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TNF activates astrocytes and catecholaminergic neurons in the solitary nucleus: implications for autonomic control

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Abstract

Tumor necrosis factor [TNF] produces a profound anorexia associated with gastrointestinal stasis. Our work suggests that the principal site of action of TNF to cause this change in gastric function is via vagal afferents within the nucleus of the solitary tract [NST]. Excitation of these afferents presumably causes gastric stasis by activating downstream NST neurons that, in turn, suppress gastric motility via action on neurons in the dorsal motor nucleus of the vagus that project to the stomach. Results from our parallel studies on gastric vago-vagal reflexes suggest that noradrenergic neurons in the NST are particularly important to the generation of reflex gastroinhibition. Convergence of these observations led us to hypothesize that TNF action in the NST may preferentially affect putative noradrenergic neurons. The current study confirms our observations of a dose-dependent TNF activation of cells [as indicated by cFOS production] in the NST. The phenotypic identity of these TNF-activated neurons in the NST was ~29% tyrosine-hydroxylase [TH]-positive [i.e., presumably noradrenergic neurons]. In contrast, less than 10% of the nitrergic neurons were activated after TNF exposure. Surprisingly, another 54% of the cFOS activated cells in the NST were phenotypically identified to be astrocytes. Taken together with previous observations, the present results suggest that intense or prolonged vagal afferent activity [induced by visceral pathway activity, action of gut hormones or cytokines such as TNF] can alter local astrocyte immediate early gene expression that, in turn, can provoke long-term, perhaps permanent changes in the sensitivity of vagal-reflex circuitry.

Keywords

Cytokines; vagus; hindbrain; autonomic; gastric function

Introduction

Tumor necrosis factor_α [TNF] is an early proinflammatory cytokine. It not only initiates a cascade of cytokine release that recruits immune effector cells but is also involved in multiple physiological and behavioral consequences of immune activation that include fever, fatigue, loss of appetite, gastric stasis, nausea, emesis, hypersensitivity to touch or pain, and significant changes in sleep patterns (Hermann et al., 2005; Turrin and Plata-Salaman, 2000). Together, these “illness behaviors” represent an attempt by the host to marshal its resources during an episode of immune activation. Systemic injection of TNF can mimic many of the autonomic

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signs associated with illness including gastric hypomotility, suppressed appetite, nausea, and vomiting (Hermann et al., 2005).

The dorsal vagal complex (DVC) of the hindbrain constitutes the basic neural circuitry of the vago-vagal reflex control of gastrointestinal function (Rogers et al., 2005) and is composed of the sensory nuclei of the solitary tract (NST) and the area postrema (AP) and their interconnections with the motor nucleus of the vagus (DMV) (Rogers et al., 1995). Not surprisingly, this region of the hindbrain is also essential for the generation of emesis (Andrews et al., 1990; Blessing, 1997).

The DVC possesses characteristics of a circumventricular organ (Broadwell and Sofroniew, 1993; Gross et al., 1990). Additionally, dendrites from neurons of the DVC penetrate the ependymal layer and enter the floor of the fourth ventricle (Rogers and McCann, 1993; Shapiro and Miselis, 1985). Thus, the DVC is in position to monitor blood- and cerebrospinal fluid-borne chemicals such as TNF and evoke appropriate physiological responses such as gastric stasis (Hermann et al., 2005).

Our previous studies [for overall review see (Hermann and Rogers, 2007)] have shown that TNF injected directly into the DVC causes a substantial reduction in gastric tone (Hermann and Rogers, 1995); activates cells in the NST and this activation is dependent on an intact glutamate signaling system (Emch et al., 2000; Emch et al., 2001). Peripherally-generated TNF evokes gastric stasis by acting within the DVC (Hermann et al., 1999; Hermann et al., 2002; Hermann et al., 2003). TNF receptors are highly concentrated on vagal afferent fibers and terminals in the NST; and TNF potentiates central vagal afferent signaling (Hermann et al., 2004; Rogers et al., 2006).

Studies of the mechanics of reflex control of the stomach indicate that noradrenergic neurons in the NST and their connections with gastric vagal efferent neurons are particularly important to inhibitory vago-vagal control of the stomach (Rogers et al., 2005). Thus, TNF action within the NST could preferentially affect noradrenergic neurons. However, nitroergic neurons in the DVC have also been implicated in gastroinhibitory control (Krowicki et al., 1997). Both phenotypes of NST neurons could be involved in feeding regulation (Rinaman, 2003) and, possibly, anorexia. Therefore, TNF-induced gastric stasis may be attributable to activation of either adrenergic or nitroergic neuronal phenotypes in the DVC.

