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CXCL12 sensitizes vago-vagal reflex neurons in the dorsal medulla

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Abstract

Previous studies from our laboratory illustrated the potential for stromal cell-derived factor one [CXCL12; also referred to as SDF-1] to act on its receptor [CXCR4] within the dorsal vagal complex [DVC] of the hindbrain to suppress gastric motility (Hermann et al., 2008). While CXCR4 receptors are essential for normal brain development, they also play a critical role in the proliferation of the HIV virus and initiation of metastatic cell growth in the brain. Anorexia, nausea, and failed autonomic regulation of gastrointestinal function are significant causes of morbidity and are contributory factors in the mortality associated with these disease states. The implication of our previous study was that CXCL12 caused gastric stasis by acting on gastric reflex circuit elements in the DVC. This hindbrain complex includes vagal afferent terminations in the solitary nucleus, neurons in the solitary nucleus (NST) and visceral efferent motoneurons in the dorsal motor nucleus (DMN) that are responsible for the regulation of digestive functions from the oral cavity to the transverse colon. In the current study, in vivo single-unit neurophysiological recordings from physiologically-identified NST and DMN components of the gastric accommodation reflex show that while injection of femtomole doses of CXCL12 onto NST or DMN neurons has no effect on their basal activity, CXCL12 amplifies the effect of gastric vagal mechanosensory input to activate the NST and, in turn, inhibit DMN motor activity.

Keywords

hindbrain; chemokines; gastric; vagus; single unit

1. Introduction

Chemokine receptors (e.g., CXCR4) in the brain are emerging as pleiotropic regulators of brain function in normal and pathological conditions (Banisadr et al., 2002; Banisadr et al., 2003). Several lines of evidence have shown that successful brain development is critically dependent on the CXCR4 receptor (Li and Pleasure, 2005; Lu et al., 2002). This agonist (CXCL12) and receptor (CXCR4) combination is also involved in neurological disorders associated with multiple sclerosis, Alzheimer's, and Parkinson's diseases (Glabinski et al., 2000; Ransohoff et al., 1996). The CXCL12 / CXCR4 interaction also appears to be critical in the initiation and maintenance of glial metastasis (Gabuzda and Wang, 2000).

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Furthermore, the CXCR4 receptor may serve as the portal of entry by the HIV virus into neurons and glia in acquired immune deficiency syndrome (Gabuzda and Wang, 2000). CXCL12 and CXCR4 are also implicated in neural, glial and vascular remodeling after ischemic brain injury (Wang et al., 2012). While CXCL12 is constitutively expressed at low-levels in the CNS, hypoxia significantly activates the chemokine's expression and release from astrocytes (Li and Ransohoff, 2008).

CNS neuroinfection, metastasis, and ischemic stroke are also accompanied by a morbidity syndrome variously comprised of nausea, anorexia, emesis and failed gastrointestinal transit (Cheema et al., 2001; Frank et al., 1989; Kaufman et al., 1986; Plata-Salaman, 2000; Schaller et al., 2006). This observation suggests that a site of action of CXCL12 may be within the neural circuitry involved in the control of gastric function. The medullary circuits responsible for coordinating autonomic reflex control of the gut are contained within the dorsal vagal complex [DVC] of the hindbrain. The DVC is composed of the nucleus of the solitary tract [NST], which receives the general visceral afferent input from the afferent vagus, and the dorsal motor nucleus of the vagus [DMN], which is the principal source of parasympathetic efferent control over the gastrointestinal tract. The NST is an important organizer and processor of visceral afferent activity and it regulates gastrointestinal function via vago-vagal reflex connections with the DMN [see (Rogers and Hermann, 2012) for review]. The NST is also critically important for the production of emesis (Andrews and Horn, 2006). Indeed, the perception of nausea associated with emesis may be the awareness of the profound gastric relaxation that precedes the emetic act (Andrews et al., 1990; Andrews and Horn, 2006; Hornbuckle and Barnett, 2000; Miller, 1999; Wolf, 1943).

