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Patterns of Convergence in Rat Zona Incerta from the Trigeminal Nuclear Complex: Light and Electron Microscopic Study

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Abstract

In contrast to the restricted receptive field (RF) properties of the ventral posteromedial nucleus (VPM), neurons of the ventral thalamus zona incerta (ZI) have been shown to exhibit multiwhisker responses that vary from the ventral (ZIV) to the dorsal (ZID) subdivision. Differences in activity may arise from the trigeminal nuclear complex (TNC) and result from subnucleus specific inputs via certain cells of origin, axon distribution patterns, fiber densities, bouton sizes, or postsynaptic contact sites. We tested this hypothesis by assessing circuit relationships among TNC, ZI, and VPM. Results from tracer studies show that, 1) relative to ZID, the trigeminal projection to ZIV is denser and arises predominantly from the principalis (PrV) and interpolaris (SpVi) subdivisions; 2) the incertal projection from TNC subnuclei overlaps and covers most of ZIV; 3) two sets of PrV axons terminate in ZI: a major subtype, possessing bouton-like swellings, and a few fine fibers, with minimal specialization; 4) both PrV and SpVi terminals exhibit asymmetric endings and preferentially target dendrites of ZI neurons; 5) small and large neurons in PrV are labeled after retrograde injections into ZI; 6) small PrV cells with incertal projections form a population that is distinct from those projecting to VPM; and 7) ~30–50% of large cells in PrV send collaterals to ZI and VPM. These findings suggest that, 1) although information to ZI and VPM is essentially routed along separate TNC circuits, streams of somatosensory code converge in ZI to establish large RFs, and 2) subregional differences in ZI response profiles are attributable in part to TNC innervation density.

Indexing terms

somatosensory; ventral thalamus; GABA; superior colliculus; attention; orienting

Several previous reports have indicated that the ascending trigeminal somatosensory system is composed of two parallel pathways, a single-whisker lemniscal pathway and a multiwhisker paralemniscal pathway (Koralek et al., 1988; Chmielowska et al., 1989; Lu and Lin, 1993; Williams et al., 1994; Veinante and Deschenes, 1999). The principalis subdivision of the trigeminal nucleus (PrV), the ventral posterior medial thalamic nucleus (VPM), and the layer IV barrels of primary somatosensory cortex (SI) constitute the former, whereas the spinal nucleus pars interpolaris of the trigeminal nuclear complex (SpVi of TNC), the rostral sector of the posterior thalamic complex (POm), and the interbarrel (septal) territories of SI represent the latter. In addition to these two well-described circuits,

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a third nucleus in the ventral thalamus, the zona incerta (ZI), has been demonstrated to receive trigeminal afferents. It is not clear how ZI fits into this current framework of connections or what it contributes to somatosensory processing. Its relationship with visual motor structures, such as the superior colliculus (Kim et al., 1992; May et al., 1997), and its unprecedented γ -aminobutyric acid (GABA)-ergic projection to layer I of nearly the entire neocortex (Lin et al., 1990, 1997) suggests that it holds a unique position within the thalamocortical network.

The fact that ZI is largely dominated by a whisker representation and strongly associated with motor nuclei suggests that ZI not only aides in the acquisition of tactile information but may direct responses to imperative somatosensory stimuli. However, it is difficult to address this notion without recognizing that ZI is a heterogeneous structure. Indeed, studies have shown that different subdivisions of ZI can be discriminated on the basis of cytochrome oxidase (CO) staining intensity (Kawana and Watanabe, 1981; Kim et al., 1992; Nicolelis et al., 1992), selective connectivity (Lin et al., 1997; Kolmac et al., 1998; Power and Mitrofanis 1999), electrophysiological properties (Nicolelis et al., 1992), and neurochemical content (Lin et al., 1990; Kim et al., 1992; Kolmac and Mitrofanis, 1999; Bartho et al., 2002; Heise and Mitrofanis, 2004). These various subregions, the rostral (ZIr), ventral (ZIV), dorsal (ZId), and caudal ZI (ZIC), constitute at least four defined sectors of ZI that may each play a distinct role in assimilating a trigeminal event. Certainly, the identification of these subgroups not only has helped to promote the involvement of ZI in nociception and somatosensory perception (Lechner et al., 1993; Nicolelis et al., 1995; Shaw and Mitrofanis, 2002) but also has been useful for implicating ZI in a range of other functions, including locomotion (Morgenson et al., 1985), sociosexual behavior (Edwards and Maillard, 1988; Edwards and Isaacs, 1991), feeding and drinking (Gonzalez-Lima et al., 1993), arousal and attention (Shammah-Lagnado et al., 1985; Berry et al., 1986; Ficalora and Mize, 1989; Hermanson et al., 1995; May et al., 1997; Mitrofanis, 2005), and seizure activity (Brudzynski et al., 1995). In fact, stimulation of the ZIC has been shown recently to be superior to stimulation of the subthalamic nucleus in improving contralateral parkinsonism (Plaha et al., 2006).

In 1992, Nicolelis et al. began to examine the subregional role of ZI in somatosensory processing by recording neuronal responses to trigeminal stimulation in two more extensively studied sectors of the ZI, the ZId and ZIV. The authors found that most ZI neurons exhibit low-threshold activation and multiwhisker receptive fields (RFs). Two somatosensory maps, corresponding to each lamina, were also demonstrated, one partial and one complete in ZId and ZIV, respectively. Neurons in ZId were furthermore observed to have lower spontaneous firing rates and were more difficult to drive. In comparison with ZIV, ZId facial RFs were often larger (many spanning the entire whisker pad) and lacked evidence of clear directionality. We postulated, based on these findings, that subnuclei of the TNC differentially contribute to the innervation of ZI and that coding within these ascending inputs contributes in large part to differences in the construct of ZI RFs. Although other investigators have examined trigeminal projections to ZI, none has looked at more discrete characteristics of terminal architecture that can significantly influence RF properties, such as intranuclear fiber distribution and terminal morphology. This is true of a study performed by Williams et al. (1994) in which the anterograde tracer rhodamine-dextran was injected into PrV and SpVi, and a study conducted by Veinante et al. (2000) in which thick, type I axons of SpVi (2–4 μ m) were reported to project preferentially to a number of sites in the thalamus and upper brainstem, including ZIV. In this latter investigation, neither photomicrographs nor line drawings within ZI were provided, despite detailed depictions of other sites in the dorsal thalamus.

Among these few reports, there is a deeper issue concerning the anatomical substrates that give rise to ZI RFs. Veinante and Deschenes (1999) have reported that there are primarily two types of cells in PrV, a small cell type that projects exclusively to VPM and a large cell type that projects to ZIv as well as other brain regions. The large cells were found to possess RFs of three to five whiskers, whereas the small cells were shown to respond to a single whisker, to exhibit directional selectivity, and to demonstrate high spontaneous activity (Jacquin et al., 1988; Veinante and Deschenes, 1999). Because some ZIv neurons share the same characteristics as PrV small cells, it is likely that ZI may receive inputs from this population of trigeminal projection neurons. The quasiintracellular morphology studies of Veinante and Deschenes (1999) would not be expected to reveal all potential sources of input to ZI, because of limitations imposed by methodology and sample size ($n = 11$). Furthermore, most ZI cells have RFs of more than five whiskers, sometimes as many as 20–30. This property cannot be automatically explained by the presence of large cell RFs of three to five whiskers. Evidence points to a convergence of input at the level of ZI. However, it remains to be determined whether this convergence is based solely on the integration of inputs from large cells or whether small cells contribute to the expansion of ZI RFs.

To address these issues, anterograde and retrograde tracing techniques were employed in the current study. Localized injections of the anterograde tracer biotinylated dextran amine (BDA) were placed into subnuclei of the TNC to examine terminal characteristics that may affect RF properties in ZI lamina, such as intranuclear fiber distribution, density, branching patterns, and orientation. Various retrograde tracers were placed into ZI and/or VPM to reveal all potential sources of trigeminal input to ZI and establish a more complete understanding of the organization of trigeminothalamic circuits. In addition, an electron microscopic analysis was performed in a fraction of cases receiving injections of anterograde tracer. This was done for two main reasons: 1) because there is a serious lack of information regarding the ultrastructure of ZI and 2) because ZI neurons may maintain different synaptic relationships with PrV, SpVi, and SpVc fibers. Evidence of the latter, whether in the form of different terminal sizes, specializations, or postsynaptic contact sites, could be used to lay the foundation for ZId and ZIv RF disparities. Morphological differences are already well-documented in VPM, where SpVi terminals tend to make synaptic contact with small dendrites and PrV terminals exhibit a greater frequency of apposition to cell bodies (Wang and Ohara, 1993; Williams et al., 1994).