This paper examines the hypothesis that TNF specifically activates catecholaminergic neurons in the NST to affect the gastric stasis evoked by the central actions of this peptide that we have demonstrated in the past (Beattie et al., 2002; Emch et al., 2000; Emch et al., 2001; Emch et al., 2002; Hermann and Rogers, 1995; Hermann et al., 2004; Hermann et al., 2001b; Hermann et al., 1999; Hermann et al., 2002; Hermann et al., 2003; Rogers et al., 2006).

Results

Direct unilateral nanoinjection of TNF α into the DVC provokes cFOS activation in a dose-dependent fashion both ipsi- and contralaterally to the injection site [Figure 1; ANOVA: $F_{7,598} = 95.98$; $P < 0.0001$]. cFOS activation counts in the NST, on both the ipsi- and contralateral sides relative to the injection, induced by either 70 or 700pg TNF were significantly greater than that seen with PBS [Dunnett's post-hoc test; $p < 0.05$]. These results compare favorably to our earlier studies of TNF effects to stimulate cFOS in the NST (Emch et al., 2001) and provide validation for the present study.

Phenotypic identification of cells in the NST was limited initially to immunohistochemical staining for TH+ (catecholamine) and nNOS+ (nitroergic) neurons. Preliminary observations suggested that many cFOS positive cells that were neither TH+ nor nNOS+ might be astrocytes.

This was confirmed with IHC staining for the astrocyte-specific marker S100. To evaluate whether astrocytes were activated [i.e., cFOS+] in response to TNF challenge, it was necessary to use antibodies generated for the S100 protein of astrocytes since this marker reveals the glial cell body. Other astrocytic markers such as glial fibrillary acidic protein [GFAP] predominately reveal the fibrous processes of the gliopil and not the astrocyte cell body. Unfortunately, the S100-labeled cell is not as “photogenic” as either TH+ or nNOS+ neurons. Therefore, additional histological sections were stained with fluorescent secondary antibodies and examined via a confocal microscope [Figures 2 and 3]. These images confirmed our identification of cFOS-positive astrocytes as well as cFOS-positive TH and nNOS neurons that we observed with bright field IHC.

Figure 2 presents examples of phenotypically identified [S100+] astrocytes in the NST. Examples of astrocytes, both cFOS+ and those not activated by TNF [i.e., cFOS-negative], are seen. cFOS-negative astrocytes, similar to cFOS-negative neurons, are identified by the nuclear “void” that would otherwise be filled with reaction product as seen in the cFOS+ cases [compare Figure 2D,E to 2F,G]. Figure 3 presents examples of phenotypically identified neurons [i.e., TH+ or nNOS+] in the NST; some were activated [cFOS+] by nanoinjections of TNF. As described above, brightfield IHC cFOS-phenotype identification was verified by confocal fluorescence imaging [compare Figure 3A to Figure 3B, C, D and Figure 3E to Figure 3F, G, H]. Arrows indicate activated-phenotype identified cells; open triangles point to examples of non-activated, phenotype identified cells; and filled triangles indicate examples of unidentified cells that have been activated [i.e., cFOS+ only]

Figure 4A presents the average number of each of these three cellular phenotypes per histological section through each side of the NST (i.e., both left and right NST areas were tabulated) without regard to the experimental condition. There are approximately twice as many astrocytes [70 ± 2.2] than either TH+ [36 ± 1.7] or nNOS+ [28 ± 1.8] neurons within the NST in any given histological section. There is no statistical difference in the numbers of TH+, nNOS+, or S100+ identified cells on the right vs left NST.

Response to nanoinjection of either PBS or TNF into the DVC provokes different distribution patterns of activated cells (i.e., cFOS+) across these three phenotypes [Figure 4B]. Clearly, more cells are activated as a consequence of TNF nanoinjection [see Figure 1], but this increase is only reflected in statistically significant increases in the number of TH+ and astrocytes+ cells that are activated. Indeed, nearly all of the increase [~89%] in cFOS+ cells can be accounted for by these two phenotypes [Table 1].

There are two perspectives one can use to evaluate cFOS/phenotype identification and each yields slightly different interpretations. For example, from the point of reference of the cFOS-positive cells, one can determine what percentage of the total activated cells are phenotypically identifiable. As presented in Table 1, in the unstimulated [PBS] condition, only a few cells [~6] are tonically active; of those few, on average only one [~10% total cFOS+ cells] can be identified as TH+, 6% are nNOS+, and ~2% are astrocytes. Thus, in the control condition, only ~18% of the activated cells were phenotypically identified. In the TNF-challenged condition, there is an eight-fold increase in the number of activated cells relative to control. Here, 54% of the cFOS+ cells are astrocytes, 29% are catecholaminergic, and 6% are nitroergic. This analysis accounts for almost 90% of all cFOS+ cells activated by TNF.

