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Subnuclear organization of parabrachial efferents to the thalamus, amygdala and lateral hypothalamus in C57BL/6J mice: a quantitative retrograde double labeling study

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Abstract

The present study investigated the subnuclear organization of collateralized efferent projection patterns from the mouse parabrachial nucleus (PbN), the second taste relay in rodents, to higher gustatory centers, including the ventroposteromedial nucleus of the thalamus (VPMpc), central nucleus of the amygdala (CeA) and lateral hypothalamus (LH). We made injections of the retrograde tracer red and green latex microspheres into the VPMpc and CeA (VPMpc-CeA group), VPMpc and LH (VPMpc-LH group) or CeA and LH (CeA-LH group, $n = 6$ for each group). Injections into these areas preferentially resulted in retrograde labeling in the ipsilateral PbN in all groups. Cells projecting to the VPMpc, CeA, and LH were generally found in all subnuclei, but were differentially distributed. VPMpc-projecting cells predominated in gustatory-related subnuclei, CeA-projecting neurons predominated in the external lateral (el) subnucleus, and concentrated labeling was observed in the dorsal lateral subnucleus (dl) following LH injection. Double-labeled neurons were found for all groups, almost entirely ipsilaterally and primarily in the medial (m), waist area (wa), ventral lateral (vl) and el subnuclei. These results suggest that PbN neurons in different subdivisions have different projection and collateralization patterns to the VPMpc, CeA and LH. Functional implications of these projections are discussed with an emphasis on their roles in taste.

Keywords

parabrachial nucleus; ventroposteromedial nucleus of the thalamus; central nucleus of the amygdala; lateral hypothalamus; taste system; mouse

Introduction

The parabrachial nucleus (PbN) surrounds the brachium conjunctivum in the dorsal pons. Among its other functions, the PbN serves as the second central relay for taste in rodents. The PbN receives axons from the rostral nucleus of the solitary tract (NST) in the medulla,

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where second-order gustatory taste neurons from the facial, glossopharyngeal and vagus nerves first synapse in the central nervous system (Norgren and Leonard, 1971, 1973). Ascending gustatory projections to the forebrain from the third-order neurons in the PbN can be somewhat arbitrarily divided into two different main routes. One is the so-called “thalamocortical pathway”, where PbN neurons send axons to the ventroposteromedial nucleus of the thalamus (VPMpc), whose neurons in turn project to the gustatory portion of the insular cortex (IC) (Bester et al., 1999; Fulwiler and Saper, 1984; Halsell, 1992; Hashimoto et al., 2009; Karimnamazi and Travers, 1998; Krout and Loewy, 2000; Krukoff et al., 1993; Norgren, 1974; Norgren and Leonard, 1973; Saper and Loewy, 1980; Voshart and van der Kooy, 1981; Yasui et al., 1989). The other route is the “ventral forebrain pathway”, which actually includes projections to a number of taste-related subcortical structures such as the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis, the lateral hypothalamus (LH), and the substantia innominata (Alden et al., 1994; Bernard et al., 1991, 1993; Bester et al., 1997; Halsell, 1992; Hermanson et al., 1998; Jhamandas et al., 1996; Karimnamazi and Travers, 1998; Krukoff et al., 1993; Norgren, R, 1976; Richard et al., 2005; Saper and Loewy, 1980; Schwaber et al., 1988; Voshart and van der Kooy, 1981). These same forebrain areas, excepting the VPMpc, project back to the PbN, suggesting the presence of feedback loops (Allen et al., 1991; Halsell, 1992; Kang and Lundy, 2009; Moga et al., 1990; Shipley and Sanders, 1982; Tokita et al., 2004, 2009; Veening et al., 1984). The PbN has been hypothesized to be a key structure in the central gustatory pathway where forebrain-brainstem interaction occurs, and from where parallel thalamocortical and ventral forebrain pathways arise (Sewards, 2004).

These pathways have been hypothesized to contribute to different functions in taste, i.e., the thalamocortical pathway is involved in “cognitive-discriminative” processing whereas the ventral forebrain pathway is involved in “hedonic-motivational” evaluation of taste (Pfaffmann et al., 1977). Although this dualistic view may be over-simplistic, there is evidence suggesting the distinct roles of these two pathways. For example, electrophysiological studies have shown that the properties of taste activity such as response magnitude and breadth of responsiveness (“entropy”, Smith and Travers, 1979) of PbN neurons that project to the VPMpc are different from those that do not (Hayama et al., 1987; Ogawa et al., 1987). It has also been shown that both amygdala and LH neurons encode information related to taste hedonics and feeding (Azuma et al., 1984; Nishijo et al., 2000; Fontanini et al., 2009). Furthermore, conditioned taste aversion modifies single unit responses to taste stimuli in the amygdala and LH but not in the VPMpc (Aleksanyan et al., 1976; Burešová et al., 1979; Yasoshima et al., 1995; Grossman et al., 2008).

