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Stress adaptation upregulates oxytocin within hypothalamo-vagal neurocircuits

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Abstract

Stress plays a pivotal role in the development and/or exacerbation of functional gastrointestinal (GI) disorders. The paraventricular nucleus of the hypothalamus (PVN) contains neurons that are part of the hypothalamic-pituitary-adrenal axis as well as preautonomic neurons innervating, among other areas, gastric-projecting preganglionic neurons of the dorsal vagal complex (DVC). The aim of the present study was to test the hypothesis that stress adaptation upregulates oxytocin within PVN-brainstem vagal neurocircuitry. The retrograde tracer cholera toxin B (CTB) was injected into the DVC of rats which, after post-surgical recovery, were pair-housed and exposed to either homo- or heterotypic stress for five consecutive days. Fecal pellets were counted at the end of each stress load. Two hours after the last stressor, the whole brain was excised. Brainstem and hypothalamic nuclei were analyzed immunohistochemically for the presence of both oxytocin (OXT)-immunopositive cells in identified preautonomic PVN neurons as well as OXT fibers in the DVC. Rats exposed to chronic homotypic, but not chronic heterotypic stress, had a significant increase of both number of CTB+ OXT co-localized neurons in the PVN as well as density of OXT positive fibers in the DVC compared to control rats. These data suggest that preautonomic OXT PVN neurons and their projections to the DVC increase following adaptation to stress, and suggest that the possible up-regulation of OXT within PVN-brainstem vagal neurocircuitry may play a role in the adaptation of GI responses to stress.

Keywords

vagus; brainstem; gastrointestinal motility

INTRODUCTION

Stress plays a significant role in the development of functional gastrointestinal disorders (FGIDs), such as functional dyspepsia (FD) and irritable bowel syndrome (IBS) (Khoo *et al.*,

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2010; Fukudo, 2013; Drossman, 2016), and stressful situations exacerbate gastrointestinal (GI) symptoms in susceptible individuals (Stengel & Tache, 2008). Both acute and prolonged unpredictable stress induce dysfunction of gastric (delayed emptying), colon (accelerated) activity (Tache *et al.*, 2009; Babygirija *et al.*, 2010a; Stengel & Tache, 2010; Babygirija *et al.*, 2011), and increases the severity of acute colitis (Reber *et al.*, 2008).

Tone and motility of the upper GI tract are modulated by vagal neurocircuits within the dorsal vagal complex (DVC), which comprise the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus (DMV) and the area postrema (Travagli & Anselmi, 2016). Visceral sensory information from thoracic and subdiaphragmatic organs, including those arising in the GI tract, are relayed by vagal afferents to the NTS (Altschuler *et al.*, 1989; Andresen & Kunze, 1994; Jean, 2001; Travagli & Anselmi, 2016). NTS neurons integrate this information with inputs from higher centers involved in autonomic control and convey the output to the adjacent vagal preganglionic DMV neurons (Browning & Travagli, 2014; Travagli & Anselmi, 2016). The efferent vagal fibers that originate in the DMV modulate tone and motility of the upper GI tract via activation of either postganglionic cholinergic excitatory or non-adrenergic non-cholinergic (NANC) inhibitory pathways (Grundy & Brookes, 2012; Travagli & Anselmi, 2016).

The activity of pacemaker DMV neurons is regulated by synaptic inputs from the adjacent NTS as well as other central nuclei, such as the paraventricular nucleus of the hypothalamus (PVN) (Browning & Travagli, 2014; Travagli & Anselmi, 2016). Central oxytocin (OXT) is synthesized primarily in the magnocellular and parvocellular subdivisions in the PVN and supraoptic nuclei of the hypothalamus (SON) (Richar *et al.*, 1991). The role of oxytocin neurons of the SON in fear, anxiety, and stress has been described in great detail by many groups (Eliava *et al.*, 2016) (Knobloch *et al.*, 2012), however, SON neurons do not project directly to the vagal complex, indeed the PVN is the sole source of oxytocin projections to the vagal complex (Voorn & Buijs, 1983; Blevins *et al.*, 2003; Browning & Travagli, 2014).

The physiological role of OXT in the DVC is well documented, and include its release following meal ingestion resulting in gastroinhibition via activation of a nitrergic pathway (Richar *et al.*, 1991; Flanagan *et al.*, 1992; Rinaman, 1998; Llewellyn-Smith *et al.*, 2012; Holmes *et al.*, 2013; Browning *et al.*, 2014). Endogenous OXT released locally in the PVN exerts an inhibitory effect on basal and stress induced activity of HPA axis (Neumann *et al.*, 2000; Jurek & Neumann, 2018) and decreases nociception via collateral projections to adjacent magnocellular neurons (Eliava *et al.*, 2016). Parvocellular OXT neurons also innervate brainstem autonomic neurons and are recognized as mediators of autonomic functions (Sawchenko & Swanson, 1982; Richar *et al.*, 1991).

