The trajectory of sensory pathways from the lamina terminalis to the insular and cingulate cortex: a neuroanatomical framework for the generation of thirst

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Hollis JH, McKinley MJ, D’Souza M, Kampe J, Oldfield BJ. The trajectory of sensory pathways from the lamina terminalis to the insular and cingulate cortex: a neuroanatomical framework for the generation of thirst. Am J Physiol Regul Integr Comp Physiol 294: R1390–R1401, 2008. First published January 30, 2008; doi:10.1152/ajpregu.00869.2007.—The pathways involved in the emotional aspects of thirst, the arousal and affect associated with the generation of thirst and the motivation to obtain satiation, have been studied but remain poorly understood. Rats were therefore injected with the neurotropic virus pseudorabies in either the insular or cingulate cortex. After 2 days of infection, pseudorabies-positive neurons were identified within the thalamus and lamina terminalis. In a separate group of rats, the retrograde tracer cholera toxin subunit b (CTb) was used in combination with either isotonic (0.15 M NaCl) or hypertonic saline (0.8 M NaCl) to label neurons in the insular cortex. Rats injected with CTb in the insular cortex and stimulated with hypertonic saline had increased numbers of Fos/CTb double-positive neurons in the paraventricular, rhomboid, and reuniens thalamic nuclei, whereas those rats injected with CTb in the insular cortex and challenged with hypertonic saline had increased numbers of Fos/CTb double-positive neurons in the medial part of the mediodorsal thalamic nuclei. Rats injected with CTb in the dorsal midline of the thalamus and challenged with hypertonic saline had increased numbers of Fos/CTb double-positive neurons in the ventral part of the laterodorsal thalamic nuclei. Rats injected with CTb in the dorsal midline of the thalamus and stimulated with hypertonic saline had increased numbers of Fos/CTb double-positive neurons in the organum vasculosum of the lamina terminalis (OVLT), median preoptic nucleus, and insular cortex but not the subfornical organ. A small proportion of the CTb-positive neurons in the OVLT were immunopositive for transient receptor potential vanilloid 1, a putative osmoreponsive membrane protein. These results identify functional thalamocortical pathways involved in relaying osmotic signals to the insular and cingulate cortex and may provide a neuroanatomical framework for the emotional aspects of thirst.

organum vasculosum; subfornical organ; hypertonic saline; pseudorabies; cholera toxin

THE LAMINA TERMINALIS, which contains the organum vasculosum of the lamina terminalis (OVLT), subfornical organ, and median preoptic nucleus, relays osmotic and endocrine signals to the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus to regulate vasopressin secretion (36, 38, 44, 45, 55, 64, 69, 70). Putative osmoreceptive membrane proteins expressed within the lamina terminalis, transient receptor potential vanilloid (TRPV) 1 and TRPV4, are important for osmoreception and subsequent vasopressin secretion following hyperosmotic stimulation (12, 30, 31, 59). The lamina terminalis contains effferent projections to regions other than the PVN and SON (34, 39, 46), including the lateral hypothalamus (4), periaqueductal gray (33), and thalamus (10, 50), suggesting that the lamina terminalis may be important for physiological responses to osmotic signals other than neuroendocrine regulation. Thirst has been described as a homeostatic or primal emotion (16, 19) whereby inner feelings (e.g., urge to breathe, hunger, thirst, desire for sleep, fatigue) provide motivating emotions essential for survival. These emotions demand behavioral responses, which in the case of thirst is the drinking of fluids. The emotional aspects of thirst therefore reflect the arousal and affect associated with the generation of thirst and the motivation to obtain satiation. Thirst has been considered in many physiological studies (36, 48, 69, 70); however, the effector regions responsible have only been alluded to (18, 19, 21, 48) and therefore require further investigation.

The thalamus has been implicated as having a role in the translation of visceral sensory information into signals of arousal and affect (9, 47, 56, 71) through intricate thalamocortical connections (9, 23, 28, 43, 51–53, 68). Two cortical regions implicated in the emotional aspects of thirst, the activity of which is altered by hyperosmotic stimulation or dehydration, are the insular and cingulate cortex (17, 20, 21, 48, 54). These two cortical regions receive direct projections from multiple thalamic nuclei (24, 28, 29, 43, 53, 68); however, only the paraventricular and mediodorsal thalamic nuclei have been implicated in osmotic signaling (17, 22, 25), albeit inconsistently (21, 60). The paraventricular and mediodorsal thalamic nuclei receive direct projections from the preoptic region of the brain containing the lamina terminalis (11, 50, 71); however, no studies have addressed the issue directly. The thalamus is therefore a potential relay for osmotic signals derived from the lamina terminalis to cortical effector regions for thirst.

