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# Phox2b expression in the aldosterone-sensitive HSD2 neurons of the NTS

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## Abstract

The transcription factor Phox2b is necessary for the development of the nucleus of the solitary tract (NTS). In this brainstem nucleus, Phox2b is expressed exclusively within a subpopulation of glutamatergic neurons. The present experiments in the adult rat were designed to test whether this subpopulation includes the aldosterone-sensitive NTS neurons, which express the enzyme 11-β-hydroxysteroid dehydrogenase type 2 (HSD2). Nuclear Phox2b was found in virtually all the HSD2 neurons (95–99%, n=6 cases). Unlike the activity-related transcription factor c-Fos, Phox2b expression in the HSD2 neurons was not influenced by dietary sodium deprivation. The ubiquitous expression of Phox2b by the HSD2 neurons suggests that they are developmentally related to other Phox2b-dependent neurons of the NTS and that they release the excitatory neurotransmitter glutamate. This finding also suggests that human Phox2b mutations, which cause the central congenital hypoventilation syndrome (CCHS, also known as Ondine's curse), may also produce deficits in central aldosterone signaling and appetitive or autonomic responses to sodium deficiency.

### Keywords

"nucleus of the solitary tract"; "nucleus tractus solitarii"; "nucleus tractus solitarius"; "sodium appetite"; "salt appetite"; "salt ingestion"; "salt intake'; aldosterone; mineralocorticoid; mineralocorticosteroid; ingestive behavior; "paired-like homeobox 2b"; central congenital hypoventilation syndrome"; CCHS; "Ondine's curse"; 11-beta-hydroxysteroid dehydrogenase type 2; 11-OHSD2; 11-HSD2

## Introduction

A unique group of neurons in the brainstem are selectively sensitive to the adrenal mineralocorticosteroid aldosterone (Geerling et al., 2006a; Geerling et al., 2006b). These cells, located in the nucleus of the solitary tract (NTS), are identifiable by their prominent expression

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*ABBREVIATIONS USED IN THIS ARTICLE: 4V, fourth ventricle; AP, area postrema; CC, central canal; CCHS, central congenital hypoventilation syndrome; DMX, dorsal motor nucleus of the vagus (Xth) nerve; GAD67, 67kDa isoform of glutamate decarboxylase; HSD2, 11-β-hydroxysteroid dehydrogenase type 2; NTS, nucleus of the solitary tract; PBel, external lateral parabrachial nucleus; pre-LC, pre-locus coeruleus; Phox2b, paired-like homeobox 2b; VGLUT2, vesicular glutamate transporter 2.

of the glucocorticoid-inactivating enzyme 11-β-hydroxysteroid dehydrogenase type 2, and are referred to as HSD2 neurons.

In addition to aldosterone-sensitivity, a number of features distinguish this group of cells from other subpopulations of neurons in the surrounding NTS. In association with sodium appetite, the HSD2 neurons are progressively activated by dietary sodium deprivation, and then inactivated when salt is ingested (Geerling et al., 2006a). This activity pattern is very different from surrounding neurons in the medial NTS, most of which are inactive during sodium deprivation, then strongly activated following salt ingestion (Geerling and Loewy, 2006c; Geerling and Loewy, 2007b). Likewise, the output connections of the HSD2 neurons are dissimilar from those of most other neurons in the surrounding NTS. Their axons target the bed nucleus of the stria terminalis in the basal forebrain as well as forebrain-projecting relay nuclei in the pons, while providing little or no input to autonomic regions of the brainstem and hypothalamus innervated by many other neurons in the NTS (Geerling and Loewy, 2006a; Geerling and Loewy, 2006b).

Other than these attributes, little is known about the molecular phenotype(s) and developmental origins of the HSD2 neurons. Initially, we were unable to find evidence for co-expression of neuropeptides, calcium-binding proteins, and enzymes that define various other subpopulations of neurons in the rat NTS (Geerling et al., 2006b). One exception was the neuropeptide galanin, which was found in a few HSD2 neurons in some cases, but only after intracerebral pre-treatment with colchicine to increase the somatic concentration of axonally-transported peptides.

