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# BRAINSTEM PROJECTIONS FROM RECIPIENT ZONES OF THE ANTERIOR ETHMOIDAL NERVE IN THE MEDULLARY DORSAL HORN

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**Abstract—Stimulation of the anterior ethmoidal nerve or the nasal mucosa induces cardiorespiratory responses similar to those seen in diving mammals. We have utilized the transganglionic transport of a cocktail of horseradish peroxidase conjugates and anterograde and retrograde tract tracing techniques to elucidate pathways which may be important for these responses in the rat. Label was seen throughout the trigeminal sensory complex after the horseradish peroxidase conjugates were applied to the anterior ethmoidal nerve peripherally. Reaction product was most dense in the medullary dorsal horn, especially in laminae I and II. Injections were made of biotinylated dextran amine into the recipient zones of the medullary dorsal horn from the anterior ethmoidal nerve, and the anterogradely transported label documented. Label was found in many brainstem areas, but fibers with varicosities were noted in specific subdivisions of the nucleus tractus solitarii and parabrachial nucleus, as well as parts of the caudal and rostral ventrolateral medulla and A5 (noradrenergic cell group in ventrolateral pons) area. The retrograde transport of FluoroGold into the medullary dorsal horn after injections into these areas showed most neurons in laminae I, II, and V. Label was especially dense in areas which received primary afferent fibers from the anterior ethmoidal nerve. These data identify potential neural circuits for the diving response of the rat.** 

**Key words: diving response, nasal mucosa, cardiovascular, respiratory, somatovisceral interactions.**

The anterior ethmoidal nerve (AEN) is a small branch of the ophthalmic division of the trigeminal nerve. In humans, the AEN innervates the anterior–superior mucosa of both the lateral and septal walls of the nasal cavity, as well as the skin of the ala, vestibule and apex of the nose; it also innervates dura in the anterior cranial fossa (Williams and Warwick, 1980). Its peripheral receptive fields have been described rarely in animals, but one study in the cat notes a distribution similar to that described in humans (Wallois et al., 1991). The receptive fields of the AEN thus surround the nares. We consider the AEN a "gatekeeper" nerve, preventing noxious or irritable gasses, or water, from entering the upper respiratory tract and potentially damaging the lungs. A high percentage of its fibers are of small diameter, and most are unmyelinated (Beidenbach et al., 1975; Wallois et al., 1992, 1993; McCulloch et al., 1999b).

Electrical stimulation of the AEN (Dutschmann and Herbert, 1996, 1997, 1998; McCulloch et al., 1999a; Dutschmann and Paton, 2002), or stimulation of the nasal mucosa with irritant vapors (White et al., 1974; McRitchie and White, 1974; White et al., 1975; Panneton, 1990, 1991b; Gieroba et al., 1994; Panneton and Yavari, 1995; Yavari et al., 1996; McCulloch and Panneton, 1997; Ho and Kou, 2000; Panneton and Gan, 2003), elicits dramatic changes in cardiorespiratory function similar to those seen in diving mammals. Either anesthetizing the tip of the nose or the nasal mucosa with local anesthetics prevents the cardiorespiratory responses usually associated with either underwater submersion or stimulation of the nasal mucosa (Dykes, 1974; McCulloch et al., 1995; Yavari et al., 1996).

The central projections of the AEN have been studied after applying tracers to the nerve using transganglionic transport techniques in the cat (Lucier and Egizii, 1986), muskrat (Panneton, 1991a), and guinea-pig (Segade, 2003), while projections after injections of tracers into the nasal mucosa have been reported in the rat (Anton and Peppel, 1991). These studies show dense projections to superficial laminae of the medullary dorsal horn (MDH) (Lucier and Egizii, 1986; Anton and Peppel, 1991; Panneton, 1991a), while projections to the rostral trigeminal sensory complex were noted only in the muskrat and cat.

