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UNMYELINATED FIBERS OF THE ANTERIOR ETHMOIDAL NERVE IN THE RAT CO-LOCALIZE WITH NEURONS IN THE MEDULLARY DORSAL HORN AND VENTROLATERAL MEDULLA ACTIVATED BY NASAL STIMULATION

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Abstract

The anterior ethmoidal nerve (AEN) innervates the nasal passages and external nares, and serves as the afferent limb of the nasopharyngeal and diving responses. However, although 65% of the AEN is composed of unmyelinated fibers, it has not been determined whether this afferent signal is carried by unmyelinated or myelinated fibers. We used the transganglionic tracers WGA-HRP, IB4-HRP, and CTB-HRP to trace the central projections of the AEN of the rat. Interpretation of the labeling patterns suggests that AEN unmyelinated fibers project primarily to the ventral tip of the ipsilateral medullary dorsal horn (MDH) at the level of the area postrema. Other unmyelinated projections were to the ventral paratrigeminal nucleus and ventrolateral medulla, specifically the Böttinger and RVLM/C1 regions. Myelinated AEN fibers projected to the ventral paratrigeminal and mesencephalic trigeminal nuclei. Stimulating the nasal passages of urethane-anesthetized rats with ammonia vapors produced the nasopharyngeal response that included apnea, bradycardia and an increase in arterial blood pressure. Central projections of the AEN co-localized with neurons within both MDH and RVLM/C1 that were activated by nasal stimulation. Within the ventral MDH the density of AEN terminal projections positively correlated with the rostral-caudal location of activated neurons, especially at and just caudal to the obex. We conclude that unmyelinated AEN terminal projections are involved in the activation of neurons in the MDH and ventrolateral medulla that participate in the nasopharyngeal response in the rat. We also found that IB4-HRP was a much less robust tracer than WGA-HRP.

Keywords

HRP; transganglionic tracer; MDH; RVLM/C1; Böttinger complex; diving response

1. INTRODUCTION

The anterior ethmoidal nerve (AEN) is a small branch of the ophthalmic division of the trigeminal nerve. The AEN innervates the nose, external nares, and the mucosa of the anterior

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internal nasal passages (Greene, 1963). Stimulation of the nose and nasal mucosa can produce apnea that prevents noxious substances from entering further into the respiratory tract or the lungs (Widdicombe, 1986). In addition, nasal stimulation can produce profound cardiovascular changes through initiation of the nasopharyngeal reflex or diving response (Butler and Jones, 1982; Daly, 1984). Because the AEN innervates the nasal passages, and electrical stimulation of the anterior ethmoidal nerve can also produce the same cardiovascular and respiratory responses (McCulloch et al., 1999a; Rybka and McCulloch, 2006), this suggests that the AEN constitutes the initial part of the afferent limb of these protective reflexes.

The projections of nerves and neurons can be traced anterogradely, retrogradely, or transganglionically, and horseradish peroxidase (HRP) is one of the most commonly used tracers for these purposes (Mesulam, 1982). HRP is often conjugated with another molecule (i.e. wheat germ agglutinin (WGA), isolectin B4 of *Bandeiraea simplicifolia* (IB4), or the B fragment of cholera toxin (CTB)), resulting in labeling that is both more intense and more extensive than when free HRP is used (Mesulam, 1982). Additionally, there can be differential transport in small and large fibers within a nerve when HRP is conjugated to these different molecules (Liu et al., 1995; Sugimoto et al., 1997). WGA-HRP is often used to label projections of both small and large fibers within a nerve, although it appears to preferentially label small diameter fibers (Liu et al., 1995; Robertson and Grant, 1985). In contrast, IB4-HRP has been shown to selectively trace small diameter fibers (Kitchener et al., 1993; Kitchener et al., 1994; Kobayashi and Matsumura, 1996), and CTB-HRP can selectively trace large diameter fibers (Robertson and Grant, 1985). Therefore, by using these selective tracers, the central projections of small, primarily unmyelinated, and large, primarily myelinated, axons within a particular primary afferent nerve can theoretically be determined (Pan et al., 2003; Sugimoto et al., 1997).

Previous studies have shown that the AEN projects primarily to the medullary dorsal horn (MDH; trigeminal nucleus caudalis) in the rat (Anton and Peppel, 1991; Panneton et al., 2006). Neurons within the ventral portion of the superficial MDH are activated, and express Fos as a measure of neuronal activation (Dragunow and Faull, 1989), after nasal or AEN stimulation (McCulloch and Panneton, 1997; McCulloch, 2005; Rybka and McCulloch, 2006). The AEN also projects to other brainstem locations in the rat that may be involved in the cardiovascular and respiratory responses to nasal stimulation (Panneton et al., 2006). Since the AEN is composed of both unmyelinated C-fibers (65%) and small myelinated A δ fibers (25%) (McCulloch et al., 1999a), we wanted to determine if the afferent limb of protective nasal reflexes project into the brainstem via unmyelinated or myelinated axons. Additionally, we wanted to determine if the central projections of the AEN show co-localization with neurons within the MDH and ventrolateral medulla that are activated during nasal stimulation.

2. RESULTS

Trigeminal Ganglion Labeling

Cell bodies within the left trigeminal ganglion contained label after injection of WGA-HRP, IB4-HRP and CTB-HRP into the left AEN, and were clustered along the edge of the ganglion (Fig 1A-C). WGA-HRP produced the most intense ganglion cells labeling (Fig 1A), and these cells had an average cross-sectional area of 374.7 ± 25.5 (Table 1). The cross-sectional areas, perimeters and diameters of the ganglion cell bodies labeled after injection of the three different tracers (WGA-HRP, total of 658 cells from 4 animals; IB4-HRP, total of 279 cells from 5 animals; CTB-HRP, total of 243 cells from 3 animals) were not significantly different from each other (Table 1).

Central Projections of the Anterior Ethmoidal Nerve

All brainstem labeling was observed ipsilaterally as a light dusting, indicative of terminal, rather than somal, labeling. The exception to this terminal label was the labeling of the cell bodies within the trigeminal ganglion and mesencephalic trigeminal nucleus. Injection of the tracer into the nerve resulted in better (more dense) labeling than dipping the cut end of the nerve, although qualitatively the labeling was similar. The pattern of labeling described is taken from results from both injection and dipping studies. Optimal labeling occurred after 3 days of transport, and the density of labeling did not increase if the transport duration was increased to 5–6 days.

