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Comparison of somatostatin and corticotrophin releasing hormone immunoreactivity in forebrain neurons projecting to taste responsive and non responsive regions of the parabrachial nucleus in rat

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Abstract

Several forebrain areas have been shown to project to the parabrachial nucleus (PBN) and exert inhibitory and excitatory influences on taste processing. The neurochemicals by which descending forebrain inputs modulate neural taste-evoked responses remain to be established. This study investigated the existence of somatostatin (SS) and corticotrophin releasing factor (CRF) in forebrain neurons that project to caudal regions of the PBN responsive to chemical stimulation of the anterior tongue as well as more rostral unresponsive regions. Retrograde tracer was iontophoretically or pressure ejected from glass micropipettes, and seven days later the animals were euthanized for subsequent immunohistochemical processing for co-localization of tracer with SS and CRF in tissue sections containing the lateral hypothalamus (LH), central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST), and insular cortex (IC). In each forebrain site, robust labeling of cells with distinguishable nuclei and short processes was observed for SS and CRF. The results indicate that CRF neurons in each forebrain site send projections throughout the rostral caudal extent of the PBN with a greater percentage terminating in regions rostral to the anterior tongue responsive area. For SS, the percentage of double-labeled neurons was more forebrain site specific in that only BNST and CeA exhibited significant numbers of double labeled neurons. Few retrogradely labeled cells in LH co-expressed SS, while no double labeled cells were observed in IC. Again, tracer injections into rostral PBN resulted in a greater percentage of double labeled neurons in BNST and CeA compared to caudal injections. The present results suggest that some sources of descending forebrain input might utilize somatostatin and/or CRF to exert a broad influence on sensory information processing in the PBN.

Keywords

parabrachial; amygdala; hypothalamus; taste; cortex; bed nucleus

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Introduction

Learning and nutritional status are important for the development and maintenance of food preferences. For instance, a conditioned taste aversion (CTA) that develops following experience with negative gastrointestinal consequences of ingesting a taste stimulus (e.g. nausea, sickness, or vomiting) produces a switch from acceptance to avoidance of that and any like tasting stimulus. In contrast, the behavioral response to a negative body sodium balance is characterized by a switch from avoidance of concentrated sodium salt to avoid ingestion. A common finding in conditions that alter taste preference is selective changes in the responses of taste neurons in the first and second central synapses of the ascending gustatory system, the nucleus of the solitary tract (NST) and parabrachial nucleus (PBN), respectively (Chang and Scott 1984; Jacobs et al. 1988; McCaughey et al. 1996; McCaughey et al. 1997; McCaughey and Scott 2000; Nakamura and Norgren 1995; Shimura et al. 1997a; Shimura et al. 1997b). In the case of CTA, neural responses to the conditioned taste stimulus are enhanced after acquisition, while expression of sodium appetite is accompanied by decreased sensitivity of NST and PBN taste cells to sodium salt, particularly at higher concentrations that are normally avoided. Whether these neural changes reflect a causal relationship is unsettled, although altered PBN taste responses induced by CTA acquisition are abolished following disruption of neural connections between the forebrain and the brainstem (Tokita et al. 2004).

Several forebrain regions that receive gustatory information like the insular cortex (IC), bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA) and lateral hypothalamus (LH) send projections back to the NST and PBN to modulate taste-evoked neural activity (Cho et al. 2003; Li and Cho 2006; Lundy, Jr. and Norgren 2001; Lundy, Jr. and Norgren 2004; van der Kooy et al. 1984; Veening et al. 1984). Despite the substantial data characterizing the influence of feedback from the LH, CeA, BNST, and IC on brainstem taste processing, the identification of the neurochemicals subserving centrifugal modulation remains in its infancy. Prior studies using stereotaxic coordinates alone to place retrograde tracer into the PBN have shown the presence of several neuropeptides like somatostatin, neurotensin, corticotrophin-releasing factor, cholecystokinin, enkephalin, substance P, and galanin in some LH, CeA, and/or BNST neurons (Moga et al. 1989; Moga et al. 1990a; Moga and Gray 1985; Veening et al. 1984). However, these studies were limited in that a precise correspondence between the neurochemical content of forebrain neurons innervating different functional segments of the PBN could not be discerned with certainty.