Our previous study (Hermann et al., 2008) supported a connection between the CXCR4 receptor in the DVC and modulation of gastric function. Both visceral sensory NST neurons and parasympathetic efferent DMN neurons exhibited strong immunohistochemical labeling for the CXCR4 receptor. Additionally, application of CXCL12 to the DVC evoked a reduction in gastric motility paired with increases in cFOS activation of cells in the NST (Hermann et al., 2008). The implication of those results was that CXCL12 may cause an increase in the sensitivity of gastric vago-vagal reflexes by acting on the neurons in NST and/or the DMN; thus explaining the gastroinhibition caused by CXCL12. Therefore, the present study was designed to investigate the effects of CXCL12 on NST and DMN neurons that are first physiologically-identified as belonging to a circuit responsible for controlling gastric relaxation.

2. Results

CXCL12 effects on identified gastric reflex NST and DMN neurons

As we have reported earlier (McCann and Rogers, 1992; Viard et al., 2012), gastric-NST neurons are essentially silent unless driven by afferent input. In contrast, gastric-DMN neurons are spontaneously active and this activity is suppressed by afferent activity [Figure 1A]. For example, mild distension (0.5ml) of the antral stomach with the gastric balloon evokes a crisp train of action potentials in gastric-NST neurons while eliciting a “mirror image” response in gastric-DMN neurons [Figure 2B], i.e., a significant reduction in ongoing activity time-locked to the distension (i.e., GAR response). The GAR response was determined under both basal and experimental condition for each identified neuron, therefore each cell served as its own control [Figure 1B, 2C].

Note that proof of single unit recording cannot be made from the slow spike train trace used to evaluate the neuron's response to the GAR [e.g., Figures 1 and 2] as the displayed spike amplitudes may appear to vary as a result of low sample rate aliasing. Therefore, to verify that neurophysiological data were obtained from the same, identified, individual neuron,

spike records were re-analyzed at a faster rate to show multiple, superimposed spikes obtained before, during, and after the period of gastric distension. Based on the uniformity of the overlapping waveforms [Figure 3], this method can clearly differentiate between single units such as the NST cell shown in Figure 1 and the DMN cell presented in Figure 2.

The percentage change in response to the same stimulus under these two conditions represents the “relative sensitization” of the neuron as a consequence of its exposure to agonist and/or antagonist [Figure 4]. Nano-injections of CXCL12 [10femtomoles] had no effect on the basal activity of either gastric-NST or -DMN neurons; e.g., see Figure 2A. One-way analysis of variance of the relative sensitization scores of either gastric-NST or -DMN neurons were statistically significant [DMN: $F_{3,55} = 7.9$; $p = 0.0002$; NST: $F_{3,44} = 5.2$; $p = 0.004$]. Dunnett’s post hoc tests using the “saline” group as the point of comparison revealed that both gastric-NST and -DMN neurons were significantly sensitized by CXCL12 [$p < 0.05$; Figure 4]. Note that the CXCL12 antagonist, AMD3100, had no effect of its own to affect changes in the sensitivity of NST or DMN neurons. However, AMD3100 injected onto either NST or DMN neurons completely blocked the sensitizing effect of CXCL12 [Figure 4].

3. Discussion

Earlier reports indicate that the CXCR4 receptor is expressed in the NST and DMN (Banisadr et al., 2002); dorsal medullary areas known to be important to the regulation of gastrointestinal function. Our previous studies (Hermann et al., 2008) demonstrated that: essentially every neuron in the NST and DMN has the CXCR4 receptor; direct injection of CXCL12 into the DVC caused a significant and dose dependent elevation in cFOS expression in the NST. Activation of the NST causes, through its connections with the DMN, significant reductions in gastric tone, motility and gastric transit; parameters normally observed in the satiated state (Bulatao and Carlson, 1924; Cato et al., 1990; Moran and Kinzig, 2004). However, severely depressed gastric tone, motility and transit are perceived as nausea and often herald emesis (Andrews et al., 1990; Andrews and Horn, 2006; Hornbuckle and Barnett, 2000; Miller, 1999; Wolf, 1943). Nanoinjection of CXCL12 injected into the DVC causes an immediate decline in stimulated gastric motility and tone (Hermann et al., 2008) not unlike that elicited by the early, proinflammatory cytokine, TNF α , which is another pro-nausea, anti-motility agent (Hermann and Rogers, 1995; Hermann and Rogers, 2008; Rogers and Hermann, 2012).

