevated plus maze. Compared to controls, intruders demonstrated significantly fewer entries into the open arms of the elevated plus maze and less time spent in the open arms while closed arm entries were comparable between groups (Fig. 4.2A). In a separate experiment, open field behavior was examined on day 6 and mice used on day 7 for electrophysiology recordings as described below. Intruders entered the central zone of the open field fewer times, spent less time in the central zone, and traveled less distance within the central zone (Fig. 4.2B). In addition, intruders traveled less distance in the entire open field arena with a slower average velocity than controls (Fig. 4.2).

While observing animals in the open field, it was noted that some mice stop to groom more than others. Stress-induced grooming is a stereotyped, displacement behavior seen in rodent models of anxiety (Kalueff and Tuohimaa, 2004b, 2005a) and compulsive disorders seen in canine and feline veterinary patients (Overall and Dunham, 2002). With a compelling parallel
Figure 4.2. Chronic social defeat induces anxiety-like behavior in intruder mice. A) In pilot experiments, intruders and controls were tested after 5-days of chronic social defeat stress. Intruders show increased anxiety-like behavior in the elevated plus compared to controls, indicated by decreased time spent in the open arms and decreased number of entries into open arms. The number of closed arm entries did not differ between intruders and controls, suggesting comparable degrees of locomotor activity in the elevated plus maze. B) In a separate experiment, mice were tested in the open field test following 5-days of social defeat. Intruders demonstrated fewer entries into the central zone, less time spent in the central zone, and a lower percentage of distance traveled within the central zone. Intruders also traveled less distance throughout the entire open field arena, suggesting decreased locomotor activity. C) Analysis of videos obtained during the open field test revealed that intruders spend more time grooming than intruders, while rearing behavior was comparable. There was a significant negative correlation between time spent grooming and distance traveled where mice that spent the most time grooming traveled the least distance throughout the open field. This suggests that stress-induced grooming may have contributed to the decrease in locomotor activity.
to human obsessive-compulsive disorder, it is attenuated by anxiolytic drugs and increased in response to anxiogenic drugs (Kalueff and Tuohimaa, 2005b). Thus, to determine whether intruders groom more than controls, videotaped behavior in the open field test was analyzed further to measure the number and duration of bouts of grooming and rearing behavior. Intruders had more bouts of grooming behavior and spent more time grooming than controls. Rather than the low-stress cephalic to caudal pattern of grooming seen just before rest, the grooming observed in the open field test occurred in short, interrupted spurts that were consistent with stress-evoked stereotypy (Fentress, 1977; Kalueff and Tuohimaa, 2004a). There was also an non-significant trend towards decreased rearing behavior in intruders, which may indicate increased neophobia in intruders, though other studies beyond the scope of these experiments would be needed to confirm this interpretation. Time spent grooming during the open field test was negatively correlated with distance traveled such that mice that groomed more, traveled less distance (Fig. 4.2C). Thus, the increased grooming behavior of intruders may partially explain the decrease in locomotor activity observed in intruders during the open field test.

An array of stress-induced changes was also observed in the body weight and weight of peripheral organs of intruder mice. There was a small but significant increase in body weight in intruders compared to controls (Fig. 4.3A). In addition, there was an increase in the bladder weight of intruders compared to controls (Fig. 4.3A) measured by both raw weight and percent of body weight. This was consistent with previous findings in a 7-day defeat paradigm in rat (Wood et al., 2009) and with a 4-week, continuous sensory contact defeat paradigm in mice (Chang et al., 2009). Bladder weight was positively correlated to behavioral measures of anxiety obtained in the open field test (Fig 4.3B), underscoring the utility of bladder weight as an indicator of the magnitude of anxious phenotype. In addition, there was also a small, but
significant increase in heart weight in intruders (Fig. 4.3A), consistent with what has been observed in subsets of mice following a 10-day continuous sensory contact defeat paradigm (Krishnan et al., 2007). The change in heart weight was positively correlated with the change in bladder weight (Fig. 4.3B). Collectively, these changes in peripheral organs point to putative sequelae of increased sympathetic tone in intruders compared to controls. Thus the chronic

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**Figure 4.3.** Chronic social defeat induces an array of stress-associated changes in periphery organs. A) The 5-day chronic social defeat paradigm induced a small but significant increase in the body of intruders compared to controls, in addition to an increase in bladder weight and heart weight. For the bladder and heart, top panels show organ weight as a percentage of body weight while bottom panels show raw weights. B) Bladder weight was correlated to other measures of anxiety seen in intruders including fewer entries into the central zone during the open field test, decreased average velocity during the open field test, and increased heart weight.
social defeat paradigm used herein induces a multifaceted, anxiety-like phenotype that makes it a useful model for the exploration of neural mechanisms underlying anxiety.

Anxious behavior following chronic social stress correlates with an increased excitability of vmDR 5-HT neurons.

As outlined above, the 5-day social defeat paradigm successfully elicited anxiety-associated changes in behavior and also induced an increase in body weight and peripheral organs in intruders. This paradigm was used to investigate the neurophysiological changes in the dorsal raphe associated with the anxious phenotype. A total of 57 mice underwent 5 days of social defeat or 5 days of the control condition, followed by assessment of open field behavior on day 6 and electrophysiology recordings in raphe brain slices on day 7. Recordings targeted 5-HT neurons of the vmDR and lwDR, as determined by YFP-label and confirmed by TPH-content in post-recording immunohistochemistry. Membrane properties and autoreceptor responses were measured in brain slices obtained from a total of 14 controls and 16 intruders. Current clamp recordings examined the membrane resistance, time constant tau, action potential (AP) characteristics, and after-hyperpolarization (AHP) characteristics of raphe slices from intruders and controls. The activation gap was the difference between resting membrane potential and AP threshold. Frequency-intensity plots were constructed to examine AP firing frequency in response to increasing depolarizing current steps. The average gain was the slope of the frequency-intensity plot. There were no significant differences in these parameters between controls and intruders in either subregion (Table 4.2). The 5-HT$_{1A}$ autoreceptor-mediated response was assessed by measuring membrane hyperpolarization and changes in resistance upon bath application of 100nM 5-Carboxyamidotryptamine (5-CT). In the vmDR, there was no difference in the 5-CT-induced membrane hyperpolarization (controls -10.9 ± 2.1, intruders -
13.0 ± 2.6, \( p = 0.566 \) or in the 5-CT induced decrease in membrane resistance (controls -270.7 ± 56.6, intruders 321.5 ± 45.6, \( p = 0.526 \)). Likewise, there was no differential effect of 5-CT on lwDR membrane hyperpolarization (controls -8.5 ± 1.3, intruders -12.0 ± 3.0, \( p = 0.305 \)) or lwDR decrease in resistance (control -203.9 ± 38.5, intruders -295.4 ± 94.5, \( p = 0.383 \)).

Table 4.2a. Membrane properties of vmDR 5-HT neurons after social defeat

<table>
<thead>
<tr>
<th></th>
<th>RMP (mV)</th>
<th>Input Res (MΩms)</th>
<th>( \tau ) (ms)</th>
<th>AHP amp (mV)</th>
<th>AHP 1/2 (ms)</th>
<th>AP thres (mV)</th>
<th>AP dur (ms)</th>
<th>AP ampl (mV)</th>
<th># AP at 80pA</th>
<th>Gain (Hz/pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>-64.3 ± 0.7</td>
<td>467.9 ± 31.1</td>
<td>27.2 ± 1.8</td>
<td>27.5 ± 1.2</td>
<td>154.1 ± 13.0</td>
<td>-22.1 ± 0.8</td>
<td>1.8 ± 0.1</td>
<td>56.3 ± 1.5</td>
<td>1.5 ± 0.4</td>
<td>0.019 ± 0.005</td>
</tr>
<tr>
<td>t-test p = 0.001</td>
<td>0.703</td>
<td>0.580</td>
<td>0.647</td>
<td>0.545</td>
<td>0.294</td>
<td>0.146</td>
<td>0.575</td>
<td>0.798</td>
<td>0.678</td>
<td>0.612</td>
</tr>
<tr>
<td>INTRUDER</td>
<td>-65.3 ± 2.1</td>
<td>441.1 ± 36.1</td>
<td>28.2 ± 1.3</td>
<td>28.4 ± 0.9</td>
<td>172.7 ± 11.5</td>
<td>-20.3 ± 1.0</td>
<td>1.8 ± 0.7</td>
<td>56.9 ± 2.0</td>
<td>1.3 ± 0.3</td>
<td>0.016 ± 0.004</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

