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The Functional Neuroanatomy of Helplessness Vulnerability

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The Functional Neuroanatomy of Helplessness Vulnerability

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Dedication

with thanks and love to my parents Jack and Dixie Shumake

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The Functional Neuroanatomy of Helplessness Vulnerability

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This dissertation had three specific aims: 1) to behaviorally characterize the congenital helplessness rat model of vulnerability to depression and post-traumatic stress disorder (PTSD); 2) to metabolically map the brains of naïve congenitally helpless adults and newborns; and 3) to map the effects of the antidepressant fluoxetine on the brains of these rats. Congenitally helpless rats, selectively bred for vulnerability to learned helplessness, were assessed on tests of exploratory activity, anxiety, circadian activity, and interest in reward; they showed increased exploration and reduced anxiety in novel environments, but also insomnia and reduced sucrose preference. This pattern of results is discussed as resembling the temperamental profile of individuals with PTSD. The brains of rats from the congenitally helpless strain were metabolically mapped with quantitative cytochrome oxidase histochemistry. Adults showed widespread metabolic abnormalities in several brain regions, but these abnormalities were bidirectional and whole-brain metabolism was not altered; the paraventricular hypothalamic nucleus (PVH), habenula, ventral hippocampus, interpeduncular nucleus, and infralimbic cortex showed increases, and the dorsomedial prefrontal cortex, basal ganglia, septal nuclei, amygdala, and ventral

tegmental area (VTA) showed decreases. In contrast, newborns showed a global reduction in brain metabolism, but the largest decreases were seen in the two regions showing the largest increases in the adult: the PVH and habenula. Newborns also showed a marked reduction in interregional functional coupling, particularly between forebrain and brainstem regions. Fluoxetine produced anti-depressant effects in the adult brain, increasing VTA metabolism and decreasing habenula metabolism. Thus, the only common region affected in adults and newborns and by fluoxetine treatment was the habenula. Functional relationships among the implicated brain systems were considered in light of the literature on depression and PTSD. It is suggested that the hypometabolism and absence of functional connectivity observed in the newborn may reflect reduced ability of forebrain regions to regulate the response of brainstem regions to stress. Consequently, the PVH may become hyperactive in the adult, and other regions may follow suit in an unsuccessful attempt to constrain its activity. The habenula is discussed as a possible nexus through which antidepressants may influence stress-responsive networks.

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Chapter 1: Modeling the Predisposition to Stress-Related Psychopathology

Helplessness, a behavioral state of passivity accompanied by feelings of uncontrollability, is a common component of several comorbid psychiatric disorders: most notably, major depression, dysthymia, post-traumatic-stress disorder (PTSD), and substance abuse. Theories concerning the role of helplessness in these disorders stem from a series of animal learning studies conducted in the early 1960s in the lab of Richard L. Solomon. Overmier and Seligman (1967) initially found that exposure to inescapable electric shocks caused dogs to switch from an active behavior—running around in a distressed manner—to a passive one: most subjects eventually lay down in a corner and whined. Subsequently, when shocks could be avoided, the dogs did not acquire the avoidance response as efficiently as control animals. The dogs appeared to have lost the behavioral flexibility to respond effectively to stressful situations. Overmier and Seligman called this phenomenon "learned helplessness" because the dogs seemed to have learned that their responses were futile. Later, Seligman and Maier (1967) validated that uncontrollability was indeed the critical factor.

LEARNED HELPLESSNESS AS A DISEASE MODEL

Based on this and other work, Seligman (1975) argued that learned helplessness in animals modeled at least some forms of human depression, noting several similar features: decreased locomotion, poor performance in appetitive tasks, decreased aggression, and loss of appetite and weight. There is even convincing evidence that learned helpless animals show attention deficits which are largely responsible for their learning difficulties (Minor, Jackson, & Maier, 1984; Lee & Maier, 1988). Indeed, if learned helpless animals have impaired attention, then the number of symptomatic

parallels would warrant a legitimate diagnosis of major depression (Table 1.1) except for one thing: most of the effects of uncontrollable shock are transient, with recovery in 2 to 3 days (Maier, 1984), whereas a diagnosis of depression requires at least 2 weeks of abnormality (American Psychiatric Association, 2000). However, learned helplessness remains a valid model of depression in other respects, including the most important one from a practical standpoint: it reliably discriminates drugs with antidepressant effects (Petty, Davis, Kabel, & Kramer, 1996; Sherman, Sacquitne, & Petty, 1982).

More recently, learned helplessness has been proposed as a model of PTSD (Foa, Zinbarg, & Rothbaum, 1992; Petty, Kramer, Wu, & Davis, 1997), a psychiatric disorder that develops following exposure to one or more stressful events. Table 1.2 shows that DSM criteria for PTSD diagnosis include four major symptom clusters: reexperiencing the traumatic event, avoidance of stimuli associated with the traumatic event, numbing of behavioral responsiveness, and increased arousal (American Psychiatric Association, 2000). Certainly the inescapable shock that leads to learned helplessness could be regarded as traumatic stress, but it would be premature to conclude that learned helplessness models PTSD exclusively. Indeed, it is not even clear that depression and PTSD are distinct entities themselves, since there is considerable phenomenological overlap between PTSD and chronic depression (Newport & Nemeroff, 2000). For example, PTSD and depression share anhedonia, sleep disturbance, and impaired concentration as core diagnostic criteria, and there is also overlap in terms of restricted

Table 1.1: Summary of *Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR)* criteria for major depression, reorganized according to signs and symptoms.

At least five symptoms must be present for at least two weeks.
One of the symptoms must be depressed mood or loss of interest or pleasure.
Behavioral Signs
Decreased interest in pleasurable stimuli (e.g., sex, food, social interactions)
Insomnia or hypersomnia
Psychomotor agitation or retardation
Decreased or increased appetite, weight loss or weight gain
Decreased ability to concentrate and think, indecisiveness
Subjective Symptoms
Depressed mood
Low energy, fatigue
Feelings of worthlessness, excessive or inappropriate guilt
Recurrent thoughts of death and suicide

Table 1.2: Summary of *Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR)* criteria for post-traumatic stress disorder.

A. Exposure to a traumatic event. Symptoms must be present for at least one month.
B. Persistent reexperience of the trauma in at least one of the following ways:
(1) Distressing recollections
(2) Distressing dreams
(3) Acting or feeling as if the traumatic event were recurring
(4) Intense distress at exposure to internal or external cues of the trauma
(5) Physiological reactivity on exposure to internal or external cues of the trauma
C. Persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness, as indicated by at least three of the following:
(1) Efforts to avoid thoughts, feelings, or conversations associated with the trauma
(2) Efforts to avoid activities, places, or people that arouse recollections of the trauma
(3) Inability to recall an important aspect of the trauma
(4) Markedly diminished interest or participation in significant activities
(5) Feeling of detachment or estrangement from others
(6) Restricted range of affect
(7) Sense of foreshortened future
D. Increased arousal, as indicated by at least two of the following:
(1) Difficulty falling or staying asleep
(2) Irritability or outbursts of anger
(3) Difficulty concentrating
(4) Hypervigilance
(5) Exaggerated startle response

range of affect, impairment in personal functioning, and guilt (Vermetten, Charney, & Bremner, 2001). Moreover, 48% of PTSD patients receive a comorbid diagnosis of major depression (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995).

Regardless of whether learned helplessness models depression, PTSD, or some aspects of both, two fundamental problems remain in using the model to understand the neurobiology underpinning human psychopathology. The first, already mentioned, is the insufficient duration of helpless behavior relative to human psychiatric disorders. The problem exists whether one is attempting to model PTSD or depression, and is even worse in the case of PTSD since its diagnosis requires a month of symptomatology as opposed to two weeks (Tables 1.1 and 1.2). The second problem is that stress per se is not sufficient to cause depression, and trauma per se is not sufficient to cause PTSD.

THE IMPORTANCE OF PREDISPOSITIONAL FACTORS

Most people do not develop depression or PTSD following stress or a traumatic event. Only 6 - 15% of women develop depression following a stressful event, while 1% develop depression even in the absence of significant stress (Kendler et al., 1995). And, while nearly half of all U.S. adults experience at least one traumatic event in their lifetime, only 10% of women and 5% of men develop PTSD (Kessler et al., 1995). This points to individual differences in susceptibility to stress, and epidemiological studies show that 40%-50% of the risk for depression is genetic (Sanders, Detera-Wadleigh, & Gershon, 1999; Fava & Kendler, 2000). This makes depression a highly heritable disorder—as heritable as type II diabetes, hypertension, asthma, and certain cancers (Nestler et al., 2002). PTSD is also partially hereditary, with at least 30% of symptoms based on genetic factors (True et al., 1993). Clearly, greater knowledge of these predispositional factors would be immensely beneficial, both to identify at-risk individuals and to develop more effective treatments. However, most studies, both animal

and human, have examined their subjects only after a stressful episode has taken its toll. These studies have accumulated a vast wealth of data, but without prospective biological measurements taken before illness onset, it is impossible to tease apart primary etiological factors from what are likely a voluminous number of irrelevant or compensatory factors that are consequential, not causal, in nature.

While it is possible to address these questions in humans—through studies of discordant identical twins or other healthy relatives of depressed patients, or through prospective longitudinal studies of at-risk subjects—the requisite experiments are difficult to execute in practice. Identical twins discordant for depression are not common, and longitudinal studies are inherently burdensome; they demand large amounts of time, funding, and manpower, and are inevitably plagued by high rates of subject drop-out. And clearly it would be unethical to genetically manipulate human subjects in an attempt to induce psychopathology. It is because of these practical and ethical limitations to human experimentation that animal models are vitally needed for elucidating the predisposing factors for depression and other stress-related affective disorders. The aim of this dissertation project is to employ one such model, the congenitally helpless rat, to map the brain systems underlying vulnerability to affective disorders.

CONGENITAL HELPLESSNESS AS A VULNERABILITY MODEL

Animals also show individual differences in vulnerability to stress. Henn, Johnson, Edwards, and Anderson (1985) showed that only 5% - 20% of rats develop learned helplessness following 20 minutes of 0.8 mA footshock distributed randomly within a 40 minute session (Henn, Johnson, Edwards, & Anderson, 1985). These percentages are remarkably in line with the prevalence rates of depression and PTSD in humans; moreover, helpless behavior in these susceptible rats can persist up to a month

following training (Henn et al., 1985). Thus, by focusing on especially vulnerable rats, both of the problems discussed earlier—prevalence and endurance—are simultaneously addressed.

Hypothesizing that individual differences in stress vulnerability had a genetic basis, Fritz Henn and Emmeline Edwards began to interbreed animals that demonstrated both greater susceptibility and resistance to helplessness. Twenty-four hours after the inescapable shock session, the rats were tested in an escape paradigm where foot shock could be eliminated with a single lever press. Animals with more than 10 failures out of 15 trials were considered helpless, and animals with less than 5 failures were considered non-helpless. Helpless and non-helpless animals, respectively, were mated for the subsequent generations, avoiding sib crosses. The first four generations of selective breeding more than doubled the percentage of helplessness-susceptible offspring and rendered the non-helpless line of rats entirely resistant to helplessness (Henn et al., 1985). Genetic vulnerability continued to increase in the helplessness-susceptible line until a plateau was reached in the 25th generation, when 95% of rats began to show spontaneous helpless behavior in the absence of a training session (Lachman et al., 1992). Henn and Edwards named their susceptible and resistant strains "congenital learned-helpless" and "congenital non-learned-helpless," respectively. For simplicity and clarity, we have shortened these to "congenitally helpless" and "congenitally non-helpless" to emphasize that the rats used in our studies did not undergo learned helplessness training. The choice of the word "congenital" as opposed to "genetic" is important because, while the helplessness-susceptibility trait appears inborn, one cannot rule out a possible contribution of the prenatal environment to the phenotype.

Behavioral Characteristics of the Congenitally Helpless Rat

Outside of their spontaneous helpless response to electric shock, congenitally helpless rats have not been extensively characterized in terms of behavior. As one might expect, following exposure to inescapable shock, vulnerable rats display all the vegetative behaviors traditionally associated with learned helplessness (Henn et al., 1985). However, prior to this year, almost no published work has examined the premorbid temperament of congenitally helpless rats by assessing behaviors early in development and prior to significant stress.

This question is the focus of the next chapter, but there are two potential criticisms of the model that can be addressed here. In breeding for escape failures following inescapable shock, one would obviously like the selected trait to be something like a depressive phenotype. However, there are two other possibilities which must be ruled out. One is that congenitally helpless rats have a general learning deficit which has nothing to do with emotional state. Two studies have dispelled this notion by showing that, under baseline conditions, congenitally helpless rats perform as well as controls in the Morris water maze (King, Abend, & Edwards, 2001; Vollmayr et al., 2004). The other alternative is that the congenital helplessness trait is somehow specific to withstanding footshock and does not reflect a generalized coping response. This possibility was dispelled by a study showing that congenitally helpless rats express elevated levels of behavioral despair in the forced swim test (Patel, Bartoszyk, Edwards, & Ashby, Jr., 2004). Behavioral despair is similar to learned helplessness in that it entails increased immobility 24 hours after inescapable stress. However, in the forced swim test the stressor is 15 minutes of swimming instead of 20 minutes of footshock. Thus, helpless rats show increased depressive behavior in another well-validated model of depression (Porsolt, 2000; Willner, 1990), which they were not specifically bred for. This, together

with the absence of a general learning deficit, argues that the selected phenotype does correspond to an emotional response to stress. This hypothesis is further supported by other behavioral tests, which are reported in Chapter 2.

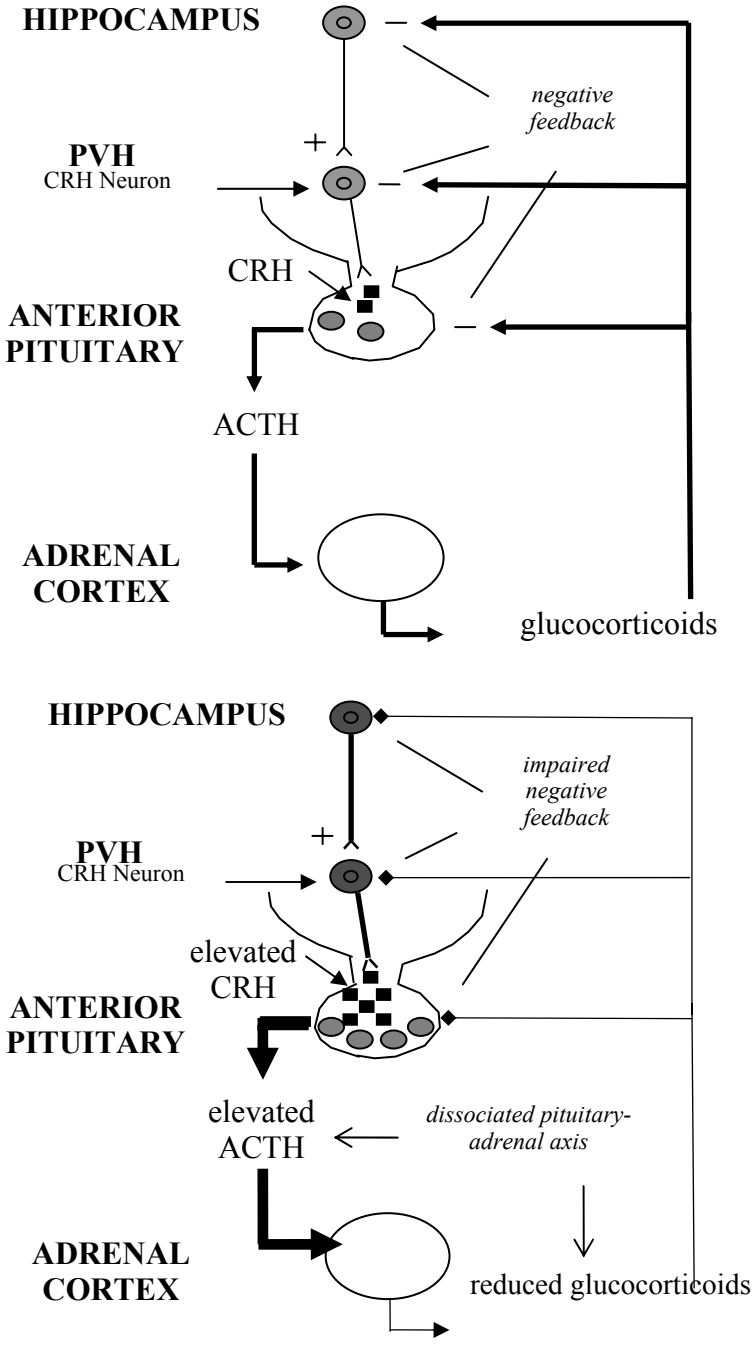
Biological Characteristics of the Congenitally Helpless Rat

Several published studies have examined the biological characteristics of the congenitally helpless strain. Their findings have fallen into one of three major themes: 1) disruption of the hypothalamic-pituitary-adrenal (HPA) axis, 2) enhanced opiod analgesia, and 3) abnormal intracellular signaling.

Disrupted Hypothalamic-Pituitary-Adrenal Axis

Several studies suggest that congenitally helpless rats have deficient glucocorticoid signaling in response to stress, and Figure 1.1 illustrates the nature of HPA disruption in congenitally helpless rats. Lachman et al. (1993) observed that, unlike control rats, congenitally helpless rats do not alter glucocorticoid receptor mRNA expression in the hippocampus or hypothalamus in response to corticosterone administration, even though their glucocorticoid receptors are normal in terms of prevalence and binding characteristics. The exact cause of this dysfunction is unknown, but appears to involve a functional modification that affects some, but not all, glucocorticoid receptors since another glucocorticoid-inducible gene, metallothionein-1 (MT-1), does increase normally in response to corticosterone administration. There is evidence that depressed humans possess similarly aberrant glucocorticoid receptors (Pariante & Miller, 2001) and that antidepressant drugs both increase expression of glucocorticoid receptors (Calfa et al., 2003) and improve their functioning (Pariante & Miller, 2001).

Figure 1.1: Dynamics of the hypothalamic-pituitary-adrenal axis in response to stress for the normal (TOP) vs. the congenitally helpless rat (BOTTOM). PVH = paraventricular hypothalamic nucleus; CRH = corticotropin-releasing hormone; ACTH = adrenocorticotropin hormone



In addition to glucocorticoid receptor dysfunction, several studies have shown reduced corticosterone secretion in congenitally helpless rats, either at baseline (Edwards, King, & Fray, 1999), in response to stress (Edwards, King, & Fray, 2000; King, Campbell, & Edwards, 1993; King & Edwards, 1999), or in response to cues associated with a prior stressful experience (King et al., 2001), but only if the animals had encountered previous stress. (Stress-naïve rats showed the same hormonal response as controls.) However, the stress does not have to be severe or protracted. For example, a mere 2 hours of isolation on postnatal day 21 was sufficient to induce a robust reduction of corticosterone secretion on postnatal day 90 (King & Edwards, 1999). While most of these studies inflicted stress early in life (Edwards et al., 1999; King et al., 1993; King & Edwards, 1999), developmental timing does not appear critical since stress first encountered in adulthood also leads to adrenal hypoactivity within as few as 24 hours (Edwards et al., 2000; King et al., 2001). However, corticosterone hyposecretion in this case is not indicative of overall HPA activity since congenitally helpless rats actually show abnormally high ACTH secretion in response to stress (King & Edwards, 1999). Similar to the congenitally helpless rats, PTSD has been linked to a dissociation between the hypothalamus and the adrenal glands. At the hypothalamic level, there is hypersecretion of CRH (Baker et al., 1999), which is similar to that reported in depression. However, cortisol levels are normal to low in PTSD (Yehuda et al., 1993; Yehuda & Antelman, 1993; Yehuda et al., 1996; Yehuda, McFarlane, & Shalev, 1998; Yehuda, 1999). In conclusion, HPA function in congenitally helpless rats resembles evidence from human studies of both PTSD and depression.

Enhanced Opiod Analgesia

King, Abend, and Edwards (2001) showed that exposure to 20 min of inescapable footshock approximately doubled the pain tolerance of congenitally helpless animals

when they were re-exposed to the shock chamber or to an odor cue associated with the shock chamber. In contrast, control rats did not experience any conditioned analgesia from the chamber or odor. Similarly, Vietnam veterans re-exposed to combat trauma through videotapes experienced a 30% decrease in pain sensitivity (van der Kolk, Greenberg, Orr, & Pitman, 1989), which was reversible with naloxone, a μ-opiod receptor antagonist (Pitman, van der Kolk, Orr, & Greenberg, 1990). Interestingly, congenitally helpless rats show 3-5 times the number of μ-opiod receptors compared to congenitally non-helpless rats (Henn, Edwards, & Muneyyirci, 1993), and suicide victims show 9 times the number of μ-opiod receptors compared to controls (Gross-Isseroff, Dillon, Israeli, & Biegon, 1990).

Abnormal Intracellular Signaling

Kohen, Neumaier, Hamblin, and Edwards (2003) assessed gene expression levels of several intracellular signaling molecules and found that congenitally helpless rats showed reduced hippocampal expression of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) mRNA. This is consistent with a helpless/depressive disposition, given that hippocampal levels of CREB mRNA increase following chronic administration of antidepressants, lithium, or electroconvulsive therapy (Nibuya, Nestler, & Duman, 1996), and over-expressing CREB in the dentate gyrus of rats reduces learned helplessness and shortens immobility time in the forced swim test (Chen, Shirayama, Shin, Neve, & Duman, 2001). Kohen et al. (2003) also found increased bcl-2 mRNA levels in the prefrontal cortex. Bcl-2 is an antiapoptotic protein that promotes neuronal survival, differentiation, and neurite outgrowth (Zhang, Westberg, Holtta, & Andersson, 1996).

CANDIDATE BRAIN SYSTEMS FOR CONFERRING DEPRESSION VULNERABILITY

Regions of Interest Suggested by Human Studies

Since congenitally helpless rats display a temperament similar to that seen in depression or PTSD (see Chapter 2), we surveyed the literature on the functional neuroanatomy of these disorders. A similar set of regions has been implicated in both, including the prefrontal cortex (PFC), anterior cingulate cortex, basal ganglia, and hippocampus, which have tended to show reduced activity, and the amygdala, medial diencephalon, and cerebellum, which have tended to show increased activity. However, the results are not completely consistent from study to study.

Studies of Depression

Frontal-cingulate cortex. Abnormalities in activation of prefrontal regions in depression have been reported more frequently than for any other brain region. Depression occurs more frequently following strokes that affect the left PFC (Morris, Robinson, Raphael, & Hopwood, 1996), and structural MRI has confirmed that depression is associated with a specific reduction of PFC volume (Coffey et al., 1993). Even depressed children and adolescents show these region-specific volume reductions (Steingard et al., 1996). However, the primary evidence linking frontal cortex to depression has come from functional neuroimaging studies. PET and fMRI have consistently shown abnormalities in frontal metabolism in depressed patients, although the specific nature of these abnormalities varies as a function of the specific subregions and the symptomatic profiles of the patients assessed.

PFC regions where dysfunction might impair the ability to modulate emotional responses in mood disorders include the dorsomedial PFC (area 9), dorsolateral PFC (areas 9/46), and the area of anterior cingulate immediately dorsal to the corpus callosum

(area 24). Abnormally decreased blood flow and metabolism in these areas have been the most extensively replicated findings in depression neuroimaging (Baxter, Jr. et al., 1989a; Bench et al., 1992; Biver et al., 1994; Ebert, Feistel, & Barocka, 1991; Mayberg et al., 1997), and these metabolic deficits appear to relate to the negative thoughts (Dunn et al., 2002) and subtle impairments of attention and memory (Bench, Friston, Brown, Frackowiak, & Dolan, 1993) that accompany major depression episodes.

Located ventral to the genu of the corpus callosum, subgenual anterior cingulate has shown both metabolic decrements (Drevets et al., 1997) and mild hyperactivity (Mayberg et al., 1999) in depression. Pregenual anterior cingulate cortex, located anterior to the genu of the corpus callosum, has shown both elevated and suppressed blood flow and metabolism in untreated depression; elevated activity predicts that a patient will respond well to antidepressant treatment, and suppressed activity predicts that a patient will respond poorly (Mayberg et al., 1997).

Similar to pregenual cingulate cortex, a complex relationship exists between depression severity and metabolic activity in the orbitofrontal cortex. Although metabolic activity is abnormally increased in outpatient or treatment-responsive depressives, more severely ill or treatment-refractory inpatients either do not differ in orbitofrontal metabolism or are decreased relative to control samples (Drevets et al., 1997; Mayberg et al., 1997). In addition, remitted depressed patients who relapse under tryptophan depletion show reduced metabolism restricted to the most medial portion of orbitofrontal cortex (gyrus rectus and medial orbital gyrus; Bremner et al., 1997a).