WGA-HRP—Sparse WGA-HRP label was located ventrally in the superficial dorsal horn of the rostral cervical spinal cord, and in the adjacent spinal tract (Fig 2A). Label was located caudal to the pyramidal decussation, disappeared at the level of the pyramidal decussation, and then reappeared just rostral to the pyramidal decussation. Between the pyramidal decussation and caudal end of area postrema there was light labeling of the ventral MDH adjacent to the spinal trigeminal tract (Fig 2B).

The most dense WGA-HRP label was located ventrally within the MDH, at the level of the area postrema (Fig 2C). At the caudal end of the area postrema, label was located in a narrow band adjacent to the spinal trigeminal tract, and was slightly more dense ventrally. There was no label found in deeper MDH locations (laminae III, IV and V). Some label was located nearby in the caudal ventrolateral medulla, between the ventral tip of MDH and the dorsolateral tip of the lateral reticular nucleus. Midway along the rostral-caudal extent of the area postrema, the trigeminal subnucleus interpolaris pushes in, displacing the MDH medially (Fig 2D).

Consequently, at the rostral end of the area postrema near to the obex (defined as the opening of the central canal into the fourth ventricle), label was located more medially, in the displaced substantia gelatinosa. At the obex, label concentrated at the ventral tip of the displaced substantia gelatinosa (Fig 2E). Also, a thin tail of label extended dorsally from the ventral tip of the displaced substantia gelatinosa along the interpolaris-caudalis transition. A thin sparse band of label also extended along the ventrolateral edge of interpolaris, and continued along the lateral side of interpolaris, adjacent to the spinal trigeminal tract.

Label was seen within the ventral spinal trigeminal tract (Fig 2F) along the full rostral-caudal extent of the area postrema. Label was more dense close to the ventral paratrigeminal nuclei, especially at the level of the obex. This spinal trigeminal tract label continued rostrally even after the substantia gelatinosa was pushed medially. Sparse label in and immediately around the ventral tip of the spinal trigeminal tract continued rostrally, and some label was present in the ventral sensory trigeminal root.

Immediately rostral to the obex, label remained concentrated in the extreme ventral tip of interpolaris (Fig 2G), but the label density diminished and quickly faded away. Sparse label was present along the ventromedial edge of subnucleus interpolaris, but did not appear in the pre-Bötzinger complex. More rostrally label was present ventrally in the rostral ventrolateral medulla, running parallel to the inner edge of the ventral spinocerebellar tract (Fig 2H) within the cardiovascular RVLM/C1 region. The origin of this label within the ventrolateral medulla appeared to be a direct medial extension of label from the spinal trigeminal tract (Fig 2I). Approximately 250 μ m caudal to the caudal pole of the 7th nucleus label extended dorsally from the spinal trigeminal tract through the Bötzinger complex towards the subcompact formation of the nucleus ambiguus (Fig 2J). At the level of the caudal subnucleus oralis, label was found along the lateral and dorsal-lateral edges of the 7th nucleus (Fig 2K), was but not found in the region of the retrotrapezoid nucleus. Cell bodies of the mesencephalic trigeminal nucleus were labeled (Fig 2L). Axonal labeling streaming toward these labeled cell bodies was sometimes seen along the medial edge of the principle trigeminal nucleus. Label was not found

outside of the trigeminal system, including the nucleus tractus solitarius (NTS), Kölliker-Fuse nucleus or any of the parabrachial subnuclei.

IB4-HRP—Although IB4-HRP label was present in the trigeminal ganglion (Fig 1B; Table 1), IB4-HRP brainstem labeling, if present, was always less intense than that of WGA-HRP, often being barely above background (compare Fig 1D with Fig 2C, and Fig 1E with Fig 2F). In many animals no label was detected within the brainstem after IB4-HRP injection into the AEN, even if transport time was increased to 6 days and/or refinements to the processing procedures were made (Kitchener et al., 1993; Olucha et al., 1985). When IB4-HRP brainstem label was present, we found that the alcohol step during tissue counterstaining caused substantial fading of the label, and that the label completely faded away within a couple of days even without counterstaining. Consequently the visualization of IB4-HRP was often problematic. However, when IB4-HRP was present within the brainstem, it was found only within the spinal trigeminal nucleus and spinal trigeminal tract, in the same locations, and with the same labeling pattern, as that of WGA-HRP. IB4-HRP label was not found in the mesencephalic trigeminal nucleus.

CTB-HRP—CTB-HRP label was found within the trigeminal ganglion (Fig 1), and within the paratrigeminal nucleus, especially at the level of the obex. CTB-HRP label was also found within the cell bodies of the mesencephalic trigeminal nucleus (Fig 1F), and in the axons leading to these cell bodies. Besides these locations, CTB-HRP label was not seen within any other area of the brainstem.

AEN Projections and Neurons Activated by Nasopharyngeal Stimulation

Cardiorespiratory responses to nasal stimulation—Nasal stimulation with ammonia vapors caused significant cardiorespiratory changes (Fig 3 and Table 2). Nasopharyngeal stimulation caused a significant decrease in HR (370 ± 14 to 236 ± 21 beats/min; $p < 0.001$), with the lowest HR during the nasopharyngeal stimulation decreasing to 137 ± 21 beats/min. Additionally there was a significant increase in MAP (120 ± 3 to 140 ± 3 mm Hg; $p < 0.001$) and significant decrease in RR (97 ± 4 to 22 ± 4 breaths/min; $p < 0.001$) during the nasal stimulation. When it occurred, the apnea duration during the nasopharyngeal stimulation was 7.8 ± 0.9 s, which was almost 50% longer than the 5 s duration of the nasal stimulation itself.

Fos-positive MDH and RVLM/C1 neurons—Stimulation of the nasal passages with ammonia vapors caused activation of brainstem neurons, indicated by the detection of Fos within the nuclei of these neurons. Brainstem locations where Fos-positive neurons were found included the ventral portions of the superficial MDH (laminae I and II; Figs 4A and D), ventral paratrigeminal nucleus, and RVLM/C1 region (Fig 4G). The number of Fos-positive neurons increased within the MDH when moving from a caudal to rostral direction (Figs 5B), peaking just caudal to the obex. Within the caudal part of the ventral MDH, each hemisection contained 3.3 ± 1.1 Fos-positive neurons, whereas more rostrally near the obex, each section contained 13.8 ± 2.8 Fos-positive neurons. The number of Fos neurons within the MDH at or 90 μ m caudal to the obex was significantly greater than the number of Fos neurons at the caudal and rostral extremes of the MDH ($p = 0.05$).