In the present study, to learn more about the molecular and developmental phenotype of the HSD2 neurons, we tested whether they express Phox2b, a transcription factor that is necessary for the development of most neurons in the NTS (Dauger et al., 2003). Phox2b is found in a restricted number of neuronal populations in the brainstem (Pattyn et al., 1997), and its expression persists into adulthood in many of these sites, including the NTS (Stornetta et al., 2006; Kang et al., 2007).

Given the considerable differences we identified in HSD2 neurons relative to other NTS groups, we were specifically interested in the possibility that these cells originate from a separate precursor population, unlike the Phox2b-derived majority in this nucleus. Also, Phox2b-expressing neurons in the NTS are exclusively glutamatergic (Kang et al., 2007), so determining whether any HSD2 neurons express this transcription factor could provide valuable information regarding their neurotransmitter phenotype. For these reasons, we used double-immunofluorescence labeling to compare the expression of HSD2 and Phox2b in the rat NTS.

## Results

Phox2b-immunoreactive nuclei in the caudal medulla were located almost exclusively within the dorsal vagal complex – the NTS, AP, and DMX (Figure 1). At levels containing these three nuclei, the medulla was virtually devoid of any other nuclear Phox2b immunoreactivity, with the exception of one or two faintly immunoreactive nuclei found occasionally within the reticular formation in a minority of sections.

In addition to neuronal nuclei, this antibody produced strong cytoplasmic labeling in non-neuronal cells bordering the medial NTS. This cross-immunoreactivity, limited to ependymal cells that line the fourth ventricle and central canal of the spinal cord, was most prominent at the obex, where the fourth ventricular ependyma invaginates to form the central canal (see arrow in Figure 1A).

All subregions of the AP and all cytoarchitectonic subdivisions of the NTS contained cells with weak-to-moderate nuclear Phox2b-like immunoreactivity. Dense clusters of strong nuclear Phox2b immunoreactivity were found in the central subnucleus of the NTS at the level of the obex and immediately rostral (see Figure 1A) as well as in the medial, dorsomedial, and commissural subnuclei of the NTS at levels extending caudally through the obex (Figure 1B–C). Further from the midline, Phox2b nuclear labeling dissipated through the lateral subdivision of the NTS (lateral to the solitary tract) where its cells merge into the adjacent medullary reticular formation. The DMX contained many faintly immunoreactive nuclei, which were generally 50–75% larger than those in the AP and NTS (this faint labeling is difficult to discriminate from background in low-magnification images such as those presented in Figure 1).

HSD2-like immunoreactivity was found in the NTS ventral and just rostral to the AP, in the same characteristic distribution we described previously (Geerling et al., 2006b). As expected from our previous experiments with dietary sodium deprivation, the intensity of HSD2 immunoreactivity was noticeably greater after dietary sodium-deprivation. This increase in immunofluorescent intensity resulted in a greater number of neurons detected in sodium-deprived cases than in non-deprived animals (158 ± 6, versus 115 ± 17; p=0.04 by one-tailed t-test). The distribution of Phox2b-immunoreactive nuclei encompassed the medial NTS subregion occupied by the HSD2 neurons. While this population accounted for only a minority of the overall population of Phox2b-immunoreactive neurons in the NTS, nearly every HSD2 neuron contained robust Phox2b immunoreactivity (Figure 2).

In contrast to their increased expression of an activity-related transcription factor, c-Fos (Geerling et al., 2006a), the ubiquitous expression of Phox2b in HSD2 neurons was unaffected by dietary sodium-deprivation. A Phox2b-immunoreactive nucleus was found in 98 ± 0.2 % of the HSD2 neurons in n=3 cases after 8d dietary sodium deprivation and in 98 ± 1.3 % in n=3 control cases fed a normal diet (see Table 1).

## Discussion

Stable Phox2b expression was found in virtually all the HSD2 neurons in adult rats, suggesting that these aldosterone-sensitive cells share a common developmental origin with most other neurons in the NTS. As discussed below, this finding also suggests an excitatory (glutamatergic) phenotype for the HSD2 neurons, similar to other Phox2b-expressing neurons in the NTS.