Table 4.2b. Membrane properties of lwDR 5-HT neurons after social defeat

<table>
<thead>
<tr>
<th></th>
<th>RMP (mV)</th>
<th>Input Res (MΩms)</th>
<th>( \tau ) (ms)</th>
<th>AHP amp (mV)</th>
<th>AHP 1/2 (ms)</th>
<th>AP thres (mV)</th>
<th>AP dur (ms)</th>
<th>AP ampl (mV)</th>
<th># AP at 80pA</th>
<th>Gain (Hz/pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>-63.7 ± 2.1</td>
<td>688.8 ± 56.7</td>
<td>35.4 ± 2.4</td>
<td>34.7 ± 1.6</td>
<td>266.5 ± 18.1</td>
<td>-24.8 ± 0.8</td>
<td>2.5 ± 0.1</td>
<td>65.7 ± 1.6</td>
<td>2.4 ± 0.4</td>
<td>0.030 ± 0.005</td>
</tr>
<tr>
<td>t-test p = 0.001</td>
<td>0.592</td>
<td>0.139</td>
<td>0.626</td>
<td>0.273</td>
<td>0.141</td>
<td>0.522</td>
<td>0.639</td>
<td>0.202</td>
<td>0.749</td>
<td>0.845</td>
</tr>
<tr>
<td>INTRUDER</td>
<td>-65.5 ± 2.3</td>
<td>522.1 ± 30.9</td>
<td>33.2 ± 3.3</td>
<td>32.2 ± 1.5</td>
<td>231.1 ± 14.8</td>
<td>-25.5 ± 0.7</td>
<td>2.4 ± 0.1</td>
<td>61.0 ± 2.8</td>
<td>2.2 ± 0.3</td>
<td>0.029 ± 0.004</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

There are several reports that highlight the variability in the behavioral and neuroendocrine effects of social defeat in populations of rodents (Krishnan et al., 2007; Walker et al., 2008; Walker et al., 2009; Wood et al., 2010). It is possible that variations in the degree of anxiety exhibited by intruders may have masked outright differences in neural properties of 5-HT cells between control and intruder mice. To address this possibility, we examined correlations between measures of anxious behavior in intruders and properties of 5-HT neurons.

The major relationship that emerged was a correlation between avoidance behavior in the open field and increased excitability of 5-HT neurons in intruders. This relationship was restricted to vmDR neurons of intruders and was not seen in lwDR neurons. In intruders, decreased time in the central zone was correlated with increased excitability elicited by an 80pA input current step, increased gain, a smaller activation gap, and an increased \( \tau \) (Fig.4.4). Previously, I reported that rostral position within the vmDR is correlated to several measures of increased...
intrinsic excitability (Crawford et al., 2010). However, the relationship between anxious behavior and 5-HT cell excitability was not due to the topographical position of the recorded vmDR neurons, as there was no coincident correlation between rostral-caudal position of recorded neurons and time in the middle zone. Thus, the most anxious intruders possessed vmDR 5-HT neurons that were more excitable than the vmDR neurons of less anxious intruders.

Figure 4.4. Increased anxiety-like behavior in the open field test was correlated to increased 5-HT neuron excitability in the vmDR of intruders. The behavior of intruder mice during the open field test was used to determine whether a pattern of membrane characteristics emerges in the most anxious mice. The most anxious mice that spent a lower amount of time in the central zone of an open field possessed vmDR 5-HT with increased gain (A) and increased excitability (B) as measured by the number of action potentials (APs) elicited by an 80pA input current step. C) The activation gap, defined by the difference between resting membrane potential (RMP) and action potential threshold (AP Thr), was also smaller in the most anxious animals. D) The 5-HT neurons of the most anxious mice also
had a larger time constant Tau. The correlation between increased excitability of 5-HT neurons and higher anxiety levels in intruders was not seen in lwDR 5-HT neurons (data not shown).

**Chronic social stress has little effect on excitatory input to 5-HT neurons**

The activity of 5-HT neurons is modulated by glutamatergic input (Pan and Williams, 1989; Adell et al., 2002). In the slice preparation, AMPA/kainate-receptor mediated sEPSCs are more abundant in lwDR 5-HT neurons than in vmDR neurons (Crawford et al., in preparation). However, the effects of stress on excitatory synaptic inputs to lwDR 5-HT neurons have never been investigated. To determine the effects of chronic social defeat on local glutamatergic input to DR neurons, voltage clamp recordings of baseline sEPSCs were conducted in slices from a total of 13 control mice and 18 intruder mice. The frequency and amplitude of sEPSC events were assessed along with rise time and decay kinetics obtained from recordings of vmDR and lwDR 5-HT neurons. There were no significant distinctions between intruders and controls in either subregion (Table 4.3).

### Table 4.3a. Excitatory events: Results of sEPSC recordings in vmDR 5-HT neurons after social defeat

<table>
<thead>
<tr>
<th></th>
<th>Frequency (Hz)</th>
<th>Decay Tau (ms)</th>
<th>Average Amplitude (pA)</th>
<th>Median Amplitude (pA)</th>
<th>Average Rise Time (ms)</th>
<th>Median Rise Time (ms)</th>
<th>Charge Per EPSC (pAms)</th>
<th>Mean Phasic Current (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=14)</td>
<td>14.7 ± 3.0</td>
<td>1.9 ± 0.2</td>
<td>17.1 ± 1.0</td>
<td>13.6 ± 0.8</td>
<td>1.4 ± 0.03</td>
<td>1.3 ± 0.03</td>
<td>47.7 ± 4.2</td>
<td>0.66 ± 0.12</td>
</tr>
<tr>
<td>t-test p val</td>
<td>0.349</td>
<td>0.917</td>
<td>0.575</td>
<td>0.417</td>
<td>0.602</td>
<td>0.318</td>
<td>0.906</td>
<td>0.843</td>
</tr>
<tr>
<td><strong>INTRUDER</strong></td>
<td>19.0 ± 3.1</td>
<td>1.9 ± 0.2</td>
<td>16.2 ± 1.0</td>
<td>12.8 ± 0.5</td>
<td>1.4 ± 0.5</td>
<td>1.3 ± 0.05</td>
<td>48.5 ± 4.1</td>
<td>0.70 ± 0.18</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

### Table 4.3b. Excitatory events: Results of sEPSC recordings in lwDR 5-HT neurons after social defeat

<table>
<thead>
<tr>
<th></th>
<th>Frequency (Hz)</th>
<th>Decay Tau (ms)</th>
<th>Average Amplitude (pA)</th>
<th>Median Amplitude (pA)</th>
<th>Average Rise Time (ms)</th>
<th>Median Rise Time (ms)</th>
<th>Charge Per EPSC (pAms)</th>
<th>Mean Phasic Current (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=16)</td>
<td>22.8 ± 3.2</td>
<td>1.4 ± 0.1</td>
<td>18.4 ± 0.5</td>
<td>14.2 ± 0.5</td>
<td>1.3 ± 0.02</td>
<td>1.2 ± 0.02</td>
<td>44.0 ± 1.8</td>
<td>0.99 ± 0.14</td>
</tr>
<tr>
<td>t-test p val</td>
<td>0.883</td>
<td>0.080</td>
<td>0.464</td>
<td>0.945</td>
<td>0.965</td>
<td>0.770</td>
<td>0.438</td>
<td>0.755</td>
</tr>
<tr>
<td><strong>INTRUDER</strong></td>
<td>22.0 ± 4.0</td>
<td>1.7 ± 0.1</td>
<td>17.5 ± 1.0</td>
<td>14.3 ± 0.8</td>
<td>1.3 ± 0.02</td>
<td>1.1 ± 0.03</td>
<td>46.8 ± 3.2</td>
<td>1.07 ± 0.23</td>
</tr>
<tr>
<td>(n=15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Chronic social stress has differential effects on inhibitory input to vmDR and lwDR 5-HT neurons

