

**EARLY LIFE EXPERIENCE ALTERS STRESS-RELATED BRAIN CIRCUITS:
EFFECTS OF REPEATED BRIEF POSTNATAL MATERNAL SEPARATION ON
CENTRAL AUTONOMIC PATHWAYS**

by

Layla Banihashemi

B.S. in Neuroscience, University of Pittsburgh, 2003

M.S. in Neuroscience, University of Pittsburgh, 2005

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This dissertation was presented

by

Layla Banhashemi

It was defended on

April 8, 2010

and approved by

J. Patrick Card, PhD, Professor

Anthony A. Grace, PhD, Professor

Susan R. Sesack, PhD, Professor

Edda Thiels, PhD, Assistant Professor

James P. Herman, PhD, Professor

Dissertation Advisor: Linda Rinaman, PhD, Associate Professor

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Layla Banihashemi, M.S., PhD

University of Pittsburgh, 2010

Early life experience has a powerful influence on later stress reactivity, which is demonstrated by the animal model, repeated brief postnatal maternal separation. In this classic paradigm, rat pups undergo a 15-minute daily separation (MS15) from their dam for approximately one to two postnatal weeks. A substantial literature has demonstrated that adult rats with a developmental history of MS15 are significantly less stress reactive compared to controls, as evidenced by decreased stress-induced hormone release. Conversely, the effects of early life experience on brain circuits that control stress responses are virtually unknown.

Descending preautonomic circuits govern the output of the autonomic nervous system, which mediates physiological responses to stress (e.g., increased heart rate and decreased digestion). These circuits begin in the paraventricular nucleus of the hypothalamus (PVN) and limbic forebrain and synaptically innervate preganglionic neurons in the brainstem dorsal vagal complex (DVC) and spinal cord that ultimately innervate body organs. A previous study from our laboratory has demonstrated that MS15 alters the developmental assembly of gastric preautonomic circuits (Card et al., 2005). These findings led us to hypothesize that MS15 rats would display altered circuit strength of gastric preautonomic circuits later in development, as assessed in juvenile rats. Indeed, the study described in Chapter 2 demonstrated that MS15

enhances the circuit strength of gastric preautonomic circuits originating within the PVN in juvenile rats. This enhanced circuit strength suggests that the function of preautonomic PVN pathways might also be altered by MS15. Thus, we hypothesized that MS15 rats would display altered stress-induced activation of the PVN to DVC pathway. The study described in Chapter 3 revealed that MS15 rats display decreased stress-induced Fos activation within the PVN and within a specific population of DVC neurons. Therefore, studies within this dissertation revealed that M15 alters the circuit strength of PVN preautonomic pathways and alters stress-induced activation of brainstem preautonomic pathways. These findings suggest that MS15 rats would display attenuated autonomic responses to stress and may provide insights into how early life experience shapes later stress reactivity.

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PREFACE

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List of Abbreviations

ACTH – Adrenocorticotrophic hormone

ANS – Autonomic nervous system

AP - Area postrema

BDNF – Brain-derived neurotrophic factor

BNST - Bed nucleus of the stria terminalis

CeA - Central nucleus of the amygdala

CORT – cortisol/corticosterone

CRH(F) – Corticotropin-releasing hormone (factor)

DbH – Dopamine-beta-hydroxylase

dBNST - Dorsal bed nucleus of the stria terminalis

DMV - Dorsal motor nucleus of the vagus

DSAP – saporin toxin conjugated to an anti-DbH antibody

DVC - Dorsal vagal complex

EE – Environmental enrichment

GR(s) – Glucocorticoid receptor(s)

HPA - Hypothalamic-pituitary-adrenal

IN - Insular cortex

LG-ABN – Licking and grooming, arched-back nursing

MS15 - Repeated brief postnatal maternal separation

NA - noradrenergic

NE - norepinephrine

NRS – No restraint stress controls

NS - Non-separated controls

NST - Nucleus of the solitary tract

P - Postnatal day

PL/IL - Prelimbic/infralimbic cortex

PVN - Paraventricular nucleus of the hypothalamus

PaDC - Paraventricular nucleus of the hypothalamus, dorsal cap

PaMP - Paraventricular nucleus of the hypothalamus, medial parvocellular

PaV - Paraventricular nucleus of the hypothalamus, ventral subnucleus

PRV - Pseudorabies virus

ROI - Region of interest

RS - Restraint stress

SES – Socioeconomic status

vBNST - Ventral bed nucleus of the stria terminalis

VLM – Ventrolateral medulla

1.0 INTRODUCTION

It is how people respond to stress that determines whether they will profit from misfortune or be miserable. – Mihaly Csikszentmihalyi

1.1 WHAT MAKES US WHO WE ARE?

Stress responses are physiological changes (e.g., stress hormone release, changes in heart and respiratory rates, and changes in digestion) that are adaptive in situations in which an organism faces an acute challenge, or stressor. As stress inevitably presents itself in everyday life, a large part of what defines individuals is how they respond to stress, or their stress reactivity. Some individuals are highly stress reactive, responding to stress with great anxiety and physiological disturbance, while others are relatively resilient to stress. Highly stress reactive individuals experience exaggerated stress responses that are larger in magnitude and/or more prolonged compared to less stress reactive individuals. Throughout life, these exaggerated stress responses have negative consequences leading to pathological changes in the body (e.g., atherosclerosis) and disease (e.g. cardiovascular disease).

How do individual differences in stress reactivity arise? A large literature examining both humans and animals demonstrates that early life experience has a profound influence on stress reactivity and vulnerability to affective disorders later in life (Levine, 2005, Heim et al.,

2008). For instance, early life neglect or trauma is associated with increased stress reactivity and increased risk for disease and affective disorders in adulthood (Heim et al., 2000, De Bellis, 2001, Meaney, 2001, Chen et al., 2004), whereas early life “enriched environments” are associated with decreased stress reactivity and risk for disease, and enhanced cognitive functioning (Meaney, 2001, Chapillon et al., 2002, Chen et al., 2004).

Importantly, circuits that connect the brain and the body govern stress responses. Individual differences in stress reactivity are likely due to individual differences in the structure and function of neural circuits that control stress responses. Despite the important role of early life experience in shaping later stress reactivity, early experience-induced alterations of stress-related brain circuits have not been examined in depth. The goal of the research presented within this dissertation is to examine how early life experience alters brain circuits that control stress responses. This research will potentially provide insights into how stress-related circuits contribute to stress reactivity and risk for affective disorders and other diseases.

1.2 STRESS AND THE STRESS RESPONSE

A stressor is any stimulus that disrupts an organism’s homeostasis – or physiological balance. Stressors are categorized broadly based on their origins - stressors like infection and hemorrhage are physiological stressors, whereas a stressor like public speaking is psychological. However, both types of stressors are ultimately physiological because the stress response is a collection of physiological changes. The stress response is an allostatic process, which coordinates body-wide physiological changes that deal with the threat and then restore homeostasis (Sapolsky, 2004). The stress response includes autonomic and neuroendocrine components, described below.

1.2.1 The stress response: autonomic

The autonomic nervous system (ANS) regulates peripheral physiology, including physiological responses to stress. The ANS includes two subdivisions, the parasympathetic and sympathetic systems. The parasympathetic system is known as the “rest and digest” division and it generally exerts an inhibitory influence on target organs (e.g. decreases heart rate) while promoting digestion (e.g., increases salivation and gastric motility, etc.). The sympathetic division is known as the “fight or flight” system and exerts an excitatory influence on target organs (e.g., increases heart rate) and inhibits digestion (e.g. inhibits salivation and gastric motility). Although these systems appear functionally distinct, both systems are active during rest and the balance between them achieves homeostasis. During stress conditions, the balance is shifted toward the sympathetic system which prepares the body to deal with the threat – blood flow is diverted away from the gut, such that digestion (and other nonessential activities, such as growth, reproduction, and immune function) are inhibited, and heart and respiratory rates increase, facilitating the transport of oxygenated blood to the muscles, thereby enabling “fight or flight.”

The general organization of the ANS is such that preganglionic neurons within the brainstem or spinal cord innervate “postganglionic” neurons within autonomic ganglia that in turn, innervate target organs. The parasympathetic system is “cranio-sacral,” which refers to the location of its preganglionic neurons. Most notably, the brainstem dorsal motor nucleus of the vagus [DMV, within the dorsal vagal complex (DVC)] houses vagal preganglionic neurons that innervate many body organs, including the entire gut from the oral cavity to the colon. Preganglionic neurons of the parasympathetic division innervate autonomic ganglia that are

proximal to and/or embedded within target organs (Fig. 1). The sympathetic division is “thoraco-lumbar;” its preganglionic neurons are located in the intermediolateral horn of the spinal cord, which extends from caudal thoracic to rostral lumbar spinal segments. Some sympathetic preganglionics innervate postganglionic neurons within the prevertebral ganglia, which innervate the viscera, including the gastrointestinal system (Fig. 1). The medulla of the adrenal gland receives input exclusively from the sympathetic nervous system and its activation leads to the release of epinephrine, or “adrenaline,” which leads to the mobilization of energy stores within various tissues to provide muscles with further nutrients and energy. This includes the breakdown of fat in adipose tissue and protein in muscle, and the synthesis of new glucose in the liver, which is accompanied by decreased glucose and amino acid uptake and decreased synthesis of new protein.

1.2.2 The stress response: neuroendocrine

The neuroendocrine component of the stress response is controlled by the hypothalamic-pituitary-adrenal (HPA) axis. The corticotropin-releasing hormone (CRH)-containing neurons of the medial parvocellular paraventricular nucleus of the hypothalamus (PVN) comprise the apex of the HPA axis. These neurons empty CRH into a portal system of capillary beds in the pituitary stalk. CRH stimulates the anterior portion of the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH stimulates the cortex of the adrenal gland to release glucocorticoids (Fig. 1), including cortisol (in humans) or corticosterone (in rats) (CORT), which then act on various body tissues to create and mobilize energy, similar to the effects of epinephrine described above. Importantly, plasma levels of CORT are regulated by negative feedback via its

actions on glucocorticoid receptors (GRs) at the level of the pituitary and various brain regions, including the hypothalamus itself, the hippocampus, and the frontal cortex.

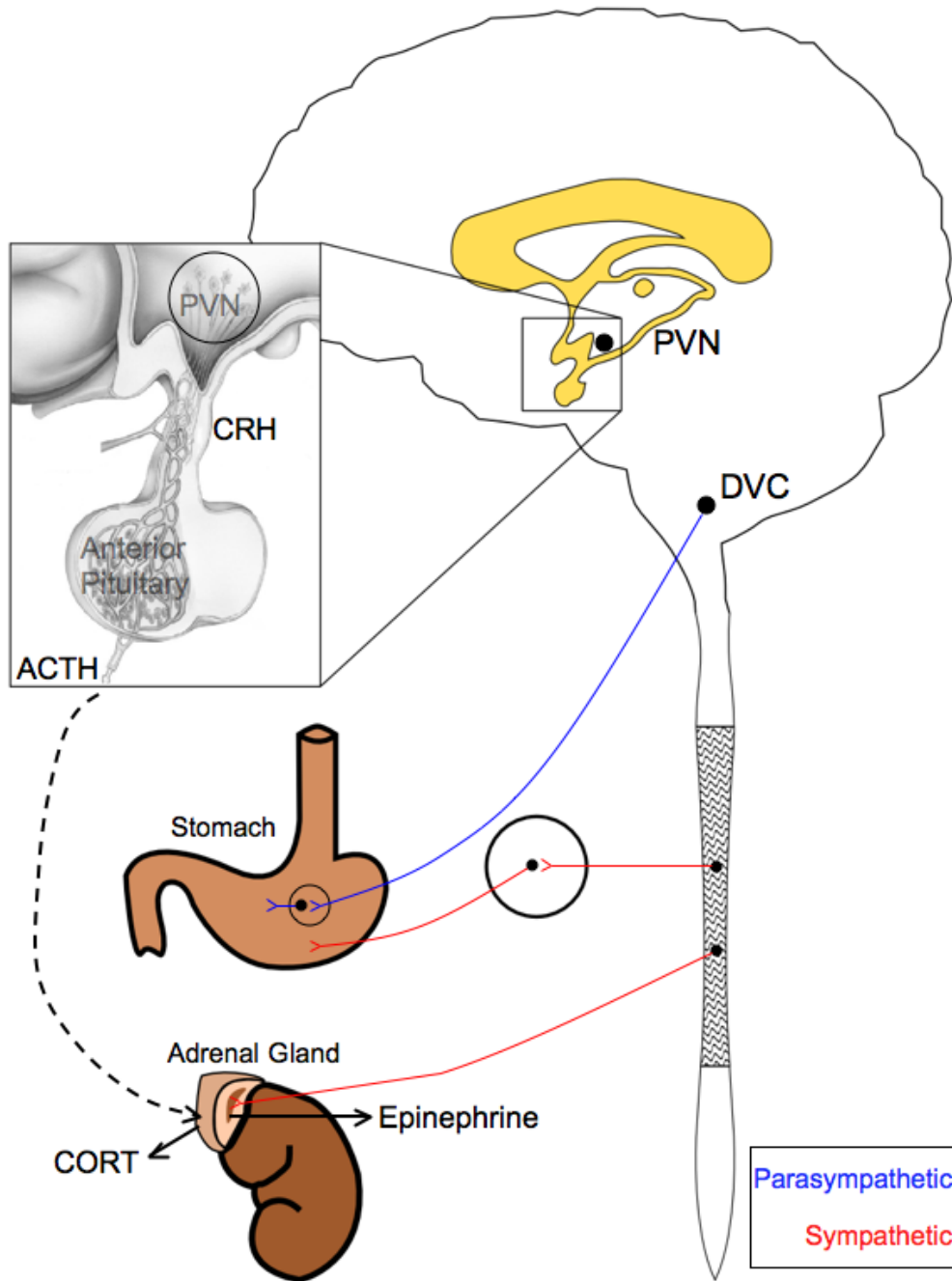


Figure 1. The components of the stress response: the autonomic nervous system and the hypothalamic-pituitary adrenal axis. The components of the HPA axis, the dual parasympathetic (blue) and sympathetic (red) innervation of the stomach, and the sympathetic innervation of the adrenal medulla are shown.

1.3 ACUTE VS. CHRONIC STRESS

The stress response is highly adaptive and necessary for survival in situations of acute stress. For example, the physiological changes that comprise the stress response would enable a person to flee from an attacker. Then, the stress response would end once the person has reached safety, allowing the body to recover and regain homeostasis. However, humans often undergo chronic psychological stress, in which the same stress response that enables fleeing from an attacker is turned on repeatedly when worrying about financial problems, conflicts at work, relationship turmoil, etc. Further, the body may not fully recover from these repeated activations of the stress response. Stress responses in chronic stress situations are maladaptive and have deleterious health-related consequences. Chronic stress is associated with obesity, gastrointestinal disorders, ulcers, attenuated growth, compromised reproductive capabilities, decreased immunity, memory dysfunction, and cardiovascular disease (Sapolsky, 2004).

Chronic stress responses may be magnified by high stress reactivity. Highly stress reactive individuals who display exaggerated responses to stress (e.g., inappropriately large-magnitude changes in blood pressure) may be even more susceptible to stress-related disease. These individuals may display inappropriately large stress responses to both acute and chronic stress over the course of their lives. Indeed, individuals who exhibit exaggerated blood pressure reactivity to acute stressors are at greater risk for hypertension and cardiovascular disease (Menkes et al., 1989, Matthews et al., 1993b, Bedi et al., 2000, Knox et al., 2002, Matthews et al., 2003, Ming et al., 2004, Stewart et al., 2006).

Both autonomic and neuroendocrine components of the stress response are controlled by brain circuits that connect the brain and body. It is likely that individual differences in stress reactivity arise from individual differences in the structure and function of stress-related brain circuits, which are described below.

1.4 STRESS-RELATED BRAIN CIRCUITS

1.4.1 Stress-related brain circuits: descending preautonomic circuits

“Preautonomic” circuits beginning in the brain directly control autonomic outflow to target organs, including stress-induced changes in peripheral physiology. Preautonomic regions innervate sympathetic and/or parasympathetic preganglionic neurons in the spinal cord and/or brainstem, respectively (Fig. 2). This anatomical organization has been elegantly identified through the use of transynaptic viral tracing studies, many of which have used attenuated strains of the retrograde transynaptic tracer, pseudorabies virus (PRV; an alpha-herpesvirus).

Central PRV transport from inoculated visceral targets that exclusively receive sympathetic inputs (e.g., adrenal medulla, kidney, spleen) identifies brainstem, pontine, midbrain, and diencephalic structures, including the caudal medial nucleus of the solitary tract (NST), ventrolateral medulla (VLM), locus coeruleus, Barrington’s nucleus, raphe nuclei, periaqueductal gray, and hypothalamic regions, including the PVN and lateral hypothalamus (Tucker and Saper, 1985, Strack et al., 1989, Cano et al., 2000, Cano et al., 2001). When injected into target organs having dual innervation from sympathetic and parasympathetic systems, such as the stomach wall, similar brainstem and hypothalamic preautonomic regions are

identified (Yang et al., 1999, Rinaman et al., 2000). However, retrograde transport to and infection of the DVC, first to the DMV and then subsequent infection of the NST and area postrema (AP), leads to infection of additional preautonomic regions, such as the PVN, bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA) and visceral cortices [i.e., prefrontal/infralimbic (PL/IL) and insular (IN) cortices] (Yang et al., 1999, Rinaman et al., 2000).

1.4.2 Stress-related brain circuits: ascending viscerosensory circuits

The NST receives viscerosensory information from the vagus, glossopharyngeal and facial nerves, and the spinothalamic tract (Kalia and Sullivan, 1982, Menetrey and Basbaum, 1987). Viscerosensory signals progress from the NST and the VLM in a bundle to hypothalamic and limbic forebrain regions via direct ascending projections that are primarily noradrenergic [NA, i.e., containing the NA synthetic enzyme dopamine-beta-hydroxylase (DbH)] (Fig. 2). Among its targets, the NST projects to the PVN, the apex of the HPA axis, as well as to other brain regions known to regulate HPA activity (Riche and DePommery, 1990, TerHorst and Streefand, 1994), such as the BNST (Choi et al., 2007).

The ascending NA pathway strongly influences stress responses (Ericsson et al., 1994, Aston-Jones et al., 1999, Rinaman, 2003a, Ritter et al., 2003). Stress increases the level of norepinephrine (NE) in PVN which is strongly correlated with plasma ACTH levels (Pacak et al., 1995b). Microinjection of NE into the PVN stimulates CRH expression and ACTH release (Itoi et al., 1994). Specific lesions of the NA input to the PVN using saporin toxin conjugated to an antibody against DbH (DSAP) have been reported to significantly attenuate stress-induced

neural activation of PVN, transcription of PVN CRH, and corticosterone secretion (Ritter et al., 2001, Ritter et al., 2003, Bienkowski and Rinaman, 2007).

Stress also increases the level of NE within BNST (Pacak et al., 1995a, Fuentealba et al., 2000, Cecchi et al., 2002). DSAP lesions of the NA input to the BNST attenuate stress-induced neural activation in the ventrolateral BNST and medial parvocellular PVN, and attenuate stress-induced corticosterone levels (Banihashemi and Rinaman, 2006).

Interestingly, the ascending NA pathway targets regions that are also preautonomic, including the PVN (Fig. 2), BNST, and CeA, and NE within these regions also influences autonomic function (Bachelard et al., 1992, Pacak et al., 1995b). Thus, ascending viscerosensory and descending preautonomic pathways are highly reciprocal.

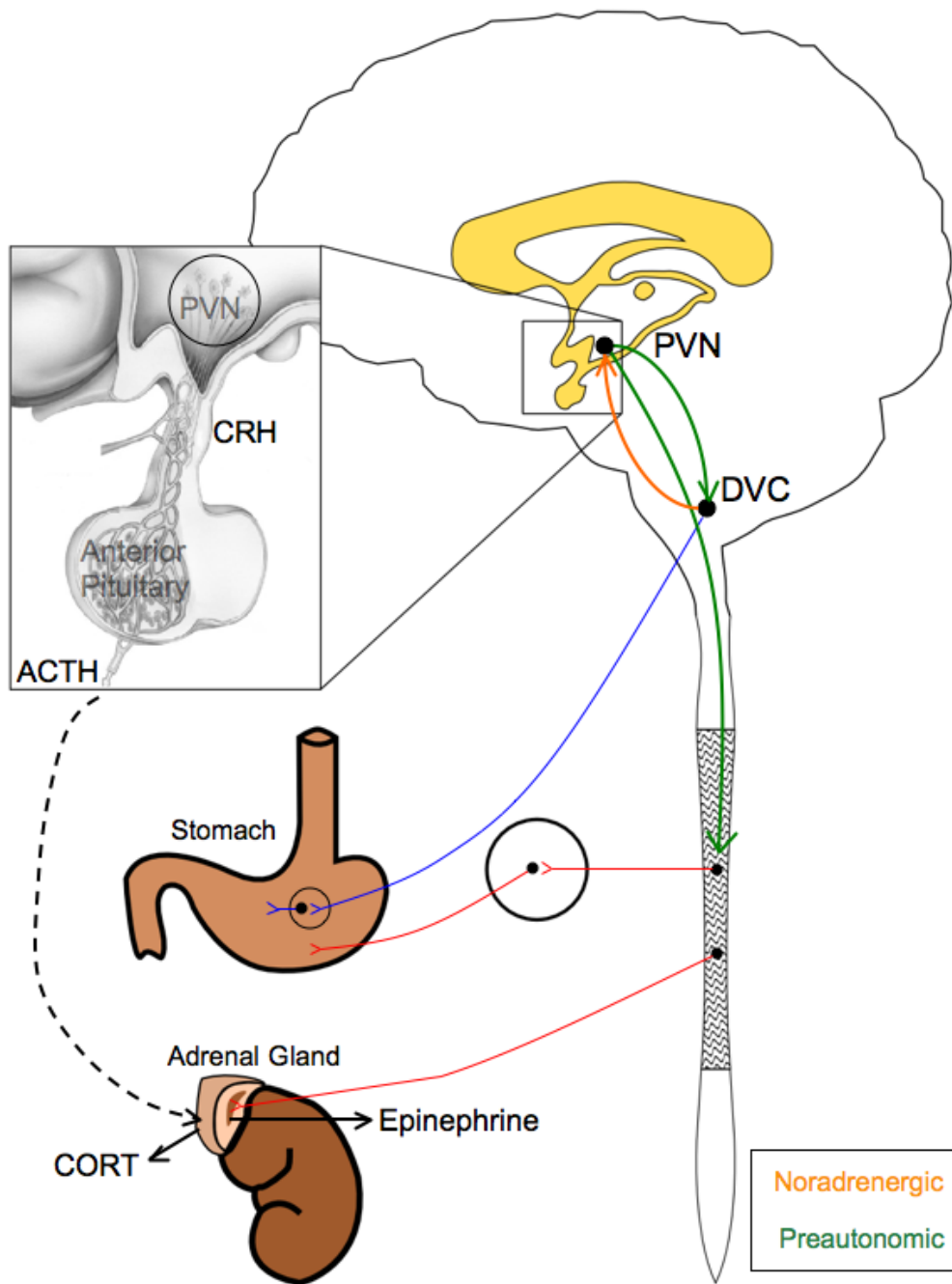


Figure 2. Stress-related brain circuits: descending preautonomic and ascending viscerosensory/noradrenergic circuits. Descending preautonomic (green) projections from the PVN to the DVC (pre-parasympathetic) and spinal cord (pre-sympathetic) and ascending viscerosensory (orange) projections from the DVC to the PVN are shown.

1.5 THE PVN: A SPECIAL REGION FOR THE CENTRAL INTEGRATION OF STRESS RESPONSES

As described above, the PVN is the apex of the HPA axis, which mediates the neuroendocrine component of the stress response. Further, the PVN synaptically innervates both brainstem and spinal preganglionic neurons (Fig. 2), and therefore controls both parasympathetic and sympathetic components of the ANS, respectively. In addition to directly innervating spinal and brainstem preganglionic neurons (Strack et al., 1989, Rinaman et al., 2000), the PVN also innervates the rostral VLM (Luiten et al., 1985), a presympathetic brainstem region that greatly influences cardiovascular function (Madden and Sved, 2003). As it is uniquely capable of controlling both the neuroendocrine and autonomic components of the stress response, the PVN can be said to exert more proximal control over stress responses than any other forebrain region.