We have shown recently that gastric-projecting vagal neurocircuits undergo a great deal of plasticity as a consequence of either acute or chronic stress (Browning *et al.*, 2014; Jiang *et al.*, 2018). Indeed, acute stress or pretreatment with the prototypical stress hormone, corticotropinreleasing factor (CRF), induces the translocation of OXT receptors to previously unresponsive GABAergic terminals impinging onto DMV neurons (Browning *et al.*, 2014). Similarly, the response to catecholamine-mediated activation of alpha2

adrenoceptors of DMV neurons, and the consequent gastric motility response differs according to the type of chronic stress induction given to the rat (Jiang *et al.*, 2018).

Activation of central oxytocinergic pathways has an anxiolytic effect and is involved in adaptive response to chronic repetitive stress (Neumann *et al.*, 2000; Ebner *et al.*, 2005). In recent years, Takahashi's group has shown that OXT plays a significant role in restoring the delayed gastric emptying and impaired gastric motility following acute and chronic heterotypic stress, and antagonism or knockout of OXT receptors prevents the adaptive GI response following chronic repetitive stress (Babygirija *et al.*, 2010a; Babygirija *et al.*, 2010b; Zheng *et al.*, 2010), suggesting an important role of central oxytocinergic pathways in mediating the response to stress. It is not clear, however, whether the gastric-related anti-stress effects of OXT depend upon its upregulation in the hypothalamic-pituitary-adrenal (HPA) axis, or an upregulation of OXT in vagal neurocircuits.

The aim of the present study was to test the hypothesis that adaptation to stress upregulates oxytocin within PVN-brainstem vagal neurocircuitry.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats (180–260g) were housed in an AAALAC accredited animal care facility, maintained at 24°C on a 12:12 hour light/dark cycle, with food and water provided ad libitum. All procedures were conducted in accordance with the National Institutes for Health guidelines, with the approval of the Penn State University - College of Medicine Institutional Animal Care and Use Committee, and according to the policies and regulations of journal policy on animal experimentation. All efforts were made to minimize the number of animals used and their suffering. The total number of rats used in the present study was 34; 10 in the control, 14 in the chronic homotypic, and 10 in the chronic heterotypic stress group.

CTB injection

Rats were anesthetized with rodent cocktail (ketamine 80mg/kg, acepromazine, 1mg/kg, xylazine 10mg/kg, intramuscular), and placed on a stereotaxic frame. After the DVC was exposed by blunt dissection, four microinjections of the retrograde tracer cholera toxin B (CTB, 0.5% in dH_2O, 60nl each injection) were made with a glass pipette into both sides of the DVC (rostro-caudal: 0.0–0.6mm from calamus scriptorius, medio-lateral: 0.2–0.4 mm from midline; dorso-ventral: 0.5–0.65 mm from the brainstem surface). The wound was closed with 5–0 suture before the animals were returned to their cage and allowed to recover for 10–15 days prior to stress inductions.

Stress paradigms

The stress procedure was used as described in detail in a previous manuscript (Jiang *et al.*, 2018). Briefly, animals were divided randomly in three groups: i) control, ii) chronic homotypic stress (CHo), and iii) chronic heterotypic stress (CHe). Animals were exposed to the following stressor for five consecutive days between 9:00am and 12:00pm: i) animals in the control group were handled each day without further stress induction; ii) rats in the CHo group underwent daily 2 hours of restraint stress in a cylinder that did not allow movement;

iii) rats in the CHe group underwent one type of stress that differed each day throughout the experimental protocol: a) restraint, i.e. 2 hours of restraint in a cylinder that did not allow movement; b) forced swim, i.e. 20 minutes in a container (36Lx26Wx24H cm) filled with water at room temperature, such that their hind paws could not touch the bottom of the container; c) water avoidance, i.e. 90 minutes on a round platform (6cm diameter) in a container with room temperature water at a level just below the top of the platform; d) cold, i.e. 45 min in a cage maintained at 4ºC; and e) repeat restraint stress as in a). For each rat, the fecal pellets excreted during stress induction were counted at the end of each session. Since in both CHo and CHe, the stress load at day 1 and 5 were the same, i.e. 2 hour restraint stress, these data were the ones compared.