However, detailed anatomical information about the discreteness of thalamocortical and ventral forebrain pathways is limited. A study by Voshart & van der Kooy (1981) demonstrated the existence of collateralized projections from the PbN to the VPMpc and CeA by utilizing two different fluorescent retrograde tracers, Evans Blue and a mixture of 4', 6'-diamidino-2-phenylindol 2 HCl and primuline. They found that apparently only small numbers of PbN neurons project to both of these areas, suggesting that the PbN-VPMpc and PbN-CeA pathways are highly discrete in the rat.

The present study was designed to precisely describe the organization of efferent projections of the mouse PbN to the VPMpc and two major components of the ventral forebrain pathway, the CeA and LH, based on quantitative and cytoarchitectural analysis by injecting fluorescent retrograde tracer latex microspheres into the three aforementioned forebrain areas. In addition to the VPMpc, we focused on the CeA and LH (among other forebrain targets) due to their aforementioned role in taste hedonics and feeding, as well as the fact that their connectivity with the PbN has been well characterized in physiological studies (e.g. Li et al., 2005). The quantitative properties and extent of collateralization within

efferent projections to these three major targets, originating across PbN subnucleus, has not been previously reported. Understanding the degree of collateralization should shed light on the functional organization of PbN efferents, i.e. whether the same information is conveyed to multiple targets from distinct subnuclei, which have been suggested to possess distinct functions (Yamamoto et al., 1994; Geerling and Loewy, 2007; Tokita et al., 2007; Hashimoto et al., 2009; Haino et al., 2010). Furthermore, these studies were conducted using mice, a species of burgeoning use in gustatory research, albeit with limited study of its central taste system physiology (McCaughey, 2007; Lemon and Margolskee, 2009) or anatomy (Sugita and Shiba, 2005; Travers et al., 2007; Zaidi et al., 2006; Hashimoto et al., 2009; Tokita et al., 2009).

Experimental Procedures

Subjects

A total of 31 male and female C57BL6/6J mice (20–31 g) were used. The animals were maintained in a temperature- and humidity-controlled colony room on a 12 h light/12 h dark cycle (lights on at 0700 h, off at 1900 h), and were given ad libitum access to normal dry pellet (22/5 rodent diet, Harlan Teklad, Madison, WI, USA) and water. Thirteen additional mice were excluded from the final number due to unsuccessful injections (i.e. one or the other injection not expelled properly, or not precisely on target). They were arbitrarily divided into Thalamus-Amygdala (VPMpc-CeA), Thalamus-Lateral Hypothalamus (VPMpc-LH), and Amygdala-Lateral Hypothalamus (CeA -LH) groups consisting of 6 mice each. This study was approved by the Animal Care and Use Committee at UTHSC, and all experiments were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996.

Surgery

Animals were anesthetized with intraperitoneal injection of Ketamine/Xylazine (100/10 ml/kg) and positioned in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). The scalp was opened with a midline incision, and the skull was leveled between bregma and lambda by adjusting the bite bar. The body temperature was maintained at 35 °C using a heating pad (Elenco electronics, Wheeling, IL, USA). Relative to the bregma, a glass micropipette (30–35 µm tip diameter) filled with red or green latex microspheres (Lumafluor, Durham, NC, USA) was lowered either into the VPMpc (anteroposterior = −1.95, mediolateral = 0.4, dorsoventral = −4.3), CeA (anteroposterior = −1.2, mediolateral = 2.7, dorsoventral = −5.1), or LH (anteroposterior = −1.2, mediolateral = 1.25, dorsoventral = −5.3) with a micromanipulator (SM-191, Narishige, Tokyo, Japan). Latex microspheres (60 nl) were injected into each region by pressure microinjector system (Picospritzer, General Valve Corp., Fairfield, NJ, USA). In the TH-AM and TH-LH groups, green beads were injected into the VPMpc and red beads were injected into the CeA or LH. In the AM-LH group, red beads were injected into the CeA and green beads were injected into the LH. The injection pipette was left in place for 10 min before and after the injection was made. Supplemental anesthetic was administered as necessary throughout the surgery to maintain the animals under deep anesthesia. After recovery from surgery, none of the mice displayed abnormal behavior or locomotion.

Perfusion and tissue preparation

After a 4-day survival period, mice were perfused transcardially with phosphate-buffered saline and 4% paraformaldehyde. The brains were removed and placed in 10% formalin and then transferred to a 30% buffered sucrose solution and stored at 4°C for at least 1 week. Coronal sections (40 µm) were cut serially using a freezing microtome and divided into two adjacent series. One series was stained with cresyl violet to reveal cytoarchitecture, and the

adjacent series was used for observation of fluorescent tracer. Both series were mounted and coverslipped on silane-coated slides (Scientific Device Laboratory, Des Plaines, IL) with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