As its anatomical connections would suggest, the PVN plays a significant role in autonomic outflow, including cardiovascular and gastric autonomic function. Experimental manipulation of the PVN leads to alterations in gastric motility and acid secretion (Tache, 1991, Flanagan et al., 1992b), and heart rate and blood pressure (Pyner, 2009). Further, experimental manipulation of the PVN in rats modifies the activity of DVC neurons (Zhang et al., 1999). In particular, the PVN appears to drive stress-induced Fos activation of a specific DVC population – the NA neurons of the NST (Buller et al., 2003, Dayas et al., 2004). As described above, these neurons receive viscerosensory information and relay it to PVN and other limbic forebrain regions to greatly influence both neuroendocrine (Banihashemi and Rinaman, 2006, Bienkowski

and Rinaman, 2008) and autonomic components of the stress response (Pacak et al., 1995b). These NA NST neurons project locally within the NST and to the DMV (Card et al., 1990, Pearson et al., 2007), and influence vagally-mediated autonomic function (Rogers et al., 2003, Herman et al., 2008).

1.6 THE DEVELOPMENT OF STRESS-RELATED BRAIN CIRCUITS

Viral transynaptic tracing has been used to determine the time course of the developmental assembly of preautonomic circuits. Studies injecting PRV into the stomach wall in neonatal rats demonstrate that spinal and brainstem preganglionic innervation of the stomach and some PVN innervation of preganglionic neurons is present at birth [postnatal day 0 (P0)] (Rinaman et al., 1999, Rinaman et al., 2000). By P4, synaptic inputs from PVN to spinal and brainstem preganglionics are more extensive, while descending preautonomic projections from CeA and BNST are modest. By P8, gastric preautonomic circuits appear adult-like with projections from CeA and BNST being more extensive and preautonomic projections from visceral cortices being present (Rinaman, 1998b, Rinaman et al., 1999, Rinaman et al., 2000). Ascending NA projections also develop during the first two weeks of postnatal life, with the density of NA terminals in PVN increasing to adult-like levels by P21 (Rinaman et al., 2000). Thus, viscerosensory and preautonomic circuits assemble throughout the one to two postnatal weeks during a potentially sensitive period of development and may be subject to the influences of early life experience.

1.7 THE INFLUENCE OF EARLY LIFE EXPERIENCE ON LATER STRESS REACTIVITY: REPEATED BRIEF POSTNATAL MATERNAL SEPARATION

Early life experience greatly influences later stress reactivity (Levine, 2005). Studies in both humans and animals have demonstrated that early life neglect (e.g., parental neglect) is associated with increased stress reactivity (Heim et al., 2000, De Bellis, 2001, Meaney, 2001, Chen et al., 2004), while early life enriched environments (e.g., parental care) are associated with decreased stress reactivity (Meaney, 2001, Chapillon et al., 2002, Chen et al., 2004).

The repeated brief postnatal maternal separation model is a classic paradigm that has clearly demonstrated the influence of early life experience on later stress reactivity (Plotsky 1993). In this model, rat pups undergo a 15-minute daily separation from their dam (MS15) for approximately one to two postnatal weeks. MS15 enhances the quality and duration of maternal care given to the pups upon their return to the dam (Liu et al., 1997, Macri et al., 2008). During this period, dams display more occurrences of “active” care-giving, including licking and grooming of the pups and the most active style of nursing, “arched-back” nursing (Liu et al., 1997, Macri et al., 2008). Adult rats with a developmental history of MS15 are far less stress reactive compared to non-separated control rats, as the effects of MS15 on the HPA component of the stress response have been very well characterized. MS15 rats display decreased CRH mRNA in the PVN and decreased stress-induced ACTH and CORT secretion compared to non-separated (NS) controls (Plotsky and Meaney, 1993, Liu et al., 2000). Further, MS15 rats also display more GRs in hippocampus and frontal cortex (Meaney et al., 1985, Plotsky and Meaney, 1993, Liu et al., 1997). This MS15-related increase in GRs is thought to contribute to a more efficient negative feedback of the HPA axis, which is supported by a more rapid decline of post-stress ACTH and CORT levels in MS15 rats compared to NS controls (Plotsky and Meaney,

1993, Liu et al., 2000). Interestingly, the decreased CRH mRNA in PVN is the earliest of the MS15-related influence on the HPA axis and is present by P9 (Avishai-Eliner et al., 2001). MS15-related alterations in stress-induced hormone release are observable by P23, while changes in GR levels in hippocampus are evident by P45 (Avishai-Eliner et al., 2001).

In addition to having an altered HPA axis, MS15 rats display fewer anxiety-like behaviors in a battery of behavioral tests. MS15 rats exhibit increased exploration of a novel environment, more time in the center of an open field, more time on the open arms of the elevated plus maze, and shorter latency to feed in a neophagia test compared to NS controls (McIntosh et al., 1999, Caldji et al., 2000, Madrugá et al., 2006, Koehnle and Rinaman, 2010).

Thus, a substantial literature has characterized the neuroendocrine and behavioral phenotype of MS15 rats, yet few studies have examined changes in brain circuitry that control stress responses. Two reports have demonstrated that MS15 attenuates stress-induced neural activation within preautonomic regions, including the PVN, BNST, and CeA (Abraham and Kovacs, 2000, Koehnle and Rinaman, 2010). Based on the ability of MS15 to decrease later stress reactivity and the early postnatal development of stress-related brain circuits, MS15 likely alters development and the later structure and function these circuits.

1.8 MS15 ALTERS THE DEVELOPMENTAL ASSEMBLY OF PREAUTONOMIC CIRCUITS

A study from Card and colleagues provided the first evidence that early life experience can influence a system of stress-related brain circuits (Card et al., 2005). Following PRV inoculation of the ventral stomach wall, 10-day-old rat pups undergoing MS15 displayed fewer PRV-

positive gastric preautonomic neurons in the PVN, BNST, CeA, and visceral cortices (IN and PL/IL) compared to NS control pups (Card et al., 2005). An intracellular threshold of infecting virions must be reached in order for PRV replication to occur, and the number of virions transported to the nucleus of a neuron is strongly correlated with the number of axon terminals in an inoculated area (Card et al., 1999). Thus, the reduced number of preautonomic neurons in these regions could represent a reduction in the number of preautonomic neurons in synaptic contact with preganglionic neurons, or a reduction in the number of axons terminals of individual preautonomic neurons (Card et al., 2005). In this manner, MS15 may decrease the circuit strength between preautonomic forebrain neurons and preganglionic neurons during the neonatal period. Alternatively, this reduced number of preautonomic neurons may reflect delayed synaptic assembly of descending preautonomic circuits during the neonatal period that may evolve throughout development.

1.9 HOW DOES MS15 ALTER PREAUTONOMIC CIRCUITS LATER IN LIFE?

Rats with an early life experience of MS15 are remarkably less stress reactive compared to NS controls, as evidenced by their well-characterized decrease in HPA and behavioral stress reactivity. However, effects of MS15 on brain circuits that influence and/or control stress responses have not been examined. Given that MS15 alters the developmental assembly of descending preautonomic circuits originating within hypothalamic and limbic forebrain regions, we hypothesized that 1) MS15 rats would display altered circuit strength of gastric preautonomic circuits later in development, as assessed in juvenile rats (Chapter 2). This study was performed using PRV inoculation of the stomach wall. The viral transynaptic tracing technique is very

powerful in that it allows identification of a system of descending circuits innervating the stomach and postnatal group differences in PRV labeling indicate alterations in the circuit strength of preautonomic circuits. Indeed, our results demonstrated that MS15 enhanced the circuit strength of the gastric preautonomic circuits originating within the PVN in juvenile rats. [The contents of chapter 2 have been published previously in Neuroscience (Banihashemi and Rinaman, 2010)]. This enhanced circuit strength suggested that the function of the preautonomic PVN pathway might also be altered by MS15.

Thus, we hypothesized that 2) MS15 rats would display altered stress-induced activation of the PVN to DVC pathway. Standard retrograde tracing was performed to identify DVC-projecting PVN neurons. This technique was used in order to assess stress-induced activation of the immediate early gene protein product, Fos (a neural marker of activation), which cannot be assessed in combination with viral tracing. This study used restraint as the experimental stressor as it has been demonstrated to produce anxiety-like behaviors (Berridge and Dunn, 1989, Casada and Dafny, 1991), activate DVC-projecting PVN neurons and DVC neurons (i.e., NA NST neurons) (Buller et al., 2003, Dayas et al., 2004), and elicit autonomic responses (Menguy, 1960, Henke, 1988, Sullivan and Gratton, 1999). Further, differences in stress reactivity between MS15 and control rats have been demonstrated using restraint stress (Abraham and Kovacs, 2000, Liu et al., 2000). This is the first study to examine MS15-related alterations in the stress-induced activation of a specific brain circuit. This research will potentially provide insights into how early life experience-induced alterations in stress-related circuits contribute to differences in stress reactivity.

2.0 REPEATED BRIEF POSTNATAL MATERNAL SEPARATION ENHANCES HYPOTHALAMIC GASTRIC AUTONOMIC CIRCUITS IN JUVENILE RATS

2.1 ABSTRACT

Maternal separation of rat pups for 15 minutes each day over the first one to two postnatal weeks (MS15) has been shown to increase the active maternal care received by pups and to decrease their later neuroendocrine and behavioral stress reactivity compared to non-separated (NS) controls. Stress responses prominently feature altered gastric secretion and motility, and we previously reported that the developmental assembly of forebrain circuits underlying gastric autonomic control, including gastric responses to stress, is delayed by MS15 in neonatal rats (Card et al. 2005, *J. Neurosci.* 25(40): 9102). To determine how this early delay affects the later organization of central gastric autonomic circuits, the present study examined the effects of neonatal MS15 on central pre-gastric circuits assessed in post-weaning, juvenile rats. For this purpose, the retrograde transsynaptic viral tracer, pseudorabies virus (PRV), was microinjected into the stomach wall of 28–30 day old male rats with an earlier developmental history of either MS15 or NS. Rats were perfused 72 hours later and tissue was processed to reveal PRV-positive cells. Transsynaptic PRV immunolabeling was quantified in selected preautonomic brainstem and forebrain regions, including the area postrema, bed nucleus of the stria terminalis, central nucleus of the amygdala, paraventricular nucleus of the hypothalamus (PVN), and visceral

cortices. Compared to NS controls, MS15 rats displayed a significantly greater amount of PRV labeling within the PVN, including both the dorsal cap and ventral subnuclei. There were no postnatal group differences in the amount of PRV labeling within any other brain region examined in this study. This effect of MS15 to enhance hypothalamic preautonomic circuit structure indicates a strengthening of this pathway and may provide insight into how early life experience produces differential effects on later stress reactivity, including gastric secretory and motor responses to stress.

2.2 INTRODUCTION

Early life experience shapes many facets of the developing organism that lay along a continuum of complexity. For instance, natural or experimentally-induced variations in maternal care received by rat pups in early life have a significant impact on gene regulation, neuron number and neurotransmitter levels in specific brain regions, and the later function of neuroendocrine and behavioral stress response systems (Liu et al., 2000, Meaney, 2001, Bredy et al., 2003, Winkelman-Duarte et al., 2007).

An established animal model that manipulates early life maternal care involves separating rat pups from their dam for 15 minutes daily for the first one to two postnatal weeks (MS15). This manipulation stimulates active maternal care (i.e., licking, grooming, and arched-back nursing) received by pups throughout the period of MS15 (Liu et al., 1997, Meaney, 2001, Macri et al., 2008). The increased maternal care is associated with decreased stress reactivity exhibited by the offspring later in life, including decreased anxiety-like behaviors and decreased

hypothalamic-pituitary-adrenal (HPA) axis tone and responsiveness to stress (Plotsky and Meaney, 1993, Levine, 2005, Plotsky et al., 2005).

The ability of MS15 to alter behavioral and neuroendocrine responses to stress led us to hypothesize that MS15 also alters autonomic emotional motor responses and underlying neural circuitry. Preautonomic neurons within the hypothalamus and limbic forebrain synapse directly onto autonomic motor neurons in the brainstem and spinal cord to powerfully modulate visceral motor outflow, including gastrointestinal responses to stressful and emotionally-evocative stimuli. Notably, Hans Selye's original description of the "general adaptation syndrome" elicited by stress included prominent alterations in gastric function (Selye, 1936). Preautonomic regions such as the bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN), and visceral cortices, [insular (IN) and prelimbic/infralimbic (PL/IL) cortex have been implicated in the central control of gastric function (Hermann et al., 1990, Aleksandrov et al., 1996, Yamamoto and Sawa, 2000, Liubashina et al., 2002, Zhang et al., 2003). In particular, experimental manipulation of the PVN in rats modifies the activity of gastric-related DVC neurons to alter gastric acid secretion and motility (Flanagan et al., 1992b, Zhang et al., 1999).

In rats, descending preautonomic pathways that control gastric function undergo significant synaptic assembly during the first one to two postnatal weeks (Rinaman et al., 2000), defining a potentially sensitive period of development during which these circuits may be influenced by experience. Indeed, we demonstrated that ongoing gastric preautonomic circuit assembly is delayed in neonatal rats during exposure to the MS15 paradigm (Card et al., 2005). That study utilized a retrograde transynaptic tracer, pseudorabies virus (PRV), to identify preautonomic hypothalamic and limbic forebrain neurons that synaptically innervate autonomic

neurons controlling visceral motor outflow to the stomach (see Fig. 3). Following PRV inoculation of the ventral stomach wall, 10-day-old rat pups undergoing MS15 displayed fewer PRV-positive gastric preautonomic neurons in the PVN, BNST, CeA, and visceral cortices (IN and PL/IL) compared to non-separated (NS) control pups (Card et al., 2005). Thus, MS15 delayed synaptic assembly between preautonomic forebrain neurons and gastric autonomic motor neurons during the neonatal period.

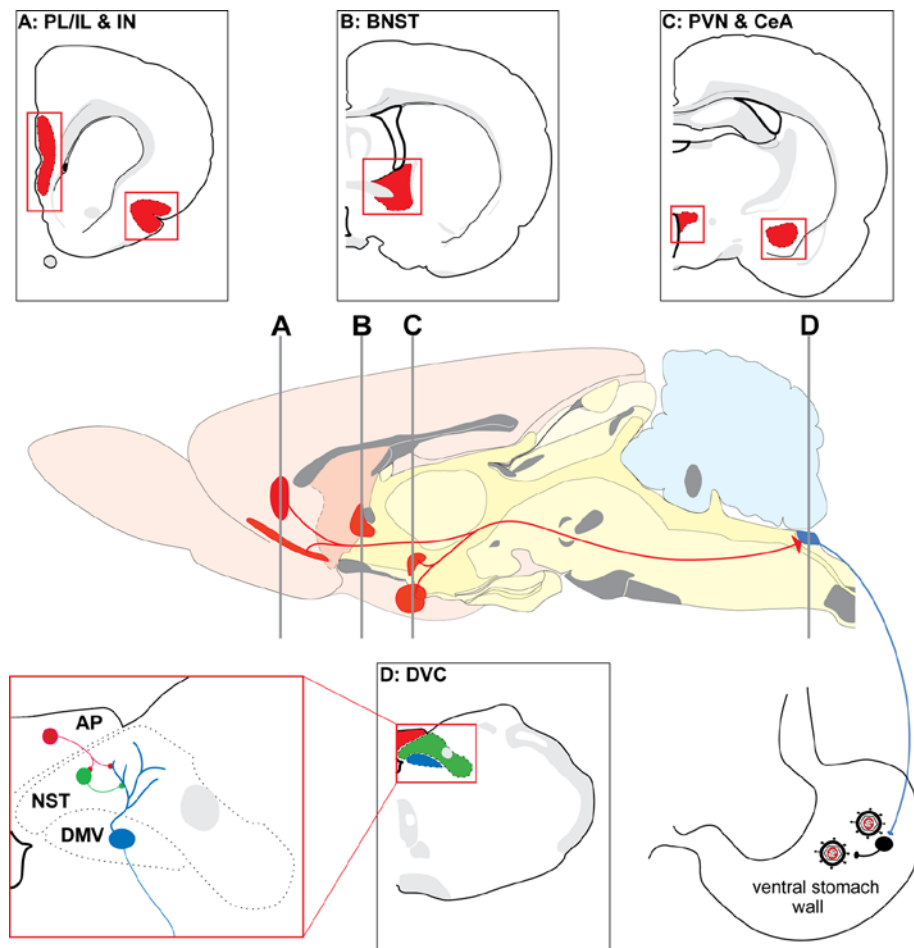


Figure 3. Schematic illustrating descending gastric preautonomic pathways and quantified regions of interest.

PRV injected into the ventral stomach wall is taken up by enteric neurons and also directly by vagal axon terminals (lower right), then is retrogradely transported to the dorsal motor nucleus of the vagus (DMV; D, coronal; blue), producing a first-order infection of parasympathetic DMV motor neurons. Replication and retrograde transynaptic transport of PRV leads to the subsequent infection of second-order neurons within the nucleus of the solitary tract (NST) (D, coronal; green) and third-order neurons within the AP (D, coronal; red). Further replication and retrograde transynaptic transport leads to passage of virus from the dorsal vagal complex (DVC) to hypothalamic and limbic forebrain regions of interest, including the PVN (C), CeA (C), BNST (B), and visceral cortices [PL/IL and IN (A)]. Sympathetic and pre-sympathetic pathways are not illustrated. Figure adapted from Card et al. 2005, based on schematics modified from Swanson (1998).

The present study sought to extend those findings by determining the influence of MS15 on gastric preautonomic circuit structure assessed in rats at a later developmental time-point, i.e., the post-weaning, “juvenile” period. Our results reveal experience-dependent structural alterations in hypothalamic preautonomic circuits, which may ultimately facilitate a more effective and/or resilient autonomic response to stress in rats with a developmental history of MS15.

2.3 EXPERIMENTAL PROCEDURES

Animals

All experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Animals were held in a controlled environment (20-22°C) with a 12-hour light dark cycle (lights on at 0700 hr) and *ad libitum* access to water and pelleted rat chow (Purina #5001, Bethlehem, PA).

The progeny of seven pregnant multiparous Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in this study. Pregnant rats arrived at the animal facility at a gestational stage between embryonic days 13 and 18, and were subsequently housed singly in opaque polyethylene cages with soft woodchip bedding and a wire lid. Pregnant rats were checked daily to determine their pups’ date of birth, designated postnatal day (P)0. Litters containing more than eight pups were culled randomly to eight pups on P0. Every litter was of mixed sex, although the PRV tracing data reported here were derived only from male rats.

All pups within each litter underwent the same postnatal treatment. Four litters were designated NS controls, and three litters were designated MS15. In total, 31 male rats from 7 litters were used for PRV tracing (NS $n = 15$, MS15 $n = 16$).

Experimental design

As in our earlier study (Card et al., 2005), pups in MS15 litters were separated from their dam for 15 minutes daily from P1 - P10, inclusive. The separation took place at approximately the same time each day (~1445 -1500 hr). The dam was briefly removed from the home cage and placed into a dedicated polyethylene tub. Using gloved hands, all of the pups were removed from their home cage along with a handful of home cage bedding and transferred together into a smaller polyethylene tub (26.7 x 15.2 x 12.7 cm). The dam was then returned to her home cage and the small tub containing the pups was promptly placed into an incubator in an adjacent room. Incubator conditions were controlled to closely match those of the home cage “nest” environment (~36°C, ~30-50% humidity). Rat pups remained in the incubator for 15 minutes and then, using gloved hands, the entire litter was returned simultaneously to their dam in the home cage. Pups in NS control litters typified the standard animal facility-reared condition, with no MS or experimental handling. However, both NS and MS15 pups and their respective dams underwent a weekly move to a clean cage with fresh bedding.

Rats were weaned from their dam on P21 and group-housed thereafter with same-sex littermates. Male rats with a developmental history of NS or MS15 underwent PRV inoculation surgery (described below) approximately one week post-weaning, between P28 and P30. According to our supplier (Harlan), males reach puberty between P45 and P48; thus, we refer to rats used for PRV tracing in this study as “juvenile”.

PRV inoculation

Group-housed rats were transferred in their home cages to the BSL-2 facility 24 hours prior to surgery. At the time of surgery, each rat was weighed and then anesthetized by halothane inhalation (Halocarbon Laboratories, River Edge, NJ; 1-2% in oxygen). After shaving and disinfecting the abdominal skin, a skin incision was made parallel to and ~ 1 cm below the lowest left rib, followed by incision of the underlying abdominal muscles. The stomach was gently exteriorized through both incisions. The attenuated Bartha strain (Bartha, 1961) of PRV (10^7 pfu/ml, provided by Dr. Lynn Enquist, Princeton University) was injected into the ventral stomach wall using a 10 μ L Hamilton syringe equipped with a fine glass tip. Injections were made tangentially between the smooth muscle layers of the ventral corpus and antrum, parallel to and between surface blood vessels. A total volume of 2 μ l was distributed equally at four injection sites. After the final injection, the surface of the stomach was gently swabbed with a saline-soaked cotton-tipped applicator, and then the stomach was returned to the abdominal cavity. The incision through the abdominal muscles was closed with silk sutures and the skin was closed with stainless steel clips. After recovery from anesthesia, rats were returned to their home cages in the BSL-2 facility where they remained for 72 hours until perfusion. This survival time was based on pilot studies demonstrating that 72 hours was sufficient to visualize a moderate amount of PRV labeling within the limbic forebrain (e.g., CeA and BNST) in rats at this age, but not long enough to reach late-stage viral infection with excessive gliosis.

Perfusion and histology

After the 72-hour post-inoculation interval, rats were deeply anesthetized by halothane inhalation (5% in oxygen) followed by intraperitoneal injection of sodium pentobarbital (Nembutal, 100 mg/kg BW). Rats were then perfused transcardially with a brief saline rinse followed by 250 ml

of fixative (4% paraformaldehyde in 0.1 M phosphate buffer with L-lysine and sodium metaperiodate) (McLean and Nakane, 1974). Brains were postfixed *in situ* overnight at 4°C, and then removed from the skull and cryoprotected for 24-72 hours in 20% sucrose solution at 4°C. Coronal 35 µm-thick tissue sections were cut from the spinomedullary junction through the rostral extent of the corpus callosum using a sliding, freezing microtome. Sections were collected serially in six adjacent sets and stored at -20°C in a cryopreservant solution (Watson et al., 1986).

Immunocytochemistry

One set of sections from each rat (sampling frequency of 210 µm) was removed from storage and rinsed for one hour in buffer (0.1 M sodium phosphate, pH 7.4) prior to immunocytochemical procedures. Antisera were diluted in buffer containing 1% normal donkey serum and 0.3% Triton-X100. Tissue sections were processed for immunoperoxidase localization of transported virus using a rabbit polyclonal anti-PRV antiserum (Rb133; provided by Dr. Lynn Enquist, Princeton University; 1:20,000), biotinylated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA), and Vectastain Elite ABC immunoperoxidase reagents (Vector Laboratories, Burlingame, CA). Sections were processed using nickel sulfate-intensified diaminobenzidine to generate a black reaction product identifying PRV-positive cells. After immunocytochemical processing, tissue sections were mounted onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA), counterstained for Nissl substance with Neutral Red, dehydrated in graded alcohols, cleared in xylene, and coverslipped using Cytoseal 60 (VWR, West Chester, PA).

Microscopic analysis and data collection: region of interest (ROI) analyses

Because PRV immunolabeling generally includes both neurons and local glial cells, and because the boundaries of individual PRV-immunolabeled cells can be difficult to discriminate, we were interested in quantifying the density of PRV immunolabeling in each brain region as an accurate index of the state of infection. Thus, we quantified the amount of PRV immunolabeling within each brain region as the area (μm^2) occupied by PRV-positive profiles within each analyzed region of interest (ROI) (e.g., one hemisphere of the CeA). For this purpose, ROIs within each section were photographed using a Zeiss Axioplan2 photomicroscope and a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu, Japan). An external red filter was used to eliminate Neutral Red counterstaining, thus producing a monochrome image, which was used for quantitative analysis (see Fig. 4, C and D). Image analysis was performed using SimplePCI imaging software (Hamamatsu Corporation, Sewickly, PA). The same microscope was used for all quantitative analyses, and illumination and filter settings were held constant across all experimental cases. All images underwent the following sequence of procedures: first, the ROI was manually outlined (see Fig. 4E and F) using the Nissl counterstain and PRV immunolabeling as a guide. Each outlined ROI fell within a Nissl-defined brain region; however, within this defined region, the ROI area itself was defined by a boundary drawn around the outer extent of PRV immunolabeling. Second, a detection threshold was applied that accurately identified PRV immunolabeling within the outlined ROI, without identifying any unlabeled areas. This detection threshold was established during preliminary work and was subsequently held constant for all labeling analyses across all experimental cases. Third, PRV immunolabeling within each outlined ROI that met or exceeded the detection threshold was automatically pseudocolored in green (see Fig. 4E and F). Finally, the program quantified the outlined ROI area (μm^2) and the

area within the ROI that was occupied by the identified (green pseudocolored) PRV-labeled profiles (“PRV labeling area,” μm^2). At the beginning of each image analysis session, PRV labeling within a specific reference section selected for each brain region was analyzed to confirm that measurements of ROI area and PRV labeling area were consistent across image analysis sessions. For each brain region, data were collected from every tissue section through that region (at a sampling frequency of 210 μm) and from both hemispheres. The rostrocaudal level of each ROI (relative to bregma) was noted during data collection. When ROIs through a given brain region did not contain any PRV immunolabeling, the ROI area and PRV labeling area for that ROI were each noted as having “0” values. Using this ROI analysis approach, PRV immunolabeling was quantified in the AP, dorsal and ventral BNST, CeA, and PVN, as described further below. PRV immunolabeling within the more sparsely infected visceral cortices was quantified using a different manual cell counting approach, as described at the end of this section.