Basal ganglia. Many human neuroimaging studies have shown reduced blood flow or metabolism in the basal ganglia of depressed patients (Austin et al., 1992; Baxter, Jr. et al., 1989b; Buchsbaum et al., 1986; Mayberg, Lewis, Regenold, & Wagner, Jr., 1994; Rogers, Bradshaw, Pantelis, & Phillips, 1998; Videbech, 2000), and structural

imaging has found reduced volume of the caudate and putamen in depressed patients (Krishnan et al., 1992; Parashos, Tupler, Blitchington, & Krishnan, 1998). Lacerda et al. (2003) further found that left putamen volumes decreased as a function of length of depressive illness, and that left globus pallidum volume increased as a function of the number of depressive episodes. Basal ganglia deficits may relate to the motor changes frequently observed in depression, and psychomotor-anhedonia symptoms correlated with decreased metabolism in the anteroventral caudate-putamen (Dunn et al., 2002).

Paraventricular hypothalamus. A prominent mechanism by which the brain reacts to acute and chronic stress is activation of the hypothalamic-pituitary-adrenal axis. Neurons in the paraventricular nucleus of the hypothalamus (PVH) secrete corticotropin-releasing hormone (CRH), which signals the pituitary to release adrenocorticotropin hormone (ACTH), which in turn activates the production of adrenal steroids. In humans, the major steroid released from adrenal cortex is cortisol (corticosterone in rats), which completes a negative feedback loop by binding to brain glucocorticoid receptors and inhibiting the secretion of CRH.

HPA-axis activity is increased in depressed patients by a number of measures (Scott & Dinan, 1998; Modell, Yassouridis, Huber, & Holsboer, 1997; Rubin, Phillips, McCracken, & Sadow, 1996; Krishnan et al., 1991b). These include elevated baseline levels of plasma cortisol and ACTH, and the failure of dexamethasone, a synthetic glucocorticoid, to suppress cortisol production (Holsboer, Liebl, & Hofschuster, 1982). Current evidence suggests that hypercortisolism and dexamethasone non-suppression stem from faulty corticosteroid receptors in regulatory structures such as the hippocampus, which fails to relay the negative feedback signal from cortisol that normally constrains the stress response (Pariante & Miller, 2001). Consequently, the paraventricular nucleus of the hypothalamus (PVH) is thought to become hyperactive,

and postmortem studies of depressed patients have demonstrated increased numbers of peptide-containing neurons in the PVH—such as arginine vasopressin (AVP)-immunoreactive neurons, oxytocin (OXT)-expressing neurons, and corticotropin-releasing hormone (CRH) neurons—as well as increased expression of CRH mRNA, which would permit more CRH synthesis in the PVH (Purba, Hoogendijk, Hofman, & Swaab, 1996; Raadsheer, Hoogendijk, Stam, Tilders, & Swaab, 1994). One would therefore expect PVH metabolism to be altered in an individual predisposed to depression.

Hippocampus. The hippocampus, with its high concentration of glucocorticoid receptors, likely mediates corticosterone negative feedback to the HPA axis (Sapolsky, Krey, & McEwen, 1986). Hippocampal volume reductions have been found in several magnetic resonance imaging (MRI) studies of patients with depression (Bremner et al., 2000; Krishnan et al., 1991a; Sapolsky, 2000; Shah, Ebmeier, Glabus, & Goodwin, 1998; Sheline, Wang, Gado, Csernansky, & Vannier, 1996); however, hippocampal function has rarely been directly assessed with functional neuroimaging. Only one study has reported reduced left hippocampal metabolism, which negatively correlated with depression severity (Saxena et al., 2001).

Amygdala. Abnormal elevations of resting regional cerebral blood flow or glucose metabolism in the amygdala have been reported in depression during both wakefulness (Drevets et al., 1992) and sleep (Ho et al., 1996; Nofzinger et al., 1999). Furthermore, metabolic rate in the amygdala correlated with depression severity (Drevets et al., 1992) or negative affect (Abercrombie et al., 1998) in depressed patients. However, amygdala changes have not been observed in most studies and may only be present in patients with comorbid anxiety disorders (Davidson et al., 2002).

Thalamus and habenula. Few studies have examined thalamic metabolism in depression, but, when it has been assessed, hypermetabolism has been found (Drevets et al., 1992; Morris, Smith, Cowen, Friston, & Dolan, 1999; Saxena et al., 2001). Of these three studies, two found that the thalamic hypermetabolism was localized to the dorsal midline (Drevets et al., 1992; Morris et al., 1999), and Morris et al. (1999) further localized the source to a region immediately superior and lateral to the posterior commissure. This region most likely corresponds to the habenula nucleus.

Studies of Post-Traumatic Stress Disorder

Compared to depression, far fewer neuroimaging studies have been conducted on PTSD, and the majority of these have employed an anxiogenic challenge paradigm (e.g., evoking traumatic memories). These studies frequently found increased activity in the premotor frontal region and decreased activity in the anterior cingulate and middle temporal gyri (Bremner et al., 1999b; Lanius et al., 2001; Liberzon et al., 1999; Rauch et al., 1996; Shin et al., 1999; Shin et al., 2001) although patients reporting dissociative symptoms showed increased activity in all these regions (Lanius et al., 2002). Some have also found amygdala activation (Rauch et al., 2000; Shin et al., 1997b; Shin et al., 1997a); however, most have not (e.g., Bremner et al., 1999b; Bremner et al., 1999a; Shin et al., 1999).

Only a few studies of resting state metabolism have been performed. Some were done on an exclusive substance abuse population and will not be discussed (Semple et al., 1993; Semple et al., 1996). Other resting state studies normalized their results to the cerebellum, which was assumed to have no relevance to PTSD (Lucey et al., 1997; Sachinvala, Kling, Suffin, Lake, & Cohen, 2000). However, because a subsequent study found this assumption to be invalid, these results will not be discussed either. This leaves only one resting state study (Bonne et al., 2003), which found that increased perfusion in

the following regions discriminated PTSD trauma survivors from healthy trauma survivors: the cerebellum, the left inferior lateral temporal lobe, and the left supramarginal and postcentral gyrus. Interestingly, the same study showed that reduced cortisol signaling predicted increased hippocampal and decreased frontal-cingulate activity in those vulnerable to PTSD. Structural MRI studies revealed reduced hippocampal volume in PTSD patients (Bremner et al., 1997b; Gurvits et al., 1996; Stein, Koverola, Hanna, Torchia, & McClarty, 1997; Villarreal et al., 2002). Interestingly, Gilbertson et al. (2002) found reduced hippocampal volume in PTSD patients' monozygotic twins who never experienced trauma, indicating that this may be a vulnerability marker.

Regions of Interest Suggested by Animal Studies

There are over a dozen animal models of depression (Willner, 1990), and reviewing the neuroscientific literature on all of them is beyond the scope of this project. Therefore, with the exception of one metabolic mapping study (which was relevant because of the methodology), our survey of the animal literature was limited to studies of brain regions which mediate the development and maintenance of learned helplessness. Collectively, the animal literature points to the hippocampus, septal nuclei, raphe nuclei, and habenula as being important players in helpless behavior.

Studies of Learned Helplessness

The hippocampus was the first brain region linked to the development of learned helplessness, which was blocked by fornix transection (Leshner & Segal, 1979). Many subsequent studies have also implicated hippocampal activation in the development of learned helplessness. For example, Petty and Sherman (1981) found that inescapable shock increased glutamate and decreased GABA release in the hippocampus. As

discussed in the first two chapters, dysfunctional glucocorticoid receptors specific to the hippocampal region appear to confer vulnerability to learned helplessness (Lachman et al., 1993; Papolos et al., 1993).

The related septal area was the second region shown to play a role in learned helplessness. Sherman and Petty (1980) found that learned helplessness was associated with decreased septal serotonin (5-HT) release and that 5-HT microinjected into the lateral septal nucleus (LS) reversed learned helpless behavior. Furthermore, rats which proved vulnerable to learned helplessness showed reduced c-fos expression in the LS (Steciuk, Kram, Kramer, & Petty, 1999), while rats which proved resistant to learned helplessness showed increased 5-HT metabolism in the LS (Ronan, Steciuk, Kramer, Kram, & Petty, 2000).

The other major region implicated in learned helplessness development is the dorsal raphe nucleus (DR). Enhanced 5-HT release from the DR appears to be a prerequisite for the development of learned helplessness (Amat et al., 2001; Grahn et al., 1999b; Grahn et al., 2002; Grahn, Watkins, & Maier, 2000; Grahn, Maswood, McQueen, Watkins, & Maier, 1999a; Greenwood et al., 2003; Hammack et al., 2003; Hammack, Pepin, DesMarteau, Watkins, & Maier, 2003; Hammack et al., 2002; Maier, Grahn, & Watkins, 1995; Maier, Kalman, & Grahn, 1994; Maier et al., 1993; Maswood, Barter, Watkins, & Maier, 1998; Sutton, Grahn, Wiertelak, Watkins, & Maier, 1997). While several neurotransmitters and neuropeptides (including norepinephrine, opiods, corticotropin-releasing factor, and urocortin) appear to mediate increased DR activity in response to inescapable stress (Grahn et al., 1999a; Grahn et al., 2002; Hammack et al., 2002; Hammack et al., 2003), the only specific brain region demonstrated to play a role in this is the habenula. Amat et al. (2001) found that lesioning the habenula prevented the

rise in DR 5-HT following inescapable shock and blocked the development of learned helplessness.

The amygdala may also play a role in some aspects of helpless behavior since lesions of the basolateral and central nuclei reduce immobility following inescapable shock. However, these lesions do not prevent the development of learned helplessness (Maier et al., 1993), but lesions of the related bed nucleus of stria terminalis (BNST) do prevent the development of learned helplessness (Hammack, Richey, Watkins, & Maier, 2004).

Metabolic Mapping of Three Models of Depression

Outside of our own work, only one other study has applied the metabolic brain mapping approach to study animal models of depression. Caldecott-Hazard, Mazziotta, and Phelps (1988) used $_{14}$C-2-deoxyglucose (2-DG) autoradiography to examine the cerebral correlates of three depression models: amphetamine withdrawal, chronic stress, and α-methyl-para-tyrosine challenge (which reduces catecholamine activity). The only region that showed elevated metabolism across all three models was the lateral habenula, but several regions showed reduced metabolism in each model: the dorsal medial prefrontal cortex, anterior ventral nucleus of the thalamus (AV), and inferior colliculus. Forebrain global metabolic rates were also reduced in each of the models. In addition, reduced caudate metabolism was observed in two of the models.

OVERVIEW

The aim of this dissertation is three-fold: first, to behaviorally characterize the congenital helplessness model; second, to metabolically map the brains of naïve congenitally helpless adults and newborns; and third, to map the effects of the antidepressant fluoxetine on the brains of these rats.

Chapter 2 discusses the behavioral performance of congenitally helpless rats on tests of exploratory activity, anxiety, circadian activity, and interest in reward. Chapter 3 discusses the general methodology used in the subsequent metabolic mapping studies and includes background on quantitative cytochrome oxidase histochemistry and the statistical techniques used to analyze functional imaging data.

The remaining chapters report the results of the metabolic mapping studies. Chapter 4 discusses the brain map of naïve congenitally helpless adults, and Chapter 5 discusses the brain map of congenitally helpless newborns. Chapter 6 discusses changes in brain metabolism induced by chronic administration of fluoxetine. Finally, Chapter 7 summarizes and integrates the major findings of the dissertation project and discusses their theoretical predictions and implications.

Chapter 2: Behavioral Characterization of the Helpless Temperament

Outside of King et al. (2001) and Vollmayr et al. (2004), no one has examined baseline behavioral differences in the congenitally helpless strain. Such behavioral tests are important for establishing possible temperamental traits that predict vulnerability to stress in these animals, and for interpreting any brain differences they might show. We assessed the rats on a variety of behavioral parameters, including exploratory activity in the open field, anxiety in the light-dark test, circadian running wheel activity, and interest in sucrose reward. We decided to focus on adolescent, as opposed to adult rats, for a number of reasons. First and foremost, from a clinical standpoint, it would be useful to identify individuals at risk for psychopathology during childhood and adolescence prior to adult onset of disease, and temperamental markers present during this period of development in congenitally helpless rats may inform this question. Second, Vollmayr et al. (2004) have already verified open-field and sucrose consumption differences in adult congenitally helpless rats. Third, for this study, we wished to avoid the influence of estrous cycling on the behaviors of female rats. Finally, rats are more exploratory and playful during this period of development, which may be an ideal time for assessing novelty-seeking behaviors (Spear, 2000).

Experimental subjects were congenitally helpless males (n = 23) and females (n = 21) bred in our laboratory, and control subjects were Sprague-Dawley males (n = 12) and females (n = 12) obtained from a commercial animal supplier (Harlan). Congenitally helpless rats were separated from their mothers on postnatal day 21, and control rats were shipped to us at the same age. All subjects were group housed 3-5 to a cage (45 × 24 × 21 cm) in a temperature-controlled room (22 ± 1°C). They were maintained on a 12/12 hour light cycle and provided continuous food and water.

Rats were handled daily for 5 min during postnatal days 22-28, and behavioral testing was conducted from postnatal day 29 to 48. Open-field and light-dark activity were assessed on postnatal days 29-31. Sucrose consumption was assessed prior to running wheel activity, but the exact start date varied due to limited testing equipment relative to number of subjects. Thus, some animals had sucrose testing on days 32-33 and running-wheel testing on days 34-35; others were delayed by intervals of 2 days, with the latest interval being sucrose testing on days 45-46 and running-wheel testing on days 47-48. Each testing period had equal numbers of each group and sex.

Note that neither these rats, nor their parents, were actually trained for learned helplessness because naïve subjects are needed in order to examine behavioral predispositions. Only their grandparents, obtained from the Central Institute for Mental Health (Mannheim, Germany), were verified as possessing the helplessness phenotype. However, it is typical for 88% of male and 94% of female offspring to express congenital helplessness (Edwards et al., 2000), and our larger sample size for these rats should compensate for the presence of a few outliers. The methodology and results of each test are discussed separately below.

OPEN-FIELD AND LIGHT-DARK ACTIVITY

The open field test is one of the most popular procedures in animal psychology (Prut & Belzung, 2003). Different versions are available, but the procedure generally consists of subjecting a rodent to an unknown environment from which escape is prevented by surrounding walls. In such a situation, rodents spontaneously prefer the periphery of the apparatus to activity in the central parts of the open field. This behavior is called thigmotaxis and is considered an index of anxiety since many anxiolytic drugs reduce it, increasing time spent in the central part of the field (Prut & Belzung, 2003). General locomotion and rearing activity appear to reflect level of arousal since stimulant

drugs increase locomotion and sedatives decrease rearing (Prut & Belzung, 2003). Based on the diagnostic criteria for depression, one might predict either increased or decreased open field activity in an animal model, since both psychomotor agitation and retardation have been linked to depression. The increased arousal observed in PTSD, however, would predict increased open field activity (American Psychiatric Association, 2000), but, because of their predisposition to become immobile when subjected to stress, one might expect congenitally helpless rats to express reduced locomotion in the open field. However, Vollmayr et al. (2004) found that they in fact show increased locomotion, but only during the first 5 minutes of the open field test. Thus, congenitally helpless rats do not show persistent hyperactivity or hypoactivity as part of their premorbid phenotype, but are temperamentally predisposed to increase locomotion in response to novelty.

The present experiment attempted to replicate this finding and extend the results to include measures of rearing and anxiety, which were not assessed by Vollmayr et al. (2004). In addition to thigmotaxis, on the second day of testing we used a variant of the open field called the light-dark test to assess anxiety. In this version, half of the field is covered by a dark box with a small opening, and the animal is allowed to move freely between the bright and dark compartments. This provides an important additional perspective on the animal's temperament since exploration of the uncovered area is voluntary. Simply put, it resembles a forced-choice test of personality type in humans: animals that show a strong preference for the dark field may be characterized as anxious or withdrawn, and animals that spend increased time in the light field have been characterized as impulsive or sensation-seeking (Kabbaj, 2004). Intuitively, one might expect that individuals predisposed to depression or PTSD would show increased withdrawal and anxiety, so we predicted that the congenitally helpless rats would show increased fearfulness on these behavioral measures. Finally, we repeated the open field

test on the third day as a more stringent test of whether any group differences observed on the first day were due to novelty.

Method

An automated activity monitoring system from MED Associates (St. Albans, Vermont, USA) recorded various locomotion indices in a testing room illuminated at 100 lux. The 43.2 cm^2 chamber consisted of clear plastic sides 30.5 cm high and a white plexiglass floor. Parallel arrays of infrared motion detectors were located 2.5 cm and 10 cm above the chamber floor to record horizontal and vertical motion, respectively. The chambers were cleaned with a mild detergent between animals.

Three 10-minute sessions were conducted over 3 consecutive days. Each session began by placing the rat in the same corner of the field. On days 1 and 3 (referred to as the novel and familiar open-field sessions, respectively), the chamber was completely open. Measures included ambulatory distance, rearing (vertical counts and time), velocity, and time spent in the periphery vs. the center, defined as 38% of the area. On day 2 (referred to as the light-dark field session), a dark plexiglass insert covered half of the chamber, and a small hole permitted free access to either the open or covered compartments. The same activity parameters were recorded as in the open field, but, instead of time spent against the walls relative to time spent in the center, fearfulness was operationalized as time spent in the dark relative to the light. In addition, latency to first exit the dark chamber was recorded.

Results

With the exception of rearing duration and exit latency measures (see below), open-field data were analyzed with a 2 × 2 × 3 × 10 (Group × Sex × Session × Time) repeated measures multivariate analysis of variance (MANOVA), with session (novel,

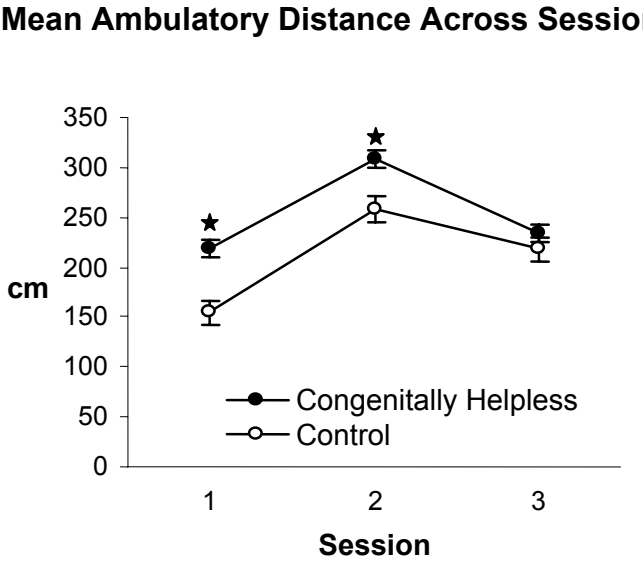
light-dark, or familiar open field) and time (in minutes) serving as repeated measures. All measures showed significant main effects of session and time: subjects always decreased activity over time within sessions, and, between sessions, they were most active in the light-dark open field, least active in the novel open field, and intermediately active in the familiar open field. However, these effects were of little interest, so the supporting data is not discussed. Sex effects were of interest; however, no significant sex differences or interactions were found for any of the analyzed variables, so the group means reported in the figures include both sexes. Due to the large number of effects and variables assessed, for sake of simplicity only group main effects and interactions showing statistical significance ($p < .05$) are reported.

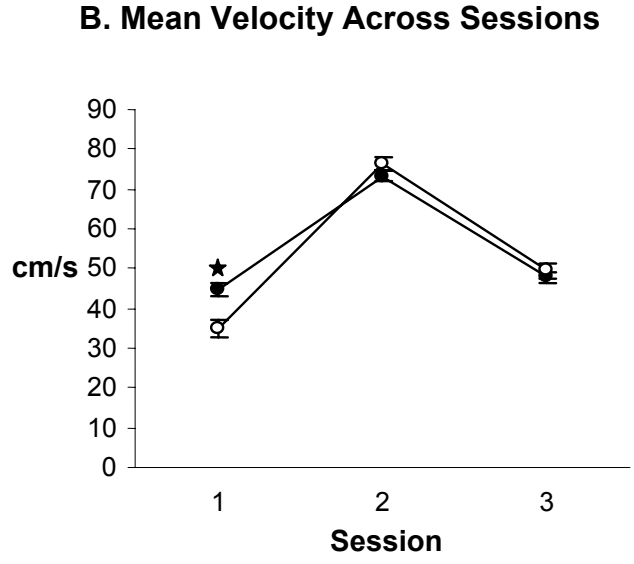
Ambulation

Repeated measures of ambulatory distance showed a significant two-way interaction (Group × Session), $F(2, 66) = 3.9$, $p = .025$. Figure 2.1A shows that within both the novel and light-dark open fields, helpless rats ambulated significantly more than controls, Fs(1, 67) = 18.9 and 9.98, ps = 0.00005 and .002, respectively. However, helpless and control rats were not significantly different in the familiar open field, $F(1, 67) = 1.16$, $p = .29$.

Concurrent measures of ambulatory velocity showed the same significant interaction (Group × Session), $F(2,66) = 7.49$, $p = .001$. Figure 2.1B shows that within the novel open field, helpless rats ambulated significantly faster than controls, $F(1,67) = 12.7$, $p = .001$. However, helpless and control rats were not significantly different in either the light-dark or familiar open fields, Fs(1, 67) = 2.37 and .436, ps = .128 and .511, respectively.

Figure 2.1: Ambulatory measures from activity chamber averaged across 10 minutes. Sessions 1, 2, and 3 refer to the novel open field, light-dark field, and familiar open field, respectively. ★$p < .01$





Rearing

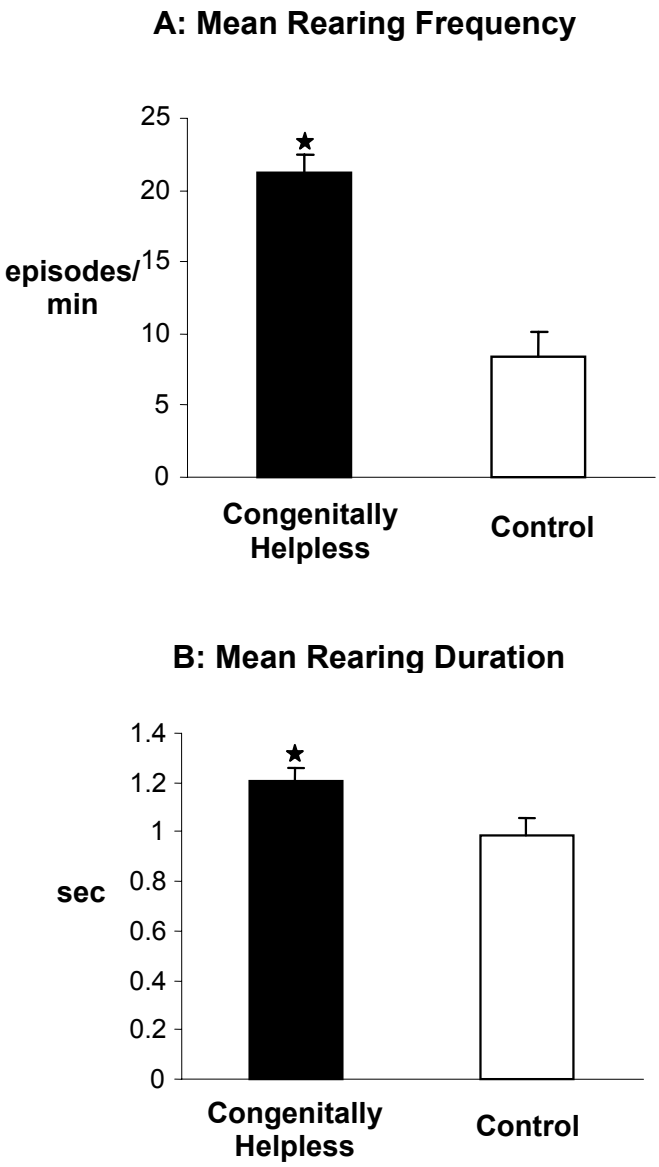
A significant three-way (Group × Session × Time) interaction was obtained for rearing counts because the two groups grew somewhat less different over time, both within and between sessions, $F(18, 50) = 2.2$, $p = .02$. However, this effect was of little interest because congenitally helpless rats continued to show significantly greater rearing than controls at every time point in every session, $F(1,67) = 35.4$, $p = 0.0000001$. Figure 2.2A shows the group means collapsed over time and session.

In addition to number of rearing episodes, the average duration of these episodes was assessed by dividing total time spent rearing by the total number of rearing episodes. Because several subjects did not rear at all during some time points, within-session repeated measures analysis could not be performed. Between-session repeated measures MANOVA showed only a main effect for group, $F(1,67) = 6.89$, $p = .01$. Figure 2.2B shows that congenitally helpless rats had longer rearing episodes than did controls.