Two hours after completion of the last stress induction, rats were anesthetized deeply with isofluorane (5% in air), euthanized via a bilateral pneumothorax, and fixed via transcardial perfusion with 0.1M phosphate buffer saline (PBS) followed by paraformaldehyde (PFA, 4%) in 0.1M PBS. Whole brains were extracted and post-fixed in 4% PFA overnight and then transferred to 0.1M PBS with 20% sucrose for 48 hours. The forebrain containing PVN and the brainstem were frozen and coronal sections (50 μm thickness) were cut using a freezing microtome. The slices were stored in long term storage buffer (PBS 0.1M, sucrose 30%, ethylene glycol 30%) at −20ºC until processed for immunohistochemistry.

Immunohistochemistry

The location and neuronal size were the main characteristics used to distinguish magno- from parvo-cellular hypothalamic neurons. In fact, magnocellular neurons are significantly larger, comprise an anular-like structure in the lateral PVN and project to the posterior pituitary but not to the brainstem, thus do not co-localize with the CTB injected in the dorsal vagal complex.

All immunohistochemical procedures were carried out at room temperature on a shaking platform as described previously (Llewellyn-Smith *et al.*, 2012). The primary antibodies used were guinea pig anti-oxytocin 1:5,000 (OXT; Peninsula Laboratories, San Carlos, CA, USA) and goat anti-CTB 1:100,000 (List Biological Labs, Campbell, CA, USA). Secondary antibodies were biotinylated donkey anti- guinea pig or -goat immunoglobulins (IgG, from Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted at 1:500. The detection complex was ExtrAvidinhorseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:1,500.

Two-color immunoperoxidase labeling was used to localize OXT-IR with a dark blue reaction product, and CTB-IR with a brown reaction in the PVN. Every fourth PVN section was rinsed with 0.1M PBS 3×10min and treated with 30% methanol in hydrogen peroxide for 30min. The sections were permeabilized by 3×10min washes in Tris-buffered PBS containing 0.3% Triton x-100 and 0.05% thimerosal (TPBS). The sections were then exposed to TPBS with 10% normal horse serum for one hour and then incubated in goat anti-CTB primary antibody diluted in TPBS with 10% normal horse serum for three days. The sections were then rinsed with TPBS for 3×10 min and incubated with TPBS containing 1% normal horse serum, followed by secondary antibody which was diluted in TPBS containing 1% normal horse serum for overnight incubation. After rinsing with TPBS

3×10min, the sections were incubated in ExtrAvidin-horseradish peroxidase for four hours. Immunoreactive neurons were visualized with an imidazole-intensified diaminobenzidine reaction with peroxide generated by glucose oxidase and producing a brown color reaction. After a second blocking step in TPBS containing 10% normal horse serum, the sections were incubated in guinea pig anti-OXT primary antibody for four days. After overnight incubation of secondary antibody and final incubation with ExtrAvidin-horseradish peroxidase, a vector SG (Burlingame, CA, USA) reaction with peroxide generated by glucose oxidase produced a dark blue reaction. Sections were washed for 3×10 min in TPBS, mounted on subbed slides, dehydrated, and coverslipped with Cytoseal (Thermo Fisher Scientific, Waltham, MA, USA).

One-color immunoperoxidase labeling was used to localize OXT-IR fibers in the DVC with a black reaction. Every fourth brainstem section was treated as above in the presence of guinea pig antiOXT for three days and stained with an enhanced imidazole-intensified Ni-Co diaminobenzidine reaction.

Data analysis

Images of both PVN and DVC immunoreactive neurons containing CTB- and/or OXT -IR were acquired with a Nikon E400 microscope equipped with a Spot® camera (Sterling Heights, MI, USA). The number of CTB-IR co-localized with OXT neurons were counted bilaterally in hypothalamic sections of the PVN spanning −0.80 to −2.30 mm from bregma as indicated by Paxinos and Watson (Paxinos & Watson, 1986).

Oxytocin immunopositive fibers in the NTS and DMV were acquired at x60 using a Nikon E400 microscope and Neurolucida® software (Microbrightfield Bioscience, Williston, VT, USA). Two to four representative sections of the brainstem were analysed at each rostral, intermediate, and caudal levels of the DVC; the OXT-IR fiber density per area was calculated using NeuroExplorer® (Microbrightfield Bioscience). Both number of neurons as well as fiber density were analysed by a researcher unaware of the treatment.

All data were checked for outliers with scatterplots and their distributions tested for normality using descriptive statistics with Graph-Pad Prism 5.01 (Graph Pad, La Jolla, CA, USA). Correction of skewness and kurtosis was done through natural log transformation of variables prior to hypothesis testing using ANOVA. Data were tested for and found normal distributions and equal variances in the sampled distributions. Student t tests were used for parametric data; inter-group comparisons were analyzed using one-way ANOVA followed by a post-hoc Tukey's comparison. Results are expressed as mean+s.e.mean with a significance defined as $p<0.05$.