The caudal classically defined pontine taste area corresponds to the locations where taste neurons responsive to chemical stimulation of the anterior oral cavity are concentrated (Nishijo and Norgren 1997; Perrotto and Scott 1976). Regions rostral to this gustatory responsive area are primarily concerned with processing nociceptive, cardiovascular, respiratory and gastric information (Baird et al. 2001a; Bester et al. 1995; Bester et al. 2000; Chamberlin and Saper 1992; Chamberlin and Saper 1994; Cohen 1971; Ezure and Tanaka 2006; Hayward 2007; Lara et al. 1994; Mraovitch et al. 1982). Nevertheless, neurons responsive to tactile stimulation of the oral cavity as well as some responsive to gastric distention are found in the caudal pontine taste area (Baird et al. 2001b; Karimnamazi et al. 2002). Moreover, a small number of neurons responsive to chemical stimulation of the posterior oral cavity reside in the external medial (MPBE) and external lateral (LPBE) subnuclei adjacent to the respiratory responsive Kolliker-Fuse nucleus (Halsell and Travers 1997). Whether any or all of the previously identified forebrain-PBN peptidergic pathways play a role in descending modulation of gustatory processing per se remains to be established.

To this end, the present study used electrophysiological techniques to compare the expression of somatostatin and corticotrophin-releasing factor immunoreactivity in LH, CeA, BNST, and

IC neurons projecting to caudal regions of PBN responsive to anterior tongue application of NaCl and more rostral regions unresponsive to anterior tongue stimulation. In one group of animals we electrophysiologically located the caudal gustatory responsive PBN and made small injections of retrograde tracer, while in a separate group we made similar injections in more rostral areas that were unresponsive to anterior tongue application of NaCl.

2. Results

2.1 Injection Sites

Figures 1A, B, C and D show photomicrograph examples of tracer injected into the taste-responsive PBN and more rostral non taste responsive regions. Microscopic examination of each gustatory-responsive injection site revealed that predominately the medial, ventral lateral and waist portions of the caudal PBN were targeted with minimal spread into the rostral regions. Injections placed rostral to this gustatory responsive area primarily targeted the medial, external lateral (medial portion), central lateral, dorsal lateral, ventral lateral and internal lateral subnuclei. A summary diagram of the tracer injections and their relative spread are shown in Figure 2.

2.2 Distribution of retrogradely labeled neurons

Since the distributions of forebrain-PBN projecting neurons were similar to those described in earlier tracing studies, the present findings are only briefly summarized (Kang and Lundy 2009; Moga et al. 1989; Moga et al. 1990b; Moga and Gray 1985; Saggi and Lundy 2008). Retrogradely labeled neurons were observed in the LH, CeA, BNST and IC following injections either in the caudal regions of PBN responsive to anterior tongue application of NaCl or rostral non responsive regions. The IC was identified as the area approximately 1 mm anterior and 0.8 mm posterior to bregma directly lateral to the claustrum. The BNST was identified as the area approximately 0.2 mm anterior and 0.4 mm posterior to bregma directly medial to the internal capsule above and/or below the anterior commissure. The CeA was identified as the area approximately 1.8 to 3.3 mm posterior to bregma ventral to the striatum, medial to the basolateral nucleus of the amygdala, and lateral to the optic tract. The LH was identified as the area approximately 2.3 to 3.8 mm posterior to bregma sandwiched between the internal capsule lateral and the fornix medial.

Although the actual numbers of labeled cells per section varied depending on the size of a given tracer injection, the number of neurons retrogradely labeled in LH, CeA, dBNST and IC did not differ as a function of PBN injection site ($F_{1,76} = 3.59$, $P = 0.062$). However, we did find two additional areas, the PVN and vBNST that consistently contained retrogradely labeled neurons following rostral injections, but not after caudal injections. Rostral PBN injections produced more retrogradely labeled neurons in the dBNST compared to the vBNST ($F_{1,5} = 63.3$, $P < 0.01$). Overall, more neurons were retrogradely labeled in the CeA following tracer injections into either caudal or rostral PBN regions compared to the other forebrain areas ($F_{3,76} = 37.3$, $P < 0.01$), which were not different from one another ($P's > 0.1$).

Immunohistochemical processing for CRF and SS resulted in robust labeling of cells with distinguishable nuclei and short processes in each forebrain site (Fig. 4, Fig. 5 and Fig. 6). Further analyses of total number and percentage of peptidergic cells that contain retrograde tracer were not performed, because only CRF- and SS-positive cells in those sections containing retrogradely labeled neurons were counted.