Alternatively, from the point of reference of the total number of phenotype-identified cells [Table 2], almost none [~4%] are cFOS-activated under the control condition of PBS nanoinjection. TNF-challenge causes cFOS activation of more than a third of the available TH-neurons and astrocytes, while only about a tenth of the available nNOS neurons are activated.

Regardless of the perspective of analysis, it is clear that TNF challenge in the NST activates a significant number of astrocytes and catecholaminergic neurons.

Discussion

We confirm one of our earlier reports that TNF evokes cFOS activation in the NST in a dose-dependent fashion (Emch et al., 2001). However, these present experiments revealed that one must be careful in the interpretation of cFOS activation data and not presume that all that is activated is neuronal. Specifically, we have phenotypically identified populations of cells in the NST, putative noradrenergic neurons and astrocytes, which account for 90% of the TNF-evoked cFOS activation of cells in this nucleus.

The dorsal vagal complex (DVC) of the hindbrain constitutes the basic neural circuitry of the vago-vagal reflex control of gastrointestinal function, e.g., gastric motility, tone, and acid secretion (Rogers et al., 2005). Most vago-vagal control of gastric motility and tone is inhibitory. That is, activation of vagal afferents by distension of the stomach, intestine, or esophagus results in a marked reduction in gastric motility and tone (McCann and Rogers, 1992; Rogers et al., 2005; Zhang et al., 1992). This effect occurs because stimulated vagal afferents activate NST neurons, which, in turn, inhibit a major subset of DMV neurons. These DMV neurons, acting through the enteric plexus, normally control the source of tonic cholinergic activation of the stomach (Grundy and Schemann, 2002; Rogers et al., 2005). A second type of DMV neuron is *activated* by the NST. This subgroup (referred to as the non-adrenergic, non-cholinergic efferent pathway; NANC) of DMV neurons normally provides gastric inhibition by activating gastroinhibitory enteric efferents. The combined result on DMV neurons is that activation of NST neurons leads to potent inhibition of gastric function (Rogers et al., 1999; Rogers et al., 2005).

Recent studies of the mechanics of reflex control of the stomach have revealed that noradrenergic neurons in the NST and their connections with gastric vagal efferent neurons are particularly important to inhibitory vago-vagal control of the stomach (Guo et al., 2001; Martinez-Pena y Valenzuela et al., 2004; Rogers et al., 1999; Rogers et al., 2005; Rogers et al., 2003). These same neurons may also be important to the development of anorexia induced by toxin ingestion or CCK exposure (Rinaman, 2003). These observations, i.e., that TNF regulates gastric function by acting within the NST and that noradrenergic-NST neurons are particularly important to the coordination of inhibitory reflex control, led us to hypothesize that TNF action within the NST may preferentially affect putative noradrenergic neurons.

Noradrenergic [NE] neurons in the NST have been implicated in the local inhibitory reflex control of gastric motility and tone. Indeed, these NE neurons in the NST may be the sole mediators of the receptive relaxation reflex in which distension of the esophagus by a bolus of swallowed food causes an anticipatory relaxation of the proximal stomach (Hermann et al., 2006; Rogers et al., 1999; Rogers et al., 2005; Rogers et al., 2003). There is also good evidence that suggests that NST-NE neurons are involved in producing emesis and anorexia when activated (Myers et al., 2005; Rinaman, 2003)].

TNF induces powerful gastric relaxation by acting within the NST (Emch et al., 2000; Emch et al., 2001; Hermann and Rogers, 1995; Hermann et al., 2005; Hermann and Rogers, 2007; Hermann et al., 1999; Hermann et al., 2002). It also produces nausea, emesis, and anorexia in both human and animal models (Esper and Harb, 2005; Moritz et al., 1989; Turrin and Plata-Salaman, 2000). The present findings, in combination with previous observations cited above, strongly suggest that many of the malaise-inducing effects of TNF are mediated via NE neurons in the brainstem. This effect is probably not direct, however. Our previous immunohistochemical studies had demonstrated that constitutive expression of TNF receptors

in the NST is limited to vagal afferent fibers and terminals that synapse on to NST neurons (Hermann et al., 2004). Our subsequent *in vitro* calcium imaging studies of identified vagal afferent terminals in the NST suggest that TNF increases vagal terminal glutamate release by regulating endoplasmic reticular calcium release (Rogers et al., 2006). Thus, a logical conclusion here is that vagal afferent fibers affected by TNF terminate principally on NST neurons of the NE phenotype. This concept is supported by the recent observations of Appleyard and colleagues (Appleyard et al., 2007) that catecholamine neurons in the NST are preferentially and directly driven by vagal afferent input and the output of these afferent fibers can be modulated by CCK; a satiety peptide also known to provoke gastric relaxation and anorexia. These actions are highly dependent on intact NE neurons in the NST (Appleyard et al., 2007; Rinaman, 2003).

