Published in final edited form as:

Neuroscience. 2010 January 13; 165(1): 265–277. doi:10.1016/j.neuroscience.2009.09.081.

Repeated brief postnatal maternal separation enhances hypothalamic gastric autonomic circuits in juvenile rats

Layla Banihashemi and Linda Rinaman

Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA, 15260, USA

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 transynaptic 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. Transynaptic 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.

Keywords

postnatal handling; MS15; preautonomic; stomach; pseudorabies virus; stress

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

Contact details for corresponding author: Layla Banihashemi, University of Pittsburgh, Department of Neuroscience, A210 Langley Hall, Pittsburgh, PA 15260, Phone: 717-880-7444, Fax: 412-624-9198, laylabanihashemi@gmail.com, labst94@pitt.edu.
Section editor: Systems neuroscience, Dr. Asla Pitkanen

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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. 1). 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.

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 timepoint, 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.

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 × 15.2 × 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 µL 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 µl 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 μ m-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 μ m) 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 (μ m²) 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. 2, 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. 2E 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. 2E 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.

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. 4). 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. 6), 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 (μm^2) and (2) PRV labeling area (μm^2). 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 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.

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. 6), 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. 1). 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, perifornical 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.

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. 2 and 3). These data indicate that postnatal treatment did not affect retrograde transynaptic PRV infection from the stomach wall to the AP.

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. 4 and 5). These data indicate that postnatal treatment did not affect retrograde transynaptic PRV infection of pre-gastric circuits within the BNST.

Central Nucleus of the Amydala—PRV labeling within the CeA was primarily localized to its medial subnucleus (see Figs. 6 and 7). 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.

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. 8 and 9A). 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. 9B), 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. 9B), which included labeling within both the dorsal cap (PaDC) and ventral subnuclei (PaV) (Fig. 8). 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.

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. 7A), with MS15 rats displaying more retrograde transynaptic PRV labeling compared NS rats.

Visceral cortices

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.

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 timecourse 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 Taché, 1987, Lenz et al., 1988, McCann and Rogers, 1990, Flanagan et al., 1992a, Flanagan et al., 1992c, Taché 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, 1998), 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.

Acknowledgments

We thank Daniel N. Lamont for constructing our database and assisting with figures, Victoria Maldovan Dzmura for expert technical assistance, Dr. John W. Wilson for advice on statistical analyses, and Dr. Lynn Enquist for providing high-titer viral stocks. This work was supported by NIH grant MH59911 (LR).

Abbreviations

AP	Area postrema
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of the amygdala
dbNST	Dorsal bed nucleus of the stria terminalis
DMV	Dorsal motor nucleus of the vagus
DVC	Dorsal vagal complex
HPA	Hypothalamic-pituitary-adrenal
IN	Insular cortex
NS	Non-separated controls
NST	Nucleus of the solitary tract
PVN	Paraventricular nucleus of the hypothalamus
PaDC	Paraventricular nucleus of the hypothalamus, dorsal cap
PaV	Paraventricular nucleus of the hypothalamus, ventral subnucleus
P	Postnatal day
PL/IL	Prelimbic/infralimbic cortex
PRV	Pseudorabies virus
ROI	Region of interest
MS15	Repeated brief postnatal maternal separation
vBNST	Ventral bed nucleus of the stria terminalis

References

- Abraham IM, Kovacs KJ. Postnatal handling alters the activation of stress-related neuronal circuitries. *European Journal of Neuroscience* 2000;12:3003–3014. [PubMed: 10971641]
- Aleksandrov VG, Bagaev VA, Nozdrachev AD, Panteleev SS. Identification of gastric related neurons in the rat insular cortex. *Neurosci Lett* 1996;216:5–8. [PubMed: 8892378]
- Balcita-Pedicino JJ, Rinaman L. Noradrenergic axon terminals contact gastric preautonomic neurons in the paraventricular nucleus of the hypothalamus in rats. *The Journal of Comparative Neurology* 2007;501:608–618. [PubMed: 17278138]
- Bredy T, Grant R, Champagne D, Meaney MJ. Maternal care influences neuronal survival in the hippocampus of the rat. *European Journal of Neuroscience* 2003;18:2903–2909. [PubMed: 14656341]
- Buller KM. Neuroimmune stress responses: reciprocal connections between the hypothalamus and the brainstem. *Stress: The International Journal on the Biology of Stress* 2003;6:11–17.