To assess whether functional connections exist between the lamina terminalis and regions of the cortex implicated in homeostatic emotions, the present study aims to 1) determine the multisynaptic connections from the lamina terminalis to the insular and cingulate cortex using the neurotropic virus pseudorabies (PRV), 2) determine the thalamic nuclei that relay osmotic signals to the insular and cingulate cortex using a combination of retrograde tracer and hypertonic saline-induced Fos expression, 3) determine the regions of the lamina terminalis that relay osmotic signals to the dorsal midline of the thalamus using a combination of retrograde tracer and hypertonic saline-induced Fos expression, and 4) determine the distribution of TRPV1 in relation to neurons of the lamina terminalis that project to the dorsal midline of the thalamus.

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**METHODS**

**Animals and Housing**

Male Sprague Dawley rats (12–16 wk, 250–350 g) were housed in groups of two to three in standard cages under a 12:12-h light-dark cycle (lights on at 0700) with free access to standard rat chow and water. For the PRV inoculation study, rats were moved into a PC3 biocontainment facility (in line with Australian Quarantine Immigration Service guidelines) and housed in groups of two to three throughout experimental procedures with the same light-dark conditions and food and water access as previously stated. All experimental procedures were approved by the School of Biomedical Sciences Animal Ethics committee at Monash University.

**Injection of PRV in the Cerebral Cortex**

PRV encoding a green fluorescent protein gene (PRV-152) was provided as a kind gift from Gary Pickard (Colorado State University), and preparation and initial characterization details have been published elsewhere (6, 8, 63). Rats were anesthetized using isoflurane and oxygen and placed on a stereotactic surgical frame fitted with a nose cone for continuous administration of anesthetic. With the use of sterile surgical procedures, the skull of each rat was exposed, bregma was located, and a small portion of skull was removed to expose the brain surface at either of two different locations. The tip of a glass micropipette (internal diameter, 30–50 μm), containing PRV (2.8 × 10^3 plaque-forming units/ml) and connected to a PicoPump (World Precision Instruments), was inserted in the brain at either of two different stereotactic coordinates (bregma and lambda were at equivalent dorsal-ventral coordinates as follows: 1) left insular cortex (1.7 mm rostral to bregma, 5.0 mm lateral to bregma, 4.8 mm ventral to the surface of brain); 2) left cingulate cortex (1.7 mm rostral to bregma, 0.5 mm lateral to bregma, 2.4 mm ventral to the surface of brain). Approximately 50–100 nl of pseudorabies virus solution was injected over the course of 5 min (~30 pounds per square inch, 5–10 ms/pulse, average of 6 pulses), with the micropipette tip maintained at the injection site for an additional 10 min.

Following a range of injection times (2 and 3 days; n = 4 per site of injection per injection time), rats were anesthetized with pentobarbital sodium (100 mg/kg ip) and perfused through the left ventricle with 100 ml of 0.05 M PBS followed by 300 ml of 0.1 M phosphate buffer (PB), pH 7.2, containing 4% paraformaldehyde. The brains were then removed from the skull and processed in preparation for sectioning.

**Combination of Retrograde Tracer and Hypertonic Saline Treatment**

The same anesthetic, surgical procedures, and stereotaxic coordinates were used for the injection of cholera toxin subunit B (CTb) solution (0.5% in dH2O; List Biological Laboratories) in the left insular (n = 16) and left cingulate cortex (n = 16), as previously described above for the pseudorabies inoculation study. In addition, the dorsal midline of the thalamus containing the paraventricular and mediodorsal thalamic nucleus (2.8 mm caudal to bregma, midline to bregma, 5.0 mm ventral to the surface of the sagittal sinus) was injected with CTb solution (n = 12). Rats were then allowed to recover for 7 days. Between the hours of 1000 and 1100, rats were given intraperitoneal injections of either warmed hypertonic saline (0.8 M NaCl, 1 ml/100 g body wt) or warmed isotonic saline (0.15 M NaCl, 1 ml/100 g body wt) and allowed free access to food and water. After injections (2 h), rats were anesthetized with pentobarbital sodium, perfused as previously described, and their brains were processed as described above.

**Tissue Processing and Immunostaining Procedures**

Following perfusion, rat brains were postfixed in PB containing 4% paraformaldehyde for 12 h at 4°C followed by 2–3 days in PB containing 30% sucrose at 4°C. Rat brains were cut in the coronal plane at 40-μm thickness, and all sections were collected, divided into sets of four, and stored in wells containing cryoprotectant solution (PB containing 30% ethylene glycol and 20% glycerol) at −20°C until required for further processing.