### Technical considerations

In each case, one or more HSD2 neurons were found without prominent nuclear Phox2b-immunoreactivity. These neurons fell into one of two groups. In most of these neurons, faint Cy3 immunofluorescence was visible within the region of the nucleus, but did not unambiguously fill out the size and shape of that cell's nucleus (seen as a void surrounded by HSD2-immunofluorescence). This first pattern of labeling could represent background immunofluorescence, a low concentration of Phox2b within the nucleus of a few HSD2 neurons, or a transient period of non-expression before newly translated Phox2b protein re-fills the nucleus.

The second type of HSD2 neuron without nuclear Phox2b labeling, representing only one or two cells in a minority of cases, was found at the section edge (evident from plane of focus), but contained no nuclear labeling whatsoever, not even at the level of background tissue fluorescence. This pattern may be interpreted as an absence of Phox2b expression in a minority of HSD2 neurons, but more likely represents the loss of a nucleus at the cut edge as a result of tissue sectioning.

In any case, nearly all the HSD2 neurons in every case in this study (over 98% across cases) exhibited unambiguous nuclear Phox2b labeling, indicating that virtually the entire population expresses this transcription factor at a level comparable to that found in other neuronal subpopulations of the medial NTS.

Finally, the larger number of HSD2 neurons detected in sodium-deprived animals likely resulted from their large increase in HSD2 immunofluorescence intensity. As discussed previously (Geerling and Loewy, 2006c), this likely reflects increased expression of HSD2 in these neurons, driven by the increase in circulating aldosterone during dietary sodium deprivation (Fukushima et al., 2005).

## Distribution of Phox2b-like immunoreactivity in the caudal medulla

In the peripheral nervous system, Phox2b expression is necessary for the development of a wide array of chemosensory cells, viscerosensory ganglia, and autonomic neurons in the peripheral nervous system (Tiveron et al., 1996; Pattyn et al., 1997; Pattyn et al., 1999; Dauger et al., 2003). In the central nervous system, expression of this transcription factor is similarly critical for the development of a select number of neuronal nuclei in the brainstem, including the AP, NTS, DMX, various noradrenergic neurons, and throughout a synaptic chain of pre-inspiratory neurons in the periphery and brainstem, which mediate chemogenic respiratory drive (Pattyn et al., 2000; Dauger et al., 2003; Stornetta et al., 2006). In humans, various mutations in the Phox2b gene cause the congenital central hypoventilation syndrome (CCHS), also known as Ondine's curse, distinguished by a reduced chemosensory drive to breathe during sleep and various other autonomic deficits (Amiel et al., 2003).

In adult rats, Phox2b expression persists within many brainstem nuclei, none more prominent than the NTS and AP (Stornetta et al., 2006; Kang et al., 2007). Phox2b is expressed in a subset of cells in every subregion of the NTS, where it has been shown to label various functionally-specific subpopulations of neurons, including A2 noradrenergic neurons (in the medial subnucleus of the NTS, just lateral to the HSD2 neurons), hypoxia-activated pre-respiratory neurons in the intermediate NTS, and barorecipient neurons in the dorsomedial and dorsal commissural NTS (Stornetta et al., 2006; Kang et al., 2007).

The general pattern of Phox2b labeling reported here is largely consistent with previous reports in adult rats. In medullary sections at and caudal to the obex, we found very little evidence for Phox2b expression outside the dorsal vagal complex, with the exception of a rare, faintly-labeled nucleus in the reticular formation. This confirms a previous report, which noted a similar paucity of labeling outside the dorsal vagal complex in the caudal medulla, with an increasing prominence in the reticular formation and in other regions further rostral to the obex (Kang et al., 2007). Within the dorsal vagal complex, the variation in the intensity Phox2b labeling shown here is also consistent with this previous report. For example, the nuclei of many DMX neurons are Phox2b-immunoreactive, but the intensity of labeling here is substantially weaker than in the dorsally-adjacent NTS and AP. Also, certain NTS subnuclei stood out with denser labeling, such as the dorsomedial and central subnuclei.