- Cano G, Sved AF, Rinaman L, Rabin BS, Card JP. Characterization of the central nervous system innervation of the rat spleen using viral transneuronal tracing. *The Journal of Comparative Neurology* 2001;439:1–18. [PubMed: 11579378]
- Card JP, Enquist LW, Moore RY. Neuroinvasiveness of pseudorabies virus injected intracerebrally is dependent on viral concentration and terminal field density. *J Comp Neurol* 1999;407:438–452. [PubMed: 10320223]
- Card JP, Levitt P, Gluhovsky M, Rinaman L. Early experience modifies the postnatal assembly of autonomic emotional motor circuits in rats. *The Journal of Neuroscience* 2005;25:9102–9111. [PubMed: 16207869]
- Card JP, Rinaman L, Schwaber JS, Miselis RR, Whealy ME, Robbins AK, Enquist LW. Neurotropic properties of pseudorabies virus: uptake and transneuronal passage in the rat central nervous system. *The Journal of Neuroscience* 1990;10:1974–1994. [PubMed: 2162388]
- Caso J, Leza J, Menchen L. The effects of physical and psychological stress on the gastro-intestinal tract: lesson from animal models. *Current Molecular Medicine* 2008;8:299–312. [PubMed: 18537637]
- Coutinho SV, Plotsky PM, Sablad M, Miller JC, Zhou H, Bayati AI, McRoberts JA, Mayer EA. Neonatal maternal separation alters stress-induced responses to viscerosomatic nociceptive stimuli in rat. *Am J Physiol Gastrointest Liver Physiol* 2002;282:G307–G316. [PubMed: 11804852]
- Diego M, Field T, Hernandez-Reif M. Vagal activity, gastric motility, and weight gain in massaged preterm neonates. *Journal of Pediatrics* 2005;147:50–55. [PubMed: 16027695]
- Dodd VL. Implications of Kangaroo Care for Growth and Development in Preterm Infants. *Journal of Obstetric, Gynecologic, and Neonatal Nursing* 2005;34:218–232.
- Dong H-W, Petrovich GD, Swanson LW. Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Research Reviews* 2001a;38:192–246. [PubMed: 11750933]
- Dong H-W, Petrovich GD, Watts AG, Swanson LW. Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. *The Journal of Comparative Neurology* 2001b;436
- Esplagues J, Barrachina M, Beltran B, Calatayud S, Whittle B, Moncada S. Inhibition of gastric acid secretion by stress: a protective reflex mediated by cerebral nitric oxide. *Proceedings of the National Academy of Sciences* 1996;93:14839–14844.
- Feldman R, Eidelman AI. Skin-to-skin contact (kangaroo care) accelerates autonomic and neurobehavioural maturation in preterm infants. *Developmental Medicine and Child Neurology* 2003;45:274–281. [PubMed: 12647930]
- Feldman R, Eidelman AI, Sirota L, Weller A. Comparison of Skin-to-Skin (Kangaroo) and Traditional Care: Parenting Outcomes and Preterm Infant Development. *Pediatrics* 2002;110:16–26. [PubMed: 12093942]
- Flanagan LM, Dohanics J, Verbalis JG, Stricker EM. Gastric motility and food intake after lesions of the hypothalamic paraventricular nucleus. *American Journal of Physiology* 1992a;263:R39–R44. [PubMed: 1322067]
- Flanagan LM, Dohanics J, Verbalis JG, Stricker EM. Gastric motility and food intake in rats after lesions of hypothalamic paraventricular nucleus. *Am J Physiol Regul Integr Comp Physiol* 1992b;263:R39–44.
- Flanagan LM, Olson BR, Sved AF, Verbalis JG, Stricker EM. Gastric motility in conscious rats given oxytocin and an oxytocin antagonist centrally. *Brain Research* 1992c;578:256–260. [PubMed: 1324762]
- Fox K. Anatomical pathways and molecular mechanisms for plasticity in the barrel cortex. *Neuroscience* 2002;111:799–814. [PubMed: 12031405]
- Grassi M, Drago F. Effects of oxytocin on emotional stress and stress-induced gastric lesions. *Journal of Physiology, Paris* 1993;87:261–264.
- Gunion MW, Taché Y. Intrahypothalamic microinfusion of corticotropin-releasing factor inhibits gastric acid secretion but increases secretion volume in rats. *Brain Research* 1987;411:156–161. [PubMed: 3496936]
- Heim C, Newport DJ, Heit S, Graham YP, Wilcox M, Bonsall R, Miller AH, Nemeroff CB. Pituitary-adrenal and autonomic responses to stress in woman after sexual and physical abuse in childhood. *JAMA* 2000;284:592–597. [PubMed: 10918705]