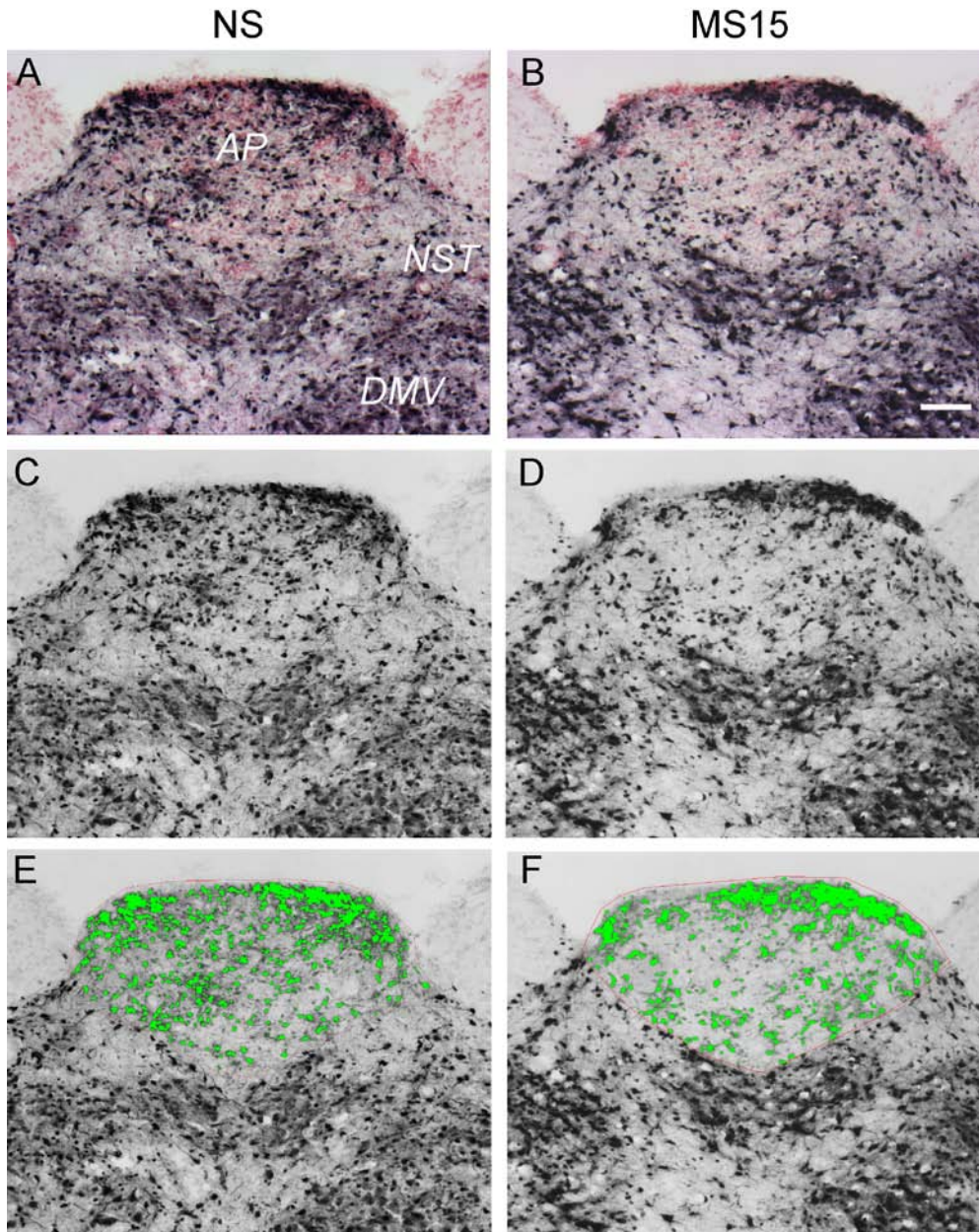


Figure 4. The method of ROI quantification and PRV labeling assessment is illustrated here for the area postrema (AP). Photomicrographs were obtained from a representative NS rat (left column) and a MS15 rat (right column). Panels A and B display black PRV immunolabeling and red Nissl counterstain. Panels C and D display monochrome images of the same sections, photographed with a red filter. Panels E and F show the same sections with manually drawn outlines of the AP ROI, with PRV labeling quantified by the image analysis program pseudocolored in green. PRV immunolabeling also is evident in the nucleus of the solitary tract (NST) and dorsal motor nucleus of the vagus (DMV). Scale bar (in B) = 200 μ m, applies to all panels.

Area postrema. In each experimental case, three sections through the area postrema (AP) were quantified that corresponded to bregma levels -14.08 through -13.68 mm (Paxinos and Watson, 1998).

Bed nucleus of the stria terminalis. Approximately three sections through the BNST were quantified in each experimental case, corresponding to bregma levels -0.72 through -0.10 mm (Paxinos et al., 1999). A natural break in Nissl staining and in PRV immunolabeling that followed the lateral and ventral curve of the anterior commissure was used to distinguish ‘dorsal’ from ‘ventral’ areas of the lateral BNST (see Fig. 6). The dorsal BNST as designated in the present study included the lateral dorsal, lateral juxtacapsular, and lateral posterior areas using Paxinos’ nomenclature (Paxinos et al., 1999), and included the oval nucleus, juxtacapsular nucleus, and the anterolateral area using Swanson’s nomenclature (Swanson, 2004). Paxinos and colleagues described lateral ventral and medial ventral areas that were included in our designation of the ventral BNST (Paxinos et al., 1999). Ventral BNST also included regions described by Swanson (2004) as comprising the rhomboid nucleus, anterolateral area, anteromedial area, and fusiform nucleus.

Central nucleus of the amygdala. Approximately six sections through the CeA were quantified in each experimental case, corresponding to bregma levels -3.30 through -1.60 mm (Paxinos and Watson, 1998). As PRV labeling was predominantly localized to the medial portion of the CeA (see Fig. 8), PRV labeling was not segregated by CeA subnuclei for quantitative analysis.

Paraventricular nucleus of the hypothalamus. Approximately six sections through the PVN were analyzed in each case, corresponding to bregma levels -2.12 through -1.30 mm (Paxinos and Watson, 1998). PRV immunolabeling within the posterior parvocellular nucleus

(PaPo) was included in an ROI only when it was visually continuous with PRV labeling in the medial parvocellular subnucleus. Rostrocaudal breakdown of PRV immunolabeling within the PVN guided additional analyses that were based on PRV labeling within specific PVN subnuclei (see Results).

Visceral cortices. PRV labeling in visceral cortical regions was more sparse than in subcortical areas, with no glial involvement. Thus, individual PRV-positive cortical neurons could be distinguished and were counted by eye using a Nikon Eclipse E200 light microscope. PRV immunolabeling was intermittent within tissue sections through the long rostrocaudal extent of the IN (i.e., approximately 7 mm from bregma levels -3.30 to +3.70 mm) (Paxinos and Watson, 1998). Conversely, PRV immunolabeling within the PL/IL spanned a much shorter rostrocaudal extent (i.e., bregma levels +2.20 through +3.70 mm) (Paxinos and Watson, 1998).

Data analysis

Data collected from each ROI within a given brain region included: (1) ROI area (mm²) and (2) PRV labeling area (mm²). For each brain region in each rat, the values for each ROI were summed across rostrocaudal levels and then divided by the number of ROIs analyzed for that brain region (average per ROI). For the visceral cortices (IN and PL/IL), the data collected in each rat included: (1) the number of hemisections through each cortical region in which PRV-positive neurons were present and (2) the number of PRV-positive neurons within each hemisection. For each brain region in each rat, the number of PRV-positive neurons was summed across hemisections and then divided by the number of hemisections in which PRV-positive neurons appeared (average per hemisection).

Data from each brain region were analyzed separately using one-way ANOVA to reveal potential effects of postnatal group (i.e., NS or MS15) on the dependent variables. Based on

significant effects of postnatal group on PRV labeling within the PVN (see Results), PVN data were subsequently binned into 4 distinct rostrocaudal levels and then further analyzed. The four rostrocaudal levels through the PVN were designated caudal (bregma levels -2.21 to -1.98 mm), mid-caudal (-1.98 to -1.88 mm), mid-rostral (-1.80 mm), and rostral (-1.6 to -1.3 mm), based on the atlas of Paxinos and Watson (1998). A repeated measures ANOVA was then performed to examine potential interactions between rostrocaudal level and postnatal group on PRV labeling area. A significant interaction between rostrocaudal level and postnatal group was followed up with independent-samples t-tests between groups at each rostrocaudal level.

Values are reported as group means \pm standard error. For all statistical analyses, differences were considered significant when $p \leq 0.05$. A priori, we considered any individual data points that lay three standard deviations beyond the group mean outliers.

2.4 RESULTS

Pre-surgical body weight

Body weight (BW) before PRV inoculation averaged $91.80 \text{ g} \pm 3.38$ for NS rats and $95.25 \text{ g} \pm 2.49$ for MS15 rats. Thus, there was no significant effect of postnatal group on juvenile BW ($F_{(1,30)} = 0.69$; $p = 0.41$).

Overview of central gastric preautonomic PRV immunolabeling

A relatively short 72 hour post-inoculation survival interval was used in this study to capture initial viral infection resulting from direct descending projections of preautonomic forebrain neurons to the dorsal vagal complex (DVC), and to avoid secondary waves of infection resulting from continued PRV replication and transport among central regions of interest [e.g., retrograde

transport from the PVN to BNST, from BNST to IL, and/or between CeA and BNST (Dong et al., 2001a, Dong et al., 2001b, Vertes, 2004)]. At this post-inoculation interval, the PVN is the only analyzed forebrain region that would be expected to contain pre-sympathetic neurons projecting to the spinal cord, whereas the other forebrain regions are presumably labeled via their projections to the parasympathetic DVC (Loewy, 1990, Cano et al., 2001). The relative densities of PRV labeling within analyzed regions and the relatively sparse amount of PRV labeling in the lateral CeA (see Fig. 8), which is known to increase at longer post-inoculation intervals (Rinaman et al., 1999, Yang et al., 1999, Rinaman et al., 2000, Card et al., 2005), indicate that the observed forebrain PRV labeling was primarily due to direct projections of preautonomic neurons to the DVC.

The distribution and relative density of hindbrain and forebrain gastric preautonomic PRV immunolabeling in juvenile male rats in the present study was consistent with previous reports of the temporal progression of retrograde PRV transport from the stomach wall to the brain in neonatal and adult rats (Rinaman et al., 1999, Yang et al., 1999, Rinaman et al., 2000, Card et al., 2005). In all cases, PRV infection included first-order preganglionic parasympathetic motor neurons in the dorsal motor nucleus of the vagus (DMV), second-order neurons in the nucleus of the solitary tract (NST) that are known to synapse onto gastric vagal motor neurons, and third-order neurons in the AP that synapse onto preautonomic NST neurons (see Fig. 3). Of the six brain regions examined quantitatively, the densest labeling was present within the hindbrain AP, consistent with its close proximity to the initially infected DMV and NST. Of the five quantified forebrain regions, the PVN displayed the most robust PRV labeling, consistent with previous findings that PVN neurons are among the first forebrain neurons to become transynaptically infected with PRV after peripheral inoculation of autonomic targets (Rinaman et

al., 1999, Rinaman et al., 2000, Cano et al., 2001). PRV labeling in the CeA and BNST was less extensive than that in the PVN, and PRV labeling in the visceral cortices was relatively sparse, consistent with the more delayed progression of PRV to these regions (Rinaman et al., 1999, Rinaman et al., 2000). In every brain region except the midline AP, PRV immunolabeling exhibited a left-side predominance, consistent with initial infection of parasympathetic motor neurons within the left DMV that innervate enteric target neurons within the inoculated ventral stomach wall. This predominantly left-sided pattern of viral labeling indicates a successful PRV injection in the ventral stomach wall as opposed to the extensive, bilateral pattern seen after intraperitoneal injection of PRV (Card et al., 1990, Rinaman et al., 1999).

In addition to PRV immunolabeling within the brain regions targeted for analysis in the present study (i.e., AP, BNST, CeA, PVN, IN, and PL/IL), retrograde transneuronal PRV immunolabeling was present within the ventrolateral medulla, caudal midline raphe nuclei (obscurus, pallidus), locus coeruleus, subcoeruleus, Barrington's nucleus, parabrachial nucleus, periaqueductal gray, ventral tuberomammillary nucleus, lateral hypothalamus, dorsomedial hypothalamus, periforniceal hypothalamus, zona incerta, and medial preoptic nucleus.

Quantitative analyses of potential group differences in PRV immunolabeling

For each brain region in which PRV immunolabeling was quantified using ROI analysis (i.e., AP, BNST, CeA, and PVN), one-way ANOVA revealed that the number of ROIs included in the analysis did not differ by postnatal group (Table 1). There also was no significant effect of postnatal group on the ROI area assayed for any brain region (Table 1). Using the criterion defined in the methods, no outliers were found in any brain region. There also were no significant litter effects within either postnatal group on PRV labeling area (average per ROI) or

the number of PRV labeled neurons (average per hemisection) within any analyzed hypothalamic or forebrain region.

Table 1. Number of ROIs and ROI area in analyzed brain regions in NS control and MS15 rats

Brain region	Postnatal group	Number of ROIs (mean±SE)	ROI area ($\times 10^5 \text{ um}^2$, avg. per ROI, mean±SE $\times 10^4$)
AP	NS	2.67±0.13	1.39±0.29
	MS15	2.75±0.14	1.39±0.60
dBNST	NS	5.87±0.36	2.21±2.26
	MS15	5.47±0.47	2.08±2.44
vBNST	NS	6.07±0.32	1.73±3.24
	MS15	5.67±0.48	1.56±2.47
CeA	NS	12.07±0.82	3.89±3.73
	MS15	11.88±0.66	3.80±2.81
PVN	NS	8.87±0.51	2.03±0.85
	MS15	8.19±0.38	2.19±0.71

Area Postrema. PRV labeling within the AP was robust and distributed throughout its Nissl-defined anatomical boundaries. One-way ANOVA revealed no significant effect of postnatal group on PRV labeling area ($F_{(1,30)} = 1.55$; $p = 0.22$) (Figs. 4 and 5). These data indicate that postnatal treatment did not affect retrograde transynaptic PRV infection from the stomach wall to the AP.

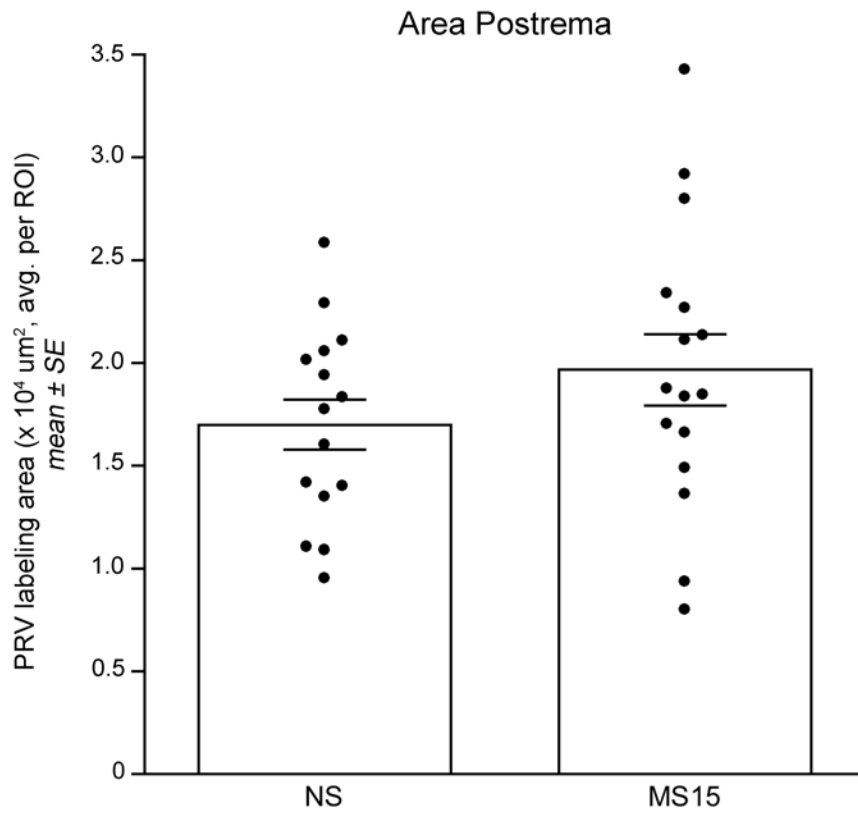


Figure 5. Bar graph illustrating PRV labeling area (mean \pm SE) within the area postrema (AP) in NS and MS15 rats. A dot plot depicting data from individual cases is overlaid on the bar plot. There is no significant difference between the two postnatal groups in PRV labeling area (See Results).

Bed Nucleus of the Stria Terminalis. PRV labeling was present within the dorsal BNST, including the lateral dorsal, lateral juxtacapsular, and lateral posterior areas, and also within the ventral BNST, including the lateral ventral and medial ventral areas (Paxinos et al., 1999). One-way ANOVA revealed no significant effect of postnatal group on PRV labeling area in either dBNST (NS: 2859.72 ± 484.61 , MS15: 3053.43 ± 464.57 ; $F_{(1,29)} = 0.08$; $p = 0.78$) or vBNST (NS: 767.08 ± 178.94 , MS15: 650.51 ± 102.42 ; $F_{(1,29)} = 0.32$; $p = 0.58$) (Figs. 6 and 7). These data indicate that postnatal treatment did not affect retrograde transynaptic PRV infection of pre-gastric circuits within the BNST.

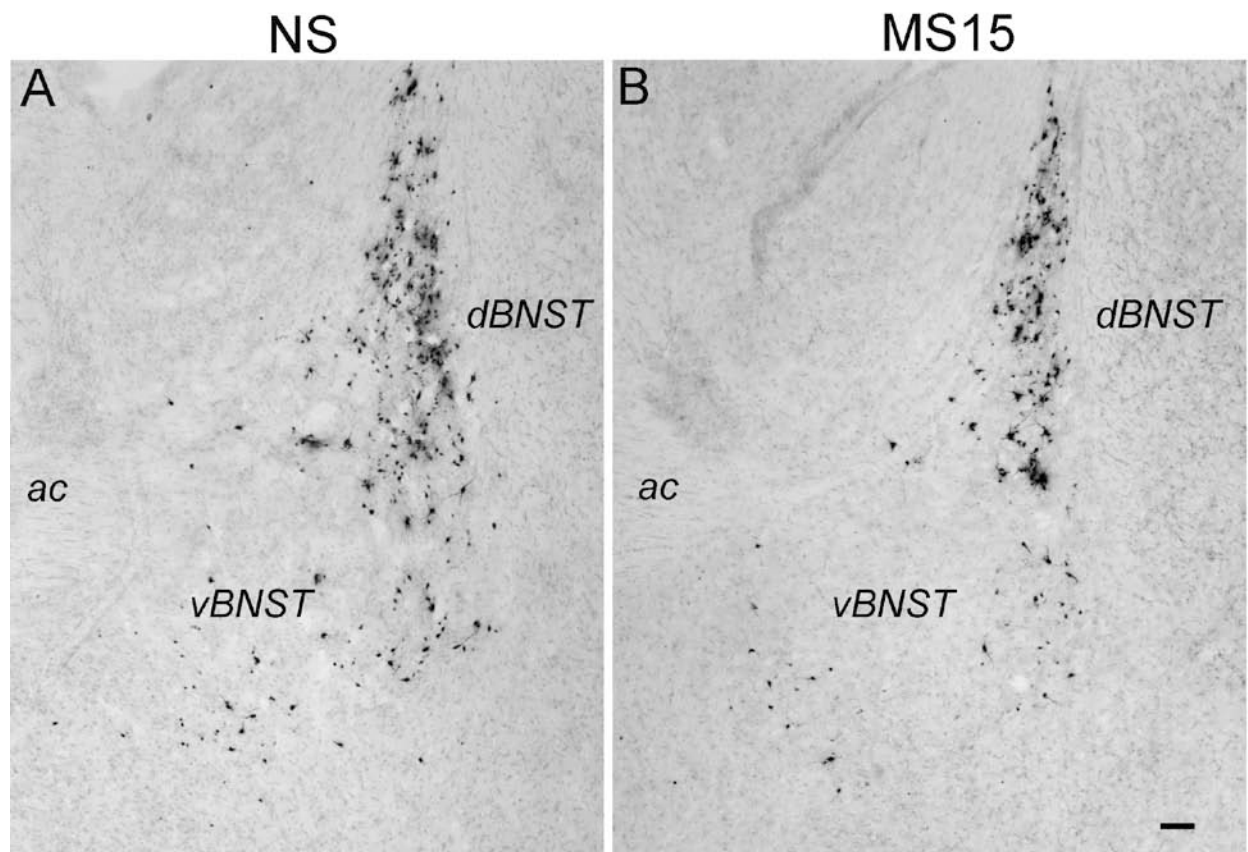


Figure 6. Photomicrographs of PRV labeling within the bed nucleus of the stria terminalis (BNST) in a representative NS rat (A) and a MS15 rat (B). PRV labeling is present within the dBNST, dorsal to the anterior commissure (ac) and within the vBNST, ventral to the ac. There is no significant difference between the two postnatal groups in PRV labeling area (See Results). Scale bar (in B) = 200 μ m, applies to both.

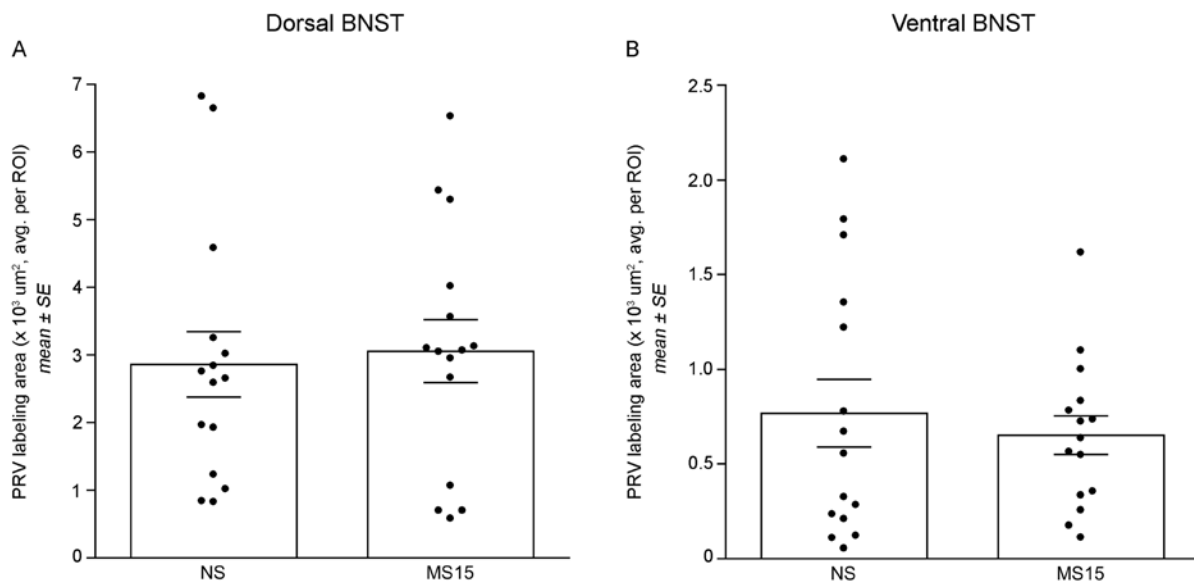


Figure 7. Bar graphs illustrating PRV labeling area (mean \pm SE) within the dorsal (A) and ventral (B) bed nucleus of the stria terminalis (BNST) in NS and MS15 rats. There are no significant postnatal group differences in PRV labeling area within the BNST (see Results).