Panneton and colleagues (Panneton, 1991b; Panneton and Yavari, 1995) showed that small injections of either lidocaine or kynurenate into the MDH of the muskrat, both of which block synaptic transmission, inhibited the cardiorespiratory sequelae of nasal stimulation, and that the effective areas were similar to the distribution of primary afferent fibers of the AEN of this species (Panneton, 1991a). Panneton et al. (2000) further showed transneuronal projections from the AEN to brainstem autonomic nuclei in the muskrat, and suggested that some of these projections may be important in the cardiorespiratory responses seen in diving mammals. Since rats also possess a brisk and dramatic diving response (McCulloch et al., 1997; Panneton and Gan, 2003), we felt it important to

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*Abbreviations:* AEN, anterior ethmoidal nerve; A1, noradrenergic cell group of caudal medulla; A5, noradrenergic cell group in ventrolateral pons; BDA, biotinylated dextran amine; BHRP, horseradish peroxidase conjugated to cholera toxin; CPA, caudal pressor area of the caudal medulla; CVLM, depressor area of the caudal medulla; C1, adrenergic cell group in pressor area of the rostral medulla; FG, FluoroGold; HRP, horseradish peroxidase; KF, Kölliker-Fuse nucleus; MDH, medullary dorsal horn; PB, phosphate buffer; PBr, parabrachial nucleus; Pr5, principal trigeminal nucleus; RVLM, pressor area of the rostral medulla; 7, facial motor nucleus; Sol, nucleus tractus solitarii; sp5, spinal trigeminal tract; Sp5I, nucleus of the spinal tract of the trigeminal nerve, interpolar part; Sp5O, nucleus of the spinal tract of the trigeminal nerve, oral part; SSN, superior salivary nucleus; WGA-HRP, horseradish peroxidase conjugated to wheat germ agglutinin.

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investigate whether trigemino-autonomic projections in the laboratory rat mimic those seen in the naturally diving muskrat. We show herein the most complete central projections of the AEN of the rat to date using the transganglionic transport of a cocktail of horseradish peroxidase (HRP) conjugates to the brainstem. We further used both anterograde and retrograde tract tracing techniques to show that projections from the MDH of the rat to brainstem autonomic nuclei are similar to those of the muskrat. A preliminary report of this work has appeared in abstract form (Panneton, 2002).

## EXPERIMENTAL PROCEDURES

All experiments were done on male Sprague–Dawley rats (~275–340 g). All protocols were approved by the Animal Care Committee of Saint Louis University and followed the guidelines published in the Guide for the Care and Use of Laboratory Animals. The animals used for this study ensured adequate labeling and allowed dissection of the various projections. The number of animals used and their pain and suffering were minimized. The rats were prepared for aseptic surgery after being anesthetized with i.p. injections of a mixture of ketamine (60 mg/kg) and xylazine (40 mg/kg); additional injections were given when required.

### Transganglionic transport experiments

Rats were anesthetized, secured in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA), and a dorsal incision made in the skin near the orbit. The AEN was isolated either via an intraorbital or intracranial approach. The AEN was seen entering the anterior ethmoidal foramen medially after retracting the eyeball laterally. Alternatively, the AEN was located *intracranially* following a craniotomy into the anterior cranial fossa where it coursed along the inferior lateral aspect of the olfactory bulb before entering the nasal mucosa. A small piece of Parafilm was placed under the AEN in either approach and 300–500 nl of a mixture [containing a 2% solution of horseradish peroxidase conjugated to wheat germ agglutinin (WGA-HRP), 1% horseradish peroxidase conjugated to cholera toxin (BHRP), 1% saponin and 0.25% poly-L-ornithine colored with the vital dye Fast Green; all chemicals from Sigma (St. Louis, MO, USA) except for BHRP which is from List Biological Laboratories, Inc. (Campbell, CA, USA)] was injected into the nerve through a micropipette cemented to a 1 μl Hamilton syringe. Any leakage of the solution out of the AEN was seen easily due to the Fast Green and immediately cleaned from the Parafilm. The AEN was injected intraorbitally in 11 rats and intracranially in 4 rats. In many of these rats, a nerve innervating the lower limb also was injected with a similar solution for a separate experiment (Panneton et al., 2005). After a 0.5-hour observation, the area was washed with saline and the wound closed.

After 48–60 h the rats were reanesthetized and perfused transcardially, first with phosphate-buffered saline containing 0.25% procaine (Sigma), and then with a mixture of 0.5% paraformaldehyde and 2.0% glutaraldehyde in phosphate buffer (PB; pH 7.3). The fixative usually was cleared from the brain with 10% sucrose in PB after approximately 1 h. The brainstems were removed and stored in the refrigerator overnight in a 20% sucrose–buffer solution. Frozen transverse sections (40–50 μm) were cut frozen through the brainstem and saved serially in 0.1 M PB. The sections were mounted on gelled slides, air-dried, and then processed histochemically for HRP using tetramethylbenzidine as the chromogen (Mesulam, 1978). After drying, the slides then were stained quickly with Thionin, dehydrated in alcohols, defatted in xylenes, and coverslipped with Permount.