Evidence suggests that GABA\_A\_R on DR 5-HT neurons are responsible for the anxiolytic effects of benzodiazepines (Gallager, 1978; Gallager et al., 1980; Soubrie et al., 1981; Thiebot et al., 1982; Maier et al., 1994) and that GABA DR neurons relay the majority of the limbic forebrain input that originates from regions such as the mPFC and lateral habenula to DR 5-HT cells (Ferraro et al., 1996; Celada et al., 2001; Allers and Sharp, 2003). Though the local GABA\_ergic input to vmDR and lwDR neurons was comparable in untreated animals (Fig. 4.1), it is unknown whether GABA\_ergic input to DR subregions is differentially altered in anxiety. Voltage clamp recordings were conducted in raphe slices from a total of 10 control mice and 11 intruder mice, revealing that GABA\_ergic inputs were altered in a topographically specific way in intruders when compared to controls. Representative traces from vmDR recordings are presented in Figure 4.5A. One clear difference between groups was that the frequency of vmDR sIPSC events was significantly lower in intruders than in controls (Fig. 4.5). In addition the average sIPSC amplitude of intruders was reduced compared to controls. Gaussian fits of histograms of event amplitudes from all cells revealed an increase in small amplitude events centered at 8.5pA (20.0% controls vs 50.7% intruders, Fig 4.5C). The peaks centered at 33.9pA and 54.0pA in the histogram from recordings in controls were absent in the histogram created from recordings in intruders. This resulted in a significant shift in the distribution of events towards smaller events in intruders (K-S test, p < 0.0001, Fig. 4.5C), indicating a loss of large-amplitude events in intruders. The rise time and decay time-50% remained comparable between intruders and controls (Fig 4.5A inset, Fig. 4.5B, Table 4.4). The inset in Figure 4.5A shows averaged IPSCs from an intruder and control mouse, demonstrating the decrease in amplitude with no difference in the rise time or decay time. Analysis of the averaged event from each recorded cell revealed a
lower charge flux per event in intruders which, combined with the decreased sIPSC frequency, resulted in a dramatic decrease in the mean phasic current in intruders (Fig. 4.5B).

Figure 4.5. Chronic social defeat leads to a decrease in sIPSC frequency and amplitude in the vmDR of intruder mice. (A) Raw data traces from representative vmDR recordings show differences in GABA$_A$AR-mediated sIPSC events. Inset contains the averaged event from each trace, overlaid to enable comparison. The lower trace of the inset shows the average event with normalized amplitude to allow comparison of rise time and decay kinetics. (B) The mean values obtained from each treatment group demonstrate a decrease in the frequency and amplitude of sIPSC events in chronically stressed intruders; this likely contributes to the observed decrease in charge transfer and in overall phasic current. There was no detectable change in event kinetics. (C) Histograms of all recorded events were constructed and fit with a multiple peak Gaussian function. This revealed a significant loss of large amplitude events in intruders, especially those contained in peaks centered at centered at 33.9pA and 54.0pA. The cumulative probability plot in the third panel demonstrates the significant shift toward small events.
Table 4.4a. Inhibitory events: Results of sIPSC recordings in vmDR 5-HT neurons after social defeat

<table>
<thead>
<tr>
<th></th>
<th>Frequency (Hz)</th>
<th>Weighted Decay Tau (ms)</th>
<th>Decay Time 50% (ms)</th>
<th>Average Amplitude (pA)</th>
<th>Median Amplitude (pA)</th>
<th>Average Rise Time (ms)</th>
<th>Charge Per IPSC (pAms)</th>
<th>Mean Phasic Current (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (n=15)</td>
<td>5.1 ± 1.2</td>
<td>3.3 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>17.3 ± 2.5</td>
<td>14.4 ± 1.9</td>
<td>1.9 ± 0.09</td>
<td>106.4 ± 16.2</td>
<td>0.79 ± 0.24</td>
</tr>
<tr>
<td>t-test p val</td>
<td>0.031</td>
<td>0.508</td>
<td>0.278</td>
<td>0.020</td>
<td>0.023</td>
<td>0.599</td>
<td>0.031</td>
<td>0.011</td>
</tr>
<tr>
<td>INTRUDER (n=15)</td>
<td>2.0 ± 0.7</td>
<td>3.9 ± 0.8</td>
<td>2.5 ± 0.2</td>
<td>11.0 ± 0.5</td>
<td>9.7 ± 0.4</td>
<td>1.9 ± 0.07</td>
<td>68.7 ± 5.2</td>
<td>0.13 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

Table 4.4b. Inhibitory events: Results of sIPSC recordings in lwDR 5-HT neurons after social defeat

<table>
<thead>
<tr>
<th></th>
<th>Frequency (Hz)</th>
<th>Weighted Decay Tau (ms)</th>
<th>Decay Time 50% (ms)</th>
<th>Average Amplitude (pA)</th>
<th>Median Amplitude (pA)</th>
<th>Average Rise Time (ms)</th>
<th>Charge Per IPSC (pAms)</th>
<th>Mean Phasic Current (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (n=15)</td>
<td>2.6 ± 0.5</td>
<td>2.7 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>13.4 ± 0.9</td>
<td>10.6 ± 0.7</td>
<td>1.8 ± 0.07</td>
<td>64.7 ± 3.9</td>
<td>0.28 ± 0.11</td>
</tr>
<tr>
<td>t-test p val</td>
<td>0.886</td>
<td>0.224</td>
<td>0.007</td>
<td>0.763</td>
<td>0.603</td>
<td>0.014</td>
<td>0.033</td>
<td>0.610</td>
</tr>
<tr>
<td>INTRUDER (n=16)</td>
<td>2.5 ± 0.7</td>
<td>3.3 ± 0.4</td>
<td>2.9 ± 0.1</td>
<td>13.0 ± 1.0</td>
<td>11.2 ± 0.9</td>
<td>2.1 ± 0.05</td>
<td>80.2 ± 5.6</td>
<td>0.21 ± .06</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

Figure 4.6. Chronic social defeat leads to distinct effects in lwDR 5-HT neurons that were limited to slower in sIPSC kinetics. (A) Raw data traces from representative lwDR recordings show sIPSC events in a neuron from a control and a neurons from an intruder. The inset demonstrates the averaged event from the respective traces. Note the differences in the shape, i.e. kinetics, of the two averaged events. (B) Comparison of the mean values of each treatment group demonstrate an increase in sIPSC event rise time, decay time 50%, and charge transfer in intruders while the frequency and amplitude remain comparable to controls.
The stress-induced changes in the lwDR were distinct from those seen in the vmDR. The frequency and amplitude of sIPSCs were comparable between intruders and controls (Table 4.4, Fig. 4.6). However, the kinetics of inhibitory events were altered by social defeat. The rise time and decay time-50% of sIPSCs in intruders were significantly slower, resulting in wider events with larger charge flux per event (Fig. 4.6B). Together with the unaltered event frequency, the wider events contributed to a trend towards an increased mean phasic current in the lwDR of intruders (Table 4.4, Fig. 4.6B).