Presence of nitric oxide synthase [nNOS]-positive neurons in the DVC (Krowicki et al., 1997) suggested the possibility that these neurons may mediate gastrointestinal relaxation. Localization of nNOS+ neurons in subnuclear regions of the NST implied second-order visceral afferent control of gastric function. Indeed, direct injection of the NO precursor, arginine, into the DVC produced tonic gastric relaxation while the NOS inhibitor, L-NAME, increased gastric tone. Both of these effects are vagally mediated (Krowicki et al., 1997). While other studies demonstrated that the DMV receives a very dense input of nNOS-ir fibers, it is not clear that these terminals originate from neurons in the NST (Rogers et al., 2003).

While nNOS-ir NST neurons have been implicated in the vagal afferent control of swallowing (Goyal et al., 2001; Rogers et al., 2005), their active role in gastric reflex control is debatable. This statement is based on the observations from previous studies that repetitive distension of the esophagus primarily activated cFOS in the TH-ir neurons in the NST while nNOS-ir NST neurons were unresponsive (Rogers et al., 2003). Secondly, this relaxation reflex was partially blocked by either α -1 or α -2 adrenoceptor antagonists, alone, and nearly eliminated by the combination of the two drugs. In contrast, antagonism of nNOS, GABA, or β -adrenoceptor actions did not affect the reflex (Rogers et al., 2003). Lastly, *in vitro* neurophysiological studies (Fukuda et al., 1987; Martinez-Pena y Valenzuela et al., 2004) (Rogers et al., 2005) on identified gastric DMV neurons have shown that these neurons may be either activated or inhibited by norepinephrine via α 1 or α 2 adrenoceptors, respectively. Thus, it appears that the nNOS-ir NST neurons are not critical to gastric vago-vagal reflex control but, perhaps, play a role in modulating gastric function in response to other afferent information.

Surprisingly, the results of the present study indicate that the nNOS neurons in the NST appear not to have a significant role in explaining the effects of TNF in the NST. Instead, it appears that TNF operates on vago-vagal *neuronal* reflex circuitry in a manner similar to vagal afferent input itself; by selectively activating catecholamine NST neurons. Clearly, TNF evoked cFOS activation in many TH+ [i.e., catecholaminergic] and a few nNOS+ neurons in the NST, however, these two phenotypes of neurons account for about 30% of the total number of cells that are activated in response to TNF microinjection. To our further surprise, the majority of the remainder was accounted for by activation of astrocytes as evidenced by the co-localization of S100-ir in ~54% of the cFOS-ir cells in the NST.

The fact that an agonist such as TNF can trigger a substantial increase in immediate early gene expression [IEG; e.g., cFOS] in astrocytes *in vivo* is a new observation. The effect has, however, also been observed in cultured astrocytes (Suh et al., 2004). The expression of IEG's in astrocytes has been associated with the detection of and response to CNS trauma, ischemia, systemic infection and inflammation (Arenander and de Vellis, 1992; Rubio and Martin-Clemente, 1999; Yu et al., 1995). IEG expression may trigger numerous responses in astrocytes including alterations in diffusional barrier function, neuroprotective as well as neuroinflammatory effects (Minagar et al., 2002; Prat et al., 2001).

The traditional role of the astrocyte as a supporter of neuronal function has been radically expanded. Astrocytes express a wide variety of receptors for transmitters, hormones, chemokines and cytokines. The action of these signaling agents can, in turn, lead to the release of glial signal molecules [including glutamate and TNF] to which neurons are sensitive (Bezzi et al., 2001; Fellin et al., 2006; Haydon and Carmignoto, 2006; Raivich et al., 1999; Vesce et al., 2007). The result is that astrocytes can encode and integrate signals within their proximity that are associated with neuronal damage or disease, and upregulate [depending on the circumstances] either pro- or anti-inflammatory gene products that produce long term changes in the function of adjacent neurons [for example, (Guo et al., 2007; Leonard, 2007; Vesce et al., 2007)].