2.3 Distribution of double labeled neurons

Corticotrophin-releasing factor (CRF-ir)—Firstly, tracer injections into caudal ($F_{1,9} = 9.4$, $P = 0.01$) and rostral ($F_{1,7} = 24.0$, $P < 0.01$) PBN regions produced significant differences in the percentage of double labeled cells between the rostral and caudal halves of CeA. This

resulted from significantly more CRF-ir neurons in cCeA compared to rCeA [P values ≤ 0.02], but comparable numbers of retrogradely labeled cells (P values ≥ 0.32). Secondly, a significant main effect for forebrain site was found following caudal ($F_{4,24} = 14.2$, $P < 0.01$) and rostral ($F_{6,25} = 15.5$, $P < 0.01$) PBN injections. Tracer injections into caudal PBN resulted in a greater percentage of retrogradely labeled dBNST cells co-expressing CRF-ir ($19.9 \pm 1.4\%$) compared to the cCeA ($11.2 \pm 3.2\%$), IC ($7.0 \pm 1.4\%$), LH ($9.1 \pm 1.8\%$) and rCeA ($1.4 \pm 0.6\%$) [P values < 0.01]. The percentages of retrogradely labeled cells co-expressing CRF-ir in IC, cCeA and LH were significantly different from rCeA (P values ≤ 0.03), but not from one another (P values ≥ 0.16).

For rostral PBN injections, post hoc analyses revealed a greater percentage of retrogradely labeled cells co-expressing CRF-ir in IC ($24.2 \pm 2.5\%$), cCeA ($26.3 \pm 1.7\%$), dBNST ($29.2 \pm 3.4\%$), LH ($24.3 \pm 1.1\%$) and vBNST ($23.6 \pm 0.9\%$) compared to rCeA ($13.0 \pm 2.8\%$) and PVN ($7.3 \pm 1.2\%$) [P values < 0.01]. Post hoc analyses of the significant interaction between PBN injection site and forebrain region ($F_{6,60} = 4.6$, $P < 0.01$) further revealed that rostral PBN injections resulted in a significantly greater percentage of double labeled neurons in each forebrain site compared to caudal PBN injections (P values ≤ 0.01). The graph in Figure 3A shows the percentages of retrogradely labeled cells in dBNST, cCeA, IC, LH, vBNST, rCeA and PVN co-expressing CRF-ir following injections centered in caudal regions of PBN responsive to taste stimulation of the anterior tongue or rostral unresponsive regions. Figure 4 and Figure 5 show photomicrograph examples of retrogradely labeled and CRF-ir forebrain neurons.

Somatostatin (SS-ir)—Significant differences between rCeA and cCeA were observed in the percentage of retrogradely labeled cells co-expressing SS-ir following tracer injections into caudal ($F_{1,9} = 13.8$, $P < 0.01$) and rostral ($F_{1,9} = 16.8$, $P < 0.01$) PBN regions. Similar to CRF-ir neurons, the cCeA exhibited a greater number of SS-ir cells (P values ≤ 0.03). A significant main effect for forebrain site was observed both for caudal ($F_{4,24} = 10.1$, $P < 0.01$) and rostral ($F_{6,31} = 23.7$, $P < 0.01$) PBN injections. Caudal injections resulted in a greater percentage of retrogradely labeled dBNST ($10.7 \pm 3.0\%$) and cCeA ($6.2 \pm 1.1\%$) cells co-expressing SS-ir compared to IC ($0 \pm 0\%$), LH ($0.6 \pm 0.4\%$), and rCeA ($2.3 \pm 0.5\%$). Following rostral injections, more double labeled neurons also were observed in the dBNST ($18.6 \pm 2.3\%$) and cCeA ($15.8 \pm 1.6\%$) compared to IC ($0 \pm 0\%$), LH ($1.7 \pm 0.9\%$), vBNST ($7.2 \pm 2.0\%$), rCeA ($5.6 \pm 2.0\%$) and PVN ($1.2 \pm 0.8\%$). Rostral PBN injections again resulted in a significantly greater percentage of double labeled neurons compared to caudal PBN injections ($F_{6,66} = 3.7$, $P < 0.01$), but only in dBNST, cCeA and vBNST (P values < 0.01). The graph in Figure 3B shows the percentages of retrogradely labeled cells in dBNST, cCeA, IC, LH, vBNST, rCeA and PVN co-expressing SS-ir following injections centered in caudal regions of PBN responsive to taste stimulation of the anterior tongue or rostral unresponsive regions. Figure 6 shows photomicrograph examples of retrogradely labeled and SS-ir forebrain neurons.

3. Discussion

The objective of the present experiments was to further delineate potential neurochemicals mediating forebrain modulation of brainstem gustatory neural processing. Our results extend prior investigations by showing that forebrain neurons expressing corticotrophin releasing factor and somatostatin immunoreactivity form pathways both to caudal regions of the PBN that receive gustatory input via the chorda tympani nerve and more rostral areas that do not.