Fearfulness

In the novel and familiar open fields, fearfulness was operationalized as the percentage of time spent against the walls (and not in the center), and, in the light-dark open field, the percentage of time spent under cover (and not in the open). While these two measures are technically different, they both reflect the relative preference of a defensive zone over a more vulnerable zone. Based on this conceptual similarity, we analyzed these fear indices as repeated measures. A significant two-way interaction (Group × Session) was obtained, $F(2, 66) = 8.423$, $p = .001$. Figure 2.3A indicates that the control group showed a substantial reduction in fearfulness following the first session, whereas congenitally helpless rats remained about the same. Thus, the groups showed a trend toward convergence across sessions. Indeed, simple-effects tests showed that, while congenitally helpless rats appeared less fearful across all three sessions, the difference

Figure 2.2: Rearing measures from activity chamber averaged across three 10 minute sessions. ★$p < .01$



was only significant in the novel and light-dark open fields, but not in the familiar open field, Fs(1, 67) = 39.8, 7.14, and 2.95, ps = 0.00000003, .009, and .09, respectively.

One additional fearfulness measure unique to the light-dark open field was the latency to first exit the dark chamber (Figure 2.3B), which was analyzed with a univariate ANOVA. This measure also indicated less fearfulness in the congenitally helpless group, with control rats taking much longer to exit the dark chamber than helpless rats, $F(1, 67) = 23.7$, $p = 0.000006$.

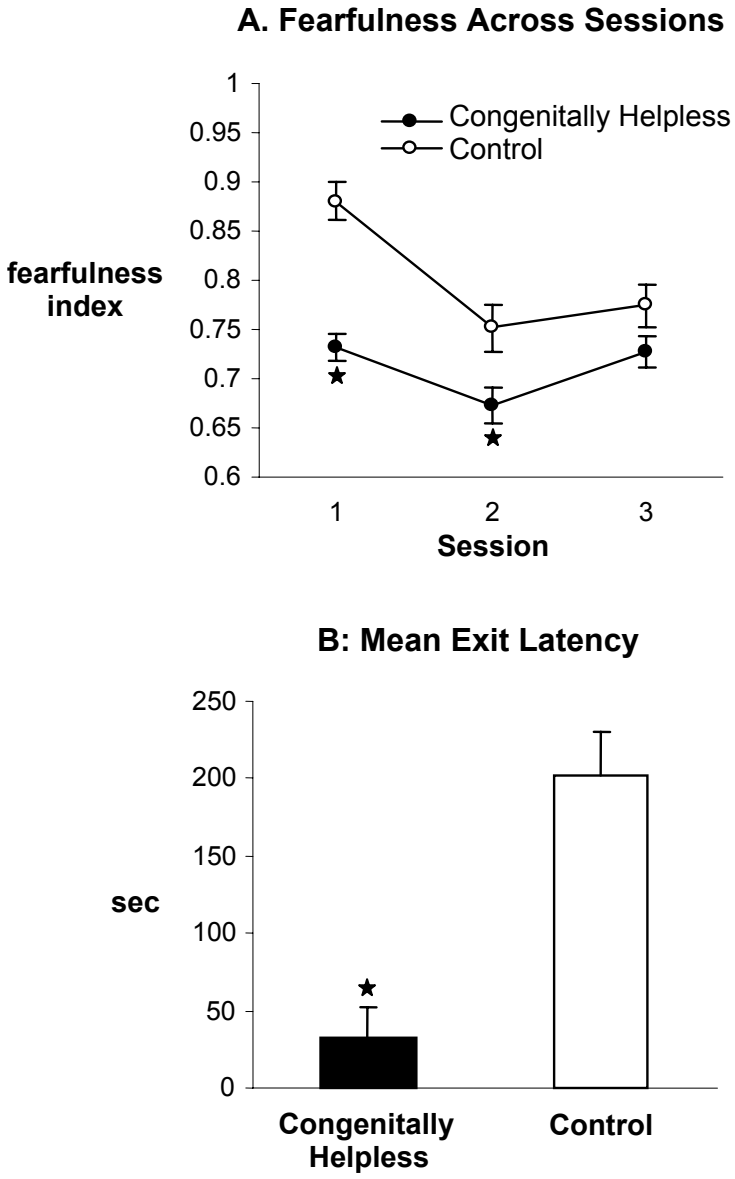
RUNNING WHEEL ACTIVITY

Monitoring voluntary activity in a running wheel for two days served as another test of general motor differences as well as a measure of circadian fluctuations in activity. Circadian rhythm has not been previously assessed in the congenitally helpless rats, but, since disruption of the sleep-wake cycle is an almost ubiquitous feature of anxiety and mood disorders (American Psychiatric Association, 2000), one might expect to observe sleep loss or a circadian phase shift in the congenitally helpless group.

Method

At the beginning of the dark cycle (1800 hours), rats were placed in the holding compartments of standard activity wheels. The door between the holding cage and wheel was open throughout the entire 48 hour session to allow continuous access to food and water. The numbers of revolutions were recorded at 600, 1000, 1400, and 1800 hours for the next 2 days. Thus, one reading was obtained for each 12 hour dark cycle, and 3 readings were obtained for each third of each light cycle.

Figure 2.3: Fearfulness measures from activity chamber. A: In sessions 1 (novel open field) and 3 (familiar open field), fear index is percentage of time spent against the walls (not in center). In session 2 (light-dark field), fear index is percentage of time spent in dark (not in light). B: In light-dark field, mean latency to first exit dark chamber after start of session. ★$p < .01$.



Results

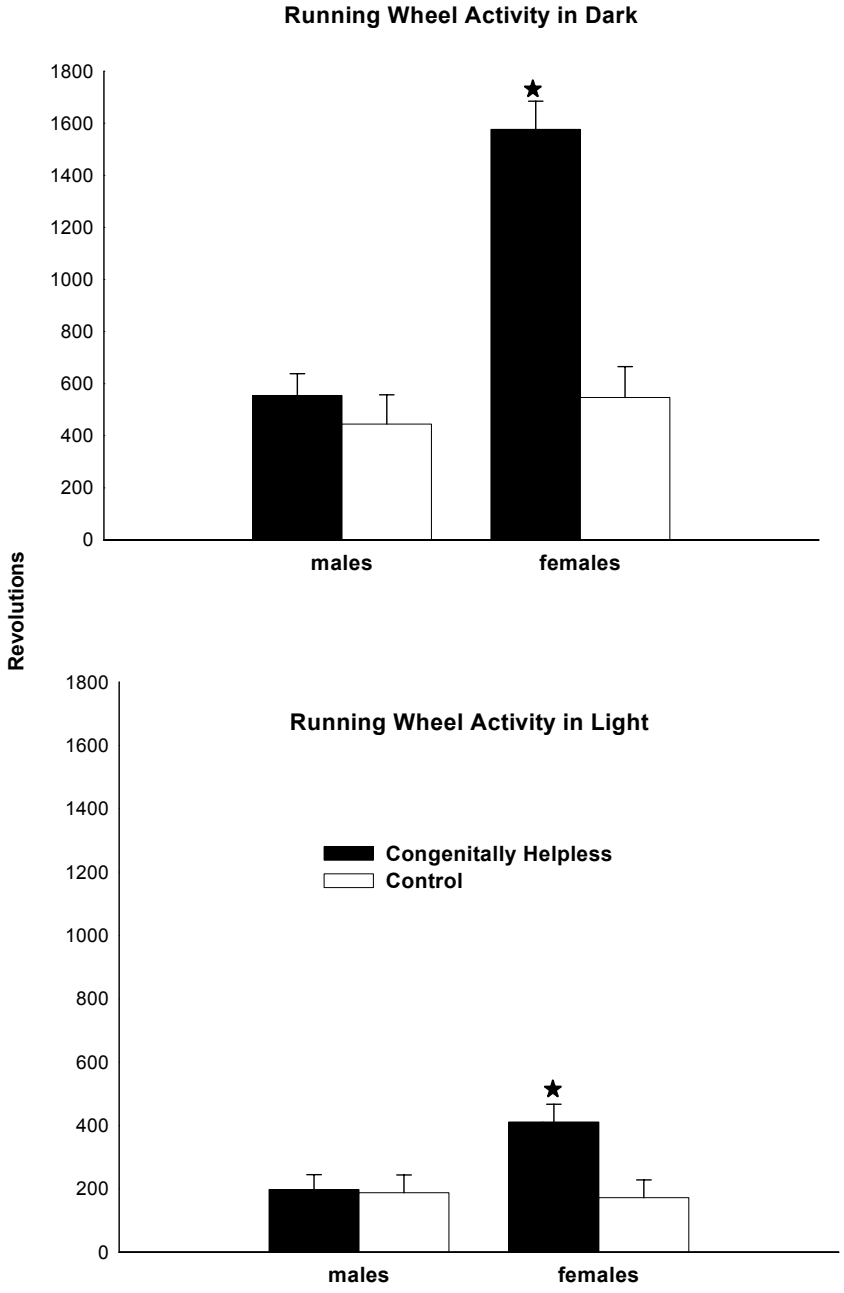
Absolute Light and Dark Activity

Data screening revealed 7 outliers (2 helpless males, 2 helpless females, 1 control male, and 2 control females), all associated with the same running wheel, which was subsequently found to have a malfunctioning counter that recorded approximately 2 revolutions for every actual revolution. These subjects were dropped from the analysis of absolute activity, which consisted of a 2 × 2 × 2 × 2 (Group × Sex × Session × Light vs. Dark) repeated measures MANOVA, with Session and Light vs. Dark serving as the repeated measures. A significant three-way (Group × Sex × Light vs. Dark) interaction was obtained, $F(1, 60) = 5.28$, $p = 0.025$. Figure 2.4 shows this interaction as 2 two-way (Group × Sex) interactions, one for the dark cycle and one for the light cycle. The three-way interaction indicates that helpless and control groups diverged more in females than males, and more in the dark than in the light. Simple-effects tests of the interaction showed that helpless males were not significantly different from control males in either the light or the dark, Fs(1, 60) = .517 and .001, ps = .47 and .97, respectively. In contrast, helpless females were significantly more active than control females in both the light and dark, Fs(1, 60) = 31.9 and 13.0, ps = 0.0000005 and 0.0006, respectively.

Relative Activity Across the Light Cycle

In order to assess circadian changes in activity independent of total activity, each subject's light-cycle activity (divided into three 4 hour intervals) was divided by its prior dark-cycle activity. Because this transformation normalized each subject for absolute differences in activity, the subjects excluded from the previous analysis were not outliers in this one. Therefore, all subjects were analyzed with a 2 × 2 × 2 × 3 (Group × Sex × Session × Time) repeated measures MANOVA, with session (first or second 24-hour

Figure 2.4: Mean revolutions in running wheel as a function of group, sex, and phase of light-dark cycle. ★p < .001.



cycle) and time (early, middle, or late day) serving as repeated measures. A significant three-way (Sex × Session × Time) interaction was obtained, $F(2, 66) = 5.02$, $p = .009$. The three-way interaction indicates that, in both groups, females showed more light-relative-to-dark activity than males across the entire first light cycle, but only in the early third of the second light cycle (data not shown). A significant two-way (Group × Time) interaction was also obtained, $F(2, 66) = 3.79$, $p = .028$. Simple-effects tests of the interaction revealed that, for both sexes in both sessions, congenitally helpless rats showed significantly more light-relative-to-dark activity than controls only in the early phase of the light cycle, $F(1, 67) = 9.23$, $p = .003$, but not in the middle or late phase of the light cycle, Fs(1, 67) = .396 and .537, ps = .53 and .47, respectively. Figure 2.5 shows that this interaction is not merely a phase shift in circadian rhythm because helpless rats, like controls, began to increase activity nearing the end of the light cycle.

SUCROSE CONSUMPTION

Anhedonia, or decreased responsiveness to rewards, is a core symptom of major depression and PTSD (American Psychiatric Association, 2000). Responsiveness to reward is usually monitored by consumption of a weak sucrose solution, a test that was developed in conjunction with the chronic mild stress model of depression (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). Vollmayr et al. (2004) previously showed that, under a progressive ratio schedule of reinforcement (in which the amount of operant responding required to receive reward is steadily increased), congenitally helpless rats were less willing to work for a 7% sucrose reward, pressing the operant bar 4 times less frequently than controls. Furthermore, congenitally helpless rats gave up entirely if more than 7 presses were required, whereas control rats were willing to press the lever up to an average of 18 times. This study already establishes that congenitally

helpless rats show reduced reward-seeking motivation. Here, we tested if this motivational deficit would also manifest as a simple reduction in sucrose drinking time.

We used a modified version of a protocol developed by Willner et al. (1987), who tested consumption of a 1% sucrose solution following 14 hours of food and water deprivation. While chronic stress reduces consumption under these conditions, the extremely weak sucrose concentration and the presence of what should be significant thirst and hunger suggest a more fundamental disruption in sensory discrimination or homeostatic drive. A pure deficit in reward seeking should, in our opinion, express itself under baseline conditions and in the face of a more potent reward. We therefore used a sweeter 5% solution, which we tested under both baseline and deprived conditions. Also, instead of measuring change in solution volume as an index of consumption, we monitored instances of licking using an automated system, which tracked changes in drinking behavior over time.

Method

Baseline consumption of a 5% sucrose solution was assessed for 1 hour in four 30 × 25 × 20 cm operant chambers (Med Associates, St. Albans, VT) where rats had free access to a bottle containing the sucrose solution. The bottles were specially designed by Med Associates for use in these operant chambers, and a light beam passing in front of the tip of the bottle registered the time the rat spent drinking, divided into six 10 minute bins. Rats were then water deprived for 20 hours, and the same procedure was repeated. Thus, rats were tested in a low (non-deprived) motivational state and a high (deprived) motivational state.

Pilot data previously showed that the four operant bottles supplied by Med Associates consistently yielded different drinking times as a consequence of different flow-rates: each bottle required significantly more or less drinking time to obtain an

equivalent volume. Therefore, bottle assignment was counterbalanced with respect to group and sex, and flow rate (inferred from the pilot data) was used as a covariate in the statistical analysis to reduce error.

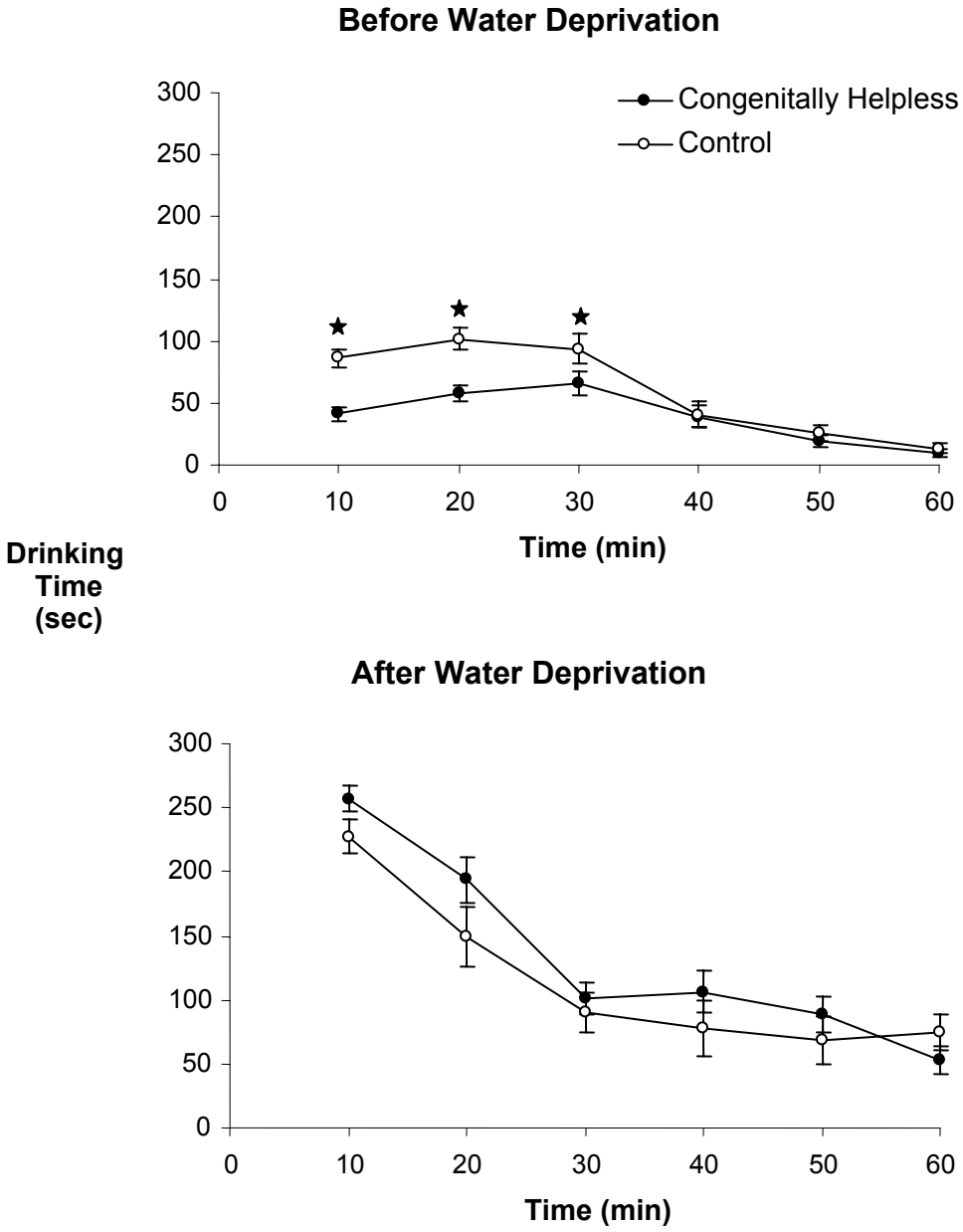
Results

Drinking time data were analyzed with a 2 × 2 × 2 × 6 (Group × Sex × Motivational State × Session Time) mixed analysis of covariance, with water bottle serving as a covariate and motivational state and session time serving as repeated measures. A significant three-way (Group × Motivational State × Session Time) interaction was obtained, $F(5, 63) = 3.63$, $p = .006$. Figure 2.6 shows this interaction as 2 two-way (Group × Session Time) interactions, one for each motivational state. The three-way interaction indicates that congenitally helpless rats spent less time drinking sucrose water than controls, but only during the first half of the baseline session, $F(1, 67) = 22.5$, $p = 0.00004$. In contrast, the groups did not differ in either the second half of the baseline session, $F(1, 67) = .405$, $p = 1$, or in the entire session following water deprivation, $F(1, 67) = 2.11$, $p = .30$.

GENERAL DISCUSSION

Congenitally helpless rats showed many behaviors consistent with diagnostic criteria for both depression and PTSD, even though they were adolescents and had never been subjected to traumatic stress. However, one potential confound is that, unlike the congenitally helpless rats, the control subjects were not bred in our laboratory but rather obtained from a commercial supplier. While it is possible that the stress of shipping could have impacted the behavior of these animals, Vollmayr et al. (2004) obtained similar results using congenitally non-helpless rats as a control. In addition, unpublished data from a separate experiment looking at the effects of maternal separation on the same

Figure 2.6: Mean time that congenitally helpless and control rats spent drinking a 5% sucrose solution, as a function of session time and level of motivation. ★ $p < .01$.



behavioral outcome measures during the same developmental period indicates that the control group, which was born in our laboratory, was similarly different from congenitally helpless rats on all of the measures. Another factor which may have contributed to behavioral differences between congenitally helpless rats and controls is a possible difference in maternal care between congenitally helpless and control mothers. It is not known whether congenitally helpless mothers differ in their maternal care, so this remains a topic for further investigation.

Perhaps the most important feature of the depressive phenotype is reduced pursuit of reward. Vollmayr et al. (2004) previously established that adult congenitally helpless rats were less motivated to work for sucrose reward. We further found that, under baseline conditions, adolescent congenitally helpless rats maintained a low but steady level of sucrose consumption throughout the testing session, in contrast to the normally observed shift from high consumption at the beginning of the session to low consumption at the end. Thus, helpless rats were less reactive to sucrose reward than controls.

We also found that congenitally helpless rats were markedly more active during the first third of their light cycle than controls, who appeared to sleep for much of this period. While both sexes of congenitally helpless rats showed this same circadian pattern, females were far more active at all time points. Despite the fact that females are 2-3 times more vulnerable to depression and PTSD than males, this was the only reliable sex difference we observed in any of the behavioral tests, and its meaning is unclear. The overall absence of sex differences may be explained by the prepubescent age at which they were tested. Thus, one should not conclude that sex differences in the helplessness phenotype do not exist because differences could still emerge later in life.

In addition to finding reward insensitivity and sleep disruption in naïve congenitally helpless rats, we replicated Vollmayr et al.'s (2004) finding of hyperactivity

in the novel open field. Unlike Vollmayr et al., we found that this hyperactivity persisted for the duration of the novel session, but novelty-dependent hyperactivity was supported by the finding that hyperactivity was no longer present by the third exposure to the activity chamber.

Surprisingly, by every measure, the congenitally helpless rats were much more exploratory and less fearful than controls. Intuitively, increased exploration of a novel environment seems inconsistent with reduced reward sensitivity and the intense immobility that these rats show in response to shock. However, it is consistent with a body of work led by Mohamed Kabbaj, in collaboration with Huda Akil, examining rats which exhibit high rates of exploratory locomotion when exposed to novel environments (Kabbaj, Devine, Savage, & Akil, 2000; Kabbaj & Akil, 2001; Kabbaj, 2004). These rats, termed high responders (HR), are virtually identical to congenitally helpless rats in terms of their open-field and light-dark behavior. Moreover, they resemble congenitally helpless rats in expressing reduced glucocorticoid receptor mRNA in the hippocampus. In fact, impaired glucocorticoid signaling appears responsible for the novelty-induced hyperactivity since injection of RU38486, a glucocorticoid antagonist, into the hippocampus essentially turned low-responding (LR) rats into HR rats (Kabbaj et al., 2000).

The HR rat was originally proposed as a model of vulnerability to substance abuse since these rats exhibit higher rates of amphetamine- and cocaine-induced locomotor activity and self-administer these drugs at lower doses than LR rats (Hooks, Jones, Smith, Neill, & Justice, Jr., 1991; Piazza, Deminiere, Le Moal, & Simon, 1989). However, their behavioral and neural similarities to congenitally helpless rats may help to account for the comorbidity of substance abuse, PTSD, and depression, with impaired glucocorticoid signaling being a common denominator. This is supported by the finding

that the same experimental manipulation which converts LR rats into HR rats—hippocampal administration of RU38486—increases vulnerability to learned helplessness, causing normal rats to respond as if they were congenitally helpless (Papolos, Edwards, Marmur, Lachman, & Henn, 1993).

It still seems paradoxical, though, that increased exploratory behavior and reduced anxiety should co-exist with reduced reward sensitivity and increased helplessness vulnerability. However, a large body of evidence from family, personality, pharmacological, and behavioral conditioning studies suggest that these behaviors correspond to dimensions of temperament which vary independently: novelty seeking, reward dependence, and behavioral inhibition (Cloninger, 1987). Operationally defined, novelty seeking is excitation in response to novel stimuli leading to exploratory behavior; reward dependence is intense responding to signals of reward leading to approach behavior; and behavioral inhibition is intense responding to aversive stimuli leading to passive avoidance. Cloninger actually refers to this last dimension as "harm avoidance," but he meant a specific kind of harm avoidance: inhibition of a punished behavior. This raises an interesting issue regarding the congenitally helpless strain, which was selected for an impairment in active avoidance, or learning to initiate a novel response to avoid harm. But this does not necessarily mean that these rats are insensitive to aversive stimuli. It is possible that they are unimpaired or perhaps even superior at passive avoidance, or learning to inhibit a punished response to avoid harm—the kind of harm avoidance that Cloninger refers to. For this reason, it is perhaps more accurate and certainly less confusing to refer to this temperament dimension as "behavioral inhibition."

Our behavioral survey of the congenitally helpless rats suggests that they are high on novelty seeking and low on reward dependence, and they were essentially bred for

high behavioral inhibition. Interestingly, two studies have found that Vietnam veterans with PTSD show an identical temperament profile when assessed with Cloninger's Tridimensional Personality Questionnaire (Richman & Frueh, 1996; Wang et al., 1997), and the authors conjecture that high novelty seeking, high behavioral inhibition, and low reward dependence confer trait vulnerability to PTSD. Moreover, all three dimensions correlated with severity of PTSD symptoms, with novelty seeking being the strongest correlate (Richman & Frueh, 1996). It seems counterintuitive that increased novelty seeking should predict vulnerability to an anxiety disorder, much as it seems counterintuitive that exploratory behavior should predict vulnerability to learned helplessness. This probably stems from the assumption that anxious individuals are stressed by unfamiliar settings, and that novelty-seeking individuals are not. However, this assumption is not supported by the facts.