Fluorescent WGA-HRP Labeling—After injection of WGA-HRP into the left AEN, immunohistochemical procedures with fluorescent labeling demonstrated the same location and pattern of label as that of the TMB visualization procedures (see above: Central Projections of the Anterior Ethmoidal Nerve). Fluorescent label was visualized within the trigeminal ganglion, ventral superficial MDH (Figs 4B and E), ventral spinal trigeminal tract, RVLM/C1 region (Fig 4H), and mesencephalic trigeminal nucleus. Within the ventral MDH, the intensity of the WGA-HRP label that surrounded activated MDH neurons increased when moving from

a caudal to rostral direction, peaking just caudal to the obex, although this trend was not significant (Fig 5A).

Co-localization of HRP Label and Fos-positive neurons—After WGA-HRP was injected into the AEN, co-localization of central terminations of the AEN and activated Fos-positive neurons was seen both within the MDH (Figs 4C and F) and RVLM/C1 region (Fig 4I). Co-localization occurred when the red terminal AEN label surrounded within close proximity a green Fos-positive neuron (arrows, Fig 4). In this co-localization situation, red-green double-labeling was not observed, as the AEN terminal label would localize to the cell body and dendrites, while Fos would be contained with the nucleus of the cell.

The average intensity of the central terminations of the AEN around Fos-positive MDH neurons varied with the rostral-caudal location. Although not showing significant rostral-caudal differences, the WGA-HRP intensity increased and peaked near the obex (Fig 5A). Also, the number of Fos-positive neurons within the MDH left hemisection peaked just caudal to the obex (Fig 5B). There was a positive correlation between the WGA-HRP AEN tracer intensity and number of Fos-positive MDH neurons (Pearson Product Moment Correlation coefficient $r = 0.91$; $p < 0.001$).

3. DISCUSSION

The present study investigated the central terminations of the AEN of the rat, and the anatomical relationship between the central projections of the AEN and second-order brainstem neurons activated by nasal stimulation. The AEN projects heavily to the ipsilateral superficial laminae of the ventral MDH, and these projections appear to be from unmyelinated fibers. The AEN central terminations co-localize with activated MDH neurons, and the density of the AEN projection reaches a peak just caudal to the obex, a location that also contains the greatest number of MDH neurons activated by nasal stimulation. The AEN also sends unmyelinated terminal projections to respiratory and cardiovascular areas of the ventrolateral medulla, specifically the Böttinger complex and RVLM/C1 region, and the AEN central terminations co-localize with activated RVLM neurons. These AEN projections to the ventrolateral medulla could be an anatomical link between stimulation of the nasal passages and the respiratory and cardiovascular reflex responses to nasal stimulation. We suggest that the afferent signals carried by unmyelinated AEN fibers are involved in the activation of neurons within both the MDH and ventrolateral medulla participating in the central integration and efferent limb of the nasopharyngeal response in the rat. Also, we found IB4-HRP to be a much less robust transganglionic tracer compared with WGA-HRP and CTB-HRP.

3.1 Technical Considerations

Several different methods may be utilized to introduce transganglionic tracer into a nerve. In the present study, we either dipped the cut AEN into a Parafilm micro-cup containing the tracer or injected the tracer directly into the AEN using a glass micropipette. Both methods resulted in a qualitatively similar pattern of brainstem labeling, but the injection of the tracer directly into the nerve produced more intense terminal labeling. Presumably this was because after nerve injection the conjugated HRP tracers were taken up by AEN axons after binding to specific membrane receptors followed by adsorptive endocytosis, whereas dipping the nerve relied upon bulk endocytosis at the cut end of an axon to uptake the tracer (Kitchener et al., 1994; Robertson and Grant, 1985). We also found that after direct injection of the tracer there was little or no tracer leakage to other structures within the orbit, as the tracer was contained within the AEN nerve sheath. Panneton et al. (2006) preferred an intracranial approach to directly inject the AEN, and produced excellent results.

HRP is one of the most common transganglionic tracers used for determining the central projections of afferent nerves, and is an enzyme that passes into neurons through adsorptive endocytosis and is then transported by cellular trafficking mechanisms (Mesulam, 1982). Neuronal labeling with HRP conjugated with wheat germ agglutinin (WGA-HRP) is both more intense and more extensive than is free HRP (Mesulam, 1982). WGA-HRP is often used to label projections of both myelinated and unmyelinated fibers within a nerve, although it appears to preferentially label small diameter fibers (LaMotte et al., 1991; Liu et al., 1995; Robertson and Grant, 1985). Presumably this is because WGA has high affinity for N-acetyl-D-glucosamine, a carbohydrate found on the plasma membrane of small diameter afferent fibers (Nagata and Burger, 1974). In contrast, HRP conjugated with Isolectin-B4 from *Bandeiraea simplicifolia* (IB4-HRP) has been used to selectively trace unmyelinated C-fibers (Kitchener et al., 1993; Kobayashi and Matsumura, 1996; Wang et al., 1998). IB4 binds to terminal α -D-galactose groups of the plasma membrane of nervous tissue (Ambalavanar and Morris, 1992; Ambalavanar and Morris, 1993), and so IB4-HRP labels only unmyelinated neurons because the myelin sheathes of myelinated axons do not contain galactose glycoconjugates (Streit et al., 1985). When HRP is conjugated with the B fragment of cholera toxin (CTB-HRP), selective tracing of large diameter myelinated afferent fibers can be achieved (LaMotte et al., 1991; Robertson and Arvidsson, 1985; Robertson and Grant, 1985). CTB binds to the cell surface monosialoganglioside GM₁ (Cuatrecasas, 1973a; Cuatrecasas, 1973b), which is present on the plasma membrane of large diameter fibers (Robertson and Grant, 1985). Thus small unmyelinated C-fibers do not appear to be labeled by CTB-HRP (Rivero-Melian and Grant, 1990; Robertson and Arvidsson, 1985; Robertson and Grant, 1985). Therefore, by selective use of WGA-HRP, IB4-HRP and CTB-HRP, one theoretically could determine the central projections of unmyelinated and myelinated axons within the AEN. Conversely, a cocktail of WGA-HRP and CTB-HRP could ensure labeling of both small (unmyelinated) and large (myelinated) fibers, respectively, within a particular afferent nerve (LaMotte et al., 1991; Panneton et al., 2005; Robertson and Grant, 1985), although this might not always be the case (Liu et al., 1995).