METHODS AND MATERIALS

Surgical procedure and perfusion: light level analysis

In total, 39 female (250–300 g) Long Evans hooded rats were used for the anterograde study. Twelve additional rats were used for retrograde tracing. Prior to surgical procedures, rats were anesthetized with Nembutal (i.p. 50 mg/kg). All animals were treated according to guidelines approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Handling and treatment procedures also conformed to NIH standards for the care and use of animals in research. The level of anesthesia was monitored by reflex testing and maintained at a surgical depth with supplementary doses. After placement in a stereotaxic apparatus, a craniotomy was performed, and tracer injections were made at the following coordinates relative to bregma (Paxinos and Watson, 1986): PrV (AP = -9.7 , ML = -2.8 , DV = -6.7 – 7.7), SpVi (AP = -12.7 , ML = -2.8 , DV = -5.3 – 6.4), SpVo (AP = -10.8 , ML = -2.7 , DV = -7.0 – 7.7), VPM (AP = -2.6 , ML = -2.7 , DV = -4.4 – 4.5), and ZI (AP = -4.0 , ML = -2.7 , DV = -7.1). The SpVc was targeted by exposing the IV ventricle and using the obex as a landmark. To avoid the possibility of tracer contamination, separate Hamilton syringes were used for the injection of different tracers. Each was flushed immediately with distilled water before and after use. Once loaded, the

needle was wiped clean to remove any excess tracer substance. Leakage from the tip of the syringe was avoided by introducing a small air bubble toward the opening of the needle just after tracer was drawn.

To characterize axon innervation patterns in ZI from TNC subnuclei, biotinylated dextran amine (BDA; 10,000 MW, 10% solution dissolved in distilled water, Molecular Probes, Eugene, OR) was used as an anterograde tracer. A small amount of BDA (0.2–0.4 μ l) was injected mechanically into different subdivisions of the TNC via a 10- μ l Hamilton syringe over a period of 30 – 40 minutes. In some cases, Hamilton syringes were modified for electrophysiological recording. This was done to verify prior to injection that the needle was positioned in a somatosensory processing subregion. The needle of the syringe was simply fashioned into a crude electrode by coating all but the tip with insulating epoxy. Extracellular responses to blunt-probe deflection of mystacial vibrissae were transmitted from the electrode to standard amplification devices. Once it was determined that the needle was placed in the target of interest, BDA was injected.

To characterize the morphology of ZI projection neurons in TNC and determine the extent to which these neurons provide collaterals to ZI and VPM, retrograde tracing studies were conducted. Because BDA also demonstrates retrograde properties, BDA, Fluoro-Gold (FG; Fluoro-chrome Inc., Denver, CO), or fluorescein-coated microspheres (FCM; Lumafluor Inc.) were used alone or in combination to label TNC-thalamus projection neurons. For single labeling, small amounts of BDA were injected into ZI mechanically, but, for double labeling, BDA deposits were made in the core of VPM. To accomplish this, micropipettes (tip diameter ~30–50 μ m) were filled with BDA [dissolved in phosphate-buffered saline (PBS)] and lowered into the dorsomedial VPM. After recording of vibrissae-evoked multiunit activity to ensure accurate target localization, the tracer was ejected by iontophoresis (positive current pulses of 150 μ A, 200 msec duration, 2 Hz frequency for 30 minutes). The second tracer, 0.4 μ l of FCM, was stereotactically applied to ZI via a 10- μ l Hamilton syringe on the same side of the brain as the VPM injection. However, the syringe was introduced at a 60° angle from the vertical in the mediolateral plane. This mode of delivery prevented the ZI injection apparatus from encroaching on the VPM injection track, and thereby problems associated with cross-contamination of tracers can be circumvented. In some cases, FG was substituted for BDA in the VPM (positive current pulses of 150 μ A, 200 msec duration, 2 Hz frequency for 15 minutes). Under such circumstances, BDA was utilized in ZI.

Anterograde transport of BDA occurred over a period of 3–7 days. At the conclusion of this interval, animals were deeply anesthetized with Nembutal (i.p. 70 mg/kg) and perfused transcardially with 0.9% saline followed by fixative containing 3.5% paraformaldehyde in 0.1 M PBS at pH 7.4. Animals that received retrograde injections were typically permitted to survive longer, approximately 2 weeks. After the perfusion, brains were removed and kept overnight at 4°C in the same fixative containing 20% sucrose.

Tissue processing and data analysis

Brains were cut coronally at 40 – 80 μ m on a freezing microtome and collected in sequential order. Cases that contained BDA were processed by incubating every third section in avidin-horseradish peroxidase (HRP; diluted 1:500 in PBS that contained 0.05–0.1% Triton X-100; Vector, Burlingame, CA) for at least 4 hours at room temperature. Tissue was further treated in a solution containing 0.1 M PBS and 0.05% diaminobenzidine HCl (DAB). Cobalt chloride and nickel ammonium sulfate (0.001%) were added to produce an intense black reaction product. The reaction was initiated by the addition of 0.01% H₂O₂ (total concentration) and terminated by three rinses in PBS when sections began to darken. Another series of sections was also stained with green or red fluorophores that were

conjugated to streptavidin, Cy2, and Cy3 (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were treated for 1 hour, rinsed, mounted on gelatin-coated slides, and then left to air dry. Slides were either coverslipped directly with DPX (fluorescent method) or counterstained with cresyl violet and coverslipped with Permount (DAB method). Labeled profiles were examined with a Nikon epifluorescent microscope that was equipped with a standard light source. As with all experiments in the present investigation, before tissue was accepted for further examination, evidence was required showing injections to be well-localized and tracers to be optimally transported. To this end, we further confirmed the location of needle tracks and the extent of tracer spread with calbindin (CB), parvalbumin (PV), and cytochrome oxidase (CO) staining (not shown). These stains can define the borders of thalamic nuclei and were useful in permitting the intranuclear distribution of trigeminal-ZI terminals to be assessed (see Table 1 for detailed antibody information). The PV stain, in particular, delineates the ZIv and VPM (Nicolelis et al., 1992; Kolmac and Mitrofanis, 1999), and CB distinguishes the ventromedial thalamic nucleus (VM; Lin et al., 1996). To identify subdivisions of the ZI and the borders of surrounding thalamic nuclei, adjacent sections through the ZI were rinsed and then incubated for 24 hours at 4°C in 0.1 M PBS containing a monoclonal antibody directed against PV (1:3,000; SWant, Belinzona, Switzerland), a monoclonal antibody directed against CB (1:2,000–3,000; Sigma, St. Louis, MO), or a polyclonal antibody directed against CB (1:1,000; SWant) or in PBS lacking primary antisera (to confirm antibody specificity). After several rinses in PBS, sections were further incubated for 1–2 hours at room temperature in a 1:200 dilution of anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch). Secondary antibodies were conjugated to Cy2 (green) or Cy3 (red, pseudocolored as magenta in the current study). Alternatively, sections were processed for CO according to the protocol of Wong-Riley (1979). Figure 1 shows the nuclear divisions of ZI and the area where we investigated trigeminal-incertal contacts. Note that the territory of ZId is immediately dorsal to the dense PV staining of ZIv (magenta) and is separated from the PV staining of the ventral posterior nucleus (VP) by the thin external medullary lamina. The medial boundaries of ZIv and ZId were approximated by the lateral margins of VM as designated by CB staining (green). In control studies, in which the CB or PV primary was omitted, sections displayed no such immunostaining.

In addition to nuclear boundary staining, the presence (or absence) of retrogradely labeled cells in particular layers of somatosensory cortex was also used to confirm the location of thalamus injections. For example, previous studies have shown that layer V pyramidal cells in neocortex project to ZI, whereas layer VI neurons project to VPM (Lu and Lin, 1993; Mitrofanis and Mikuletic, 1999). With this information, we were able to ascertain the placement of tracer substance and assess the overall accuracy of the injection. In fact, for cases that received injections of ZI only, it was common that cortical layer V be screened for evidence of retrograde labeling. Likewise, for the case to be deemed a successful ZI injection, an additional lack of layer VI retrograde labeling was required.

For purposes of analysis, DAB-labeled trigemino-incertal fibers were reconstructed with a camera lucida attachment and a $\times 40$ or $\times 100$ objective lens. This permitted a number of axonal features to be assessed, including evaluations of individual fibers for intranuclear trajectory/directionality and propensity to branch. Terminal densities in ZId and ZIv were also quantified according to this method. In these latter examinations, the main focus was on animals that received injections of SpVi ($n = 4$) and PrV ($n = 4$). Two or three of the most densely labeled tissue sections through ZI were selected from each case and subjected to analysis under high magnification ($\times 100$, oil). The locations of individual bouton terminals were recorded within two circular fields per section. Each field measured 240 μm in diameter and corresponded to one subdivision of ZI. The total number of bouton-like swellings found in each area was counted and averaged across cases in the same injection group. Mean values were then compared using the SigmaStat paired-sample *t*-test.