In actuality, HR rats experience an abnormally intense activation of the HPA axis in response to their self-initiated exploratory activity (Kabbaj et al., 2000; Kabbaj & Akil, 2001). Similarly, Kendler, Karkowski, and Prescott (1999) found that individuals genetically predisposed to major depression paradoxically select themselves into high-stress environments which put them at even greater risk of developing depression. Thus, it seems that animals and humans prone to stress-related psychopathology are likely to be novelty seekers, and novelty seeking is likely to trigger stress-related psychopathology.

From a folk psychology standpoint, this conclusion appears nonsensical. However, from a neuroendocrine standpoint the combination of high novelty seeking, low reward dependence, and vulnerability to helplessness, depression, and PTSD makes complete sense, since all these things have been linked to reduced glucocorticoid signaling. As already discussed, glucocorticoid antagonism increases novelty seeking and vulnerability to helplessness in rats (Kabbaj et al., 2000; Papolos et al., 1993). In

addition, urinary cortisol levels negatively correlate with novelty-seeking scores on the Cloninger Tridimensional Personality Questionnaire (Wang et al., 1997). Reduced glucocorticoid function could also lead to low reward dependence since glucocorticoids appear to potentiate the reinforcing effects of many rewards, including food, sex, and drugs of abuse (Piazza & Le Moal, 1997), and a general anhedonic state is one of the major symptoms of pathologies, such as Addison disease, in which hypocortisolism is observed (Reiser & Reiser, 1995). Moreover, several human studies point to the role of reduced glucocorticoid signal in conferring vulnerability to PTSD and depression. Individuals who demonstrate low cortisol levels after an acute trauma, such as motor vehicle accident, are at higher risk of subsequently developing PTSD symptoms (Delahanty, Raimonde, & Spoonster, 2000). Cortisol levels are also negatively correlated with a number of risk factors for depression, such as neuroticism, introversion, and perceptions of uncontrollability; in contrast, cortisol levels are positively correlated with self esteem, hardiness, and affective stability—factors which confer resistance to depression (McCleery & Goodwin, 2001; Pruessner, Hellhammer, & Kirschbaum, 1999; Scarpa & Luscher, 2002; Zorrilla, DeRubeis, & Redei, 1995).

In conclusion, congenitally helpless rats show behaviors consistent with a temperament of high novelty seeking, high behavioral inhibition, and low reward dependence, which most closely resembles the temperament observed in combat veterans with PTSD. However, just as PTSD patients are markedly inclined to depression and substance abuse, the temperament of helpless rats may best be viewed as conferring risk to stress-related psychopathology in general. Paradoxically, part of this vulnerability may lie in a predisposition to seek out the very kinds of stressful situations which are likely to provoke helplessness and depression.

Chapter 3: Metabolic Brain Mapping Methods

Functional neuroimaging technologies, such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), have enabled the in vivo investigation of the functional neuroanatomy of depression. Given the current wealth of functional imaging data from depressed patients, analogous techniques used in animal models could provide an excellent bridge to the human disorders they are purported to simulate. In particular, PET measurements of regional glucose metabolism are directly comparable to the autoradiographic deoxyglucose method developed in animals. However, the use of metabolic mapping in animal models of depression has been rare, and this provides the major impetus for my dissertation work.

Metabolic mapping provides an opportunity to compare an animal model with a human disorder using a similar in vivo brain measure, which may speak more to a model's validity than any apparently shared behavior. Furthermore, since current human brain-imaging technology cannot accurately localize signals to specific subcortical nuclei, metabolic brain mapping of animal models provides a means for inferring functional activity in these undetectable regions of humans: if a model shares a pattern of cortical activity with a human disorder, one may speculate that the animal's subcortical pattern of activity would be shared as well. Finally, and perhaps most important, metabolic mapping provides a circuit-level framework for integrating the plethora of cellular-molecular data that animal research has generated.

QUANTITATIVE CYTOCHROME OXIDASE HISTOCHEMISTRY

Background

Cytochrome oxidase histochemistry, like 2-deoxyglucose (2-DG) and fluoro-deoxyglucose (FDG) autoradiography (Gonzalez-Lima, 1992), provides a functional marker for energy metabolism, and therefore neuronal activity (Wong-Riley, 1989; Wong-Riley, Nie, Hevner, & Liu, 1998). As the final step in the electron transport chain, the catalytic activity of the mitochondrial enzyme cytochrome oxidase is critical for glucose oxidization and the production of adenosine triphosphate (ATP). Furthermore, as neuronal activity increases or decreases (and the need for ATP increases or decreases), the number of mitochondria and the amount of cytochrome oxidase within mitochondria adjust accordingly to match the activity-dependent need for ATP (Wong-Riley, 1989; Wong-Riley et al., 1998). Thus, cytochrome oxidase activity within a brain region is tightly coupled to the neuronal activity of that region.

While many PET studies have used deoxyglucose radiotracers, cytochrome oxidase has never been measured in depressed humans. The question arises then, if the goal is to provide a common brain measure between the helplessness model and human depression, why use cytochrome oxidase histochemistry instead of deoxyglucose autoradiography? To be sure, there is not a one-to-one correspondence between a region's deoxyglucose uptake and its cytochrome oxidase activity (Gonzalez-Lima & Cada, 1998b), but both provide an index of oxidative metabolism. In addition, cytochrome oxidase has several advantages for mapping changes in neurobiological predispositions. Namely, cytochrome oxidase is unique in that it marks cumulative, long-term neuronal activity, while FDG uptake reflects state-dependent activity spanning approximately 45 minutes following tracer injection (Gonzalez-Lima & Cada, 1998b). In contrast, cytochrome oxidase in naïve animals from different genetic strains reflects

stabilized baseline differences (Gonzalez-Lima & Sadile, 2000; Papa, Sadile, Sergeant, Shumake, & Gonzalez-Lima, 1998). Thus, with naïve rats from the congenitally helpless strain, deoxyglucose would mark state-dependent differences, while cytochrome oxidase would mark trait-dependent differences in brain metabolism. Certainly traits (inherited, stable characteristics) and states (dynamic brain conditions) are related, and one would expect to see both overlap and distinctions among cytochrome oxidase and deoxyglucose measures. However, our initial goal has been to examine the neurobiological predisposition to develop helpless behavior—not the brain's evoked response to stress. Thus, quantitative cytochrome oxidase histochemistry provides a unique way to isolate this innate component by identifying the brain regions affected in the congenitally helpless rats.

Method

Following decapitation, brains were removed intact and frozen rapidly in isopentane. Using a cryostat (Reichert-Jung) at -20°C, brains were sectioned at 40 μm and kept frozen at –40°C until they were processed using quantitative cytochrome oxidase histochemistry, as described previously (Gonzalez-Lima & Cada, 1994; Gonzalez-Lima & Garrosa, 1991). In brief, the cytochrome oxidase staining procedure involves a series of chemical exposures, the first of which (0.1 M phosphate buffer with 10% wt/vol sucrose and 0.5% vol/vol glutaraldehyde, pH 7.6, for 5 min) facilitates tissue adherence to the slides. The next series of exposures (four changes of 0.1 M phosphate buffer with 10% wt/vol sucrose, for 5 min each) removes red blood cells. Then the slides undergo metal intensification (0.05 M Tris buffer, pH 7.6, with 275 mg/l cobalt chloride, 10% wt/vol sucrose, and 0.5% vol/vol dimethylsulfoxide, for 10 min) to enhance staining contrast and reduce time spent in the following incubation procedure (350 mg diaminobenzidine tetrahydrochloride, 52.5 mg cytochrome c, 35 g sucrose, 14 mg

catalase, and 1.75 ml dimethylsulfoxide in 700 ml of oxygen-saturated 0.1 M phosphate buffer, at 37 °C for 1 hour). The reaction is stopped by fixing the tissue in buffered formalin (for 30 min at room temperature with 10% wt/vol sucrose and 4% vol/vol formalin). Finally, the slides are dehydrated in a series of ethanol baths (increasing from 30% to 100% vol/vol ethanol), cleared with xylene, and coverslipped with Permount.

To quantify enzymatic activity and to control for staining variability across different batches of cytochrome oxidase staining, sets of tissue-homogenate standards were included with each batch of slides. This tissue homogenate was obtained from 12 normal adult Sprague-Dawley male rats, whose brains were removed after decapitation, stored at 4°C (in sodium phosphate buffer, pH 7.6), and then homogenized at 4°C. The enzymatic activity of cytochrome oxidase in this homogenate was assayed using spectrophotometry, as described by Gonzalez-Lima and Cada (1994), and activity units were defined at pH 7 and 37°C, where 1 unit oxidizes 1 μmol of reduced cytochrome c per min (μmol/min/g tissue wet weight). Remaining tissue homogenate was frozen and stored at –40°C.

Immediately prior to each cytochrome oxidase staining procedure, the standard homogenate was sectioned at varying thickness (10, 20, 40, 60, and 80 μm), and these sections were thaw-mounted onto a slide and stained for cytochrome oxidase along with the experimental tissue. Activity values determined from the spectrophotometric procedure were assigned to each standard, and these values were correlated with corresponding optical density measurements of the cytochrome oxidase chromatic indicator, taken with an image-processing system as described by Gonzalez-Lima and Cada (1994). The resulting linear regression equations ($r^2 > .90$) were used to convert optical density readings from brain regions of interest into cytochrome oxidase activity values, which were used in all statistical analyses in this study.

Using an image-processing system (JAVA, Jandel Scientific, Corte Madera, CA), optical density was sampled from the regions of interest. The size of the square-shaped sampling window was adjusted for each region so that it was as large as possible while still allowing four, non-overlapping readings to be taken bilaterally. For each region, optical density was sampled across three adjacent sections and averaged. These optical density values were then converted to cytochrome oxidase activity units, which were determined by spectrophotometry of cytochrome oxidase standards as described above. In addition to the regions of interest, whole-brain metabolism was assessed by taking the mean of the average activity for each section of the brain.

ANALYZING METABOLIC MAPPING DATA

Background

Two competing assumptions govern how imaging data is used to infer the involvement of brain regions in a behavioral function. The first assumption presupposes a modular brain, in which specific functions are localized to specific regions. The alternative assumption holds that functions are distributed across networks of interacting brain regions. Most likely, the reality lies somewhere in between. That is, brain regions are neither modular, nor fully interconnected. Rather, they are selectively connected, or "semiconnected" (McIntosh, 2000), and there are mathematical demonstrations that such semiconnected systems maximize complexity and computational power (Tononi, Sporns, & Edelman, 1994). Thus, a semiconnected brain integrates much more information much more quickly than a fully interconnected or modular brain would.

The statistical analysis of such complex systems is rife with challenge. By far the most popular approach is to simply ignore the complexity and apply a univariate linear model for each region of interest, followed by a correction for multiple comparisons.

However, brain imaging data clearly has multivariate features, since activity measured at different regions covaries, and the standard Bonferroni correction overestimates the number of independent tests and may lead to the rejection of many real differences (McIntosh, Bookstein, Haxby, & Grady, 1996). Alternatively, one could assume the other simple extreme—that the brain is fully interconnected—and apply a multivariate linear model or treat all regions as repeated measures of a unitary system. However, multivariate models require that the number of subjects exceed the number of variables. This may be the case if one is conducting a narrow sample of regions, but usually one is conducting a brain-wide exploratory analysis. And including all ROIs in a single omnibus repeated-measures ANOVA does not result in a further reduction of error variance, despite the gain in degrees of freedom (McIntosh & Gonzalez-Lima, 1994).

This dilemma led McIntosh & Gonzalez-Lima (1994a) to a strategy that is essentially a compromise between the two extremes: conduct separate repeated measures ANOVAs on small subsets of brain areas, grouped on the basis of functional connectivity. This approach may have greater theoretical validity than the alternatives mentioned so far since it explicitly reflects the balance of segregation and integration present in brain organization. Still, the delineation of functional groups is somewhat arbitrary. Even though brain regions are not fully interconnected and it is clear that unconnected regions should be assigned to separate groups, brain connectivity is still sufficiently extensive to defy strict bounds. No matter which parceling system one chooses, there are bound to be a large number of regions that bridge two or more anatomical groups. For example, regions comprising frontal cortex could form a functional group, or these regions could be segregated and grouped with the subcortical structures with which they share the most connections. Once again, one can choose to ignore this troublesome complexity and proceed with one's favorite grouping. Or, if one

were extremely zealous, one could analyze all possible anatomical groupings. Even so, potentially important covariance information about regional interactions remains neglected. Because metabolic mapping makes it possible to obtain functional data from many regions in a single brain, one would ideally like to consider the interactions among brain regions when inferring which ones are important to a particular behavioral function. Perhaps by examining the covariance of brain activity between different brain areas, one can infer which areas are important nodes and how they are functionally connected.

Thus, regional means and network covariances are complementary ways of looking at functional neuroimaging data. Both are necessary for a thorough understanding of metabolic mapping results.

Method

The general strategy I have chosen for analyzing the data sets reported in the next three chapters is as follows: First, I divide the regions into small functional subgroups. I then perform repeated measures ANOVA on each subgroup, followed by simple-effects tests as necessary. I also perform a step-wise discriminant analysis on each subgroup of regions, as used by McIntosh and Gonzalez-Lima (1994a), to identify regions which might differentiate the experimental groups based not on mean differences, but on interregional covariance differences. I then assess a smaller number of regions for functional connectivity using pair-wise correlations. The regions entering into this analysis are limited to those showing significant mean differences from the ANOVAs and those identified by the discriminant analyses. If there are numerous regions, I limit the regions to the most influential ones, arbitrarily defined as the top 10 regions in terms of absolute mean difference, the top 10 regions in terns of univariate effect sizes, and the top 10 regions in terms of standardized discriminant coefficients.

Regional Mean Differences

Each group of regions was analyzed with a separate repeated measures ANOVA, followed by simple-effects tests adjusted by a Bonferroni correction. (Correcting for the smaller number of regions within each ANOVA is a compromise between correcting for all possible comparisons and not correcting at all.) An alpha level of .05 was used to establish statistical significance.

Estimates of Regional Influence

In order to infer the relative contribution of each region to the helpless phenotype, three different statistics were calculated. The first is simply the magnitude of the mean difference between groups, expressed as the activity difference between groups divided by the control group's activity. The second estimate is the η_p^2 statistic, which is the proportion of the effect + error sum-of-squares (SS) variance that is attributable to the effect:

$$\eta_p^2 = SS_{effect} / (SS_{effect} + SS_{error})$$

In addition to these indices of univariate effects, there might also be a pattern of interregional relationships, reflected by the between-group covariances, that differentiates the experimental groups. Because a discriminant function is a weighted linear combination of variables, a stepwise discriminant analysis can reveal regions which are influential in combination, but which may not show individual mean differences (McIntosh & Gonzalez-Lima, 1994). Discriminant analyses were conducted using a forward step-wise procedure with the minimization of Wilk's Lambda (a measure of residual variance) as the criteria for deriving the discriminant equation. A criterion of $p < .05$ was used for entry of a brain area into the discriminant function. The total number of potential areas that could be entered into an equation was limited to the total sample size minus two (n - 2), so all regions could not be assessed simultaneously. Therefore, regions

were arbitrarily regrouped and reanalyzed. Only brain areas that were able to consistently distinguish between the experimental groups are reported.

Functional Connectivity

Functional connectivity was assessed by calculating the Pearson correlation coefficient for each pair of regions. To ensure the reliability of correlations and to protect against the effects of outliers, all correlations were subjected to a modified jackknife procedure. In this modified procedure, an individual is removed from the data set, and correlations are computed on the data set with n-1 subjects. Significance tests are then done on the correlation using n-1 subjects. The individual is then replaced back into the data set, and another individual is removed. Correlations and significance tests are again computed on the data set with n-1 subjects. This procedure is repeated until all individuals have been taken out once. Correlations are considered to be reliably significant from zero if they remain significant ($p < 0.05$) throughout each iteration.

Chapter 4: The Untreated Congenitally Helpless Adult

Evidence from human neuroimaging and post-mortem studies of patients with depression or PTSD, as well as other animal models of depression, was used to develop a list of candidate brain systems to be explored.

METHOD

Subjects

Subjects were 10 congenitally helpless and 10 congenitally non-helpless males, age-matched and weighing 250-300 g. The rats were housed at the University of Maryland under constant humidity (50 ± 10%) and temperature (23 ± 1°C), on a 12 hour light/dark cycle, and with food and water available continuously. Following decapitation of the subjects, brains were removed intact, frozen rapidly in isopentane, and shipped frozen to the University of Texas (Austin, TX). These naïve rats were neither subjected to inescapable shock, nor any other experimental manipulations. The only independent variable differentiating the two groups was their ancestry. All animal protocols were approved by the institutional care and use committee (IACUC) at the University of Maryland School of Pharmacy, and conform to all NIH and USDA guidelines.

Tissue Processing

Tissue processing proceeded according to the protocol for quantitative cytochrome oxidase histochemistry outlined in Chapter 3.

Delineating Regions of Interest

In addition to the regions of interest discussed in the first section of this chapter, readings were taken from some regions sharing prominent anatomical connectivity. ROIs

were located using Paxinos and Watson's (1997) and Gonzalez-Lima and Cada's (1998a) atlases of the rat brain.

Unfortunately, a generally accepted nomenclature and boundaries for the prefrontal cortical areas in rats is still lacking (Uylings, Groenewegen, & Kolb, 2003). To avoid confusion, names and abbreviations from different anatomists are matched in Table 4.1, and the boundaries and terms used in our study are outlined in Figure 4.1. I will follow the terminology of Uylings and van Eden (1990), except that I refer to ACd and Fr2 collectively as dorsomedial PFC, and to the ACv as simply anterior cingulate. Dorsomedial PFC, prelimbic, and medial orbital cortex were imaged at Bregma 3.7 mm. Although not regions of interest, other cortical regions were also imaged at this level, including lateral frontal, agranular insular, and lateral orbital cortex. Infralimbic cortex was imaged at Bregma 2.2 mm, and anterior cingulate was imaged at Bregma 0.7 mm. Because frontal-cingulate cortex shows consistently greater cytochrome oxidase staining in the superficial layers compared to the deep layers, these two divisions were analyzed separately.

For the basal ganglia, cytochrome oxidase readings were averaged across rostral (Bregma –0.8 mm) and caudal (Bregma –3.8 mm) samples of the caudate-putamen. The globus pallidus and ventral pallidum were assessed at Bregma -0.3 mm, and the nucleus accumbens was measured at Bregma 0.7 mm.

The lateral septal nucleus (LS) and medial septal/vertical diagonal band nucleus (MSDB) were imaged at Bregma +0.2 mm.

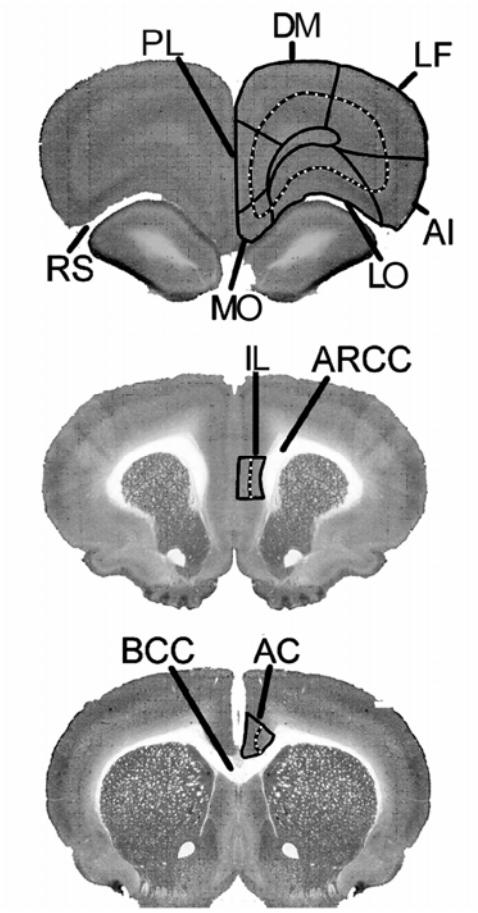
For the hippocampal complex, the dentate gyrus was imaged at Bregma –3.8 mm, and the subiculum was imaged at Bregma –5.8 mm. Fields CA1 and CA3 were assessed at both Bregma –3.8 and –5.8 mm.

Table 4.1: Various terminologies of subdivisions of medial prefrontal cortex in the rat.

(Paxinos & Watson, 1998)	M2	Cg1	Cg2	PL	IL	MO
(Krettek & Price, 1977)	PrCm	ACd	ACv	PL	IL	MO
(Vogt & Peters, 1981)	4	24b	24a	32	25	14
(Zilles & Wree, 1985)	Fr2	Cg1	Cg2	Cg3	IL	MO
(Uylings & van Eden, 1990)	Fr2	ACd	ACv	PL	IL	MO

Note. M2 = secondary motor area; PrCm = medial precentral cortex; Fr2 = frontal cortex area 2; Cg1-Cg3 = cingulate cortex; ACd = dorsal anterior cingulate; ACv = ventral anterior cingulate; PL = prelimbic; IL = infralimbic)

Figure 4.1: Cytochrome oxidase-stained sections of frontal-cingulate regions of the rat brain. Segmented lines indicate division between superficial and deep layers of cortex. Abbreviations: AC, anterior cingulate; AI, agranular insular; ARCC, anterior radiation of the corpus callosum; BCC, body of the corpus callosum; DM, dorsomedial frontal; IL, infralimbic; LF, lateral frontal; LS, lateral orbital; MO, medial orbital; PL, prelimbic**;** RS, rhinal sulcus.



The basolateral and central amygdala were imaged at Bregma –2.3 mm. The anterior amygdala was imaged at Bregma –1.3 mm, and the medial amygdala was imaged at Bregma –2.3 mm. In addition, the bed nucleus of the stria terminalis (BNST), considered to be part of the "extended amygdala," was grouped with these structures and imaged at Bregma -0.3 mm.

The main hypothalamic area of interest was the paraventricular nucleus (PVH), which was imaged at Bregma –1.8 mm. In addition, various other hypothalamic areas implicated in the regulation of eating, sexual behavior, and circadian rhythm were imaged: the medial preoptic area (Bregma –0.3 mm), the lateral hypothalamic area and suprachiasmatic nucleus (Bregma –1.3 mm), and the ventral medial hypothalamic nucleus (Bregma –2.3 mm).

The following nuclei from the midline thalamic region were imaged: the reuniens (Bregma -1.8 mm), paraventricular, medial dorsal, and centromedial (Bregma -2.3 mm) nuclei. In addition, the anterior ventral nucleus (AV) was imaged at Bregma -1.8 mm.

The habenula (lateral and medial nuclei combined) was imaged at Bregma -3.8 mm. In addition, three brainstem regions which receive prominent projections from the habenula were imaged: the interpeduncular nucleus (IPN) and ventral tegmental area (VTA) at Bregma –6.3 mm, and the dorsal raphe (DR) at Bregma –8.0 mm.

RESULTS

Means Analysis

Means and standard errors for all regions of interest are reported in Tables 4.2 and 4.3.

Whole-Brain Metabolism

A univariate ANOVA showed no significant difference in whole-brain metabolism between groups, $F(1,18) = .015$, $p = .91$.

Frontal-Cingulate Cortex

Frontal and cingulate cortical regions were analyzed with a 2 × 8 × 2 (Group × Region × Layers) repeated measures ANOVA. A significant three-way (Group × Region × Layers) interaction was obtained, $F(7, 126) = 2.63$, $p = .014$. Simple-effects tests of the interaction showed significant decreases in helpless rats in the superficial layers of dorsomedial PFC, $F(1, 18) = 5.0$, $p = 0.038$, medial orbital, $F(1, 18) = 5.3$, $p = 0.034$, and anterior cingulate, $F(1, 18) = 5.3$ $p = 0.03$, whereas a significant increase was found in the superficial layers of infralimbic cortex, $F = 8.46$, $p = 0.009$. No significant differences were observed in prelimbic cortex or in the inner layers of any region.

Basal Ganglia

Basal ganglia regions were analyzed with a 2 × 2 × 2 (Group × Striatum-Pallidum × Dorsal-Ventral) repeated measures ANOVA, so that striatal regions (CP and Acb) could be contrasted with pallidal regions (GP and VP) and dorsal regions (CP and GP) could be contrasted with ventral regions (Acb and VP). However, only a significant main effect of group was obtained, $F(1,18) = 24.9$, $p = 0.0001$, indicating that metabolism throughout the basal ganglia was reduced in the helpless group (256 vs. 296 μmol/min/g).