In general, our data are in good agreement with prior studies using only stereotaxic injections to examine the neuropeptide content of LH, CeA and BNST neurons projecting to the PBN. Two studies have investigated descending CeA peptidergic pathways with one study reporting that 30 – 50% of retrogradely labeled cells were immunoreactive for SS and 54 – 66% CRF-

ir (Moga and Gray 1985), while the other study reported only a few double labeled SS-ir cells (Veening et al. 1984). Both studies mentioned that double labeled neurons tended to be concentrated in the caudal CeA. For the LH, 21% of retrogradely labeled cells were found to be immunoreactive for CRF, while less than 1% were SS-ir (Moga et al. 1990b). Moreover, these authors found retrogradely labeled neurons in the PVN, as did the present study following injections into rostral regions of PBN; however, we observed a greater percentage of double labeled CRF-ir (7% vs. <1%). Finally, 14% of retrogradely labeled cells in the BNST were CRF-ir, while only 4% were SS-ir (Moga et al. 1989). Together with the present data, it appears that a greater portion of descending CRF and/or SS inputs originating from the LH, CeA, and BNST target rostral PBN regions that do not process chorda tympani derived gustatory information compared to caudal regions that do, and CRF neurons comprise a greater portion of the descending pathways compared to SS cells. In fact, few, if any, SS-ir neurons in LH project to PBN. The present data also provide, to the best of our knowledge, the first description of the neurochemical profile of IC neurons projecting to PBN. Numerous retrogradely labeled IC cells co expressed CRF-ir, while none were immunoreactive for SS. Similar to the other forebrain regions discussed above, a greater portion of descending CRF input originating from IC targeted rostral PBN regions compared to caudal regions.

Although the present injections into the caudal gustatory responsive PBN correspond to the locations where taste neurons are known to be concentrated, evidence indicates that regions more rostral also contain taste responsive cells (Halsell and Travers 1997). Specifically, a small number of neurons responsive to chemical stimulation of the posterior oral cavity reside in the external medial (MPBE) and external lateral (LPBE) subnuclei adjacent to the respiratory responsive Kolliker-Fuse nucleus. Two of our rostral injections (i.e. #0841 and #0835) included the most medial aspect of LPBE, but did not appear to include any of MPBE (Fig. 2). Moreover, a representation of other sensory modalities is intermingled with gustatory input. The caudal classically defined pontine taste area, for instance, contains some neurons that receive gastric input as well as tactile input from the oral cavity (Baird et al. 2001b; Hermann and Rogers 1985; Karimnamazi et al. 2002). Thus, we cannot precisely identify the nature of the neurons targeted by forebrain peptidergic pathways to the caudal and rostral PBN as delineated in the present study. However, the rostral placement of injections into areas unresponsive to anterior tongue stimulation and limited spread into posterior oral cavity responsive subnuclei makes it unlikely that they process gustatory information.

Each of the forebrain areas investigated in the present study have been implicated in the control of ingestive behavior (Bielavska and Roldan 1996; Caulliez et al. 1996; Currie et al. 2001; Lamprecht et al. 1997; Roldan and Bures 1994; Roth et al. 1973; Schwartz and Teitelbaum 1974; Zardetto-Smith et al. 1994). Furthermore, centrally administered CRF and its homologue urocortin have been shown to diminish intake in a variety of species including rat, while SS administration augmented intake (Ciccocioppo et al. 2003; Feifel and Vaccarino 1994; Fekete et al. 2007; Heinrichs et al. 1993; Jones et al. 1998; Parrott 1990; Spina et al. 1996). These opposing effects might be due, in part, to modulation of gastric function, because gastric motility is decreased by CRF and increased by SS administration to the dorsal motor complex [DMC] (Lewis et al. 2002; Martinez et al. 2000; Yoneda and Tache 1995). Several experimental treatments that augment food intake have been shown to have parallel excitatory effects on gastric motility and emptying, while those inhibiting intake have parallel inhibitory effects (Cato et al. 1990; Flanagan et al. 1989; McCann et al. 1989). Both CRF- and SS-ir neurons in BNST and CeA have been shown to project to the DMC, while only CRF-ir cells in LH do so (Gray and Magnuson 1987; Saha et al. 2002). Furthermore, activation of LH, CeA, BNST and PVN modulates DMC neurons and/or gastric function (Hermann et al. 1990; Jiang et al. 2003; Liubashina et al. 2002; Zhang and Fogel 2002).