RESULTS

CHo rats showed adaptation to stress.

The number of fecal pellets excreted on the first day of stress induction was not significantly different between rats from the homotypic (CHo), 5.2±0.56 (N=14), or from the heterotypic (CHe), 6.2±0.42 (N=10) stress groups ($t_{22}=1.316$ $p>0.05$). At the end of the fifth day, however, the number of fecal pellets expelled by rats from the CHo stress group, 1.5±0.60,

was significantly lower than the number of pellets expelled on day 1 (t_{13}=4.252 $p<0.05$), and lower than the number of fecal pellets secreted by rats from the CHe group, 6.7±1.02 (t_{22}=4.663 $p<0.05$ vs CHo; t_9=0.4596 $p>0.05$ vs day 1), suggesting that stress adaptation occurred in rats in the CHo stress group (fig. 1A).

At the end of the last day of stress, the weight of the rats was similar among the groups, i.e. control 299±8.4g, CHo 282±15.2g, and CHe 292±23.4g (one way ANOVA $F_{2,31}$=0.4591 Tukey's multiple Comparison test $p>0.05$ for all).

PVN preautonomic neurons in the homotypic group have an increased co-localization of CTB and oxytocin immunoreactivity.

The total number of OXT-IR neurons in the PVN was similar among the three groups, i.e. 654±40, 567±32, and 553±29 for control (N=10), CHo (N=8), and CHe (N=6) rats, respectively (one way ANOVA $F_{2,21}$=2.445, Tukey's multiple Comparison test $p>0.05$ for all). There was a negative correlation between the number of OXT-IR neurons in the PVN area and the number of fecal pellet expelled at the end of the session at day 5 (fig. 1B).

By applying retrograde tracing techniques and immunochemical staining with specific antibodies against OXT, we analysed the rostro-caudal span of the PVN for co-localization of CTB labeled and OXT-IR positive neurons, i.e. preautonomic oxytocin-IR neurons projecting to the DVC. No CTB-positive neurons were located in SON (not shown).

As can be noted in the micrographs in figure 2 A-D, most of the CTB-IR neurons were located in the ventral parvocellular PVN, i.e. the area known to project to autonomic medullary areas (Sawchenko & Swanson, 1982). The number of CTB positive neurones/PVN slice was 167±8, 165±15, and 144±10 from control (N=10), CHo (N=8), and CHe (N=6) rats, respectively (one way ANOVA $F_{2,21}$=1.119, Tukey's multiple Comparison test $p>0.05$ for all).

The number of parvocellular PVN neurons that co-localized CTB and oxytocin-IR, however, was significantly higher in CHo rats, i.e. 8±0.9 in control and 12±1.0 co-localized neurons/slice in CHo (t_{16}=2.554 $p<0.05$; fig. 2). Conversely, the number of parvocellular PVN neurons that co-localized CTB and oxytocin-IR were comparable to controls in CHe rats, i.e. 6±0.6 co-localized neurons/slice (t_{14}=1.472 $p>0.05$ vs control; t_{12}=3.951 $p<0.05$ vs CHo; fig. 2E). The total number of CTB-OXT co-localizing neurons, as counted in one every four PVN slices, was 61±6.4, 90±7.6, and 53±5.4 in control, CHo and CHe, respectively. Thus there were approximately 240 and 360 PVN neurons that projected to the vagal complex in control and after CHo, respectively.

These data indicate that in rats that showed adaptation to stress, the number of preautonomic oxytocin-IR neurons that project from PVN to DVC is increased.

The density of oxytocin axons in the DVC is increased in CHo rats.

As described previously (Llewellyn-Smith *et al.*, 2012), OXT-IR axons occurred throughout the rostrocaudal extent of the DVC.

The density of OXT-IR fibers/ DVC area was increased significantly in CHo rats (N=5), i.e. from 58.7±4μm/1000μm^2 in the caudal DVC of controls (N=4) to 87.3±11 μm/1000 μm^2 in the caudal DVC of CHo (t_7=2.469 $p<0.05$; fig. 3). Conversely, the number of oxytocin-IR fibers/DVC area was comparable to controls in the caudal DVC of CHe rats, i.e. 60.3±9μm/1000μm^2 (N=5, t_8=0.2056 $p>0.05$ vs control; fig. 3E). Similar results were also observed in the intermediate portion of the DVC (fig. 3F). The rostral portion of the DVC, however, showed comparable fiber density in all groups, i.e. 62.1±1.6, 76.2±1.0, and 73.3±1.4 μm/1000μm^2 in control, CHo and CHe respectively (data not shown).