Septal Region

The septal region was analyzed with a 2 × 2 (Group × Region) repeated measures ANOVA. Only a significant main effect of group was obtained, $F(1,18) = 16.5$, $p = .001$, indicating that metabolism was similarly reduced in both the LS and MSVDB of helpless rats (285 vs. 324 μmol/min/g).

Table 4.2: Mean ± S.E. of baseline cytochrome oxidase activity (μmol/min/g tissue) for frontal-cingulate cortex in adult males. *p < .05.

Cortical Region	Helpless		Non-helpless	
	Superficial Layers	Deep Layers	Superficial Layers	Deep Layers
Lateral frontal	298 ± 15	254 ± 11	323 ± 19	269 ± 18
Dorsomedial PFC	266 ± 9*	249 ± 10	315 ± 20	278 ± 14
Prelimbic	202 ± 12	209 ± 10	229 ± 11	232 ± 13
Medial orbital	212 ± 7*	197 ± 6	242 ± 11	224 ± 12
Lateral orbital	248 ± 7	217 ± 8	277 ± 13	233 ± 13
Anterior insular	257 ± 8	239 ± 7	284 ± 13	256 ± 15
Infralimbic	244 ± 13*	224 ± 10	198 ± 9	224 ± 11
Anterior cingulate	256 ± 6*	200 ± 5	291 ± 14	213 ± 9

Table 4.3: Mean ± S.E. of baseline cytochrome oxidase activity (μmol/min/g) for subcortical regions in adult males. *p < .05.

Region	**Helpless**	**Non-helpless**
Basal ganglia		
Caudate putamen (CP)	219 ± 9*	261 ± 10
Nucleus accumbens (Acb)	313 ± 8*	364 ± 7
Ventral pallidum (VP)	284 ± 14*	329 ± 3
Globus pallidus (GP)	192 ± 7*	212 ± 5
Septal nuclei		
Lateral septum (LS)	308 ± 9*	349 ± 8
Medial septum/Vertical diagonal band (MSVDB)	248 ± 10*	290 ± 10
Hippocampal complex		
CA1 field, dorsal	259 ± 7	254 ± 10
CA1 field, ventral	310 ± 8*	257 ± 8
CA3 field, dorsal	254 ± 7	252 ± 7
CA3 field, ventral	321 ± 16*	278 ± 6
Dentate gyrus (DG)	345 ± 25	330 ± 10
Subiculum (Sub)	399 ± 14*	300 ± 18
Amygdaloid nuclei		
Bed nucleus stria terminalis (BNST)	229 ± 8*	262 ± 8
Basolateral amygdala (BlA)	359 ± 9*	420 ± 11
Central amygdala (CeA)	333 ± 9*	398 ± 7
Anterior amygdala (AA)	202 ± 9	199 ± 12
Medial amygdala (MeA)	322 ± 15	341 ± 7
Hypothalamus		
Paraventricular nucleus (PVH)	262 ± 22*	145 ± 6
Lateral nucleus (LH)	205 ± 8	206 ± 10
Medial preoptic area (MPA)	232 ± 20	250 ± 15
Suprachiasmatic nucleus (SCH)	232 ± 17	241 ± 11
Ventromedial nucleus (VMH)	245 ± 20	252 ± 29
Limbic thalamus		
Anteroventral nucleus (AV)	286 ± 32	210 ± 9
Centromedial nucleus (CM)	295 ± 23	254 ± 17
Medial dorsal nucleus (MD)	299 ± 20	266 ± 10
Paraventricular nucleus (Pv)	247 ± 26	237 ± 15
Reuniens nucleus (Reu)	251 ± 27	175 ± 11
Habenula and Brainstem Efferents		
Habenula	338 ± 46*	198 ± 14
Interpeduncular nucleus (IPN)	461 ± 10*	368 ± 20
Ventral tegmental area (VTA)	158 ± 14*	218 ± 16
Dorsal raphe (DR)	178 ± 4	194 ± 5

Hippocampus

The hippocampal region was analyzed with a 2 × 2 × 3 (Group × Anterior-Posterior × Region) repeated measures ANOVA. A significant two-way (Group × Anterior-Posterior) interaction was obtained, $F(1, 18) = 9.27$, $p = .007$. Simple-effects tests of the interaction showed that mean cytochrome oxidase activity in the congenitally helpless strain was significantly increased throughout the posterior hippocampus, $F(1, 18) = 26.8$, $p = 0.00006$, but not in the anterior hippocampus, $F(1, 18) = .442$, $p = .52$.

Amygdala

The amygdala region was analyzed with a 2 × 5 (Group × Region) repeated measures ANOVA. A significant two-way (Group × Region) interaction was obtained, $F(4, 72) = 4.61$, $p = .002$. Simple-effects tests of the interaction showed that mean cytochrome oxidase activity in the congenitally helpless strain was significantly reduced in the BNST, basolateral, and central amygdala, Fs(1,18) = 8.42, 18.3, 30.9, ps = .01, 0.0005, 0.00003, respectively; but not in the medial or anterior amygdala, Fs(1, 18) = 1.4, .086, ps = .25, .77, respectively.

Hypothalamus

Hypothalamic nuclei were analyzed with a 2 × 6 (Group × Region) repeated measures ANOVA. A significant two-way (Group × Region) interaction was obtained, $F(4, 72) = 4.61$, $p = .002$. Simple-effects tests of the interaction showed that mean cytochrome oxidase activity in the congenitally helpless strain was significantly increased in the PVH, $F(1, 18) = 28.2$, $p = .$ 0.00005. No other regions were significantly different.

Midline and Anterior Dorsal Thalamic Regions

Thalamic nuclei were analyzed with a 2 × 5 (Group × Region) repeated measures ANOVA. No significant effects were obtained, either for the group main effect, $F(1, 18) = 2.48$, $p = .14$, or for the interaction (Group × Region), $F(1, 18) = 1.46$, $p = .24$.

Habenula and Efferent Brainstem Nuclei

The habenula and its efferent brainstem targets were analyzed with a 2 × 4 (Group × Region) repeated measures ANOVA. A significant two-way (Group × Region) interaction was obtained, $F(3, 54) = 10.2$, $p = 0.00002$. Simple-effects tests of the interaction showed that mean cytochrome oxidase activity in the congenitally helpless strain was significantly increased in the habenula and IPN, Fs(1, 18) = 9.31 and 18.0, ps = .007 and .0005, respectively; and significantly decreased in the VTA, $F(1, 18) = 8.33$, $p = .01$. The dorsal raphe was not significantly different between groups, $F(1, 18) = 2.74$, $p = .12$.

Estimates of Regional Influence

Estimates of regional influence are given in Tables 4.4 and 4.5. Percent mean differences and partial-eta-squared (η_p^2) estimates give very different assessments for some regions. For example, the habenula and VTA are ranked high in terms of difference magnitude but low in terms of η_p^2 because the larger group difference for these regions was accompanied by a disproportionate increase in error, or unexplained variance. Vice versa, the basal ganglia and amygdala regions showed relatively small mean differences but very large η_p^2 because group membership accounted for a far greater portion of their variance. For other regions, the two statistics are more consistent. For example, both suggest that the PVH, IPN, and ventral hippocampus are among the most influential regions, whereas frontal-cingulate cortex and BNST are among the least.

Table 4.4: Top 10 regions in terms of effect size, ranked according to relative magnitude of difference (left) or partial eta-squared (right).

Region	**% Dif.**	**Region**	η_p^2
1. PVH	.80	1. Central amygdala	.63
2. Habenula	.68	2. PVH	.62
3. VTA	-.28	3. Ventral hippocampus	.60
4. IPN	.25	4. Basal ganglia	.58
5. Ventral hippocampus	.20	5. Basolateral amygdala	.50
6. ACd	-.19	5. IPN	.50
7. Central amygdala	-.16	7. Septal nuclei	.48
8. Basolateral amygdala	-.15	8. ACd	.36
8. Infralimbic cortex	.15	9. Habenula	.34
10. BNST	-.14	9. Infralimbic cortex	.34

Table 4.5: Standardized discriminant coefficients for regions selected by a stepwise discriminant analysis.

Region	**Standardized Discriminant Coefficients**
1. Ventral CA1	.89
2. VTA	-.82
3. Central amygdala	-.74
4. Habenula	.57

In addition to these univariate estimates of effect size, a multivariate stepwise discriminant analysis selected four regions on the basis of their ability to discriminate significantly between the two groups: the CA1 field of the ventral hippocampus, the ventral tegmental area, the habenula, and the central amygdala. Note that all of these regions also showed significant mean differences.

In summary, the PVH and habenula showed the largest mean differences between groups. The central amygdala and PVH showed the largest effect sizes, and ventral CA1 and the VTA showed the largest discriminant coefficients. These appear to be the regions most affected by the selective breeding for helplessness susceptibility.

Analysis of Functional Connectivity

Pearson product-moment correlations were performed for the regions showing the ten largest mean differences and ten largest effects sizes. The basal ganglia regions (CP, Acb, GP, VP) were considered as a collective, as were the septal nuclei (MS and LS) and posterior hippocampal regions (CA1, CA3, and subiculum). This is because, in addition to showing similar mean differences, regions within each collective were highly correlated with each other in both helpless and non- helpless groups (data not shown). However, no other significant correlations were found in either group ($p > .05$ following jackknife, data not shown).

DISCUSSION

Using cytochrome oxidase as a marker for metabolic capacity, the results indicate that congenitally helpless rats, as compared to congenitally non-helpless rats, show reduced metabolism in dorsomedial prefrontal cortex, infralimbic cortex, basal ganglia, septal nuclei, basolateral and central amygdala, and ventral tegmental area. In contrast, elevated metabolism was found in the paraventricular hypothalamus, habenula,

interpeduncular nucleus, and ventral hippocampus. In interpreting these findings, it is important to keep in mind the following issues. First, it should be emphasized that the assayed animals never experienced the learned helplessness training paradigm, nor any other stressful challenges. Therefore, the results reported here reflect endogenous differences in brain metabolism. While these differences likely influence the neurobehavioral response to stress, they should not be interpreted as reflecting the stress response itself.

A second issue concerns the fact that this is a comparison between two genetically altered strains, not a comparison between an altered strain and a randomly bred strain. Although biochemical assays have shown congenitally non-helpless rats to be similar to randomly bred Sprague-Dawley rats (Henn & Edwards, 1994), the possibility exists that any given cytochrome oxidase difference between strains may be due as much to deviation from normal of the non-helpless rats as the helpless rats. However, we have cytochrome oxidase data available from normal control rats from other studies in this lab (Cada, Gonzalez-Lima, Rose, & Bennett, 1995; Gonzalez-Lima & Cada, 1998b), and they are similar to values in non-helpless rats, with the exception of the PVH and habenula. In these regions, normal rats appear to have activity intermediate to that of the congenitally helpless and non-helpless rats. Thus, a mutual divergence in activity may account for the unusually large groups differences observed in the PVH and habenula. Still, the absence of a random control group does not permit a conclusive answer to the question of which strain is driving which regional difference, and it is best to interpret the findings in light of a resistance-vulnerability continuum. That is, from the perspective of the congenitally helpless rats, strain differences reflect increased vulnerability to helplessness (even if the increased vulnerability is technically due to an absence of resistance).

Prefrontal Cortex (PFC)

Congenitally helpless rats showed changes in frontal-cingulate regions comparable to those that have demonstrated differences in human depression, and the direction of these changes are the same as those seen in human studies. Located ventral to the genu of the corpus callosum, subgenual anterior cingulate has shown both metabolic decrements (Drevets et al., 1997) and mild hyperactivity (Mayberg et al., 1999) in depression. This apparent discrepancy may be resolved by the finding that depressed patients show a marked reduction in glial cells within the subgenual region (Ongur, Drevets, & Price, 1998), giving a false impression of hypometabolism when measured with low-resolution PET. Computer simulations that correct PET data for this volume effect conclude that metabolic activity in the remaining subgenual tissue is actually increased in these patients (Drevets, 2000). Moreover, the subgenual cingulate was the only region that showed a correlation between activity and overall depressive symptoms in both unipolar and bipolar patients (Dunn et al., 2002), and increased subgenual metabolism is a well-replicated correlate of normal human sadness (Liotti et al., 2000). Infralimbic cortex, the homologous region in the rat, was likewise hypermetabolic in the congenitally helpless rat, indicating that excessive activity in this region may be a susceptibility trait that exists prior to the onset of depression.

Subgenual/infralimbic cortex appears to be important for modifying the activity of the autonomic nervous system (Bacon & Smith, 1993; Frysztak & Neafsey, 1991). Humans with lesions that include the subgenual cingulate show abnormal autonomic responses to emotionally provocative stimuli and are unable to experience emotion related to concepts that ordinarily evoke emotion (Damasio, Tranel, & Damasio, 1990). Likewise, rats with infralimbic lesions show significantly reduced freezing and little or no ultrasonic vocalizations when presented with a conditioned stimulus predicting shock

(Frysztak & Neafsey, 1991). If hyperactivity of this region reflects the converse of the lesion effects, then increased subgenual/infralimbic metabolism may confer increased emotionality.

Other PFC regions where dysfunction may impair the ability to modulate emotional responses in mood disorders include the dorsomedial PFC (area 9), dorsolateral PFC (areas 9/46), and the area of anterior cingulate immediately dorsal to the corpus callosum (area 24). Abnormally decreased blood flow and metabolism in these areas have been the most extensively replicated findings in depression neuroimaging (Baxter, Jr. et al., 1989a; Bench et al., 1992; Biver et al., 1994; Ebert et al., 1991; Mayberg et al., 1997), and these metabolic deficits appear to relate to the negative thoughts (Dunn et al., 2002) and subtle impairments of attention and memory (Bench et al., 1993) that accompany major depression episodes. The congenitally helpless rats also showed metabolic suppression in homologous dorsomedial PFC. Since helpless rats also show learning deficits related to impaired attention (Lee & Maier, 1988; Minor et al., 1984), diminished functioning of their dorsomedial PFC may likewise be to blame.

The pattern of reduced metabolism in dorsomedial frontal and elevated metabolism in infralimbic cortex generally agrees with the human literature. It is also consistent with a circuit model put forth by Helen Mayberg (1997), who suggests there is a ventral-dorsal, limbic-cortical opposition in effect in depression, as well as normal sadness. Her hypothesis is based on a series of neuroimaging studies showing that cognitive tasks activate the dorsal PFC and anterior cingulate while suppressing more ventral, limbic structures, and that, vice versa, emotional tasks activate more ventral limbic structures while suppressing dorsal regions (Mayberg, 1997). Interestingly, similar reciprocal relationships in brain metabolism appear to exist in rodents. Mice that have learned to suppress an emotional response show increased metabolism in dorsomedial

PFC which is strongly correlated with decreased metabolism in multiple subcortical areas related to emotional expression (Barrett, Shumake, Jones, & Gonzalez-Lima, 2003). The implication is that depressed humans and helpless rats may experience a brain state in which negative emotions interfere with new learning and new learning is unable to change negative emotions.

Regions Regulating the Hypothalamic-Pituitary-Adrenal (HPA) Axis

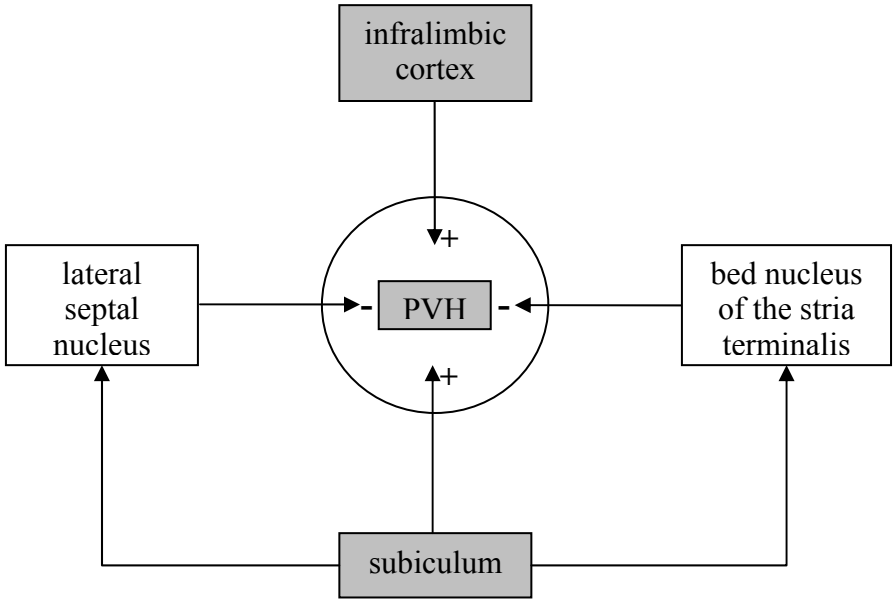
The PVH of the congenitally helpless rat showed a large elevation of cytochrome oxidase activity, more than five times the difference observed in frontal and cingulate cortex. Indeed, it is the largest cytochrome oxidase difference we have ever detected in any study. (Originally, magnocellular and parvocellular divisions of the PVH were imaged separately, but, because both showed the same magnitude of difference, the PVH data is reported and discussed as a single region.) Since stress activates the PVH (Palkovits, 2000) and since chronic stress can lead to depression, one might expect the PVH to become hyperactive in stress-induced depression. What is interesting about the congenitally helpless rats is that they show PVH hypermetabolism in the absence of any explicit stress, indicating that PVH hyperactivity can also be triggered genetically. This finding suggests a novel theoretical mechanism linking the contributions of heredity and stress in the etiology of depression: if sustained hyperactivity of the PVH leads to depression, then individuals with higher baseline levels of PVH activity would simply be closer to this threshold.

Although the CRH-producing neurons of the PVH receive few direct forebrain afferents, several forebrain regions influence PVH activity via local interneurons scattered in the area immediately surrounding the PVH. These include glutamate projections from the ventral subiculum and infralimbic cortex and GABA projections from the lateral septal nucleus (LS), bed nucleus of the stria terminalis (BNST), medial

amygdaloid nucleus, paraventricular thalamic nucleus, suprachiasmatic nucleus, and ventromedial hypothalamic nucleus (Canteras & Swanson, 1992; Moga, Weis, & Moore, 1995; Risold & Swanson, 1997; Watts, Swanson, & Sanchez-Watts, 1987). Of these, infralimbic cortex and the ventral subiculum showed metabolic increases in the congenitally helpless rat, and the LS and BNST showed metabolic decreases. This metabolic pattern complements the transmitter pattern: the structures sending excitatory projections to the PVH are overactive, and the structures sending inhibitory projections are underactive (Fig. 4.2). However, the intervening neurons in the PVH-surround are both GABAergic and glutamatergic, so incoming inhibitory and excitatory signals could potentially be reversed by the GABA interneurons. Thus, increased activity of infralimbic cortex and hippocampus coupled with decreased activity of LS and BNST could either be a cause of PVH hyperactivity or an adaptive attempt to constrain a hyperactive PVH.

Even though congenitally helpless rats were not subjected to shock or any other experimental stress, their dissociative septo-hippocampal pattern fits with data obtained from learned helpless rats that have undergone inescapable shock. First, fornix transection completely blocks the development of learned helplessness (Leshner & Segal, 1979), suggesting that hippocampal output facilitates helpless behavior. Moreover, the hippocampi of learned helpless rats show increased glutamate activity and decreased GABA activity (Petty & Sherman, 1981). This is consistent with our finding of hippocampal hypermetabolism, which may be related to functional changes in the processing of corticosterone feedback; chronic antagonism of hippocampal glucocorticoid receptors increases vulnerability to helplessness (Papolos et al., 1993), and these receptors appear dysfunctional in congenitally helpless rats (Lachman et al., 1993). Hippocampal hypermetabolism is consistent with some human neuroimaging data, which has shown elevated hippocampal metabolism in PTSD patients with low cortisol levels

Figure 4.2: Regions projecting to interneurons surrounding the PVH. Shaded regions are hypermetabolic, and unshaded regions are hypometabolic. + = excitatory glutamate influence; - = inhibitory GABA influence.



(Bonne et al., 2003) and reduced hippocampal metabolism following six weeks of antidepressant treatment (Mayberg et al., 2000).

Our finding of reduced septal metabolism also fits with data from learned helplessness experiments. Reduced c-fos activity was observed in the lateral septum of rats that became helpless following inescapable shock (Steciuk et al., 1999). Interestingly, the same study found that resistant rats, which received the same inescapable shock but did not become helpless, showed no change in septal activity. This indicates that the septal effect was due, at least in part, to phenotypic differences, perhaps similar to those expressed by the congenitally helpless rats. It also raises the possibility that a baseline septal difference may exist prior to stress exposure in rats that go on to become learned helpless.

Reduced septal metabolism may also reflect an impairment in reward motivation. The medial and lateral septal nuclei, the nucleus of the diagonal band, and the BNST all support electrical self-stimulation (Cazala, Galey, & Durkin, 1988; Lithgow & Barr, 1984). Furthermore, stimulation of the medial forebrain bundle increases cytochrome oxidase activity in the LS (Bielajew, 1991), lateral hypothalamic self-stimulation increases c-fos activity in the BNST (Arvanitogiannis, Flores, Pfaus, & Shizgal, 1996), and stimulation of the ventral tegmental area increases glucose uptake in both the LS and BNST (Esposito et al., 1984). In addition, several different antidepressant treatments—including drugs, sleep deprivation, and electroshock—were all found to increase neuronal firing rates in the LS (Contreras, Alcala-Herrera, & Marvan, 1989).

Evidence also suggests that the LS and BNST play a role in regulating the PVH, which receives significant projections from both regions (Silverman, Hoffman, & Zimmerman, 1981). Since these projections are thought to be predominantly GABAergic and inhibitory (Swanson & Risold, 2000), decreased activity in the LS and BNST could

disinhibit PVH activity, contributing to its hyperactivity in congenitally helpless rats. This idea is further supported by evidence showing that adrenalectomy reduces BNST input to the PVH, particularly to the corticotropin-releasing hormone neurons (Mulders, Meek, Hafmans, & Cools, 1997), and that the hippocampus may mediate adrenal feedback via its projections to the LS and BNST (Cullinan, Herman, & Watson, 1993). In conclusion, the dissociation between hippocampal and septal activity in congenitally helpless rats may reflect a disruption in negative feedback to the PVH, which may be a key predisposing factor for the development of helpless behavior.

Habenula, VTA, and Related Regions

The results suggest that the habenula (reported and discussed as a single region because both medial and lateral nuclei showed equivalent differences) may be an especially important region affected in helpless rats, yet it has been overlooked by many depression researchers, in spite of its major role in regulating monoamine transmission (Christoph, Leonzio, & Wilcox, 1986; Ho et al., 1996; Lisoprawski, Herve, Blanc, Glowinski, & Tassin, 1980; Matsuda & Fujimura, 1992; Nishikawa, Fage, & Scatton, 1986; Speciale, Neckers, & Wyatt, 1980; Wang & Aghajanian, 1977). In fact, animal studies have implicated the habenula in a variety of processes disrupted in depression, including the sleep-wake cycle (Haun, Eckenrode, & Murray, 1992; Valjakka et al., 1998), reward mechanisms (Boyd & Celso, 1970; Gallistel, Gomita, Yadin, & Campbell, 1985; Sutherland & Nakajima, 1981), antinociception (Benabid & Jeaugey, 1989; Fuchs & Cox, 1993), and behavioral inhibition (Lee & Huang, 1988). In addition, numerous reports have linked the habenula with mating behavior, avoidance learning, maze learning, feeding behavior, and hormonal response to stress (Sandyk, 1991; Sutherland, 1982). Three other models of depression—amphetamine withdrawal, chronic stress, and α-methyl-para-tyrosine challenge—were shown to increase habenula metabolism

(Caldecott-Hazard, Mazziotta, & Phelps, 1988), and it appears that selective breeding for helplessness has produced the same result. At least one human neuroimaging study has found a strong correlation between habenula activity and depression severity (Morris et al., 1999). Perhaps most striking, though, is the finding that habenula ablation completely blocks the development of learned helplessness (Amat et al., 2001).