Central Nucleus of the Amygdala. PRV labeling within the CeA was primarily localized to its medial subnucleus (see Figs. 8 and 9). There was no effect of postnatal group on PRV labeling area within the CeA (NS: 6278.13 ± 860.90 , MS15: 7819.77 ± 943.30 ; $F_{(1,30)} = 1.45$; $p = 0.24$). These data indicate that postnatal treatment did not affect retrograde transynaptic PRV infection of pre-gastric circuits within the CeA.

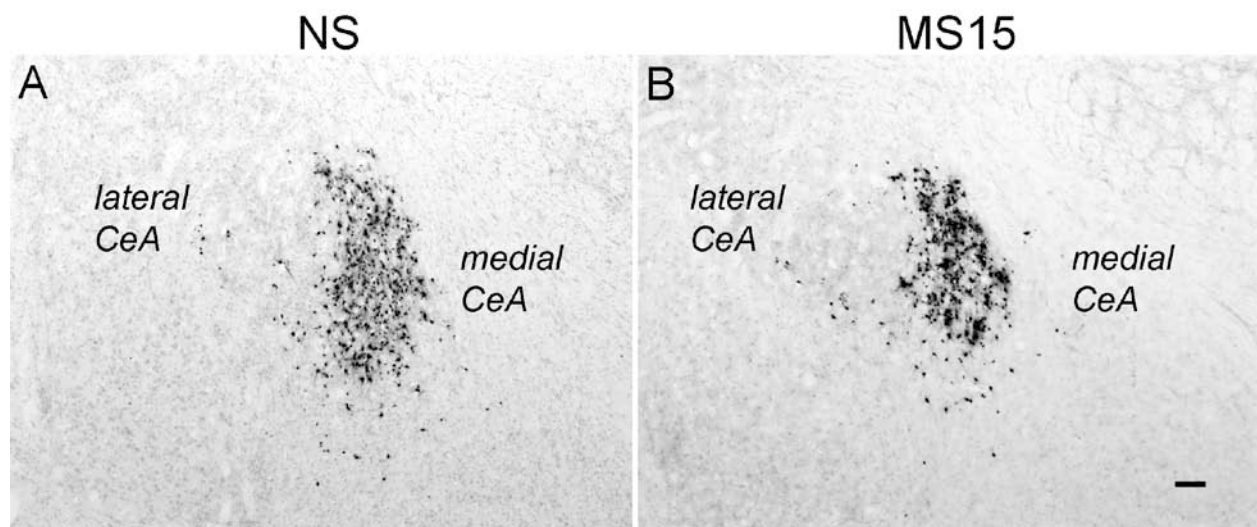


Figure 8. Photomicrographs of PRV labeling within the central nucleus of the amygdala (CeA) from representative NS (A) and MS15 rats (B). There is no significant difference between the two postnatal groups in PRV labeling area (See Results). Scale bar (in B) = 200 μ m, applies to both.

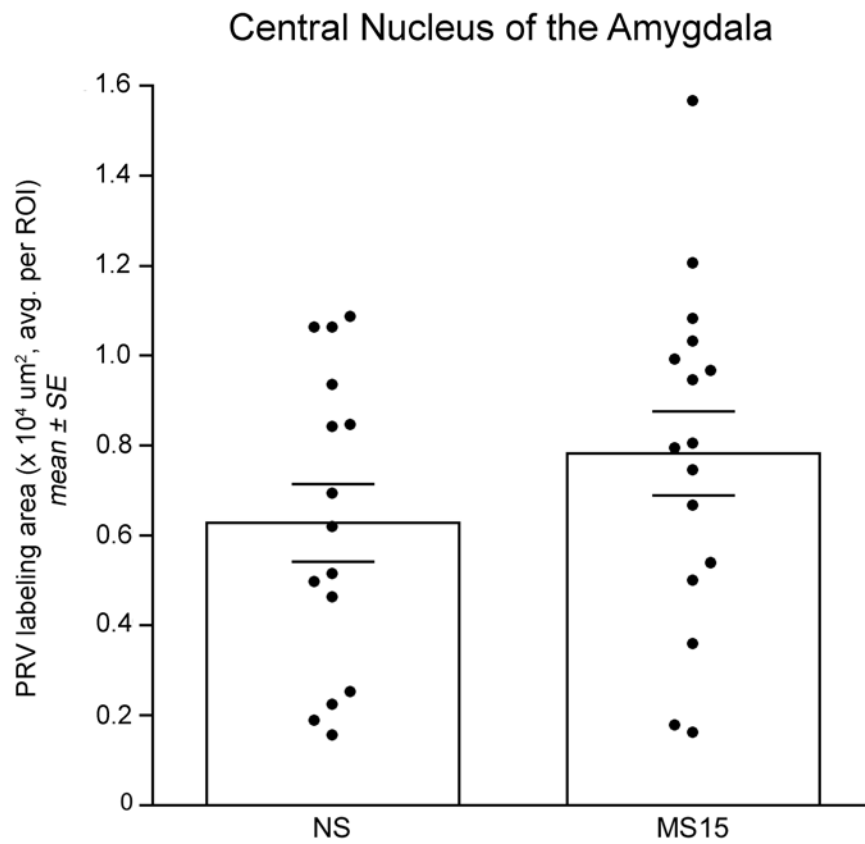


Figure 9. Bar graph illustrating PRV labeling area (mean \pm SE) within the central nucleus of the amygdala (CeA) in each NS and MS15 rats. A dot plot depicting data from individual cases is overlaid on the bar plot. There is no significant difference between the two postnatal groups in PRV labeling area (See Results).

Paraventricular Nucleus of the Hypothalamus. One-way ANOVA revealed a significant effect of postnatal group on PRV labeling area within the PVN ($F_{(1,30)} = 10.24$; $p = 0.00$), in which MS15 rats displayed greater PRV labeling area compared to NS rats (Figs. 10 and 11A). These data suggest that MS15 exerted a unique effect within the PVN to increase retrograde transynaptic PRV infection of pre-gastric circuits.

PRV labeling area data were subsequently binned into four rostrocaudal levels through the PVN in an effort to localize the effect described above. Repeated measures ANOVA revealed a significant interaction between rostrocaudal level and postnatal group on PRV labeling area within the PVN ($F_{(1,21)} = 5.34$; $p = 0.00$). Independent-samples t-tests at each rostrocaudal level revealed a significant between-group difference at only the mid-caudal level ($p = 0.01$, Fig. 11B), in which MS15 rats displayed greater PRV labeling area compared to NS rats. Interestingly, the group-related difference in PRV labeling area was observed at the rostrocaudal level containing especially dense PRV immunolabeling (Fig. 11B), which included labeling within both the dorsal cap (PaDC) and ventral subnuclei (PaV) (Fig. 10). In an effort to further localize the postnatal group effect to specific preautonomic PVN subnuclei, the PaDC and PaV were quantified separately at this mid-caudal level.

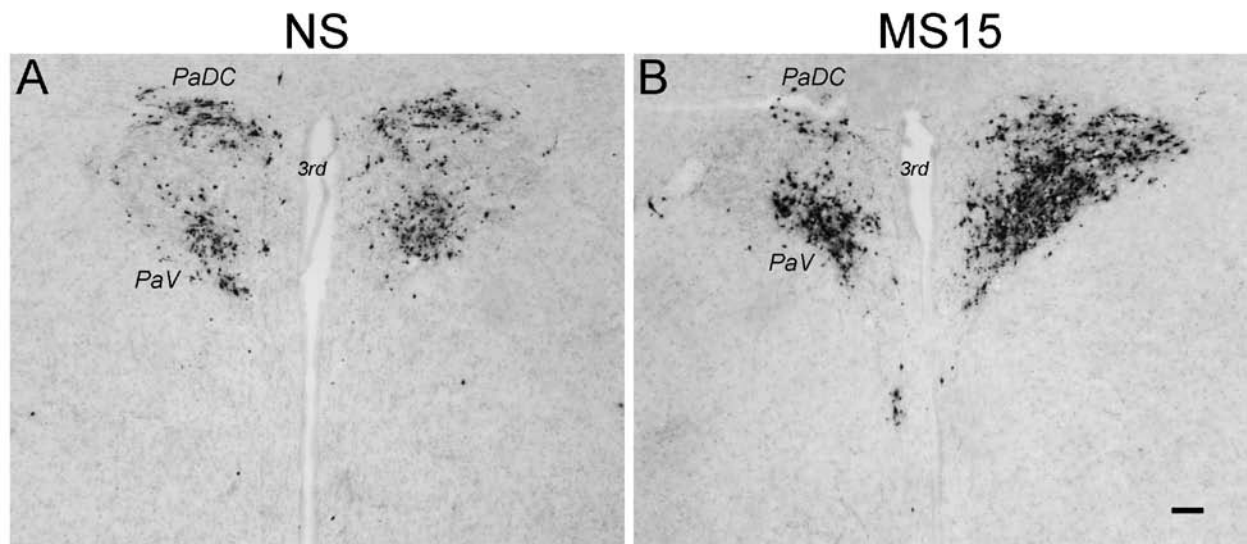


Figure 10. Photomicrographs of PRV labeling within the mid-caudal paraventricular nucleus of the hypothalamus (PVN) in representative NS (A) and MS15 rats (B). PRV labeling is evident within both the dorsal cap (PaDC) and ventral (PaV) subnuclei. There is a significant difference in PRV labeling area between the two postnatal groups (See Results and Fig. 9). Scale bar (in B) = 200 μ m, applies to both panels.

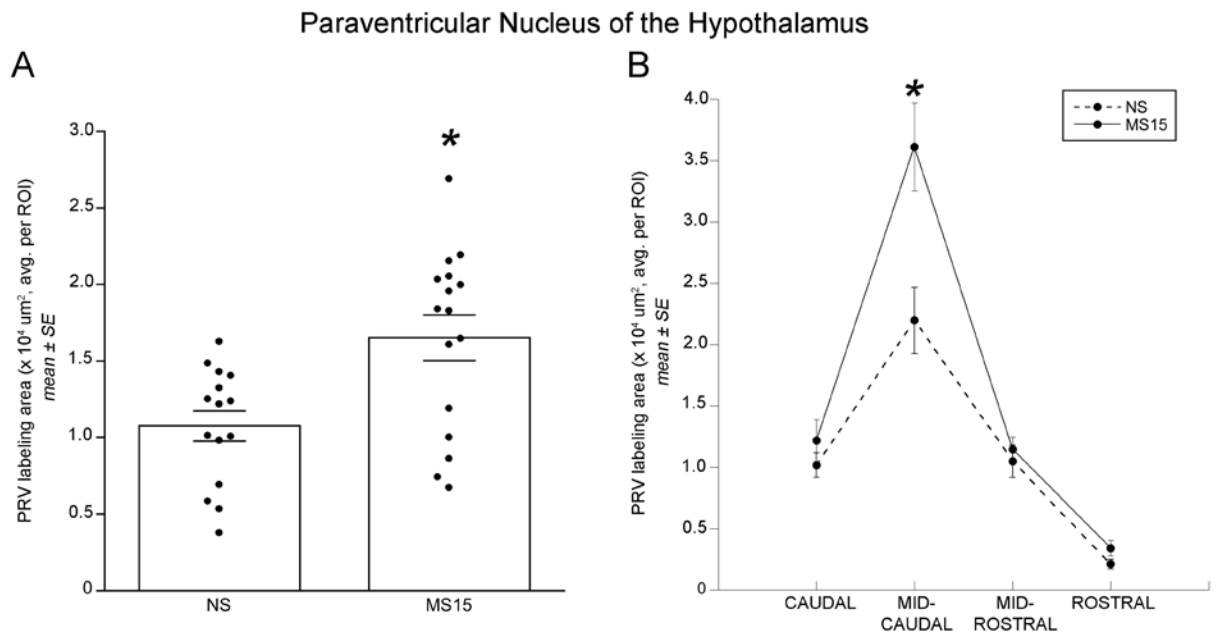


Figure 11. PRV labeling within the PVN. A. Bar graph illustrating PRV labeling area (mean \pm SE) within the paraventricular nucleus of the hypothalamus (PVN) in NS and MS15 postnatal groups. Individual data from rats within each group are overlaid in the dot plots. MS15 rats displayed significantly greater PRV labeling area compared to NS controls. (*, $p < 0.05$) B. Line plot illustrating a breakdown of PRV labeling area across four rostrocaudal levels of the PVN, depicted on the x-axis from caudal (left) to rostral (right). At the “mid-caudal” level, MS15 rats displayed significantly greater PRV labeling area compared to NS controls. (*, $p < 0.05$)

PaDC: One-way ANOVA revealed a significant effect of postnatal group on PRV labeling area within the PaDC (NS: 3339.36 ± 441.45 , MS15: 5481.05 ± 774.03 ; $F_{(1,26)} = 5.54$; $p = 0.03$), in which MS15 rats display greater PRV labeling area compared to NS rats.

PaV: One-way ANOVA revealed a significant effect of postnatal group (NS: 20018.17 ± 2958.29 , MS15: 31378.80 ± 3232.53 ; $F_{(1,26)} = 6.66$; $p = 0.02$) on PRV labeling area within the PaV. Thus, PRV labeling area within both the PaDC and PaV subnuclei of the PVN at the mid-caudal level reflected the same overall result as the PVN data collapsed across all rostrocaudal levels (Fig. 9A), with MS15 rats displaying more retrograde transynaptic PRV labeling compared NS rats.

Insular cortex. One-way ANOVA revealed no significant effect of postnatal group on the number of hemisections in which PRV-positive neurons appeared (NS: 14.20 ± 1.75 , MS15: 17.50 ± 1.69 ; $F_{(1,30)} = 1.84$; $p = 0.19$) or the number of PRV-positive neurons (average per hemisection) in the IN (NS: 3.99 ± 0.37 , MS15: 3.91 ± 0.32 ; $F_{(1,30)} = 0.03$; $p = 0.87$).

Prelimbic/Infralimbic cortex. One-way ANOVA revealed no significant effect of postnatal group on the number of hemisections in which PRV-positive neurons appeared (NS: 5.25 ± 0.82 , MS15: 7.00 ± 0.85 ; $F_{(1,30)} = 2.18$; $p = 0.15$) or on the number of PRV-positive neurons (average per hemisection) in the PL/IL (NS: 1.90 ± 0.29 , MS15: 1.88 ± 0.26 ; $F_{(1,30)} = 0.00$; $p = 0.97$).

These visceral cortex cell count data indicate that postnatal group did not significantly affect retrograde transynaptic gastric PRV infection within either the IN or the PL/IL.

Effect of postnatal group on PRV labeling area: repeated measures analysis by brain region

When each brain region was analyzed separately, as described above, PRV labeling area was the only dependent variable that showed a significant effect of postnatal group, and only within the PVN. To further examine the relative size of this localized effect when all brain regions are considered together, we performed a repeated measures ANOVA with brain region (AP, BNST, CeA, and PVN) as the repeated measure and PRV labeling area as the sole dependent variable. This analysis confirmed a significant main effect of postnatal group on PRV labeling area ($F_{(1,28)} = 4.53$; $p = 0.04$). There was a significant within-subjects effect of brain region on PRV labeling area ($F_{(4,28)} = 146.58$; $p = 0.00$), as expected, and a significant interaction between postnatal group and brain region ($F_{(4,28)} = 4.22$; $p = 0.00$). Post-hoc tests (corrected for multiple comparisons) confirmed that the effect of postnatal group on PRV labeling area was significant only within the PVN ($F_{(1,28)} = 10.98$; $p = 0.00$). Thus, the effect of MS15 to increase PRV labeling area within the PVN was large enough to emerge as a significant main effect of postnatal group within this repeated measures analysis.

2.5 DISCUSSION

In humans, the quality of early life experience influences later neuroendocrine and autonomic functions, including responses to stress (Heim et al., 2000, Feldman and Eidelman, 2003, McCain et al., 2005, Heim et al., 2008). For example, providing active and passive tactile stimulation to premature human infants improves behavioral development, visceral function, and

sympatho-adrenal maturation (Kuhn and Schanberg, 1998, Feldman et al., 2002, Feldman and Eidelman, 2003, Diego et al., 2005, Dodd, 2005, McCain et al., 2005). Experimental evidence from rat studies also supports the view that early life experience influences visceral motor responses to stressful stimuli (Coutinho et al., 2002, Sanders and Anticevic, 2007). It is well known that gastric secretory and motor activity is markedly altered by acute and chronic stress in humans and experimental animals (Selye, 1936, Caso et al., 2008). Because MS15 during postnatal development alters the assembly of gastric preautonomic circuits (Card et al., 2005) and later stress reactivity (Plotsky and Meaney, 1993, Levine, 2005, Plotsky et al., 2005), we hypothesized that differences in gastric autonomic circuits would be revealed by transneuronal PRV tracing in older rats. The most striking result from the present study is that MS15 enhanced gastric preautonomic PVN circuitry in post-weaning, juvenile rats.

There was no general effect of postnatal group on initial retrograde viral transport from the stomach wall to the DVC, as evidenced by the lack of a between-group difference in PRV immunolabeling within the AP in either neonatal (Card et al., 2005) or juvenile rats (present study). These findings are consistent with other results indicating that caudal medullary autonomic circuits involved in gastric vagal control are already well-established in newborn rats (Rinaman et al., 1999, Rinaman et al., 2000) and are, therefore, perhaps less susceptible to potential alteration by postnatal experience. Thus, the observed experimental differences in PVN transneuronal labeling within the PVN are not directly related to differences in caudal brainstem PRV labeling.

An intracellular threshold of infecting virions must be reached in order for PRV replication to occur, and there is a direct correlation between the number of axon terminals available for virion invasion and the number of virions transported to the nucleus of a neuron

(Card et al., 1999). Thus, having more axon terminals available for viral uptake results in faster rates of retrograde viral infection. Further, PRV immunolabeling within a brain region that becomes infected at a somewhat earlier time point will contain larger numbers of neurons at more advanced stages of viral infection, which will recruit relatively more resident glial cells to become PRV-positive (Rinaman et al., 1993, Rinaman et al., 1999). Although glial cells likely contributed to quantified PRV labeling in the present study, postnatal group differences in PRV labeling still reflect differences in the time-course and/or magnitude of retrograde transneuronal infection.

The enhanced hypothalamic retrograde transneuronal PRV labeling observed in MS15 rats could reflect increased numbers of PVN neurons in synaptic contact with infected postsynaptic target neurons within the DVC and/or spinal cord. In this regard, there is evidence that early life “handling” alters cell number in the parvocellular PVN (Winkelman-Duarte et al., 2007). However, changes in transneuronal PRV labeling also could reflect changes in the number of synaptic connections formed by individual PVN neurons with their infected postsynaptic targets (Card et al., 1999). In either case, our results suggest an altered ability of the PVN to modulate gastric function, including secretory and motor responses to stress.

Potential functional consequences of enhanced hypothalamic preautonomic circuits

The PVN is known to alter the activity of gastric-related DVC neurons, which in turn alters vagally-mediated gastric acid secretion and motility (Flanagan et al., 1992b, Zhang et al., 1999). DVC-projecting parvocellular PVN neurons are activated in response to stress (Buller, 2003), and include subpopulations that are immunoreactive for corticotropin-releasing factor or oxytocin (OT) (Sawchenko, 1982, 1987, Olson et al., 1992). Corticotropin-releasing factor and OT receptor signaling within the DVC and hypothalamus contribute importantly to vagally-

mediated gastric stress responses (Gunion and Tache, 1987, Lenz et al., 1988, McCann and Rogers, 1990, Flanagan et al., 1992a, Flanagan et al., 1992c, Tache et al., 2001). Interestingly, central administration of OT protects against gastric ulceration that occurs during cold-plus-restraint stress in rats (Grassi and Drago, 1993), evidence that stress-induced recruitment of OT projections from the PVN to the DVC may comprise a protective physiological defense mechanism (Esplugues et al., 1996). Thus, increases in the number or connectivity of gastric preautonomic PVN neurons in MS15 rats, potentially including OT neurons, could form the basis for differential effects of stress on gastric autonomic functions. In this regard, it is significant that OT-positive inputs to the NST and DMV (which originate exclusively in the PVN) increase by approximately 15-fold and 60-fold, respectively, during the first two postnatal weeks in rats (Rinaman, 1998a), the same developmental period during which MS15 exerts its lasting effects on stress responsiveness.

The observed increase in PRV labeling in MS15 rats in both the PaDC (primarily pre-spinal) and PaV (primarily pre-vagal) PVN subnuclei suggests that both sympathetic and parasympathetic functions could be altered by MS15 (Luiten et al., 1985). The observed lack of statistically significant experimental effects on PRV immunolabeling in the BNST, CeA, or visceral cortices was unexpected, as these brain regions are also implicated in the central control of gastric function (Hermann et al., 1990, Aleksandrov et al., 1996, Yamamoto and Sawa, 2000, Liubashina et al., 2002, Zhang et al., 2003), and PRV transneuronal transport indicates that the developmental assembly of neuronal projections from these regions to gastric autonomic neurons is delayed by MS15 in neonatal rats (Card et al., 2005). However, potential PRV labeling differences within these relatively late-infected forebrain regions might emerge with longer post-inoculation intervals that produce more extensive transneuronal labeling. Indeed, the most

robust experimental differences in the present study were evident within PVN subregions that displayed the densest viral labeling (i.e., the mid-caudal PVN), and the PVN itself displays retrograde transneuronal infection at earlier post-inoculation time points than the BNST, CeA, and visceral cortices (Rinaman et al., 1999, Rinaman et al., 2000).

Activity-dependent plasticity

Early life sensory experience is known to influence the anatomical structure of neural circuits that respond to various sensory modalities (Hubel et al., 1977, Merzenich et al., 1984, Fox, 2002, Nakahara et al., 2004). The MS15 manipulation used in our study is known to increase active maternal care [e.g., licking, grooming, and arched-back nursing (Meaney, 2001)], and hence to increase the somatic and visceral sensory stimulation received by neonatal rats. Thus, MS15 may produce activity-dependent changes in central ascending viscerosensory projections from the caudal brainstem to the hypothalamus and limbic forebrain (Riche and DePommery 1990). These projections are primarily noradrenergic and undergo significant structural development in rats during the first two postnatal weeks (Rinaman, 2001), suggesting that they could be susceptible to the influence of early life experience. In this regard, adult rats with a developmental history of MS15 display decreased stress-induced norepinephrine levels in the PVN (Liu et al., 2000). MS15 rats also display attenuated stress-induced neural activation in the PVN, CeA, and BNST (Abraham and Kovacs, 2000), which may be related to altered noradrenergic signaling in these regions. Central visceral circuits are highly reciprocal, such that preautonomic hypothalamic and limbic forebrain regions are subject to direct and relayed viscerosensory modulation. Indeed, noradrenergic terminals have been shown to synapse directly onto identified gastric preautonomic neurons within the PVN of adult rats (Balcita-Pedicino and Rinaman, 2007). These findings lead us to hypothesize that MS15-induced

alterations in preautonomic circuitry demonstrated in this study and in our previous report (Card et al., 2005) are partially due to early experience-induced alterations in the activity of ascending viscerosensory inputs to hypothalamic and limbic forebrain regions that provide descending control over autonomic outflow. Thus, early life experience may result in differential interactions between ascending viscerosensory and descending autonomic projections that shape the functional organization of these pathways.

Interpretations and future directions

Results from our previous work suggested that MS15 promotes a temporal delay in gastric preautonomic neural circuit assembly during early postnatal development (Card et al., 2005). In light of the present results, the early temporal delay appears to lead to a later enhancement of PVN gastric preautonomic circuits in juvenile MS15 rats. Additional studies are necessary to determine the basis of altered PRV labeling (e.g., alterations in PVN neuron number and/or density of PVN neural inputs to infected target neurons), to investigate potential interactions of postnatal treatment and sex on PRV transneuronal labeling, to examine the physiological consequences of altered central autonomic circuits, and to compare MS15 with other natural variations in maternal care or experimental manipulations that alter early maternal care. Current studies are examining the how MS15 alters the stress-induced recruitment of DVC-projecting PVN neurons, including the OTergic population, in juvenile rats.