Rat brain sections used only for microscopic investigation of pseudorabies virus localization were washed briefly in PB followed by mounting on glass slides and coverslipped using fluorescent mounting medium (DAKO). For investigation of Fos protein and CTb, rat brain sections were washed for 15 min in PB followed by 30 min in PB containing 0.1% Triton X-100 and 10% normal horse serum. Sections were then incubated overnight (12–16 h) in PB containing 0.1% Triton X-100, 1% normal horse serum, and anti-GFAP raised against Fos protein (rabbit anti-Fos, 1:4,000; Merck Biosciences) and CTb (goat anti-choleragenoid; 1:2,000; List Biological Laboratories). Sections were then incubated in PB containing affinity-purified antibodies raised against rabbit and goat (Texas Red-conjugated donkey anti-rabbit and fluorescein isothiocyanate-conjugated donkey anti-goat, 1:400 each; Jackson Immunoresearch) for 90 min. Sections were then washed two times in PB for 15 min between and following incubation steps and then mounted on glass slides and coverslipped using fluorescent mounting medium (DAKO). Rat brain sections used for investigation of CTb and TRPV1 were immunostained as previously described except for the use of serum containing antibodies raised against TRPV1 (guinea pig anti-TRPV1, 1:250; Neuroimmics) and the addition of affinity-purified antibodies raised against guinea pig (aminomethylcoumarin acetate-conjugated donkey anti-guinea pig, 1:400; Jackson Immunoresearch). According to the manufacturer, the TRPV1 antibody selectively and specifically recognizes TRPV1 of rat and mouse origin, but the most compelling evidence of specificity comes from the absence of TRPV1-positive staining when the antibody is used on tissue from TRPV1 knockout mice (59).

**Tissue Analysis and Cell Counting**

The boundaries of injection sites were analyzed by photographing the rostrocaudal extent of the injection site using a fluorescent microscope (Image.Z1; Zeiss, Australia) with an appropriate bandpass filter (to observe PRV-infected neurons or CTb-positive neurons). The outlines of injection sites from each rat were then overlaid together on templates based on a rat brain stereotaxic atlas (49) to assess the topographical extent of the PRV (data not shown) or CTb injections. PRV labeling at the injection site estimated at the time of death is predominantly dependent on the concentration and volume of virus injected (7). To estimate the extent of the PRV injection site, preliminary injections were performed in rats not included in the experimental analysis using a cocktail mixture of PRV/CTb (CTb final concentration 0.1%), and the extent of the injection site as determined by PRV and CTb labeling was compared (42). Following PRV/CTb injection (2–3 days), the extent of the injection site as determined by CTb immunostaining was approximately two times as large as that from PRV labeling. The brain sections from rats in which the topographical extent of the injection sites was predominantly outside of the intended injection targets (left insular cortex, left cingulate cortex, or dorsal midline of the thalamus) were removed from analyses.

To assess the distribution of PRV- or CTb-positive neurons within the thalamus following PRV or CTb injection in the insular or cingulate cortex, 10 rostrocaudal levels of the thalamus between bregma levels −1.60 and −3.60 mm were analyzed. Similarly, qualitative analysis of the distribution of CTb-positive neurons within the preoptic area following CTb injection in the dorsal midline of the thalamus was derived from selected sections containing the lamina terminalis and adjacent regions. The numbers of CTb-positive and Fos/CTb double-positive neurons projecting to either the insular or cingulate cortex were determined within thalamic nuclei along the rostrocaudal extent of the thalamus. Counts of Fos-positive neurons...
shown by their CTb content to project to discrete parts of the cortex were counted within thalamic nuclei along their rostrocaudal extent. The numbers of CTb-positive and Fos/CTb double-positive neurons were also determined within the lamina terminalis, surrounding preoptic area, and select regions outside of the thalamus and lamina terminalis. In the case of cell counts in the thalamus, counts taken from each rostrocaudal level (but within the same thalamic nucleus) were summed. In Figs. 1–8, the cell counts are represented as the percentage of CTb-positive neurons that are Fos-positive.