In comparison with both WGA-HRP and CTB-HRP, we found IB4-HRP to be a much less robust transganglionic tracer. Because we found labeling of trigeminal ganglion cells, this indicates that we successfully injected IB4-HRP into the AEN and that IB4-HRP retrogradely transported from the peripheral AEN injection site to the trigeminal ganglion. However, the IB4-HRP brainstem labeling, if present, was always lighter than the other two HRP conjugated tracers. This would indicate that IB4-HRP anterograde transport from the trigeminal ganglion to the brainstem was markedly reduced, and could not be improved upon by increasing transport time. We found this somewhat puzzling, and at present have no explanation for the differences in transganglionic transport between WGA-HRP, IB4-HRP and CTB-HRP. However, others have also reported weaker labeling when IB4-HRP was used to trace the central terminations of small trigeminal (Kobayashi and Matsumura, 1996; Sugimoto et al., 1997) and sciatic afferent neurons (Kitchener et al., 1994). However, subtracting the CTB-HRP pattern of termination (consisting primarily of myelinated fibers) from the WGA-HRP pattern of termination (containing both unmyelinated and myelinated fibers) suggests that the label found in brainstem areas labeled by WGA-HRP but not CTB-HRP are the central terminations of unmyelinated AEN fibers. Certainly these findings would be stronger if IB4-HRP labeling had more reliably demonstrated selective central transport of unmyelinated fibers. However, any IB4-HRP labeling we did see in the brainstem supported our conclusions generated from analysis of the WGA-HRP and CTB-HRP labeling patterns. Thus the faint brainstem labeling generated with IB4-HRP becomes confirmatory, rather than primary, evidence of unmyelinated AEN brainstem projections.

3.2 Brainstem Projections of the AEN

Similar labeling patterns for the central terminations of the AEN were seen regardless of whether visualization was achieved using a TMB procedure with light microscopy or an immunohistochemical procedure with fluorescent microscopy. The central brainstem projections of the AEN were exclusively ipsilateral, confirming similar results for the rat (Anton and Peppel, 1991; Panneton et al., 2006) and cat (Lucier and Egizii, 1986). However, sparse contralateral dorsal horn labeling has been described in the muskrat (Panneton, 1991).

3.2.1 Trigeminal Ganglion—Injection of the different HRP conjugates directly into the trigeminal ganglion produces differing labeling patterns of trigeminal ganglion cells, as CTB-HRP injected into the trigeminal ganglion primarily labels medium to large cells ($> 600 \mu\text{m}^2$), while IB4-HRP labels mostly small cells ($< 400 \mu\text{m}^2$), and WGA-HRP labels neurons of all sizes (Sugimoto et al., 1997). This suggests that neurons having myelinated axons have larger cell bodies, and neurons having unmyelinated axons have smaller cell bodies. However, after trigeminal nerves are injected peripherally with different HRP tracers, cell bodies labeled with each of the different tracers have wide ranges of cross-sectional areas and often do not show significant differences in areas or diameters (Anton and Peppel, 1991; Robertson and Arvidsson, 1985; Roche and Kajander, 1998; Sugimoto et al., 1986). We found that there were no differences in the cross-sectional area, perimeter or diameter of trigeminal ganglion cell bodies after labeling the AEN with WGA-HRP, IB4-HRP, or CTB-HRP. Additionally, the range of labeled trigeminal ganglion cell body areas labeled with the three different tracers suggests that the AEN cell body size does not have a high degree of correlation with axonal myelination.

3.2.2 Medullary Dorsal Horn—After WGA-HRP labeling of the AEN, it was found that the AEN projects primarily to the ventral superficial laminae (laminae I and II) of the MDH between the pyramidal decussation and the obex. Significant AEN projections to the ventral superficial MDH were previously reported in the rat (Anton and Peppel, 1991; Panneton et al., 2006), cat (Lucier and Egizii, 1986), and muskrat (Panneton, 1991; Panneton et al., 2000). We found these MDH projections after tracing the central terminations of the AEN with WGA-HRP (and, when present, with IB4-HRP), but found no MDH projections after tracing the AEN with CTB-HRP. Subtracting the CTB-HRP results from the WGA-HRP results therefore suggests that the label found in the superficial laminae of the ventral MDH are the central terminations of unmyelinated AEN fibers. Approximately 65% of the AEN is composed of unmyelinated C-fibers (McCulloch et al., 1999a), and based on the density of the AEN projections to the MDH, we suggest that a significant proportion of these unmyelinated fibers project to the MDH.

In the present study neurons within the ventral tip of the superficial MDH expressed Fos, as a measure of neuronal activation (Dragunow and Faull, 1989), after nasal stimulation with ammonia vapors. The activation of superficial ventral MDH neurons after nasal stimulation has previously been seen and characterized in anesthetized preparations using both rats (Rybka and McCulloch, 2006) and muskrats (McCulloch and Panneton, 1997). Neurons within the ventral MDH of rats also express Fos after repetitive voluntary dives (McCulloch, 2005). The number of Fos-positive neurons within the ventral MDH peaked near the obex, which was previously observed after nasal stimulation in both rats and muskrats (McCulloch and Panneton, 1997; Rybka and McCulloch, 2006). Neurons within the superficial ventral MDH presumably are involved in production of the cardiovascular and respiratory responses to nasal stimulation (McCulloch and Panneton, 1997; McCulloch, 2005; Rybka and McCulloch, 2006). These second-order MDH neurons could be afferent integrative and/or secondary projection neurons. If the ventral MDH is injected with the local anesthetic lidocaine, the cardiorespiratory consequences of nasal stimulation are abolished (Panneton and Yavari,

1995). If the AEN is cut bilaterally, thus preventing the afferent AEN signal from reaching the MDH, the cardiorespiratory responses to nasal stimulation are severely attenuated (Rybka and McCulloch, 2006). Additionally, the ventral MDH projects to important cardiovascular and respiratory control centers within the brainstem (Panneton et al., 2000; Panneton et al., 2006).

In the present study the MDH Fos-positive neurons co-localized with the central projections of the AEN. Indeed, after tracing the AEN with WGA-HRP, the greater the intensity of the AEN central terminations that surrounded that Fos-positive neuron, the greater the number of Fos-positive neurons within the MDH. We assume that the greater intensity of WGA-HRP label indicates that more central projections of the AEN terminate in the region of the MDH near the obex, rather than the central terminations of the AEN near the obex contained more fluorescent label. These results, along with the AEN tracer results, suggest that afferent signals traveling in unmyelinated, but not myelinated, AEN fibers after nasal stimulation induce activation of second-order neurons within the MDH. Although this may be a logical conclusion, electron micrographic analysis would be needed to confirm the presence of synaptic connections between central AEN terminals and MDH second-order neurons.

AEN projections to deeper MDH laminae (laminae III, IV, and V) were not found in the present study or by Anton and Peppel (1991). However, reaction product was seen in the most ventral parts of laminae III and IV of the rat by Panneton et al. (2006), although this label might have been passing to other sites. Sparse AEN projections to the deeper MDH laminae were reported in the muskrat (Panneton, 1991) and were much more extensive in the cat (Lucier and Egizii, 1986).