Supplemental Data

It is known that mild stress, including handling or exposure to an open field can increase markers of neuronal activation in the DR in behavioral controls, particularly in lwDR non-5-HT neurons that are presumably GABAergic (Martinez et al., 1998; Chung et al., 2000; Roche et al., 2003; Johnson et al., 2004; Abrams et al., 2005; Gardner et al., 2005; Berton et al., 2007; Hale et al., 2008; Johnson et al., 2008). To my knowledge, there are few stressors that do not activate the lwDR. In my social defeat experiments, controls were handled, exposed to a clean empty cage and wire partition, and exposed to the open field in the days preceding electrophysiological recordings. It is possible that the mild stress induced by manipulations of controls activated GABA inputs to 5-HT neurons. To understand the effects on GABAergic sIPSCs induced by experimental manipulation outside of the actual defeat, we conducted a comparison of sIPSC activity recorded from DR neurons of the unhandled, unmanipulated mice in the first experiment (Fig. 4.1, Table 4.1) with the controls of social defeat experiments. In the vmDR, sIPSC frequency was 3.2 ± 1.2 Hz in unmanipulated mice (n=14) and was 5.1 ± 1.2 Hz in control mice (n=15). Though there was a trend towards an increase in control sIPSC frequency, a comparison between control mice and unmanipulated controls revealed no significant
Supplemental Figure 4.S1.
The vmDR 5-HT neurons of control mice show a trend towards increased sIPSC frequency compared to unmanipulated controls. A comparison was conducted between the sIPSC frequency recorded in 5-HT neurons from control mice of social defeat experiments and unmanipulated mice (YFP). Data from intruder mice are shown for clarity but were not included in the statistical comparison. A) There was no significant difference between the average sIPSC frequency of controls and unmanipulated mice in the vmDR (Mann-Whitney test p = 0.214) or in the lwDR (Mann-Whitney test p = 0.421). B) Cumulative plots of all-event histograms were constructed for vmDR data, revealing a significant shift towards lower inter-event interval (increased frequency) when comparing recorded events in unmanipulated mice to events in controls (K-S test, p <0.0001).

In the lwDR, sIPSC frequency of unmanipulated mice was $2.4 \pm 0.7$ Hz (n=14) while it was $2.6 \pm 0.5$ Hz (n=15) in control mice, revealing no significant difference (p=0.421, Mann-Whitney test). These data are summarized in Supplemental Figure 4.S1.

DISCUSSION

The lwDR and vmDR 5-HT neurons are postulated to have distinct roles in stress circuits in the normal, healthy brain and may be differentially affected in stress-related pathology (Abrams et al., 2004; Johnson et al., 2004; Johnson et al., 2008; Lowry et al., 2008). In relation to vmDR 5-HT neurons, lwDR 5-HT neurons in the healthy brain exhibit increased intrinsic excitability (Crawford et al., 2010) and increased excitatory synaptic input (Crawford et al., in
preparation), while data presented above shows that baseline inhibitory synaptic input remains comparable. However, no one, to my knowledge, has examined changes in 5-HT neuron physiology and modulatory input induced by chronic stress. A 5-day chronic social defeat paradigm induced an array of anxiety-associated changes in behavior and peripheral organ systems in intruders compared to behavioral controls. Probing the neurophysiological changes induced by 5-days of defeat revealed a distinct pattern of changes in vmDR versus the lwDR 5-HT neurons. In the vmDR chronic social defeat induced an increased excitability of vmDR neurons in the most anxious mice, few changes in excitatory input, and a decrease in the frequency and amplitude of inhibitory synaptic input. However these changes were not seen in the lwDR. Rather, chronic social defeat induced distinct alterations in the lwDR that were limited to slower kinetics of inhibitory synaptic events and a trend towards an increase in mean inhibitory current. The distinct stress-induced changes in inhibitory tone to vmDR or lwDR neurons suggest that the 5-HT output in brain regions targeted by either subfield is differentially affected in anxiety disorders.

The behavioral and physiological sequelae of chronic social defeat

The 5-day social defeat paradigm produces an anxiety-like syndrome that included anxiety-like avoidance behavior in the elevated plus maze and open field tests, an increase in stress-induced grooming behavior, and an increase in body weight, relative bladder weight, and relative heart weight. While our findings are supported by some reports in the literature (Denmark et al.; Kudryavtseva et al., 1991; Avgustinovich et al., 1997; Wood et al., 2009; Crawford et al., 2010) there are other reports, particularly of open field behavior and changes in body weight, that are inconsistent with the effects of social defeat I observed (Bhatnagar and Vining, 2003; Bhatnagar et al., 2006; Barsy et al., 2010; Wood et al., 2010). It is unclear what
causes the discordance in the literature as to the behavioral and physiological changes induced by chronic social defeat, though it likely stems in part, from the variation in defeat protocols used. In addition to the chronic episodic defeat paradigm used in our experiments, some studies use a continuous subordination paradigm, also referred to as a sensory contact model. The continuous subordination paradigm induces a syndrome that is unique in some ways to that induced by chronic episodic defeat (Miczek et al., 2008). For example rats that have encountered chronic episodic defeat show an escalated pattern of cocaine intake thought to be linked to stress-induced behavioral sensitization while rats that have endured continuous subordination show a suppressed pattern of cocaine intake, thought to be linked to stress-induced anhedonia (reviewed in Miczek et al., 2008). Other factors that may contribute to this variability include the duration of the defeat paradigm, the type of controls used, whether controls experience auditory and olfactory cues from residents and intruders during defeats, the conditions of the behavioral test (high-light versus low light, etc), and the species and strain of animal used. Despite the varied nuances of the paradigms used across the literature, the common characteristic of these paradigms is that social defeat is a potent, salient stressor that induces a phenotype that is comparable in many ways to human anxiety and depression, and serves as a model for the dysfunction in peripheral organ systems that is often co-morbid with these disorders. While the experiments described above only assessed anxiety-related measures, it is clear that there may be implications for the mechanisms underlying depression and other stress-associated disorders as well.

Avoidance of aversive environments is a cardinal feature of anxiety disorders and was significantly increased in intruders, as demonstrated by the two tests of anxiety-like behavior used in this study. This suggested that the effects of chronic social defeat were not restricted to social interactions as has been reported in other variations of the paradigm (Barsy et al., 2010)
but were generalized beyond the social environment. The increased grooming behavior of intruders was consistent with previous reports of grooming in response to stressors and anxiogenic drugs (Kudryavtseva et al., 1991; Kalueff and Tuohimaa, 2004b, 2005b, a) and is reminiscent of the role of grooming and other stereotypies seen in compulsive disorders in veterinary patients (Stein et al., 1992; Woods-Kettelberger et al., 1997; Overall and Dunham, 2002) and obsessive-compulsive disorders in humans. While chronic social defeat is commonly associated with a decreased weight gain or weight loss in some paradigms (Kudryavtseva et al., 1991; Bhatnagar and Vining, 2003; Bhatnagar et al., 2006; Krishnan et al., 2007; Wood et al., 2010), other reports have described an increase in food intake (Bhatnagar et al., 2006) and an increase in body weight (Bartolomucci et al., 2004) in defeated animals that would be consistent with the phenotype of intruders in our paradigm. As dysfunction of the HPA axis, and especially elevated corticosterone levels have been demonstrated following chronic social defeat (Bhatnagar and Vining, 2003; Bhatnagar et al., 2006; Wood et al., 2010) it is possible that the appetite enhancing effects of corticosterone are a contributing factor to the weight gain in intruders, although measures of food intake would be needed to test this hypothesis. In addition to the HPA axis dysfunction, anxiety disorders are also characterized by an increased sympathetic tone that contributes to the heightened responses to innocuous stressors. The increase in relative bladder weight and relative heart weight of intruders are likely the sequelae of a robust, long-lasting increase in the sympathetic tone in intruders compared to controls.

Though it lies beyond the scope of our studies, the mechanisms underlying the pathological changes in bladder and heart will likely lend insight as to the interaction between anxiety and cardiovascular disease or urogenital tract pathology such as interstitial cystitis, conditions that are known to be comorbid with human anxiety (Clemens et al., 2008; Roy-Byrne et al., 2008).
**Chronic social stress induces selective presynaptic changes in inhibitory input to vmDR neurons.**

While the GABAergic, inhibitory input to 5-HT neurons has long been implicated in the mechanisms of anxiolytic drugs, no one to my knowledge has examined the changes in GABAergic input to DR 5-HT neurons induced by a model of anxiety. The vmDR 5-HT neurons of mice that underwent chronic social defeat received a lower frequency of sIPSC input than vmDR cells of control mice. This decreased inhibitory input could be due to a decrease in the activity of GABA neurons present in the slice, a decrease in the number of inhibitory synapses formed, a decrease in the number of GABA neurons, or a combination of these possibilities. Previous reports (Lemos et al., 2006) have demonstrated that the frequency of sIPSC and mIPSC do not differ in recordings of vmDR 5-HT neurons in the rat. While it is unknown if chronic social stress would increase the activity of GABA neurons within the slice preparation, the finding by Lemos et al. suggests that at least under baseline conditions, the action potential-dependent release of GABA onto 5-HT neurons is negligible in vmDR neurons. It has been demonstrated in other brain regions that chronic stress can alter dendritic morphology and thereby alter potential synaptic contacts (Cook and Wellman, 2004; Izquierdo et al., 2006; Liu and Aghajanian, 2008; Martinez-Tellez et al., 2009). The effects of chronic social defeat on 5-HT neuron morphology are not known. Given the lack of changes in sEPSC frequency or amplitude, it is likely that any contribution of potential morphological changes would selectively affect the domain of the dendritic tree where inhibitory synapses are found.