In conclusion, our results demonstrate that MS15 enhances the development of hypothalamic gastric preautonomic circuitry. Our previous study in neonates (Card et al., 2005) and the present study in juvenile rats are the first to demonstrate that early life experience can alter the anatomical organization of central neural circuits that govern visceral motor function,

including visceral responses to stress. Early experience-induced alterations within these circuits may contribute to individual differences in stress reactivity.

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3.0 REPEATED BRIEF POSTNATAL MATERNAL SEPARATION ATTENUATES STRESS-INDUCED ACTIVATION OF NORADRENERGIC NEURONS WITHIN THE NUCLEUS OF THE SOLITARY TRACT

3.1 ABSTRACT

Repeated brief postnatal maternal separation is a paradigm in which rats pups undergo a 15-minute daily separation (MS15) from their dam for the first one to two postnatal weeks. This manipulation enhances active maternal care, which is associated with decreased hypothalamic-pituitary adrenal axis responses to stress. The study described in the previous chapter demonstrated that MS15 enhances the circuit strength of gastric preautonomic circuits originating within the paraventricular nucleus of the hypothalamus (PVN) in juvenile rats. This enhanced circuit strength suggested that the function of the preautonomic PVN pathway might also be altered by MS15. Thus, we hypothesized that MS15 rats would display altered stress-induced activation of the PVN to dorsal vagal complex (DVC) pathway. To this end, rats were given DVC injections of a standard retrograde tracer (Fluorogold) on postnatal day 30. After one week, these rats and their surgically intact littermates were exposed to a no restraint stress (NRS) control condition or a 15-minute restraint stress (RS) and perfused after one hour. Tissue from DVC-injected rats was processed for Fluorogold and Fos, a neural marker of activation, to identify DVC-projecting PVN neurons activated in response to restraint stress. Additionally,

tissue from surgically intact littermates was processed for Fos (single labeled) and dopamine-beta-hydroxylase [DbH, the noradrenergic (NA) synthetic enzyme] and Fos (double labeled). Compared to non-separated (NS) controls, MS15 rats display decreased restraint stress-induced Fos activation within the PVN, which was not accompanied by a decrease in stress-induced Fos activation within DVC-projecting PVN neurons. Further, MS15 rats display decreased stress-induced Fos activation of a specific population of NA neurons within the NST, without a decrease in overall Fos activation within the NST. These findings have interesting implications for the anatomical organization of the PVN to DVC pathway and for the physiological consequences of MS15. This study will potentially provide insights into how early life experience-induced alterations in stress-related circuits contribute to differences in stress reactivity.

3.2 INTRODUCTION

The paraventricular nucleus of the hypothalamus (PVN) can be said to exert more proximal control over the stress response than any other forebrain region, as it is uniquely capable of direct control over both neuroendocrine and autonomic outflow (Herman et al., 2002). The PVN houses the corticotropin-releasing hormone (CRH) -containing neurons that comprise the apex of the hypothalamic-pituitary-adrenal (HPA) axis, which ultimately controls basal and stress-induced glucocorticoid secretion (Herman et al., 2002). Further, anatomical studies injecting viral transynaptic tracers into peripheral targets have clearly demonstrated that PVN neurons synaptically innervate both sympathetic preganglionic neurons in the spinal cord and

parasympathetic preganglionic neurons in the dorsal vagal complex (DVC) (Rinaman et al., 1999, Yang et al., 1999, Rinaman et al., 2000). The DVC is composed of the area postrema (AP), the nucleus of the solitary tract (NST), and the dorsal motor nucleus of the vagus (DMV), which houses the parasympathetic vagal motor neurons that innervate multiple thoracic and abdominal viscera, including the entire gut from the oral cavity to the colon (Kalia and Sullivan, 1982).

The PVN modulates the activity of DVC neurons (Flanagan et al., 1992b), and in turn, alters autonomic function. For example, the PVN alters vagally-mediated gastric acid secretion and motility (Zhang et al., 1999). The PVN stimulates DVC neurons that activate inhibitory vagal efferents that inhibit gastric motility (McCann and Rogers, 1991). Importantly, DVC-projecting PVN neurons are activated in response to stress and contribute to stress-induced DVC activation, including the activation of noradrenergic (NA) neurons within the NST (Buller et al., 2003, Dayas et al., 2004).

In rats, synaptic inputs from the PVN to the DVC form during the first postnatal week (Rinaman et al., 2000), defining a potentially sensitive period in which this pathway may be subject to the influence of early life experience. It is well known that early life experience is an important factor in shaping later stress reactivity. Natural or experimentally-induced variations in maternal care received by rat pups in early life are associated with differential stress reactivity assessed later in life (Liu et al., 1997, Meaney, 2001, Levine, 2005). Repeated brief maternal separation, a paradigm in which rat pups undergo a 15-minute daily separation from their dam (MS15) for the first one to two postnatal weeks, enhances active maternal care (e.g., licking, grooming, and arched-back nursing) received by pups (Liu et al., 1997, Macri et al., 2008), which is associated with decreased HPA and behavioral stress reactivity exhibited by the

offspring later in life (Plotsky and Meaney, 1993, Caldji et al., 2000). However, potential MS15-induced alterations of central circuits that control stressor-evoked autonomic responses have been largely unstudied.

Our laboratory had previously demonstrated that MS15 alters the developmental assembly of gastric preautonomic circuits in neonates (Card et al., 2005). In a more recent report, we tested the hypothesis that the influence of MS15 on these circuits would be manifested as altered circuit strength later in development, in juvenile rats. We used the retrograde transynaptic tracer pseudorabies virus (PRV) to determine whether central gastric autonomic circuits differed in juvenile rats with a developmental history of MS15 compared to non-separated (NS) controls (Banihashemi and Rinaman, 2010). MS15 rats displayed a significant increase in the amount of transynaptic PRV labeling within the PVN, indicating enhanced circuit strength of PVN gastric autonomic circuits (Banihashemi and Rinaman, 2010).

The present study examines whether the demonstrated enhancement of PVN gastric preautonomic circuitry in MS15 rats is accompanied by differences in the stress-induced Fos activation of the PVN to DVC pathway. The Fos activation technique cannot be accurately assessed using viral tracing. Thus, DVC-projecting PVN neurons were identified using standard retrograde tract tracing techniques. In order to examine stress-induced activation of the PVN, DVC, and its NA NST population, we examined Fos activation in surgically-intact rats. We used restraint stress in this study as it activates significant proportions of DVC-projecting PVN neurons and NST catecholamine neurons (Dayas et al., 2004, Crane et al., 2005). We hypothesized that juvenile rats with a developmental history of MS15 would display less restraint stress-induced activation of within both the PVN and the DVC, including the NA NST neurons, compared to NS controls, consistent with the MS15 phenotype of low stress reactivity.

Early experience-induced differences in the stress-induced recruitment of the descending preautonomic pathway from PVN to DVC may contribute to individual differences in stress reactivity.

3.3 EXPERIMENTAL PROCEDURES

Animals

All experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Animals were held in a controlled environment (20-22°C) with a 12-hour light dark cycle (lights on at 0700 hr) and *ad libitum* access to water and pelleted rat chow (Purina #5001, Bethlehem, PA).

The progeny of 12 pregnant multiparous Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in this study. Pregnant rats arrived at the animal facility at a gestational stage between embryonic days 13 and 18, and were subsequently housed singly in opaque polyethylene cages with soft woodchip bedding and a wire lid. Pregnant rats were checked daily to determine their pups' date of birth, designated postnatal day (P)0. On P1, anogenital distance of each pup was measured in order to determine sex (Jackson, 1912). Litters were then culled to eight pups while trying to shift the litter composition toward a majority of male rat pups. Each litter contained at least two females; however, only male rats were used for the experiments in the present report. All pups within each litter underwent the same postnatal treatment. Litters were designated as either NS controls (6 litters) or MS15 (6 litters).

Experimental design

On P1, NS litters were returned to their dam promptly after the litter was sexed and culled whereas MS15 litters were placed in the incubator for the remainder of 15 minutes. This served as the MS15 litters' first maternal separation day. As in our previous studies (Card et al., 2005, Banihashemi and Rinaman, 2010), pups in MS15 litters were separated from their dam for 15 minutes daily from P1 - P10, inclusive. The separation took place at approximately the same time each day (~1445 -1500 hr). The dam was briefly removed from the home cage and placed into a dedicated polyethylene tub. Using gloved hands, all of the pups were removed from their home cage along with a handful of home cage bedding and transferred together into a smaller polyethylene tub (26.7 x 15.2 x 12.7 cm). The dam was then returned to her home cage and the small tub containing the pups was promptly placed into an incubator (~36°C, ~30-50% humidity) in an adjacent room. Rat pups remained in the incubator for 15 minutes and then, using gloved hands, the entire litter was returned simultaneously to their dam in the home cage. Aside from being sexed and culled on P1, NS control litters underwent no MS or experimental handling. However, both NS and MS15 pups and their respective dams underwent a weekly move to a clean cage with fresh bedding.

Rats were weaned from their dam on P21 and group-housed thereafter with same-sex littermates. On P30, a subset of male rats with a developmental history of NS or MS15 underwent DVC injection of the retrograde tracer, FG, as described below (see "DVC FG injections," NS $n = 18$, MS15 $n = 21$). The remaining male littermates were left surgically intact (NS $n = 11$, MS15 $n = 10$) to be used for later assessment of stress-induced Fos expression within the DVC. On P37, one week after surgery, FG-injected rats and their surgically-intact

littermates were either exposed to a 15-minute restraint stress (RS) or to a no restraint stress (NRS) control condition (see “Restraint stress exposure,” below).

DVC FG injections

Rats were anesthetized by isoflurane inhalation (~2% in oxygen) and mounted into a stereotaxic frame with the neck ventroflexed. After shaving and disinfecting the dorsal neck surface, a longitudinal incision was made through the skin overlying the occipital ridge. Dorsal neck muscles were gently blunt dissected and retracted to reveal the atlanto-occipital membrane overlying the roof of the fourth ventricle. With the aid of a surgical microscope, a 25-gauge needle was used to pierce and open the membrane and create a window revealing the AP on the caudal floor of the fourth ventricle. A 1- μ l Hamilton syringe equipped with a fine glass tip filled with FG (Fluorochrome, Inc. Englewood, CO; 2.0% in 0.15 M NaCl) was attached to the stereotaxic arm, positioned on the dorsal medullary surface on one lateral edge of the AP at its mid-rostrocaudal extent (corresponding to bregma level -13.80 mm), and then lowered 0.25 mm into the medial NST-DMV. Once lowered, the syringe was left in place for two minutes, and then a total volume of 50 nl FG was pressure-injected over 2.5 minutes. The syringe was left in place for two minutes after tracer injection and then withdrawn. The incision through the dorsal neck muscles was closed with silk sutures and the skin was closed with stainless steel clips. After surgery, rats were returned to their home-cage with their same-sex littermates.

Restraint stress exposure

In a terminal study on P37, FG-injected rats and their surgically-intact littermates were either exposed to 15-minute RS in a clear plastic cylindrical tube with a black nose cone (Kent Scientific Corporation, Torrington, CT), or served as NRS controls (DVC FG injections: NS-NRS $n = 7$, NS-RS $n = 11$, MS15-NRS $n = 7$, MS15-RS $n = 14$; surgically-intact: NS-NRS $n = 5$,

NS-RS $n = 6$, MS15-NRS $n = 5$, MS15-RS $n = 5$). Experiments were performed in six cohorts, with each cohort including rats derived from one NS and one MS15 litter. During each experiment, rats designated to receive RS were removed from their home cage individually, placed into a polyethylene cage with fresh bedding, taken to an adjacent room, and put into the restrainer. Rats were left undisturbed in the restrainer within the fresh cage for 15 minutes. After the RS procedure, the nose cone was removed from the restrainer and the rats crawled out. Rats remained in the cage in which the RS was performed, which was returned to a shelf in the adjacent animal facility and remained there until perfusion. Rats were anesthetized and perfused with fixative (see “Perfusion and histology,” below) an hour after the end of the 15-minute RS in order to capture stress-induced neural Fos expression, which typically peaks one hour after treatment (for review see Kovacs, 1998). Restraints were staggered such that perfusions took place every 20 minutes. Rats designated as NRS controls were perfused during each experiment either before the RS procedures or after completing the RS perfusions and this order was counterbalanced across cohorts. Restraints and perfusions were performed beginning in the morning and ending by noon (~0900 - 1200).

Perfusion and histology

Rats were anesthetized by isoflurane inhalation (5% in oxygen) followed by intraperitoneal injection of sodium pentobarbital (Nembutal, 100 mg/kg BW). Rats were then perfused transcardially with a brief saline rinse followed by 250 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer with L-lysine and sodium metaperiodate) (McLean and Nakane, 1974). Brains were removed from the skull, postfixed overnight at 4°C, and then cryoprotected for 24-72 hours in 20% sucrose solution at 4°C. Coronal 35 μm -thick tissue sections were cut from the spinomedullary junction through the rostral extent of the corpus callosum using a sliding,

freezing microtome. Sections were collected serially in six adjacent sets (sampling frequency of 210 μm within each set) and stored at -20°C in a cryopreservant solution (Watson et al., 1986).

Immunocytochemistry

One set of sections from each DVC FG injected rat and two sets from each surgically-intact rat were removed from storage and rinsed for one hour in buffer (0.1 M sodium phosphate, pH 7.4) prior to immunocytochemical procedures. Antisera were diluted in buffer containing 1% normal donkey serum and 0.3% Triton-X100. Tissue sections from both DVC FG injected and surgically-intact rats were processed for immunocytochemical localization of Fos protein by using a rabbit polyclonal antiserum (1:50,000; provided by Dr. Philip Larsen, Denmark) and Vectastain Elite ABC immunoperoxidase reagents (Vector Laboratories, Burlingame, CA). The specificity of this antibody for Fos has been reported (Rinaman et al., 1997). Sections were processed with a nickel sulfate-intensified diaminobenzidine (DAB) reaction to generate a blue-black reaction product in the nuclei of Fos-positive cells. In DVC FG injected rats, Fos-labeled tissue sections were subsequently processed for immunoperoxidase localization of FG (rabbit anti-FG: Chemicon; 1:30,000). In surgically intact rats, the brainstem sections from one set of Fos-labeled tissue were subsequently processed for immunoperoxidase localization of the noradrenergic synthetic enzyme, dopamine-beta-hydroxylase (DbH) using a monoclonal anti-DbH antibody (1:30,000; Millipore, Temecula, CA). DAB was used to generate a brown reaction product at the DVC injection site and in the cytoplasm of retrogradely labeled and DbH-positive neurons. After immunocytochemical processing, single and dual-peroxidase labeled tissue sections were mounted onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA), dehydrated in graded alcohols, cleared in xylene, and coverslipped using Cytoseal 60 (VWR, West Chester, PA).

Microscopic analysis and data collection

Quantification of PVN labeling in FG-injected rats. Tissue sections that were double-labeled for FG and Fos were analyzed with a light microscope to determine the number of retrogradely-labeled, FG-positive neurons and the proportion that were Fos-positive. Criteria for counting a neuron as FG-positive included the presence of brown cytoplasmic immunoreactivity and a visible nucleus. FG-positive neurons were considered Fos-positive if their nucleus contained blue-black immunolabel, regardless of intensity, and Fos-negative if their nucleus was unlabeled. Retrogradely labeled, FG-positive neurons were counted bilaterally in two sections through the PVN (bregma levels -2.12 through -1.80 mm) where their distribution was most dense (Paxinos and Watson, 1998). Labeling was quantified by mapping the distribution of FG-positive and double-labeled (i.e., FG- and Fos-positive) neurons using an image analysis system and StereoInvestigator software (Microbrightfield, Inc., Williston, VT). Tissue section boundaries and landmarks were outlined at low magnification (2.5X objective). At higher magnification (40X objective), each section was systematically examined and each FG-positive and double-labeled neuron was plotted individually. The Stereoinvestigator software tallied the number of single and double-labeled plotted neurons within each section.

Quantification of Fos activation within the PVN and NST in surgically-intact rats. The total amount of Fos labeling was quantified in tissue sections single-labeled for Fos using region of interest (ROI) analysis. In each experimental case, two PVN sections (bregma levels -1.88 through -1.80 mm) and three NST sections through the level of the AP (bregma levels -14.08 through -13.68 mm) were analyzed bilaterally (Paxinos and Watson, 1998). PVN and NST regions within each tissue section were photographed using an Olympus photomicroscope and a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Image analysis was

performed using SimplePCI imaging software (Hamamatsu Corporation, Sewickly, PA, USA). Our procedure for performing ROI analyses has been previously described (Banihashemi and Rinaman, 2010). Briefly, the ROI was manually outlined and then two thresholds were set, one for the detection of labeling intensity and another for the detection of size. The threshold for labeling intensity was set to quantify darkly-labeled Fos-positive neurons, thereby excluding profiles that were lightly labeled. The threshold for size was set to exclude objects too small to be considered a Fos-positive nucleus. The size threshold was also intended to avoid situations in which one Fos-positive nucleus could be quantified as multiple “objects.” Thus, labeling that was supra-threshold for intensity would be excluded if sub-threshold for size. The program then provided an “object count” equal to the number of Fos-positive neurons exceeding both detection thresholds. Thresholds were held constant for all analyses within each brain region and across all cases.

Quantification of DbH and Fos within the NST in surgically-intact rats. Tissue sections that were double-labeled for DbH and Fos were analyzed with a light microscope at 40X magnification to determine the number of double-labeled neurons within the NST. Criteria for counting double-labeled neurons were the same as described above for FG and Fos-positive neurons within the PVN (see above). NST sections were quantified through the same rostrocaudal levels of the AP as for the NST Fos analysis. We have confined our NST quantification to sections through the AP level because we have only observed a difference in restraint stress-induced Fos activation of DbH-positive NST neurons at this rostrocaudal level (unpublished observations).

Data analysis

Individual data points were considered outliers and removed from analysis if they lay beyond one and a half times the interquartile range. One-way ANOVA was used to examine potential effects of postnatal group on retrograde FG labeling within the PVN.

Both the PVN and NST contain distinct subdivisions and/or specific topography through multiple rostrocaudal levels (Paxinos, 2004, Simmons and Swanson, 2009). Further, our previous report demonstrated a postnatal group difference in PVN autonomic circuits that was localized to only one rostrocaudal level (Banihashemi and Rinaman, 2010). For these reasons, data for all other labeling (i.e., single-labeled Fos within the PVN and NST, double-labeled FG and Fos within the PVN, and double-labeled DbH and Fos within the NST) were averaged within each group according to PVN or NST rostrocaudal level. For each brain region, potential effects of postnatal group (NS control vs. MS15) and/or pre-perfusion stress condition (NRS control vs. RS) were examined at each rostrocaudal level using two-way ANOVA. When F values indicated significant main effects and/or interactions between experimental variables, ANOVAs were followed by independent samples t-tests. When initial analysis by rostrocaudal level revealed no significant trends, data from each brain region were collapsed across rostrocaudal levels and then examined using two-way ANOVA as above.

3.4 RESULTS

DVC FG injection sites

Of the 39 rats that underwent DVC FG injection, 11 were excluded as missed injections. The remaining 28 rats (NS-NRS: $n = 7$, NS-RS: $n = 7$, MS15-NRS: $n = 7$, MS15-RS: $n = 7$) were included in data analyses. Tracer injection sites targeted the mid-NST/DMV and were intentionally large in order to maximize the amount of retrograde labeling within the PVN (see Fig. 12A for a representative injection site). This did not contribute to non-specific PVN retrograde labeling, as previous reports have demonstrated that the PVN does not project to regions surrounding the DVC (Luiten et al., 1985). Labeling results from missed injections in our study confirmed this by demonstrating little to no retrograde labeling within the PVN when the injection site targeted nuclei dorsal (i.e., in the gracile or cuneate nuclei) or ventral (i.e., in the hypoglossal nucleus) to the mid-NST/DMV. Thus, these cases were excluded from analyses as “missed” injection sites.

Cases having injection sites targeting the mid-NST/DMV produced retrograde labeling in regions previously reported to project to the DVC (Gray and Magnuson, 1987, Danielsen et al., 1989, Rinaman, 2003b, Gabbott et al., 2007), including the PVN (see Fig. 13), bed nucleus of the stria terminalis (Fig. 12B), central nucleus of the amygdala (Fig. 12C), insular cortex (Fig. 12D), and medial prefrontal cortex (prelimbic and infralimbic cortices, Fig. 12E).

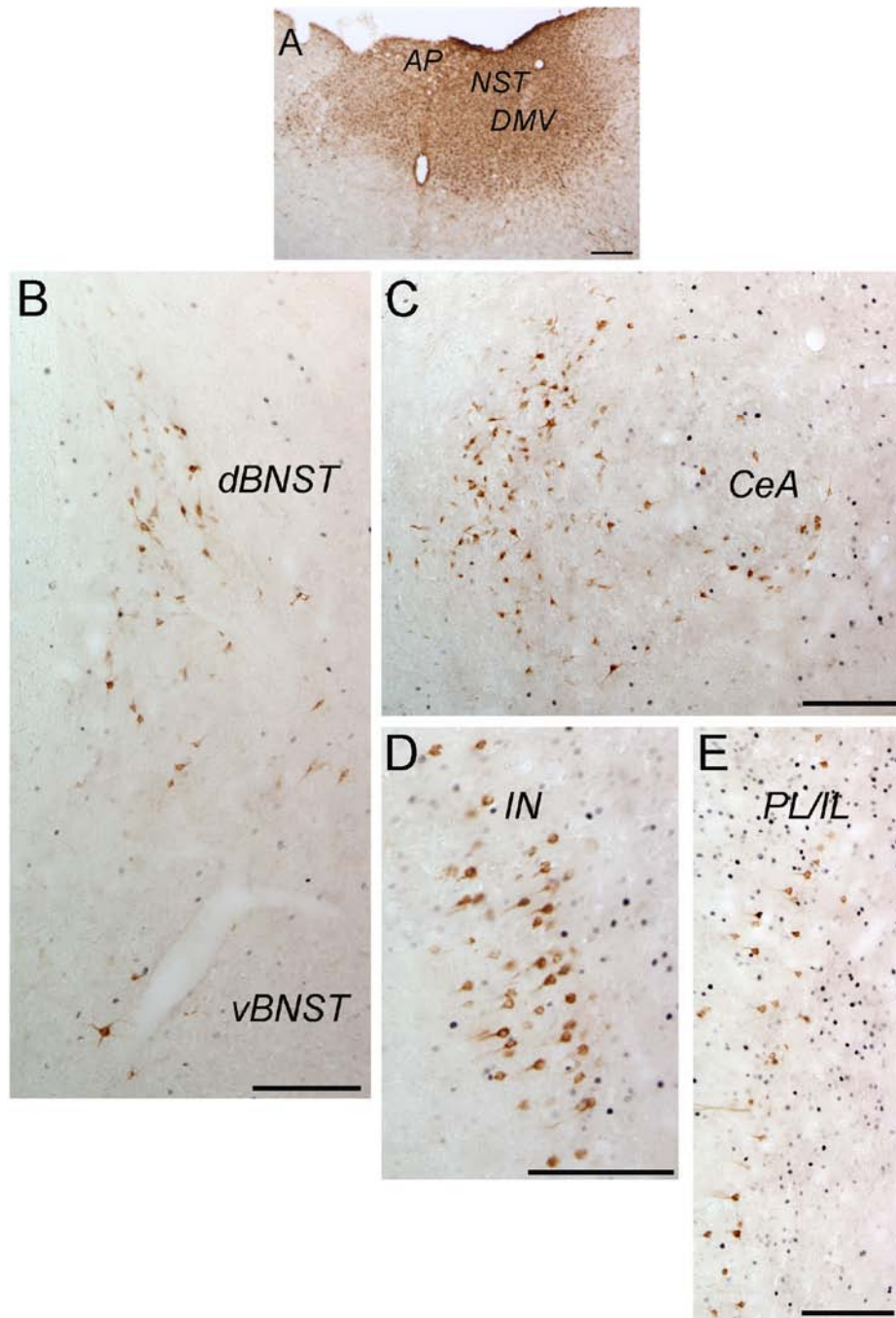


Figure 12. Photomicrographs of retrograde labeling from a representative DVC-injection site. Retrograde Fluorogold labeling within the (B) dorsal (dBNST) and ventral BNST (vBNST) (C) central nucleus of the amygdala (CeA) (D) insular cortex (IN) and (E) prelimbic/infralimbic cortex (PL/IL) from the same DVC injection site (A). Scale bars = 200 μ m.