Statistical Analysis

All statistical analyses used Statistical Package for the Social Sciences (SPSS) version 14.0 (SPSS). The number of Fos/CTb double-positive neurons is represented as the mean percentages of CTb-positive neurons that are Fos-positive ± SE. Statistical analyses were performed on the total numbers of Fos/CTb double-positive neurons sampled within each thalamic nucleus. For analysis of Fos/CTb double-positive neurons in the thalamus, a single multifactor ANOVA with repeated measures was performed using treatment (hypertonic vs. isotonic saline) and the site of injection (insular vs. cingulate cortex) as the between-subject factors and region (thalamic nucleus) as the within-subjects factor for repeated-measures analysis. For analysis of either Fos-positive (CTb-negative) nuclei or Fos/CTb double-positive neurons in other regions of the brain, a single ANOVA was performed using treatment as the between-subject factor. When appropriate, post hoc pairwise comparisons were made using Fisher’s protected least-significance difference tests for analysis of the thalamus or post hoc two-tailed t-test for analysis of all other brain regions. In all cases, significance was accepted at \( P < 0.05 \).

Imaging and Figure Preparation

All photographic images were captured using a Zeiss Imager.Z1 microscope with appropriate bandpass filters, Apotome (Zeiss), grayscale digital camera, and AxioCam image capture software v4.5 (Zeiss). Images shown in Figs. 1–8 were created by the digital merging of a series of 10 focal planes (≈3–4 µm apart) along the z-axis using AxioCam image software. All graphs were made using SigmaPlot 8.0 software (Systat), and Figs. 1–8 and schematics were created and assembled in Adobe Illustrator CS2 12.0.1 (Adobe Systems).

RESULTS

Localization of PRV

Injection sites. The rostrocaudal spread of PRV within the insular cortex and cingulate cortex was estimated to extend from +2.70 to −0.26 mm bregma, and the cross-sectional spread of PRV was estimated to be contained within the cytoarchitectonic boundaries of each region. These estimates are based on the preliminary injections using the estimated spread of the PRV and CTb immunoreactivities in relation to the center of the injection.

Fig. 1. Photomicrographs of pseudorabies virus (PRV)-positive neurons located within the median preoptic nucleus (A and B), organum vasculosum of the lamina terminalis (OVLT; C and D), and subfornical organ (E and F) following injection of PRV in either the insular (A, C, and E) or cingulate (B, D, and F) cortex. Scale bar, 50 µm.
Fig. 2. Schematic depicting the topographical extent of the cholera toxin subunit b (CTb) injection site from each rat included in the analysis. The injection sites depicted include the insular and cingulate cortices and the dorsal midline of the thalamus. Rostrocaudal levels relative to bregma are given in mm. Brain schematics adapted from a standard rat brain stereotaxic atlas (49).
Distribution of transported PRV. The topographical distribution of PRV-positive neurons was consistent between rats, although the extent of the infection as evidenced by the numbers of virally infected neurons in each nucleus varied to some extent in individual rats. Following PRV injection in either the insular (n = 4) or cingulate (n = 4) cortex (2 days), presumptive first-order infected neurons (monosynaptic projections to the insular or cingulate cortex) were identified within overlapping populations present along the rostrocaudal extent of the thalamus. The predominant regions of the thalamus included the paraventricular, mediodorsal (medial, central, and lateral parts), intermediodorsal, interanteromedial, antero- medial, ventromedial, rhomboid, reunions, and ventral reunions thalamic nuclei (data not shown). Presumptive first-order neurons were also identified within the lateral hypothalamus and perifornical area. At 3 days of infection following PRV injection (n = 4/injection site), the relative number of PRV-positive neurons along the rostrocaudal extent of the thalamus, lateral hypothalamus, and perifornical area was greater than in rats infected for 2 days. At 3 days of infection, putative second and possibly third-order PRV-positive neurons were identified within other hypothalamic regions, including the arcuate nucleus, ventromedial nucleus, suprachiasmatic nucleus, and the tuberomammillary nuclei. Putative second and possibly third-order PRV-positive neurons were also identified within the medial and lateral preoptic areas and within all regions of the lamina terminalis, that is, the median preoptic nucleus, OVLT, and the outer portion of the subfornical organ (Fig. 1).

CTb Localization

Injection sites. The extent of the CTb injection site was predominantly confined to the targeted regions (insular or cingulate cortex, PVN/mediodorsal thalamic nucleus; Fig. 2). Specifically, the rostrocaudal spread of the CTb within the insular and cingulate cortex extended from −2.70 to 0.26 mm bregma and within the PVN/mediodorsal thalamic nucleus from −1.40 to −3.60 mm bregma.