The other morphological analyses of trigeminal afferents were based on reconstructions of single axons in ZIv after separate injections of BDA into PrV (n = 4), SpVi (n = 4), and SpVc (n = 4). Typically, 10 axons were plotted per case, and each fiber was traced throughout multiple planes of focus to track its appearance over an extended distance. Branching patterns were assessed by determining the average frequency with which collaterals extended from an ~80- μ m segment of axon (60–100 μ m), and fiber orientation of each TNC-incertal projection was evaluated by using semiquantitative angle analysis. To perform each of these analyses, individual axons were reconstructed from areas of ZI that lacked dense meshworks of fibers (where it was more feasible to discern single axon profiles). Each fiber was drawn to preserve the orientation of the process within the boundaries of ZI. For angle analysis, each fiber was represented by a straight line plot that extended through the main trunk of the fiber. In practice, the line was often generated by simply joining the beginning and end point of the axon segment. A set of axes was then placed on top of each drawing to determine the pitch of each line. The axes spanned a distance of 180° and consisted of two 90° quadrants that were subdivided into 30° intervals. The angle of each fiber was assessed by lining the x-axis (0°) in parallel with the long axis of ZI (estimated from the medial and lateral extremes of the ZI ventral boundary) and positioning the most medial aspect of the fiber at the intersection of the x and y axes. A protractor was used to categorize the subquadrant (0–30°, 30–60°, 60–90°, 0° to –30°, –30° to –60°, –60° to –90°) to which each fiber belonged (Fig. 2). This was done for each of two sections (five to seven axons/section) per case. The number of axons in each subsector was then counted, and values were pooled across cases with similar injections. If the number of axons in a subdivision exceeded the number of axons in an adjacent subdivision by more than 20%, fibers were deemed to maintain a directional preference. If the number of axons appeared more evenly distributed within several quadrants and there was less than a 20% difference between adjacent subdivisions, fibers were deemed to be randomly oriented within the nucleus. Ultimately, fiber distribution was illustrated by capturing images of labeled profiles with a conventional camera or a Sensys digital microscope attachment (Roper Scientific, Inc.). Fluorescent photomicrographs were acquired on a system that was equipped with the appropriate filters and MetaMorph imaging software (Molecular Devices, Sunnyvale, CA). Adobe Photoshop 8 was used to pseudocolor images and add identification tags (i.e., abbreviations, arrows, lines, and scale bars). This software was also used in a few instances to enhance brightness and contrast when it was determined that the viewed/captured image did not adequately represent the microscopic appearance. In these particular cases, values were modestly rescaled from original settings to provide better resolution of fluorescent markers and to reduce the visual impact of background emissions.

In the analysis of retrograde data, a major advantage was conferred by the utilization of BDA. BDA labels cells in a Golgi-like manner, often revealing cells that are almost completely filled. Thus, the overall size and morphology of ZI projection neurons in TNC could be assessed. We were particularly interested in discriminating the small, oval neurons with polarized elaborations of dendrites from the large, multipolar neurons with expansive dendritic branches that have been described previously for PrV (Veinante and Deschenes, 1999). For each stained section, we plotted the intranuclear distribution of labeled cells and then acquired images of these profiles by photographing at least three sections per subnucleus per case. At this point, cells in PrV were categorized according to shape and dendritic branching (number of processes). To ensure that cell fragments were not included in the analysis procedure, only neurons that demonstrated the presence of a nucleus were accepted for analysis. Thus, our assessments are on the conservative side. Cells were classified as multipolar if one or both of the following conditions were met: 1) large dendritic processes were observed extending in several directions from a cell body that was 2) distorted or irregularly shaped. Conversely, cells were determined to be ovoid if they exhibited a rounded cell body and/or one or two thin dendritic processes on opposite sides of

the soma. The Metamorph surface area function was used to select individual neurons, derive their diameters, and verify that small ovoid cells were approximately 8 μm in size and large multipolar neurons were approximately 25 μm . After these analyses were conducted, the numbers of small and large retrogradely labeled PrV projection neurons were each expressed as a percentage of the total PrV retrogradely labeled population. Values from each injection were then averaged across cases to determine the relative proportion of small and large PrV cells that contribute to the innervation of ZI.

Double labeling of individual TNC projection neurons was examined in cases that received paired tracer injections into ZI and VPM ($n = 5$). Most (if not all) double-labeled cells were found in PrV and were characteristic of one morphological subpopulation (i.e., large multipolar cells), so double-labeled cells were counted in each PrV subnucleus and expressed as a percentage of the total population of large, retrogradely labeled cells. In manner like that described above, values were then averaged across cases to arrive at an overall estimate of double labeling. These cells were photographed using the appropriate filters and were demonstrated by merging complementary single-wavelength images into a composite overlay.

Surgical procedure and perfusion: electron microscopic analysis

To identify synaptic relationships between specific subpopulations of trigeminal afferents and ZI neurons, anterograde tracer injections of BDA were placed into individual subnuclei of the TNC. In total, 23 female (250–300 g) Long Evans hooded rats were used, and cases included the following distribution of injections: PrV = 10, SpVi = 8, and SpVc = 5. The administration of anesthetics, surgical coordinates, tracer volume, and interval to sacrifice were the same as those described above. At the time of perfusion, animals were deeply anesthetized with Nembutal (70 mg/kg) and flushed transcardially with 0.9% saline followed by 3.5% paraformaldehyde and 0.25–0.5% glutaraldehyde in 0.1 M PBS at pH 7.4. The brains were postfixed in the same solution overnight at 4°C.

Tissue processing and data analysis

Brains were cut coronally into 50–100- μm sections with a vibratome. Tissue was then processed to reveal tracer material by using a modified EM techniques (Smith and Bolam, 1991; Chen and May, 2000). Briefly, sections were treated with 0.05% Triton X-100 in 0.1 M PBS at pH 7.2 overnight at 4°C. Under these same conditions, tissue was then further incubated for 24 hours in avidin D-HRP (1:500), with agitation. After consecutive rinsing in PBS, sections were reacted in a solution containing 0.05% DAB, 0.01% nickel-ammonium sulfate, 0.01% cobalt chloride, and 0.01% H_2O_2 in 0.1 M PBS at pH 7.2. This procedure yields a black, electron-dense product that appears homogeneous in labeled axon terminals at the EM level. Samples containing labeled terminals were dissected with a No. 10 blade and subjected to standard EM osmication with en bloc staining procedures. They were then flat embedded in epon, attached to beam capsules, trimmed, and cut. Semithin (1–2 μm) sections were stained with toluidine blue to permit further trimming of the block face before cutting into ultrathin sections. Tissue was collected onto grids coated with Formvar, then further stained with lead citrate and uranyl acetate.

Materials were examined and photographed with a Leo Biological transmission electron microscope. EM evaluations initially included a survey of terminal labeling in ZId and ZIv, but later analyses were concentrated in ZIv. The reasons for this approach were twofold: 1) the trigeminal innervation of ZId is significantly less dense than that of ZIv and 2) no obvious differences were noted between ZId and ZIv in terms of their ultrastructural relationship with different TNC subnuclei. The goal here was to determine the size of labeled axon terminals from different TNC subnuclei, to identify the postsynaptic structure,

and to assess the size of the corresponding target site. This latter objective was undertaken primarily to establish the preponderance of trigeminal synapses with distal vs. proximal dendrites. To obtain measurements, BDA-labeled terminals from different subnuclei of the TNC were randomly photographed. In total, 350 profiles were analyzed (PrV = 120; SpVi = 134; SpVc = 96). The diameter of the labeled bouton and apposing element were then calculated by examining the cross-sectional view of each profile and measuring the transverse width along the perpendicularly oriented long and short axes of the structure (Wang and Ohara, 1993). The mean of these values was calculated and averaged with data from other cases that received similar injections. Results from each subdivision of the TNC were then compared via one-way ANOVA. In addition, labeled axon terminals from each TNC subdivision were examined for evidence of synaptic specialization, as a third criterion for discriminating trigeminal inputs to ZI. These terminals were categorized under the classification of symmetrical or asymmetrical synapses according to definitions established by Peters et al. (1991). A bouton was recorded as belonging to an asymmetric synapse (Gray's type I) if it demonstrated thick, postsynaptic densities, round clear vesicles, and a wide synaptic cleft (~20 nm) relative to its postsynaptic target. In contrast, boutons engaged in symmetric synapses (Gray's type II) exhibited thin, nearly identical pre- and postsynaptic densities, pleomorphic vesicles, and associations with narrower (~12 nm) synaptic clefts. In the present investigation, synaptic specializations were frequently verified by screening serial sections (three or four) through the synapse.