These data indicate that in rats that showed adaptation to stress, i.e CHo rats, the density of oxytocin-IR fibers that project from PVN to DVC is increased.

DISCUSSION

In the present study, we have shown that following adaptation to chronic homotypic stress, there is an upregulation of oxytocin neurons and fibers within the PVN-brainstem vagal neurocircuitry. Our data suggest the possibility that these oxytocinergic neurocircuits may play a role in GI adaptation to stress.

Our data in fact indicate a negative correlation between the number of fecal pellets excreted during stress induction and the total number of OXT-IR neurons in the PVN. Indeed, rats that show stress adaptation, and thus excrete fewer fecal pellets, have an increased number of PVN OXT-IR neurons. Furthermore, rats that underwent chronic, repetitive, homotypic stress display a higher number of OXT-IR neurons that co-localize with CTB-IR, and a higher density of OXT-IR fibers in the caudal and intermediate DVC, suggesting that, at least in part, the adaptation to stress is determined by an upregulation of the oxytocinergic PVN- brainstem- vagal neurocircuitry. Previous studies by Takahashi's group showed that adaptation to stress, as measured by gastrointestinal motility, increased OXT mRNA in PVN (Babygirija *et al.*, 2012). It is possible, therefore, that stress adaptation engages neurons in PVN that, under control conditions, either do not express OXT, or express OXT at levels below the detection limit of immunohistochemistry. The role of OXT in the interaction with the HPA axis is well documented and recognized as playing a prominent role in stress/stress adaptation (Windle *et al.*, 1997; Neumann *et al.*, 2000; Dabrowska *et al.*, 2011; Herman *et al.*, 2016; Herman & Tasker, 2016; Jurek & Neumann, 2018), however, the role of OXT projections from the PVN to the DVC and their relation to GI functions related to stress have been investigated to a lesser extent.

It is well accepted that rodents exposed to either acute or chronic variable, i.e. heterotypic, stress show both a delayed gastric emptying and increased colonic motility (Babygirija *et al.*, 2010b; Babygirija *et al.*, 2011). Conversely, rodents exposed to chronic repetitive, i.e. homotypic, stress undergo a series of adaptive changes that restore, within five days, gastric emptying and colonic motility to pre-stress levels (Zheng *et al.*, 2010).

In a series of brilliant experiments, Takahashi's group correlated the GI adaptive changes observed upon chronic homotypic stress to increased levels of OXT-IR and OXT mRNA containing neurons in the PVN (Zheng *et al.*, 2010; Babygirija *et al.*, 2012). Whether the

increased OXT levels modulate the HPA axis or the brainstem autonomic neurons that regulate visceral targets, such as the brainstem vagal neurons innervating the GI tract, was yet unclear.

In the present study, we used retrograde labeling from the DVC to provide direct anatomic evidence that these adaptive changes are, at least in part, determined by increased hypothalamic oxytocinergic projections to the DVC.

OXT-IR axons originating exclusively from the PVN are present in the DVC region at birth, and increase markedly with age (Rinaman, 1998). In adult rats, oxytocin axons occur throughout the rostrocaudal extent of the NTS and DMV, i.e. the DVC, and appose closely to GI-projecting DMV neurons (Llewellyn-Smith *et al.*, 2012). The direct innervation of the DVC by OXT-IR neurons suggests that OXT modulates NTS-DMV neuronal activity, hence vagal GI-regulating circuitry. Indeed, electrophysiological studies have shown that OXT excites a subpopulation of DMV neurons (Raggenbass & Dubois-Dauphin, 1987), and enhances glutamatergic, but not GABAergic, neurotransmission in the NTS and DMV (Peters *et al.*, 2008; Browning *et al.*, 2014), indicating that OXT modulates vagal neurons via both pre- and post-synaptic mechanisms. Furthermore, microinjection of OXT into the DVC induces gastric relaxation through the activation of a postganglionic nitric oxide-mediated pathway (Holmes *et al.*, 2013; Browning *et al.*, 2014), confirming the pivotal role of OXT in modulating gastric functions.