The present in vivo electrophysiological results suggest that CXCL12 acts as a positive neuromodulator on DVC neurons that are involved in gastric motility control. That is, CXCL12 has no apparent effect to increase the firing rate of NST neurons or decrease the firing of DMN neurons, per se. However, CXCL12 increases the reflex responsiveness of these vago-vagal reflex neurons; this effect can explain how this chemokine can produce gastric stasis.

CXCL12, the natural agonist for the CXCR4 receptor, is released as a consequence of several neuropathological processes, especially neuroinfection and metastasis (Gabuzda and Wang, 2000; Glabinski et al., 2000; Ransohoff et al., 1996). CXCL12 has been implicated in the onset of dementia and in movement disorders that occur in the late stages of these disease states. Our results suggest that the gastric stasis, nausea and anorexia that occur during neuroinfection and metastasis could be caused, in part, by CXCL12 action to sensitize brainstem vago-vagal control circuits in the dorsal medulla.

4. Experimental Procedure

4.1 Animal care and preparation

Male or female Long-Evans rats [N = 38; 200-500g body weight], obtained from the breeding colony located at Pennington Biomedical Research Center, were used in these studies. All animals were maintained in a room with a 12:12 hour light-dark cycle with constant temperature and humidity, and given food and water ad libitum. All experimental protocols were performed according to the guidelines set forth by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees at the Pennington Biomedical Research Center.

Rats were adapted to a palatable liquid diet [Ensure] two days prior to the experiment. The liquid diet helped facilitate complete gastric emptying prior to instrumentation of the stomach on the day of the experiment. The night before the experiment, the rat was fasted (approximately 16hr fast). Animals were anesthetized with thiobutabarbital (Inactin®, Sigma, 150 mg/kg, intraperitoneal). This anesthetic was chosen for its lack of interference with autonomic reflexes (Buelke-Sam et al., 1978). A cannula was secured in the trachea to ensure a free airway throughout the duration of the experiment. A midline abdominal laparotomy allowed access to the stomach and adjacent portion of the duodenum. A small balloon, made from the little finger of a surgical glove, was then inserted through the pylorus and secured in the antrum of the stomach. This gastric balloon was attached to a piece of Silastic tubing (0.065" O.D.) which was exteriorized through the duodenal incision and secured via purse-string ligature. The midline abdominal opening was then sutured closed with the gastric balloon tubing exiting via the incision.

The instrumented rat was then mounted in a stereotaxic frame. A midline incision was made in the scalp; the musculature retracted to expose the foramen magnum. The occipital plates were removed; foramen magnum was opened and removal of the dura and arachnoid membranes allowed exposure of the floor of the fourth ventricle.

The gastric balloon was then attached to a pressure transducer (Isotec, Harvard Instruments) to monitor balloon pressure. A 1ml syringe attached to the pressure transducer permitted delivery of air (0.1 to 1.0ml) to the gastric balloon for measured stimulation of gastric mechanoreceptors.