Descending CRF and SS peptidergic pathways also might influence ingestive behavior by regulating ascending gustatory and visceral evoked neural signals in the PBN. Evidence for this assertion comes from studies showing that 1) the anorexic effects of dehydration increased CRF mRNA in LH neurons, many of which project to the medial and lateral parts of PBN (Kelly and Watts 1998), 2) injections of CRF into the lateral PBN inhibits sodium chloride intake in sodium depleted rats, while injections of a CRF receptor antagonist had the opposite effect, increasing sodium chloride intake (De Castro e Silva et al. 2006) and 3) intra PBN infusion of SS inhibited the spontaneous activity of thalamic neurons responsive to vagal stimulation (Saleh and Cechetto 1993; Saleh and Cechetto 1995). Prior electrophysiological studies also demonstrate that CTA and sodium appetite selectively alter taste elicited responses in the PBN (Shimura et al. 1997a; Shimura et al. 1997b; Tokita et al. 2004). In the case of CTA, neural responses to the conditioned taste stimulus are enhanced after acquisition, while expression of sodium appetite is accompanied by decreased sensitivity of taste cells to sodium salt. Importantly, stimulation or inactivation of forebrain sites projecting to PBN similarly produces inhibitory and/or excitatory effects on PBN taste cells (Dilorenzo and Monroe 1992; Li et al. 2005; Li and Cho 2006; Lundy, Jr. and Norgren 2001; Lundy, Jr. and Norgren 2004). Thus, CRF and/or SS forebrain-PBN pathways might be involved in mediating these neurophysiological changes thought to play a role in the elaboration of gustatory preference/aversion and, consequently, ingestive behavior. In the case of SS, evidence suggests a direct inhibitory action on PBN neurons involved in processing visceral signals (Saleh and Cechetto 1993; Saleh and Cechetto 1995). To the best of our knowledge the action(s) of CRF on PBN neural activity has not been determined; however, in several other brain regions like the amygdala, dorsal raphe nucleus and hippocampus it has a predominately excitatory influence (Blank et al. 2003; Lowry et al. 2000; Ugolini et al. 2008).

The CRF and SS expressing forebrain neurons also have extensive input to more rostral regions of PBN primarily concerned with processing nociceptive, cardiovascular, respiratory and gastric information (Baird et al. 2001a; Bester et al. 1995; Chamberlin and Saper 1992; Chamberlin and Saper 1994; Lara et al. 1994; Mraovitch et al. 1982). Stimulation of the LH, CeA, BNST and IC have been shown to influence cardiovascular and/or respiratory function (Galeno and Brody 1983; Harper et al. 1984; Ruggiero et al. 1987; Saleh and Connell 2003). One interpretation is that forebrain induced pressor and respiratory responses are mediated by descending projections to rostral PBN. This notion gains support from experiments showing that cardiovascular and/or respiratory responses produced by electrical or chemical stimulation of the CeA, IC, posterior portion of the anterior hypothalamus, dorsal periaqueductal gray and somatic nociceptors were reduced or completely blocked following inhibition of rostral medial and lateral PBN subnuclei (az-Casares et al. 2009; Boscan et al. 2005; Hayward 2007; Saleh and Connell 2003). Interestingly, the number of LH, CeA, dBNST and IC neurons retrogradely labeled in the present study did not differ as a function of PBN injection site (i.e. rostral vs. caudal), but the incidence of peptide co-expression was significantly greater following rostral PBN injections compared to caudal injections. The significance of this finding is not entirely clear, but might be related to the greater functional diversity of rostral PBN regions.

The present finding that rostral PBN injections, but not caudal injections, produced retrogradely labeled cells in two additional nuclei are consistent with previous studies using anterograde tracer to examine the output of the BNST and PVN. Specifically, *Phaseolus vulgaris*-leucoagglutinin (PHA-L) injections into the dBNST resulted in terminal label throughout the rostrocaudal extent of the PBN. In contrast, injections centered in the vBNST revealed dense label in the rostral PBN, but only very light label in the caudal region corresponding to the present gustatory responsive sites (Dong et al. 2001). A similar pattern of anterograde label was observed following PHA-L injection into the PVN (Luiten et al. 1985). Similar to the LH, CeA, BNST and IC, the PVN has been shown to influence visceral, cardiovascular and respiratory function (Rogers et al. 1996; Rogers and Hermann 1986). Projections from these

forebrain regions to rostral PBN are well suited to "gate" the flow of ascending autonomic information that might be mediated in part by CRF and/or SS. Clearly, further research is needed to determine the identity of additional descending peptidergic pathways to the brainstem, their synaptic organization and their function(s). Although each of the circuits investigated in the present study are known to participate in ingestive behavior and autonomic control, the precise role(s) of specific descending peptidergic inputs is not yet defined.