3.2.3 Other Trigeminal Locations—Extensive WGA-HRP label was found in the spinal trigeminal tract, especially at the same rostral-caudal levels as the MDH label and when juxtaposed with the ventral paratrigeminal nucleus. These projections apparently contain both unmyelinated and myelinated fibers, as paratrigeminal labeling was seen after injection of the AEN with both WGA-HRP and CTB-HRP, and occasionally with IB4-HRP. AEN projections to the ventral paratrigeminal nucleus have been previously reported (Panneton, 1991; Panneton et al., 2000). Trigeminal fibers enter the brainstem at the level of the caudal pons, descend within the spinal trigeminal tract, and terminate within the spinal trigeminal nucleus (Torvik, 1956). However, the ventral paratrigeminal nucleus is a group of scattered interstitial cells within the spinal trigeminal tract, and is most likely a lateral extension of the MDH (Phelan and Falls, 1989). Like some MDH neurons, there are neurons within the ventral paratrigeminal nucleus that are activated by nasal stimulation (Rybka and McCulloch, 2006).

Rostral to the obex, there was a concentration of AEN projections located in the ventral tip of subnucleus interpolaris, but not in other dorsal locations within the subnucleus interpolaris (present study; Panneton et al., 2006). Similar findings have been reported in the muskrat (Panneton, 1991; Panneton et al., 2000) and cat (Lucier and Egizii, 1986). At the level of the area postrema, the superficial MDH projections are pushed medially by the appearance of subnucleus interpolaris. In the rat, this occurs approximately midway between the pyramidal decussation and the obex. Anton and Peppel (1991) reported nucleus interpolaris labeling rostral to the obex in the rat. We suggest that the thin band of nucleus interpolaris labeling rostral to the obex reported by Anton and Peppel (1991; i.e. see their Fig 5) is MDH labeling that has been pushed medially by the emerging subnucleus interpolaris. More rostrally, label within the subnucleus oralis and trigeminal principal nucleus of the rat was found by Panneton et al. (2006), but was not found in the present study or by Anton and Peppel (1991).

Label was observed in the dorsal mesencephalic nucleus, a projection noted in the rat (Panneton et al., 2006), cat (Lucier and Egizii, 1986), and muskrat (Panneton, 1991). This projection is significant in that cell bodies were labeled, rather than terminal projections, and axonal label

was sometimes observed streaming toward the labeled cells bodies. The mesencephalic trigeminal nucleus is the only nucleus located within the central nervous system that contains the cell bodies of primary afferent neurons (Shults, 1992). Because the labeling of the mesencephalic trigeminal nucleus was seen after injection of the AEN with WGA-HRP and CTB-HRP, but not with IB4-HRP (present study; (Sugimoto et al., 1997)), this suggests that only myelinated fibers within the AEN project to the mesencephalic trigeminal nucleus. The peripheral processes of the mesencephalic trigeminal nucleus carry proprioceptive information from the muscles of mastication and extrinsic ocular muscles (Shults, 1992), and their central processes project mainly to the trigeminal motor nucleus to provide reflex control of biting movements (Wilson-Pauwels et al., 1988). Why the mesencephalic trigeminal nucleus was labeled after tracer injection into the AEN is, at present, unknown. However, Lucier and Egizii (1986) suggested that labeled cell bodies in the mesencephalic nucleus after AEN labeling in the cat could be those of nasal proprioceptors.

3.2.4 Other Brainstem Locations—Anton and Peppel (1991) concluded that the AEN projects bilaterally to the interstitial NTS near the pyramidal decussation, based on results seen after WGA-HRP gel was applied to the nasal mucosa of the rat. However neither we nor Panneton et al. (2006) found evidence of AEN projections to the NTS after application of WGA-HRP directly into the AEN. Thus direct projections from AEN to the interstitial NTS cannot be confirmed. Panneton et al. (2006) did however see AEN terminal label in the nearby Probst's tract, and showed by anterograde and retrograde tract tracing that the MDH sends projections to the ventrolateral subdivisions of the NTS.

AEN terminal label, presumably from unmyelinated fibers, was found just medial to the ventral tip of the MDH, in the reticular formation of the caudal ventrolateral medulla (present study; Panneton et al., 2006). Additionally, this area receives projections from the ventral MDH (Panneton et al., 2006). The caudal ventrolateral medulla contains neurons involved in blood pressure control (Natarajan and Morrison, 2000; Sun and Panneton, 2002), the baroreceptor reflex (Masuda et al., 1991; Schreihofer and Guyenet, 2003), and parasympathetic innervation of the heart (Panneton et al., 1996). It is possible that the direct AEN, or indirect MDH, projections to the caudal ventrolateral reticular formation are involved in the intense cardiovascular responses seen after nasal stimulation (Fig 3; (McCulloch and Panneton, 1997; Rybka and McCulloch, 2006), electrical stimulation of the AEN (Dutschmann and Herbert, 1997; McCulloch et al., 1999a), or voluntary diving (McCulloch et al., 1997).

AEN terminal label was also found in more rostral locations of the ventrolateral medulla. Similar to previously observed projections in the rat (Panneton et al., 2006) and muskrat (Panneton, 1991; Panneton et al., 2000), we found AEN terminal projections within the RVLM/C1 region (Haselton and Guyenet, 1989; Schreihofer and Guyenet, 1997). Adrenergic C1 neurons express Fos during voluntary diving in rats (McCulloch and Panneton, 2003), and we found that the central terminations of presumably unmyelinated AEN fibers co-localize with Fos-positive RVLM neurons that had been activated by nasal stimulation. The RVLM contains neurons that are an important source of tonic excitatory drive to sympathetic vasomotor neurons controlling peripheral vasculature (Dampney, 1994; Guyenet, 1990), and the sympathoexcitatory response to nasal stimulation with ammonia vapors is largely due to activation of bulbospinal presympathetic neurons within the RVLM (McCulloch et al., 1999b). Because central terminations of the AEN co-localize with Fos-positive RVLM neurons, we suggest that direct AEN terminal projections to the RVLM are at least partially responsible for the sympathetic activation caused by stimulation of the nasal mucosa. However this AEN-RVLM connection cannot be completely responsible for the sympathetic activation after nasal stimulation, because injection of local anesthetic lidocaine or excitatory amino acid receptor antagonist kynurenate into the ventral MDH blocks the cardiorespiratory responses to nasal stimulation (Panneton and Yavari, 1995).