Microscopic analysis of sections

Fluorescent labeling in all sections was imaged and analyzed using a Leica (DMRXA2, Leica Microsystems, Bannockburn, IL) episcopic-fluorescence microscope equipped with a digital camera (Hamamatsu ORCA-ER) and imaging software (SimplePCI). Red beads were visualized with a rhodamine filter and green beads with a fluorescein filter. Beads of either color labeled clearly labeled round or fusiform shaped cells in the mouse PbN; viewed under higher power, the labeling had a distinctive granular appearance (e.g. Figure 3). Single-labeled (red or green) and double-labeled neurons (yellow) were plotted from high-resolution digital microscopic images (using 10x or 20x objective) onto 9 PbN subdivisions in representative sections (e.g. Figures 7–9). Areas containing double-labeled cells were also examined using a high-powered objective (63x oil-immersion), focusing up and down through the section under both filters in order to clearly differentiate labeling. It was possible in this way to discriminate cell profiles containing both colors of microspheres (double-labeled) from those that were single-labeled but superimposed (Figure 3).

Cytoarchitectonic identification of PBN subnuclei was made with the use of Nissl-stained sections, and according to previous studies in the rat, hamster and mouse (Fulwiler and Saper, 1984; Halsell and Frank, 1991; Hashimoto et al., 2009). In the present study labeled cells in the Kölliker-Fuse nucleus were not examined because this area has not been reported to be involved in taste processing.

Data analysis

In all PbN subdivisions quantified, numbers of single-labeled neurons for each injection site were compared with respect to ipsilateral–contralateral location (relative to injection site) via repeated measures ANOVA (subdivision x side) followed by planned comparisons of ipsilateral vs. contralateral counts at each subdivision. With asymmetry in counts across subnucleus established, overall numbers of single-labeled neurons (sum of labeled neurons in ipsilateral and contralateral side) with respect to injection sites were compared at each subnucleus with a one-way ANOVA followed by post-hoc comparison (bonferroni). The numbers of double-labeled neurons and percentages of overall labeling from a particular forebrain site, were assessed via repeated measures ANOVA (either subdivision x side, or subdivision x forebrain area), followed by planned comparisons. The statistical rejection criterion for all tests was set at $p < 0.05$.

Results

Injection sites

Microscopic observation revealed that in the 18 animals included in this study injections were centered in each target site (VPMpc, CeA and LH) with minimal spread into surrounding areas (Figure 1). The location and spread of tracer injections in each mouse in all groups are summarized in Figure 2. In many cases the boundary of injected microspheres was very sharp and tracer was observed along with pipette penetration, in contrast to Fluorogold or cholera toxin subunit B, injections of which are characterized by round-shaped injection sites and somewhat blunt boundaries (e.g. Tokita et al., 2009).

Subnuclear organization of the PbN

We divided the PbN into 9 subdivisions based on our own Nissl-stained sections as well as previous studies using the rat, hamster and mouse (Fulwiler and Saper, 1984; Halsell and

Frank, 1991; Hashimoto et al., 2009). These 9 subdivisions include medial (m), dorsal medial (dm), external medial (em), central lateral (cl), dorsal lateral (dl), external lateral (el), internal lateral (il), ventral lateral (vl) subnuclei and waist area (wa). In the present study the m was not divided into the central medial and ventral medial subnuclei, and the el was not divided into inner and outer parts, as has been done in some previous studies (e.g. Halsell and Frank, 1991; Hashimoto et al., 2009). This was due to the fact that in some cases it was difficult to delineate the cytoarchitectonic boundary marking these particular subdivisions of subnuclei. Although we do not disagree with the validity of these cytoarchitectonic classifications in previous studies we, adopted a more conservative approach, and did not subdivide the m or els.

Subnuclear distribution of retrogradely labeled neurons

Figure 3 shows high magnification images of single-labeled and double-labeled neurons. Based on the different shapes of their respective soma, it was possible to reliably discriminate real double-labeled neurons from overlapping single-labeled neurons.

Examples of retrogradely labeled neurons located in major PbN subnuclei from each combination of injection sites (VPMpc-CeA, VPMpc-LH, and CeA-LH) are shown in Figures 4–6. In the VPMpc-CeA group, both VPMpc and CeA injections resulted in numerous retrogradely labeled neurons in taste-related PbN subdivisions including those in the m, wa, and vl, as well as in the el (Figure 4). Concomitant with this overlap was a fairly large number of double-labeled cells, indicating that neurons in these regions often collateralize to both the VPMpc and CeA. In contrast, lateral hypothalamic-projecting cells were found more sparsely distributed in these regions (Figures 5 and 6). Strikingly, a distinct cluster of LH-projecting cells was found in the dl; VPMpc- or CeA-projecting cells were rarely found in this subnucleus.

In all groups, retrogradely labeled neurons were distributed throughout the rostral-caudal extent of the PbN and found preferentially ipsilateral to the injection site (Figures 7–9). The greatest concentrations of cells double-labeled from the VPMpc and CeA were found caudally in the ipsilateral m, wa, and vl, and more rostrally in the ipsilateral el (Figure 7). Neurons that collateralize to the LH and either the VPMpc or CeA were found in these regions ipsilaterally (Figure 8 and 9). Double-labeled neurons were only rarely found on the contralateral side for any groups.