4.2 Extracellular electrophysiological recordings

4.2.1 Micropipette preparation—Triple-barrel micropipettes were used for the electrophysiological recordings as previously described (Emch et al., 2000; Emch et al., 2002; Viard et al., 2012). The center micropipette (1micron tip diameter; filled with 2M NaCl plus 1% pontamine sky blue for iontophoretic marking of recording sites) was used in the identification and recording of neuronal activity of cells in the NST and DMN. The other two micropressure injection pipettes [10micron tip diameters], were used to deliver agonists (CXCL12; Peprotech, Rocky Hill NJ) or antagonists (AMD3100; Sigma-Aldrich, St Louis, MO) in nanoliter volumes. These microinjection pipettes were glued to the recording pipette such that the recording pipette extended beyond the microinjection pipettes by ~50microns. The volume of fluid ejected from the micropressure pipettes was measured directly by monitoring the movement of the fluid meniscus in the pipette with a microscope equipped with a reticle calibrated in nanoliter units (Emch et al., 2000; Emch et al., 2002; Viard et al., 2012).

Extracellular signals from the recording micropipette were amplified [5000X; WPI DAM 50 Differential Amplifier] and band-pass filtered [300-3000Hz; Warner Instruments LPF 202A]

before being displayed on an oscilloscope and stored for later analysis on an AM Systems LabChart 7 PC-based waveform analysis system [AD Instruments].

4.2.2 Identification of gastric reflex NST and DMN neurons—Antral distension either physiologically by chyme or experimentally by a gastric balloon initiates a vago-vagal gastric relaxation reflex referred to as the gastric accommodation reflex [GAR]. Antral distension activates vagal afferent mechanosensors that, in turn, activate second order NST neurons in the hindbrain. Normally quiescent NST neurons that are activated by gastric distension inhibit spontaneously active DMN neurons, thus removing cholinergic activation from the gastric enteric plexus (McCann and Rogers, 1992; McCann and Rogers, 1994; Rogers et al., 1995; Rogers and Hermann, 2012). In response, the proximal stomach then relaxes in proportion to the magnitude of the initial distension (Grundy and Schemann, 2002; Rogers and Hermann, 2012).

The compound micropipette was directed toward the dorsal vagal complex (approx. 300microns anterior to calamus and 300microns off midline) in the hindbrain with the aid of an hydraulic microdriver (David Kopf instruments). NST neurons are encountered between 250-600 microns below the brainstem surface. Gastric vago-vagal reflex NST neurons are readily identified by their brisk response to a 0.5mL inflation of the antral balloon (McCann and Rogers, 1992) (Viard et al., 2012). Gastric vagal DMN neurons are located immediately ventral to the NST; are spontaneously active [0.1 – 5Hz] and are abruptly inhibited by antral inflation (McCann and Rogers, 1992; Viard et al., 2012).

Proof of single unit recording cannot be made from the slow spike train trace used to evaluate the neuron's response to the GAR [see Figures 1 and 2] as the displayed spike amplitudes may appear to vary as a result of low sample rate aliasing. Therefore, to verify that neurophysiological data were obtained from the same, identified, individual neuron, spike records were re-analyzed at a faster rate to show multiple, superimposed spikes obtained before, during, and after the period of gastric distension. Based on the uniformity of the overlapping waveforms [Figure 3], this method can clearly differentiate between single units such as the NST cell shown in Figure 1 and the DMN cell presented in Figure 2. Note that this method readily identifies multi-unit recordings of identified neurons of similar amplitudes [Figure 3C; these multi-unit data were not used for analyses.]

4.3 Experimental Design

4.3.1 Effects of CXCL12 on NST or DMN neurons involved in the GAR—Gastric NST or DMN neurons were initially identified, as described above, by recording neuronal responses to 0.5ml gastric distensions over 10sec. [Our previous study has demonstrated that 0.5ml distension volume elicits an approximately half-maximal response by gastric-DVC neurons (Viard et al., 2012).] This elicited response to gastric distension served as the basal GAR response of the identified neuron for comparison purposes [Figures 1 and 2]. After this physiological identification of the neuron, saline was microinjected [1nL] onto the gastric-NST or gastric-DMN neuron and the GAR was evaluated again to ensure there was no volume effect of saline on its response. Several minutes later, 10femtomoles total dose CXCL12 [1nL of 10uM] was microinjected into the same location. After monitoring the basal neuronal firing rate [FR] of the identified neuron for approximately 1min, antral gastric distension stimulation was again applied for 10sec and the resultant FR of the identified neuron was recorded. The percentage change in FR of the gastric-NST or -DMN neuron in response to gastric distension was compared between the pre- and post-CXCL12 microinjection to determine if there was modification of the GAR reflex; thus each cell served as its own control [Figure3].