Nasal stimulation can produce apnea and other alterations of respiratory rhythm (Widdicombe, 1986), and terminal unmyelinated AEN projections were found in specific areas of the ventrolateral medulla that have been shown to have respiratory-related activity (Alheid et al., 2002). The Böttinger complex received AEN terminal projections, but the pre-Böttinger complex, rostral ventral respiratory group, and retrofacial nucleus did not. The Böttinger complex contains neurons that primarily fire during the late expiratory phase (Schreihofer et al., 1999; Tian et al., 1998) and inhibit phrenic motoneurons during the late part of expiration (Tian et al., 1998). This conceivably could be important for the production of apnea during both the diving response and nasal stimulation, as Böttinger complex neurons may suppress other respiratory neurons during reflexes that interrupt respiratory rhythm (Hopkins and Ellenberger, 1994). However, nasal stimulation has been shown to inhibit, rather than excite, neurons located within the Böttinger region (McCulloch et al., 1999b). Further electrophysiological research could determine the relationship between AEN activation and neuronal responses within the Böttinger complex.

Projections to the Kölliker-Fuse and external lateral and external medial parabrachial subnuclei have been found in the guinea pig (Segade, 2003). Parabrachial labeling was reported in the rat (Panneton et al., 2006) and muskrat (Panneton, 1991), but Kölliker-Fuse or parabrachial subnuclei labeling was not found in the present study.

Unmyelinated AEN fibers also project to the superficial dorsal horn of the rostral cervical spinal cord. AEN projections to the rostral cervical spinal cord have also been previously reported for the muskrat (Panneton, 1991; Panneton et al., 2000) and the rat (Panneton et al., 2006).

3.3 Conclusions

The present results show that fibers within the AEN project heavily to the ipsilateral superficial laminae (laminae I and II) of the ventral MDH at the level of the area postrema and the ventral spinal trigeminal tract at the level of the area postrema. Interpretation of the observed labeling after use of WGA-HRP, IB4-HRP and CTB-HRP suggests that the MDH receives unmyelinated AEN fibers, while the ventral paratrigeminal nucleus within the ventral spinal trigeminal tract receives both unmyelinated and myelinated AEN fibers. The central terminations of the unmyelinated AEN fibers show co-localization with MDH neurons that were activated by nasal stimulation. Although it would require further research for confirmation, it is likely that the afferent signals carried by the AEN activate these secondary trigeminal neurons that are important in the production of the cardiorespiratory response to stimulation of nose and nasal passages. Additionally, unmyelinated AEN fibers project to cardiovascular and respiratory areas of the ventrolateral medulla. AEN projections co-localize with RVLM neurons that are activated by nasal stimulation, and this direct AEN-RVLM connection may be at least partially responsible for the sympathetic activation caused by stimulation of the nasal mucosa. Additionally, AEN projections to the Böttinger complex could be important for the production of apnea during both the diving response and nasal stimulation, although this could only be confirmed by further histological and electrophysiological studies.

4. EXPERIMENTAL PROCEDURES

The Midwestern University Animal Care and Use Committee approved all experimental procedures, which were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and to minimize their pain and suffering.

4.1 Tracing the Central Projections of the Anterior Ethmoidal Nerve

Male Sprague-Dawley rats (260–390 g; Harlan, Indianapolis IN) were anesthetized with an intraperitoneal injection of Ketamine (80 mg/kg; Ketaset, Fort Dodge, Fort Dodge IA) and Xylazine (10 mg/kg; Butler, Dublin OH), and placed into a stereotaxic head holder (Kopf, Tujunga CA). If necessary, a supplemental dose of anesthetic (half the original dose) was administered. A supraorbital incision was made lateral to the sagittal suture to expose the left orbit. Small cotton balls were positioned at the base of the orbit to move the eyeball and Hardarian gland laterally, exposing the left AEN and anterior ethmoidal artery as they exit the anterior ethmoidal foramen. The AEN was then carefully isolated from the artery and surrounding fascia.

One of three tracers (WGA-HRP, Vector [PL-1026], Burlingame CA; IB4-HRP, Sigma [L-5391], St. Louis MO; or CTB-HRP, Sigma [C-3741]) was used to trace the central projections of the left AEN. In some cases (N=12) the AEN was cut and the proximal end of the nerve was placed in a Parafilm micro-cup and bathed in a pool of 2% WGA-HRP for 1–2 hr. In other cases approximately 200 nl of the tracer (WGA-HRP, N=15; IB4-HRP, N=18; CTB-HRP, N=10) was injected directly into the intact nerve using a pulled glass micropipette attached to a 1 µl Hamilton syringe. A bulge created by the injected tracer could usually be seen within the nerve sheath. After uptake of the tracer by the nerve, the surgical incision was closed, and the analgesic buprenorphine (0.045 mg/kg; Reckitt Benckiser, Hull UK) was given subcutaneously in the hind leg. After recovery from the anesthesia the rats were returned to the animal quarters for 3–5 days to allow tracer transport. A second dose of buprenorphine was given 24 hr after the surgery.

After the transport period, the rats were deeply anesthetized with a euthanasia solution (0.3 ml, Sleepaway, Fort Dodge). The rats were then perfused transcardially with 50 ml 0.9% saline, followed by 500 ml fixative (1.25% glutaraldehyde + 1% paraformaldehyde), followed by 500 ml of a buffered sucrose solution. The brains (and in some cases the left trigeminal ganglion: WGA-HRP, N=4; IB4-HRP, N=5; CTB-HRP, N=3) were removed and placed overnight in a buffered sucrose solution at 4°C. The following day the brainstem and trigeminal ganglion were cut at 50 µm on a freezing microtome fitted with a Pellitier cooling device. The brain tissue was then reacted for the presence of HRP according to Mesulam's procedure (1982). Briefly, the sections underwent a histochemistry reaction using tetramethylbenzidine (TMB; Sigma) as a chromagen to visualize the HRP, sodium nitroferricyanide (Sigma) as a stabilizer, and hydrogen peroxide (Sigma) as a catalyst. This procedure produced a black dust-like labeling. In some cases, variations of the reaction procedure followed those described by Olucha et al. (1985) and Kitchener et al. (1993) by increasing reaction time and changing reaction solutions and H₂O₂ concentrations every 5 min. Every brain section was mounted on double-gelled subbed slides in 1:1 serial order and allowed to air dry for 2 days. After drying, sections were counterstained with Neutral Red (Sigma), dehydrated in alcohols, and cleared with xylene. The slides were then coverslipped with Permount (Fisher Scientific).