The anatomy of local DR circuitry is still poorly understood. The topographical pattern of neurons with stress-induced changes in sIPSC frequency yields insight into the local GABAergic circuitry. The decrease in sIPSC frequency was limited to vmDR 5-HT cells and did not affect lwDR 5-HT cells. If the decrease in sIPSC frequency seen in intruders is indeed due to
decreases in GABA release, our data suggest that lwDR neurons are innervated by GABA afferents that are differentially regulated than those that innervate vmDR neurons. While there may be GABAergic inputs to vmDR cells with decreased GABA release after chronic stress, there could be a separate population of GABA neurons not altered by chronic stress that innervate lwDR 5-HT neurons. The differential GABAergic innervation of DR subregions provides a mechanism for differential regulation of vmDR 5-HT neurons, whose projections include forebrain regions, and of lwDR 5-HT neurons, whose projections include midbrain and hindbrain regions. The potential effects of this differential control are more fully understood by looking at post-synaptic changes induced by chronic stress.

**Chronic social stress induces distinct post-synaptic changes in vmDR and lwDR 5-HT neurons.**

While the major defining membrane properties of 5-HT neurons (Vandermaelen and Aghajanian, 1983; Kirby et al., 2003; Beck et al., 2004) remained unaltered by chronic social stress, there were several post-synaptic changes in subpopulations of 5-HT neurons that likely contribute to stress-induced pathology. Though there were no differences in membrane characteristics between intruders and controls, social defeat did induce changes whereby the vmDR 5-HT neurons of the most anxious intruders demonstrated a pattern of intrinsic membrane properties contributing to an increased excitability. Interestingly this pattern consisting of increased excitability, larger tau, and decreased activation gap is the same pattern of intrinsic properties that is present in populations of vmDR 5-HT neurons with increased propensity to be activated by stressors (Crawford et al., 2010). This pattern of changes was not present in lwDR neurons of intruders. In addition, sIPSC data suggested distinct post-synaptic changes in 5-HT neurons of vmDR and lwDR neurons. In the vmDR there was a decrease in the amplitude and a consequent decrease in charge flux of events mediated by GABA$_A$R. In the
In lwDR there were slower rise time and decay kinetics and a trend toward increase in charge flux of events mediated by GABA$_A$R. This could be due to differential regulation of GABA$_A$R subunit expression, receptor trafficking, post-translation modification, or a combination of these factors. In the vmDR chronic stress may induce a downregulation of functional GABA$_A$R that synergizes with the decreased GABA release and pattern of increased intrinsic excitability of neurons in the most anxious intruders. In contrast, stress-induced altered subunit composition or modification of GABA$_A$R in the lwDR may lead to longer, more slowly decaying inhibitory events and an increased charge mediated by GABA$_A$R. Thus, in the anxious brain, vmDR 5-HT output is likely increased due to the decreased inhibitory tone and increased excitability of 5-HT neurons while lwDR 5-HT output is likely unchanged or decreased, due to the increased potency of GABA$_A$R found on lwDR neurons.

*Functional consequences of the neurophysiological changes in the anxious brain*

Because the DR subregions have distinct efferent projections, the distinct stress-induced changes in vmDR or lwDR neurons suggest that the 5-HT output in brain regions targeted by either subfield is differentially affected in anxiety disorders. Our data suggest that in the anxious brain, 5-HT output in vmDR target regions may be increased due to decreased inhibitory tone and increased excitability of vmDR 5-HT neurons. This would result in a stress-induced increase in 5-HT release in regions that include the amygdala or the mPFC, regions that are preferentially targeted by midline DR neurons but not the lwDR (Hale et al., 2008; Meloni et al., 2008). In fact several studies have demonstrated an increase in 5-HT levels in amygdala, ventral hippocampus, and medial prefrontal cortex after uncontrollable stress in behavioral models of anxiety and depression (Amat et al., 1998b, a; Bland et al., 2003). Our data also suggest that lwDR plays a distinct role in stress circuits and the pathology underlying anxiety. An understanding is emerging that identifies the 5-HT neurons of the lwDR as a crucial inhibitory
regulators of cardiovascular and behavioral responses to innocuous stressors (Johnson et al., 2004; Johnson et al., 2008; Lowry et al., 2008). This role in dampening panic responses depends upon lwDR 5-HT projections to target regions such as the dorsolateral periaqueductal gray (dIPAG) and rostral ventrolateral medulla (RVLM) and the 5-HT\textsubscript{1A} heteroreceptor found in those regions (Bago and Dean, 2001; Bago et al., 2002; Johnson et al., 2004; Johnson et al., 2008). Our findings suggest that lwDR 5-HT neurons have normal or increased inhibitory feedback after chronic social stress, which could potentially lead to insufficient attenuation of panic responses due to decrease release of 5-HT in lwDR target regions. It is unclear how stress alters levels of 5-HT in regions preferentially targeted by the lwDR but consistent behavioral effects have been demonstrated: A loss of neuronal activation of lwDR 5-HT neurons is associated with increased vasopressor responses and exaggerated panic in a rat model of panic disorder (Johnson et al., 2008). This insufficient attenuation of panic responses may contribute to the increased sympathetic tone and the associated changes in the heart and bladder observed in our anxious mice exposed to chronic social defeat. The changes in the physiology of lwDR cells and other descending 5-HT neurons therefore have implications for the somatic pathology that is often comorbid with anxiety and other mood disorders.

In summary, chronic social defeat had effects on the 5-HT system that extended beyond the 5-HT neuron itself. This model of anxiety produced differential effects on vmDR and lwDR 5-HT cells and on the local inhibitory circuits that modulate their activity. The local GABAergic circuits that modulate DR 5-HT activity were selectively altered by chronic social stress such that vmDR 5-HT cells demonstrated a loss of inhibitory input while lwDR 5-HT neurons did not. Our findings help fill the gaps left by other studies as to the mechanisms underlying anxiety and may yield insight into other stress-related disorders including depression. Although current approaches to pharmacotherapy regard the entire 5-HT system as a unitary target, this study
demonstrates that targeting specific components of the serotonin system, namely midline 5-HT neurons versus lwDR 5-HT neurons may aid in the understanding and treatment of specific components of the anxiety disorders and pathological sequelae.

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CHAPTER 5

General Discussion

The experiments described in this document add crucial information to fill the gap in our understanding of the local circuitry of the DR, the heterogeneity of 5-HT neurons within the DR, the distinct regulation of vmDR and lwDR neurons in stress circuits, and some of the mechanisms underlying anxiety.

