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SEROTONERGIC INPUTS TO FOXP2 NEURONS OF THE PRE-LOCUS COERULEUS AND PARABRACHIAL NUCLEI THAT PROJECT TO THE VENTRAL TEGMENTAL AREA

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Abstract—The present study demonstrates that serotonin (5-hydroxytryptamine, 5-HT)-containing axons project to two sets of neurons in the dorsolateral pons that have been implicated in salt appetite regulation. These two neuronal groups are the pre-locus coeruleus (pre-LC) and a region in the parabrachial nucleus termed the external lateral-inner subdivision (PBel-inner). Neurons in both regions constitutively express the transcription factor Forkhead protein2 (FoxP2), and become c-Fos activated after prolonged sodium depletion. They send extensive projections to the midbrain and forebrain, including a strong projection to the ventral tegmental area (VTA)—a reward processing site. The retrograde neuronal tracer cholera toxin β-subunit (CTb) was injected into the VTA region; this was done to label the cell bodies of the pre-LC and PBel-inner neurons. After 1 week, the rats were killed and their brainstems processed by a triple-color immunofluorescence procedure. The purpose was to determine whether the CTb-labeled pre-LC and PBel-inner neurons, which also had FoxP2 immunoreactive nuclei, received close contacts from 5-HT axons. Neurons with these properties were found in both sites. Since the origin of this 5-HT input was unknown, a second set of experiments was carried out in which CTb was injected into the pre-LC or lateral PB. One week later, the rats were perfused and the brainstems from these animals were analyzed for the presence of neurons that co-contained CTb and tryptophan hydroxylase (synthetic enzyme for 5-HT) immunoreactivity. Co-labeled neurons were found mainly in the area postrema and to a lesser degree, in the dorsal raphe nucleus. We propose that the 5-HT inputs to the pre-LC and PBel-inner may modulate the salt appetite-related functions that influence the reward system.

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Abbreviations: AP, area postrema; CTb, cholera toxin-β subunit; DR, dorsal raphe nucleus; dscp, decussation of the superior cerebellar peduncle; fx, fornix; Gr, gracile nucleus; IC, inferior colliculus; KPBS, potassium phosphate buffered saline; LC, locus coeruleus; MeV, mesencephalic trigeminal nucleus; MGN, medial geniculate nucleus; ml, medial lemniscus; mlf, medial longitudinal fasciculus; MnR, median raphe nucleus; mtV, tract of the mesencephalic trigeminal nucleus; NTS, nucleus of the solitary tract; OVLT, organum vasculosum of the lamina terminalis; PAG, periaqueductal gray matter; PBcl, parabrachial nucleus, central lateral subnucleus; PBdl, parabrachial nucleus, dorsal lateral subnucleus; PBel, parabrachial nucleus, external lateral subnucleus; PBel-inner, parabrachial nucleus, external lateral subnucleus—inner portion; PBel-outer, parabrachial nucleus, external lateral subnucleus—outer portion; PBl, parabrachial nucleus, lateral region; PBm, parabrachial nucleus, medial subnucleus; PBvl, parabrachial nucleus, ventral lateral subnucleus; pre-LC, pre-locus coeruleus; RN, red nucleus; SC, superior colliculus; scp, superior cerebellar peduncle; SFO, subfornical organ; SNr, substantia nigra, pars reticulate; t, solitary tract; VTA, ventral tegmental area; 4n, trochlear nerve; X, dorsal vagal nucleus; XII, hypoglossal nucleus.

Key words: area postrema, dorsal raphe nucleus, parabrachial nucleus, pre-locus coeruleus, salt appetite, ventral tegmental area.

Serotonin (5-hydroxytryptamine, 5-HT)-containing central neurons have been implicated in a wide variety of neurological functions including the regulation of sodium appetite (Reis, 2007). Drug-induced depletion of central serotonin stores induces an increase in sodium appetite (Lima et al., 2004). In addition, third ventricle injections of 5-HT pharmacological agents that activate 5-$HT_{2B/2C}$ and 5-HT_3 receptors cause a decrease in sodium appetite in sodium-depleted rats, but this drug treatment does not affect salt intake in normal animals (Castro et al., 2003). These global experiments raise the possibility that 5-HT acts centrally to modulate sodium intake, but the site of action remains unknown.