Distribution of transported CTb following injection in the cerebral cortex. The distribution of CTb-positive neurons within the thalamus was consistent between rats that received injection of CTb in either the insular or cingulate cortex in different experiments (Fig. 3). A large proportion of thalamic nuclei contained overlapping populations of CTb-positive neu-

Fig. 3. Schematic depicting the topographical distribution of CTb-positive and Fos/CTb double-positive neurons located along the rostrocaudal extent of the thalamus following injection of CTb in the insular or cingulate cortices followed by ip injection of hypertonic saline. Rostrocaudal levels relative to bregma are given in mm. Brain schematics adapted from a standard rat brain stereotaxic atlas (49).
rons projecting to the insular and cingulate cortex. These included the paraventricular, the medial, central, and lateral parts of the mediodorsal, intermediodorsal, centrolateral, middle portion of paracentral, paratenial, rostral and middle portion of central medial, interanteromedial, anteromedial, ventromedial, rhomboid, reuniens, and ventral reuniens thalamic nuclei. The majority of thalamic nuclei within the more dorsal lateral divisions of the thalamus contained CTb-positive neurons projecting only to the cingulate cortex, the most prominent of these being the rostral portion of paracentral, anterodorsal, anteroverentral, ventral anterior, ventrolateral, and dorsomedial parts of the laterodorsal and the mediorostral part of the lateral posterior thalamic nuclei. The ventrolateral, caudal portion of central medial, and caudal portion of paracentral thalamic nuclei contained CTb-positive neurons projecting only to the insular cortex. The zona incerta, along the ventral border of the thalamus, contained CTb-positive neurons projecting to the insular and cingulate cortex. CTb-positive neurons were also identified within the lateral hypothalamus and perifornical area.

**Distribution of transported CTb following injection in the dorsomedial thalamus.**

Following injection of CTb in the dorsal medial region of the thalamus encompassing the paraventricular and mediodorsal thalamic nuclei, labeled neurons were found within the median preoptic nucleus, the dorsal cap and lateral aspects of the OVLT, the outer part of the subfornical organ, and within surrounding regions of the preoptic regions that include the medial and lateral preoptic area. CTb-positive neurons were also located within the medial portions of the insular cortex and claustrum. CTb-positive neurons were also identified within hypothalamic regions, including the lateral hypothalamus, perifornical area, arcuate nucleus, ventromedial nucleus, suprachiasmatic nucleus, and the tuberomammillary nuclei; CTb-positive neurons were not present within the PVN and SON (data not shown).

**Fos Expression Induced by Hypertonic Saline After CTb Injection**

Compared with isotonic saline-treated controls, intraperitoneal injection of hypertonic saline increased the numbers of Fos-positive nuclei within the lamina terminalis, the PVN, and SON (Table 1), and, as described in detail below, in discrete regions of the thalamus and cerebral cortex.

Hypertonic saline treatment increased the numbers of Fos/CTb double-positive neurons within subdivisions of the thalamus that was dependent on the cortical site of CTb injection. Multifactorial ANOVA with repeated measures revealed a treatment effect [$F(1,20) = 8.651; P < 0.01$] and treatment × site of injection × region interaction [$F(26,520) = 1.531; P < 0.05$; Figs. 3, 4, and 5]. Hypertonic saline treatment increased the numbers of Fos/CTb double-positive neurons within the paraventricular (18 ± 4.3 vs. 5 ± 2.8), rhomboid (20 ± 5.8 vs. 3 ± 1.1), and reuniens (3 ± 1.6 vs. 0 ± 0) thalamic nuclei of rats that received CTb injections in the insular cortex. In contrast, hypertonic saline treatment increased the numbers of Fos/CTb double-positive neurons within the medial part of the mediodorsal (1 ± 0.8 vs. 7 ± 2.4), interanteromedial (2 ± 1.6 vs. 26 ± 9.5), anteromedial (1 ± 0.7 vs. 20 ± 6.3), and ventrolateral part of the laterodorsal (1 ± 0.3 vs. 8 ± 2.2) thalamic nuclei of rats that received CTb injections in the cingulate cortex.

Rats that received both CTb injections in the paraventricular/mediodorsal thalamic nuclei and intraperitoneal hypertonic saline were also examined in the hypothalamus where hypertonic saline treatment increased the numbers of Fos-positive nuclei within selected brain regions (Table 1).