- Heim C, Newport DJ, Mletzko T, Miller AH, Nemeroff CB. The link between childhood trauma and depression: insights from HPA axis studies in humans. *Psychoneuroendocrinology* 2008;33:693–710. [PubMed: 18602762]
- Hermann GE, McCann MJ, Rogers RC. Activation of the bed nucleus of the stria terminalis increases gastric motility in the rat. *Journal of the Autonomic Nervous System* 1990;30:123–128. [PubMed: 1973425]
- Hubel DH, Wiesel TN, LeVay S. Plasticity of ocular dominance columns in monkey striate cortex. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* 1977;278:377–409.
- Kuhn CM, Schanberg SM. Responses to maternal separation: mechanisms and mediators. *International Journal of Developmental Neuroscience* 1998;16:261–270. [PubMed: 9785122]
- Lenz HJ, Raedler A, Greten H, Vale WW, Rivier JE. Stress-induced gastrointestinal secretory and motor responses in rats are mediated by endogenous corticotropin-releasing factor. *Gastroenterology* 1988;95:1510–1517. [PubMed: 2846402]
- Levine S. Developmental determinants of sensitivity and resistance to stress. *Psychoneuroendocrinology* 2005;30:939–946. [PubMed: 15958281]
- Liu D, Caldji C, Sharma S, Plotsky PM, Meaney MJ. Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinephrine release in the hypothalamic paraventricular nucleus. *Journal of Neuroendocrinology* 2000;12:5–12. [PubMed: 10692138]
- Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM, Meaney MJ. Maternal Care, Hippocampal Glucocorticoid Receptors, and Hypothalamic-Pituitary-Adrenal Responses to Stress. *Science* 1997;277:1659–1662. [PubMed: 9287218]
- Liubashina O, Bagaev V, Khotiantsev S. Amygdalofugal modulation of the vago-vagal gastric motor reflex in rat. *Neuroscience Letters* 2002;325:183–186. [PubMed: 12044651]
- Loewy, AD. Central Autonomic Pathways. In: Loewy, AD.; Spyer, KM., editors. *Central Regulation of Autonomic Functions*. New York: Oxford University Press; 1990.
- Luiten PGM, Ter Horst GJ, Karst H, Steffens AB. The course of paraventricular hypothalamic efferents to autonomic structures in medulla and spinal cord. *Brain Research* 1985;329:374–378. [PubMed: 3978460]
- Macri S, Chiarotti F, Wurbel H. Maternal separation and maternal care act independently on the development of HPA responses in male rats. *Behav Brain Res* 2008;191:227–234. [PubMed: 18468700]
- McCain GC, Ludington-Hoe SM, Swinth JY, Hadeed AJ. Heart Rate Variability Responses of a Preterm Infant to Kangaroo Care. *Journal of Obstetric, Gynecologic, and Neonatal Nursing* 2005;34:689–694.
- McCann MJ, Rogers RC. Oxytocin excites gastric-related neurones in rat dorsal vagal complex. *Journal of Physiology* 1990;428:95–108. [PubMed: 2231433]
- McLean IW, Nakane PK. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *Journal of Histochemistry and Cytochemistry* 1974;22:1077–1083. [PubMed: 4374474]
- Meaney MJ. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Annual Reviews Neuroscience* 2001;24
- Merzenich MM, Nelson RJ, Stryker MP, Cynader MS, Schoppmann A, Zook JM. Somatosensory cortical map changes following digit amputation in adult monkeys. *The Journal of Comparative Neurology* 1984;224:591–605. [PubMed: 6725633]
- Nakahara H, Zhang LI, Merzenich MM. Specialization of primary auditory cortex processing by sound exposure in the “critical period”. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101:7170–7174. [PubMed: 15118079]
- Olson BR, Hoffman GE, Sved AF, Stricker EM, Verbalis JG. Cholecystokinin induces cFos expression in hypothalamic oxytocinergic neurons projecting to the dorsal vagal complex. *Brain Research* 1992;569:238–248. [PubMed: 1371708]
- Paxinos, G.; Kus, L.; Ashwell, KWS.; Watson, C. *Chemoarchitectonic Atlas of the Rat Forebrain*. San Diego: Academic Press; 1999.
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academic Press; 1998.