RESULTS

Axonal distribution patterns from TNC to ZI

In total, 24 cases were analyzed after it was confirmed that the anterograde tracer, BDA, was placed within the boundaries of a particular TNC subnucleus, i.e., PrV (n = 8), SpVi (n = 8), SpVo (n = 3), and SpVc (n = 5). Our data revealed that BDA-labeled trigeminal afferents mainly target the contralateral ZI and contribute a weak projection to the ipsilateral ZI (Fig. 3). Among the four major TNC subdivisions that were examined, PrV and SpVi were found to provide the densest projection to ZI. Although inputs from SpVc were notable, they were much less robust by comparison. In contrast, very few afferents, if any, were detected in ZI from SpVo. Inspection of individual ZI lamina further revealed that trigeminal terminals are more prevalent in ZIv than ZId. This differential distribution was consistently found irrespective of whether brain tissue was reacted according to DAB methods or for fluorescence (Fig. 4).

Overall, BDA-labeled terminals from TNC were found to innervate nearly the entire extent of ZIv. For example, after injections of PrV, labeled terminals were observed throughout the mediolateral axis of ZIv (Fig. 5). The densest concentrations, however, tended to avoid the flanking regions of the nucleus and target more centralized portions between the medial and the lateral margins. Occasionally, these fibers crossed the boundaries of ZIv and continued into the overlying ZId. In addition to the bouton-studded, medium-sized fibers that composed the main incertal projection, a few fine-caliber fibers were also noted to ascend to the level of ZIv (Fig. 6A,D). These fibers were found scattered among dense patches of labeling and often appeared long and straight, displaying little evidence of axonal branching or varicose formations. Such morphological diversity suggests that two different subpopulations of PrV neurons provide input to ZI. Although a similar, but more tortuous, pattern of labeling was observed after injections of SpVi (Fig. 6B), the presence of fine caliber axons in ZI was not a shared characteristic (Fig. 6E). In contrast to the TNC projection from PrV and SpVi, labeled terminals from SpVc were found to maintain a rather loose arrangement throughout ZIv and ZId. Individual fibers coursed in a discrete array that showed little evidence of intersection (Fig. 6C,F). When other sectors of ZI were examined,

TNC input was found to be sparse. Both ZIr and ZIc exhibited only a limited number of labeled fibers.

Relative density of trigeminal terminals in ZIv and ZId

Since the densest trigeminoincertain input was found to arise from PrV and SpVi, quantitative analyses were performed to determine whether these two subnuclei contribute differentially to the innervation of ZI. This was achieved by plotting and counting the number of labeled bouton-like swellings in each ZI lamina after injection of BDA into PrV or SpVi. It was revealed not only that ZIv is in receipt of a greater number of trigeminal terminals but also that the difference in number between ZIv and ZId is substantial ($P < 0.001$) and not contingent on the location of tracer injection. In other words, a labeling bias for ZIv was obtained, regardless of whether BDA was placed in PrV or SpVi. A second major finding was that there was no significant difference between the densities of terminals supplied by PrV or SpVi to ZIv or the densities of terminals supplied by PrV or SpVi to ZId. The mean number of bouton-like swellings in ZIv from PrV was 322 ($n = 4$ cases, $SD \pm 51$), whereas the mean number from SpVi was 298 ($n = 4$ cases, $SD \pm 69$). Comparisons with ZId showed the dorsal lamina to contain a mean number of 29 and 22 bouton-like swellings from PrV ($n = 4$ cases, $SD \pm 11$) and SpVi ($n = 4$ cases, $SD \pm 6$), respectively. This translates into PrV-ZIv (612 boutons/100 μm^2), PrV-ZId (55 boutons/100 μm^2), SpVi-ZIv (566 boutons/100 μm^2), and SpVi-ZId (42 boutons/100 μm^2). Figure 7 demonstrates representative cases in which a differential distribution of labeled varicosities from PrV and SpVi in ZId and ZIv was observed. The histogram in Figure 8 and the raw data in Table 2 complement Figure 7 by illustrating the weighted input of PrV and SpVi to ZIv relative to ZId in terms of population values. In this histogram, the number of bouton-like swellings in each ZI subdivision is expressed as an average of data collected from animals in similar injection groups.

Morphological characteristics of TNC terminals in ZI: light level analysis

Because most labeled terminals from TNC were concentrated in ZIv and axon profiles appeared similar in ZIv and ZId after a particular subnucleus injection, our morphological analyses were focused on ZIv. As mentioned earlier, TNC inputs share a few common features, but they also exhibit some striking differences. Closer inspection of PrV and SpVi fibers revealed that projections from both subregions are endowed with a number of bouton-like swellings. Along the length of an individual axon, one bouton can be found approximately every 5–10 μm , whereas SpVc fibers demonstrate bouton-like swellings much less frequently. In this fiber population, one bouton occurs approximately every 30 μm . A second distinction that emerged was noted between PrV and SpVi axon processes. BDA-labeled PrV projections tended to travel along or parallel to the long axis of ZIv and rarely displayed evidence of branching. SpVi fibers, however, branched regularly, and coursed in different directions. Representative line drawings and photomicrographs in Figure 6 depict these differences. After semiquantitative assessment, it was revealed that SpVi fibers branch approximately every 30 μm , whereas PrV processes display only one point of collateralization or less over the entire 80- μm span of axon. Furthermore, after angle analysis, it became evident that most PrV axons ($n = 50$) maintain a consistent intranuclear orientation within ZI. The trajectory of most axons fell within 0–30° of the line delineating the ZI long axis. In fact, 98% of those axons sampled were found within this subquadrant. Although 74% of SpVi axons ($n = 50$) also demonstrated similar directional tendencies, 26% were aligned in a more vertical position. 14% traversed the ZI at a 30–60° angle, whereas 12% of SpVi fibers extended within the 60–90° subquadrant. In consideration of these axonal arrangements, SpVc fibers were observed to resemble PrV fibers more closely. Although very fine in caliber, SpVc processes displayed a characteristic continuity and typically exhibited a straight path in association with the long axis of ZI.

Ultrastructural characteristics of TNC terminals in ZI

Organizational principles at the ultrastructural level may dictate differences in the response profiles of ZI neurons, so we undertook an electron microscopic study of trigeminal terminals in ZIv. We examined the overall morphology of these inputs but were specifically interested in determining whether PrV, SpVi, and SpVc axons could be discriminated on the basis of their terminal bouton size or by their target selectivity. Upon inspection, BDA-labeled terminals were found to contain numerous small, round vesicles and several mitochondria. It was also noted that, regardless of origin, the prevailing type of synaptic specialization was asymmetric (Fig. 9). In general synaptic contacts were mostly axodendritic in nature, but on occasion axosomatic relationships were observed. A more detailed description of terminal endings from different TNC subnuclei is given in the following paragraph.

As mentioned in Materials and Methods, 120 profiles from PrV in ZIv were analyzed. The mean diameter of these terminals was 1.23 μm , with sizes ranging from 0.6 to 3.5 μm (SD ± 0.4 μm). The majority (>90%) formed synaptic contacts with dendrites, having a mean diameter of 1.36 μm and ranging from 0.4 to 6.0 μm (SD ± 0.8 μm) in size. Axosomatic contacts were rare and were noted in only 2.5% of the surveyed sample. For the 134 BDA-labeled SpVi terminals that were analyzed, data revealed an average bouton size of 1.28 μm , although a range of 0.6–2.4 μm (SD ± 0.4 μm) was observed. As with PrV endings, >90% of SpVi terminals were in synaptic contact with dendrites. The diameter of these dendrites ranged from 0.6 to 4.8 μm (SD ± 0.7 μm) but on average was 1.58 μm . Again, somal contacts were infrequent; only small numbers of SpVi terminals were found in apposition to ZI cell bodies (~3.7%). When they were compared, no statistical difference was found between PrV and SpVi in terms of the size of their terminal inputs or the size of their postsynaptic dendritic targets.

In contrast to the above-described findings, BDA-labeled SpVc terminals in ZIv were found to be much smaller. Based on the analysis of 96 terminals, data revealed the mean diameter of SpVc terminals to be 0.87 μm and the mean diameter of their postsynaptic dendrites to be 0.7 μm . A range of 0.2–1.5 μm (SD ± 0.3 μm) and 0.1–1.7 μm (SD ± 0.4 μm) was observed for each synaptic profile, respectively. Interestingly, no SpVc terminals were found in contact with ZI cell bodies. In comparison with other TNC inputs, these differences were significant ($P < 0.001$; Table 3). Figure 10 demonstrates the overall size distribution of TNC terminals in ZIv and shows that PrV and SpVi terminals are clearly larger than SpVc endings.

Retrograde labeling of TNC neurons with projections to ZI and/or VPM

Because morphological analysis revealed two subtypes of axon in ZI after anterograde tracer placement in PrV, we decided to assess all potential sources of afferent input to this ventral thalamus structure from the TNC by conducting retrograde tracer studies. We were specifically interested in determining whether ZI neurons are innervated by a limited subgroup of PrV neurons (i.e., large, multipolar cells), as suggested by Veinante and Deschenes (1999), or whether other neuronal subtypes contribute to the widely pervasive incertal projection. We, therefore, targeted the ZI with our injections and assessed the origin of trigemino-incertal connections in terms of both their location within a specific trigeminal subnucleus and their morphological identity.