4. Experimental Procedures

4.1. Subjects

Ten male Sprague-Dawley rats weighing 350–450 g [CrL: CD (SD) BR; Charles River Breeding Laboratories] were used in this study. The animals were maintained in a temperature-controlled colony room on a 12-h light/dark cycle and allowed free access to normal rat chow (Teklad 8604) and distilled water. All procedures conformed to NIH guidelines and were approved by the University of Louisville Institutional Animal Care and Use Committee.

4.2. Surgery

The rats were anesthetized with a 50-mg/kg intraperitoneal injection of pentobarbital sodium (Nembutal). Atropine was administered to reduce bronchial secretions. Additional doses of Nembutal (10-mg/kg) were administered as necessary to continue a deep level of anesthesia. The animals were placed on a feedback-controlled heating pad and rectal temperature was monitored to maintain body temperature at $37\pm 1^{\circ}\text{C}$. Animals were secured in a stereotaxic instrument and the skull was exposed with a midline incision then leveled with reference to bregma and lambda cranial sutures. A small hole was drilled through the bone overlying the cerebellum to allow access to the parabrachial nucleus. The analgesic buprenex (0.1-mg/kg) was administered for at least two days post surgery.

4.3. Electrophysiological recording

A 0.1M NaCl solution was applied to the anterior 2/3 of the tongue for 10 sec using a wash bottle to elicit extracellular recorded neural responses using a glass-insulated tungsten microelectrode oriented 20° off vertical with the tip pointing rostral (1–3M Ω). Only the anterior two-thirds of the tongue was stimulated because numerous studies have demonstrated that forebrain activation has a profound influence on brain stem taste cells that receive input via the chorda tympani nerve (Cho et al. 2003; Li et al. 2002; Li et al. 2005; Lundy, Jr. and Norgren 2001; Lundy, Jr. and Norgren 2004). Further, the concentration of NaCl used in the present study has been shown to produce a significant neural response in each "best-stimulus" class of NST and PBN neurons (Boughter, Jr. et al. 1999; Di Lorenzo et al. 2003; Lundy, Jr. and Norgren 2001; Lundy, Jr. and Norgren 2004; Smith et al. 2000). Once the gustatory PBN was located, the tungsten electrode was replaced by a micropipette (ID 10 – 20 μm) filled with 4% Fluorogold mixed in saline (FG, Biotium Inc) or undiluted green fluorescent latex microspheres suspended in distilled water (GFM, Lumafluor, Inc.). For gustatory PBN injections, the taste responsive area was electrophysiologically relocated and retrograde tracer was injected iontophoretically (FG n=4, +2 μA for 20 min; 2 min on and 1 min off) or by pressure (GFM n=1, 10–20 ms pulses at 20 psi). The rostral PBN injection sites were identified by first electrophysiologically locating the taste responsive area and then moving the recording electrode rostral and re sampling neural responsiveness to NaCl. Injections were made at sites that were unresponsive to anterior tongue stimulation with NaCl typically 500 – 700 μm rostral to the caudal taste responsive area (FG n=4; GFM n=1). Cambridge Electronic Design's Spike2 hardware and software was used to monitor NaCl-evoked neural responses (Lundy and Norgren, 2001). No attempt was made to isolate single neurons for analysis of response rate; rather, responsiveness to NaCl applied to the anterior tongue was monitored visually relative to baseline discharge during pre stimulus water flow.

4.4. Colchicine treatment

Five days after surgery, the animals were re-anesthetized using Nembutal (50-mg/kg) and 2.5 µl of colchicine (10mg/500 µl dissolved in 0.1 M NaCl, Tokyo Kasei Co. Ltd) was infused into each lateral ventricle using a 25-µl Hamilton syringe to arrest axonal transport thereby enhancing the immunohistochemical staining of neuropeptides in cell bodies. The coordinates were 0.85 mm anterior to bregma, 1.5 mm lateral to midline and 3.7 mm ventral to dura (Paxinos and Watson 1982). Buprenex (0.1-mg/kg) was administered for the next two days.

4.5. Perfusion and histology

Two days after colchicine treatment, the animals were administered a lethal dose of Nembutal (150-mg/kg) and perfused through the ascending aorta, initially with 250ml of 0.9% saline containing 5 ml of 100 units/ml heparin followed by 500ml of 4% paraformaldehyde with 1% Sucrose in 0.1 M phosphate buffer (pH 7.4) then 150 ml of 20% Sucrose in 0.1 M PBS. The brains were removed, blocked just rostral to the PBN, and post fixed overnight at 4°C in 30% sucrose. Coronal (20 µm) sections were cut using a cryostat and two series of sections were collected for somatostatin (SS) and corticotrophin-releasing factor (CRF) immunoreactivity.