We quantified the mean number of all labeled and double-labeled cells in each mouse, according to subnucleus and side (Figure 10). As expected, the subnuclear distribution of cells retrogradely labeled from each forebrain target was remarkably consistent in each experimental group (e.g. similar distribution and counts of VPMpc-projecting neurons in both VPMpc-CeA and VPMpc-LH groups). Groups were therefore combined to examine the effects of side (ipsilateral vs. contralateral) in each subnucleus, and analyzed with repeated measures ANOVA (subnucleus) with a factor for side. Significant effects were found in each group for subnucleus ($F_{[8,176]} = 39.9$, $p < 0.00001$) and side ($F_{[1,22]} = 27.3$, $p < 0.0001$). Significant interactions between these variables were also found for each group ($p < 0.00001$), most indicative of the uneven distribution of labeled neurons across subdivision. VPMpc-projecting neurons were found preferentially located on the ipsilateral side in the m, cl, dl, and el (side effects confirmed with planned comparisons, $p < 0.05$). In contrast, CeA-projecting neurons were found in significantly greater numbers on the ipsilateral side in all but 2 subnuclei (dl, il). LH-projecting neurons were found in significantly greater numbers on the ipsilateral side in all but 2 subnuclei (dm, il, vl).

We next combined cells counts across both side and experimental groups in order to describe variation in subnuclear organization of projections to each forebrain area (Figure

11). Data were analyzed with a series of one-way ANOVAs (one per subnucleus) followed by multiple comparisons ($p < 0.05$). Significantly more VPMpc-projecting cells were found than CeA- or LH-projecting cells in the m, wa, and vl, i.e. the gustatory region of the PBN ($F_{s [2,33]} = 11.2$; $ps < 0.002$). Greater numbers of VPMpc-projecting neurons were also found in the em ($F_{[2,33]} = 5.6$; $p < 0.008$). On the other hand, more LH-projecting neurons were found in the dl and il relative to VPMpc or CeA-projecting cells ($F_{s [2,33]} = 3.8$; $ps < 0.04$). In the el, each forebrain target differed from one another, with the order of retrogradely labeled neurons $CeA > VPMpc > LH$ ($F_{[2,33]} = 20.6$; $p < 0.00001$).

The amount of double-labeled neurons were counted and analyzed separately within each experimental group (Figure 12). For all three groups, double-labeled cells were found almost entirely on the ipsilateral side (repeated measures ANOVA, side x subnucleus, main effects of side: $F_{s [1,10]} = 9.19$; $ps < 0.01$). A significant effect of subnucleus ($F_{s [8,80]} = 11.98$; $ps < 0.00001$), as well as a significant side x subnucleus interaction ($F_{s [8,80]} = 11.67$; $ps < 0.00001$), was found for each group. The greatest number of double-labeled cells, irrespective of forebrain target, was generally located in the m, wa, el and vl (Figure 12). Examination of double-labeled cells as a percentage of cells labeled from each forebrain area reveals that cells collateralizing to each pair of targets were found concentrated in the gustatory region and in the els (Figure 12D–F). In some regions, the number and percentage of double-labeled cells essentially reflects the overall number of cells that project to each region. For example, in the el, a greater percentage of LH-projecting neurons were double-labeled than either VPMpc-projecting (Figure 12E) or CeA-projecting cells, reflecting the fact that there are fewer LH-projecting neurons in this subnucleus (c.f. Figure 11). The results also indicate an uneven distribution of double-labeled cells across subnuclei. For example, the distribution and number of projection cells located in the wa and em is extremely similar (Figure 11), yet far more double-labeled cells were found in the wa (Figure 12).

Discussion

These studies characterized and quantified the efferent projections from PbN subnuclei to gustatory-related forebrain centers in mice, including the VPMpc, CeA, and LH. In addition, using paired injections of retrograde tracers allowed us to determine numbers of neurons that collateralized to two areas. Overall, we found that retrogradely labeled neurons were found predominantly on the ipsilateral side. Double-labeled neurons were found for each pairing of tracer, especially in the caudal medial (m), waist area (wa), and ventral lateral (vl) subnuclei and in the external lateral subnucleus (el), indicating that some PbN neurons in these areas collateralized to at least two different forebrain regions. These results most likely reflect actual projection patterns as an advantage of latex microspheres is minimal uptake by fibers of passage (Katz et al., 1984; Richmond et al., 1994; Apps and Ruigrok, 2007). The bulk of the work investigating PBN efferents has been done in rats and hamsters; our study with mice revealed a similar organization, although some species differences are elaborated below.