- Plotsky PM, Meaney MJ. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Molecular Brain Research* 1993;18:195–200. [PubMed: 8497182]
- Plotsky PM, Thrivikraman KV, Nemeroff CB, Caldji C, Sharma S, Meaney MJ. Long-term consequences of neonatal rearing on central corticotropin-releasing factor systems in adult male rat offspring. *Neuropsychopharmacology* 2005;30:2192–2204. [PubMed: 15920504]
- Rinaman L. Oxytocinergic inputs to the nucleus of the solitary tract and dorsal motor nucleus of the vagus in neonatal rats. *The Journal of Comparative Neurology* 1998;399:101–109. [PubMed: 9725704]
- Rinaman L. Postnatal development of catecholamine inputs to the paraventricular nucleus of the hypothalamus in rats. *The Journal of Comparative Neurology* 2001;438:411–422. [PubMed: 11559897]
- Rinaman L, Card JP, Enquist LW. Spatiotemporal responses of astrocytes, ramified microglia, and brain macrophages to central neuronal infection with pseudorabies virus. *Journal of Neuroscience* 1993;13:685–702. [PubMed: 8381171]
- Rinaman L, Levitt P, Card JP. Progressive postnatal assembly of limbic-autonomic circuits revealed by central transneuronal transport of pseudorabies virus. *The Journal of Neuroscience* 2000;20:2731–2741. [PubMed: 10729354]
- Rinaman L, Roesch MR, Card JP. Retrograde transsynaptic pseudorabies virus infection of central autonomic circuits in neonatal rats. *Developmental Brain Research* 1999;114:207–216. [PubMed: 10320760]
- Sanders BJ, Anticevic A. Maternal separation enhances neuronal activation and cardiovascular responses to acute stress in borderline hypertensive rats. *Behavioural Brain Research* 2007;183:25–30. [PubMed: 17604851]
- Sawchenko PE. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* 1982;205:260–272. [PubMed: 6122696]
- Sawchenko PE. Evidence for differential regulation of corticotropin-releasing factor and vasopressin immunoreactivities in parvocellular neurosecretory and autonomic-related projections of the paraventricular nucleus. *Brain Research* 1987;437:253–263. [PubMed: 3325130]
- Selye H. A syndrome produced by diverse nocuous agents. *Nature (London)* 1936;138:32.
- Swanson, LW. *Brain Maps: Structure of the Rat Brain*. San Diego: Academic Press; 2004.
- Taché Y, Martinez V, Million M, Wang L. Stress and the Gastrointestinal Tract. III. Stress-related alterations of gut motor function: role of brain corticotropin-releasing factor receptors. *American Journal of Physiology Gastrointestinal and Liver Physiology* 2001;280:G173–G177. [PubMed: 11208537]
- Vertes RP. Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse* 2004;51:32–58. [PubMed: 14579424]
- Watson RE Jr, Weigand SJ, Clough RW, Hoffman GE. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* 1986;7:155–159. [PubMed: 3520509]
- Winkelman-Duarte EC, Todeschin AS, Fernandes MC, Bittencourt LC, Pereira GAM, Samios VN, Schuh AFS, Achaval ME, Xavier LL, Sanvitto GL, Mandarim-de-Lacerda CA, Lucion AB. Plastic changes induced by neonatal handling in the hypothalamus of female rats. *Brain Research* 2007;1170:20–30. [PubMed: 17692831]
- Yamamoto T, Sawa K. Comparison of c-fos-like immunoreactivity in the brainstem following intraoral and intragastric infusions of chemical solutions in rats. *Brain Res* 2000;866:144–151. [PubMed: 10825490]
- Yang M, Card JP, Tirabassi RS, Miselis RR, Enquist LW. Retrograde, transneuronal spread of pseudorabies virus in defined neuronal circuitry of the rat brain is facilitated by gE mutations that reduce virulence. *Journal of Virology* 1999;73:4350–4359. [PubMed: 10196333]
- Zhang X, Cui J, Tan Z, Jiang C, Fogel R. The central nucleus of the amygdala modulates gut-related neurons in the dorsal vagal complex in rats. *J Physiol* 2003;553.3:1005–1018. [PubMed: 14555729]
- Zhang X, Fogel R, Renehan WE. Stimulation of the paraventricular nucleus modulates the activity of gut-sensitive neurons in the vagal complex. *American Journal of Physiology Gastrointestinal and Liver Physiology* 1999;277