A total of seven cases received an injection of tracer into ZI. In support of our anterograde findings, retrogradely labeled neurons were found mainly in the contralateral PrV, SpVi, and SpVc. SpVo, however, was devoid of labeling. On average, about 10 – 40 retrogradely labeled neurons were observed in individual sections through PrV or SpVi. In contrast, only

5–10 ZI projection neurons were found per section through SpVc. The majority of labeled cells residing in PrV were small and oval (Fig. 11D–F), although a few were large and multipolar. Calculations based on more than 1,000 cells from five cases revealed that >90% of the retrogradely labeled cell population consisted of the small subtype, whereas <10% was composed of the large subtype. Surface area assessments were in agreement with earlier reports indicating the size of PrV projection neurons to be ~8 and ~25 μm , respectively. Unlike the projection from PrV, only one population of SpVi neurons was labeled after ZI injections (Fig. 11A–C). This group of neurons tended to be larger in diameter than the small cells in PrV but smaller in diameter than the large cells in PrV. Quantitative analysis revealed this group of multipolar neurons to be 15–20 μm in size, which is similar to the population of POM projecting neurons described by Pierret et al. (2000) and implicated by Veinante to send collaterals to ZI. In terms of topography, it was revealed that the different morphological subtypes in PrV and SpVi did not cluster or maintain unique positions within the boundaries of each subnucleus. Neurons appeared to be distributed in a random fashion and showed no labeling biases for a particular subregion in rostral divisions of the trigeminal complex. However, in SpVc, small, spindle-shaped neurons were restricted to superficial laminae (I/II), whereas multipolar cells were located predominantly in deep layers (III–V).

In light of a previous report that has suggested ZI to receive selective innervation from large, multipolar PrV neurons and VPM to receive exclusive innervation from small, oval PrV cells (Veinante and Deschenes, 1999), we explored the possibility that small PrV cells, labeled in the current study after retrograde injections of ZI, provide collateral input to VPM. Phrased differently, in the final portion of the study, we investigated whether individual small cells in PrV send axonal branches to both ZI and VPM. To accomplish this goal, dual retrograde tracers were injected into ZI and VPM. In total, five cases with restricted injections were analyzed. In general, about 40–100 cells per section were labeled in PrV after injection of the VPM compared with the 10–40 labeled neurons after ZI injection. Over the total population of labeled projection neurons, a majority (~90%) of cells were small and oval and displayed one or two thin, polarized dendrites. Neurons containing either of the two tracers were found to distribute within overlapping territories of PrV. However, despite their close intranuclear association, none of the neurons was double labeled. Interestingly, among the large, multipolar neurons that were labeled after ZI injections (3–10 cells per section), at least two of every 5–10 cells were double labeled (Fig. 12). The distribution of single- and double-labeled projection neurons in PrV is shown in Figure 13. This figure also demonstrates the location and extent of tracer spread after such paired injections.

DISCUSSION

To our knowledge, the present findings are the first to demonstrate a differential and distinctive pattern of TNC afferent inputs to ZI at the light and ultrastructural level. Our data more specifically suggest that ascending trigeminal somatosensory inputs are distinguished by their innervation density and the sublaminar specificity of their efferent projections to ZI as well as their fiber morphology and the size of their bouton-like swellings. Among the four subdivisions of TNC, the major contributors to the trigeminothalamic pathway are PrV and SpVi, providing an especially dense set of inputs to ZIv compared with ZId. At the synaptic level, these terminals tend to primarily form asymmetric contacts and preferentially target dendrites of ZI neurons rather than somata. Based on results from our dual retrograde tracer experiments, there appear to be two populations of small cells in PrV that supply ascending projections to the thalamus; one that targets VPM and the other that is directed toward ZI. Although evidence of collateral branching was not observed across this collection of smaller neurons, a subgroup of large, multipolar cells was detected in PrV that did exhibit double labeling of individual neurons following paired injections of ZI and VPM.

Technical considerations

In the present study, a small amount (0.2–0.4 μ l) of BDA was injected via Hamilton syringe into the TNC. With this volume, it is possible to restrict the spread of tracer to less than 0.7 mm. Because the overall dimensions of an individual trigeminal subnucleus are larger than 0.7 mm, tracer spread outside the target area was not a confounding variable in the present investigation. It is also important to note that anterograde tracers may be taken up by fibers of passage and that ascending trigeminothalamic axons from SpVi may send axon collaterals to PrV (Jacquin et al., 1986). This being the case, there is the potential for injections of PrV to yield tracer uptake into SpVi collaterals and produce subsequent labeling of SpVi fibers in ZI. However, we found that labeled fibers in VPM (data not shown) exhibited different axonal distribution patterns and terminal morphologies following BDA tracer injections into PrV or SpVi. These results suggest that anterogradely labeled axons in ZI from BDA injections placed into PrV did not occur as a consequence of tracer uptake into SpVi fibers of passage. Morphological differences were also detected in ZI between labeled SpVi and SpVc fibers. These findings further suggest that, after tracer deposition in SpVi, SpVc axons were not artifactually labeled and mistaken for SpVi fibers.

Comparison with previous studies at the light microscopic level

In the present investigation, a rather distinctive pattern of TNC afferent input to the ventral and dorsal sublaminae of ZI was observed. Our data suggest that the most robust innervation of ZI from TNC originates in the contralateral PrV and SpVi and that together these afferent fibers distribute throughout almost the entire extent of ZIv. In contrast, SpVc gives rise to a weak incertal projection with fine-caliber fibers, and SpVo contributes little, if any, to the ascending trigeminoincertal pathway. These findings are in overall agreement with previous reports. For example, taking advantage of the retrograde transport of HRP, Roger and Cadusseau (1985) reported that some retrogradely labeled neurons are found within PrV and SpVi after tracer injection into the contralateral ZI. Similarly, by using BDA as an anterograde tracer, Veinante et al. (2000) described dense terminal fields in ZI following tracer injection into PrV and SpVi. Although SpVo was shown to provide major inputs to POn, it was found to lack projections to ZI.

Despite the fact that several similarities can be observed between studies, there is a considerable gap in the literature where trigeminal inputs to ZId are concerned. This point can be demonstrated by comparing the subregional distribution of trigeminal afferents between the current report and Veinante et al. (2000). The latter study emphasized the innervation of ZIv, with no description of an input to ZId. Such missing information is likely to be attributable to the small size of the tracer injection (~200–500 μ m) or to the fact that the smaller proportion of terminals in ZId was inadvertently overlooked.

A second issue worthy of note relates to the matter of topography within ZI. More specifically, through a series of anatomical experiments, Mitrofanis and colleagues have advanced the notion that sensory information from the body surface is transmitted to ZI via somatotopically arranged ascending and descending projections. In particular, Shaw and Mitrofanis (2002) report that, after anterograde tracer injections of cholera toxin subunit B or biotinylated dextran into parietal cortex, brainstem trigeminal (V) nuclei, and spinal cord, labeled terminals are distributed in a medial to lateral pattern. Inputs conveying information from the head concentrate in medial portions of the somatosensory sector of ZI (observed most distinctly in ZIv; see also Mitrofanis, 2005), whereas inputs transmitting information about progressively more caudal body regions target more lateral aspects of ZI. Our data from PrV and SpVi injections support the notion that a significant portion of ZIv is tuned to the processing of somatosensory inputs and that afferent inputs from different sources may show tendencies for innervating certain subregions of ZIv. It is evident from the current

anatomical study that, despite considerable overlap in the middle portion of ZIv, there is a noticeable shift in terminal labeling within ZIv. However, in our hands, this shift in ZI labeling is seen relative to inputs from different subnuclei within the trigeminal complex; i.e., inputs from SpVi are distributed slightly lateral to inputs from PrV. Such labeling differences were not noticed following comparable injections by Shaw and Mitrofanis (2002), possibly because their tracer injections did not cover the Vs (spinal V) as completely as our SpVi penetrations did (see their Fig. 4B). It is also conceivable that differences in labeling between the two studies resulted from the use of different tracer substances.

Despite clear evidence of somatotopy and similarities that exist between VP and ZI, it is difficult to reconcile the notion that the ZI body map is constructed in a way that follows the basic organizing principles of VP. Indeed, Shaw and Mitrofanis (2002) demonstrate that cortical inputs from different regions of the body distribute within discrete patches of ZI and that patches of incertal projections from functionally similar somatosensory centers terminate “in register,” i.e., in the same region of ZI. However, the fact remains that labeled fibers from forelimb areas of cortex and thoracic cord injections overlap in broad regions of ZI, areas that are in receipt of face/whisker information. ZI cells are in a position to be influenced by a variety of tactile inputs, so it is critical that we consider whether this area of the brain is truly wired in a manner that resembles a primary sensory relay. The need to explore this issue is underscored by the physiological findings of Nicolelis et al. (1992). In these experiments, individual cells were observed that had large and variable receptive fields. It should be emphasized that the whiskers/face were represented throughout most of the mediolateral extent of ZI (not just within the medial subregion) and that the most noticeable shifts in topographical organization occurred within the dorsal-ventral domain (see Fig. 2D of Nicolelis et al., 1992).