Gustatory regions of the PbN

In rats, taste responsive neurons are predominantly found in the caudal “waist” region, including clusters of cells just medial or lateral to, and within, the brachium conjunctivum (described in Lundy and Norgren, 2004). In mice, the homologous area includes the m, wa, and vl subnuclei as delineated in Figure 4. Additionally, in rats, Halsell and Travers (1997) and Geran and Travers (2009) recorded taste responses from cells in the el and em. Intraoral delivery of tastants has been shown to elicit c-Fos in all of the aforementioned regions, as well as in the dl. Comparable studies in mice have not been done, with the exception of a recent study by Hashimoto and colleagues (2009), where intraoral delivery of 0.1 M NaCl

evoked c-Fos in several different PBN regions, roughly similar to rats (e.g. Yamamoto et al., 1994). In ongoing work in our lab, we have recorded gustatory responses from neurons located in the m, wa, and vl in C57BL/6J mice (Tokita and Boughter, unpublished). We surmise that the subnuclear organization of the mouse PbN with respect to gustatory responsiveness is at least similar to that of the rat or hamster, and thus efferent projections are discussed with respect to this organization.

PbN–VPMpc projection

Previous anatomical studies in rat and hamster indicated a predominantly ipsilateral projection from the PbN to both the CeA and LH, as opposed to a more strongly bilateral projection to VPMpc (Bester et al., 1999; Cechetto and Saper, 1987; Halsell, 1992; Karimnamazi and Travers, 1998; Krukoff et al., 1993; Loewy and Saper, 1980; Norgren, 1974, 1976; Voshart and Van der Kooy, 1981; Yasui et al., 1987, 1989). These bilateral projections to VPMpc were also characterized physiologically in taste-responsive and mechanoreceptive PbN neurons using antidromic activation techniques (Norgren, 1974, 1976; Ogawa et al., 1987). The present experiment confirmed the results of the original study reporting a strong projection from the gustatory region of the PbN (Figures 7 and 8) to the VPMpc (Norgren, 1973). However, the ipsilateral dominance of the VPMpc projection, especially in the m and el subnuclei, is stronger in mice than in the rat or hamster (Halsell, 1992; Voshart and Van der Kooy, 1981; Yasui et al., 1989).

Overall we found robust numbers of VPMpc-projecting cells in the ipsilateral m, wa, and vl, but not dl, subdivisions, generally confirming the results of earlier studies utilizing retrograde tracer injections into the VPMpc in the rat (Fulwiler and Saper, 1984; Voshart and Van der Kooy, 1981; Yasui et al., 1989), hamster (Halsell, 1992) and mouse (Hashimoto et al., 2009). One of the most striking differences from mouse to rat is that there is strong labeling bilaterally (and actually stronger contralaterally) in the em subnucleus following Fluorogold or wheat germ agglutinin–conjugated horseradish peroxidase (WGA-HRP) injections into the VPMpc in the rat (Cechetto and Saper, 1987; Fulwiler and Saper, 1984; Yasui et al., 1989). By contrast, labeling in the em was weak in both sides in our study. Recently, Hashimoto et al. (2009) electrophysiologically identified the location of the VPMpc by recording taste responses prior to iontophoretic injection of the retrograde tracer WGA-HRP and showed labeling in the contralateral em (in their terminology, MPBE) in the mouse. This contralateral projection was not seem very apparent in the Halsell (1992) hamster study. It is not clear if these discrepant results are due to differences in tracer efficacy, extent of injection, or species used. Further studies utilizing both anterograde and retrograde tracers with small, physiologically guided injections are needed to clarify the properties of the em–VPMpc pathway in the mouse.

The numbers of VPMpc-projecting neurons in the el subdivision in the present study was found to be more akin to the hamster where numerous retrogradely-labeled cells are observed in the el (Halsell, 1992), than to the rat where sparse el labeling was found (Fulwiler and Saper, 1984; Yasui et al., 1989). The recent mouse study showed moderate labeling in the el (Hashimoto et al., 2009), but they also reported that VPMpc-projecting neurons in the PbN in general are mainly observed at the boundary of the brachium conjunctivum, and inside the brachium. However, our results show many retrogradely labeled cells in the regions distant from the brachium conjunctivum as well as at the boundary, as in hamster or rat (Figures 4–8). When we substituted Fluorogold for microspheres, the distribution of cells retrogradely labeled from VPMpc did not differ (data not shown).

PbN–CeA projections

Previously, it was shown that neurons in the PbN project directly to the CeA with ipsilateral dominance in the rat and hamster (Bernard et al., 1993; Fulwiler and Saper, 1984; Halsell, 1992; Krukoff et al., 1993; Moga et al., 1990; Norgren, 1976; Saper and Lowey, 1980; Karimnamazi et al., 1998). To the best of our knowledge, there has been no report describing the distribution of cells retrogradely labeled from the CeA in the mouse. Consistent with rat and hamster studies, retrograde labeling in the mouse PbN was predominantly ipsilateral and strongest by far in the el subdivision, followed by the m and vl. Only a few CeA-projecting cells were observed in the dl and il subnuclei (Figures 8–10). Overall, our results indicate that ascending information from the PbN to the CeA is principally conveyed by an ipsilateral projection in rodents.