The only discrepancy between the labeling reported here, relative to previous investigations using the same Phox2b antiserum, was the prominent immunoreactivity found in ependymal cells lining the caudal fourth ventricle and central canal of the spinal cord (Figure 1). This labeling may represent off-target cross-reactivity because all other brainstem Phox2b labeling in this and previous studies has been localized to neuronal nuclei. This cross-reactivity may have been less apparent in previous reports due to differences in tissue processing or immunohistology (such as tissue storage in cryoprotectant solution and the use of tyramide signal amplification; Kang et al., 2007). Regardless of the cause, this ependymal labeling did not affect the primary subject of interest – nuclear Phox2b immunoreactivity in the NTS.

## Phox2b in the NTS: developmental origins and glutamatergic phenotype

Other than their location inside the NTS, much of what was previously known about the HSD2 neurons suggested that they are unlike most other neurons in this nucleus. These unusual features include: their restricted location in a blood brain barrier-compromised region beneath the AP (Geerling et al., 2006b), aldosterone-sensitivity and -selectivity (Geerling et al., 2006a), selective activation by sodium deprivation and inactivation by salt ingestion (Geerling et al., 2006a; Geerling and Loewy, 2006c; Geerling and Loewy, 2007b; Geerling and Loewy, 2007a), and a unique pattern of rostral output projections (Geerling and Loewy, 2006b; Geerling and Loewy, 2006a). Also, their lack of immunoreactivity for various enzymes, neuropeptides, and calcium-binding proteins that mark various other neuronal subpopulations in the NTS (Geerling et al., 2006b) begged the question: what, if any, developmental relationship do the HSD2 neurons share with other cells in the NTS?

Detailed answers to this question will require additional work, but examining Phox2b expression in adult animals was a logical first step, as this transcription factor represents perhaps the most widely expressed marker of principal neurons in this brainstem nucleus. The stable expression of Phox2b across two different states of neuronal activation suggests that the HSD2 neurons are derived from the same precursor population as most other neurons in the NTS.

In the NTS, Phox2b selectively labels glutamatergic neurons. It is not expressed in inhibitory NTS neurons, evident from a lack of co-localization with the GABA synthetic enzyme GAD67 in all but two from a sample of more than 1000 Phox2b-ir neurons at various rostrocaudal levels of the NTS (Kang et al., 2007). Conversely, every cell in the NTS with a Phox2b-ir nucleus also contained mRNA for the vesicular glutamate transporter VGLUT2 (Kang et al., 2007), a reliable marker for glutamatergic neurons in this nucleus (Stornetta et al., 2002).

These findings suggest that HSD2 neurons excite their synaptic targets using the neurotransmitter glutamate. While unambiguous confirmation of this hypothesis will require direct testing for VGLUT2 and GAD67 mRNA in HSD2-immunoreactive neurons, as well as electrophysiologic recordings with pharmacologic receptor blockade in identified post-synaptic targets, additional lines of converging evidence support this conclusion.

First, although some GABAergic NTS neurons project to extra-nuclear targets (Takakura et al., 2007), many inhibitory cells in this nucleus are local interneurons. In detailed morphological studies within this nucleus, most larger neurons that project outside the NTS (like the HSD2 neurons) were not GABAergic (Kawai and Senba, 1996; Kawai and Senba, 1999). Second, electron microscopic analysis of anterograde axonal projections from the NTS to the external lateral parabrachial nucleus (PBel, the inner subdivision of which is a target of the HSD2 neurons; Geerling and Loewy, 2006b) revealed that virtually all labeled synapses were asymmetric (Gray's type I) axodendritic contacts (Jia et al., 1994), which are associated with fast excitatory transmitter release (Gray, 1959; Uchizono, 1965). This finding is particularly compelling as the subregion of the caudal NTS injected with anterograde tracer overlapped the distribution of HSD2 neurons (see Fig. 2C, Jia et al., 1994). Third, we have found that neurons receiving input from HSD2 neurons in this region (the pre-locus coeruleus and the inner subdivision of PBel) are activated in association with the HSD2 neurons during dietary sodium deprivation (Geerling and Loewy, 2007b). As their connection with the NTS is almost exclusively one-way (NTS→ pre-LC/PBel), this finding is most consistent with excitatory transmitter release by the HSD2 neurons.