4.6. Immunohistochemistry

First, brain sections were incubated in 5% normal donkey serum (NDS; Jackson Labs) mixed in 0.3% triton-x phosphate buffer saline (TPBS) for 1 hr. Tissue sections were then incubated for 24 hrs in a TPBS cocktail of rabbit anti-FG (1:250; Chemicon; antigen, fluorogold) and monoclonal mouse IgG anti-SS (1:20; GeneTex; antigen, human somatostatin conjugated to a protein carrier; reactivity, human and rat; purity, protein A affinity purified; positive control, pancreas islet cells) or guinea pig IgG anti-CRF (1:200; Bachem; antigen sequence, H-Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-Ile-NH₂, specificity for CRF (human, mouse, rat) determined by manufacturer using radioimmunoassay applications). Sections were then rinsed several times in TPBS followed by 2 h incubation in a cocktail of FITC donkey anti-rabbit (1:50; Jackson ImmunoResearch) and Cy-3 donkey anti-mouse (1:10; SS; Jackson ImmunoResearch) or Cy-3 donkey anti-guinea pig (1:100; CRF; Jackson ImmunoResearch) mixed in TPBS. Following several rinses, the tissue sections were then cover slipped using Fluoromount-G mounting media. For tissue labeled with microspheres (n=2), only SS and CRF primary antibodies were used. In one series of tissue sections the SS and CRF antibody was omitted and, consequently, Cy-3 immunofluorescence. In one animal that received a rostral PBN injection, the tissue destined for CRF immunohistochemistry was damaged resulting in n=4 for this data set.

4.7. Data analysis

Cell bodies positive for FG or GFM (FITC; excitation filter: 490 nm; barrier filter: 550 nm) and SS or CRF (Cy-3; excitation filter: 520–554 nm; barrier filter: 580 nm) immunoreactivity in the insular cortex (IC), central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST), and lateral hypothalamus (LH) were identified using sequential scanning with an Olympus confocal microscope. The number of immunoreactive cells per section (sum of cells divided by the number of sections) was calculated for each forebrain site and used for statistical analyses. The color segmentation function in Image-Pro Plus software was used to separate and count retrogradely labeled, peptide-ir, and double labeled neurons. Briefly, confocal images were opened in Image-Pro Plus and invert contrast applied, which changed the black background to white, the green color of retrogradely labeled cells to pink, the red color of peptide-ir cells to turquoise, and double labeled cells to dark blue/purple. The threshold for counting a cell as singly or double labeled was set to >10 adjacent pixels exhibiting the same color. For each neurochemical, separate One-Way ANOVAs were used to compare

differences between forebrain sites resulting from caudal or rostral PBN injections, while a Two-Way ANOVA was used to examine differences between PBN injection sites (SPSS 17.0). In some instances, post hoc analyses (LSD) were used to determine the source of statistically significant differences. The results are presented as mean $\pm$ s.e. A value of $P < 0.05$ was considered statistically significant.

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Abbreviations

3V, third ventricle
 7n, facial nerve
 ac, anterior commissure
 cCeA, caudal central nucleus of the amygdala
 rCeA, rostral central nucleus of the amygdala
 CnF, cuneiform nucleus
 dBNST, dorsal bed nucleus of the stria terminalis
 DMC, dorsal motor complex
 fx, fornix
 ic, internal capsule
 IC, insular cortex
 KF, Kolliker-Fuse nucleus
 LH, lateral hypothalamus
 LPBC, parabrachial nucleus central lateral
 LPBCr, parabrachial nucleus lateral crescent
 LPBD, parabrachial nucleus dorsal lateral
 LPBE, parabrachial nucleus external lateral
 LPBI, parabrachial nucleus internal lateral
 LPBV, parabrachial nucleus ventral lateral
 LC, locus coeruleus
 Me5, mesencephalic trigeminal nucleus
 MPB, medial parabrachial nucleus
 MPBE, parabrachial nucleus external medial
 Mo5, motor trigeminal nucleus
 NST, nucleus of the solitary tract
 ot, optic tract
 PBN, parabrachial nucleus
 PBW, parabrachial nucleus, waist part
 Pr5, principle sensory trigeminal nucleus
 PVN, paraventricular nucleus
 scp, superior cerebellar peduncle
 Su5, supratrigeminal nucleus
 vBNST, ventral bed nucleus of the stria terminalis

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Figure 1.