It has been further proposed that the ZI is an area of integration, a diencephalic interface that links diverse sensory inputs to appropriate visceral, arousal, attention, and posture-locomotive responses (for review see Mitrofanis, 2005). We agree that ZI is a site for information assimilation. In our view, this is best underscored by the fact that the ZI is the only region of thalamus where the lemniscal and trigeminal systems intersect. Certainly, one way for the ZI to become multimodal is for the nucleus to receive a large number of varied inputs. Reports indicate that afferents to ZI originate from a number of sources (for review see Mitrofanis, 2005). However, with the results of the current study, we believe that the processing capacity of ZI is dependent not only on the number of inputs that it receives but also on how these inputs distribute within the nucleus and the preponderance of contacts that are established. Our data show that, indeed, ascending inputs from PrV and SpVi are organized into streams of patterned axonal arrays. BDA-labeled PrV projections tend to sweep across broad domains of ZI neural territory and travel as a continuous band of fibers parallel to the long axis of ZIv. As such, the medial to lateral reach of individual fibers within this projection is in a position to influence sequentially a string of ZI neurons and thereby distribute fine-grain receptive field information over an extended population of cells. A circuit organization of this type could explain how the relatively restricted receptive field properties of PrV neurons are translated into the multiwhisker responses of ZI cells; especially if individual ZI neurons are innervated by several PrV afferents with different principle whisker affiliations. The preferential orientation of ZI dendrites along the main axis of the ZI (dorsolateral to ventromedial), in combination with their extended length, would seem to further indicate that ZI neurons are poised to collect and analyze a broad spectrum of inputs (Iwahori and Mizuno, 1980; Ma et al., 1992; Richard et al., 2002). The tendency for SpVi fibers to branch suggests that they facilitate the transfer of multiwhisker information across the ZI and may even exert a greater influence over ZI activity than PrV. Although much work remains in determining the exact mechanisms responsible for signal encoding in the ZI, the anatomical substrate discussed here not only considers the robust

representation of the trigeminal system in ZI but provides a connectional means to account for the crude somatotopy of ZI that is observed during electrophysiological recording.

Comparison with other studies at the ultrastructural level

At present, very limited information is available regarding the ultrastructural characteristics of ZI, and even less is known about synaptic contacts between ascending trigeminal terminals and ZI neurons in rodent. The only detailed study of the ultrastructural organization of ZI was conducted in the monkey (Ma et al., 1997). The authors categorized synaptic contacts into three subtypes. By comparing the fine features of trigeminal terminals in our data and the synaptic endings described in their study, it appears that the trigeminal inputs observed in the present investigation resemble the type 3 profiles described by Ma and colleagues. These features include 1) numerous small, round vesicles; 2) an active zone that is either flat or concave; and 3) some asymmetric synaptic densities. However, some slight differences were noted between the type 3 synapse reported in monkey and our BDA-labeled trigeminal terminals in ZI. In the monkey, type 3 synapses tend to have mitochondria that contain only round and indistinct cristae. In the present study, BDA-labeled trigeminal terminal mitochondria appeared as long, parallel sheets. Such minor discrepancies may simply reflect species differences between the rodent and the primate or, alternatively, the type of fixative utilized.

By revealing differences in the morphology of terminal endings in ZI and VPM, ultrastructural analysis may also be used as an approach to confirm the existence of different PrV neuronal subpopulations. More specifically, it has been shown in previous studies that ascending PrV terminals not only are rather large but, in apposition to VPM neurons, form synaptic contacts with multiple active zones (see Figs. 1, 2 of Wang and Ohara, 1993; see Figs. 16, 17 of Williams et al., 1994). Such synaptic specialization was rarely noted in the ZI tissue that we evaluated following BDA tracer injection into PrV or SpVi. Our present findings support the notion that a second subpopulation of small PrV neurons may in fact exist and exclusively project to ZI (discussed further below).

At this point, it is worthwhile to note that another recent study has examined SpVi projections to ZI (Lavalley et al., 2005). The authors report that SpVi terminals are medium-sized to large (1–5 μ m) and form asymmetrical synapses with multiple release sites on the somata and proximal dendrites of GABAergic ZIv neurons. These findings differ from ours on the basis that we 1) primarily observed SpVi contacts on small dendrites of ZI neurons and 2) report rare occasions when terminals demonstrate multiple release sites. We can make the following comments. First, trigeminal terminals in ZI do not exhibit multiple release sites that are as clearly defined as those in VPM. Close scrutiny of our EM material (serial ultrathin sections included) indicates some instances in which terminals may have a faint appearance of more than one release site, but we have decided to treat this issue conservatively given the disparities with VPM noted above. Second, we have noted trigemino-incertal contacts on the soma of ZI neurons, but in our sample these contacts represent a minority. Indeed, the axosomatic relationships that Lavalley et al. (2005) document surely provide a necessary anatomical framework for justifying their physiological findings, i.e., explaining how SpVi can drive ZI to exert a feedforward inhibitory influence over POn activity. However, caution is warranted before the synaptology described by Lavalley et al. (2005) is accepted as characteristic of SpVi-ZI ultrastructure, insofar as sample size is not indicated in this report.

Ascending projection neurons in PrV and SpVi

Previous studies have suggested that different populations of projection neurons in PrV and SpVi (each identifiable on the basis of morphological appearance) provide innervation to

VPM and POm (Chiaia et al., 1991; Williams et al., 1994; Veinante and Deschenes, 1999; Pierret et al., 2000; Veinante et al., 2000). For instance, Veinante and Deschenes (1999) reported that PrV neurons responsive to a single whisker send axons to corresponding barreloids in VPM. These neurons were found to possess small somata (~8–12 μ m) and to exhibit restricted dendritic trees. In contrast, multiwhisker responsive cells in PrV were observed to send axons to POm and to be characterized by large somata (~25 μ m) as well as a collateral innervation of ZIv. In the current study, it is important to note that our dual tracer experiments also revealed the evidence of small cells in PrV with projections to VPM. However, we obtained two unexpected findings. First, we encountered large multipolar cells in PrV that were double labeled after injections of VPM and ZI. Second, and perhaps the more intriguing of the two, was the detection of small PrV neurons that project solely to ZI. In brief, these data suggest that 1) multiwhisker information from PrV large cells is relayed in parallel to VPM and ZI (and POm) and 2) single whisker information is acquired by two different populations of PrV small cells and conveyed to ZI and VPM along two separate channels. The functional relevance of these neuronal subgroups remains to be determined, but future combined anatomical-electrophysiological examinations may be able to elucidate whether these small neurons play a critical role in the discrimination and forwarding of particular stimulus attributes to somatosensory thalamus.

In terms of SpVi, Veinante et al. (2000) reported that there are two subtypes of thalamus-bound axons. Type I fibers are thick, fast-conducting processes that target POm, superior colliculus, and ZIv. Type II axons are thin, slow-conducting projections that innervate VPM. By injecting the retrograde tracer Fluoro-Gold into VPM and POm, Pierret et al. (2000) further showed that these axons likely arise from one of two different subgroups of SpVi projection neurons. Large, multipolar cells were found to target POm, whereas small cells were observed to innervate VPM. Based on these lines of evidence, it could be assumed that SpVi neurons with ascending projections to POm and ZIv belong to the class of large, multipolar cells. In the present study, our data indicate that SpVi neurons with projections to ZI are large and multipolar (15–20 μ m), compared with those that were slightly smaller (~10–15 μ m) and found to project to VPM.

Correlation between trigeminal afferent patterns and ZI receptive field properties

In 1992, Nicolelis et al. provided the first account of somatosensory maps within the confines of ZI and suggested further that there is one partial and one complete representation of the contralateral rat face/body within ZId and ZIv, respectively. Analysis of neuronal recordings within these two sectors revealed that ZId neurons exhibit much weaker and larger facial RF properties compared with ZIv. Our present anatomical findings complement electrophysiological data by demonstrating that trigeminal afferent terminals predominantly innervate ZIv and that (unlike connections with ZId) these terminals distribute throughout nearly the entire extent of the ventral division. Such observations lead us to believe that differences in receptive field properties between ZId and ZIv may be attributed in part to disparities in trigeminal innervation density and that the higher proportion of input to ZIv may account for a relative amount of refinement in the ZIv neuronal response to somatosensory stimulation. With regard to which of the inputs from the trigeminal complex influences ZI activity more, it has been shown that ZIv neurons tend to have high spontaneous activity. They display robust low-threshold cutaneous RFs (contralateral) and on occasion exhibit directional selectivity (Nicolelis et al., 1992). Previous electrophysiological studies have revealed that most PrV neurons exhibit direction selectivity (Shipley, 1974; Jacquin et al., 1988; Veinante and Deschenes, 1999; Minnery and Simons, 2003) and demonstrate high rates of spontaneous activity (Minnery and Simons, 2003), so it is very likely that PrV contributes significantly to the RF characteristics observed in ZIv.