Stress, however, induces neuroplasticity in many brain areas, including the vagal neurocircuits that control GI motility (Herman, 2013; Herman & Tasker, 2016; Travagli & Anselmi, 2016). In naïve, i.e. non-stressed rats, OXT-mediated modulation of the otherwise unresponsive GABAergic currents between NTS and DMV is uncovered by pretreatment with the prototypical stress hormone, corticotropin released factor (CRF) (Holmes *et al.*, 2013; Browning *et al.*, 2014). Indeed, acute exposure of brainstem slices to CRF uncovers an OXT-mediated inhibition of the GABAergic currents impinging on DMV neurons (Browning *et al.*, 2014). Furthermore, OXT microinjection into the DVC of CRF-exposed rats induces a gastric relaxation that is attenuated, abolished, or even reversed compared to the gastroinhibition observed in naïve rats (Browning *et al.*, 2014). This inverted effect of OXT is determined by the involvement of postganglionic VIP- and cholinergic- vagal pathways, indicating that stress induces plasticity and rearrangement of the OXT modulation of vagal neurocircuits controlling GI motility (Holmes *et al.*, 2013; Browning *et al.*, 2014).

Although the mechanisms of chronic stress-induced neuroplasticity that occur in vagal neurocircuits are still largely unknown, there seems to be a common mechanism that rearranges brainstem wiring. Indeed, we have shown recently that the response of these neurocircuits to catecholamines varies according to the type of chronic stress load to which the rats are exposed (Jiang *et al.*, 2018). Here we provide anatomical evidence that OXT projections to the DVC increase following chronic homotypic stress, indicating that the structural plasticity that occurs in response to stress adaptation may contribute to the adaptive behavioral response. The cellular mechanisms and the relationship between such structural plasticity and the response of vagal neurocircuits following chronic stress exposure, however, need further investigation.

Functional gastrointestinal disorders (FGID), such as FD and IBS are defined as disorders of the brain-gut axis, and are strictly correlated with stress. Indeed, stress triggers and exacerbates GI symptoms (Fukudo, 2013; Van & Aziz, 2013; Drossman, 2016). Stressful situations stimulate CRF release, which inhibits gastric motility profoundly through actions that involve vagal motoneurons in the DVC (Lewis *et al.*, 2002; Tache & Bonaz, 2007). The anxiolytic and anti-stress effects of OXT play an important role in attenuating the pathophysiological responses to stress, including restoring impaired GI motility. In fact, central administration of OXT restores impaired gastric emptying and colonic motility following acute stress or chronic stress maladaptation (Zheng *et al.*, 2010; Babygirija *et al.*, 2012). OXT is also involved in the GI-related adaption mechanisms to chronic homotypic stress (Zheng *et al.*, 2010; Babygirija *et al.*, 2011).

Although we report an increased oxytocinergic input to DVC neurons following stress adaptation, the beneficial effects on GI motility by OXT via its action to reduce CRF release from PVN neurons, as well as the prominent systemic effect on the HPA axis, have to be borne in mind (Neumann *et al.*, 2000; Bulbul *et al.*, 2011); Windle et al., 1997; Dabrowska et al., 2011; Herman et al., 2016; Herman & Tasker, 2016; Jurek & Neumann, 2018).

In conclusion, our data provide anatomic evidence of an upregulation of OXT in the hypothalamicvagal neurocircuits that may contribute to restoring GI motility following stress adaptation. Although the physiological effect of those upregulated OXT in GI motility needs further investigation, our data provide new insights into the role played by OXT within the hypothalamovagal neurocircuits in GI-associated stress adaptation.

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Yanyan Jiang and F. Holly Coleman: performed the experiments and analysed the data.

Yanyan Jiang and R. Alberto Travagli: conception and design of experiments.

Yanyan Jiang, Kim Kopenhaver Doheny and R. Alberto Travagli: drafted the article or revised it critically for important intellectual content.

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Abbreviations

OXT	oxytocin
CRF	corticotrophin-release factor
PVN	paraventricular nucleus of the hypothalamus
FD	functional dyspepsia
IBS	irritable bowel syndrome
FGID	functional gastrointestinal disorders

GI	gastrointestinal
DVC	dorsal vagal complex
CTB	cholera toxin B
DMV	dorsal motor nucleus of the vagus
NTS	nucleus tractus solitaries
NANC	non-adrenergic and non-cholinergic
HPA	hypothalamic-pituitary-adrenal axis
CHo	chronic homotypic stress
CHe	chronic heterotypic stress
PBS	phosphate buffer saline
PFA	paraformaldehyde
IgG	immunoglobulin
TPBS	tris-buffered PBS
IR	immunoreactive

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Highlights

- The prototypical anti-stress hormone, oxytocin, has been shown to participate in stress adaptation of the GI tract.
- OXT-IR preautonomic neurons in the PVN were increased in rats that adapted to stress.
- Similarly, in rats that adapted to stress there was an increased density of oxytocin-IR fibers in the dorsal vagal complex.
- The upregulation of oxytocin in the hypothalamic- vagal circuit may play a role in stress adaptation of GI functions.