Effect of postnatal group on PVN retrograde FG labeling

FG retrograde labeling was predominantly localized to two sections through the PVN (corresponding to bregma levels -2.12 through -1.80 mm) and was heavy in its ventral “pre-parasympathetic” subnuclei (Luiten et al., 1985). Overall, FG labeling within the PVN appeared remarkably similar between NS and MS15 groups (see Fig. 13, A vs. B or C vs. D). One-way ANOVA revealed no significant differences between postnatal groups (NS controls vs. MS15) on the total number of FG-positive neurons within the PVN ($F_{(1,25)} = 2.72$; $P = 0.11$; Figs. 13 and 14A). Thus, postnatal group did not influence the number of DVC-projecting PVN neurons.

Effect of postnatal group on stress-induced Fos activation of DVC-projecting PVN neurons

NRS cases within both postnatal groups displayed little Fos activation of DVC-projecting PVN neurons (Fig. 13A and B, and Fig. 14B). Two-way ANOVA revealed a significant main effect of stress condition (NRS vs. RS) to increase the number of FG and Fos-positive neurons within the PVN ($F_{(1,27)} = 28.50$; $P = 0.00$; Figs. 2 and 3B). However, there was no main effect of postnatal group ($F_{(1,27)} = 0.13$; $P = 0.73$) and no interaction between postnatal group and stress condition ($F_{(1,27)} = 0.16$; $P = 0.70$) on the number of FG and Fos-positive PVN neurons. Independent samples t-tests demonstrated that RS significantly increased the number of PVN FG and Fos-positive neurons compared to the NRS condition within both NS and MS15 groups (NS-NRS vs. NS-RS: $P = 0.00$; MS15-NRS vs. MS15-RS: $P = 0.01$). Thus, postnatal group did not influence the number of DVC-projecting PVN neurons that displayed stress-induced Fos activation.

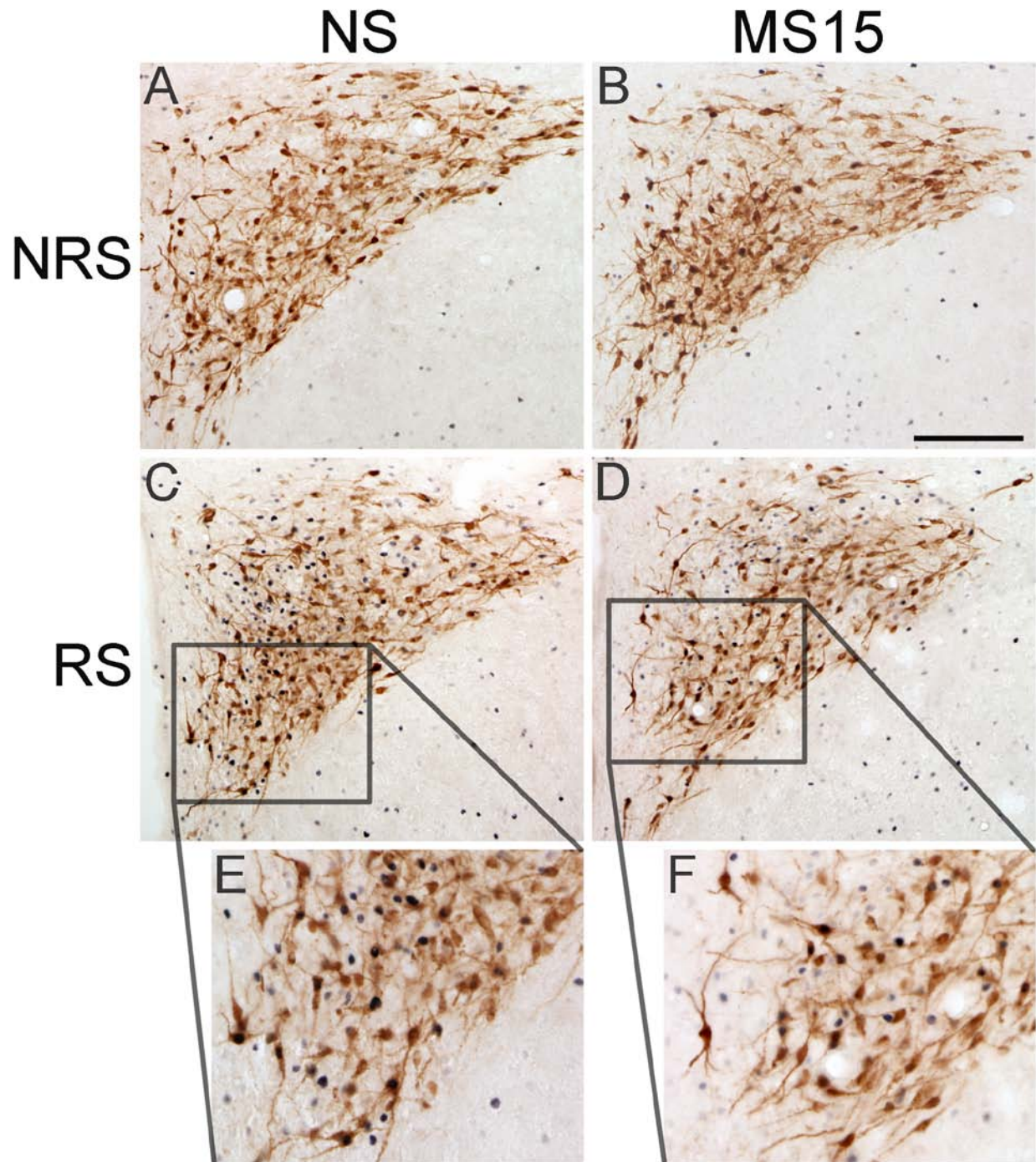


Figure 13. Photomicrographs of Fluorogold and Fos labeling within the PVN in NS (A and C) and MS15 (B and D) cases. There is no postnatal group difference in the number of FG-positive neurons (A vs. B) or in the number of FG-positive neurons expressing Fos in response to restraint stress (C vs. D). See also larger images of outlined areas in C and D (E vs. F). Scale bar (in B) = 200 μ m, applies to A-D.

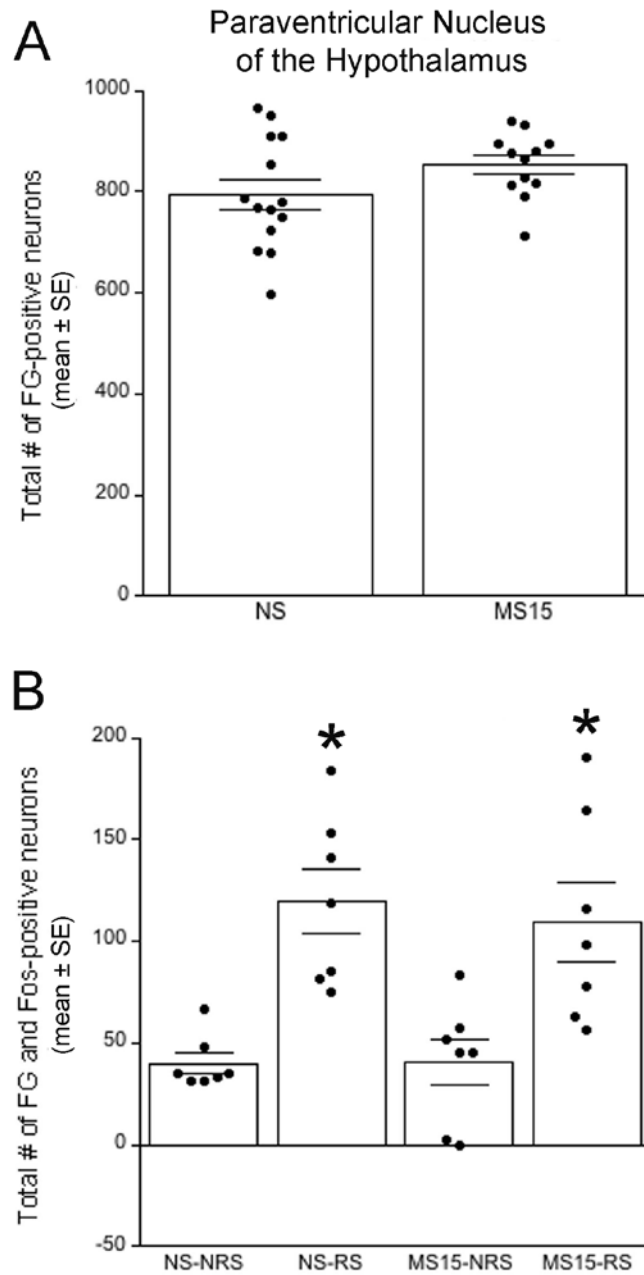


Figure 14. DVC-projecting PVN neurons and the number that express Fos in response to restraint stress. (A) Bar graph depicting the total number of FG-positive neurons within the PVN (with overlaid dot plot depicting data from individual cases.) There is no significant difference between the two postnatal groups in FG-positive neurons. (B) Bar graph depicting the total number of FG and Fos-positive neurons within the PVN in no restraint stress (NRS) control and restraint stress (RS) cases within both postnatal groups. RS increases the number of double-labeled neurons similarly in both NS and MS15 groups. (* $P < 0.05$, within postnatal group)

Effect of postnatal group on total stress-induced Fos activation within the PVN

Twenty-one surgically-intact rats were included in the following analyses (NS-NRS $n = 5$, NS-RS $n = 6$, MS15-NRS $n = 5$, MS15-RS $n = 5$). RS-induced Fos labeling was densest in two sections through the PVN (corresponding to bregma levels -1.88 through -1.80 mm) which contained both the medial parvocellular (PaMP) and dorsal cap subnuclei (PaDC); Fos labeling appeared to be densest in the PaMP subdivision (Fig. 15). NRS cases within both postnatal groups displayed little Fos activation within the PVN (Figs. 16A and B). ROI analysis of the total number of Fos-positive neurons within the PVN revealed a clear trend for MS15-RS rats to display fewer Fos-positive neurons compared to NS-RS rats at both rostrocaudal levels examined (Fig. 16A). However, this trend was only statistically significant within the caudal PVN level (Fig. 16A). At the caudal level of the PVN, two-way ANOVA revealed significant main effects of postnatal group ($F_{(1,20)} = 6.12$; $P = 0.02$) and stress condition ($F_{(1,20)} = 116.28$; $P = 0.00$) on the number of Fos-positive neurons, as well as an interaction between postnatal group and stress condition ($F_{(1,20)} = 4.59$; $P = 0.05$; Figs. 15 and 16B). T-tests at the caudal level revealed that while RS significantly increased the number of Fos-positive neurons within the PVN in both NS and MS15 groups (NS-NRS vs. NS-RS: $P = 0.00$; MS15-NRS vs. MS15-RS: $P = 0.00$), this effect was less pronounced in the MS15 group compared to the NS group (NS-RS vs. MS15-RS: $P = 0.04$).

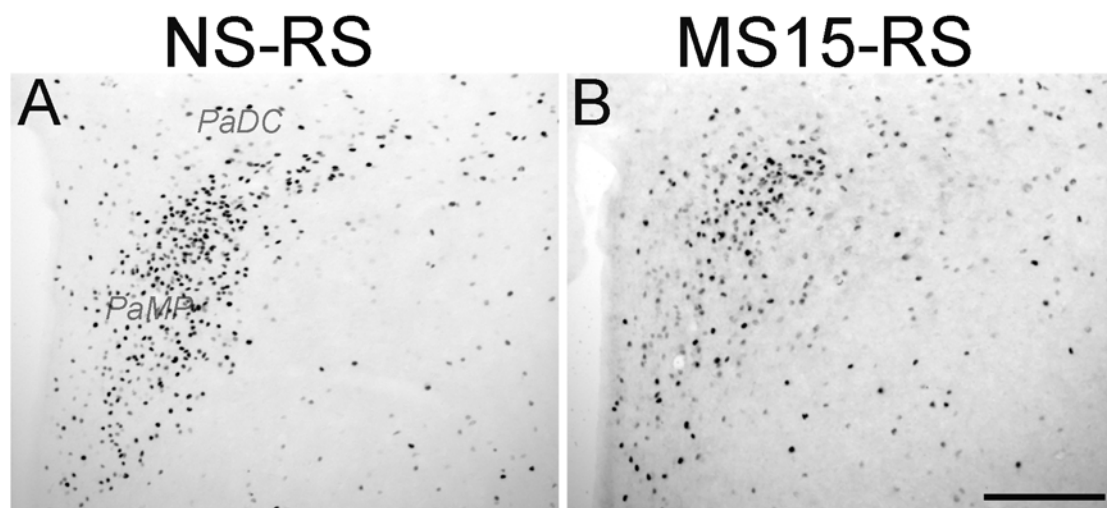


Figure 15. Photomicrographs depicting restraint stress-induced Fos activation within the PVN in (A) NS and (B) MS15 cases. MS15 rats display decreased restraint stress (RS)-induced Fos activation within the PVN, which is significant at the rostrocaudal level depicted in these photomicrographs. Scale bar (in B) = 200 μ m, applies to both. PVN dorsal cap (PaDC), PVN medial parvocellular (PaMP)

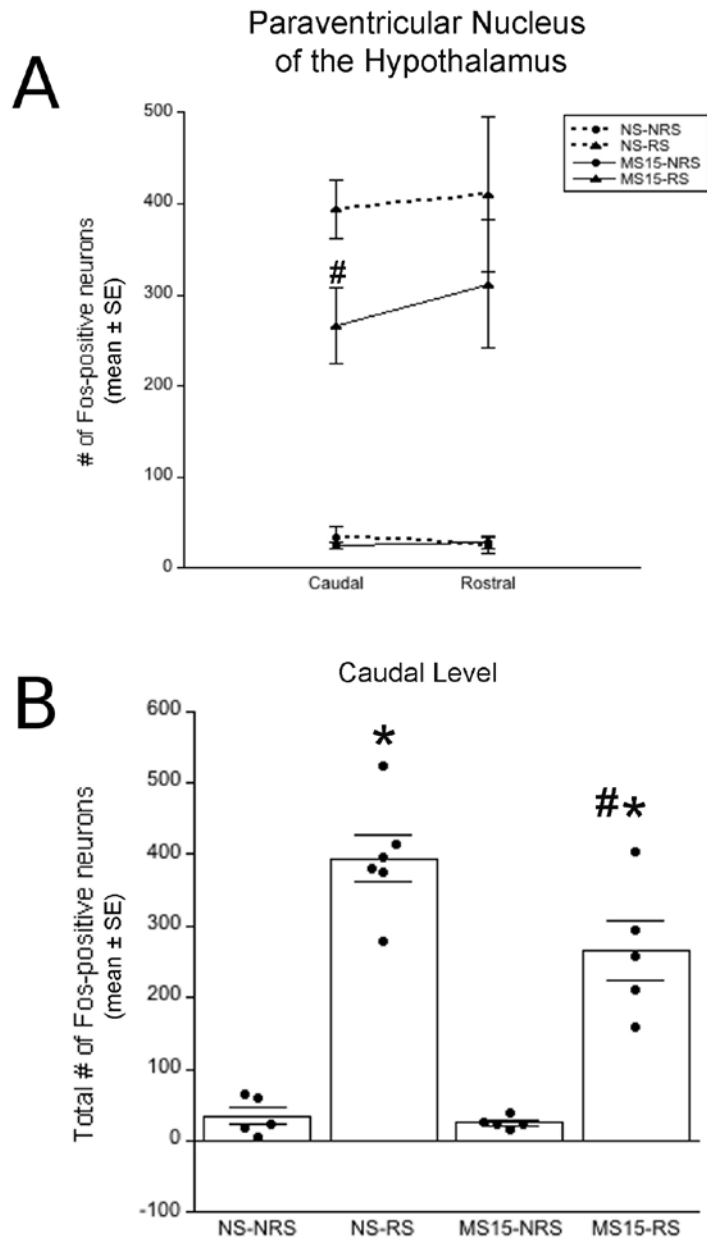


Figure 16. Fos-positive neurons within the PVN. (A) Line graph depicting Fos-positive neurons within the two PVN rostrocaudal levels examined. There was a significant difference between the NS and MS15 groups in the number of restraint stress (RS)-induced Fos-positive neurons within the caudal level. (B) Bar graph depicting the total number of Fos-positive neurons within caudal level of the PVN in no restraint stress (NRS) control and RS cases within both postnatal groups. RS significantly increases the number of Fos-positive neurons in both postnatal groups; however, this effect was attenuated in the MS15 group. (# $P < 0.05$, between postnatal groups, * $P < 0.05$, within postnatal groups)

Effect of postnatal group on total stress-induced Fos activation within the NST

With both postnatal groups, RS-induced DVC Fos activation was primarily located in the NST, with moderate Fos labeling in the AP and little to no Fos labeling in the DMV (Fig. 17). NRS cases within both postnatal groups displayed little Fos in the DVC, including the NST (Fig. 18). Two-way ANOVA revealed a significant main effect of stress condition (NRS vs. RS) to increase the number of Fos-positive neurons within the NST ($F_{(1,20)} = 41.11$; $P = 0.00$). However, there was no effect of postnatal group on the number of Fos-positive NST neurons ($F_{(1,20)} = 0.68$; $P = 0.42$) and no interaction between postnatal group and stress condition ($F_{(1,20)} = 0.00$; $P = 0.97$; Fig. 17 and 18). T-tests confirmed a significant difference between NRS and RS conditions within both NS and MS15 postnatal groups (NS-NRS vs. NS-RS: $P = 0.00$; MS15-NRS vs. MS15-RS: $P = 0.00$).

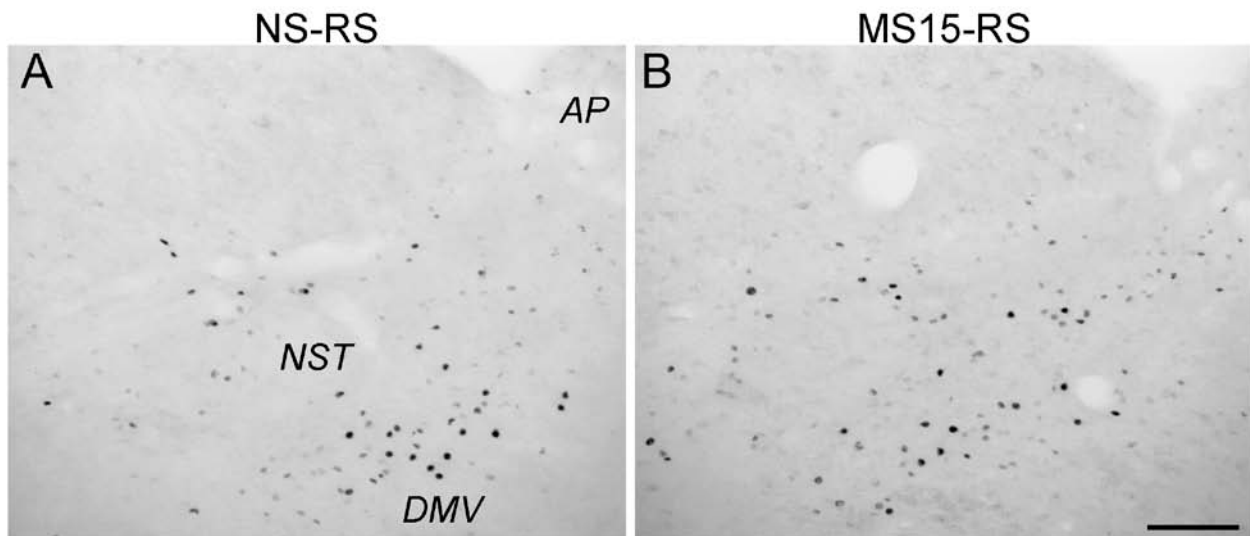


Figure 17. Photomicrographs depicting restraint stress-induced Fos activation within the NST in (A) NS and (B) MS15 cases. There is no postnatal group difference in the restraint stress (RS)-induced Fos activation within the NST. Scale bar (in B) = 200 μ m, applies to both. Area postrema (AP), nucleus of the solitary tract (NST), dorsal motor nucleus of the vagus (DMV)

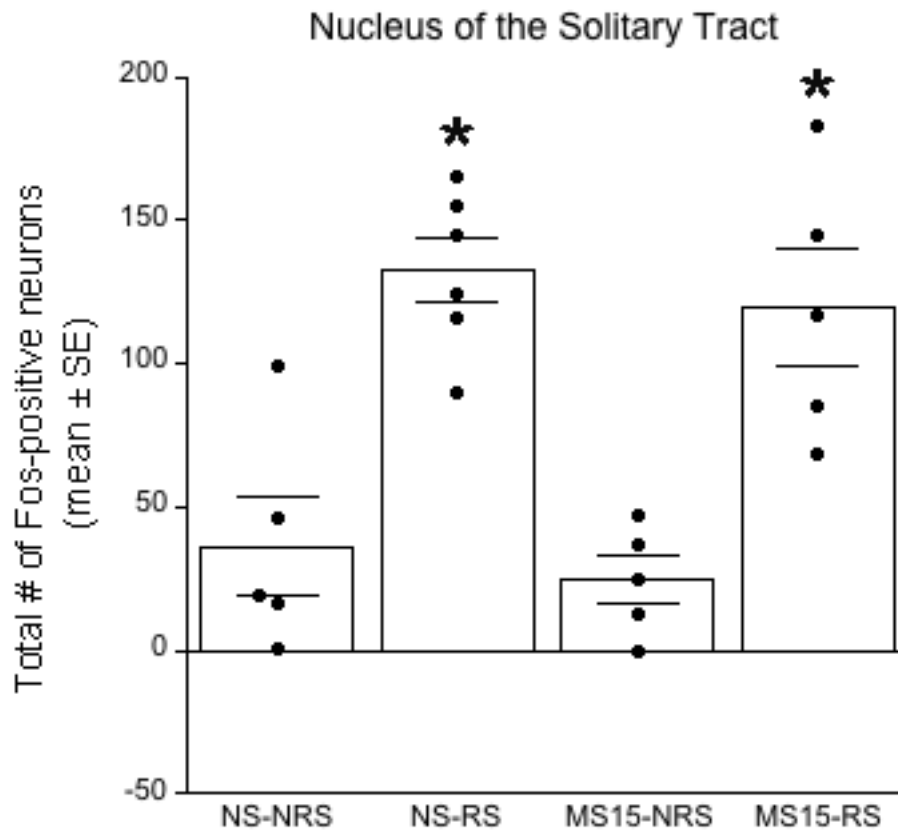


Figure 18. Bar graph depicting the number of Fos-positive neurons within the NST in no restraint stress (NRS) control and restraint stress (RS) cases within both postnatal groups. RS increases the total number of Fos-positive neurons within the NST similarly within both postnatal groups. (* P<0.05, within postnatal groups)

Effect of postnatal group on stress-induced Fos activation of noradrenergic NST neurons

There were few DbH and Fos-positive NST neurons in NRS cases in both NS and MS15 groups (Fig. 19, A vs. B and Fig. 20A). MS15-RS rats exhibited a trend toward having fewer double-labeled NST neurons compared to NS-RS rats at all three rostrocaudal levels examined (Fig. 20A). However, this postnatal group difference was only significant at the most caudal level of the AP. At this level, two-way ANOVA revealed significant main effects of postnatal group ($F_{(1,19)} = 9.73$; $P = 0.01$) and stress condition ($F_{(1,19)} = 66.63$; $P = 0.00$) on the number of DbH and Fos-positive neurons, as well as an interaction between postnatal group and stress condition ($F_{(1,19)} = 7.02$; $P = 0.02$; Figs. 19 and 20B). T-tests at this caudal NST level revealed that while RS significantly increased the number of double-labeled neurons in both NS and MS15 groups (NS: $P = 0.00$; MS15: $P = 0.00$), this effect was attenuated in the MS15 group (NS-NRS vs. MS15-RS: $P = 0.00$).