On the other hand, SpVi is also in a position to play a key role in influencing ZI RFs but may do so through an alternative mechanism. It has been determined that most ZIv neurons are activated by the displacement of many whiskers (Nicolelis et al., 1992). This is also true of SpVi neurons that display slightly smaller RFs and carry multiwhisker information to VPM, (Woolston et al., 1982; Jacquin et al., 1986, 1988, 1989; Friedberg et al., 1999). The possibility therefore exists that, in establishing contacts with ZI neurons, SpVi cells transfer information about broad territories of the periphery. In preliminary studies, we have found that this is a plausible means for constructing multiwhisker RFs in ZI (Kuo et al., 2004).

Functional considerations

The present study is valuable and important because it provides the first detailed description of the organization of afferent inputs from the trigeminal complex to ZI. With the current findings, we have gained a deeper understanding of the circuitry that governs the function of the ventral thalamic nucleus, ZI, and are in a position to postulate on signal processing mechanisms in this region based on what we know about better studied relays in the dorsal thalamus (see diagram in Fig. 14). Specifically, the well-described lemniscal (PrV to VPM barreloids) and paralemniscal (SpVi to POM) pathways have been shown recently to encode spatial and temporal information, respectively (Ahissar et al., 2000; Petersen and Diamond, 2000). One distinct difference between ZI and dorsal thalamic nuclei is that the single and multiwhisker inputs of PrV and SpVi converge extensively in ZI. Hence, in instances in which the mystacial vibrissae have been deflected, individual neurons in ZI might not only process spatial information regarding the number(s) and position(s) of whiskers that are involved but these cells might simultaneously analyze temporal sequences of events related to the stimulus. This being the case, trigemino-ZI interactions would be expected to differ from functional relationships that have been established in another, more newly described pathway, the extralemniscal pathway. Indeed, it has been speculated that this projection, which originates in SpVi and terminates in the ventrolateral sector of VPM (VPMvl), serves in the temporal processing of object location by working with the paralemniscal system and conveying contact/touch information (Pierret et al., 2000; Yu et al., 2006).

Studies show that output from the ZI may establish a critical inhibitory control over higher order thalamic nuclei, such as POM (Bartho et al., 2002; Trageser and Keller, 2004; Lavallee et al., 2005). This projection appears to have the ability to regulate peripheral sensory signals that reach cortex through a feedforward gating mechanism. Given the findings of Yu et al. (2006), data suggest that ZI may in fact exert a limiting effect on sensor motion transmissions related to whisking. Certainly, the interposition of ZI between POM and SC supports the notion that ZI plays a key role in coordinating sensory-directed motor commands (Lavallee et al., 2005).

For now, we are left to consider the consequences of somatosensory activation on ZI. Certainly anatomical and physiological investigations of the incertotectal pathway have provided fairly strong evidence in support of a role for ZI in attention (Kawamura and Hasikawa, 1978; Moschovakis and Karabelas, 1985; Olivier et al., 1991; Kim et al., 1992; Nicolelis et al., 1992; May et al., 1997). However, the projections from ZI to SC are GABAergic, and available data indicate that ZI activation leads to the suppression of saccade onset (Hikosaka and Wurtz, 1983; Ficalora and Mize, 1989; Ma, 1996). It is, therefore, difficult to reconcile the prevailing view that somatosensory stimulation of the ZI elicits orienting movements of the head and eyes toward a perturbed area of the body surface (Kaelber and Smith, 1979; Mitrofanis, 2005). There is the possibility, though, that GABAergic projections from ZI activate predorsal bundle cells through disinhibition. This mode of action has been advanced in other regions of the SC, such as the stratum griseum superficiale (SGS; Mize, 1992; Schmidt et al., 2001). In further support of this notion, Lu et al. (1985), found that the soma and proximal dendrites of individual intermediate gray layer

cells are virtually covered with GAD-immunoreactive terminals. More importantly, Harting et al. (1997) have shown that corticotectal terminals engage in synapses with profiles resembling inhibitory interneurons. Although ultrastructural investigations of the ZI projection to SC have thus far only confirmed appositions of incertal terminals to SC somata, proximal, and distal dendrites (Kim et al., 1992), it is conceivable that further investigation will provide information concerning the relationship of incertotectal terminals with local GABAergic interneurons.

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Fig. 1. Immunohistochemical characterization of nuclei in the dorsal and ventral thalamus. **A:** Neurons in VP and ZIv are strongly PV-immunoreactive (-ir). Concentrations of Cy3 fluorescence (pseudocolored as magenta) help to define the boundaries of both ZIv and ZId. Note that only a few PV-ir neurons are labeled in ZId. **B:** CB staining in adjacent sections can be used to assess the ZId medial border. The medial extent was approximated at the point where densely packed CB-positive neurons were evident in VM. CB-ir neurons were labeled with Cy2, and appear green. **C:** A merged photograph containing images from A and B. VP, ventral posterior nucleus; VM, ventromedial nucleus; STh, subthalamic nucleus; LH,

lateral hypothalamus; PV, parvalbumin; CB, calbindin; ZId, zona incerta dorsal lamina; ZIv, zona incerta ventral lamina. Scale bar = 300 μ m.

Fig. 2.

Diagram demonstrating the procedure used to obtain a semiquantitative measure of trigeminal fiber orientation in ZI. The procedure was termed *angle analysis*, because it involved overlaying a set of axes over axon profiles that were individually reconstructed via camera lucida line drawings. The trajectory of each fiber was ultimately determined by evaluating where within a range of subsectors (90° to -90°) a fiber was positioned relative to the long axis of ZI. During these assessments, close attention was paid to the ventral and dorsal boundaries of ZI, the former of which was critical for estimating the ZI long axis.

Fig. 3. Comparison of PrV innervation density between the contralateral (**A**) and the ipsilateral (**B**) ZIv. Note that, in this cresyl violet-stained section, BDA-filled fibers project mainly to the contralateral ZIv, whereas labeled processes in the ipsilateral ZIv are quite sparse.

Fig. 4.

Comparison of axon profiles from PrV to ZId (**A,C**) and ZIv (**B,D**). A and B are photomicrographs taken from a DAB-stained section. C and D are images taken from a section processed with Cy2-conjugated streptavidin. Morphological characteristics of labeled PrV fibers in ZId are similar to those of ZIv, but ZIv is more densely innervated. Scale bars = 50 μ m.

Fig. 5.

Line drawings representing axon distribution patterns in ZId and ZIv following BDA transport from different subdivisions of the trigeminal nuclear complex. **A:** PrV injection. **B:** SpVi injection. **C:** SpVc injection. Note that labeled SpVi axons tend to prevail along the lateral extent of ZIv at rostral levels. The distance between sections is approximately 200 μ m. SpVi, interpolaris subdivision of the TNC; SpVc, caudalis subdivision of the TNC.

Fig. 6. Comparison of the BDA-labeled projection in ZIv from PrV, SpVi, and SpVc. **A–C:** Photomicrographs. **D–F:** Line drawings. Most labeled axons from PrV are medium-sized, possessing large boutons (A,D). However, a few fine-caliber fibers with a limited number of small, bouton-like swellings (arrow) were noted. Axons originating in SpVi appeared as dense as the major fiber type from PrV and exhibited similarly sized bouton-like swellings (B,E). One major distinction between SpVi and PrV fibers was that SpVi fibers tended to branch more extensively. Afferents from SpVc are quite sparse in ZIv. Branching rarely, their varicosities are among the smallest of the three input sources examined (C,F). Scale bar = 50 μ m.

Fig. 7. Terminal density plots illustrating number of bouton-like swellings in ZId (**A,C**) and ZIv (**B,D**) following BDA injections of PrV (**A,B**) and SpVi (**C,D**). Note that labeled terminals are much more prevalent in ZIv than ZId under both injection conditions.

Fig. 8.

Bar graph shows density of labeled terminals in ZId and ZIv from unilateral tracer injections placed into PrV ($n = 4$) and SpVi ($n = 4$). There is no statistically significant difference between PrV and SpVi in terms of the mean number of bouton-like swellings in ZIv, with the same holding true for ZId. However, the mean number of bouton-like swellings in ZId and ZIv from PrV is statistically different ($P < 0.001$). A similar difference between ZId and ZIv is observed with respect to input from SpVi.