The IPN, which receives a major projection from the medial habenula (Contestabile & Flumerfelt, 1981), may also play an important role in helplessness susceptibility. Like the habenula, it is metabolically hyperactive in congenitally helpless rats, and input from the habenula likely contributes to IPN hypermetabolism. In addition, the IPN receives more acetylcholine input than any other region in the mammalian brain (Woolf & Butcher, 1985). This is interesting in light of evidence implicating excessive cholinergic tone in the etiology of depression (Charles et al., 1994; Dilsaver & Coffman, 1989; Dube, 1993; Janowsky, el Yousef, Davis, & Sekerke, 1972; Janowsky, Risch, & Gillin, 1983; Steingard et al., 2000). Cholinergic agonists increase the drive for REM sleep, and excessive REM sleep is a reliable marker of endogenous depression (Riemann, Berger, & Voderholzer, 2001). Furthermore, the FSL rat, which was selectively bred for sensitivity to cholinergic agonists, shows many features of depression, including the characteristic REM sleep abnormality (Yadid et al., 2000), which may be related to IPN function. In particular, cholinergic innervation of the IPN is necessary for maintaining REM sleep episodes (Haun et al., 1992), and deafferentation of the IPN reduces time spent in REM sleep by 79%, without affecting non-REM sleep (Valjakka et al., 1998). A reasonable prediction is that the converse—cholinergic stimulation of the IPN—would increase the drive toward REM sleep. Thus, IPN hypermetabolism might reflect a localized increase of cholinergic activity and a corresponding alteration in REM sleep.

Assessing electroencephalographic parameters of the sleep cycle in congenitally helpless rats remains an important experiment yet to be done.

The finding of habenula hypermetabolism is also consistent with the finding of diminished VTA activity, which may reflect defective dopamine innervation. Numerous studies have shown that habenula metabolism is sensitive to changes in dopamine transmission and that the relationship is consistently an inverse one. Dopamine agonists and stimulation of the medial forebrain bundle strongly inhibit metabolism in the habenula (Trugman, James, & Wooten, 1991; Weissman, Marquis, Moreton, & London, 1989; Wilkerson & London, 1989), while dopamine antagonists strongly promote it (Gomita & Gallistel, 1982; Pizzolato, Soncrant, & Rapoport, 1984). Interestingly, a recent human fMRI study revealed analogous habenula responses to positive and negative feedback in a motion prediction test; blood flow to the habenula decreased following successful trials and increased following failures (Ullsperger & Von Cramon, 2003). The authors relate this finding to animal studies showing increased dopamine activity following reward and decreased dopamine activity following omission of expected reward (Schultz, 2000; Schultz & Dickinson, 2000; Schultz, 2002), and they speculate that the habenula may inhibit dopamine activity in response to failure. However, the causality between VTA activity and habenula activity could be reciprocal. The VTA and nucleus accumbens have been shown to project to the habenula (Felton, Linton, Rosenblatt, & Morell, 1999), and these projections may be responsible for dopamine-mediated changes in habenula activity. However, the habenula also sends projections to the VTA and can indeed inhibit the firing of dopamine neurons (Christoph et al., 1986; Lisoprawski et al., 1980; Matsuda & Fujimura, 1992; Nishikawa et al., 1986).

The major targets of VTA projections also showed metabolic differences in this study, with almost equivalent metabolic decreases in the striatum and basal and central regions of the amygdala. Moreover, destruction of VTA dopamine neurons leads to an identical pattern of activity as that found here: increased metabolism in the habenula and reduced metabolism in the striatum and amygdala (Kozlowski & Marshall, 1980). Decreased metabolism in nucleus accumbens, caudate-putamen, ventral pallidum, and globus pallidus is consistent with evidence implicating basal ganglia dysfunction in depression. There appears to be a connection between major depression and other neurological disorders affecting the basal ganglia (Rogers et al., 1998). For example, symptoms of major depression often accompany loss of forebrain dopamine in Parkinson's disease (Mayeux, Stern, Rosen, & Leventhal, 1981; Stern & Langston, 1985), and depression frequently precedes the onset of the cardinal motor signs of Parkinson's disease (Austin & Mitchell, 1996). Many human neuroimaging studies have also shown reduced blood flow or metabolism in the basal ganglia of depressed patients (Videbech, 2000).

The finding of decreased basolateral and central amygdala metabolism is also consistent with reduced VTA activity. Although we anticipated the amygdala would be hypermetabolic based on human neuroimaging studies, these findings appear to be restricted to a subtype of depression known as "familial pure depressive disease" and also non-psychotic bipolar depression (Drevets, 2000). However, in patients meeting the criteria for major depressive disorder, right amygdala metabolism predicted negative affect, but neither left nor right amygdala metabolism predicted depression severity or differentiated depressives from controls (Abercrombie et al., 1998). In addition, decreased metabolism has been observed in anterior paralimbic areas of depressed patients, around and encompassing some of the amygdala (Kimbrell et al., 2002).

Although the role of the amygdala in fear and anxiety has dominated the literature, there is also a substantial literature linking the amygdala with reward-related processing (Everitt et al., 1999). Indeed, in rats, rewarding intracranial self-stimulation and administration of dopamine agonists increase glucose metabolism in the amygdala (Porrino et al., 1984), while aversive, arousing stimulation of the midbrain reticular formation decreases amygdala metabolism (Gonzalez-Lima & Scheich, 1985). Likewise, human studies have shown that amphetamine and cocaine administration increase blood flow to the amygdala (Devous, Sr., Trivedi, & Rush, 2001; Breiter & Rosen, 1999), and cocaine withdrawal, which mimics many symptoms of major depression, was accompanied by amygdala hypometabolism in rats (Hammer, Jr., Pires, Markou, & Koob, 1993). The projection fields of the VTA are also consistent with the hypothesis that amygdala hypometabolism may reflect reduced dopamine innervation in the helpless rats: the VTA sends substantial projections to the basal and central amygdala, which showed significant differences, but not to the anterior and medial amygdala, which did not (Fallon & Ciofi, 1992). Furthermore, depressed humans show decreased expression of dopamine transporters in the basal and central amygdala and increased expression of D2/D3 receptors in the basal, lateral, and central amygdala, consistent with a loss of dopamine innervation (Klimek, Schenck, Han, Stockmeier, & Ordway, 2002).

The habenula is also poised to play a major role in regulating serotonin neurons of the DRN since habenula afferents account for the largest excitatory amino acid input to the DRN (Kalen, Karlson, & Wiklund, 1985; Kalen, Pritzel, Nieoullon, & Wiklund, 1986). However, the effects of this input are complicated by the fact that the habenula excites both serotonin neurons and local GABA neurons, which in turn inhibit the serotonin neurons (Ferraro, Montalbano, Sardo, & LaGrutta, 1997). The net effect seems to be an excitation of serotonin neurons with low-frequency habenula stimulation, but an

inhibition with high-frequency stimulation (Ferraro et al., 1997). While dorsal raphe activation appears to be necessary for the development of learned helplessness, its baseline activity appears relatively normal in congenital helplessness. It is still possible that the amygdala, DRN, or other regions could become activated if the rats were exposed to inescapable shock or more severe stress.

Conclusion

The current findings suggest that the genetic predisposition to helpless behavior is more linked to the habenula and VTA, which showed the second largest increase and decrease, respectively, and which were identified by a stepwise discriminant analysis as reliably distinguishing congenitally helpless rats from non-helpless rats. The PVH, central amygdala, and ventral hippocampus also appear to be important. These regions appear to be critical nodes in a broader circuit underlying helplessness susceptibility and, as such, may be particularly effective targets for therapeutic intervention.

Chapter 5: The Congenitally Helpless Newborn

In the previous chapter, congenitally helpless rats were found to possess activity patterns in prefrontal (PFC) and cingulate cortex similar to those detected in human imaging studies of depression. The paraventricular hypothalamic nuclei (PVH) showed an 80% increase in metabolism, and the septal-hippocampal regions regulating the PVH appeared dissociated, with hypermetabolic hippocampal regions and hypometabolic septal regions. The habenula and interpeduncular nucleus (IPN) also appeared hyperactive, while metabolism was decreased in the ventral tegmental area (VTA), basal ganglia, and amygdala.

By using the metabolic marker cytochrome oxidase, which reflects brain activity averaged over a long period of time (Wong-Riley, 1989), we reasoned that any phasic stress an animal might experience in day-to-day life (e.g., moving to a new cage) would have minimal impact on cytochrome oxidase levels. Thus, innate pathology could theoretically be viewed apart from stress pathology. However, this is likely an over-idealized scenario for several reasons. First, research has shown that quality of maternal care affects the neural circuitry governing stress reactivity, and maternal behavior itself appears to be learned (Meaney, 2001). It is not known whether congenitally helpless mothers are deficient in maternal care, but, if so, this could further contribute to the pattern of brain activity seen in adult helpless animals. Second, what we might regard as mild, phasic stress might not be perceived as such by an animal that becomes helpless after a single electric shock. If the helpless phenotype involves high reactivity to stress, then even "mild" stress such as cage changing may lead to profound brain changes. Finally, even if postnatal stress were not a factor, many brain differences seen in congenitally helpless adults may reflect compensatory developmental processes; a few

regions showing pathology may, over time, confer abnormal activity to many interconnected structures.

We attempted to address these issues by examining congenitally helpless brains collected at birth. The neonatal brain provides an opportunity to examine pathology before functional connections are well established, and this may shed light on which brain regions are at the core of the activity pattern in adults. In addition, examining newborn brains is a simple way to avoid the potential confound of postnatal stress and establish which brain changes, if any, are purely congenital—that is, determined by genetics and/or prenatal environment.

METHODS

Subjects

Subjects were congenitally helpless (n = 16) and non-helpless (n = 20) newborn rats bred in our laboratory from 4 breeding pairs (2 of each strain) obtained from the Central Institute for Mental Health (Mannheim, Germany), courtesy of Prof. Fritz Henn. In order to avoid excessive inbreeding, first and second order relatives were excluded from mating. Breeding pairs were composed of first or second cousins.

When not mating, rats were individually housed in polycarbonate cages (45 × 24 × 21 cm) in a temperature-controlled room (22 ± 1°C). They were maintained on a 12/12 hour light cycle and provided continuous food and water. Males were introduced into the female cages daily at the beginning of the dark cycle, and sexual behavior was monitored for 1 hour. If a female proved receptive, the pair was left together until the beginning of the next dark cycle when they were separated. Otherwise, males were returned to their home cages at the close of the hour.

Females were weighed three times a week in order to monitor pregnancy progress, and they were moved to clean cages twice per week. Otherwise, they were left undisturbed during the pregnancy period. With the mating period considered Day 0, females rats who gave birth did so invariably during the light cycle on Day 22. On the expected delivery date, the pregnant rat was checked once per hour, and pup brains were harvested within one hour of birth. Sex of pup was determined by measuring anogenital distance. There were 7 male and 9 female helpless pups from 2 litters (2 males and 1 female from one breeding pair, 5 males and 8 females from the other), and 10 male and 10 female non-helpless pups from 3 litters (3 males and 5 females from one breeding pair and 2 males and 5 females from the first litter and 5 males culled from a second litter of 7 males and 6 females from the other breeding pair). These methods were approved by the Institutional Animal Care and Use Committee.

Tissue Processing

Following decapitation, brains were removed intact and frozen rapidly in isopentane. Using a cryostat (Reichert-Jung) at -20°C, brains were sectioned at 40 μm and kept frozen at –40°C until they were processed using quantitative cytochrome oxidase histochemistry, as described in Chapter 3.

Delineating Regions of Interest

Regions of interest were limited to regions showing significant effects in the adult. The Paxinos, Törk, Tecott, and Valentino (1991) Atlas of the Developing Rat Brain was used to delineate regions of the newborn brain. In the adult helpless brain, significant differences in cortical regions were limited to the outer layers. Therefore, we divided the cortical regions of the newborn into equal outer and inner halves, which were sampled separately.

Dorsomedial PFC was sampled across the dorsomedial quadrant of atlas level P0-3, which combined the medial half of frontal cortex and the dorsal half of cingulate cortex. Medial orbital (MO) cortex was defined as the medial half of the orbital region, which was sampled at the same level.

Infralimbic cortex (IL) was sampled at atlas level P0-5, adjacent to the portion of the forceps minor corpus callosum where the medial side runs roughly parallel to the midline. Readings were taken from the dorsal most part of this region, which should correspond to the IL. However, the boundary with dorsal peduncular cortex was extremely close and undefined, so it is possible that some readings overlapped with this more ventral region.

The anterior cingulate (AC) was sampled at atlas level P0-10, where it is bounded by white matter and an imaginary perpendicular line bisecting the cingulum. Also imaged at this level were the nucleus accumbens core and shell, sampled just lateral and medial, respectively, to the anterior commissure.

The septal area was imaged at atlas level P0-11. Here, the lateral septal nucleus (LS) is bounded dorsally by the corpus callosum and laterally and ventrally by the septal neuroepithelium (spt). Readings were taken adjacent to these structures to insure no overlap with the medial septal nucleus (MS). The dorsal limit of the MS was defined by an imaginary horizontal line through the most lateral point of the LS, and the ventral limit was defined by an imaginary horizontal line through the most ventral point of the spt. MS readings were taken adjacent to the midline to insure no overlap with the LS. Vertical diagonal band (DB) readings were also taken adjacent to the midline, immediately ventral to the MS, and extending to the base of the brain.

Basal ganglia regions and the bed nucleus of the stria terminalis (BNST) were sampled at atlas level P0-16, which provides the best anatomical landmarks for

delineating these regions. The BNST was sampled within an area delimited dorsally by the caudate putamen neuroepithelium, medially by the anterior commissure, laterally by the internal capsule epithelium (ic), and ventrally by an imaginary horizontal line extending through the ventral most point of the ic. This same horizontal line was also used to roughly divide the globus pallidus (GP) and ventral pallidum (VP), with readings taken lateral and ventral, respectively, to the ic. The boundaries of the caudate putamen (CPu) were visually obvious.

The basolateral (BLA) and central (CeA) amygdaloid nuclei were imaged at atlas level P0-23, where the external capsule (ec) and stria terminalis (st) could serve as landmarks, with readings for the BLA taken immediately ventromedial to the ec and readings for the CeA taken immediately dorsolateral to the st. The PVH was also imaged at this level. This nucleus was well delineated by the cytochrome oxidase stain although magnocellular and parvocellular divisions could not be discriminated. The medial (MHb) and lateral (LHb) habenula, imaged at atlas level P0-29, were also readily discerned from the cytochrome oxidase stain itself.

Hippocampal regions (CA1, CA3, and subiculum) were imaged at atlas level P0-37. (It was only at this more posterior level that hippocampal differences were found in the adult.) In the neonate hippocampus, the pyramidal cell bodies show the darkest cytochrome oxidase staining (Di Rocco, Kageyama, & Wong-Riley, 1989), appearing as a thin line which, at this level, defines the dorsal-ventral span of the hippocampus proper. If this line is trisected, dividing the hippocampus proper into three parts, then CA1 readings can be taken within the lateral half of the most dorsal part and CA3 readings from the medial half of the most ventral part. Subicular (sub) readings can be taken adjacent to either the dorsal or ventral boundaries of CA1 and CA3, respectively.

Midbrain regions were imaged at atlas level P0-44. The interpeduncular nuclei (IPN) stained darkly against background, while readings for the VTA were taken between the IPN and medial lemniscus. Dorsal raphe nuclei (DR) readings were taken at atlas level P0-49, adjacent to the midline and immediately ventral to the aqueduct.

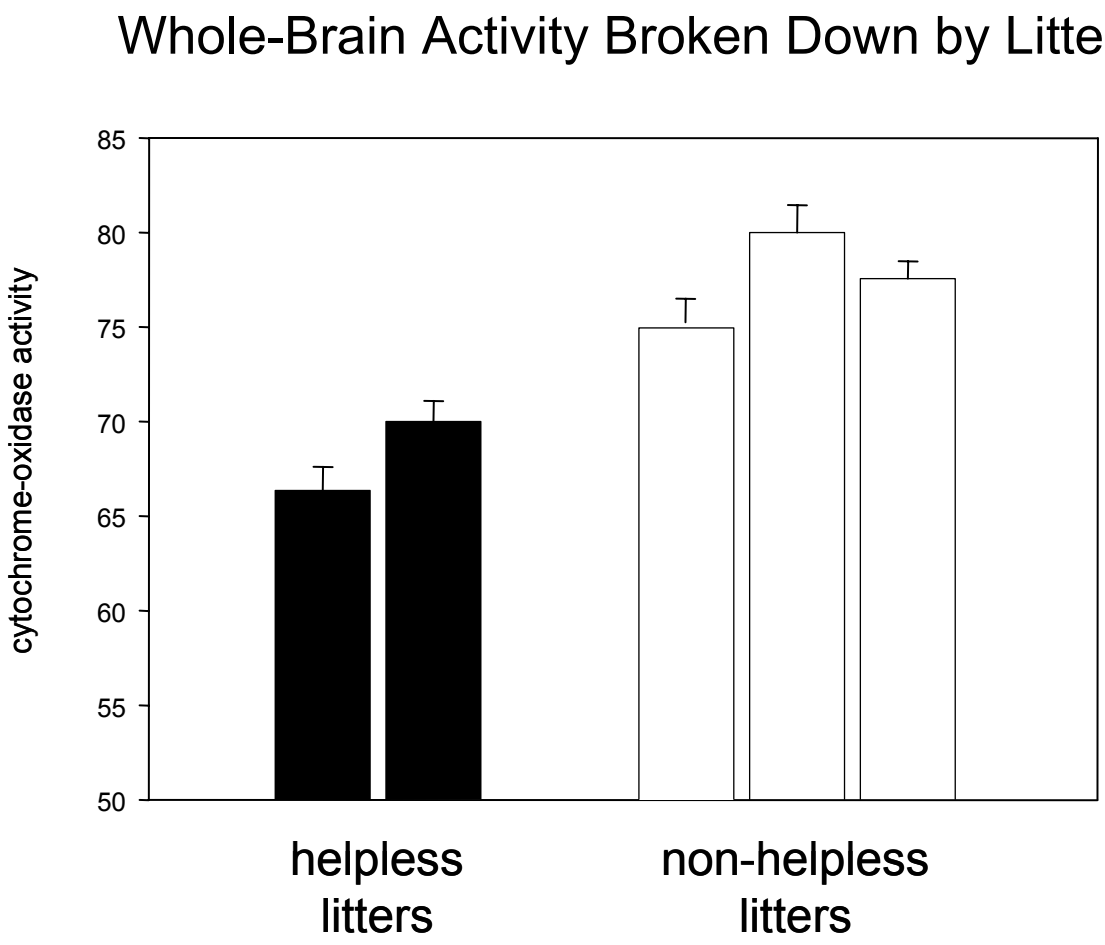
RESULTS

Means Analysis

Due to the small size of the newborn brains, sections which were randomly lost or damaged during sectioning had a greater impact on our ability to match anatomical levels across subjects. This resulted in several missing values, more or less randomly distributed across all subjects and regions. Because of this, the usual method of repeated measures ANOVAs could not be performed as this would have resulted in the list-wise exclusion of too many subjects. Therefore, all mean group data were analyzed using separate two-factor (Group × Sex) univariate ANOVAs for each region. This also meant that the compromise of applying the Bonferroni correction to each repeated measures ANOVA could not be used. Instead, corrections for multiple comparisons were not performed, but a more stringent alpha level ($p < .01$) was used to assess statistical significance.

There were no significant sex differences for any of the variables assessed, nor did sex interact with group to influence any of these measurements (data not shown). Birth weight was not significantly different between groups, $F(1, 30) < 1$, suggesting there were no gross differences in fetal development. There was, however, an overall difference in brain metabolism between groups, $F(1, 30) = 41.2$, $p = 0.0000004$, with congenitally helpless rats showing an 11% reduction in whole-brain cytochrome oxidase activity (Figure 5.1). Unlike adult congenitally helpless rats, which show a balance of severe hypermetabolism in a few regions and mild hypometabolism in several regions,

Figure 5.1: Whole-brain cytochrome oxidase activity values broken down by litter. Each bar is a separate litter.



congenitally helpless newborns showed unidirectional decreases in brain metabolism. Separate ANOVAs for each brain region revealed, though, that some regions were more hypometabolic than others, as displayed in Table 5.1. Estimates of regional influence, shown in Table 5.2, further revealed that the regions most affected in adult congenitally helpless rats—PVH, hippocampal system, and habenula—were the regions showing the largest effects in the newborn. Following these were the lateral septal nuclei and several medial PFC regions.

Functional Connectivity Analysis

Because the ANOVAs indicated no sex interactions, male and female subjects were pooled for the covariance analysis. Since a discriminant analysis could not be used to narrow the focus of the analysis (because of too many missing values), Pearson correlations were computed for all measured regions, with missing values dropped on a pair-wise basis. For ease of interpretation, several simplifications were made in reporting these correlations in Tables 5.3 and 5.4 and Figure 5.1:

1) Regions showing no significant correlations in either group were omitted. Only basal ganglia regions (caudate-putamen, globus pallidus, and ventral pallidum) met this criterion.
2) Cortical regions were not broken down into inner and outer layers because, in each group, layers within a region were highly correlated with each other, $r(13\text{-}15) > .87$, and did not differ in their significant correlations with other regions. Thus, correlations with cortical regions were averaged across layers.
3) The same averaging was done for other regions highly correlated with each other and not differentially correlated with other regions if they could also be regarded as functional units based on anatomical proximity. Thus, the core and shell of the accumbens, $r(15) = .71$ for helpless group, $r(16) = .92$ for non-helpless, were

Table 5.1: Mean ± S.E. cytochrome oxidase activity (μmol/min/g tissue) in brains of newborn helpless and non-helpless rats. * p < .01

Region	***n***	**Helpless**	***n***	**Non-helpless**	***p***
Amygdala, basolateral nucleus	13	77 ± 5	18	80 ± 4	.564
Amygdala, central nucleus	13	81 ± 5	18	87 ± 4	.354
Anterior cingulate, outer cortex	15	79 ± 3	16	92 ± 4	.014
Bed nucleus of the stria terminalis	13	65 ± 2	17	73 ± 2	.010
Caudate putamen	12	73 ± 2	14	72 ± 4	.859
Globus pallidus	14	70 ± 2	18	72 ± 2	.511
Habenula	13	89 ± 3*	18	107 ± 3	.000
Hippocampus, CA1	12	75 ± 3*	16	91 ± 2	.000
Hippocampus, CA3	12	86 ± 2*	16	96 ± 2	.001
Infralimbic, outer cortex	14	66 ± 2*	16	75 ± 2	.002
Interpeduncular nucleus	12	90 ± 4	15	101 ± 5	.099
Lateral septal nucleus	14	67 ± 2*	18	78 ± 2	.001
Medial orbital, outer cortex	14	63 ± 2*	14	71 ± 2	.005
Medial septal diagonal band	14	67 ± 3	18	74 ± 2	.119
Nucleus accumbens	15	50 ± 4	16	59 ± 4	.084
Paraventricular hypothalamus	13	62 ± 3*	19	81 ± 3	.000
Prefrontal, dorsomedial outer cortex	13	64 ± 2	14	70 ± 2	.048
Subiculum	12	82 ± 2*	16	95 ± 2	.000
Ventral pallidum	14	67 ± 2	18	74 ± 2	.045
Ventral tegmental area	12	67 ± 4	15	77 ± 3	.031

Table 5.2: Top 10 regions in terms of effect size for congenitally helpless newborns, ranked according to relative magnitude of difference (left) or partial eta-squared (right).

Region	**% Dif.**	**Region**	η_p^2
1. PVH	-.23	1. PVH	.63
2. Habenula	-.18	2. Ventral subiculum	.62
2. Ventral CA1	-.18	3. Ventral CA1	.60
4. Ventral subiculum	-.14	4. Habenula	.58
4. Lateral septal nucleus	-.14	5. Ventral CA3	.50
6. Infralimbic	-.12	5. Lateral septal nucleus	.50
7. Medial orbital	-.11	7. Infralimbic	.48
7. BNST	-.11	8. Medial orbital	.36
9. VTA	-.10	9. BNST	.34
9. Ventral CA3	-.10	9. Anterior cingulate	.34

Table 5.3: Correlations of cytochrome oxidase activity, non-helpless newborns. Significant correlations ($p < .05$) appear in bold.