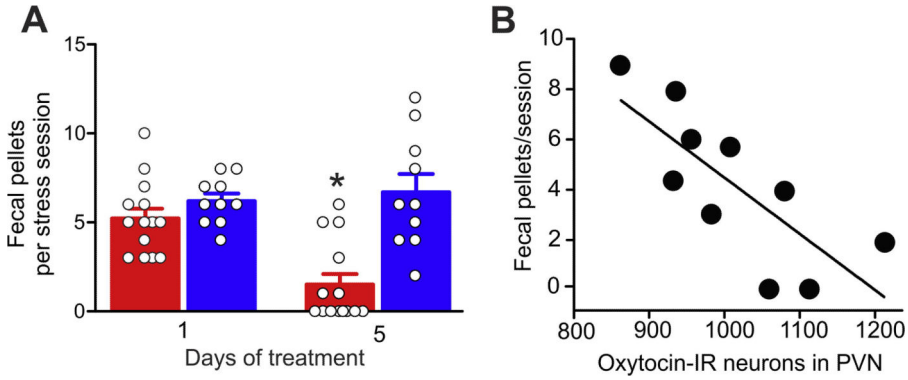

Figure 1. The decreased fecal pellet output in CHo rats correlates with a larger number of oxytocin immunoreactive neurons in the PVN.

A: graphical summary illustrating the number of fecal pellets excreted during the periods of stress loading.

B: graphic representation showing the negative correlation (r^2=0.563) between the number of oxytocin-immunoreactive neurons in the PVN and the fecal pellets during the stress loading. Although the regression was calculated using the entire data set, for reasons of clarity only a few data points have been included in the graph.

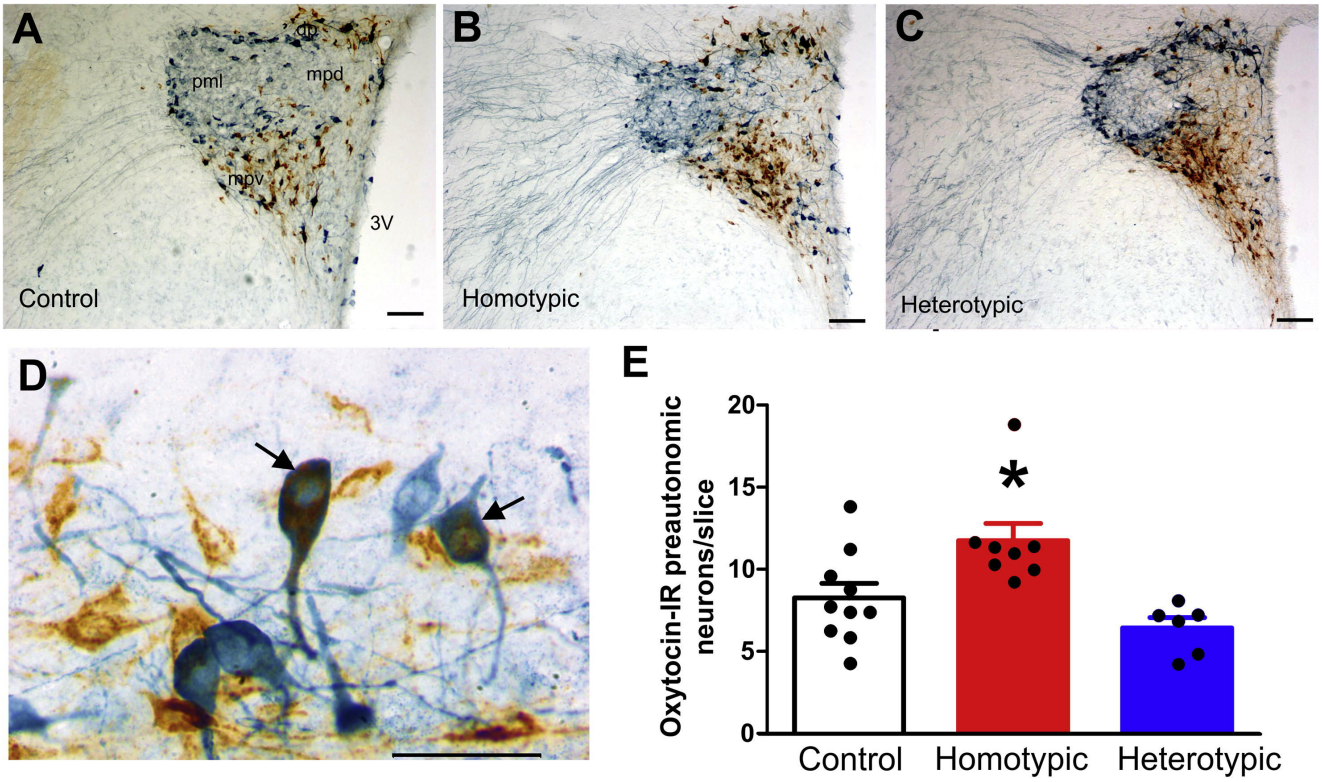

Figure 2. CHo increases co-localization of CTB- and OXT-IR in preautonomic PVN neurons.