**Table 1. Effects of ip hypertonic saline injection on the numbers of Fos-positive nuclei within selected brain regions**

<table>
<thead>
<tr>
<th>Region</th>
<th>Isotonic Saline</th>
<th>Hypertonic Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVLT</td>
<td>17.8±3.0</td>
<td>80.1±9.5*</td>
</tr>
<tr>
<td>MnPO</td>
<td>22.4±2.7</td>
<td>80.5±8.6*</td>
</tr>
<tr>
<td>SFO</td>
<td>1.1±0.3</td>
<td>20.9±5.0*</td>
</tr>
<tr>
<td>PVN</td>
<td>42.0±7.9</td>
<td>277.8±28.8*</td>
</tr>
<tr>
<td>SON</td>
<td>19.0±7.3</td>
<td>211.2±16.0*</td>
</tr>
</tbody>
</table>

Values are represented as means ± SE. OVLT, organum vasculosum of the lamina terminalis; MnPO, medial preoptic nucleus; SFO, subfornical organ; PVN, paraventricular nucleus; SON, supraoptic nucleus. *P < 0.001 vs. isotonic saline-treated controls.
saline treatment exhibited increased numbers of Fos/CTb double-positive neurons within the median preoptic nucleus (3 ± 0.3 vs. 0 ± 0) and OVLT (8 ± 0.9 vs. 0 ± 0) but not the subfornical organ (2 ± 1.0 vs. 0 ± 0) (Figs. 6 and 7). Double-positive neurons were also notably present in the insular cortex (18 ± 4.3 vs. 5 ± 2.8), consistent with the presence of reciprocal projections between this region of the cortex and the dorsomedial thalamus, both limbs of which were activated by osmotic stimuli.

Fig. 5. Photomicrographs depicting the effects of ip injection of either hypertonic saline (C, F, I, L, and O) or isotonic saline (B, E, H, K, and N) on Fos expression within retrogradely labeled CTb-positive neurons. CTb had been injected previously in either the insular (A–C and J–L) or cingulate (D–I and M–O) cortex. The regions are depicted in coronal brain schematics highlighted by red squares (A, D, G, I, and M) and as photomicrographs of the paraventricular (A–C), medial part of the mediodorsal (D–F), interanteromedial (G–I), reuniens (J–L), and ventrolateral part of the laterodorsal (M–O) thalamic nuclei. Higher-magnification photomicrographs of CTb-positive neurons either positive or negative for Fos protein are shown as insets in the bottom right of each photomicrograph. Scale bar: 50 μm; higher-magnification insets, 20 μm.
TRPV1 Localization

TRPV1 immunoreactive neurons were identified within the PVN, SON and OVLT but not the median preoptic nucleus or subfornical organ (Fig. 8). Within the PVN, the TRPV1-positive neurons were predominantly localized in the lateral magnocellular portion. Similarly, TRPV1-positive neurons were found throughout the SON, consistent with an association with vasopressin- and oxytocin-containing neurons (3). Within the OVLT, the TRPV1-positive neurons were dispersed throughout but were not present within the surrounding preoptic region. A small proportion of TRPV1 immunoreactive neurons within the dorsal cap of the OVLT were also CTb-positive (<5%) in rats that received CTb injections in the dorsal midline of the thalamus.

DISCUSSION

The present study identifies for the first time circuitry linking the lamina terminalis to the insular and cingulate cortex that relay osmotic signals. PRV injected in either the insular or cingulate cortex results in putative first-order (directly projecting) neurons within all regions of the lamina terminalis, including the OVLT and subfornical organ. Hypertonic saline challenge in rats that had received CTb injection in either insular or cingulate cortex resulted in increased Fos expression within CTb-positive neurons of multiple thalamic nuclei. These thalamic regions include the paraventricular, rhomboid, and reuniens nuclei that relay osmotic signals to the insular cortex and the medial part of the mediodorsal, interanteromedial, anteromedial, and ventrolateral part of the lateralodorsal thalamic nuclei that relay osmotic signals to the cingulate cortex. In addition, the combined use of CTb and Fos immunostaining has identified the dorsal midline of the thalamus containing the paraventricular and medial part of the mediodorsal thalamic nuclei as recipient of osmotic signals from the OVLT, median preoptic nucleus, and the insular cortex.

Immunostaining positive for the putative osmoreponsive membrane protein TRPV1 was present within the PVN, SON, and OVLT; a small proportion of TRPV1-positive neurons within the OVLT project to the PVN/mediodorsal thalamic nucleus. The present study highlights for the first time specific regions of the thalamus that are important for relaying osmoregulatory signals from the OVLT to the insular and cingulate cortex. These thalamocortical circuits may be responsible for changes in arousal and affect (56, 71), that is, the emotional aspect of thirst in response to osmotic stimuli.