Numerous retrogradely labeled neurons in the PbN gustatory region, especially in the m subnucleus, were found following injection of microspheres into the CeA. Similar results were obtained from rats (Herbert and Bellintani-Guardia, 1995; Loewy and Saper, 1984; Moga et al., 1990; Richard et al., 2005; Schwaber et al., 1988) and hamsters (Halsell, 1992). In electrophysiological studies, many taste-responsive neurons in the PbN are antidromically activated by electrodes located in the ipsilateral CeA in the rat (Norgren, 1976) and hamster (Li et al., 2005). In these studies, CeA-projecting taste neurons were recorded from the areas that correspond to the m, wa and vl (Norgren, 1976) or m subdivisions (Li et al., 2005) in our study. These results clearly show that the PbN–CeA pathway conveys substantial gustatory information.

Prominent labeling in the el was consistent with previous studies utilizing retrograde tracer injection into the CeA in the rat (Herbert and Bellintani-Guardia, 1995; Loewy and Saper, 1984; Richard et al., 2005; Schwaber et al., 1988) and hamster (Halsell, 1992). The function of the el has been primarily ascribed to viscerosensory and nociceptive processing (Hermann and Rogers, 1985; Richard et al., 2005). Detailed mapping of the hamster PbN gustatory region showed that taste responses evoked by anterior tongue stimulation were recorded from the m (in their terminology, central medial subnucleus) and vl, but not from the el (Halsell and Frank, 1991). In rats, however, taste-activated neurons (especially those responsive to posterior oral cavity stimulation) have been found in the el (Geran and Travers, 2009; Halsell and Travers, 1997). Furthermore, immunohistochemical studies demonstrate that oral, but not intragastric, delivery of a quinine solution evokes robust c-Fos expression in this subnucleus in the rat (King et al., 2003; Yamamoto et al., 1994; Yamamoto and Sawa, 2000).

The el subnucleus has also been shown to be involved in other functions than gustation. For example, sodium deprivation activates the inner el neurons, and subsequent NaCl solution intake activates the outer el neurons, indicating a role of the el in both sodium need state and sodium detection (Geerling and Loewy, 2007). These el neurons may in turn project to the same CeA neurons onto which aldosterone-sensitive NST neurons expressing the enzyme 11- β -hydroxysteroid dehydrogenase type 2 directly synapse (Geerling and Loewy, 2006). Lesions of the CeA attenuate sodium appetite (Zardetto-Smith et al., 1994). Bernard and Besson (1990) reported the involvement of the el in nociceptive processes, showing that the majority of el–CeA projection neurons (69%) were activated by noxious mechanical and/or thermal stimuli applied to the limb, tail, face and tongue of rats. These el neurons lay rostral to those activated by taste. Overall, these findings suggest a close correspondence in function of the el subnucleus and one of its main target sites, the CeA.

PbN–LH projections

Similar to the PbN–CeA pathway, the PbN–LH projections are primarily ipsilateral in the rat and hamster (Bester, 1997; Fulwiler and Saper, 1984; Halsell, 1992; Krukoff et al., 1993; Moga et al., 1990; Norgren, 1976; Saper and Lowey, 1980; Karimnamazi et al., 1998). These previous studies report that cells retrogradely labeled from LH were mainly observed in the superior lateral (this region is not investigated in the present study), cl and dl subdivisions, whereas labeling in the m, wa and vl was modest (Fulwiler and Saper, 1984; Moga et al., 1990). Although our results generally confirm these earlier rat studies, we found that labeling in the cl was modest whereas the dl labeling was particularly strong (Figures 8–10). Proof that the PbN–LH pathway conveys gustatory information is reflected in the fact that as many as 80% of taste-responsive neurons can be antidromically activated by ipsilateral LH stimulation in the hamster (Li et al., 2005).

The strongest labeling from the LH was a distinct cluster of labeling in the dl subnucleus (Figures 5,6,8 and 9). Neurons in this subnucleus show c-Fos expression by intraoral delivery of palatable taste stimuli such as sucrose, saccharin, MSG + IMP, and relatively low concentrations (0.1 – 0.2 M) of NaCl (Yamamoto et al., 1994; Yamamoto, 2006; Hashimoto et al., 2009). Significantly, c-Fos expression in the dl was not evoked by quinine or HCl, or by intragastric delivery of saccharin (Yamamoto and Sawa, 2000). Neurons in the dl or rats express dynorphin, an opioid peptide shown to play a role in appetite and feeding (Morley and Levine, 1981; Hermanson et al., 1998; Maeda et al., 2009). These facts implicate the dl–LH projection in taste-related modulation of feeding behavior.