### Tract tracing experiments

The animals were anesthetized (vide supra) and secured prone in a flat skull position in a stereotaxic device. Their skulls and muscles were exposed via a dorsal incision and the medulla oblongata exposed after cutting the dura. Micropipettes (20~25 μm OD) were filled with 10% biotinylated dextran amine (BDA; Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA; 10,000 MW) in saline and lowered into the ventral MDH. These injections were made with the carrier angled anteriorly at 24° from vertical; the coordinates were 0.5 mm rostral to calamus scriptorius, 2.4–2.6 mm lateral to the midline and 1.4 mm ventral from the dorsal surface of the brainstem. [It should be noted that the calamus scriptorius (or "point of a pen") is situated approximately 650 μm caudal to the rostral edge of the area postrema and 600 μm caudal to the obex, the beginning of the central canal of the spinal cord. Apparently, the calamus scriptorius is labeled the "obex" in the rat atlas of Paxinos and Watson (1998).] In other rats, micropipettes filled with 1.5% FluoroGold (FG; Fluorochrome, Inc., Denver, CO, USA) were lowered into the nucleus tractus solitarii (Sol; 5 rats), the caudal ventrolateral medulla near the obex (6 rats), the rostral ventrolateral medulla (6 rats), the A5 area (7 rats), the parabrachial complex (PBr; 11 rats), or the trigeminal sensory complex (7 rats), respectively. Coordinates for the more rostral injections were derived from the stereotaxic atlas of Paxinos and Watson (1998) and were approached with the micropipette fixed in standard planes. Tracers were deposited in the brain by passing a positive current (5 mA; 7s on/off) via a silver wire inserted into the micropipette for 10 min using a constant current device (Midguard, Cole-Parmer Instrument Corp., Vernon Hills, IL, USA). The micropipette was left in place for 5 min after the injections. Wounds were washed with sterile saline and closed with silk.

After survivals of 8–10 days, the animals were deeply anesthetized and perfused through the heart with a peristaltic pump (Masterflex) first with phosphate-buffered saline, followed immediately by a fixative of 4% paraformaldehyde in 0.1 M PB (pH 7.3). Brains and spinal cords were removed and stored in the fixative with 20% sucrose at 4 °C. The brains were blocked in the transverse plane using a precision brain slicer prior to cutting frozen transverse sections (40 μm) with a microtome. A 1:3 series was processed for either BDA or FG.

For the BDA cases, sections were washed three times with 0.1 M PB for 10 min, and then in 0.1 M PB with 0.3% triton for at least 5 min. The sections then were incubated in Vectastain ABC Elite solution (1:200; Vector Laboratories, Burlingame, CA, USA) for 1 h, washed in three rinses of PB, and reacted with diaminobenzidine dihydrochloride (DAB) intensified with nickel ammonium sulfate for 4–10 min. Hydrogen peroxide at a concentration of 0.06% catalyzed the reaction. Sections containing FG were floated in buffer containing rabbit anti-FG (1:20,000; Chemicon, Temecula, CA, USA) overnight on a shaker at room temperature. The following morning, the sections were washed three times in PB with 0.3% Triton and incubated for 1 h in a solution containing goat anti-rabbit immunoglobulin (Sigma) at a dilution of 1:400. After three washes in PB containing 0.3% triton, sections were incubated in Vectastain ABC Elite solution (1:200) for 1 h, and then reacted as above. The sections from both groups were then rinsed, mounted on gelatinized slides, air-dried, counterstained with Neutral Red, dehydrated in alcohols, defatted in xylenes, and coverslipped with Permount.

Sections from all experiments were examined with a Nikon E800 microscope equipped with bright- and dark-field optics, photographed digitally (MicroImager II, QImaging Corp., Burnaby, BC, Canada), and processed and saved on a computer with Northern Eclipse software (Empix Imaging, Inc., Mississauga, ON, Canada). The location of label was reconstructed using a Neurolucida System (MicroBrightField, Inc., Cochester, VT, USA) interfaced with a Nikon E600 microscope. Reaction product aligned linearly