The neurotropic virus PRV has been used within the central nervous system to identify neural systems that possess multisynaptic connections to either the insular or cingulate cortex. The distribution of putative first-order (directly projecting) PRV-positive neurons within the thalamus of rats infected for 2 days that project to either the insular or cingulate cortices in this study are largely consistent with previous monosynaptic tracing studies (2, 9, 23, 28, 43, 51–53, 68). The majority of the overlapping regions of the thalamus that project to both the insular and cingulate cortex are localized to the midline thalamic region (2, 9, 28, 43, 51–53, 68). The exceptions are the ventrolateral, posterior, caudal portions of the paracentral, and centromedial thalamic nuclei that project to the insular but not the cingulate cortex (24, 28, 53) and the dorsal lateral thalamic region containing the anterodorsal, anteroventral, ventral anterior, and the ventrolateral and dorsomedial parts of the laterodorsal thalamic nuclei that project to the cingulate but not the insular cortex (24, 28, 53). The lateral and ventral lateral regions of the caudal thalamus are largely devoid of projections to the insular or cingulate cortex (24, 28, 53). The presence of putative first-order PRV-positive neurons within the lateral hypothalamus is also consistent with previous monosynaptic tracing studies (57). At 3 days of infection, putative second-order PRV-positive neurons that project indirectly to either the insular or cingulate cortex are observed within all regions of the lamina terminalis. Previous monosynaptic tracing studies suggest that regions of the thalamus and hypothalamus are potential relay sites for osmotic signaling to the cortex (14, 15, 26, 61), consistent with the present results.

The majority of the thalamic regions that relay osmotic signals to the insular and cingulate cortex are located along the thalamic midline and are nonoverlapping, suggesting some degree of functional organization within the thalamus in relation to osmotic stimuli. The functions of the thalamic midline are largely attributed to the translation of viscerosensory sig-
nals into signals of arousal and affect (9, 47, 56, 71). The dorsal midline of the thalamus in particular has been implicated in viscero-sensory awareness, whereas the lateral portion of the dorsal midline has been implicated in cognitive awareness. Both of these thalamic regions have previously been shown to be involved in osmotic signaling (18, 22, 25). The present study further characterizes these regions by identifying the paraventricular and medial part of the mediodorsal thalamic nuclei as potentially both relaying osmotic signals to the insular and cingulate cortex, albeit the significance was not reached for both nuclei (see Fig. 4). The present study identifies the rhomboid and reuniens as relaying osmotic signals to the insular cortex and the anteromedial and interanteromedial as relaying osmotic signals to the cingulate cortex. There is neuroanatomical evidence showing reuniens thalamic efferents to the hippocampus in addition to the insular cortex, suggesting a role in memory consolidation (13, 43, 71); however, there is a lack of functional evidence. The anteromedial and interanteromedial thalamic nuclei also contain efferents to the hippocampus in addition to the cingulate cortex, and functional studies suggest a role in behavioral learning and spatial memory (1, 27, 41). The ventrolateral part of the laterodorsal thalamic nucleus is the only thalamic region in the present study not along the thalamic midline found to relay osmotic signals; these signals were only directed toward the cingulate cortex. Functional evidence on the laterodorsal thalamic nucleus, similar to the rhomboid and reuniens thalamic nuclei, is lacking.

The dorsal midline of the thalamus integrates both ascending osmotic signals and descending cortical efferent signals. The lamina terminalis is now well established as a primary site for osmoreception in the brain (12, 36, 37, 55, 69, 70). Furthermore, based on injections of tracers in the OVLT but not restricted to it, there appear to be efferent connections from the region to the dorsal midline of the thalamus (4, 10, 14, 15, 23, 61). The present study provides functional evidence, in the form of Fos labeling following osmotic challenges, that the OVLT relays osmotic signals directly to the dorsal midline of the thalamus, which contains the paraventricular and medial part of the dorsomedial thalamic nucleus. In light of the aforementioned osmotic signaling by the dorsal midline of the thalamus to the insular and cingulate cortex, it is possible that the OVLT-thalamocortical relay operates to translate osmotic signals into arousal and affect. The dorsal midline of the thalamus is also integrated into what is described as the behavioral control column whereby cortical efferents drive motivated behavior (65, 66). Previous studies that have alluded to the thalamus as relaying both signals of thirst and the motivation to obtain satiation (18, 21, 48) are consistent with