### Conclusions

In the NTS of adult rats, the aldosterone-sensitive HSD2 neurons stably express the transcription factor Phox2b. This finding implies that the HSD2 neurons share a common origin with other glutamatergic Phox2b-derived neuronal subpopulations in the NTS. In this regard, it would be interesting to learn whether human CCHS patients and Phox2b-mutant animal models exhibit any deficits in central aldosterone signaling, particularly changes in sodium deficiency-related hedonic and autonomic functions.

## Experimental procedure

Experimental protocols were approved by the Washington University School of Medicine Institutional Animal Care and Use Committee and conformed to NIH guidelines.

### Animals and dietary sodium deprivation

Adult male Sprague-Dawley rats (n=6, 205–324g; Harlan, Indianapolis, IN) were individually caged and provided *ad libitum* access to tap water and either standard rat chow (PicoLab rodent diet #20, containing 0.33% sodium; LabDiet, Richmond, IN; n=3) or an extremely low-sodium chow (<0.01% Na, #85292, Harlan-Teklad; Madison, WI; n=3). Rats in the dietary sodium deprivation group were provided a fresh cage every day to minimize any ingestion of excreted sodium. All rats were maintained on an automated 12h-12h light-dark schedule (lights-on at 6:30 AM; lights-off at 6:30 PM), and perfused between 7–9am after 8d.

### HSD2 and Phox2b antisera

HSD2 was labeled using an affinity-purified polyclonal antiserum raised in sheep (product #1296; Chemicon International; Temecula, CA). This antiserum was raised against a synthetic peptide sequence generated from nucleotides 385–1204 of the rat HSD2 gene (Gomez-Sanchez et al., 2001). At a dilution of 1:40,000, it produces optimal signal-to-noise for staining the restricted group of HSD2-expressing neurons located in the NTS, consistent with prior localization of HSD2 mRNA by *in situ* hybridization (Roland et al., 1995). For further details regarding HSD2 immunoreactivity in the rat brain see (Geerling et al., 2006b).

Phox2b was labeled (in combination with HSD2) using a polyclonal antiserum provided by J. F. Brunet. This antiserum was raised in rabbit against a C-terminal 14 amino acid sequence from murine Phox2b with an additional N-terminal tyrosine (amino acid sequence: YPNGAKAALVKSSMF; Pattyn et al., 1997). Its specificity has been supported *in vitro* by ELISA (Pattyn et al., 1997) and by pre-incubation with a peptide representing the C-terminal 14AA of Phox2b (3.7μg/mL), which completely eliminated labeling with this antiserum (Kang et al., 2007). Its specificity was further verified *in vivo* by the correspondence between Phox2b immunostaining and labeling of its mRNA by *in situ* hybridization (Pattyn et al., 1997), and by the absence of immunolabeling in Phox2b knockout mice (J. F. Brunet, unpublished data).

### Perfusions and immunofluorescence labeling

All rats were anesthetized with pentobarbital (50 mg/kg, i.p.) prior to perfusion in the morning (7–9 AM). Each animal was perfused through the ascending aorta with 500 mL isotonic saline followed by 200 mL 4% paraformaldehyde prepared in 0.1 M sodium phosphate buffer (pH 7.4). Brains were post-fixed in 4% paraformaldehyde overnight, sectioned on a freezing microtome the following morning, and placed in primary antibody solution the same afternoon.

Double-immunofluorescence staining for HSD2 + Phox2b was performed on a 1-in-5 series of 50 μm frozen sections cut in the transverse plane. Tissues from normal and sodium-deprived groups of rats were run simultaneously in order to eliminate any possibility that differences in labeling resulted from different histochemical treatment. Immunofluorescence staining was