Fig. 9.

Representative examples of trigeminal terminals in ZIv. **A,B:** BDA-labeled terminals from PrV that make asymmetric synaptic contacts (arrows) with unlabeled dendrites (d). Note the existence of small, round vesicles and mitochondria within these trigemino-incertal boutons. Similar synaptic characteristics were noted in BDA-labeled terminals that arise from SpVi (C) and SpVc (D). Scale bar = 1 μ m.

Fig. 10.

Size distribution of trigeminal terminals in Zlv from each subdivision of the trigeminal nuclear complex. It is evident that, among the three sources, SpVc terminals are some of the smallest. There is a statistically significant difference in terminal size between SpVc and SpVi as well as between SpVc and PrV ($P < 0.001$ for both comparisons). No such difference was noted between SpVi and PrV.

Fig. 11.

Photomicrographs and line drawings of retrogradely labeled cells in SpVi (A–C) and PrV (D–F) that project to ZI. These BDA-containing cells were revealed after staining with streptavidin-conjugated Cy2. Arrows indicate dendritic processes that are emerging from the cell bodies of labeled multipolar SpVi cells and ovoid (bipolar) PrV cells. Note that the interpolaris cell population (A,B) is clearly larger than the principalis contingency (D,E) and that the somas of the former group are more irregularly shaped. The asterisk in A denotes a cell that was sectioned through the nucleus and is characteristic of cells that were accepted for analysis. The interpolaris cell in B is representative of a multipolar neuron having a majority of dendrites in the same focal plane. Two dimensional reconstructions of labeled interpolaris (C) and principalis (F) neurons help demonstrate the overall cell morphology and dendrite distribution of incertal projection neurons in each trigeminal subdivision. Scale bar = 50 μ m.

Fig. 12.

Color photomicrographs illustrate subpopulations of retrogradely labeled neurons in PrV that project to the core of VPM and/or ZI after paired tracer injections into dorsal and ventral thalamus. **A:** BDA-labeled VPM projection neurons as revealed by Cy3 staining. **B:** FCM-labeled ZI projection neurons. Note that both large cells (large arrows) and small cells (small arrows) are evident after dual tracer injections into VPM and ZI. **C:** Superimposed photograph from a case that received a paired injection of FG and BDA. FG was injected into the core region of VPM and retrogradely labeled cells were pseudocolored with a shade of green. BDA was injected into ZI, and retrogradely labeled cells were pseudocolored magenta. Note that each tracer primarily labels small cells in PrV, but none of these cells appear double labeled. **D–F** show an example of a double-labeled large cell (arrowhead) that was observed after injections of FG and BDA were placed into VPM and ZI, respectively. Although not every large cell that was labeled contained both tracers, when double labeling did occur (~30% of the labeled large cell population), it was revealed only within this

morphological subtype of cells. BDA labeling via Cy3 is represented in D by the color magenta, and FG labeling is demonstrated by pseudocoloring with green (E). The overlay of D and E in F further demonstrates single-cell double labeling through the merged white profile. Note that all of the small green cells (PrV-VPM FG projection neurons) lack magenta staining. VPM, ventral posteromedial thalamic nucleus; FG, Fluoro-Gold. Scale bar = 50 μ m

Fig. 13.

Representative line drawings depict tracer deposition sites in ZI and VPM following paired injections of thalamus and show the location of retrogradely labeled cells in PrV. **A** shows placement of BDA in ZI with limited spread to the underlying cerebral peduncle (CP). Overflow of tracer substance into the dorsal thalamus was avoided by angling the trajectory of the needle (long arrow). Vertical injections of FG (short arrow) were well-localized in VPM, with minimal involvement of the adjacent posterior thalamic nucleus (POm). Each illustration in **B** represents a section through the rostrocaudal domain of an individual PrV nucleus (roughly 300 – 400 μ m apart) and demonstrates the typical distribution of labeled cells at each level. Each symbol represents two to five cells. Solid circles denote cells that

project to ZI, whereas open circles indicate cells that project to VPM. Asterisks represent large, double-labeled neurons in PrV. LP, lateral posterior thalamic nucleus; HYP, hypothalamus.

Fig. 14.

Diagram illustrating a simplified functional circuit that involves the trigeminal nuclear complex, somatosensory thalamus, cortex, and superior colliculus. In addition to the parallel lemniscal (green) and paralemniscal (brown) trigeminodorsal thalamic pathways, PrV and SpVi afferents converge in a third projection (orange) to the ZI. Output from ZId is directed toward layer I of nearly the entire cerebral cortex, whereas ZIv targets higher order thalamic relays such as POm as well as the intermediate layer of superior colliculus (blue). Through associations with predorsal bundle cells, this latter projection is thought to play a major role in orienting behaviors along with the nigrotectal pathway. It is interesting to note that both the incertotectal and the nigrotectal pathways contain the neurotransmitter GABA and that these connections may differentially utilize inhibitory mechanisms to govern the activity of superior colliculus neurons.

TABLE 1

Information on Antibodies Used in This Study

Antibody	Manufacturer	Catalog/lot No.	Species	Specifications on structure of immunizing antigen and recognition site
Primary				
Parvalbumin	SWant Co. Bellinzona, Switzerland	235	Mouse	Produced by hybridization of mouse myeloma cells with spleen cells from mice immunized with parvalbumin purified from carp muscles; specifically stains the binding spot of parvalbumin (MW 12,000 and IEF 4.9) in two-dimensional immunoblot; produced against recombinant rat calbindin
Calbindin	SWant Co.	CB-38	Rabbit	D-28k; in immunoblots, has 10% cross-reactivity with calretinin
Calbindin	Sigma St. Louis, MO	C9848	Mouse	Derived from the CB-955 hybridoma, produced by the fusion of mouse myeloma cells and splenocytes from BALB/c mice immunized with a purified recognizes calbindin-D-28K (28 kD) using immunoblotting; antibody does not react with calbindin-D-9K, calretinin, myosin light chain, parvalbumin, or other members of the EF-hand family bovine kidney calbindin-D-28K;
Secondary				
Anti-mouse IgG-Cy2	Jackson ImmunoResearch West Grove, PA	115-226-003 lot No. 62913	Goat	Based on immunoelectrophoresis, antibody reacts with heavy chains on mouse IgG and with light chains common to most mouse immunoglobulins; does not detect nonimmunoglobulin serum proteins but may cross-react with immunoglobulins from other species (see above)
Anti-mouse IgG-Cy3	Jackson ImmunoResearch	115-166-003 lot No. 65766	Goat	Antibody reacts with heavy chains on rabbit IgG and with light chains common to most rabbit immunoglobulins (see above)
Anti-rabbit IgG-Cy2	Jackson ImmunoResearch	111-226-003 lot No. 59480	Goat	
Anti-rabbit IgG-Cy3	Jackson ImmunoResearch	111-166-003 lot No. 65352	Goat	

TABLE 2

Density of BDA-Labeled Trigeminal Terminals in ZId and ZIv Following Unilateral Tracer Injections Into PrV or SpVi¹

Section	PrV/ZIv	PrV/ZId	SpVi/ZIv	SpVi/ZId
1	290	7	241	24
2	250	48	290	25
3	312	24	303	19
4	413	22	273	20
5	322	28	395	37
6	394	33	280	14
7	315	16	407	18
8	300	39	188	23
9	306	37	306	20
Mean	322	29	298	22

¹There is no statistically significant difference between the density of terminals from PrV and SpVi to ZIv or the density of terminals from PrV and SpVi to ZId. However, there is a statistically significant difference in terminal density between ZIv and ZId from either PrV or SpVi ($P < 0.001$).

TABLE 3
SIZE COMPARISON BETWEEN BDA-LABELED TRIGEMINAL TERMINALS AND POSTSYNAPTIC DENDRITES IN ZIV

Input	No. of terminals	Terminal diameter (mean $\pm$ SD; μ m)	No. of dendrites ²	Dendrite diameter (mean $\pm$ SD; μ m)	Input comparison	Terminal diameter comparison	Dendrite diameter comparison
SpVc	96	0.87 $\pm$ 0.3	67	0.70 $\pm$ 0.4	SpVc vs. SpVi	$P < 0.001$	$P < 0.001$
SpVi	134	1.28 $\pm$ 0.4	102	1.58 $\pm$ 0.7	SpVc vs. PrV	$P < 0.001$	$P < 0.001$
PrV	120	1.23 $\pm$ 0.4	100	1.36 $\pm$ 0.8	SpVi vs. PrV	No significant difference	No significant difference

¹ The terminals of PrV and SpVi inputs are larger than those of SpVc. The same holds true for dendrites that are postsynaptic to these afferents.

² Denotes only those dendrites that were observed to be in contact with BDA-labeled trigeminal terminals. Not all BDA-labeled terminals that were analyzed formed synaptic associations.