In accordance with my hypothesis, lwDR 5-HT neurons were distinct from vmDR 5-HT neurons in terms of membrane characteristics and spontaneous glutamatergic synaptic input. A subset of lwDR 5-HT neurons demonstrated enhanced 5-HT_{1A}R-mediated responses that were significantly larger than those of vmDR cells. Contrary to my hypothesis, the baseline spontaneous GABAergic synaptic input to vmDR and lwDR 5-HT neurons was largely comparable. I also hypothesized that the chronic stress induced by a social defeat paradigm would differentially alter the membrane characteristics of lwDR and vmDR neurons and decrease the negative feedback onto both vmDR and lwDR 5-HT cells. In support of my hypothesis, GABAergic input to vmDR neurons was decreased in mice after enduring chronic social defeat; however this was not the case in the lwDR. GABAergic input to lwDR neurons was unaltered in defeated mice, although there was a non-significant trend towards an increase in inhibitory charge mediated by lwDR GABA_{A} receptors. Contrary to my hypothesis, there were no observed changes in the 5-HT_{1A}R-mediated response in either DR subregion of defeated mice.
Implications of the distinct functional characteristics of 5-HT neurons of the vmDR and lwDR

Although our understanding of the local circuitry of the DR is incomplete, the intricacies of my findings yield much insight into the functional correlates of the topographical organization of 5-HT neurons and the inputs that modulate their activity. My findings add to what is known about vmDR neurons, including the rostral-caudal patterning of intrinsic excitability and the subpopulations of vmDR with unique inward rectification and delayed-onset rectification ($I_h$) currents. In addition, I have characterized the membrane characteristics, 5-HT1A-R-mediate responses, and excitatory and inhibitory synaptic inputs in the mouse, which had never been done, to my knowledge, in immunohistochemically identified 5-HT neurons. Our quantification of dendritic morphology of vmDR neurons, in particular the correlation of 2nd order branch length with the frequency of excitatory synaptic input, clarifies the role of 5-HT dendrite morphology in mediating synaptic input.

Though the 5-HT neurons of the lwDR were poorly characterized, my findings have underscored their unique role in stress circuits: lwDR 5-HT neurons seem to function as prime integrators of synaptic input. An extended, complex dendritic morphology is the medium for increased selective innervation by glutamatergic and stress-resistant GABAergic afferents. These synaptic factors can potentially synergize with the increased resistance and lower AP threshold that afford an increased intrinsic excitability of the lwDR neuron. Collectively these factors enable the lwDR to integrate these excitatory and inhibitory synaptic inputs with driving excitatory noradrenergic input and other modulatory input including afferents from the central amygdala and corticotropin releasing factor (CRF) fibers, which are more highly concentrated in the lwDR of the middle and caudal DR (Kirby et al., 2000; Valentino et al., 2001; Lee et al., 2007; Waselus and Van Bockstaele, 2007). The increased gain enables the lwDR neuron to integrate
these inputs and produce increased output in target regions selectively innervated by the lwDR, such as the panic and cardiovascular response centers of the midbrain and hindbrain.

The distinctions between vmDR and lwDR 5-HT neurons also bring to light the prospect of being able to selectively target subpopulations of 5-HT neurons. Electrophysiology data has highlighted a number of ion channels and receptors that may potentially differ between vmDR and lwDR neurons. The increased presence of inward rectifying currents and $I_h$ currents in the vmDR suggest a higher prevalence of the channels that mediate these currents in vmDR 5-HT neurons. The demonstration of longer AP duration and longer AHP with larger amplitude in lwDR cells compared to vmDR cells suggests increased levels of $Ca^{2+}$ channels and the Ca-dependent K$^+$ SK-channels in lwDR neurons. I have recently observed that depolarization-induced Ca-spikes are seen more often in lwDR neurons (5 of 7) than in vmDR neurons (1 of 6) in the presence of the AP blocker TTX (data not shown), supporting the conclusion of increased Ca-current in lwDR 5-HT neurons. Pharmacotherapy aimed at modulating the ion channels that mediate these functional differences between vmDR and lwDR 5-HT cells could potentially allow one to alter the activity of a subset of 5-HT neurons and thereby selectively alter 5-HT output in brain regions innervated by that subset. Interestingly a recent study targeted SK channels as an potential adjunct to selective serotonin reuptake inhibitor therapy (Crespi, 2010). They found that a blocker of SK$_{2/3}$ channels, apamin, increases vmDR 5-HT neuron firing and partially overcomes the feedback inhibition that usually decreases DR 5-HT neuron firing induced by acute SSRI treatment. Because I found that apamin had a larger effect in the lwDR, completely eliminating the AHP while vmDR neurons maintain a small apamin-resistant AHP (Crawford et al., 2010), I would expect this combined therapy would have a larger effect in lwDR neurons with clear implications for lwDR target regions. Unfortunately, because the Crespi study did not include the lwDR, the differential effect of apamin on lwDR activity still needs to be confirmed.
Additional potential distinctions of vmDR and lwDR cells indicated by electrophysiology recordings include differences in the expression or modification of GABA receptor subunits after chronic stress, baseline differences in AMPA receptor subunit composition and increased AMPA receptors in lwDR 5-HT neurons, and increased levels of the autoreceptor in a subset of high-resistance lwDR neurons that demonstrated significantly larger 5-HT$_{1A}$R-mediated responses. Future experiments are needed to verify the differential functional expression of these receptors by way of characterizing subregion specific expression patterns as well as subregion-specific differences in the regulation of membrane localization, coupling to effector signaling pathways, and post-translational modification of these receptors. Furthermore, subregional differences in target molecules need to be confirmed in the human brain. The lwDR of the human and non-human primate brain actually contains a higher abundance of 5-HT neurons than the vmDR subregion (Charara and Parent, 1998; Austin and O'Donnell, 1999), which underscores the importance of subregional differences in the DR when envisioning the translation of novel approaches to anxiolytic and antidepressant pharmacotherapy.

The potential benefit of pharmacological modulation of specific subpopulations of 5-HT neurons lies in the ability to manipulate 5-HT output in projection regions targeted by those subpopulations. The 5-HT neurons of the lwDR are known to act through 5-HT$_{1A}$R to inhibit the rostral ventral lateral medulla (RVLM) and dorsolateral periaqueductal gray (dPAG) leading to dampening of sympathoexcitation in response to mild, innocuous stressors such as intravenous lactate (Bago et al., 1999; Bago and Dean, 2001; Bago et al., 2002; Johnson et al., 2004; Johnson et al., 2008). Thus, by targeting lwDR 5-HT neurons, novel therapeutics may be able to selectively attenuate the cardiovascular and behavioral panic responses, especially in patients prone to panic attacks or for whom sympathetic excitation is an especially undesirable symptom e.g. patients with pre-existing hypertension or severe cardiovascular disease. Findings from my
social defeat experiments support the hypothesized role of lwDR neurons in the symptoms associated with anxiety disorders: The distinctive features of lwDR neurons revealed in my early experiments were intact in the anxious brain, despite the stress-induced deficiency of GABAergic input to vmDR neurons. My data suggested that there may even be an increase in inhibitory charge flux in lwDR GABA\textsubscript{A}R, such that GABA input to lwDR neurons in the intact brain could elicit an increased inhibitory effect. This would tend to disinhibit sympathoexcitation and lead to the increased panic responses, pathological cardiovascular effects, and other sequelae of increased sympathetic tone that accompanies anxiety. My findings also highlight a potential role in anxiety and psychogenic pathology of peripheral organs for other 5-HT neurons with descending projections, i.e. caudal raphe nuclei found in the pons and medulla. In fact 5-HT neurons in all major raphe nuclei are activated by uncontrollable stressors (Takase et al., 2004). Although caudal 5-HT nuclei have traditionally received less attention in studies on anxiety and affective disorders due to their lack of forebrain projections, they may yet play an important role.

An additional key finding from my social defeat experiments included subregion specific, presynaptic changes in inhibitory input to 5-HT neurons. The fact that chronic stress selectively decreased the sIPSC frequency to vmDR neurons is the first evidence of its kind to suggest distinct sources of GABAergic inputs to vmDR and lwDR neurons. Several markers such as parvalbumin, calbindin, and calretinin have been used to identify subtypes of GABA neurons in other brain regions, revealing distinct topographical distribution, distinct functional role in modulating the activity of principal cells, and distinct roles in mental and neurological pathology (Bouilleret et al., 2000; Cotter et al., 2002; Bastianelli, 2003; Jinno and Kosaka, 2006; Schwaller, 2009). These and other interneuron markers are found in discrete patterns in the DR (data not shown), though their contribution to modulation of 5-HT neuron activity is unknown. Future
experiments will investigate whether certain subtypes of GABA cells die off as a result of chronic social stress, as this may clarify the relative contribution of those subtypes to differential modulation of vmDR and lwDR 5-HT neurons. The selective loss of subtypes of interneurons could also underscore their importance in mediating stress-related behavior and may introduce novel targets for medical treatment of anxiety.