performed using standard double-labeling procedures with Cy2- and Cy3-conjugated secondary antibodies for visualizing HSD2 and Phox2b, respectively. Nine free-floating 50μm sections from a 1-in-5 series containing the caudal NTS (extending from roughly 500 μm rostral to the area postrema back through the the spinomedullary transition) were gently agitated overnight at room temperature in a 5% donkey serum solution into which both primary antisera were diluted – sheep anti-HSD2 (1:40,000) and rabbit anti-Phox2b (1:10,000). After two washes in KPBS, sections were transferred for 2.5 hours into 5% donkey serum containing a mixture of two fluorophore-conjugated secondary antisera – Cy2-conjugated donkey anti-sheep and Cy3-conjugated donkey anti-rabbit (each 1:500; Jackson Immunochemicals, West Grove, PA). After two more rinses in KPBS, sections were mounted on double-dipped gelatin-coated slides, air-dried, and coverslipped using a fade-retardant glycerol mounting solution with azide and n-propyl gallate.

### Data analysis and Photomicrographs

After immunohistochemical staining, all HSD2 neurons containing a clear nucleus within the plane of section were counted at 400x magnification and scored for the presence or absence of nuclear Phox2b-ir using a digital X-Y plotter system (Accustage, Minneapolis, MN). The number of HSD2 neurons containing immunofluorescent nuclei was divided by the total number of HSD2 neurons, providing a percent estimate of Phox2b expression within this population. The HSD2 neurons were always found in the same distribution within the NTS (described in Geerling et al., 2006a), spanning 6–8 transverse sections rostrocaudally (out of 9 sections immunostained for HSD2+c-Fos in every case). In all cases from this study, Phox2b-ir nuclei were found in a similar distribution as described as part of the present Results. No counting correction algorithms were used because the same systematic sample was measured in each animal, the average size of measured objects (HSD2 neuronal nuclei) did not differ between samples, and the purpose of these measurements was to test for relative changes between groups, not to estimate the absolute number of objects in a particular group (Saper, 1996).

All data are presented as group mean ± SEM. Statistical comparisons for %Phox2b+HSD2 between control and sodium-deprived groups were performed using a Student's two-tailed t-test with a type I error probability of $p<0.05$ considered significant. Based on previous observations, we anticipated a larger total number of HSD2 neurons visible for counting after dietary sodium-deprivation due to more intense HSD2-immunoreactivity in these cases, so a one-tailed t-test was used to evaluate whether this sample was significantly larger.

Sections were imaged using an Olympus Fluoview FV500 laser-scanning confocal microscope. The panels of Figure 1 are montages of maximum-intensity projection image stacks combining 30–50 confocal z-planes per stack (spaced 0.3 μm apart) acquired using a 20x water objective. Figure 2 is a maximum-intensity projection combining 39 confocal planes spaced 0.3 μm apart (11.7 μm through the tissue section) acquired using a 60x oil objective (1.4 NA). Manipulation of confocal image stacks and z-frame compression (maximum intensity projection) were performed using MetaMorph software (Molecular Devices, Sunnyvale, CA). Image cropping, resizing, montages, and adjustments in contrast were performed in Adobe Photoshop CS.

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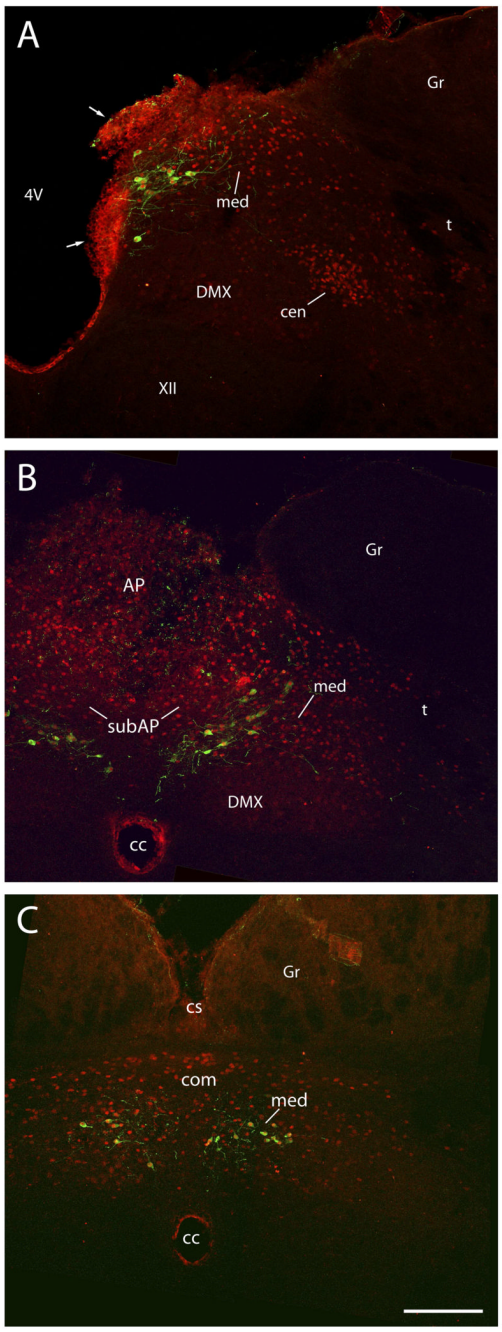