The parabrachial nucleus (PB), a brainstem region implicated in a range of visceral functions, is one site where focal injections of 5-HT drugs affect salt appetite. For example, when the nonselective 5-$HT_{1/2}$ antagonist methysergide is injected bilaterally into the lateral PB, rats increase their sodium intake (Menani and Johnson, 1995; Colombari et al., 1996; De Gobbi et al., 2000; Menani et al., 2000; Andrade-Franzé et al., 2010; Davern and McKinley, 2010). Bilateral activation of 5-HT_{1A} receptors in the PB increases sodium appetite (De Gobbi et al., 2005), yet a similar treatment with pharmacological agents that act on 5-HT_{2A} and 5-HT_{2c} receptors inhibits sodium ingestion (De Gobbi et al., 2007). Finally, Tanaka and co-workers (2004) measured the levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the PB following sodium intake and depletion. 5-HT and 5-HIAA levels increased following the saline intake, and both decreased after sodium depletion (Tanaka et al., 2004). Collectively, when one considers all of these data, a strong case can be developed to suggest that 5-HT has a modulatory function in the PB that affects salt appetite. These 5-HT agents also modulate other visceral functions including those that affect the cardiovascular system (Menani and Johnson, 1995).

The exact site(s) in the PB complex where these pharmacological agents act is unknown. There are two sites in the dorsolateral pons that become c-Fos activated after prolonged sodium depletion (Geerling and Loewy, 2007; Geerling et al., 2011). One is a subregion within the PB, which is termed the PB external lateral-inner subdivision (PBel-inner) and the other is the nearby pre-locus coer-

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uleus (pre-LC). Only about 55–65% of the neurons in these two respective sites become Fos-activated eight days after sodium deprivation (Geerling et al., 2011). The reason(s) for this remains unclear, but several possibilities may explain these findings. One is that the sodium depletion was incomplete, and perhaps it would require longer periods of sodium deprivation than 1 week to get maximal Fos labeling in these two cell groups. Another possibility is that other functional types of neurons exist within these two regions, besides those that become Fos-activated after sodium depletion. While we have assumed in this and previous studies (Geerling et al., 2011; Shin et al., 2011) that the majority of neurons in these two sites contribute to an ascending projection system that regulates salt appetite, some of these neurons may not be activated by sodium depletion but respond to other types of visceral afferent information. For example, Fig. 3 in a paper published by Rocha and Herbert (1996) shows that c-Fos activated neurons are present in areas in the dorsolateral pons that appear to correspond to pre-LC and PBel-inner; these cells were activated after 60 min of hypotension (Rocha and Herbert, 1996), and thus, raise the possibility that these or a separate set of neurons respond to cardiovascular information.

Both the pre-LC and PBel-inner contribute to an afferent pathway that projects to numerous midbrain and forebrain sites (Shin et al., 2011). Neurons in both of these regions constitutively express the transcription factor Forkhead protein2 (FoxP2) (Geerling et al., 2011), and this property provides a unique cellular marker for the immunohistochemical localization of these two groups (Geerling et al., 2011). Even with the limitations described above, the analysis of the inputs and outputs of the FoxP2+ neurons in both of these cell groups represents a new approach for deciphering the connections of the PB region.

Few reports have been published describing the source(s) of the 5-HT input to the PB region, but one of them found that the 5-HT neurons of the area postrema project to the lateral PB (Lança and van der Kooy, 1985). Surprisingly, little is known about the distribution or origin of the 5-HT inputs to the PB or the nearby pre-LC beyond the report by Steinbusch who analyzed the distribution of 5-HT fibers in the rat brain (Steinbusch, 1981). This issue is now important in light of the pharmacological data discussed above.