A-C: representative micrographs showing oxytocin-immunoreactive (OXT-IR, blue) and CTBimmunoreactive (CTB-IR, brown) neurons in the PVN of control (A), CHo (B), and CHe (C) stressed rats. Bar, 100μm.

D: Enlarged area showing co-localization (arrow) of OXT- and CTB-IR. Bar, 50μm.

E: summary graphic showing the number of OXT-IR preautonomic neurons in the PVN. Note the increased OXT-IR in rats that underwent CHo, but not CHe, stress.

*P<0.05 vs. control and CHe.

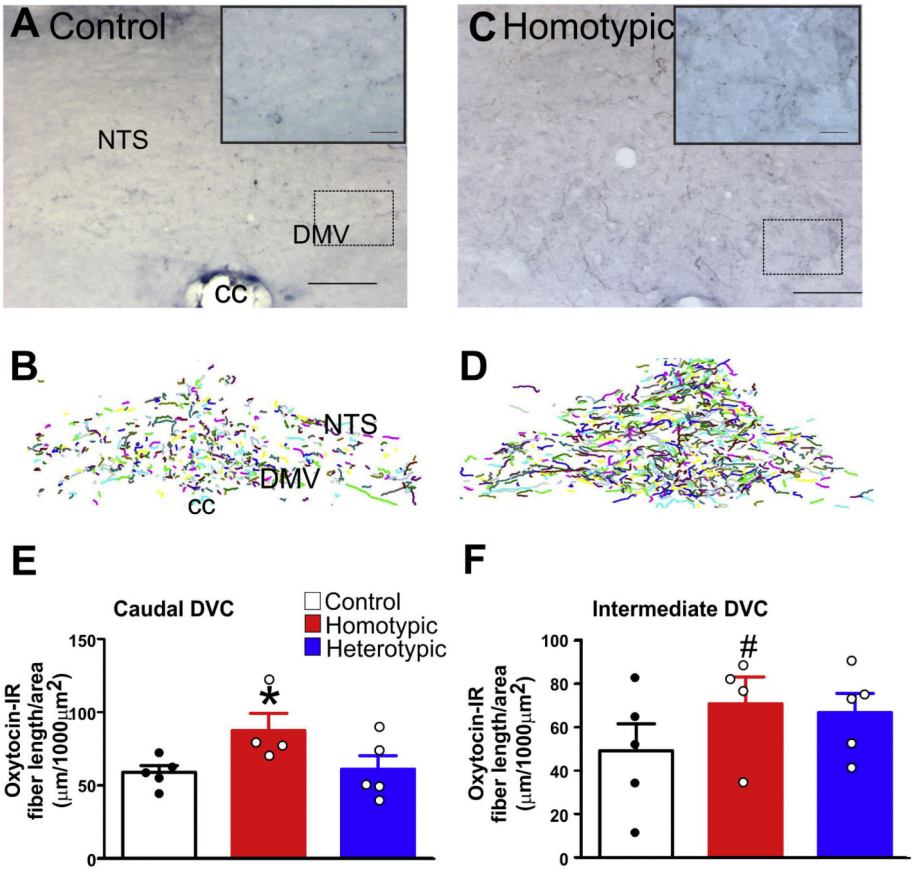

Figure 3. CHo increases the density of OXT axons in the DVC.

A: representative micrographs showing OXT-IR fibers in the dorsal vagal complex (DVC) from a control rat. Insert at the top right shows a higher magnification of the dotted area. Bar, 100μm.

B: representative image showing reconstruction of OXT-IR fiber tracing from the image in A. Bar, 20μm.

C: representative micrographs showing OXT-IR fibers in the DVC from a rat that underwent CHo stress. Insert at the top right shows a higher magnification of the dotted area. Bar, 100μm.

D: representative image showing fiber reconstruction tracing from the image in C. Bar, 20μm.

E-F: summary graphic showing the density of OXT-IR fibers in the caudal (E) and intermediate (F) DVC. Note the increased oxytocin-IR fibers in rats that underwent CHo, but not CHe, stress.

*P<0.05 vs. control and CHe. #P<0.05 vs control.