4.2 AEN Projections and Trigeminal Neurons Activated by Nasopharyngeal Stimulation

In 7 rats WGA-HRP tracer was injected into the left AEN as above. After 3–5 day transport of the tracer, the nasal passages of the rats were stimulated to induce a nasopharyngeal reflex (see Rybka and McCulloch (2006) for complete surgical and stimulation details). Briefly, under Isoflurane anesthesia (Webster, Sterling MA) the right femoral vein and artery were cannulated for the administration of drugs and recording of blood pressure, respectively. The trachea was transected caudal to the larynx, and two polyethylene tubes were then inserted into the trachea. One tube was inserted caudally to aid in respiration and the other tube was inserted rostrally to the choana to aid in stimulation of the nasal mucosa.

The rats were then switched from Isoflurane to urethane anesthesia (Sigma; 1.3 mg/kg, iv). At least 1 hr separated the end of urethane injection and the start of nasal stimulation with ammonia vapors (see below). To record blood pressure, the arterial cannula was connected to a pressure transducer (Statham P23XL, Spectramed, Oxnard CA). To record respiration, a temperature probe was placed in the tracheal cannula. All electronic signals were then amplified (Grass 7P122, West Warwick RI), and transmitted through an A/D board (micro 1401; CED, Cambridge UK) to a computer where they were stored and analyzed using the appropriate software (Spike 2; CED). Heart rate was determined from pulse pressure intervals.

An ammonia trial consisted of placing a cotton swab soaked in 100% ammonia approximately 2–3 mm in front of the external nares of the rat for 5 s. Ammonia vapors were drawn through the nasal passages by connecting a suction pump to the nasopharyngeal tube. Approximately 5 min separated each ammonia trial, and the rats were stimulated with ammonia vapors 24 times over a period of 2 hr.

One hour after the completion of each stimulation experiment, each rat was deeply anesthetized (Sleepaway) and perfused transcardially with 100 ml 0.9% saline with 0.25% procaine followed by 450 ml 4% paraformaldehyde in 0.1M phosphate buffered saline. The brain was removed and placed overnight in a buffered sucrose solution at 4°C. The brainstem was then cut at 30 µm using a cryostat. A 1:3 series of the tissue was immunohistologically processed using fluorescent chromagens (Dederen et al., 1994; Ericson and Blomqvist, 1988) for both the WGA-HRP tracer and Fos as a measure of neuronal activation (Dragunow and Faull, 1989). After washing with phosphate buffered saline, the tissue was incubated in a 10% normal donkey serum for 1 hr. The tissue was then incubated overnight in a cocktail of primary antibodies (rabbit anti-HRP; 1:4000; Sigma [P7899] and goat anti-Fos 1:1000; Santa Cruz Biotechnology, Santa Cruz CA [SC-052G]). The tissue was next incubated in 10% bovine serum albumin (BSA) for 1 hr, and then incubated in the dark with donkey anti-rabbit secondary antibodies conjugated with AF594 (1:1000; Molecular Probes, Eugene OR [A21207]). Following a second incubation with BSA, the tissue was incubated in the dark with donkey anti-goat conjugated secondary antibodies conjugated with AF488 (1:500; Molecular Probes [A11055]). This labeling procedure produced red fluorescent labeling of WGA-HRP and green fluorescent labeling of Fos-positive nuclei. The sections were then mounted in serial order on glass slides, and coverslipped using glycerol as a mounting solution and clear nail polish to seal the coverslip.

4.3 Analysis of Histological Data

All brainstem (from the rostral cervical spinal cord caudally to the parabrachial region rostrally) and trigeminal ganglion histological sections were viewed with a Nikon E600 microscope. A light microscope configuration was used to view all non-fluorescent tissue, while an epi-fluorescent microscope attachment (X-Cite 120, EXFO, Mississauga CAN) was used to view fluorescent tissue. Color photomicrographic images were made with a digital camera (Micropublisher, Q-Imaging, Burnaby CAN) attached to the microscope, and microscope imaging software (Northern Eclipse; Empix, Mississauga CAN). The software features of this imaging program were also used to measure the area, perimeter and diameter of labeled trigeminal ganglion cells.

For the fluorescent WGA-HRP tracer, hemisections from the left side of the brain were photographed using the red and then the green cube to fluoresce the HRP tracer and the Fos-positive nuclei, respectively. For consistency between sections and between animals, the same exposure time was used while imaging each red-only photomicrograph. To create fluorescent photomicrographs, the red and green images were overlapped using the Boolean addition feature of the microscope imaging software. To measure the intensity of AEN central terminations surrounding activated MDH neurons, a 25 µm diameter circle (490 µm²) was

created around each Fos-positive neuron within the left MDH from the pyramidal decussation caudally to the obex rostrally, using the spot density function of the microscope imaging software (see Fig 5C center column). The average density of the red within this circle was then measured on the red-only fluorescent photomicrograph (see Fig 5C right column). Subtraction of background red from the labeled intensity gave a corrected average density of the HRP label around that Fos-positive neuron. Based on the range of densities observed, each corrected average density was given a 1–5 score, with 5 being the most intense label, and 1 being the lightest label (see Fig 5C). Each HRP density score was then related to the rostral-caudal location of that particular activated MDH neuron. This scoring estimated the density of the fluorescent WGA-HRP labeling, and therefore the amount of AEN terminal projections, around each Fos-positive neuron within the left MDH.

4.4 Presentation of data

Data were tested for statistical significance using SigmaStat (SPSS, Chicago IL). Statistical significance was set at $P < 0.05$. One-way ANOVAs were used to compare the area, perimeter and diameter of labeled trigeminal ganglion cells. Repeated measures one-way ANOVAs were used to compare differences between pre-stimulation, nasal stimulation, and post-stimulation cardiorespiratory parameters. In the case of significant F-values, Tukey's Multiple Comparison Procedure *post-hoc* testing was used to make pairwise comparisons. Repeated measures one-way ANOVAs were used to compare differences between the WGA-HRP tracer intensities along the caudal-rostral axis of the MDH. Pearson Product Moment Correlation was used to determine if there was a correlation between tracer density and the number of Fos-positive neurons along the rostral-caudal extent of the MDH. Graphs were created with SigmaPlot (SPSS). Data are presented as mean $\pm$ standard error. Heart rate (HR, beats/min), mean arterial blood pressure (MAP, mm Hg) and respiratory rate (RR, breaths/min) are presented immediately before, during, and after nasal stimulation. The duration of the apnea, if present, was also measured during the period of nasal stimulation. Figures and photomicrographic panels were composed and labeled using CorelDraw (Corel, Ottawa CAN).