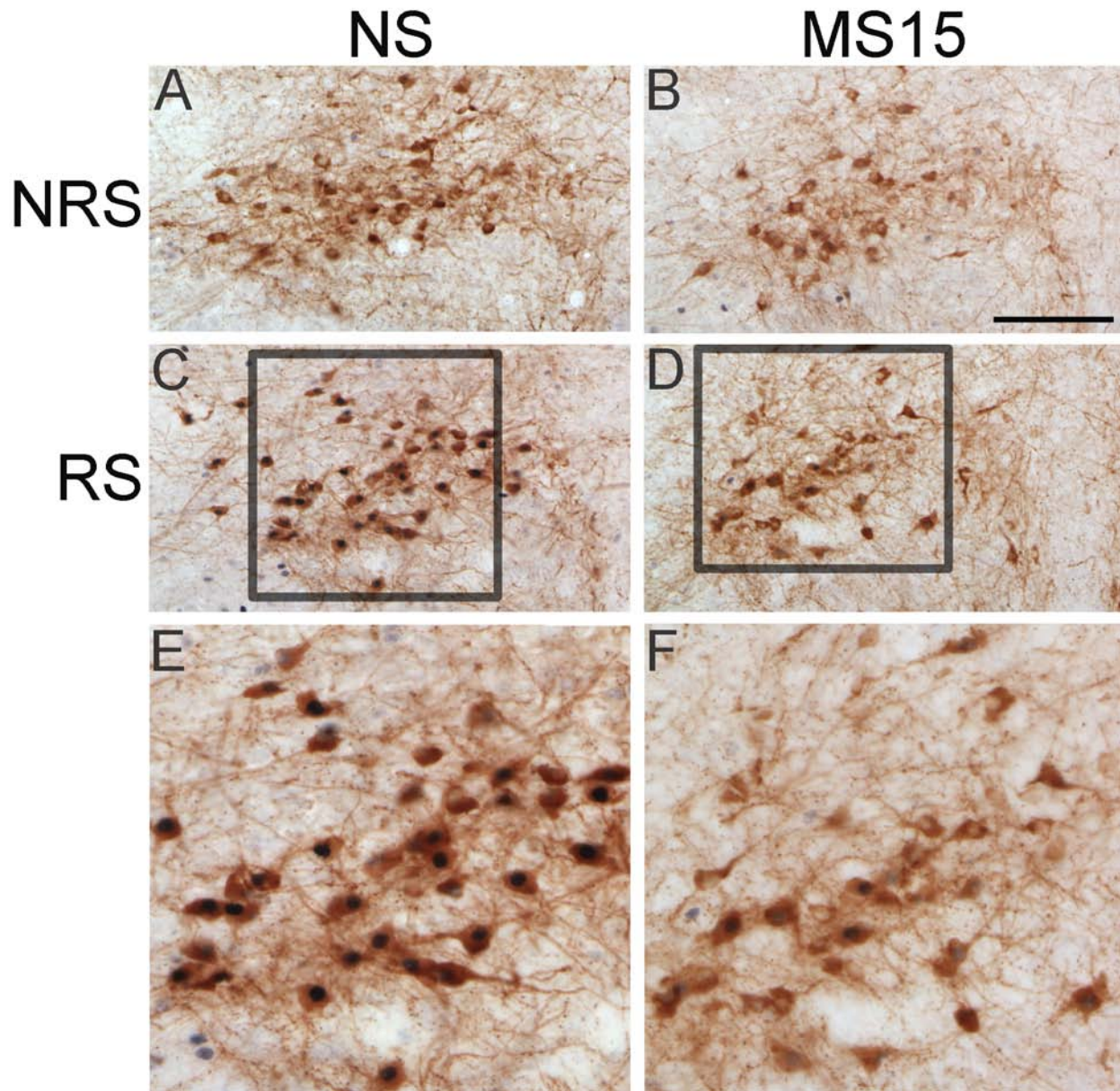


Figure 19. Photomicrographs of DbH and Fos labeling within the NST in NS (A and C) and MS15 (B and D) cases. There was no postnatal group difference in the number of double-labeled neurons among no restraint stress (NRS) controls (A vs. B). However, MS15 rats display significantly fewer double-labeled neurons in response to restraint stress (RS) compared to NS controls (C vs. D). See also larger images of outlined areas in C and D (E vs. F). Scale bar (in B) = 200 μ m, applies to A-D.

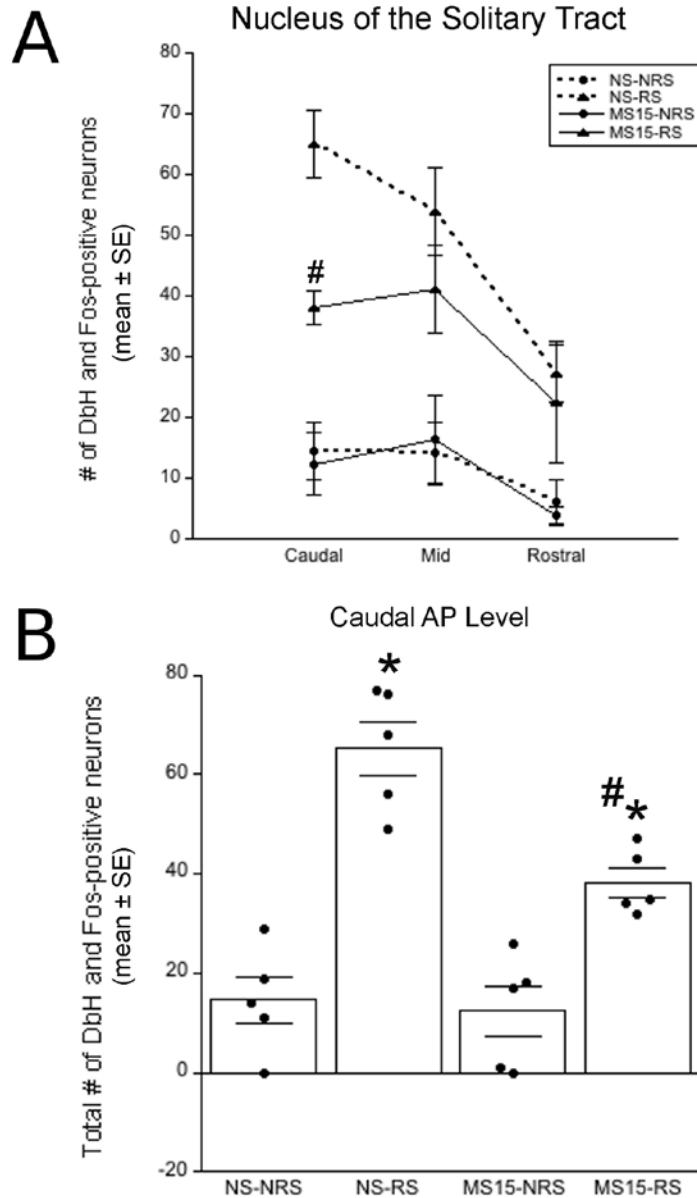


Figure 20. Fos and DbH-positive neurons within the NST. (A) Line graph depicting double-labeled neurons within the three NST rostrocaudal levels examined. There was a significant difference between the NS and MS15 groups in the number of Fos-expressing DbH neurons in response to restraint stress (RS) within the caudal level. (B) Bar graph depicting the total number of double-labeled neurons within the caudal level of the PVN in no restraint stress (NRS) control and RS conditions within both postnatal groups. RS significantly increased the number of double-labeled neurons in both postnatal groups; however, this effect was attenuated in the MS15 group. (# $P < 0.05$, between postnatal groups, * $P < 0.05$, within postnatal groups)

3.5 DISCUSSION

Previous work in our laboratory demonstrated that MS15 alters the developmental assembly of descending gastric preautonomic circuits originating within limbic forebrain and hypothalamic regions in neonates (Card et al., 2005). Further, a more recent report from our laboratory demonstrated that juvenile rats with a developmental history of MS15 display enhanced PVN gastric preautonomic circuitry compared to NS controls (Banihashemi and Rinaman, 2010). This enhancement was reflected by an increase in PVN retrograde transynaptic viral labeling from the stomach, evidence for enhanced circuit strength of the descending pathway from the PVN to spinal and brainstem preganglionic neurons.

PVN stimulation replicates the physiological effects of acute stress, in that it inhibits gastric motility and increases gastric acid secretion (McCann and Rogers, 1991). The PVN contributes importantly to DVC activity (Zhang et al., 1999), including stress-induced activation of NA NST neurons (Buller et al., 2003, Dayas et al., 2004). DMV preganglionic neurons innervate postganglionic neurons in the enteric plexus that are inhibitory (non-adrenergic, non-cholinergic) or excitatory (cholinergic) (Travagli et al., 2006). The PVN appears to modulate NST and DMV activity, such that activation of the PVN results in activation of the inhibitory pathway and inhibition of the excitatory pathway, a pattern of activation that results in inhibition of gastric motility (McCann and Rogers, 1991). Thus, we hypothesized that MS15 rats would display less stress-induced Fos activation of DVC-projecting PVN neurons and in turn, less stress-induced Fos activation of the NST, including NST NA neurons. These hypotheses were based on the idea that dampened stress-induced recruitment of the PVN to DVC pathway would contribute to the overall phenotype of decreased stress reactivity exhibited by MS15 rats (Plotsky

and Meaney, 1993, Liu et al., 2000). Interestingly, although results from the present study did not reveal a postnatal group difference in the number of DVC-projecting PVN neurons that expressed Fos activation after restraint stress, the results did reveal that MS15 rats displayed less stress-induced Fos activation of noradrenergic (NA) neurons within the NST compared to the NS group. This finding supports the view that MS15 alters the functional impact of stress on the recruitment of the DVC.

MS15 did not alter the number of DVC-projecting PVN neurons

Our previous finding that MS15 rats display increased retrograde transynaptic viral labeling from the stomach compared to NS controls could be the result of an increase in the number of PVN neurons in synaptic contact with brainstem or spinal preganglionic neurons, an increase in the number of axon terminals of individual preautonomic PVN neurons synapsing on infected postsynaptic targets, or a combination of the two (Banihashemi and Rinaman, 2010). Our present study showed that retrograde FG labeling within the PVN was remarkably similar between NS and MS15 postnatal groups, suggesting that the number of DVC-projecting PVN neurons is equivalent between postnatal groups. Taken together, the increased viral labeling within the PVN of MS15 rats is likely due to an increase in the number of axon terminals of individual PVN neurons in synaptic contact with infected targets. However, it is important to note that the retrogradely labeled PVN neurons in the current study are a general population of DVC-projecting PVN neurons, only a subset of which are directly preautonomic and/or pre-gastric. It is possible that there is a difference in the number of preautonomic PVN neurons that is not revealed by standard retrograde labeling of the general DVC-projecting population.

MS15 decreases stress-induced Fos activation within the PVN but not within the DVC-projecting population

Our results demonstrated that juvenile rats with a developmental history of MS15 displayed significantly less stress-induced Fos activation within the caudal PVN. Interestingly, this rostrocaudal level includes the “mid-caudal” level through the PVN at which MS15 rats displayed greater retrograde transynaptic labeling in our previous study (Banihashemi and Rinaman, 2010). These results concur with those of a previous report showing that restraint stress-induced Fos activation within the PVN is attenuated in adult MS15 rats (Abraham and Kovacs, 2000). Qualitatively, the MS15-related decrease in stress-induced Fos within the PVN appears to be primarily localized to the medial parvocellular subnuclei (PaMP), which contains the CRH neurons of the HPA axis. Thus, it is likely that decreased activation of CRH neurons within the PVN contributes importantly to the decrease in overall stress-induced Fos activation within MS15 rats observed in the present study. This would correspond with previous findings that MS15 rats display markedly attenuated stress-induced activation of the HPA axis, such as lower stress-induced ACTH and corticosterone levels (Plotsky and Meaney, 1993, Liu et al., 2000). It is also possible that decreased stress-induced activation of pre-sympathetic (spinally-projecting) neurons within the dorsal cap subnuclei of the PVN (Luiten et al., 1985) also contribute to the observed decrease in overall stress-induced Fos activation within the PVN in the MS15 group.

Despite the MS15-related decrease in stress-induced Fos activation within the PVN, our findings demonstrate that the primarily pre-parasympathetic, DVC-projecting PVN neurons are similarly activated by restraint stress within both NS and MS15 postnatal groups. Further, the proportion of DVC-projecting PVN neurons that was activated in response to restraint stress is

similar in juvenile (present study) and adult rats (Dayas et al., 2004). The possibility remains open that stress-induced activation of a specific phenotypic sub-population of DVC-projecting PVN neurons is different between postnatal groups. For example, DVC-projecting oxytocin and CRH neurons within the PVN both contribute to DVC neuron activity and have important influences on gastric autonomic outflow (Gunion and Tache, 1987, McCann and Rogers, 1990, Flanagan et al., 1992c, Monnikes et al., 1992). Further, other DVC-projecting, preautonomic regions, such as the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA), play a role in autonomic function (Hermann et al., 1990, Aleksandrov et al., 1996, Liubashina et al., 2002, Zhang et al., 2003, Hatam and Nasimi, 2007), and may express postnatal group differences in stress-induced Fos activation.

MS15 decreases the stress-induced recruitment of noradrenergic NST neurons

Our previous finding that PVN gastric preautonomic circuit strength is enhanced in MS15 rats (Banihashemi and Rinaman, 2010) suggests that that stress-induced recruitment of the PVN to DVC pathway is altered to result in less stress-induced activation of the NST. Indeed, the present study demonstrated that MS15 rats display less stress-induced Fos activation of NA neurons with the NST compared to NS controls. This reduction appears to be specific to this phenotypic population, as total stress-induced Fos activation within the NST was not different between postnatal groups. Further, this postnatal group difference was predominantly localized to the caudal level of the AP, which receives vagal sensory input from the stomach (Altschuler et al., 1989) and contains stress-responsive neurons that co-express DbH and other peptides, such as prolactin-releasing peptide (Morales and Sawchenko, 2003). Additionally, NA NST neurons innervate gastric-projecting DMV neurons (Pearson et al., 2007) and norepinephrine (NE) within the DMV inhibits gastric motility (Rogers et al., 2003, Herman et al., 2008).

Previous research has demonstrated the importance of the PVN in the stress-induced activation of NA neurons within the NST, as PVN lesions result in a significant reduction of stress-induced Fos activation of NA NST neurons (Buller et al., 2003, Dayas et al., 2004). Thus, one might predict that the potential increase of axon terminals of PVN neurons innervating the DVC would result in increased, not decreased, activation of NST NA neurons. One possible interpretation of our findings is that increased synaptic contacts from PVN neurons target local GABAergic neurons within the NST that, in turn, inhibit stress-induced activation of NST NA neurons. Indeed, GABAergic immunoreactive neurons are present within the caudal visceral area of the NST (Meeley et al., 1985, Pickel et al., 1996). Further, previous studies have reported alterations in both NA and GABAergic receptors within the NST in rats with a developmental history of MS15. These reports have demonstrated that MS15 rats have substantially greater GABA_A receptor mRNA and binding within the NST (Caldji et al., 2000). MS15 rats also display increased levels of α_2 adrenergic receptor binding in the NST compared to NS rats (Liu et al., 2000). Interestingly, the presynaptic α_2 receptor acts as an autoreceptor, which inhibits NE release (Wozniak et al., 1995). Previous work from our laboratory suggests that NA NST neurons collateralize locally (Rinaman, 2003a); thus, MS15 rats potentially have less NE release within the NST itself. Overall, the lines of evidence suggest that MS15 rats display increased GABAergic and decreased NA tone within the NST. It is also possible that the decrease in stress-induced activation of NA NST neurons observed in MS15 rats in the present report is due to inhibition from other DVC-projecting regions. Indeed, GABAergic efferents from the BNST (Dong et al., 2001b) and the CeA innervate the DVC (Saha et al., 2000). The CeA, in particular, has been shown to form inhibitory-type synapses with catecholaminergic neurons within the mid-NST (Pickel et al., 1995), a subset of which are NA.

MS15 potentially influences the interaction between ascending viscerosensory and descending autonomic pathways

The PVN receives viscerosensory signaling from the NST via ascending NA pathways (TerHorst et al., 1989). Stress has been shown to significantly increase the level of NE within the PVN (Pacak et al., 1995b). Further, NA terminals have been shown to synapse directly onto PVN gastric preautonomic neurons in adult rats (Balcita-Pedicino and Rinaman, 2007), a pathway by which NA signaling can influence stress-induced autonomic responses. Indeed, NE within the PVN has a stimulatory effect on both neuroendocrine and autonomic responses (Bachelard et al., 1992, Pacak et al., 1995b). Interestingly, MS15 rats display strikingly lower levels of restraint stress-induced NE release within the PVN (Liu et al., 2000), which likely corresponds to the observed decrease in stress-induced Fos activation of NA NST neurons in MS15 rats in the present study. Ascending NA projections from the NST and the ventrolateral medulla (VLM) form the “ventral NA bundle.” Thus, it is possible that an MS15-related decrease in stress-induced Fos activation within the VLM also contributes to decreased stress-induced NE levels in the PVN.

Interpretations and future directions

The most striking new finding from the present report was that juvenile rats with a developmental history of MS15 display less stress-induced Fos activation in NA neurons within the caudal visceral NST compared to NS controls. The increased circuit strength of PVN gastric preautonomic circuits observed in our previous report (Banihashemi and Rinaman, 2010) suggests that DVC-projecting PVN neurons target and strongly activate local GABAergic neurons within the NST, which then inhibit stress-induced activation of NA NST neurons. However, the stress-induced recruitment of inhibitory inputs from other preautonomic areas,

such as the BNST and CeA, to NA NST neurons could also be enhanced in MS15 rats, contributing to decreased stress-induced Fos activation of NA NST neurons. We predict that the decrease in stress-induced activation of NA NST neurons in MS15 rats corresponds to diminished recruitment of vagal motor outflow to the stomach that leads to stress-induced inhibition of gastric motility. Further research is necessary to directly test these hypotheses; however, our research has established that MS15 alters the developmental assembly of gastric preautonomic circuits in neonatal rats (Card et al., 2005), and enhances the circuit strength of PVN gastric preautonomic circuits (Banihashemi and Rinaman, 2010) and decreases stress-induced activation of NA NST neurons that receive preautonomic inputs later in development, in juveniles (present study). This is the first study to examine MS15-related alterations in stress-induced activation within a specific neural circuit and our findings may provide insights into how early life experience shapes later stress reactivity.

4.0 GENERAL DISCUSSION

4.1 SUMMARY AND INTERPRETATIONS OF THE FINDINGS

The decreased hypothalamic-pituitary adrenal (HPA) stress responsiveness of MS15 rats has been well established (Meaney et al., 1985, Plotsky and Meaney, 1993, Liu et al., 2000, Levine, 2005). However, the HPA axis is only one component of the stress response – the autonomic nervous system is also an essential component. Further, circuits within the brain influence and control both components of stress responses. For instance, descending preautonomic circuits that synaptically innervate preganglionic neurons in the brainstem and/or spinal cord directly control autonomic responses to stress. Thus, if MS15 alters stress reactivity, it is highly likely that it also alters neural circuits that regulate stress responses. Indeed, a previous report from our laboratory has demonstrated that MS15 alters the developmental assembly of gastric preautonomic circuits in neonates (Card et al., 2005). Neonatal rats undergoing MS15 display reduced circuit strength of - and/or a delay in the synaptic assembly of gastric preautonomic circuits (Card et al., 2005). These findings led us to hypothesize that MS15 rats would display altered circuit strength of gastric preautonomic circuits later in development, as assessed in juvenile rats (Chapter 2). This study demonstrated that:

1. MS15 rats display increased retrograde transynaptic PRV labeling from the ventral stomach wall within the PVN, which indicates enhanced circuit strength of gastric preautonomic circuits originating within the PVN.
2. The increased PVN PRV labeling is primarily due to an increase within one specific rostrocaudal level, containing both the dorsal cap (PaDC) and ventral (PaV) subnuclei. The PaDC and PaV contain primarily pre-sympathetic and pre-parasympathetic neurons, respectively.
3. MS15 rats display increased PRV labeling in both PaDC and PaV subnuclei, suggesting that the circuit strength of both the pre-sympathetic and pre-parasympathetic PVN pathways are enhanced.
4. Surprisingly, MS15 rats did not display altered PRV labeling within any other gastric preautonomic region examined. However, potential PRV labeling differences may be revealed with longer post-inoculation intervals (see Chapter 2, Discussion).

The enhanced circuit strength of the PVN gastric preautonomic circuit suggested that the function of the preautonomic PVN pathway might also be altered by MS15 at the same developmental time-point. Thus, we hypothesized that MS15 rats would display altered stress-induced activation of the PVN to DVC pathway (Chapter 3). The second study demonstrated that:

1. MS15 did not alter the number of DVC-projecting PVN neurons.
2. MS15 rats display decreased restraint stress-induced Fos activation within the PVN. Unexpectedly, this was not accompanied by a decrease in stress-induced Fos activation within DVC-projecting PVN neurons, suggesting that the stress-induced activation of

CRH neurons of the HPA axis and/or pre-sympathetic neurons within the PaDC may be decreased.

3. MS15 rats display decreased stress-induced Fos activation of a specific population of noradrenergic (NA) neurons within the NST, without a decrease in overall Fos activation within the NST. This suggests that there is less norepinephrine (NE) acting within the NST and DMV to elicit stress-induced inhibition of gastric motility.

A proposed model of MS15-related alterations in preautonomic circuits

Thus, MS15 rats display enhanced circuit strength of the PVN gastric preautonomic circuits without a change in the number of DVC-projecting PVN neurons. Together, these findings could suggest that increased PRV labeling within PVN in MS15 rats is due to individual PVN neurons having more axons terminals in synaptic contact with preganglionic neurons compared to controls. Further, MS15 rats display decreased stress-induced activation of NA NST neurons, without a decrease in stress-induced activation of DVC-projecting PVN neurons. Thus, DVC-projecting PVN neurons are similarly activated by restraint stress in both MS15 and NS groups. However, the enhanced circuit strength of the PVN gastric preautonomic circuits suggests that this pathway is still functionally altered in MS15 rats. Perhaps, in MS15 rats, DVC-projecting PVN neurons have greater synaptic innervation of GABAergic neurons within the NST (Fig. 21). Thus, PVN activation during stress would strongly activate GABAergic neurons, which would then inhibit stress-induced activation of NA NST neurons (Fig. 21). Further, enhanced inhibitory projections from limbic forebrain regions, such as the CeA and BNST (Pickel et al., 1996, Saha et al., 2000, Dong et al., 2001b), could also contribute to the observed stress-induced inhibition of NA NST neurons in MS15 rats. This proposed model is supported by data in the literature

suggesting that NST GABAergic tone is increased (Caldji et al., 2000), while NST NA tone is decreased (Liu et al., 2000), in MS15 rats (see Chapter 3, Discussion).

Potential MS15-related functional consequences of alterations in preautonomic circuits

The effects of MS15 on autonomic function and stress-induced autonomic responses have not yet been examined in depth. One study has reported that MS15 increases stress-induced changes in blood pressure and decreases stress-induced changes in heart rate (Sanders and Anticevic, 2007). However, this study used a borderline hypertensive rat strain and a variation of the MS15 paradigm in which pups were separated from their dam and their littermates. Thus, the effects of MS15 on autonomic function and responses to stress must be characterized using a standardized MS15 protocol and a non-hypertensive rat strain. However, other animal models that manipulate early life experience have demonstrated alterations in gut mucosa (Gareau et al., 2005) and altered somatic motor reflexes to noxious visceral stimuli (Mayer et al., 2001, Coutinho et al., 2002, Welting et al., 2005).

In addition to the above findings, the results reported in this dissertation also suggest that autonomic function and stress-induced autonomic responses are altered by MS15. PVN stimulation mimics the physiological effects of acute stress, in that it inhibits gastric motility and increases gastric acid secretion (McCann and Rogers, 1991). The PVN has been shown to modulate the activity of DVC neurons (Zhang et al., 1999) and contributes to the stress-induced activation of NA NST neurons (Buller et al., 2003, Dayas et al., 2004). These NA NST neurons have local projections within the NST and also project to the DMV (Card et al., 1990, Pearson et al., 2007). NE has various effects on DMV neurons that are related to their visceral targets (y Valenzuela et al., 2004) but NE within the DMV results in relaxed gastric tone and inhibition of gastric motility (Rogers et al., 2003, Herman et al., 2008).

Thus, enhanced circuit strength of PVN gastric preautonomic circuits (Chapter 2) and decreased stress-induced activation of NA NST neurons (Chapter 3) in MS15 rats suggest that MS15 rats would display attenuated stress-induced inhibition of gastric motility. In the context of the proposed MS15 model, stress-induced PVN activation would strongly activate GABAergic NST neurons, inhibiting stress-induced activation of NA NST neurons. Decreased stress-induced activation of the NA NST neurons would potentially lead to less stress-induced NE release within the DMV, resulting in less stress-induced inhibition of gastric motility (Fig. 21). The observed MS15-related decrease in stress-induced activation of NA NST neurons was primarily localized to the caudal AP level of the NST, an area which receives vagal sensory inputs from the stomach (Altschuler et al., 1989), suggesting altered gastric autonomic function in these rats. However, decreased stress-induced activation of NA NST neurons also suggests that pre-parasympathetic control over other visceral targets (e.g., the heart and lungs) may also be altered by MS15. Thus, MS15-related alterations in stress-induced autonomic function must be examined in greater depth.