A deeper understanding of the mechanisms underlying anxiety and resilience

Beginning with the knowledge that the anxiolytic benzodiazepines are agonists at the GABA_A, GABAergic input has been a key feature of the hunt for a mechanism underlying anxiety. The finding that the DR is the site of benzodiazepine action gave rise to the theory that GABAergic input to the DR 5-HT neurons are central in the mechanisms of anxiolytic activity (Gallager, 1978; Soubrie et al., 1981; Thiebot et al., 1982; Maier et al., 1994) and may likewise be part of the circuitry that mediates anxiety itself, a hypothesis that is now supported with evidence presented in my experiments using the social defeat model of anxiety. Dorsal raphe GABA neurons are also crucial mediators of the inhibitory feedback onto 5-HT neurons that originates from limbic forebrain regions including the mPFC. Uncontrollable stress activates DR 5-HT neurons and leads to learned helplessness, a behavioral state that has also been characterized as behavioral inhibition, anxiety-like avoidance behavior, or behavioral depression (Maier, 1984; Maier and Watkins, 1998; Maswood et al., 1998; Grahn et al., 1999; Maier and Watkins, 2005). Controllable stress, on the other hand, activates mPFC pyramidal neurons that project to and activate DR GABA neurons, thereby inhibiting 5-HT activity and inducing normative behavior in lieu of learned helplessness (Amat et al., 2005; Maier and Watkins, 2005; Maier et al., 2006; Amat et al., 2008; Baratta et al., 2009; Christianson et al., 2009).
Together with what is known about the mechanisms of anxiolytic drugs and the neural correlates of controllable stressors, the effects of chronic social stress on vmDR and lwDR neuron physiology have enabled the development of a proposed mechanism underlying anxiety, as summarized in Figure 5.1. The circuitry involving vmDR neurons is clarified in the context of other findings in the literature: In the normal, healthy brain vmDR 5-HT neurons receive inhibitory feedback mediated by GABA input and the 5-HT$_{1A}$ autoreceptor, along with glutamatergic input and a range of other modulatory input. In response to a stressor, there may be an increase in both excitatory and inhibitory modulatory inputs to 5-HT neurons. The GABA neurons of the DR are activated by a wide range of stressors even as mild as handling (Martinez et al., 1998; Chung et al., 2000; Roche et al., 2003; Abrams et al., 2004; Johnson et al., 2004; Gardner et al., 2005; Johnson et al., 2005; Berton et al., 2007; Bouwknecht et al., 2007; Hale et al., 2008; Johnson et al., 2008; Johnson et al., 2010). This may have induced the increase in sIPSC input revealed when comparing handled, manipulated control mice to unmanipulated YFP mice from earlier experiments (Supplemental Figure 4.S1). GABAergic input may also have increased due to local release of 5-HT, as GABAergic input to 5-HT neurons is increased by activation of 5-HT$_{2A/C}$ receptors expressed on GABA neurons (Liu et al., 2000; Boothman and Sharp, 2005; Serrats et al., 2005; Boothman et al., 2006). However, this GABAergic inhibition may be outweighed by excitatory influences on 5-HT neurons. In addition to a stress-induced increase in glutamatergic input to vmDR neurons (Grahn et al., 2000), increased release of CRF may activate CRF R2 receptors and increase 5-HT activity (Hammack et al., 2002; Hammack et al., 2003; Takase et al., 2005; Waselus et al., 2009) and noradrenergic drive of 5-HT activity may increase due to stress-induced increased activation of LC neurons (Grahn et al., 2002; Takase et al., 2005; Valentino and Van Bockstaele, 2008; McDevitt et al., 2009). The stress-activated GABAergic input to 5-HT neurons becomes overwhelmed by the excitatory input from other
modulatory systems and vmDR 5-HT activity is increased, leading to increased 5-HT output in target regions including, but not limited to, the mPFC, the amygdala, and ventral hippocampus (Amat et al., 1998b, a).

During exposure to a stressor that is perceived to be controllable, mPFC projections to the DR are activated, increasing GABAergic inhibition of vmDR 5-HT activity and decreasing 5-HT output in target regions including the mPFC, the amygdala, and ventral hippocampus (Amat et al., 1998a, b). Activation of this endogenous mechanism of resilience is crucial at the time of exposure to the stressor in order to avoid the anxiety-inducing effects of stress, i.e. to have the effect of a controllable stressor (Amat et al., 2006; Amat et al., 2008). So long as the circuitry is intact, prior exposure to controllable stressors can even be protective, mitigating the effects of a subsequent exposure to uncontrollable stress (Amat et al., 2006; Amat et al., 2010). Input from the lateral habenula may play a similar role to that of the mPFC (Amat et al., 2001).

My findings now add to the understanding of how this circuitry is altered in the anxious brain. With chronic exposure to uncontrollable stress, this circuitry is altered, especially at the level of the DR GABA neuron. In the anxious brain, there is likely a reduction of synaptic vmDR \( \text{GABA}_A \) receptors in addition to a presynaptic loss of GABAergic input to 5-HT neurons, perhaps due to a loss of GABA neurons. Due to continuous exposure to stress and continued activation by local release of 5-HT, GABA neurons are continually activated which could eventually lead to maladaptive changes, excitotoxicity and apoptotic cell death. In fact, in a genetic model of maladaptive stress responsivity, non-5-HT neurons of the DR are lost due to apoptotic death following exposure to chronic stress (McEuen et al., 2008). Due to the loss of the GABA mediator of mPFC feedback, even innocuous stressors previously perceived as controllable now induce the effects of uncontrollable stressors.
A. The resilient brain
- mPFC, LHb afferents
- GABA
- vmDR 5-HT neuron
- Glut

Decr 5-HT in mPFC, Amyg, vHpc

Normal emotional responses and cognitive processing

B. The anxious brain
- mPFC, LHb afferents
- GABA
- vmDR 5-HT neuron
- Glut

Incr 5-HT in mPFC, Amyg, vHpc

Dysregulated emotional responses enhanced fear processing
C. **The resilient brain**

- mPFC, LHb afferents
- IwDR 5-HT neuron
- GABA
- Glut

**Incr 5-HT in dIPAG, RVLM**
- Dampered panic responses
- Decreased sympathoexcitation

D. **The anxious brain**

- mPFC, LHb afferents
- IwDR 5-HT neuron
- GABA
- Glut

**Decr 5-HT in dIPAG, RVLM**
- Uninhibited panic responses
- Increased sympathoexcitation
- Sequelae of prolonged, heightened sympathetic tone
Figure 5.1. The summarized effects of stress on the inputs that modulate 5-HT activity. vmDR 5-HT neurons are depicted in (A) and (B); lwDR neurons are depicted in (C) and (D). Normal effects of controllable stress on 5-HT activity are shown in (A) and (C) while the alterations in 5-HT activity due to the pathological effects of chronic stress are shown in (B) and (D). While GABAAR and AMPAR are present on many cell types in the DR, they are only represented on 5-HT neurons for clarity; 5-HT receptors play an important role in feedback regulation but are omitted for clarity. A) In the vmDR of the resilient brain, controllable stress activates mPFC afferents, increasing activation of DR GABA neurons which increase GABAergic inhibition of 5-HT neurons. This limits 5-HT release in vmDR projection regions of the forebrain and contributes to normative emotional processing and cognitive function. B) After chronic stress, there is a loss of GABA input to vmDR 5-HT neurons as well as a decrease in the number GABAAR, resulting in loss of inhibitory tone and, thus, increased 5-HT output in target regions. This contributes to changes in emotional processing and altered fear responses mediated by projection regions such as the mPFC, amygdala and vHpc. Exposure to a controllable stressor, which would activate mPFC projections, no longer has the same effect due to the loss of the DR GABA neuron and therefore causes the same emotional and cognitive responses as an uncontrollable stressor. C) The circuitry regulating lwDR 5-HT neurons is distinct. In the normal, resilient brain, controllable stress activates the lwDR 5-HT neuron. This is due to the increased intrinsic excitability of the lwDR neuron and the increased excitatory synaptic modulation that is present even in baseline conditions. The afferent input from the mPFC to lwDR neurons is poorly understood but may contribute via direct projections to 5-HT cells. This activation leads to dampening of panic responses and minimizes sympathetic activation via projections to the dIPAG and RVLM. D) In the anxious brain, i.e. after chronic stress, the GABAAR of lwDR neurons are altered such that the slower kinetics results in a more potent charge flux through the ion
Any GABA that is released now has a more potent effect and causes the inhibition of the 5-HT cell to outweigh excitatory influences. 5-HT output is decreased in lwDR projection regions leading to uninhibited panic responses, increased sympathetic tone, and resultant changes in peripheral organ systems. Amyg – amygdala, dIPAG – dorsolateral periaqueductal grey, LHb – lateral habenula, mPFC – medial prefrontal cortex, RVLM – rostral ventrolateral medulla, vHpc – ventral hippocampus.