However, previous observations by our laboratory suggest that TNF effects on IEG expression in NST astrocytes may, also, not be direct. That is, *in vivo* TNF effects to elevate cFOS in cells [presumably both neurons and glia] in the NST were eliminated by blocking glutamate AMPA receptors (Emch et al., 2001). We have shown that TNF activates vagal afferent terminals (Rogers et al., 2006) [which are predominantly glutamatergic] and, in other brain areas, TNF immediately upregulates AMPA receptor function post synaptically (Pickering et al., 2005; Stellwagen et al., 2005). Several studies report that AMPA [kainate] and metabotropic receptor stimulation can provoke rapid IEG expression in isolated or cultured astrocytes (Condorelli et al., 1993; McNaughton and Hunt, 1992) though the mechanism of cFOS activation is different in glial cells than in neurons (Edling et al., 2007). Altogether, these factors suggest that TNF-induced cFOS expression in NST neurons and glia could be triggered entirely by augmenting glutamate release from vagal afferents not unlike the “tri-partite synapse” that has been proposed by Haydon and colleagues (Araque et al., 1999; Fellin et al., 2006; Haydon and Carmignoto, 2006). Indeed, preliminary calcium imaging studies performed in this laboratory [2008 Neuroscience Abstracts] suggest that electrical stimulation of vagal afferents in a slice preparation evoke calcium spikes in both NST astrocytes and neurons.

Alternatively, the presence of glutamate may be required as a gating cofactor for TNF to provoke IEG expression in astrocytes and neurons. We have observed a similar cooperativity between TNF and glutamate in augmenting the expression of IEG's in the *in vivo* spinal cord (Hermann et al., 2001b). In those studies, microinjection of low doses of either TNF or kainate, alone, into the spinal grey elicited limited activation of cFOS in neurons and glia. However, co-injection of these same doses of TNF with kainate evoked dramatic increases in cFOS expression in neurons and glia along with preliminary signs of neurodegeneration.

Conclusion

Taken together with these previous observations, the present results suggest that intense or prolonged vagal afferent activity [induced by visceral pathway activity, the action of gut hormones or the action of cytokines such as TNF] can alter local astrocyte IEG expression that, in turn, can provoke long-term, perhaps permanent changes in the sensitivity of vagal-reflex circuitry [Figure 5]. Such modulation of neuronal responsiveness as a consequence glial-neuronal communication may have precedence in migraine pathology (Thalakoti et al., 2007), enteric celiac disease (Esposito et al., 2007), and astrocyte-neuron vulnerability to prenatal stress effects on brain development (Barros et al., 2006). This relationship between glia and adjacent neurons may provide another target for therapeutic intervention in autonomic disease states.

Experimental procedures

Long-Evans rats [250–500g] of either sex, obtained from the breeding colony located at Pennington Biomedical Research Center, were used in these studies. Animals were maintained

in a room with a 12–12 hour light-dark cycle, constant temperature and humidity, and provided with food and water ad libitum. Rats were randomly assigned to one of four different experimental groups. 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 anesthetized with thiobutabarbital (Inactin; 150mg/kg; Sigma, St. Louis, MO). This long-lasting anesthetic does not depress autonomic reflexes (Buelke-Sam et al., 1978) and will neither induce nor interfere with cFOS activation (Hermann et al., 2001a). Using aseptic technique, the trachea was cannulated for maintenance of an open airway; the animal was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The scalp on the back of the head was opened, the musculature attached to the back of the occipital plate was detached, the skull plate removed, and the floor of the fourth ventricle was exposed.

Capillary glass micropipettes pulled on a vertical microelectrode puller (PE-2; Narishige Scientific, Tokyo, Japan) were beveled (BV-10, Sutter Instrument Co., Novato, CA) to a tip diameter of 20µm. Pipettes were filled with either phosphate buffered saline (PBS) or tumor necrosis factor- α (TNF; R and D Systems, Minneapolis, MN; 10^{-6} , 10^{-7} or 10^{-9} M; diluted in PBS). Micropipettes were then mounted to a stereotaxic carrier and directed into the left medial NST under visual guidance; stereotaxic coordinates relative to calamus scriptorum: 0.3mm rostral to scriptorum, 0.3mm lateral to midline and 0.35mm below brainstem surface. Unilateral nanoinjections (40nL volume) of one of the experimental solutions were made into the left medial NST (thus, total doses of injected TNF were: 0, 0.7pg, 70pg, or 700pg). Ninety minutes after this stimulation correlates with maximal cFOS production (Rinaman et al., 1993). At this time, the anesthetized rats were transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Brainstems were removed to a 30% sucrose-PBS solution overnight. The next morning, brainstems were sectioned on a freezing microtome into 40µm thick sections. All brainstems were then processed for the presence of nuclear cFOS protein. Only those brainstems [N = 31] that had received a unilateral nanoinjection of either TNF [700pg; 40nL volume] or PBS [40nL volume] were immunohistochemically [IHC] processed for both cFOS activation and phenotypic identification of the presumed activated cell types [i.e., immunostain for tyrosine hydroxylase (TH), neural nitric oxide synthase (nNOS). Preliminary results convinced us to add the phenotypic identification of astrocytes as possible targets for TNF activation. Therefore, we also used IHC staining for the S100 protein which is a specific marker for astrocyte cell bodies and proximal processes (Ghandour et al., 1981; Haan et al., 1982).