4.3.2 Specificity of CXCL12 effect on gastric-NST or –DMN neurons GAR; effect of CXCL12 antagonist, AMD3100—

In this set of experiments, all surgical and instrumentation preparations were made as described above. However, in this case the micropipette was filled with either the antagonist of CXCL12 (AMD3100; 0.3mM; Sigma-Aldrich, St Louis, MO) or the combination of CXCL12 plus AMD3100. [The concentration of AMD3100 used in these studies was derived from the effective agonist/antagonist ratio [3:100] determined by Schols (Schols et al., 1997) in their in vitro observations concerning AMD3100 ability to block the calcium fluxes and chemotaxis in THP-1 cells elicited by CXCL12.]

It was important to verify that microinjection of the antagonist, AMD3100 [1nL; 0.3mM] alone onto identified cells did not have an independent role in the sensitization of the gastric-NST and –DMN neurons. Additionally, to replicate the same effective dose of CXCL12 as seen in the preceding experiments, the total injection volume of the combination of agonist plus antagonist was 2nL. Thus, similar to the procedure described above, after the basal response to antral distension was determined, saline was microinjected onto the identified neuron and the evoked GAR was determined. Basal neuronal FR of the identified neuron was monitored for approximately 1min, either AMD3100 alone or the agonist/antagonist combination was microinjected onto the identified neuron and the evoked GAR was evaluated. The percentage change in FR of the gastric-NST or –DMN neuron in response to antral distension was compared between the pre- and post- CXCL12/AMD3100 injection [OR AMD3100 alone] to determine if there were changes in the GAR reflex. Thus, each cell served as its own control.

At the end of the recording session, the final location of the identified gastric neurons could be marked by applying 2uA positive direct current for 1min to the pontamine-filled recording micropipette using a stimulus isolation unit (WPI, Sarasota, FL, USA). The anesthetized rat was transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. The brainstem was removed and post-fixed overnight in 4% paraformaldehyde, then cryoprotected in 10% sucrose solution. Histological sections [40micron] were cut on a freezing microtome, mounted onto slides and photographed with a Nikon E800 microscope equipped with a Jenoptic C7 camera. Thus, in addition to our physiological identification of gastric-related neurons, we could verify the location of our recording tracks.

4.4 Data Analysis

The evoked response [i.e., change in FR] of gastric-NST or –DMN neurons to antral distension was determined under basal conditions and then compared to the evoked response after exposure to either saline, CXCL12 alone, AMD3100 alone, or the combination of CXCL12+AMD3100. The percentage change in FR of the identified neuron in response to this stimulus was compared to the response under basal conditions; that is, each cell served as its own control. This comparison of magnitude of responses was meant to represent the “relative sensitization” of the neuron as a consequence of its exposure to agonist and/or antagonist. Thus, if there was no change in FR (as seen with those neurons exposed to saline), the relative sensitization of the reflex would be “1.0” [Figure 3]. These relative sensitization values were subjected to a one-way analysis of variance; p values < 0.05 were considered statistically significant. Dunnet’s post-hoc tests with the saline group used as control were used.

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Abbreviations

DMN	dorsal motor nucleus of the vagus
DVC	dorsal vagal complex
GAR	gastric accommodation reflex
nL	nanoliter
NST	nucleus of the solitary tract
TNF	tumor necrosis factor

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Highlights

- Chemokine CXCL12 acts in hindbrain to suppress gastric motility
- Physiologically-identified NST and DMN neurons are sensitized by CXCL12
- Net effect of CXCL12 is to amplify gastric accommodation reflex

Figure 1.