The circuitry involving lwDR neurons is less clear, due in part to the fact that experiments delineating the mechanisms underlying controllable stress and characterization of mPFC projections to the DR have not included lwDR 5-HT neurons. Some evidence suggests a distinct circuitry for lwDR neurons, as 5-HT efflux in the lwDR target region dIPAG, is increased in controllable but not uncontrollable stressors, contrary to the pattern seen in vmDR projection regions (Amat et al., 1998b). It could be that mPFC projections directly stimulate lwDR neurons. A small percentage of DR neurons are activated in response to mPFC stimulation (Hajos et al., 1998; Celada et al., 2001; Varga et al., 2001) and a small percentage of PFC terminals do contact 5-HT neurons (Jankowski and Sesack, 2004), though the topographical location of this minority of 5-HT neurons was not reported. In addition, lwDR neurons with a more ventral position within the subfield receive higher amplitude glutamatergic synaptic input (Figure 3.7). It may be that closer juxtaposition of ventral lwDR neurons to the high density region of vGlut1 puncta (Figure 3.1) enables direct contact with glutamatergic terminals of cortical projections via synapses positioned closer to the 5-HT cell soma. The mPFC input to lwDR 5-HT neurons must be further characterized in future experiments.
Though the mPFC input to the lwDR remains unclear, a model for the contribution of lwDR to anxiety can be pieced together from my data and prior studies in the literature. In response to a mild stressor, GABAergic input to lwDR may be unchanged, since control mice and unmanipulated mice demonstrated comparable sIPSC input. Together with the increased baseline glutamatergic input seen in lwDR recordings, other stress-activated excitatory modulatory inputs from CRF fibers and LC afferents may activate lwDR neurons, as these fibers are present in the lwDR (Kirby et al., 2000; Valentino et al., 2001; Kim et al., 2004). This would lead to an increase in 5-HT output in lwDR target regions including the dIPAG and RVLM where the effects of 5-HT are to reduce sympathomotor and panic responses. However, with chronic exposure to stress, maladaptive changes ensue. In lwDR neurons this included slower kinetics of GABA<sub>A</sub> receptor which contributed to a trend towards an increased potency of GABAergic synaptic input. If, in response to chronic stress, lwDR neurons receive increased inhibitory input, this would decrease 5-HT output in lwDR target regions, thereby disinhibiting sympathoexcitation and increasing the potential for panic responses. In the anxious brain, this would contribute to the hallmark signs of exaggerated panic responses to otherwise mild or controllable stressors.

**Novel approaches to anxiolytic therapeutics**

The vast majority of studies described above that investigated the role of the DR in the effects of benzodiazepines focused on midline 5-HT cells and did not include lwDR 5-HT neurons. Collectively, this suggests that benzodiazepines are effective as anxiolytics due to their ability to increase inhibitory feedback onto forebrain projecting vmDR 5-HT neurons, mimicking endogenous resilience mechanisms. The effect of benzodiazepines on lwDR neurotransmission is not clear, as studies have either excluded the lwDR or failed to delineate whether experiments
targeting the DR included the lateral subdivision. It is not known if lwDR 5-HT neurons demonstrate the same sensitivity to benzodiazepines as seen in vmDR 5-HT neurons and whether this sensitivity changes with exposure to chronic stress. The distinct post-synaptic changes seen in vmDR and lwDR neurons of stressed animals suggest subregional differences in the GABA_A R in the anxious brain. Further experiments are needed to clarify the effect of benzodiazepines and other anxiolytics on lwDR neuron physiology and to clarify the role of lwDR neurons in the circuits mediating resilience and susceptibility to stress.

It seems that many drugs that are effective at quelling anxiety either mimic the endogenous resilience mechanisms of the mPFC-vmDR circuit, as do benzodiazepines through activation of DR GABA_A R, or introduce parallel methods of inhibiting vmDR 5-HT neurons, as do 5-HT_1A -agonists. Novel approaches to the treatment of anxiety that follow this trend should prove to be promising in reducing anxiety. In addition to GABA_A R and 5-HT_1A -agonists, there are additional neurotransmitter and neuropeptide systems that modulate inhibitory feedback onto 5-HT neurons, including endogenous opioids and CRF which can act through GABA neurons to inhibit 5-HT neurons or inhibit 5-HT neurons directly (Kirby et al., 2000; Roche et al., 2003; Valentino and Commons, 2005; Waselus et al., 2005; Kirby et al., 2008; Nazzaro et al., 2010). It could be that these peptidergic systems play a differential role in the vmDR or lwDR subregion. With an ever-increasing knowledge base of the distinctions between subpopulations of 5-HT neurons, it will become possible to selectively target inhibitory feedback to vmDR neurons or activation of lwDR neurons to obtain anxiolytic therapeutic effects.

The growing understanding of the mechanisms underlying resilience to anxiety also shed light on potential approaches for preventative therapy. Recent studies have demonstrated that exposure to a controllable stressor can attenuate the effects of subsequent uncontrollable stressors, so long as the activation of mPFC during the first exposure is intact (Amat et al., 2006;
Amat et al., 2010). Stimulating the mPFC during a stressor gives the illusion of controllability, that is to say an inescapable stressor consequently has the behavioral effect of escapable, controllable stressor (Amat et al., 2008; Christianson et al., 2009). It is unknown how long-lasting the effects of this “behavioral immunization” are and whether it can serve a protective function in models of anxiety or depression. However, with the advent of noninvasive methods of activating brain regions in human patients (i.e. transcranial magnetic stimulation and transcranial direct current stimulation), this presents an intriguing avenue for the prevention of the harmful effects of stress in patients prone to stress-induced panic attacks or in anticipation of intense deleterious stress as with soldiers approaching combat. The success of this type of approach would depend upon normal levels of GABAergic neurotransmission within the DR. Thus, therapies that selectively target inhibitory transmission to vmDR neurons or stimulatory transmission to DR GABA neurons may complement this type of approach.

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APPENDIX 1

Abbreviations

5-CT – 5-carboxyamidotryptamine, 5-HT_{1A}R agonist
5-HT – 5- hydroxytryptamine, serotonin
aCSF – artificial cerebrospinal fluid
AHP – after-hyperpolarization
AHP t_{1/2} – AHP duration measured at half-height
Amp – amplitude
AP – action potential
AP thr – action potential threshold
CdCl_{2} – cadmium chloride
CPG 55845 – GABA_{A} receptor antagonist
CRF – corticotrophin releasing factor
DL-AP5 – NMDA receptor antagonist
dIPAG – dorsolateral PAG
dmDR – dorsomedial DR
DNQX – AMPA/kainate receptor antagonist
DR – dorsal raphe
Dur – duration
GABA – gamma- Aminobutyric acid
GAD65/67 – glutamate decarboxylase, marker of GABA neurons
LHb – lateral habenula
lwDR – lateral wings of the DR
mPFC – medial prefrontal cortex
RMP – resting membrane potential
RVLM – rostral ventrolateral medulla
PAG – periaqueductal gray
sEPSC – spontaneous excitatory post-synaptic current
sIPSC – spontaneous inhibitory post-synaptic current
TPH – tryptophan hydroxylase, marker of 5-HT neurons
vGlut – vesicular glutamate transporter
vlPAG – ventrolateral PAG, overlaps with lwDR
vmDR – ventromedial DR
ZD 7288 – I(h) current blocker