		MO	IL	AC	Acb	LS	MSDB	BNST	Amyg	Hab	PVH	IPN
IL	*R*	.34										
	N	13										
AC	*R*	-.26	-.01									
	N	11	14									
Acb	*R*	-.38	.15	**.71**								
	N	13	13	14								
LS	*R*	-.21	.52	.42	**.68**							
	N	14	16	15	15							
MSDB	*R*	-.38	.48	.47	**.76**	**.79**						
	N	14	16	15	15	18						
BNST	*R*	-.09	.60	.11	.28	.60	**.68**					
	N	14	15	14	15	17	17					
Amyg	*R*	-.04	.17	.10	.08	.16	.46	**.71**				
	N	13	15	16	16	17	17	16				
Hab	*R*	-.26	.34	-.20	.26	**.68**	.51	.42	.29			
	N	13	15	16	16	17	17	16	18			
PVH	*R*	-.23	.02	.23	.37	**.64**	.46	.36	.07	.58		
	N	14	16	16	16	18	18	17	18	18		
IPN	*R*	.03	.57	.16	.53	**.70**	**.67**	**.71**	.45	**.72**	.43	
	N	12	13	14	14	14	14	13	15	15	15	
DR	*R*	.46	.76	.25	.49	**.78**	.60	.69	.40	.36	.29	**.71**
	N	10	10	12	13	12	12	12	13	13	13	12

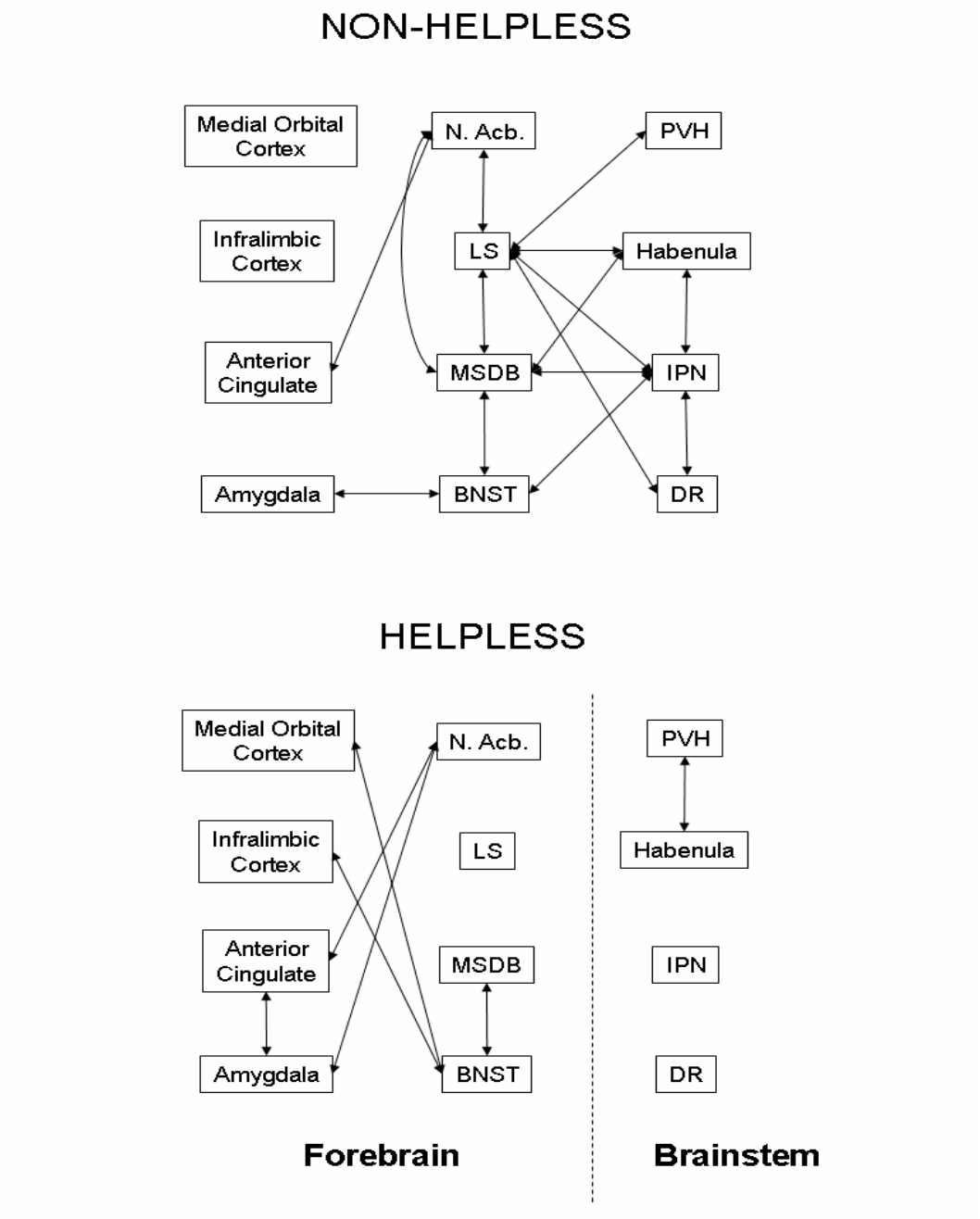
Abbreviations: MO = medial orbital cortex, IL = infralimbic cortex, AC = anterior cingulate, Acb = accumbens nucleus, LS = lateral septal area, MSDB = medial septal/diagonal band nuclei, BNST = bed nucleus of the stria terminalis, Amyg = amygdala, Hab = habenula, PVH = paraventricular hypothalamus, IPN = interpeduncular nucleus, DR = dorsal raphe

Table 5.4: Correlations of cytochrome oxidase activity, helpless newborns. Significant correlations ($p < .05$) appear in bold.

		MO	IL	AC	Acb	LS	MSDB	BNST	Amyg	Hab	PVH	IPN
IL	*r*	.68										
	N	13										
ACv	*r*	.28	.30									
	N	14	14									
Acb	*r*	.11	-.01	**.69**								
	N	14	14	15								
LS	*r*	.49	.42	.26	.13							
	N	13	13	14	14							
MSDB	*r*	.64	.54	.24	.23	.61						
	N	13	13	14	14	14						
BNST	*r*	**.72**	**.79**	.26	-.02	.55	**.79**					
	N	12	12	13	13	13	13					
Amyg	*r*	.16	.24	**.73**	**.71**	.60	.40	.44				
	N	12	12	13	13	13	13	12				
Hab	*r*	-.15	-.29	-.20	-.48	-.19	-.15	-.25	-.13			
	N	12	12	13	13	13	13	12	12			
PVH	*r*	.40	.03	-.07	.06	.21	.22	.18	.08	**.82**		
	N	12	12	13	13	13	13	12	13	12		
IPN	*r*	.24	-.41	-.04	-.10	.18	.30	.04	.15	.45	.53	
	N	11	11	12	12	11	11	10	11	11	11	
DR	*R*	-.06	-.62	-.38	.22	-.32	.04	-.08	-.14	-.30	.07	.62
	N	10	10	11	11	11	11	10	11	10	11	9

Abbreviations: MO = medial orbital cortex, IL = infralimbic cortex, AC = anterior cingulate, Acb = accumbens nucleus, LS = lateral septal area, MSDB = medial septal/diagonal band nuclei, BNST = bed nucleus of the stria terminalis, Amyg = amygdala, Hab = habenula, PVH = paraventricular hypothalamus, IPN = interpeduncular nucleus, DR = dorsal raphe.

Figure 5.2: A diagram of the significant correlations among brain regions in helpless vs. non-helpless rats. All correlations are positive. The dashed line in the helpless diagram emphasizes the functional decoupling between forebrain and brainstem regions in this group.



unified as the nucleus accumbens (Acb); the basolateral and central nuclei of the amygdala, $r(13\text{-}18) > .90$ for both groups, were unified as the amygdala (Amyg); the medial septal and diagonal band nuclei, $r(14) = .84$ for helpless group, $r(18) = .71$ for non-helpless, were unified as the MSDB.

4) Isolated correlations (when two regions were correlated exclusively with each other) were also omitted from the tables and figure. These consisted of, in the helpless group, coupling between CA1 and subiculum, $r(12) = .76$, and between the VTA and PAG, $r(12) = .85$, and, in the non-helpless group, coupling between CA1 and CA3, $r(16) = .79$.
5) Dorsomedial PFC was omitted from the tables because, in both groups, it was correlated only with medial orbital cortex and the correlations were similarly high: $r(13) = .90$ for helpless group, $r(13) = .80$ for non-helpless.

Following these simplifications, Tables 5.3 and 5.4 and Figure 5.1 show that non-helpless rats had twice as many significant correlations as helpless rats, and, with the exception of coupling between the anterior cingulate and nucleus accumbens, and between the MSDB and BNST, the regions showing significant correlations were completely different across groups. Notably, the non-helpless newborns showed extensive correlations both within the basal forebrain (Acb, LS, MSDB, BNST) and between the basal forebrain and much of the rest of the brain (amygdala, PVH, habenula, IPN, and DR). These correlations were strikingly absent in the helpless newborns: instead, more correlations appeared between the BNST and medial orbital and infralimbic cortex, between the amygdala and anterior cingulate and nucleus accumbens, and between the habenula and the PVH.

In particular, the LS and IPN appeared to be network hubs in the non-helpless brain; together they accounted for two-thirds of the significant correlations in this group.

In marked contrast, the LS and IPN showed no significant correlations with any regions in the helpless group.

DISCUSSION

Despite decreased whole-brain metabolism in congenitally helpless newborns, only a third of the regions examined showed a statistically reliable difference, and, more important, the regions most hyperactive in the adult showed the largest effect sizes in the newborn. In particular, the PVH showed the largest group difference in both adults and newborns. This means in effect that the brain regions most underactive at birth go on to become the most hyperactive in adulthood. What could account for this radical shift? One consideration is that congenitally helpless rats show abnormal reversals of HPA-axis responsiveness across development. Relative to controls, adrenocorticotropin hormone (ACTH) response to cold stress is hypoactive at postnatal day 7 but hyperactive at postnatal day 14 (King et al., 1993), and ACTH responsiveness in adulthood may be either blunted or hyper depending on the type and timing of stress encountered during early life (King et al., 1993; King & Edwards, 1999). Thus, a developmental difference in stress responsiveness could account for the metabolic reversal.

Another important consideration is that, in the barely sensate newborn, cytochrome oxidase may predominantly reflect morphogenetic intrauterine differences. This is because most brain pathways still being laid down at birth have yet to be functionally activated, such that cytochrome oxidase activity may simply reflect the genesis and maintenance of synapses, independent of the sensorimotor activation of those synapses (Di Rocco et al., 1989). In this light, reduced cytochrome oxidase in the PVH may reflect a loss of connectivity with other regions.

The current covariance analysis of functional connectivity corroborates this story. Before discussing this, it is important to qualify what functional connectivity means in

the context of cytochrome oxidase data. With most functional imaging techniques, which assess blood flow or metabolism in the here and now, brain regions couple and decouple on a dynamic basis according to stimulus or task, forming different networks to accomplish different functions (Horwitz, 1994). However, because cytochrome oxidase is a historical marker, reflecting past metabolic demands instead of current ones, its functional couplings are relatively static. Transient dynamic couplings will tend to average out over time, leaving correlations that reflect stable anatomical relationships between regions. Thus, cytochrome oxidase correlations should be interpreted as reflecting behavioral predispositions conferred by anatomical wiring (Sakata, Coomber, Gonzalez-Lima, & Crews, 2000). This is expected to be even more true in the case of neonates since metabolic activity at this phase of development is primarily concerned with the establishment of anatomical connections, rather than the recruitment of specific connections for specific behaviors (Di Rocco et al., 1989).

In the congenitally helpless newborns, the correlations suggest that the PVH is cut off from forebrain regulation, along with every other brainstem region (LHb, MHb, IPN, DRN). Of all the forebrain inputs to the PVH, perhaps the most important comes from the subiculum, which projects not only to the zone surrounding the PVH, but also to other regulatory regions, such as the LS and BNST (Herman et al., 2003). The subiculum is thus well-positioned to translate the activation of glucocorticoid receptors, which the hippocampus has in abundance (McEwen, Weiss, & Schwartz, 1968), into feedback inhibition of the PVH, but the subiculum may also inhibit the PVH response to certain stressors even in the absence of glucocorticoid signaling (Herman, Dolgas, & Carlson, 1998).

Neither strain of newborns showed hippocampal coupling with the PVH or with any other regions. This is likely because of the relative immaturity at birth of the

hippocampus, which undergoes extensive postnatal development and begins to show functional coupling with other regions only a few days prior to weaning (Nair & Gonzalez-Lima, 1999). However, next to the PVH, the subiculum and CA1 field showed the largest effect sizes in the newborns, perhaps indicating their future importance in determining the helpless phenotype. Again, hypometabolism in the immature hippocampus suggests that it is developing fewer axons and/or dendrites to link it with other regions (Di Rocco et al., 1989).

Assuming that primary causal pathology is more likely to be present at birth and secondary consequential adaptation is more likely to occur during postnatal development, the results may help to resolve some competing causal scenarios suggested by the adult brain map. For example, reduced VTA metabolism accompanied increased habenula metabolism in congenitally helpless adults. Since the habenula and VTA share mutually inhibitory connections, it was possible that habenula hyperactivity had decreased VTA activity, or that VTA hypoactivity had increased habenula activity. (See Chapter 4.) The present results suggest the former scenario since the habenula showed a more significant difference in newborns. Thus, deficits in psychomotor and reward-related circuitry may stem from the habenula, which showed the second largest mean difference in both neonates and adults. In addition, the habenula appears decoupled in helpless newborns from the IPN, its primary efferent target through which it influences various brainstem nuclei (Contestabile & Flumerfelt, 1981). Perhaps the most important of these brainstem targets is the DR (Wang & Aghajanian, 1977), which is also decoupled from the IPN in helpless newborns. Interestingly, helpless newborns showed novel coupling between the habenula and PVH that was not present in non-helpless newborns. We have found no evidence in the literature for projections which might directly link these two regions, nor were they functionally coupled to any of the sampled regions which could serve as

intermediaries. Intraventricular corticotropin-releasing hormone (CRH) administration was found to increase c-fos expression in the habenula (Imaki, Shibasaki, Hotta, & Demura, 1993), but it is unclear how this functional link is established since the habenula does not express CRH receptors (Van Pett et al., 2000). It does appear, however, that the habenula and PVH operate as a functional unit in congenitally helpless rats, since both regions were severely hypermetabolic in the adult.

Among those regions which remain hypometabolic in the congenitally helpless adult, the LS showed the largest effect size in the newborn. It also showed the greatest difference in functional coupling, being connected to the nucleus accumbens, MSDB, and every single brainstem nucleus in non-helpless newborns while being completely disconnected in helpless newborns. The LS contributes a significant number of GABA projections to the PVH (Herman et al., 2003), and the relevance of LS dysfunction to depression vulnerability is supported by the effects of septal lesions, which increase susceptibility to mild stress (Brown, Uhlir, Seggie, Schally, & Kastin, 1974; Seggie, Uhlir, & Brown, 1974; Uhlir, Seggie, & Brown, 1974).

Another causal question stems from the relationship between PVH hyperactivity and frontal-cingulate metabolism: does a hyperactive HPA axis alter PFC function, or do PFC deficits promote a hyperactive HPA axis? The current results argue against the former scenario since a PFC abnormality exists prior to the onset of PVH hyperactivity. However, the smaller effect sizes for PFC regions suggest that, relative to the hippocampus, habenula, and LS, the PFC plays more of a supporting role in determining the helpless phenotype. Interestingly, helpless newborns showed increased functional connectivity between medial PFC regions and the BNST, and between the amygdala, anterior cingulate, and nucleus accumbens. It is unclear what role these extra functional connections play in the helpless predisposition, but a human PET study showed that

positive coupling between the amygdala and anterior cingulate in posttraumatic stress disorder (PTSD) was one of the few functional connections that differentiated PTSD patients from healthy trauma survivors (Gilboa et al., 2004).

In conclusion, the major findings were that the PVH was the most altered brain region in congenitally helpless newborns and that it, along with all other brainstem regions, was functionally decoupled from the forebrain. These results suggest that the essence of the helplessness vulnerability does not reside in an endogenously hyperactive HPA axis per se, but rather in the limbic networks which regulate it. This implies that when a congenitally helpless animal encounters a stressful situation, no matter how mild, regulatory regions such as the hippocampus, LS, and medial PFC may be unable to constrain the response of the PVH. Because congenitally helpless animals also lack functional glucocorticoid receptors (Lachman et al., 1993), which ordinarily might exert some local negative feedback, the PVH, once activated, would be unable to turn itself off. This could, over time, lead to the chronic PVH hypermetabolism seen in congenitally helpless adults.

Chapter 6: Response to Treatment

All current antidepressant medications require several weeks of administration before clinical efficacy is observed (Hickie, Scott, & Davenport, 1999; Nurnberg, Thompson, & Hensley, 1999). This response delay follows myriad neurophysiological adaptations to enhanced aminergic transmission, including autoreceptor desensitization, multiple increases and decreases of postsynaptic receptor sites, altered second-messenger pathways, and enhanced neurotrophism (Blier & de Montigny, 1999; Duman, Malberg, & Thome, 1999; Hyman & Nestler, 1996). However, the brain regions mediating these events are not well characterized.

The study reported in this chapter examined changes in regional brain cytochrome oxidase activity associated with 2 weeks of fluoxetine treatment in adult congenitally helpless males. Adult males were chosen as subjects so that their brain maps could be compared to those of the untreated adult males reported in Chapter 4. Fluoxetine was chosen because it has become a first-line drug for the treatment of affective disorders and it is the only drug of its class—the selective serotonin reuptake inhibitors (SSRIs)—whose effects have been metabolically mapped in the rat brain following chronic administration. Freo, Ori, Dam, Merico, and Pizzolato (2000) examined resting glucose metabolism in over fifty regions following two weeks of daily fluoxetine administration. They found changes in only two regions: the lateral habenula and CA3 field of the hippocampus both showed significant decreases.

Although the human imaging literature on antidepressant treatment effects is highly variable, studies using SSRIs have most often found increases in prefrontal and anterior cingulate cortex and decreases in the hippocampus and in ventromedial frontal, subgenual cingulate, and anterior insular cortex (Brody et al., 1999; Buchsbaum et al.,

1997; Mayberg et al., 1999). For purposes of comparison with our study, we have focused on one exceptional study by Mayberg et al. (2000), who examined glucose metabolism in depressed males both one week and six weeks after initiating fluoxetine treatment. The authors found some changes that were common at both time points, some that were opposite, and some that were only present after depression remission. Changes present at one week which persisted at six weeks included increased metabolism in the rostral brainstem, premotor cortex, and inferior parietal cortex, and decreased metabolism in the caudate, medial thalamus, insular cortex, and cerebellum. Initial increases were observed in the hippocampus, medial temporal cortex, putamen, and pallidum, but net decreases were observed in these same regions at six weeks. The opposite was seen in the posterior cingulate, where an initial decrease was followed by a final increase. Changes in prefrontal and cingulate cortex were only observed after depression remission. These changes constituted a normalization of prefrontal cortex and dorsal anterior cingulate metabolism, but also a decrease of subgenual cingulate metabolism to below normal.

METHODS

Subjects

Twenty congenitally helpless males were selected from the cohort of subjects used in the behavioral characterization study reported in Chapter 2. These rats were randomly assigned for either fluoxetine treatment (n = 10) or vehicle control (n = 10). Drug administration began when the subjects weighed 450-550 g, approximately 3 months after the end of behavioral testing which took place between postnatal days 29 and 48. Rats were individually housed at this time in 45 × 24 × 21 cm cages in a temperature-controlled room (22 ± 1°C). They were maintained on a 12/12 hour light cycle and provided continuous food and water.

Drug Administration

Fluoxetine hydrochloride (Sigma) was dissolved in a 25% dimethylsulfoxide (DMSO)-75% saline vehicle (2.5 mg/ml). For 2 weeks, rats received daily i.p. injections of either 5 mg/kg fluoxetine/vehicle or vehicle. Injections were administered during the last hour of the light cycle.

Forced-Swim Test

On the last day of drug administration, rats were placed in a glass cylinder (46 cm high × 20 cm in diameter) containing a 30-cm water column (24 ± 1 °C). Two swimming sessions were conducted: an initial 15-min pretest, followed by a 5-min test 24 h later. Test sessions were video-taped for scoring. A time-sampling technique was employed to score several behaviors during a single viewing. This previously described method has proven reliable and valid for detecting the effects of different antidepressant drugs (Detke, Rickels, & Lucki, 1995). At the end of each 5-s period during the 5 min test, the scorer rated the rat's behavior as belonging to one of the following behavioral categories: (1) immobility—floating passively in the water and only making slight movements to keep the head above the water line (Porsolt, Le Pichon, & Jalfre, 1977); (2) swimming—making active swimming motions, more than necessary to merely keep the head above water; or (3) climbing—making active movements with forepaws in and out of the water, usually directed against the walls. Two raters blind to the treatment conditions scored all of the behaviors. Scores for each behavior were expressed as percentages of time.

Tissue Processing

Immediately after the conclusion of the forced-swim test, rats were taken to a separate room and decapitated. Two brains were damaged during decapitation, so the *n*s for the brain analysis were 9 for each group. Brain extraction and processing proceeded

according to the protocol for quantitative cytochrome oxidase histochemistry outlined in Chapter 3.

Delineating Regions of Interest

Regions of interest were limited to regions showing significant effects in the previous studies, except for the dorsal raphe (DR). Although the DR did not show significant mean differences in either congenitally helpless adults or newborns, it was of interest because fluoxetine, an SSRI, might be expected to alter its serotonergic output. Regions were imaged according to the specifications given in Chapter 4.

Statistical Methods

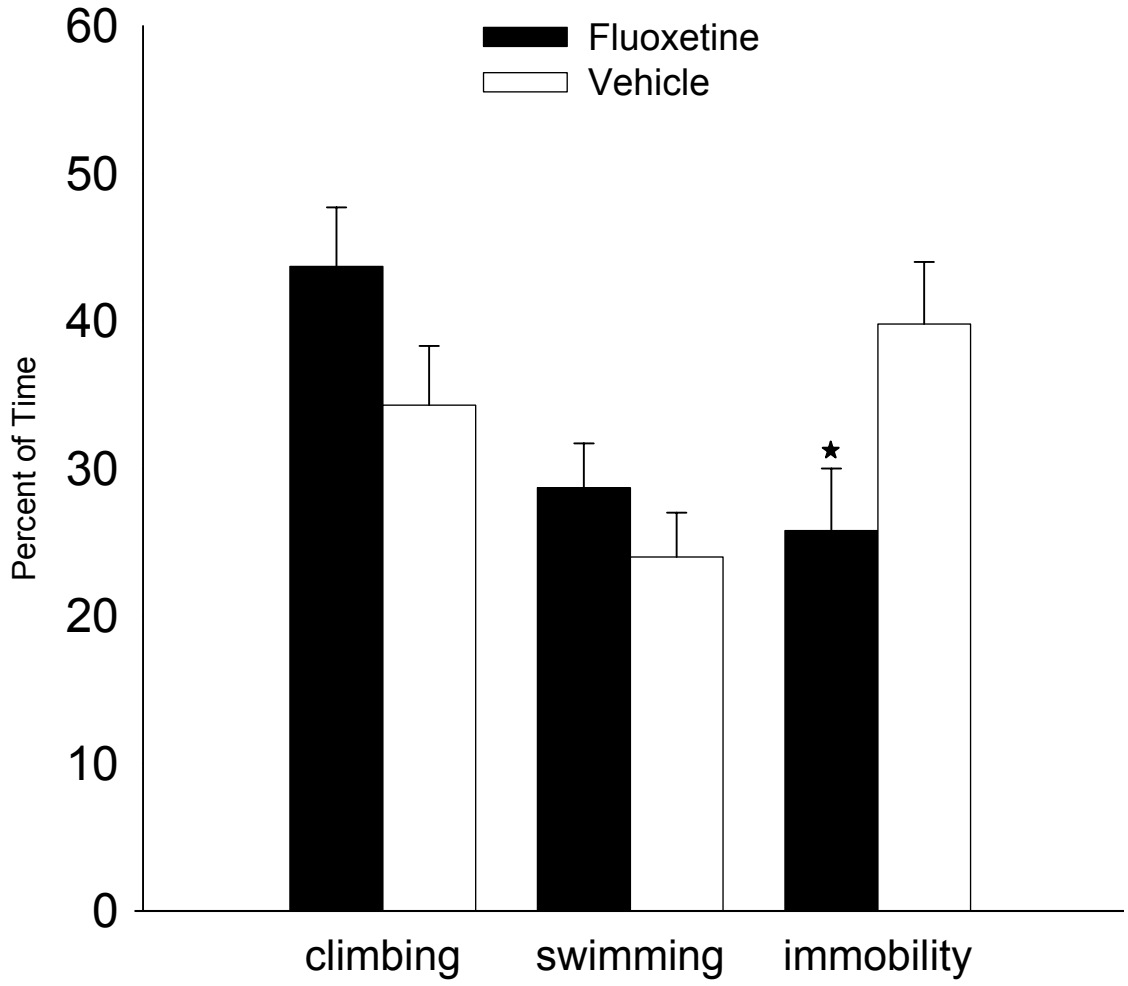
Repeated measures ANOVAs were performed according to the same groupings given in Chapter 4. A multivariate discriminant analysis and pair-wise correlations were also performed according to the criteria outlined in Chapter 3.

