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Mediation of osmoregulatory influences on neuroendocrine corticotropin-releasing factor expression by the ventral lamina terminalis

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ABSTRACT **Chronic exposure to a hyperosmolar challenge invokes coordinate, differential, and ostensibly adaptive alterations in the expression of mRNA encoding corticotropin-releasing factor (CRF) in the endocrine hypothalamus. Rats maintained on 2% (wt/vol) saline for 7 days displayed the expected reduction in CRF mRNA levels in the parvocellular neurosecretory compartment of the paraventricular nucleus, as well as a concomitant increase in CRF transcripts in oxytocin-containing magnocellular neurosecretory neurons. Also detected in salt-loaded animals was a prominent induction of the immediate-early gene product Fos in magnocellular neurosecretory cell groups and in several brain regions that are known to provide major projections to the endocrine hypothalamus. These included a triad of cell groups making up the lamina terminalis of the third ventricle, and, to a lesser extent, catecholaminergic cell groups in the caudal brain stem. Discrete transections of descending projections from structures associated with the lamina terminalis, as well as excitotoxin lesions centered in one lamina terminalis-associated structure, the organum vasculosum, abolished the effects of salt loading in both the magno- and parvocellular neurosecretory systems. Knife cuts in the lamina terminalis complex that spared only projections from the organum vasculosum region or cuts that disrupted ascending catecholaminergic projections failed to modify either effect of salt loading. The results suggest the existence of a simple circuit through which osmotic influences on gene expression in the magnocellular and parvocellular neurosecretory systems are effected.**

Though best known for its obligate role in initiating pituitary–adrenal responses to stress (1), corticotropin-releasing factor (CRF) is a neuropeptide that is broadly distributed within the central nervous system (2). Even within the paraventricular nucleus (PVH), the acknowledged seat of parvocellular neurosecretory neurons that deliver CRF to the hypophyseal–portal circulation for the initiation of the stress cascade, additional cell types are capable of expressing the peptide. These include a subset of magnocellular neurosecretory neurons, whose dominant secretory product is the posterior pituitary hormone oxytocin (3). Although levels of CRF gene and peptide expression in the magnocellular system are normally low, the increased plasma osmolality associated with salt loading promotes a prominent increase in CRF expression in magnocellular neurons, which is accompanied by a down-regulation of CRF expression in the parvocellular neurosecretory system (4–7). The net result is a pattern of CRF expression in neurosecretory hypothalamus that is virtually indistinguishable from that of oxytocin. These influences are accompanied by decreased circulating levels of adrenocorticotropic hormone and corticosterone and increased CRF content in the posterior pituitary (8). Both effects may contribute via distinct mechanisms (9, 10) to the antidiuresis and natriuresis that represent major adaptive responses to a hyperosmolar challenge.

The neural pathways that convey sensory information to the magnocellular and parvocellular neurosecretory systems to effect alterations in CRF expression in response to hyperosmotic challenge are unknown. Moreover, the coordinate changes in the expression of a single peptide in two neuroendocrine cell types that are seen in this model provide an opportunity to investigate how integrative aspects of hypothalamic function may rely upon common versus divergent afferent pathways. Here we provide evidence to indicate that a single region, the ventral lamina terminalis, mediates the effects of salt loading on CRF expression in both neuroendocrine cell types.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley albino rats, maintained under standard laboratory conditions, were used in all experiments. Salt loading involved substitution of 2% (wt/vol) saline for tap water as a sole source of fluids for 7 days.

Surgeries. All surgeries were carried out under pentobarbital anesthesia (40 mg/kg, i.p.). Fiber transections in the coronal plane designed to isolate the hypothalamus from descending projections of the lamina terminalis were produced stereotaxically by using a retractable wire knife fashioned from a microliter syringe (11). Cuts were positioned immediately caudal to the organum vasculosum of the lamina terminalis (OVLT) and extended 2 mm laterally from the midline and 3 mm dorsally from the ventral surface of the brain. Based on the trajectories of these pathways (12–15), only data from animals bearing cuts that extended to the midline and reached the base of the brain were analyzed. Cuts of similar size and orientation, but positioned immediately rostral to the OVLT, were used as controls.

A similar unilateral transection approach was used to assess the potential role of ascending medullary catecholaminergic projections in the salt loading-induced effects on CRF mRNA. These cuts were placed in the coronal plane in the rostral medulla as described (11). Entry into the analysis was based on detection of accumulation of dopamine β-hydroxylase immunoreactivity in fibers on the proximal side of the transection and significant depletion of the dopamine β-hydroxylase innervation of the PVH and supraoptic nucleus (SO) on the ipsilateral side (11).