Because the 5-HT system has been implicated in reward processing (Kranz et al., 2010), the present experiments were designed to analyze the 5-HT inputs to two subsets of FoxP2+ neurons localized in the pre-LC and PBel-inner that project to the ventral tegmental area (VTA)—an area implicated as part of the reward system. Here, we show that 5-HT axons make “close contacts” with the pre-LC and PBel-inner neurons that project to the VTA. Then, we found two brainstem sites which contain 5-HT neurons that project to these dorsolateral pontine areas; one originates from the area postrema (AP) and the other from the dorsal raphe nucleus (DR).

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Washington University School of Medicine Animal Care Committee, conformed to NIH guidelines, and were performed on Sprague–Dawley rats (male and female; 250–350 g; Charles River Laboratories, Wilmington, MA, USA) under sodium pentobarbital (50 mg/kg, i.p. injections) anesthesia. At the termination of each experiment, anesthetized rats were killed by transcardiac perfusion with 200 ml of saline, followed by 500 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH=7.4). Brains were removed and stored in this fixative for 1–2 weeks.

Double-color immunofluorescence histochemistry: distribution of 5-HT axons in relationship to the FoxP2 neurons in the pre-LC and PB

Brainstems from three normal female rats (250–300 g) were cut in the transverse plane at 50 μm on a freezing microtome and the sections were stored as a one-in-five series in 0.1 M sodium phosphate buffer (pH=7.4) containing 0.1% sodium azide. Sections through the PB region were processed by a double-color immunofluorescence method for 5-HT and FoxP2. Sections were incubated overnight in a solution containing rabbit anti-5-HT (1:1000; S-5545; Sigma, St. Louis, MO, USA) and sheep anti-FoxP2 (1:5000; AF5647; R&D Systems, Minneapolis, MN, USA). The antibodies were added to a solution containing 0.1 M sodium phosphate buffer (pH=7.4), 5% donkey serum, and 0.3% Triton X-100 (Sigma). This solution was used in all the subsequent primary and secondary antibody solutions described in this report. The sections were washed in potassium phosphate buffered saline (KPBS; 0.01 M; pH=7.4) and transferred to a solution of secondary antibodies Cy3-donkey anti-rabbit (1:500; Jackson ImmunoResearch, West Grove, PA, USA) and Cy2-donkey anti-sheep (1:200; Jackson), washed in KPBS (2×), mounted on glass slides, and coverslipped using a fade-retardant glycerol mount solution containing sodium azide and n-propyl gallate.

The polyclonal 5-HT antibody used here was raised in rabbit against 5-HT creatinine sulfate conjugated to bovine serum albumin, and was found to be optimal for immunostaining of 5-HT axons. The polyclonal sheep antibody to FoxP2 has been used in previous publications from our laboratory (Stein and Loewy, 2010; Geerling et al., 2011).

CTb injections in the ventral tegmental area

Anesthetized rats (n=43) were placed in a stereotaxic apparatus, their skull was leveled, and then, a dorsal craniotomy was performed. Using an operating microscope, glass micropipettes (tip≈25 μm) were backfilled under direct visualization with a 0.1% CTb (cholera toxin-β subunit) solution made in distilled water (Product # 104; List Biological, Campbell, CA, USA). The pipettes were mounted on a micromanipulator and advanced into the VTA using stereotaxic coordinates taken from a rat brain atlas (Paxinos and Watson, 2005). The coordinates were bregma=−4.1 mm, lateral=1.1 mm and deep=7.2 mm.

CTb was iontophoresed for 30 min using 7 μA on/off positive pulses delivered from a Midgard precision current source (Stoelting, Wood Dale, IL, USA). The pipette was left in place for 5 min and then removed. The wound was closed in layers. The rats were allowed to survive for 1 week and were reanesthetized, killed by vascular perfusion, and their brains were removed and stored in fixative for 1 week.

Transverse frozen sections (50 μm) were cut through the region of the CTb injection site. A one-in-five series of midbrain sections were incubated for 16 h in a goat polyclonal antiserum directed against CTb (1:25,000; Product #703; List Biologicals, Campbell, CA, USA), washed in KPBS, and colorized by the