RESULTS

Forced-Swim Test

Due to animal welfare concerns, we agreed to terminate the swim test if at any time a subject remained completely submerged for 5 consecutive seconds. Unfortunately, this meant that during the initial swim session, only 11 out of 20 subjects were able to swim the entire 15 minutes. Of the remaining subjects, the test was terminated between 5.5 and 14.5 minutes (mean = 11.5 min). Three of these subjects were from the fluoxetine-treated group (mean = 11.8 min), and six were from the vehicle-control group (mean = 10.9 min). This, of course, introduces a possible bias into the interpretation of the results, in that some rats experienced a shorter duration of swim stress than others. Thus, rats experiencing less stress might show reduced immobility in the final test, regardless of group assignment. However, this appears not to have been the case since a

Figure 6.1: Effect of two weeks of administration of fluoxetine or vehicle on immobility, climbing, and swimming in the forced swim test in adult male congenitally helpless rats. ★ $p < .05$



linear regression indicated that, if anything, rats with earlier sink times on day 1 tended to show more, not less, immobility on day 2, $r(9) = -.65$, $p = .06$. Therefore, all subjects were included in the univariate ANOVAs of the forced-swim scores. These tests indicated that fluoxetine-treated rats showed significantly less immobility than vehicle-control rats, $F(1,18) = 5.5$, $p = .03$, while swimming and climbing measures were not significantly different between groups, Fs(1, 18) = 1.2 and 2.7, ps = .29 and .12, respectively (Figure 6.1).

Regional Effects

Means and standard errors for all regions of interest are reported in Table 6.1. A univariate ANOVA of whole-brain activity found no significant difference between groups, $F(1, 16) = .091$, $p = .70$. Separate repeated measures ANOVAs were performed for each group of regions (frontal-cingulate cortex, basal ganglia, septal nuclei, ventral hippocampus, PVH, and the habenula and its efferent targets). The only significant omnibus finding was a two-way (Group × Region) interaction involving the habenula and VTA, $F(3, 48) = 5.3$, $p = 0.003$. Simple-effects tests showed that mean cytochrome oxidase activity in the fluoxetine-treated group was significantly decreased in the habenula, $F(1, 16) = 10.4$, $p = .005$, and significantly increased in the VTA, $F(1, 16) = 9.1$, $p = .008$.

The habenula and VTA had almost equivalent univariate effect sizes, $\eta_p^2 = .39$ and .36, respectively. Moreover, the mean differences in activity differed by only a couple of points, although this translated into a larger percent change for the VTA since it stains much lighter than the habenula (15% vs. 8%). Likewise, a multivariate stepwise discriminant analysis selected only the VTA and habenula, which had equal and opposite standardized discriminant coefficients. However, despite the opposite changes in the

Table 6.1: Effects of fluoxetine on cytochrome oxidase activity (μmol/min/g tissue). Data are mean ± S.E. *p < .05.

Region	Fluoxetine	Vehicle
Amygdala, basolateral nucleus	244 ± 9	249 ± 7
Amygdala, central nucleus	226 ± 13	230 ± 9
Anterior cingulate, outer cortex	278 ± 15	293 ± 10
Bed nucleus of the stria terminalis	228 ± 9	231 ± 3
Caudate putamen	315 ± 10	335 ± 8
Globus pallidus	160 ± 7	157 ± 4
Habenula	282 ± 5*	308 ± 6
Hippocampus, CA1	206 ± 8	224 ± 3
Hippocampus, CA3	235 ± 2	252 ± 5
Infralimbic, outer cortex	229 ± 5	241 ± 7
Interpeduncular nucleus	392 ± 7	383 ± 10
Lateral septal nucleus	286 ± 13	310 ± 10
Dorsal raphe nucleus	212 ± 8	212 ± 9
Medial septal diagonal band	213 ± 12	223 ± 2
Nucleus accumbens	260 ± 9	271 ± 9
Paraventricular hypothalamus	156 ± 7	173 ± 5
Prefrontal, dorsomedial outer cortex	290 ± 6	289 ± 6
Subiculum	251 ± 9	254 ± 8
Ventral pallidum	202 ± 8	202 ± 4
Ventral tegmental area	172 ± 5*	149 ± 6

VTA and habenula, the regions were not functionally coupled in either the treated or untreated groups, *r*s(9) = .22 and .13, respectively.

DISCUSSION

There are two principal findings of this study. The first is that 2 weeks of fluoxetine treatment reduced immobility in the forced-swim test. The second is that, out of several regions which showed cytochrome oxidase differences in congenitally helpless adults and newborns, only the habenula and VTA responded significantly to fluoxetine treatment, and both changes were in the direction of metabolic normalization—a decrease in the habenula and an increase in the VTA.

Forced-Swim Test

Two weeks of fluoxetine administration (5 mg/kg/day) reduced immobility in the forced swim test, a finding consistent with studies in normal rats (Vazquez-Palacios, Bonilla-Jaime, & Velazquez-Moctezuma, 2004). However, the results were unusual in a number of ways. First, fluoxetine and other SSRIs typically reduce immobility by increasing swimming without affecting climbing (Detke et al., 1995; Vazquez-Palacios et al., 2004), whereas antidepressants targeting the norepinephrine system do just the opposite (Detke et al., 1995). The congenitally helpless rats in our experiment did not show a significant difference in either swimming or climbing, indicating an additive effect of both behaviors in reducing immobility. More important, climbing is typically the least prevalent behavior in both treated and untreated groups (Detke et al., 1995; Vazquez-Palacios et al., 2004), whereas in our study it was the most prevalent. The reason for this aberration is unclear, but it does not appear to be caused by the congenital helplessness phenotype since the only other study assessing forced swim behavior in this strain found the typical pattern in response to an experimental 5-HT_{2A} receptor

antagonist: increased swimming was solely responsible for decreased immobility, while climbing remained the least frequent behavior (Patel et al., 2004). Even the congenitally non-helpless rats in Patel et al.'s experiment climbed far less than our untreated congenitally helpless rats.

One possible explanation for the discrepancy is that we erroneously identified as climbing many behaviors which another investigator would have considered swimming. While we relied on published descriptions of these swim behaviors for our scoring, climbing is a dramatic behavior, involving thrashing and splashing which should be hard to mistake for swimming. Another possible explanation is that our rats were larger than those typically used (450-550 g vs. 250-300 g). Given the narrowness of the cylinder, the larger rats were forced into a mostly vertical posture which restricted their horizontal-rotational range of motion. This may have led to an artifactual increase in climbing and decrease in swimming. However, Patel et al. (2004) used congenitally helpless rats of approximately the same size (400-500 g), which apparently had no bearing on their swim-test results. One final possibility is that the extensive handling and behavioral testing conducted when these rats were 3-6 weeks old may have constituted a form of early environmental enrichment, which may have had a long-lasting beneficial impact on these animals. However, the increased propensity for these animals to sink during the initial 15 minutes of swimming would seem to indicate vulnerability to this test, not resistance. But, again, Patel et al. (2004) did not report any abnormalities during the initial forced-swim training with their congenitally helpless animals. In conclusion, all of these explanations are possible, but none of them are obvious. The experiment would need to be repeated under many conditions (different scorers of behavior, presence vs. absence of behavioral testing in early life, varying ages and weights, or smaller vs. larger cylinders) in order to resolve this issue.

Regional Effects

Cytochrome oxidase changes identified the habenula and VTA as the only regions responding to 2 weeks of fluoxetine treatment. These results are fairly consistent with the 2-DG study of Freo et al. (2000), who found baseline changes in only the CA3 pyramidal layer and in the habenula in response to the same duration of treatment. (They did not assess the VTA.) Like Freo et al., we found habenula metabolism to be significantly reduced in treated rats. One human imaging study has also implicated the habenula in the therapeutic response to antidepressant treatment. Using a group of depressed patients who had recovered under antidepressant medication, Morris et al. (1999) demonstrated that habenula metabolism increased in response to tryptophan depletion, but only in subjects who experienced a relapse of depressive symptoms. This suggests that antidepressant effects dependent on serotonin are related to an inhibition of habenula activity. These are the only two studies to my knowledge which have examined the habenula in connection with antidepressant response. Thus, the current finding of a fluoxetine-induced reduction of habenula metabolism adds to an extremely small but consistent literature.

The finding of increased VTA metabolism concurrent with decreased habenula metabolism adds to a large number of metabolic mapping studies which have consistently found opposite changes in these two regions (see pages 70-71), but increased VTA activity appears inconsistent with several studies showing that fluoxetine acutely antagonizes the dopamine system and suppresses the firing rates of VTA neurons (Esposito, 1996; Prisco & Esposito, 1995). However, after 3 weeks of chronic fluoxetine administration, acute administration of fluoxetine no longer affected the firing rates of VTA neurons (Prisco & Esposito, 1995), suggesting a compensatory response had occurred. Increased metabolic capacity of excitatory synapses innervating the VTA might account for its resistance to fluoxetine inhibition after chronic administration.

Alternatively, fluoxetine may have a normalizing effect on VTA metabolism in congenitally helpless rats which might not be seen in normal rats.

While our results are consistent with those obtained from normal rats (Freo et al., 2000), we had hypothesized that we might see more widespread changes in line with those observed in human imaging studies of depression treatment, such as normalization of metabolic deficits in prefrontal cortex. This was not the case. There are several possible explanations for out negative findings, the most simple being that the rat brain may not respond to antidepressants in the same way the human brain does, at least not in terms of regional metabolism. Alternatively, we may have observed more widespread changes if we had administered fluoxetine at a higher dose or for a longer time. For example, Mayberg et al. (2000) did not observe normalization of cortical metabolism until after 6 weeks of treatment. Although still more widespread than our observations, changes that Mayberg et al. (2000) observed after 1 week did include decreased metabolism in the medial thalamus and increased metabolism in the brainstem. The habenula and VTA, respectively, might be involved in these effects, in which case the directions of change are consistent with our findings. Yet another possibility is that, despite a modest response to fluoxetine in the forced-swim test, congenitally helpless rats might be resistant to fluoxetine treatment or might respond better to another class of drug. Regardless, the results suggest that the habenula and VTA are highly sensitive to chronic fluoxetine treatment and are among the first regions to adapt to it. These regions are then well-positioned to affect the function of many other systems.

Chapter 7: Conclusion

FROM THE NEWBORN BRAIN MAP TO THE ADULT BRAIN MAP

In comparing the brain maps of newborn and adult congenitally helpless rats, the effects may be categorized as follows: 1) brain regions affected at birth and in adulthood, 2) brain regions affected in adulthood but not at birth, and 3) functional couplings present at birth but not in adulthood.

Brain Regions Affected in Both Adults and Newborns

Among those brain regions showing significant differences in both adults and newborns, all of the regions (except for the IPN) which showed significant hypermetabolism in adulthood showed significant differences at birth; however, the changes were in the opposite direction. In contrast, almost none of the regions which showed hypometabolism in adulthood were significantly different at birth, the exceptions being the LS and medial orbital cortex.

Regions Showing Opposite Effects

The PVH, habenula, ventral hippocampus, and infralimbic cortex were hypometabolic at birth but hypermetabolic in adulthood, but, interestingly, the relative magnitudes of the differences showed a strikingly similar pattern. Namely, the PVH and habenula showed, respectively, the greatest and second greatest mean difference in both adult and newborn congenitally helpless rats. This means in effect that the regions most hypometabolic at birth become the most hypermetabolic in adulthood.

Possible explanations for this finding were discussed extensively in Chapter 5. In summary, the most likely interpretation stems from developmental differences in the cytochrome oxidase stain. During the prenatal period, cytochrome oxidase is functionally coupled to the growth and development of synapses, but during the postnatal period,

there is a shift to functional coupling with synaptic activity. This difference between *connectivity* on the one hand and *activity* on the other was judged to underlie the shift from hypometabolism to hypermetabolism. In essence, impoverished connectivity of a region could causally lead to hyperactivity of that same region, particularly if there were a selective loss of inhibitory inputs. For example, if the PVH received reduced regulatory input from forebrain regions (as the correlational study in the newborns seems to suggest), then it would have a propensity to overreact to environmental stimuli which normally recruit it. This would likely be reflected by reduced regional cytochrome oxidase activity at birth and increased activity following postnatal experience. However, while our brain mapping results are consistent with this explanation, they do not prove it. A more direct follow-up experiment would be to inject a retrograde tracer into the zone surrounding the PVH; if there were reduced labeling in the forebrain and hippocampus of congenitally helpless rats, the reduced connectivity hypothesis would be supported.

Regions Showing Common Effects

The LS and medial orbital cortex showed reduced metabolism in both adults and newborns. However, this does not mean that they are hypometabolic for the same reasons. As with the regions showing opposite effects, reduced cytochrome oxidase activity in the newborn likely reflects reduced connectivity with other regions. The interregional correlations in the newborn suggest this is especially true for the LS, which was the most extensively interconnected region in the non-helpless newborn but was completely disconnected in the helpless newborn. Indeed, the metabolic deficit was even larger in the newborn than in the adult for this region, the only region to show such an effect. As for why these regions remain hypometabolic in adulthood, there are two possibilities. One is that there is a modular malfunction in these regions, independent of their connections with other regions, and certainly the work of Petty and colleagues

points to the LS as a key player in the development of helplessness (Ronan et al., 2000; Sherman & Petty, 1984; Steciuk et al., 1999). The other possibility is that adult hypoactivity follows from newborn hypoconnectivity. This would be similar to the scenario suggested for the regions showing opposite effects, except in this case a relative loss of excitatory influence to the LS could result in hypometabolism at both developmental time points.

Brain Regions Affected in Adults but not in Newborns

In attempting to account for why some regions affected in adults were not significantly affected in newborns, one must first acknowledge that several of these regions came close to achieving significance ($p < .10$) in the newborn, such as the VTA, BNST, IPN, nucleus accumbens, and ventral pallidum. It would therefore be premature to make too much of the "absence" of these effects although some regions, namely the amygdala and basal ganglia, were much more equivocal between newborn groups.

There are two general explanations for the apparent involvement of additional regions in the congenitally helpless adult. One is "spreading activation": that is, due to abnormalities in some structures at birth, additional regions may alter their activity during development as a consequence of receiving more or less input from these regions. The possible scenarios here are almost infinite and impossible to determine from the current data, but, as an example, decreased inhibitory input to the habenula (reflected by neonatal hypometabolism) could translate into increased habenula output to the IPN and VTA. Because the habenula appears to have a net excitatory influence over the IPN and inhibitory influence over the VTA (see Chapter 4, Discussion), this could translate into the IPN hypermetabolism and VTA hypometabolism seen in the adult. Reduced VTA output to the basolateral and central nuclei of the amygdala could then cause hypometabolism in these regions. Indeed, as discussed in Chapter 4, the basolateral and

central nuclei (which receive VTA projections) showed changes in the adult, whereas the other amygdala nuclei (which do not receive VTA projections) did not. However, one would need to perform additional metabolic mapping experiments at numerous developmental time points to test this spreading-activation hypothesis.

The other general explanation for the appearance of new effects in the adult may be postnatal experience, including possible differences in maternal care and peer interaction. These two explanations—spreading activation and postnatal experience—are not mutually exclusive and may in fact be synergistic. For example, brain regions altered at birth may predispose the animals to react to certain experiences and engage in certain behaviors in a way which feeds back on the brain and alters its function. Indeed, this was suggested to be the case for explaining PVH and habenula hypermetabolism, which is hypothesized to result from an interaction between reduced regulatory input from other brain regions and some minimal form of environmental stress. Similar diathesis-stress interactions may produce additional changes in other brain regions not primarily altered at birth.

Functional Couplings Present in Newborns but not in Adults

Unlike both the helpless and non-helpless newborns, which showed extensive interregional correlations, neither the helpless nor non-helpless adults showed any significant interregional correlations. There are several explanations for this, one of which may simply be that there was an insufficient number of subjects to detect significant correlations in the adults. (Because male and female subjects were pooled, the sample size was about twice as large for the newborns.) A more likely explanation may again have to do with the functional meaning of cytochrome oxidase staining in the newborn, which, as discussed previously, is more indicative of the growth of anatomical connections. Thus, regions forming synapses with each other may be more likely to show

correlations in their cytochrome oxidase activity. However, once cytochrome oxidase activity becomes coupled to synaptic activations, transient dynamic couplings between regions, which might be revealed by correlations in deoxyglucose uptake, might go undetected by correlations in cytochrome oxidase, which reflect a much longer time span.

FROM BRAIN MAPS TO BEHAVIOR

Congenitally helpless rats, by design, are highly susceptible to develop learned helplessness. In addition, the behavioral characterization experiments in Chapter 2 showed two principle findings: increased novelty seeking but decreased reward seeking. How do the brain maps relate to these behavioral predispositions? As discussed in Chapter 2, one biological marker that has been linked to both vulnerability to learned helplessness and increased novelty seeking is impaired glucocorticoid signaling in the hippocampus (Kabbaj et al., 2000; Papolos et al., 1993). Glucocorticoid receptor activation has been shown to suppress the excitability of hippocampal neurons (de Kloet, 2004), to reduce blood flow to the hippocampus (Endo, Nishimura, Kobayashi, & Kimura, 1997), and to decrease glucose uptake by hippocampal cells (Horner, Packan, & Sapolsky, 1990). Vice versa, adrenalectomy was shown to increase blood flow to the hippocampus (Endo, Nishimura, & Kimura, 1994). These lines of evidence are consistent with the increase in cytochrome oxidase activity found in the ventral hippocampus of adult congenitally helpless rats and their increased predisposition to novelty seeking and learned helplessness. Markedly increased expression of CRH mRNA was also found in the PVH of high novelty-seeking rats (Kabbaj et al., 2000), which suggests that the PVH hypermetabolism in adult congenitally helpless rats may also be related to their novelty-seeking temperament (and may also stem from reduced glucocorticoid signal). The other brain region most consistently linked with exploratory behavior, especially rearing, is the septal area. However, the literature suggests that septal

activation increases rearing and septal lesions decrease rearing (Monmaur, Sharif, & M'Harzi, 1997b; Lee et al., 1988; Myhrer, 1989a; Poplawsky & Isaacson, 1983b), which is not consistent with the finding of decreased septal metabolism in both congenitally helpless adults and newborns. However, decreased activity of the LS is consistent with increased vulnerability to helplessness (Ronan et al., 2000; Steciuk et al., 1999), as is its apparent functional decoupling from other brain regions, as observed in the newborn helpless rats.

Hypometabolism of the basal ganglia would seem to predict psychomotor retardation; indeed, this is how the finding in humans has generally been interpreted. However, the congenitally helpless rats do not show any evidence of psychomotor retardation, and, if anything, appear more active than non-helpless rats. Hypometabolism in the ventral striatum and pallidum, however, is consistent with the reduced reward-seeking behavior (in the form of sucrose consumption) of congenitally helpless rats, as is hypometabolism in the VTA and the basolateral and central amygdala (see Chapter 4, discussion). In addition, reduced amygdala metabolism may be related to the role of this region in fear and anxiety, which congenitally helpless rats appear to show less of, at least in the open-field and light-dark test (see Chapter 2). And, as already discussed repeatedly, habenula hypermetabolism may be a state marker of depression (Caldecott-Hazzard et al., 1988) and habenula activation appears necessary for the development of learned helplessness (Amat et al., 2001).

In the end, though, it is premature at this point to relate specific regional cytochrome oxidase changes to specific behaviors. Ideally, one would like to show that differences in habenula metabolism, for example, correlate with some of the behavioral measures shown to distinguish helpless from non-helpless rats. However, attempts to demonstrate this with the vehicle control subjects used in the fluoxetine study were

unsuccessful. This could be due to any number of reasons, including non-linear effects or insufficient sample size. Future studies aimed at correlating individual differences in regional cytochrome oxidase differences with individual behavioral differences in a large normative sample should shed more light on this question.

THE HABENULA AS A THERAPEUTIC TARGET

Caldecott-Hazard et al. (1988) showed that habenula hypermetabolism was the common denominator across three animal models of depression, and this finding has now been extended to a rat strain that models the predisposition to depression and PTSD. Moreover, the habenula was the only region showing a significant decrease in metabolism following 2 weeks of antidepressant treatment. As shown in the circuit diagramed in Fig. 8.1, the habenula → IPN pathway appears to serve as an interface between frontostriatal and septohippocampal systems and the brainstem monoaminergic nuclei, of which the most important for helplessness susceptibility may be the VTA. The habenula → IPN is linked to PVH regulatory regions via a major input to the habenula from the entire septal area, including LS and BNST (Felton et al., 1999), and via a major output from the IPN to the hippocampus (Montone, Fass, & Hamill, 1988). The IPN further receives cholinergic input from the medial septal nucleus and reciprocates a serotonergic projection to the basal forebrain and ventral hippocampus (Groenewegen & Steinbusch, 1984; Montone et al., 1988).

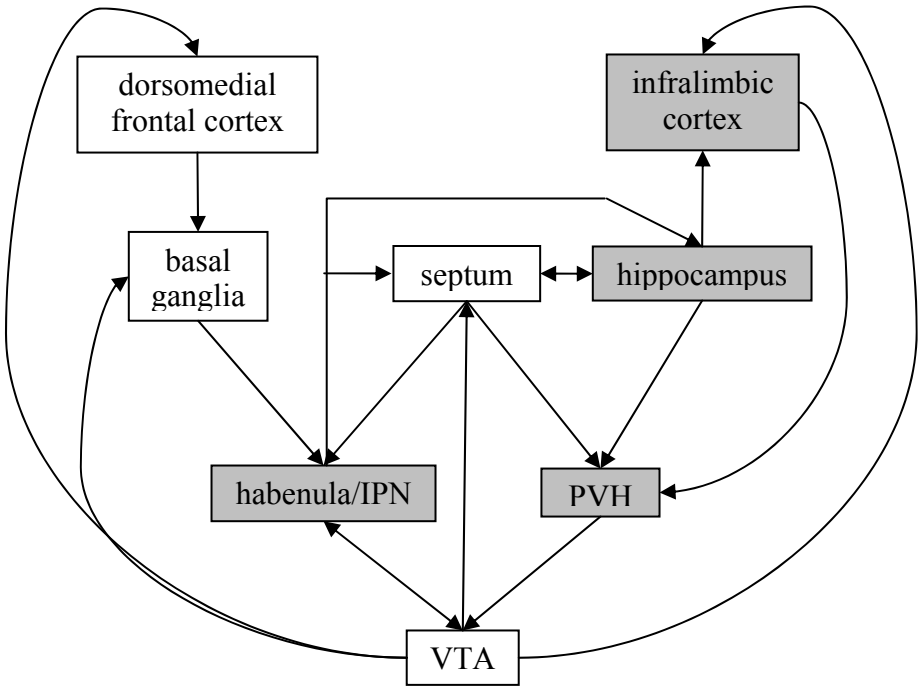
The chemical neuroanatomy of the medial and lateral habenula is rich with a considerable number of different neurotransmitters and neuropeptides, including somatostatin, substance P, neurotensin, serotonin, agmatine, parathyroid hormone, neuromedin, Leu-enkephalin, dopamine, acetylcholine, and urocortin III (Freed et al., 1995; Geisler, Andres, & Veh, 2003; Hervieu & Emson, 1998; Honzawa, Sudoh, Minamino, Tohyama, & Matsuo, 1987; Jennes, Stumpf, & Kalivas, 1982; Li, Vaughan,

Sawchenko, & Vale, 2002; Otake et al., 1998; Schafer, Eiden, & Weihe, 1998; Shinoda, Inagaki, Shiosaka, Kohno, & Tohyama, 1984; Shinoda & Tohyama, 1987; Wang, Palkovits, Rusnak, Mezey, & Usdin, 2000). In addition, there are at least two receptor types which are relatively specific to the habenula. First, the medial habenula is especially rich in several types of nicotinic receptors, including one with a selective affinity for epibatidine that appears uniquely expressed there and in the fasciculus retroflexus and IPN (Plenge, Mellerup, & Wortwein, 2002). Even more promising is the finding of an orphan G-protein-coupled receptor (GPCR-2037), the mRNA for which was exclusively expressed throughout both the medial and lateral habenula (Berthold, Collin, Sejlitz, Meister, & Lind, 2003). It is considered an "orphan" because a ligand for it has not yet been discovered. Further research elucidating the structure and function of this receptor could eventually provide a way of selectively targeting the habenula with a systemically administered drug, which could be of immense therapeutic utility.

CONCLUSIONS

This body of research has provided several unique insights. One is that novelty-seeking behaviors may be an early temperamental indicator of vulnerability to stress-related psychopathology, and that novelty seeking, helplessness susceptibility, and reduced adrenal signaling may go hand in hand. Another is the suggestion that animals characterized by these behavioral and endocrine features have extensively altered brains even at birth, particularly in terms of how stress-responsive regions appear functionally disconnected. Finally, the habenula may be a nexus through which antidepressants may influence these dysfunctional neural networks, and its unique pharmacology offers great promise as a therapeutic target for treating emotional disorders in the future.

Figure 7.1: Model of major circuitry disruption in adult congenitally helpless rats. Shaded regions are hypermetabolic, and unshaded regions are hypometabolic.



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Vita

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