Relatively fewer numbers of LH-projecting neurons compared to the VPMpc- and CeA-projecting neurons (Figure 10) is in accordance with a weaker density of parabrachial axon terminals in the LH found in previous research. Studies using anterograde tracing or axonal degeneration techniques consistently showed that PbN–LH projections are weaker than PbN–VPMpc and PbN–CeA projections in the rat and hamster (Norgren and Leonard, 1973; Norgren, 1976; Halsell, 1992; Bernard et al., 1993; Krukoff et al., 1993; Travers et al., 1998; Bester et al., 1997,1999). It is also possible that individual double-labeled neurons that collateralize to the LH and either/both the VPMpc and CeA are difficult to recognize due to a paucity of label transported to the soma from the LH relative to the other areas. This is a limitation of using microspheres for retrograde label, as the punctate label may obscure the extent of co-localization.

Comparison of PbN projections to forebrain sites

Significantly more VPMpc-projecting neurons than CeA- or LH-projecting neurons were observed in the m, wa and vl subdivisions (Figure 11), corresponding to the gustatory area. These facts add to the literature implicating the VPMpc as a more salient taste “relay” than the CeA or LH. It is also not surprising that the cells retrogradely labeled from the CeA predominate in the el subnucleus, considering the dominant roles of the el and CeA in autonomic, viscerosensory and nociceptive functions (Richard et al., 2005). The el has also been shown to have substantial projections to other autonomic centers including the bed nucleus of the stria terminalis, insular cortex, and substantia innominata in the rat (Fulwiler and Saper, 1984; Moga et al., 1990). We also found a notable el projection to the VPMpc. Hashimoto et al. (2009) suggest that the inner part of the el conveys taste and viscerosensory signals, whereas the outer part of this subnucleus conveys only viscerosensory signals, based on their results that VPM-projecting cells were found only in the inner part of the el and 0.1 NaCl evoked weak but noticeable c-Fos expression in this subnucleus. Finally, only LH-projecting neurons were found in significant numbers in the dl. In other PbN subnuclei such as the dm, cl, and il where no taste function has been previously ascribed, there were generally no significant differences in the number of labeled cells across injection sites.

Figure 13 contains a schematic detailing the projection patterns and degree of collateralization to the three forebrain sites. In all groups, double-labeled cell were almost exclusively found ipsilateral to the injection site, which may be in part due to fewer labeled neurons in the contralateral side. In the VPMpc-CeA group, taste-related subdivisions such as the m, wa, vl and el showed a substantial proportion of collateralization. These results suggest that the CeA shares ascending taste information from the PbN with the VPMpc. This contrasts with anatomical data from the rat showing rare double labeling in the PbN following VPMpc and CeA retrograde tracer injection (Voshart and Van der Kooy, 1981). It is possible that in the mouse, PbN projections to the VPMpc and CeA are less segregated both in terms of anatomy and function. Further experiments in the mouse utilizing methods such as in vivo electrophysiology or c-Fos immunohistochemistry are needed to clarify the functions of PbN projections to the VPMpc, CeA, and both. Furthermore, future neuroanatomical studies examining potential collateralization to other forebrain areas such as the BNST and the substantia innominata will help complete the picture of PbN connectivity.

The pattern of collateralization in the VPMpc-LH group was similar to that of the VPMpc-CeA group in that proportion of collateralization was relatively high in the m, wa, el and vl subdivisions although the total number of double-labeled cells were less. In the CeA-LH group, both number and percentage of double-labeled cells were low compared to the other two groups. This finding suggests that the CeA- and LH-projections are relatively segregated, although both CeA and LH are categorized as belonging to the ventral forebrain pathway. However, it was demonstrated in an electrophysiological study that 56.4% (57/101) of taste-responsive neurons in the PbN both project to the CeA and LH in the hamster (Li et al., 2005). Since we have not characterized neurons as responding to taste, a comparison between studies is not straightforward. It is noteworthy to indicate that in the Li et al. study (2005) the collision test for antidromicity was performed only with taste-responsive cells but not non-taste-responsive cells, which may account for the higher percentage of collateralization.

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Abbreviations

BLA	basolateral nucleus of the amygdala
CeA	central nucleus of the amygdala
CeL	central nucleus of the amygdala, lateral division
CeM	central nucleus of the amygdala, medial division cp, cerebral peduncle
f	fornix
fr	fasciculus retroflexus
ic	internal capsule
mt	mammillothalamic tract
LA	lateral nucleus of the amygdala
LH	lateral hypothalamus
ml	medial lemniscus

opt	optic tract
NST	nucleus of the solitary tract
PbN	parabrachial nucleus
cl	central lateral subnucleus
dl	dorsal lateral subnucleus
dm	dorsal medial subnucleus
el	external lateral subnucleus
em	external medial subnucleus
il	internal lateral subnucleus
m	medial subnucleus
vl	ventral lateral subnucleus
wa	waist area
PF	parafascicular thalamic nucleus
VPM	ventroposteromedial nucleus of the thalamus
VPMpc	parvocellular division of the ventroposteromedial nucleus of the thalamus

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Figure 1.