Montage photomicrograph images of tracer injections into the caudal region of the PBN responsive to NaCl stimulation of the anterior tongue (panels A & B) and the rostral unresponsive region of PBN (panels C & D). The dark areas outlined by closely spaced white bars in panels A, B and C represent the core of fluorogold injection, while the bright area in panel D represents that of green fluorescent microbead injection. The approximate boundary of the superior cerebellar peduncle (scp) is outlined by longer more widely spaced bars (white, panels A, B and D; black, panel C). The scale bar in each panel equals approximately 100 μm .

Figure 2.

The different fill patterns represent the extent of individual tracer injections concentrated in the caudal and rostral regions of PBN. Sections are arranged from rostral (top) to caudal (bottom) and lateral is to the right. The approximate levels relative to bregma are indicated at the top left of each figure (Paxinos and Watson, 1998). For each level, a separate diagram immediately to the right is labeled with PBN subnuclei as defined in Paxinos and Watson (1998). The circumference of all injections at each PBN level is outlined by large black dots and superimposed over PBN subnuclei. The scale bar equals approximately 1 mm.

Figure 3.

The mean percentage of retrogradely labeled neurons in the dBNST, cCeA, IC, LH, vBNST, rCeA and PVN co-expressing CRF-ir (A) or SS-ir (B) following injections into the caudal (open bars) or rostral (solid bars) PBN. For caudal PBN injections, only dBNST, cCeA, IC, LH and rCeA were not included in analyses of significance because no retrograde labeled cells were observed in vBNST and PVN. Caudal PBN Injections: **, significantly different from all other groups; *, significantly different from rCeA. Rostral PBN Injections: ****, dBNST and cCeA significantly different from all other groups; ***, significantly different from rCeA and PVN; **, significantly different from IC, LH and PVN; *, significantly different from PVN. #, rostral PBN injections significantly different from caudal PBN injections.

Caudal PBN Injection

Figure 4.

Representative photomicrographs of neurons projecting to caudal PBN and immunoreactive for CRF in the IC, cCeA, dBNST and LH. In each panel, the image at top left (green dotted box) shows fluorogold (FG) retrogradely labeled neurons only (green), top right (red dotted box) CRF-ir cells (red) only and bottom middle (yellow dotted box) the merged confocal images. Yellow indicates double labeled neurons. Double headed arrows point to examples of green FG labeled cells that were not CRF-ir, while single headed arrows point to neurons immunoreactive for FG and CRF. In each panel, a scale bar is shown in the merged image (white, 50 μm) and the magnification is given in the lower right corner. Below the photomicrographs are two schematic representations showing the general location of

retrogradely labeled neurons in each forebrain area. The approximate levels relative to bregma are indicated at the top left corner in each drawing (Paxinos and Watson, 1998).

Rostral PBN Injection

Figure 5.

Representative photomicrographs of neurons projecting to rostral PBN and immunoreactive for CRF in the IC, dBNST, vBNST, cCeA, LH and PVN. In each panel, the image at top left (green dotted box) shows fluorogold (FG) retrogradely labeled neurons only (green), top right (red dotted box) CRF-ir cells only (red) and bottom middle (yellow dotted box) the merged confocal images. Yellow indicates double labeled neurons. Double headed arrows point to examples of green FG labeled cells that were not CRF-ir, while single headed arrows point to neurons immunoreactive for FG and CRF. In each panel, a scale bar is shown in the merged image (white, 50 μ m) and the magnification is given in the lower right corner. The inset from the merged images in LH and PVN shows the area between the ends of the dotted white lines re sampled at 300% the original size. Below the photomicrographs are two schematic representations showing the general location of retrogradely labeled neurons in each forebrain area except PVN. The approximate levels relative to bregma are indicated at the top left corner in each drawing (Paxinos and Watson, 1998).

Figure 6.

Representative photomicrographs of neurons projecting to caudal or rostral PBN and immunoreactive for SS in the IC, dBNST, vBNST, cCeA, LH and PVN. In each panel, the image at top left (green dotted box) shows fluorogold (FG) retrogradely labeled neurons only (green), top right (red dotted box) SS-ir cells only (red) and bottom middle (yellow dotted box) the merged confocal images. Yellow indicates double labeled neurons. Double headed arrows point to examples of green FG labeled cells that were not SS-ir, while single headed arrows point to neurons immunoreactive for FG and SS. In each panel, a scale bar is shown in the merged image (white, 50 μ m) and the magnification is given in the lower right corner. The

inset from the merged images in dBNST shows the area between the ends of the dotted white lines re sampled at 300% the original size.