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Comprehensive List of Abbreviations

7N	facial nucleus
AEN	anterior ethmoidal nerve
BSA	bovine serum albumin
CTB	B fragment of cholera toxin
CTB-HRP	B fragment of cholera toxin conjugated to HRP
dl	dorsolateral fasciculus

IB4	isolectin B4 of <i>Bandeiraea simplicifolia</i>
IB4-HRP	isolectin B4 of <i>Bandeiraea simplicifolia</i> conjugated to HRP
HR	heart rate
HRP	horseradish peroxidase
MAP	mean arterial blood pressure
MDH	medullary dorsal horn
Me5	mesencephalic trigeminal nucleus
NA	nucleus ambiguus
NTS	nucleus tractus solitarius
Para5	paratrigeminal nucleus
RR	respiratory rate
RVLM	rostral ventrolateral medulla
RVLM/C1	adrenergic C1 neurons of the rostral ventrolateral medulla
SDH	spinal dorsal horn
Sp5	spinal trigeminal tract
Sp5I	spinal trigeminal subnucleus interpolaris
TMB	tetramethylbenzidine
WGA	wheat germ agglutinin
WGA-HRP	wheat germ agglutinin conjugated to HRP

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Figure 1.

Cell bodies within the left trigeminal ganglion after injecting the AEN with (A) WGA-HRP, (B) IB4-HRP, and (C) CTB-HRP. After injecting the AEN with IB4-HRP, central projections were located within the ventral medullary dorsal horn (arrows in D) and paratrigeminal nucleus (arrow in E). After injecting the AEN with CTB-HRP, central projections were located within the mesencephalic trigeminal nucleus (arrows in F). Abbreviations: Sp5, spinal trigeminal tract; Para5, paratrigeminal nucleus. Scale bars: in C = 250 μ m for A–C; in E = 50 μ m; in F = 100 μ m for D and F;

Figure 2.

Central projections of the AEN after labeling with WGA-HRP. (A) The spinal dorsal horn (SDH) of the rostral cervical spinal cord, adjacent to the dorsolateral fasciculus (dl). (B) Within the ventral medullary dorsal horn (MDH) of the caudal medulla, between the pyramidal decussation and the area postrema. (C) Within the ventral MDH at the level of the caudal area postrema. (D) Within the ventral MDH at the mid area postrema level, as the spinal trigeminal subnucleus interpolaris (Sp5I) emerges to displace the MDH medially. (E) Within the ventral MDH at the level of the obex. (F) The ventral paratrigeminal nucleus (Para5) within the spinal trigeminal tract (Sp5), at the level of the caudal area postrema. (G) Ventral to Sp5I, rostral to the obex. (H) Within the ventrolateral medulla, just lateral to the RVLM/C1 region. (I) Within the ventrolateral medulla, as the label exits the spinal trigeminal tract. (J) Within the ventrolateral medulla, as label crosses the Böttinger complex and extends towards the nucleus ambiguus. (K) at the caudal end of the facial nucleus (7N). (L) Cell bodies labeled within the mesencephalic trigeminal nucleus (Me5). Scale bar in L = 100 μ m for A–D, F and L; in K =

250 μm for E and G–K. Other abbreviations: Sp5, spinal trigeminal tract; NA, nucleus ambiguus.

Figure 3.

Cardiorespiratory responses to a 5 s stimulation of the nasal passages with ammonia vapors. Nasal stimulation produced a substantial and sustained bradycardia, an increase in arterial pressure, and an apnea that lasted longer than the period of nasal stimulation. From top: heart rate; mean arterial blood pressure (MAP); pulsatile arterial blood pressure (BPa); and respiration (up, inspiration (Insp); down, expiration (Exp)). Solid bar underneath tracing indicates period of nasal stimulation.

Figure 4.

Fluorescent photomicrographs after WGA-HRP injection into the AEN and stimulation of the nasal passages with ammonia vapors. (A–C) the ventral MDH between the pyramidal decussation and the area postrema; (D–F) the ventral MDH at the mid area postrema level, as Sp5I emerges to displace the MDH medially; and (G–I) within the RVLM/C1 region. Left column: After stimulation of the nasal passages with ammonia vapors, Fos-positive neurons were immunologically processed to fluoresce green. Middle column: after injection of the AEN with WGA-HRP, the central terminations of the AEN were immunologically processed to fluoresce red. Right column: Boolean addition of the left and middle columns show co-localization of activated Fos-positive neurons and central terminations of the AEN within the MDH (C and F) and RVLM/C1 (I), as indicated by arrows. Scale bar in F = 100 μ m for A–F. Scale bar in I = 50 μ m for G–I.

Figure 5.

The number of Fos-positive neurons within the MDH left hemisection (B) and the average density of WGA-HRP labeling around each of those Fos-positive neurons (A) presented in a caudal (left side) to rostral (right side) profile of the MDH. The density of the WGA-HRP labeling surrounding each activated neuron had been assigned a 1–5 score (C). The intensity of the WGA-HRP label, and therefore the amount of AEN terminal projections at that rostral-caudal level, positively correlated with the number of Fos-positive neurons. Both the number of Fos-positive neurons and intensity of label surrounding those neurons peaked just caudal to the obex. Number of Fos neurons within MDH at this rostral-caudal level was significantly less than: ¹ at -90 μm; ² at obex.

TABLE 1

Area (mean and range), perimeter and diameter of ipsilateral trigeminal ganglion cells labeled after injection of tracer (WGA-HRP, N=4; IB4-HRP, N=5; or CTB-HRP, N=3) into the left anterior ethmoidal nerve. There were no significant differences between the ganglion cells labeled with the three different tracers.

	Area (μm^2)		Perimeter (μm)	Diameter (μm)
	Mean	Range		
WGA-HRP	374.7 $\pm$ 25.5	(127–1145)	77.5 $\pm$ 2.6	27.7 $\pm$ 0.9
IB4-HRP	390.2 $\pm$ 41.1	(92–841)	77.4 $\pm$ 4.3	27.2 $\pm$ 1.7
CTB-HRP	299.8 $\pm$ 25.8	(103–1107)	71.5 $\pm$ 3.9	25.8 $\pm$ 1.8

TABLE 2

Cardiovascular responses to stimulation of the nasal passages with ammonia vapors. These animals previously had received WGA-HRP injections into the left AEN. Heart Rate (HR) in beats/min, mean arterial blood pressure (MAP) in mm Hg, and respiratory rate (RR) in breaths/min. Nasopharyngeal stimulation had a significant effect (*; $p < 0.05$) on HR, MAP and RR.

	HR	MAP	RR
Pre-Stimulation (N=9)	370 $\pm$ 14	120 $\pm$ 3	97 $\pm$ 4
Stimulation (N=9)	236 $\pm$ 21*	140 $\pm$ 3*	22 $\pm$ 4*
Post-Stimulation (N=9)	386 $\pm$ 13	118 $\pm$ 3	97 $\pm$ 6