Representative images of injection sites in the parvicellular division of the ventroposteromedial nucleus of the thalamus (VPMpc) (A), central nucleus of the amygdala (CeA) (B) and lateral hypothalamus (LH) (C). The left side of each panel shows fluorescent images and the right side of each shows the adjacent cresyl violet stained sections. Scale bar = 1 mm.

A: VPMpc-CeA **B: VPMpc-LH** **C: CeA-LH** **Figure 2.**

Schematic illustrations of the injection sites in each mouse in the VPMpc-CeA (A), VPMpc-LH (B) and CeA-LH (C) groups. In panel A the upper row shows injection sites in the VPMpc and the lower row shows injection sites in the CeA. In panel B the upper row shows injection sites in the VPMpc and the lower row shows injection sites in the LH.

Figure 3.

Fluorescent images of double-labeled (upper row) and single-labeled neurons overlapping (bottom row) in the el subnucleus in the CeA-LH group. Double-labeled neuron (yellow arrowhead) and overlapping single-labeled neurons (red and green arrowheads) could be reliably discriminated based on the different shapes of their soma with high magnification pictures. Scale bar = 20 μm .

Figure 4.

Fluorescent images of retrogradely labeled neurons in the m, wa, vl (upper row), el (middle row) and dl (bottom row) subdivisions in the PbN of mice in the VPMpc-CeA group. Photomicrographs of Nissl-stained sections demarcating the boundary and location of these subdivisions are also shown. Injection of retrograde tracers in both VPMpc and CeA resulted in strong labeling in the m, wa, vl and el but not in the dl. Numerous double-labeled neurons were also observed in these subdivisions. Scale bars in fluorescent images = 100 μ m. Scale bar in Nissl-stained sections = 200 μ m

Figure 5.

Fluorescent images of retrogradely labeled neurons in the m, wa, vl (upper row), el (middle row) and dl (bottom row) subdivisions in the PbN of mice in the VPMpc-LH group. Injection of microspheres in the VPMpc (green) resulted in strong labeling in the m, wa, vl and el but not in the dl. Injection of microspheres (red) in the LH resulted in moderate labeling in the m, wa, vl and el, and concentrated labeling in the dl. There was moderate double labeling in the m, wa, vl and el. Scale bars = 100 μ m.

Figure 6.

Fluorescent images of retrogradely labeled neurons in the m, wa, vl (upper row), el (middle row) and dl (bottom row) subdivisions in the PbN of mice in the CeA-LH group. Injection of microspheres in the CeA (red) resulted in strong labeling in the m, wa, vl and el but not in the dl. Injection of microspheres (green) in the LH resulted in moderate labeling in the m, wa, vl and el, and concentrated labeling in the dl. There was moderate double labeling in the el. Scale bars = 100 μ m.

Figure 7.
Plots showing bilateral distributions of neurons in the PbN retrogradely labeled from the VPMpc (blue), CeA (green) and both of these areas (red). Sections are spaced 80 μ m apart.

Figure 8.
Plots showing bilateral distributions of neurons in the PbN retrogradely labeled from the VPMpc (blue), LH (green) and both of these areas (red). Sections are spaced 80 μ m apart.

Figure 9.

Plots showing bilateral distributions of neurons in the PbN retrogradely labeled from the CeA (blue), LH (green) and both of these areas (red). Sections are spaced 80 μ m apart.

Figure 10.

Mean counts of neurons retrogradely labeled from the VPMpc (A), CeA (B), and LH (C). Counts ($n = 12$ for all groups) were made on both ipsilateral (black bars) and contralateral (white bars) sides relative to injection site. Significant differences (ipsi vs. contra) were found for all forebrain areas (asterisks indicate significant difference via planned comparisons, $p < 0.01$).

Figure 11.

Total mean counts of neurons retrogradely labeled from the VPMpc (black bars), CeA (gray bars), or LH (white bars) across 9 PBN subnuclei (n = 12 mice/group). Data for each subnuclei were analyzed with one-way ANOVA followed by multiple comparison post-hoc tests (Bonferroni). Two asterisks indicates a significance level $p < 0.01$, one asterisk indicates significance $p < 0.05$.

Figure 12.

Mean counts of double-labeled (DL) cells in each experimental group ($n = 6$ per group). Counts of ipsilateral (black bars) vs. contralateral (white bars) double-labeled cells were made for the VPMpc-CeA group (A), the VPMpc-LH group (B), and the CeA-LH group (C). For all three groups, significantly more cells were located ipsilaterally (planned comparisons $p < 0.01$; asterisks). Mean counts of DL cells, expressed as a percentage of total retrogradely labeled cells for each forebrain area, are shown for each group in D-F). Double-labeled cells were in general found more frequently in the m, wa, el and vl (Bonferroni multiple comparisons tests; one dagger for $p < 0.05$, two daggers for $p < 0.01$)

Figure 13. Schematic diagrams showing collateralized projection patterns from the m (blue), vl (green), dl (purple) and el (red) subnuclei to the VPMpc, CeA and LH. The thickness of lines indicates relative robustness of the projection, and arrows indicate terminal fields.