Histological processing of the medullary brainstem for cFOS production required: primary cFOS antibody [AB-5; rabbit cFOS, 1:5000; Oncogene Science Diagnostics (now Calbiochem, San Diego, CA)] and Biotin-SP donkey anti-rabbit IgG [1:500; Jackson ImmunoResearch, West Grove, PA]. Amplification of antibody-antigen reactions required incubation with Vector Elite avidin - biotin -peroxidase complex [Vector Labs, Burlingame, CA] followed by Vector Nova Red peroxidase detection reagents [Vector Labs]. Phenotypic identification required mouse anti-rat tyrosine hydroxylase [TH; 1:500] primary antibody [cat. no. 22941, ImmunoStar, Hudson, WI], mouse anti-rat neuronal nitric oxide synthase [nNOS; 1:500] primary antibody [N2280, Sigma, St Louis, MO], or mouse anti-rat astrocytic marker [S100; 1:3000] primary antibody [AB4066, Abcam, Cambridge, MA] and Biotin-SP donkey anti-mouse [1:500; Jackson ImmunoResearch] as secondary antibody. Amplification of antibody-antigen reactions required incubation with Vector Elite avidin-biotin-peroxidase complex [Vector Labs] followed by Vector SG peroxidase detection reagents [Vector Labs]. Alternatively, sections were prepared for immunofluorescent detection by substituting RRX-conjugated to donkey, anti-rabbit antibodies [1:500; JacksonImmuno cat. 711-295-152] to tag

cFOS+ cells and AF488 conjugated to donkey, anti-mouse antibody [1:500; Invitrogen, cat. A21202] to tag TH+, nNOS+, or S100+ cells.

All sections through the medullary brainstem that contained the area postrema (i.e., this area corresponds with the maximal rostrocaudal spread of the nanoinjections) were saved and processed for the demonstration of nuclear cFOS protein, a marker for prolonged and significant neuronal excitation (Rinaman et al., 1993). This protocol is available in detail elsewhere (Hermann et al., 2001a; Rinaman et al., 1993). Briefly, all tissue sections were rinsed in PBS and incubated in sodium borohydride and hydrogen peroxide to eliminate remaining fixative and to block endogenous peroxidase, respectively. Sections were then rinsed and blocked in 10% normal donkey serum with 0.3% Triton X-100 [MP Biomedicals, LLC, Aurora, OH], re-rinsed in PBS and then incubated in rabbit, anti-rat cFOS primary antibody overnight at room temperature on a shaker table. Sections were rinsed in PBS and incubated with biotinylated donkey, anti-rabbit secondary antibody. Sections were rinsed in PBS and incubated in Vector ABC peroxidase reagent, followed by the Vector peroxidase chromogen Nova Red. cFOS staining was revealed in this protocol as brick-red nuclei. Omission of primary antibody or incubation with inappropriate secondary antibody produced no cFOS label.

In addition to immunostaining for cFOS activation, each brainstem that was exposed to the high dose nanoinjection of TNF [700pg] was processed for one of the following phenotypes: tyrosine hydroxylase [TH], neuronal nitric oxide synthase [nNOS], or the astrocytic marker, [S100]. Sections were rinsed and blocked with 10% normal donkey serum plus 0.3% Triton X-100, re-rinsed between each of the following reaction steps: (1) mouse, anti-rat TH primary antibody, (2) mouse, anti-rat nNOS primary antibody, or (3) mouse, anti-rat S100. Sections were incubated in a humidity chamber on a shaker table overnight at room temperature followed by incubation with Biotin-SP donkey anti-mouse IgG; then incubated in Vector ABC reagent and, finally, reacted with the Vector peroxidase chromogen SG. Cytoplasmic staining for TH, nNOS, or S100 is blue-black using this method. This cytoplasmic chromogen contrasts with the nuclear Nova Red stain that identifies cFOS activated neurons [Figures 2 and 3]. To help verify our chromagen-based, bright field IHC results [which were the basis of our quantification data], random sections were reacted with fluorescent secondary antibodies [refer to preceding details of fluorescence conjugated antibodies]. Images of S100+, TH+, and nNOS+ cells in the NST with and without cFOS+ nuclei were taken with a Zeiss Axioskop equipped with a PerkinElmer Ultraview CSU10 confocal illuminator [Figures 2 and 3].