**Figure 1.**

Low-magnification photomontages showing the relative distributions of Phox2b-like nuclear immunoreactivity (red) and HSD2 neuronal labeling (green) throughout the caudal nucleus of the solitary tract (NTS). Phox2b-immunoreactive nuclei of widely varying intensities were observed throughout the NTS, dorsal motor nucleus of the vagus nerve (DMX), and area postrema; it is difficult to discriminate the more faintly-labeled nuclei from background tissue fluorescence at these low magnifications, particularly in the DMX (for a more detailed account of Phox2b labeling in this region, see Kang et al., 2007). Panel (A) shows the rostralmost cluster of HSD2 neurons, which border the open fourth ventricle (4V) at the obex. At this level, HSD2 neurons comprise the ventromedial extent of intensely Phox2b-immunoreactive neurons found

in the medial subnucleus of the NTS (med). Note also the apparent cytoplasmic cross-immunoreactivity in ependymal cells near the obex (arrows), as well as the prominent Phox2b labeling throughout the central subnucleus of the NTS (cen). (B) At levels containing the area postrema (AP), HSD2 neurons comprise the ventromedial extent of Phox2b-labeled cells found in NTSmed. Also, intensely Phox2b-labeled nuclei were found throughout the dorsally-adjacent subpostremal subnucleus of the NTS (subAP). (C) Caudal to the AP, HSD2 neurons comprise part of the ventromedial Phox2b-labeled cells within the caudal NTSmed and commissural (com) subnuclei. Scale bar in (C) is 200 μm, and applies to all three panels. Other abbreviations: 4V, fourth ventricle; cc, central canal; cs, calamus scriptorius; Gr, gracile nucleus/fasciculus; XII, hypoglossal nucleus.