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**Fig. 7. Photomicrographs depicting the effects of ip injection of either hypertonic saline (C, F, and I) or isotonic saline (B, E, and H) on Fos expression within retrogradely labeled CTb-positive neurons. CTb had been injected previously in the dorsal midline of the thalamus. The regions are depicted in coronal brain schematics highlighted by red squares (A, D, and G) and as photomicrographs of the OVLT (A–C), subformical organ (D–F), and insular cortex (G–I). Higher-magnification photomicrographs of CTb-positive neurons either positive or negative for Fos protein are shown as insets in the bottom right of each photomicrograph. Scale bar: 50 μm; higher-magnification insets, 20 μm.**
the present study in which the thalamus relays osmotic signals transferred through the insular cortex to the dorsal midline of the thalamus. Together these studies highlight a functional portion of a larger, distributed neural network consisting of collaterals to the midline thalamus and the nucleus of the solitary tract (47), which are important for thalamocortical integration of viscerosensory reflexes.

The present study highlights a series of lamina terminalis-thalamo-cortical pathways with the use of both PRV and the conventional retrograde tracer CTb; however, inherent limitations exist with both techniques. The present neuroanatomic data using PRV provides evidence of direct multisynaptic connections between the lamina terminalis and cerebral cortices. Functional studies in combination with PRV, however, are complicated by the fact that PRV infection in itself results in production of Fos protein (62); using separate groups for multisynaptic tracing and assessing function is an acceptable, alternative approach (5). In contrast, CTb can easily be used in combination with Fos protein in functional studies, albeit CTb is only useful for tracing monosynaptic relays in the brain. The lamina terminalis-thalamo-cortical connections illustrated in the present study through the use of CTb are not necessarily synaptically connected relays, as illustrated through the use of PRV. Nevertheless, the present study clearly provides novel evidence of functional lamina terminalis-thalamo-cortical connections that may be important for the emotional aspects of thirst.

PRV-labeled neurons in the subfornical organ that were connected polysynaptically to the insular and cingulate cortices were confined to the outer part of this circumventricular organ. It is likely that thirst-inducing stimuli act through neurons in this outer part and not the core of the subfornical organ because the outer part rather than the core of the subfornical organ is activated by dipsogenic stimuli such as systemic infusion of hypertonic saline or relaxin (35). The latter stimulus exerts its dipsogenic effect entirely through relaxin receptors in the subfornical organ (64). In regard to angiotensin-induced drinking, although systemic infusion of angiotensin activates neurons in both the core and outer parts of the subfornical organ, it is likely that its dipsogenic action is also mediated by neurons in the outer part. This is because direct efferent neural connections from the subfornical organ to the median preoptic nucleus signal angiotensin-mediated drinking (32), and the neurons within the periphery of the subfornical organ give rise to this connection (40, 58, 67). As well, we have observed that low systemic doses of ANG II, while activating neurons in the core of the subfornical organ, did not stimulate drinking, but higher doses that activated neurons in the outer part of the subfornical organ were dipsogenic (35).

Conclusion

The present study identifies functional lamina terminalis-thalamocortical circuitry that may be important for the translation of osmotic signals into arousal and affect. The use of PRV provides evidence that the lamina terminalis contains multisynaptic projections to both the insular and cingulate cortex. The use of CTb in combination with the osmotic stimulus has further identified specific nuclei involved in relaying osmotic signals along the lamina terminalis-thalamocortical circuitry. In particular, the dorsal midline of the thalamus is important for efferent signals to the insular and cingulate cortex, and additional afferent signals from the insular cortex, highlighting reciprocal thalamocortical circuitry likely involved in influencing viscerosensory reflexes and behavior. These data identify thalamocortical circuitry that may provide a neuroanatomical framework for the generation of thirst, the physiological need to drink, and the motivational drives to obtain satiation.
Perspectives and Significance

These data significantly expand our view of the neuronal circuitry that translates body fluid homeostatic information into states of arousal and affect, which ultimately result in drinking behavior. While necessarily incomplete, the proposed schema, which includes relays in the dorsal midline of the thalamus that direct information to cortical sites, represent frontiers that have not been well investigated outside imaging studies. This expanded view may complement lesion studies or cortical and subcortical pathologies that impact on drinking behavior and provide clues as to the long-sought-after loci of thirst. In a more general sense, these data also elucidate neuronal circuitry responsible for the translation of homeostatic drivers to effector mechanisms related to motivated behaviors. It should be recognized that the final cortical effector pathways identified here in relation to dehydration and thirst may be intermingled with other pathways responsive to a range of homeostatic stimuli that give rise to feelings as varied as hunger for food or the urge to inhale air. In fact it is possible given the number of modalities that have been associated with the insular and anterior cingulate cortex that motivated behaviors are derived from a mosaic of cells in these regions that are influenced by specific sensory input.

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