Double immunostained sections were mounted on glass Plus® slides [Electron Microscopy Sciences, Hatfield, PA] and coverslipped using Entellan mountant [Electron Microscopy Sciences]. For the sake of consistency, only the chromagen stained sections were included in the quantification of labeled cells. Sections containing the NST at the level of the area postrema [i.e., the position of the injection site] were evaluated with the aid of a Nikon E800 microscope equipped with a Zeiss Axiocam CCD camera. An investigator unaware of the experimental condition being analyzed counted cFOS stained nuclei; a second observer verified counts. The agreement between counts of the two observers was within 10%. Data in Figure 1 represent the dose-dependent response to unilateral nanoinjections of TNF into the DVC, i.e., the averaged number of cFOS-activated cells in the NST (both ipsilateral and contralateral to the site of injection) per histological section per animal across all four experimental groups. A one-way analysis of variance (ANOVA) was performed across all four groups; Dunnett's post-test comparisons were made against the PBS injection as a control. Statistical significance was defined at $P < 0.05$.

In those cases where the brainstems were exposed to unilateral nanoinjections of either TNF [700pg] or PBS, the total numbers of cFOS labeled nuclei, specific phenotype labeled cells [i.e., TH+, nNOS+, or S100+], and double-labeled cells [i.e., cFOS+/phenotype+] were

counted for all histological sections. Photomicrographs of representative double IHC stained sections [i.e., cFOS+/phenotype+] are shown in Figure 2 and 3. The average numbers of specific cellular phenotypes in the DVC per histological section are presented in Figure 4A. The average numbers of each of the identified phenotypes [i.e., TH+, nNOS+, or S100+] that were activated [i.e., cFOS+/phenotype+] under these two conditions [i.e., TNF or PBS] are presented in Figure 4B; ANOVA analysis of these activated cell types were subjected to Bonferroni post-hoc tests between appropriate subgroups. For example, number of TH+ neurons activated after PBS exposure were compared to number of TH+ neurons that were activated after TNF stimulation; statistical significance was defined at $P < 0.05$. The distribution or percentage of each identified phenotype that accounts for the total number cFOS+ cells in response to either of these stimulus conditions is presented in Table 1 and 2.

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Figure 1.

cFOS activation of cells in the NST by TNF.

Direct unilateral nanoinjection of TNF_α into the DVC provokes cFOS activation in a dose-dependent fashion both ipsi- and contralaterally to the injection site [ANOVA: $F_{7,532} = 95.98$; $P < 0.0001$]. cFOS activation counts in the NST induced by either 70 or 700pg TNF were significantly greater than that seen with PBS [Dunnett's post-hoc test with PBS = control, * = ipsilateral; # = contralateral comparisons; $p < 0.05$].

Figure 2.

TNF activation of astrocytes in the NST

A: Low power photomicrograph of TNF injection site in the medial NST illustrating both cFOS-activated nuclei [red-brown] and S100+ labeled astrocytes [blue-black].

B: Control PBS injection site [portion of the pipette track indicated by chevrons] in the NST. Abbreviations: AP = area postrema; gr = gracile fasciculus; sol = solitary tract.

C: Field of cFOS+ nuclei and S100+ astrocytes activated in response to TNF injection. Examples of cFOS-activated astrocytes [i.e., dual labeled cFOS+/S100+] are indicated by arrows; non-activated astrocytes [S100+ only] indicated by open triangle; un-identified cFOS + nuclei indicated by filled triangles.

D, E: higher magnification views of cFOS+/S100+ double labeled astrocytes [arrows] as well as un-identified cFOS+ nuclei [filled triangle]

F, G: comparison views of non-activated [i.e., not cFOS+] S100-identified astrocytes.

H, I, J: confocal examples of fluorescently labeled cells in the NST following TNF injection.

H, S100 signal; **I,** cFOS signal; **J;** composite image. Double-labeled cells appear yellow/orange.

Scale bars: A,B = 200micron; C, H, I,J = 75 micron; D,E,F,G = 10micron

Figure 3.

TNF activation of neurons [TH+ or nNOS+] in the NST

A: Example of a field of double-labeled TH+ [blue-black] and cFOS [red-brown] neurons in the NST activated following TNF injection. A relatively high percentage of TH+ neurons in the NST were activated [i.e., cFOS+] by TNF; examples indicated by arrows. Non-activated TH+ neurons are indicated by open triangles.