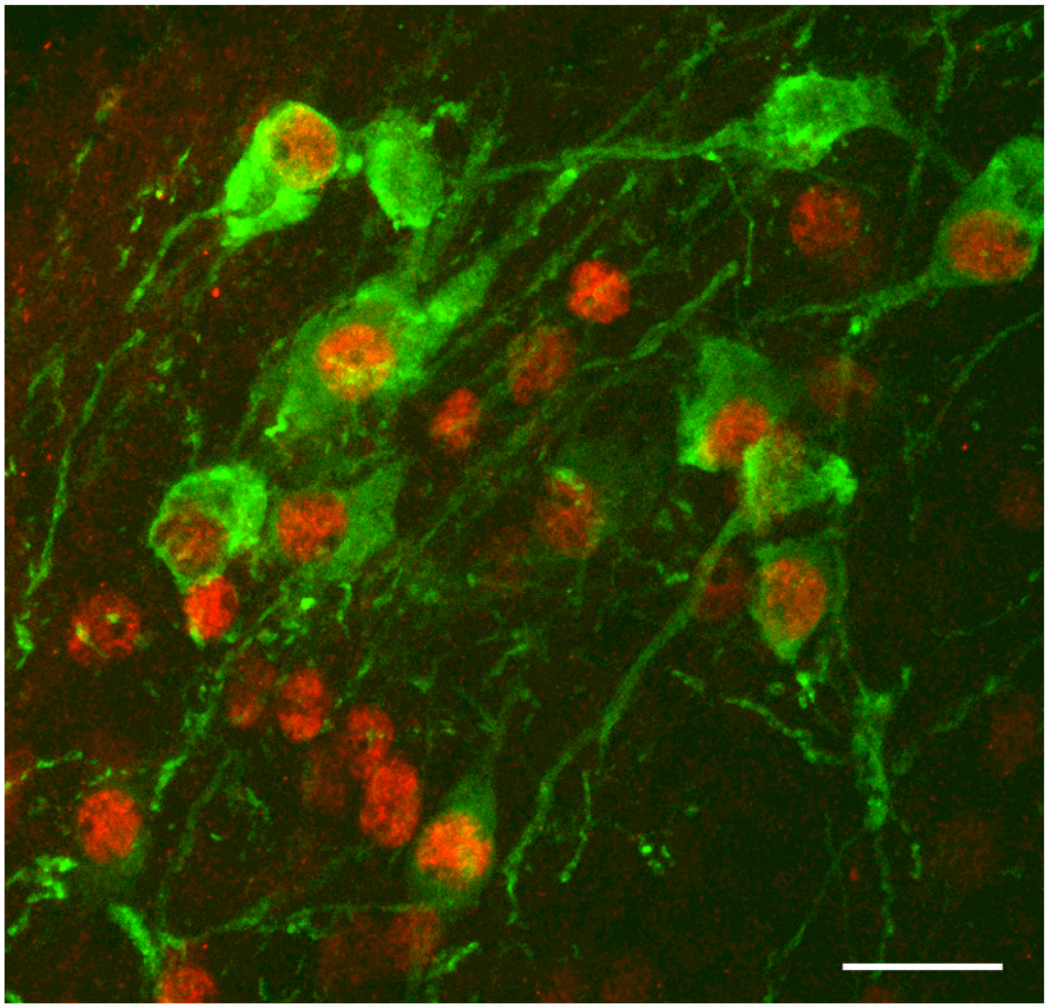

**Figure 2.**
Nuclear Phox2b-like immunoreactivity (red) was co-localized in nearly every neuron with cytoplasmic HSD2-immunoreactivity (green) with a nucleus present in the tissue section. A few Phox2b-ir nuclei were also found in other neurons intermingled with the HSD2 group. This high-magnification (60x) image was re-constructed from a stack of 39 confocal planes (maximum-intensity projection) spaced 0.3 μm apart and spanning ~12 μm through the z-axis. Scale bar is 25 μm.

**Table 1**

Number of HSD2-ir neurons counted in each case (one-in-five series of transverse sections through the caudal NTS), along with number and percentage containing a Phox2b-ir nucleus. Cases 2233–2235 were fed a normal diet (non-deprived), while 2262–2264 were fed a sodium-deficient diet (8d Na-deprived).

| Case# | Dietary Na | HSD2 neurons | +Phox2b-ir nucleus | % HSD2 neurons with Phox2b-ir nucleus |
|---|---|---|---|---|
| **2233** | non-deprived | 87 | 83 | 95.4% |
| **2234** | non-deprived | 146 | 145 | 99.3% |
| **2235** | non-deprived | 113 | 112 | 99.1% |
| Mean ± SEM | | 115 ± 17.1 | 113 ± 17.9 | **97.9 ± 1.3%** |
| **2262** | 8d Na-deprived | 155 | 153 | 98.7% |
| **2263** | 8d Na-deprived | 170 | 167 | 98.2% |
| **2264** | 8d Na-deprived | 149 | 147 | 98.7% |
| Mean ± SEM | | 158 ± 6.2 | 156 ± 5.9 | **98.5 ± 0.2%** |
| p-value (*non-deprived vs. 8d Na-deprived*) | | *0.04** | | *0.7 (NS)* |

* significant by one-tailed t-test; more HSD2 neurons were visible for counting in Na-deprived cases due to an increase in HSD2-ir intensity (as discussed previously, see Geerling and Loewy, 2006c), resulting in a larger total number of Phox2b-labeled HSD2 neurons as well. The percentage of Phox2b-ir HSD2 neurons was not significantly different between groups.