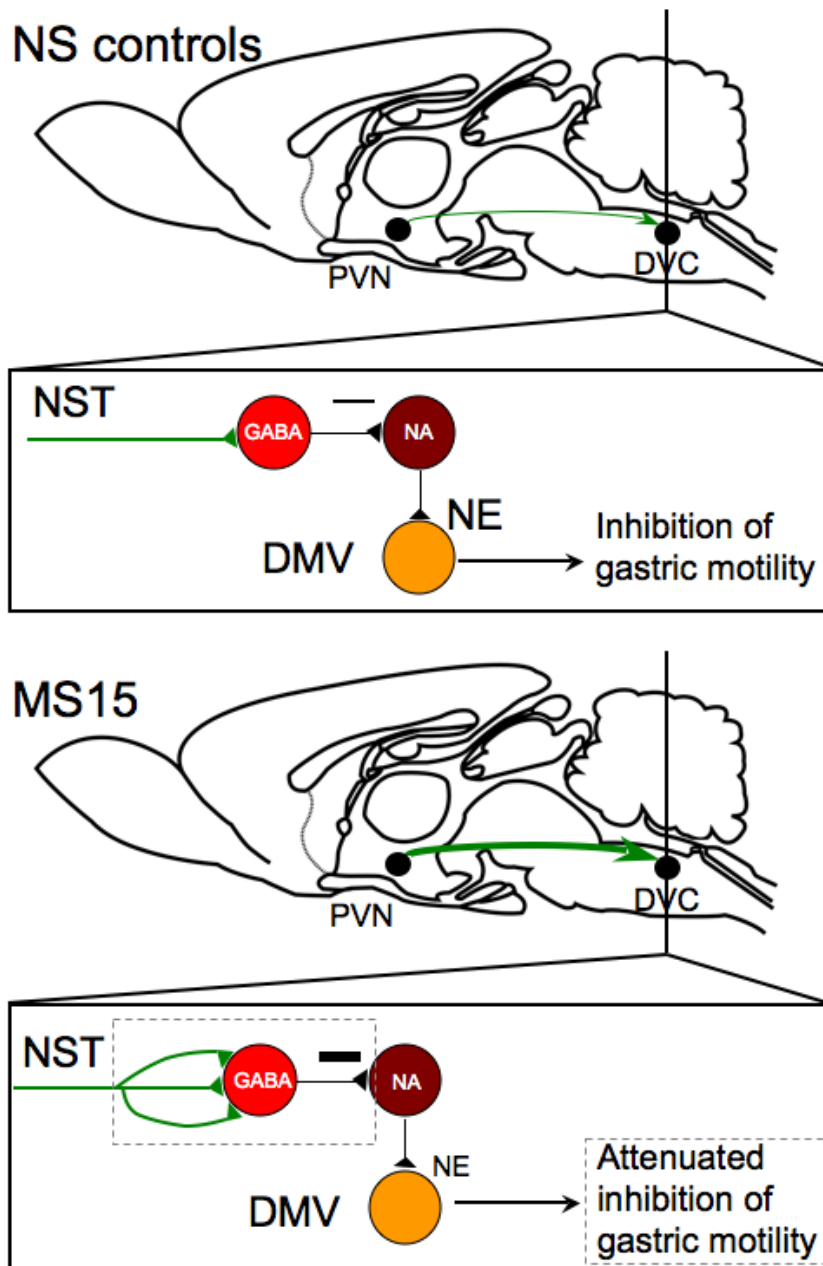


Figure 21. Proposed model of MS15-related alterations in PVN to DVC circuits and their potential functional consequences. It is possible that MS15 rats have increased synaptic contact with GABAergic neurons within the NST, which could result in the observed decrease in stress-induced activation of NA NST neurons in MS15 rats. These findings have important functional implications, namely that decreased norepinephrine (NE) release within the DMV may result in an attenuated stress-induced inhibition of gastric motility.

Potential MS15-related alterations of additional stress-related brain circuits

The studies in this dissertation have focused on the influence of MS15 on descending stress-related pathways that are primarily preautonomic. However, given that MS15 greatly decreases stress-induced HPA responses (Plotsky and Meaney, 1993, Liu et al., 2000), it is likely that MS15 also alters the structure and/or function of stress-related pathways that strongly influence PVN activity and hence, HPA responses.

Ascending viscerosensory, NA projections from the NST and the ventrolateral medulla (VLM) densely innervate the PVN (Ter Horst et al., 1989, Rinaman, 2001) and the ventrolateral BNST (Aston-Jones et al., 1999, Fendt et al., 2005). Stress has been shown to significantly increase the level of NE within both of these regions (Pacak et al., 1995b, Fuentealba et al., 2000, Cecchi et al., 2002). Further, NE within both the BNST and PVN is associated with stimulation of HPA responses (Bachelard et al., 1992, Pacak et al., 1995b, Banihashemi and Rinaman, 2006). Interestingly, many of the NA neurons innervating the BNST have axon collaterals that also project to the PVN (Fig. 22) (Banihashemi and Rinaman, 2006). Thus, the ascending NA projections from the caudal brainstem appear to co-regulate the BNST and the PVN.

The ventrolateral BNST sends a dense and direct projection to the PVN (Moga and Saper, 1994, Dong et al., 2001b) and the BNST stimulates stress-induced PVN activity and subsequent HPA responses (Fig. 22) (Gray and Piechowski, 1993, Herman et al., 1994, Crane et al., 2003a, Sullivan et al., 2004, Choi et al., 2007). Conversely, the medial prefrontal cortex (PFC) inhibits stress-induced PVN activity and corticosterone levels (Crane et al., 2003b, Spencer et al., 2005). The PFC does not directly innervate the PVN; however, it does densely

innervate the BNST (Vertes, 2004) and inhibits stress-induced BNST activation (Spencer et al., 2005).

Thus, direct projections from the BNST and NST/VLM (NA projections) strongly influence stress-induced PVN activity and HPA responses. Further, the PFC indirectly influences stress-induced PVN activity and HPA responses via its input to the BNST (Fig. 22). The structure and/or function of these principal connections are most likely altered by MS15 exposure. Interestingly, restraint stress-induced NE levels within the PVN are substantially lower in MS15 rats compared to NS controls (Liu et al., 2000), which may be related to the decreased stress-induced activation of NA NST neurons (Chapter 3), which give rise to these ascending projections. Given the collateralization of the ascending NA input to both the BNST and PVN (Banihashemi and Rinaman, 2006), it is likely that stress-induced NE release within the BNST is also decreased in MS15 rats. Decreased stress-induced NE release within the BNST and/or PVN would result in decreased stress-induced HPA responses (Fig. 22). Further, MS15 rats may have an enhanced PFC to BNST pathway (i.e., either through an increase in circuit strength or an increase in stress-induced recruitment) that would result in less stress-induced activation of the BNST and PVN and hence, less stress-induced HPA responses (Fig. 22). MS15-related alterations in these pathways must also be examined in future studies.

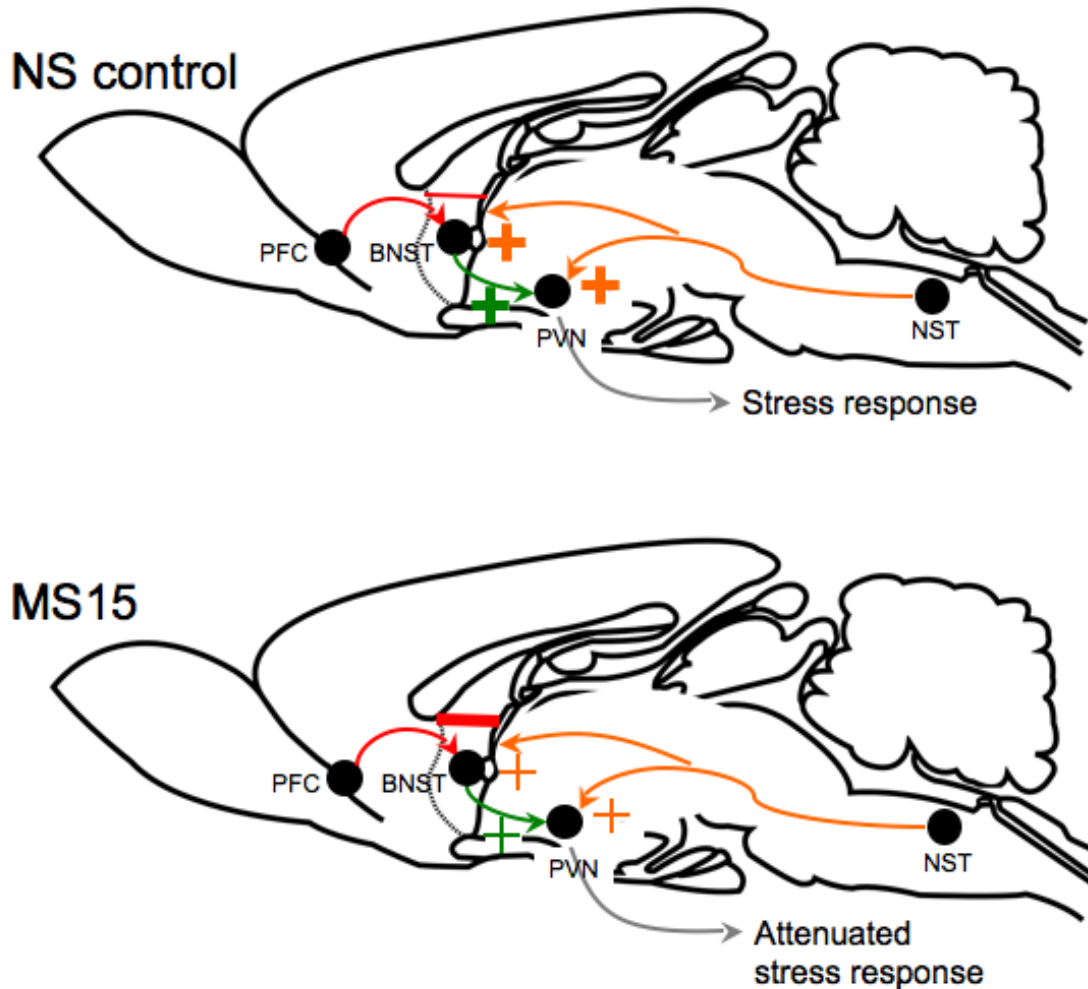


Figure 22. Potential postnatal group differences in additional stress-related circuits. This figure illustrates how MS15-related alterations in ascending viscerosensory projections (orange), and in the BNST to PVN (green) and PFC to BNST (red) pathways could result in attenuated HPA responses to stress. Less stress-induced activation of the NA NST neurons (Chapter 3) may lead to less NE release within the BNST and PVN (observed by Liu and colleagues), meaning less activation of these regions and less activation of the BNST to PVN pathway. Further, the inhibitory influence of the PFC on the BNST, and thus, on the BNST to PVN pathway, may be enhanced in MS15 rats. Ultimately, these potential MS15-related alterations would result in attenuated stress responses in MS15 rats.

4.2 MULTI-LEVEL MECHANISMS BY WHICH MS15 MAY INFLUENCE STRESS-RELATED BRAIN CIRCUITS

In this portion of the discussion, I will describe how MS15 may be altering the structure and function of stress-related circuits at multiple levels - from a systems-level perspective (maternal care and activity dependent-plasticity) down to a molecular level (gene regulation). I will briefly discuss how these mechanisms may be at play within the context of the dissertation findings.

MS15 enhances active maternal care

MS15 enhances the quality and duration of maternal care given to the pups upon their return to the dam (Liu et al., 1997, Macri et al., 2008). During this period, dams display more occurrences of “active” care-giving, including licking and grooming the pups and providing the most active style of nursing, “arched-back” nursing, to the pups (Liu et al., 1997, Macri et al., 2008). Thus, sensory experience associated with maternal care may influence developing brain circuits. For instance, nursing provides neonatal rat pups with somatosensory stimulation (Blass, 1994). Further, anogenital licking by the dam promotes digestion (a parasympathetic function) and also stimulates visceral reflexes, such as urination and defecation in neonatal pups (Hofer, 1984). Therefore, maternal care provides the pups with sensory stimulation that influences the activity of viscerosensory and autonomic circuits during a time in which these circuits are developing (Rinaman et al., 2000, Rinaman, 2001). Enhanced sensory stimulation could result in repeated coordinated activation of ascending viscerosensory and descending preautonomic pathways which may strengthen their interaction and contribute to the observed enhancement in circuit strength of PVN gastric preautonomic circuits (Chapter 2). Thus, alterations in the developmental assembly and later circuit strength of preautonomic circuits may be activity-dependent, not unlike activity-dependent changes known to occur in other brain sensory systems

(e.g., visual, auditory, somatosensory) during critical periods of development (Hubel et al., 1977, Merzenich et al., 1984, Nakahara et al., 2004, Fox et al., 2006).

Activity-dependent plasticity and trophic factors

Experience-dependent changes in sensory cortices during development have been studied extensively. These studies have manipulated early life sensory experience to examine structural and functional changes within circuits. For example, in the visual system, early life monocular deprivation leads to an increase in the number of neurons within cortical ocular dominance columns that represent the non-deprived eye (Hubel et al., 1977). Additionally, the deprived eye loses cortical space as the terminals from the lateral geniculate thalamic nucleus of the non-deprived eye expand (Hubel et al., 1977). These experience-dependent changes in the circuits are referred to as “activity-dependent plasticity.” Activity-dependent plasticity is attributed to Hebbian competition, in which activity influences the strength of the synaptic connections, potentiating those that repeatedly participate in firing one another and eliminating those which do not (Bi and Poo, 2001). Hebbian competition is in part due to synapses competing for trophic support and there is evidence for the activity dependent release of trophic factors (Bonhoeffer, 1996). For instance, blocking retinal activity or dark-rearing during the critical period for ocular dominance column formation is associated with decreased brain-derived neurotrophic factor (BDNF) mRNA levels within the primary visual cortex and disruption of ocular dominance column formation (Lein and Shatz, 2000).

Similar to MS15, the environmental enrichment (EE) paradigm is an animal model of enhanced sensory stimulation. EE generally consists of group housing in large cages, with include items used for exercise (e.g., running wheels) and other novel objects that are often removed and replaced with other novel objects. The EE paradigm is often compared to MS15, in

that animals reared in enriched environments display fewer anxiety-like behaviors compared to controls (Chapillon et al., 2002, Fernandez-Teruel et al., 2002). Additionally, both paradigms enhance performance on cognitive/spatial memory tasks like the morris water maze, and attenuate age-related hippocampal and cognitive declines (Meaney et al., 1988, Falkenberg et al., 1992, Pham et al., 1999, van Praag et al., 2000, Fenoglio et al., 2005). Changes in morphology and structure associated with EE may be due in part to activity dependent alterations in trophic support. EE has been shown to increase levels of nerve growth factor, BDNF, and glial derived neurotrophic factor within the hippocampus (Falkenberg et al., 1992, Pham et al., 1999, van Praag et al., 2000). EE-induced increases in trophic factors lends support to the view that MS15-related alterations of the development and/or later structure of viscerosensory and autonomic circuits may be due in part to Hebbian competition and activity-dependent release of trophic factors. Thus, the enhanced circuit strength of PVN gastric preautonomic circuits in MS15 rats (Chapter 2) may be due to increased activity-dependent release of trophic factors, stimulated by enhanced sensory stimulation accompanying enhanced maternal care. For example, higher trophic factor levels within the DVC could prompt PVN neurons to extend more axons terminals into the DVC.

Gene regulation

The MS15 paradigm experimentally induces an enhancement in maternal care, which is associated with decreased stress reactivity later in life. Similarly, offspring of dams that are naturally more active maternal care-givers, (“high licking and grooming and arched-backed nursing,” or high LG-ABN) are less stress reactive and display increased levels of glucocorticoid receptors (GRs) in hippocampus (Meaney, 2001, Champagne et al., 2003). This increased level of GRs is associated with well-characterized changes in the regulation of the GR gene.

Specifically, the offspring of high LG-ABN dams display increased expression of a GR transcription factor, nerve-growth-factor-inducible factor-A (NGFI-A), and more efficient binding between NGFI-A and its promoter sequence within the GR gene compared to controls (Meaney and Syzf, 2005, Kaffman and Meaney, 2007).

Changes in GR gene regulation in high-LG-ABN offspring also occur via changes in DNA methylation. DNA methylation results in gene silencing directly, by interfering with recognition elements, and indirectly, by facilitating inactive chromatin structure. Methylation at a specific site within the NGFI-A promoter sequence of the GR gene interrupts binding between NGFI-A and the GR promoter resulting in lower GR levels. The offspring of high-LG-ABN dams rats display little methylation of this site, again, resulting in a higher level of GRs compared to controls (Meaney and Syzf, 2005, Kaffman and Meaney, 2007). Together, these changes in gene regulation result in increased GR levels within the hippocampus resulting in more sensitive negative feedback of the HPA axis, in high LG-ABN offspring.

Adult rats with a developmental history of MS15 also display some changes in GR gene regulation. For example, MS15 rats display increased expression of GR mRNA splice variants that contain the NGFI-A promoter sequence (McCormick et al., 2000) and increased NGFI-A mRNA in the hippocampus (Meaney et al., 2000), supporting the view that MS15-related changes in stress reactivity could be due in part to changes in gene regulation. As the primary proponents of this work state, "... [T]o date these findings have been restricted to the study of a single promoter in but one gene in one brain region (Meaney and Syzf, 2005)." However, MS15-related changes in gene regulation could influence a wide range of developmental processes (e.g., neuronal proliferation and differentiation, axon outgrowth, synapse formation

and elimination) that may result in altered developmental assembly and the later structure and function of stress-related circuits.

4.3 RELEVANCE TO THE HUMAN LITERATURE

In the following section, I will review the literature examining early life experience and its relationship to later stress reactivity in humans and its parallels with the animal literature. Further, I will briefly discuss the relevance of early life experience-induced alterations in stress-related circuits to the neural correlates of individual differences in stress reactivity in human brain.

Effects of early life experience in humans

In humans, early life experience has also been shown to have important consequences for later stress reactivity, health outcomes, and disease risk. Much of the human literature has focused on early life adverse experience or trauma, which includes parental separation or neglect (e.g., children in foster care or orphanages), or physical or sexual abuse. Early life adverse experience/trauma is associated with altered HPA and autonomic function, both at baseline and in response to stress (for review see De Bellis, 2001), and increased susceptibility to anxiety or affective disorders in adulthood (Furukawa et al., 1999, Heim et al., 2000). Early life trauma also increases the risk for gastrointestinal disorders, such as irritable bowel syndrome (IBS), in adulthood (Lydiard, 2001, Mayer et al., 2001). Stress plays a key role in initiating and/or aggravating IBS and individuals with IBS tend to display increased stress reactivity (Heitkemper et al., 1996, Mayer et al., 2001). Thus, early life adverse experience/trauma in humans is

associated with greater stress reactivity and increased risk for affective and gastrointestinal disorders.

Another index of early life experience in the human literature is socioeconomic status (SES), which is assessed by education, occupation, and/or income. Early life SES is thought to be good indicator of the environment a child is raised in, for instance, the quality of the child's housing, neighborhood, and family relationships (Evans and Kantrowitz, 2002). Low SES is associated with a more unpredictable lifestyle and exposure to more unhealthy practices (i.e., smoking, drinking alcohol, poor diet, etc.) (Van de Mheen et al., 1998, Evans et al., 2005). Low early life SES is also associated with higher stress reactivity and greater risk for several diseases, including cardiovascular disease, later in life (Poulton et al., 2002, Chen et al., 2004). Certainly, SES and early life adverse experience share some common factors. For instance, parental neglect and overall lack of sensory stimulation may be a factor in both assessments.

Thus, early life environments characterized by neglect or deprivation, or by traumatic or frequent stress-inducing experiences are associated with higher stress reactivity and risk for disease later in life. However, healthy or enriched early life environments are associated with better outcomes. For instance, individuals with a developmental history of high early life SES are less stress reactive and have better adult health outcomes (Poulton et al., 2002, Chen et al., 2004). Further, sensory stimulation can improve the otherwise deleterious effects of early life adverse conditions. For instance, providing active and passive tactile stimulation (e.g., through neonatal massage or skin-to-skin contact, respectively) to isolated premature infants improves physical and behavioral development and significantly enhances sympatho-adrenal maturation (Kuhn and Schanberg, 1998, Feldman et al., 2002, Feldman and Eidelman, 2003, Dodd, 2005, McCain et al., 2005).

The human early life adverse experience/trauma and SES literatures parallel studies performed with animal models in which maternal care is low and/or pups are deprived from their dam repeatedly for prolonged durations. These animals consistently display increased HPA reactivity to stress (Plotsky and Meaney, 1993, Meaney, 2001) and lower GR levels in the hippocampus compared to controls (Meaney, 2001, Kaffman and Meaney, 2007). These rats also display heightened visceral motor sensitivity to noxious stimuli (Mayer et al., 2001, Coutinho et al., 2002), a symptom of IBS. Conversely, animal models in which maternal care is high (high LG-ABN and MS15), or in which there is greater environmental sensory stimulation (rats reared in an enriched environment), are associated with decreased stress reactivity and enhanced cognition (Meaney, 2001, Fernandez-Teruel et al., 2002, Levine, 2005). Thus, there are many parallels between human and animal literatures, strongly supporting the clinical relevance of the use of animal models to describe changes in stress-related circuits associated with early life experience.

Experimentally-induced vs. naturally-occurring differences in stress reactivity

Individual differences in stress reactivity are related to health and disease risk. For instance, individuals who display exaggerated blood pressure reactivity to stress (i.e., a large magnitude stress-induced change in blood pressure) are at much higher risk for developing cardiovascular disease because repeated exaggerated stress responses are associated with pathological changes in the vasculature (Matthews et al., 1993a, Treiber et al., 2003). Gianaros and colleagues have shown that individual differences in stress-induced changes in blood pressure covary with amygdala activation and the coordinated activity of the amygdala with the pons and the perigenual anterior cingulate cortex (Gianaros et al., 2008). Thus, individuals displaying greater stress-induced changes in blood pressure show greater activation within the amygdala and

greater coordinated activity of the amygdala with the pons and the perigenual anterior cingulate cortex (Gianaros et al., 2008). Thus, individual differences in stress reactivity are associated with individual differences in the function of stress-related circuits and examining early life experience-induced differences in stress-related circuits may provide insights into how individual differences in stress related circuits shape later stress reactivity. For instance, while the MS15 paradigm experimentally induces a decrease in stress reactivity, the structure and function of stress-related circuits MS15 rats may mirror the structure and function of stress-related circuits in humans that are more resilient to stress.

4.4 CONCLUSIONS AND FUTURE DIRECTIONS

To conclude, findings presented in this dissertation demonstrate that MS15 influences the structure and/or function of hypothalamic and brainstem preautonomic circuits. MS15 rats display enhanced circuit strength of gastric preautonomic circuits originating within the PVN, within pre-sympathetic and pre-parasympathetic PVN pathways (Chapter 2). The enhanced circuit strength of PVN gastric preautonomic circuits suggested that stress-induced activation of the PVN to DVC pathway would also be altered in MS15 rats. Indeed, MS15 rats display decreased stress-induced Fos activation of NA neurons within the NST (Chapter 3). These findings suggest further MS15-related changes in the anatomical organization and/or stress-induced recruitment of descending preautonomic circuits, potentially including increased PVN inputs to NST GABAergic neurons and/or increased stress-induced recruitment of limbic forebrain inhibitory inputs to the DVC. Further studies are necessary to test these hypotheses

and further examine MS15-related changes in other stress-related brain circuits, including ascending viscerosensory/NA pathways.

Our findings strongly suggest that MS15 attenuates stress-induced gastric autonomic function and also alters stress-induced parasympathetic control over other visceral targets. Thus, the functional consequences of MS15 on autonomic responses to stress must also be examined. As the studies in this dissertation focused on juvenile rats, further experiments are necessary to determine how the observed MS15-related alterations in preautonomic pathways evolve into adulthood.

MS15-related alterations in stress-related brain circuits may mirror the neural correlates of stress resilience in humans. Thus, these and future experiments examining early life experience-induced changes in stress-related circuits will provide insight into how early life experience shapes later stress reactivity and how individual differences in circuit structure and function are related to vulnerability to affective disorders and other diseases.

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