BCD: confocal examples of fluorescently labeled TH+ neurons in the NST following TNF injection. B = TH+ signal; C = cFOS signal; D = composite image.

E: Example of a field of double-labeled nNOS+ [blue-black] and cFOS [red-brown] neurons in the NST activated following TNF injection. A relatively low percentage of nNOS+ neurons

in the NST were activated [i.e., cFOS+] by TNF. Double-labeled neurons are indicated by arrows; non-activated nNOS+ neurons are indicated by open triangles.

FGH: confocal examples of fluorescently labeled nNOS+ neurons in the NST following TNF injection. F = nNOS+ signal; G = cFOS signal; H = composite image. Double-labeled cells appear yellow/orange.

Scale bars: A = 75micron; B,C,D,E,F,G,H = 150micron

Figure 4.

Identified and activated phenotypes in the NST

A: The average number of each of these three cellular phenotypes per histological section through each side of the NST is presented (i.e., both left and right NST areas were tabulated). There are approximately twice as many astrocytes than either TH+ or nNOS+ neurons within the NST in any given histological section.

B: Activation of cells (i.e., cFOS+) in response to nanoinjection of either PBS or TNF into the DVC provokes different distribution patterns across these three phenotypes. Clearly, more cells are activated as a consequence of TNF nanoinjection [see Figure 1], but this increase is only reflected in statistically significant increases in the number of TH+ and astrocytes+ cells that

are activated. Indeed, nearly all of the increase [$\sim 89\%$] in cFOS⁺ cells can be accounted for by these two phenotypes [Table 1].

Figure 5.

Cartoon illustrating potential relationship between vagal afferent fibers, astrocytes, and neurons to elicit specific motor or behavioral responses such as we have observed with TNF and gastric stasis. Our previous work demonstrated that TNFR1 receptors are located predominately on vagal afferents (Hermann et al., 2004) and that glutamate antagonism blocks activation of cells [apparently both neural and glial] in NST by TNF (Emch et al., 2001). Activation of catecholaminergic [i.e., TH-ir]-NST neurons (Rinaman, 2003) would explain the gastric stasis and anorexia evoked by TNF. Perhaps this tri-partite relationship may cause long term change in synaptic sensitivity that could produce chronic reflex hypersensitivity?

Table 1

FOS activation of cells in the NST in response to central nanoinjection [40nL] of either PBS or TNF. Data are presented as percentage of total number of FOS-activated cells that were phenotypically identified by immunohistochemistry. Only ~18% of the cells that were FOS⁺ after PBS-challenge could be phenotypically identified. In contrast, ~89% of the cells activated following TNF-challenge were identified and the majority [54%] were astrocytes

NST stimulus	average number FOS ⁺ cells per histo section	average number TH ⁺ /FOS ⁺ cells per histo section	average number NOS ⁺ / FOS ⁺ cells per histo section	average number SI00 ⁺ / FOS ⁺ cells per histo section	summation of % FOS ⁺ cells phenotype identified
PBS	6.0 ± 0.7	0.6 ± 0.2 [10% of 6 FOS ⁺ cells]	0.3 ± 0.2 [6%]	0.1 ± 0.1 [~2%]	~18%
TNF	48.0 ± 2.4	14.0 ± 0.9 [29% of 48 FOS ⁺ cells]	3.0 ± 0.3 [6%]	26.0 ± 1.9 [54%]	~89%

Table 2

FOS activation of cells in the NST in response to central nanoinjection [40nL] of either PBS or TNF. Here, data are presented as percentage of total number of phenotypically identified FOS⁺ cells relative to the total number of each phenotype available within a given histological section. For example, of the 37 TH⁺ neurons in the NST for a given section, less than one [on average] was activated after PBS challenge, while 14 TH⁺ neurons were activated by TNF-challenge. These data demonstrate the relative percentage of each phenotype that was activated in response to the two challenges

NST stimulus	average number TH ⁺ /FOS ⁺ cells/ section relative to average number of TH ⁺ cells/section	average number NOS ⁺ /FOS ⁺ cells/section relative to average number of NOS ⁺ cells/section	average number S100 ⁺ /FOS ⁺ cells/section relative to average number of S100 ⁺ cells/section
PBS	0.6 ± 0.2 [~2% of 37 TH ⁺ cells]	0.3 ± 0.2 [~1% of 28 NOS ⁺ cells]	0.1 ± 0.1 [<<1% of 70 S100 ⁺ cells]
TNF	14.0 ± 0.9 [39% of 37 TH ⁺ cells]	3.0 ± 0.3 [~11% of 28 NOS ⁺ cells]	26.0 ± 1.9 [~37% of 70 S100 ⁺ cells]