Figure 1. 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).

Figure 2.

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.

Figure 3.

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).

Figure 4.

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 5.

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).

Figure 6. 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 7.

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).

Figure 8.

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. 7). Scale bar (in B) = 200 μ m, applies to both panels.

Figure 9.

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. (*, $p < 0.05$)

Table 1

Number of ROIs and ROI Area in Analyzed Brain Regions in NS control and MS15 rats.

Brain Region	Postnatal Group (n)	Number of ROIs (mean $\pm$ SE)	ROI Area ($\times 10^5 \text{ um}^2$, avg. per ROI, mean $\pm$ SE $\times 10^3$)
AP	NS (15)	2.67 $\pm$ 0.13	1.39 $\pm$ 0.29
	MS15 (16)	2.75 $\pm$ 0.14	1.39 $\pm$ 0.60
dBNST	NS (15)	5.87 $\pm$ 0.36	2.21 $\pm$ 2.26
	MS15 (15)	5.47 $\pm$ 0.47	2.08 $\pm$ 2.44
vBNST	NS (15)	6.07 $\pm$ 0.32	1.73 $\pm$ 3.24
	MS15 (15)	5.67 $\pm$ 0.48	1.56 $\pm$ 2.47
CeA	NS (15)	12.07 $\pm$ 0.82	3.89 $\pm$ 3.73
	MS15 (16)	11.88 $\pm$ 0.66	3.80 $\pm$ 2.81
PVN	NS (15)	8.87 $\pm$ 0.51	2.03 $\pm$ 0.85
	MS15 (16)	8.19 $\pm$ 0.38	2.19 $\pm$ 0.71

Values reported are the number of ROIs (left, mean $\pm$ SE) included in data analysis and the ROI area (right, um^2 , average per ROI, mean $\pm$ SE) within each analyzed brain region in NS and MS15 rats. There were no significant differences between postnatal groups (NS and MS15) in either value (See Results). AP, Area postrema; dBNST, dorsal bed nucleus of the stria terminalis; vBNST, ventral bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; PVN, paraventricular nucleus of the hypothalamus