An example of raw neurophysiological data from a gastric-NST neuron responding to gastric distension. [Note: Demonstration of verification that data are obtained from the same, individual neuron is seen in Figure 3].

Gastric-NST neurons are typically quiescent until stimulated. In these experiments, 0.5ml distension of the antral balloon for 10sec was used to activate gastric-NST neurons. Each identified neuron served as its own control. **[A]** Microinjection of 1nL saline into the NST did not affect the responsiveness of gastric-NST neurons to the antral distension stimulus. **[B]** Microinjection of 1nL CXCL12 [10femtomole total dose] increased the responsiveness of the identified gastric-NST neurons. Ten second scale bars in A and B denote onset and offset of gastric distension. Quantitated results from all NST neurons examined are presented in Figure 3A.

Figure 2.

Example of raw neurophysiological data from a gastric-DMN neuron responding to gastric distension. [Note: Demonstration of verification that data are obtained from the same, individual neuron is seen in Figure 3].

Most gastric-DMN neurons display ongoing basal activity rates that are inhibited in response to gastric stimulation. **[A]** Basal activity of gastric-DMN neurons were not affected by 1nL nanoinjections of either saline or CXCL12 [10femtomole total dose]. Scale bars are 2mV by 10sec.

In these experiments, gastric stimulation was mimicked by 0.5ml distension of the antral balloon for 10sec. This stimulus elicited an inhibition of gastric-DMN neuronal firing rate. Each identified neuron served as its own control. **[B]** Microinjection of 1nL saline into the DMN did not affect the responsiveness of gastric-DMN neurons to the antral distension stimulus. **[C]** Microinjection of 1nL CXCL12 [10femtomole total dose] increased the responsiveness of the identified gastric-DMN neurons. Scale bars [10sec] in B and C denote onset and offset of gastric distension. Quantitated results from all DMN neurons are presented in Figure 3B.

Figure 3.

Verification that data are obtained from the same, individual neuron. Proof of single unit recording cannot be made from the slow spike train trace used to evaluate the neuron's response to the GAR [see Figures 1 and 2] as the displayed spike amplitudes may appear to vary as a result of low sample rate aliasing. Therefore, spike records are re-analyzed at a faster rate to show multiple, superimposed spikes obtained before, during, and after the period of gastric distension. Based on the uniformity of overlapping waveforms, this method can clearly differentiate between single units such as [A] NST cell shown in Figure 1 and [B] the DMN cell presented in Figure 2 and [C] multi-unit recordings of identified neurons of similar amplitudes. These multi-unit data were not used.

Figure 4.

Quantification of changes in responsiveness of gastric-NST or -DMN neurons due to CXCL12.

The GAB response was determined under both basal and experimental condition for each identified neuron, therefore each cell served as its own control [see examples of raw data in Figures 1 and 2]. The percentage change in response to the same stimulus under these two conditions represents the “relative sensitization” of the neuron as a consequence of its exposure to agonist and/or antagonist. Nano-injections of CXCL12 [10femtomoles] had no effect on the basal activity of either gastric-NST or -DMN neurons; e.g., see Figure 2A. One-way analysis of variance of the relative sensitization scores of either gastric-NST or -DMN neurons were statistically significant [DMN: $F_{3,55} = 7.9$; $p = 0.0002$; NST: $F_{3,44} = 5.2$; $p = 0.004$]. Dunnett’s post hoc tests using the “saline” group as the point of comparison revealed that both gastric-NST and -DMN neurons were significantly sensitized by CXCL12 [$*p < 0.05$]. The CXCL12 antagonist, AMD3100, had no effect of its own to affect changes in the sensitivity of NST or DMN neurons. However, AMD3100 injected onto either NST or DMN neurons completely blocked the sensitizing effect of CXCL12.