Cuts in the horizontal plane designed to eliminate the influences of specific lamina terminalis-associated cell groups were produced by using a similar knife construction and involved rotating the 2.0-mm knife assembly positioned

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Abbreviations: CRF, corticotropin-releasing factor; PVH, paraventricular nucleus; OVLT, organum vasculosum of the lamina terminalis; SFO, subfornical organ; MePO, median preoptic nucleus; SO, supraoptic nucleus.

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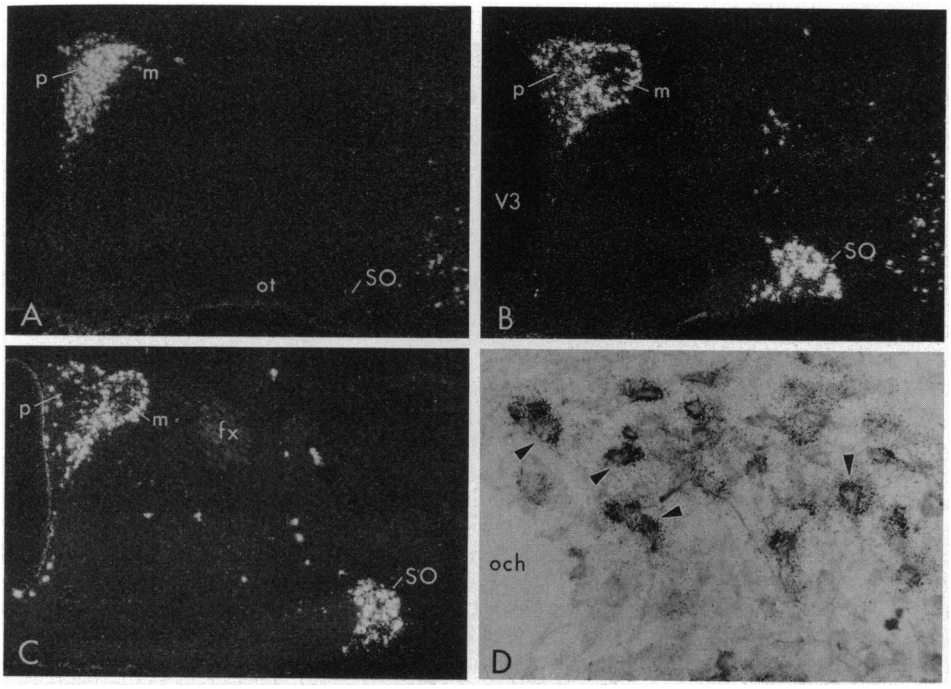

FIG. 1. Effects of salt loading on CRF mRNA in neuroendocrine neurons. Dark-field photomicrographs of hybridization histochemical localization of CRF mRNA in hypothalami of control (*A*) and salt-loaded (*B*) rats. Salt loading prompts a reduction in signal intensity in the parvocellular division (p) of the PVH and an increase in the magnocellular compartment (m), as well as in the SO, a pure magnocellular cell group. The resultant pattern of expression is similar to that of oxytocin mRNA (*C*). (*D*) Bright-field photomicrograph of the supraoptic nucleus of a salt-loaded animal in which hybridization histochemical localization of CRF mRNA is imposed on a background of immunoperoxidase staining for oxytocin. Reduced silver grains representing the CRF mRNA signal are localized principally over oxytocin-immunoreactive neurons. fx, fornix; och, optic chiasm; V3, third ventricle; ot, optic tract. (*A–C*, ×20; *D*, ×130.)

just caudal to the intended target pathway through a 180° rostrally directed arc. These are illustrated schematically in Fig. 3 and were intended to sever projections from the subfornical organ (SFO; cut 2) or the SFO and the median preoptic nucleus (MePO; cut 3) to the hypothalamus.

Excitotoxin lesions were produced by microinjection of 200 nl of 160 μM ibotenic acid (Sigma) in saline. Animals similarly injected with 200 nl of saline served as controls.

Upon recovery of preoperative body weight, animals were assigned to groups and subjected to either maintenance on 2% saline (salt loading) or tap water (controls). All rats were then anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M borate buffer at pH 9.5. Multiple regularly spaced series of 20- to 30-μm-thick frozen sections were saved. One series from each was stained with thionin for reference and/or to aid in reconstruction of lesion/knife cut placements. Histochemical processing of tissue to be compared was carried out concurrently, using common batches of antisera or radiolabeled cRNA probes.

Histochemistry. Localization of Fos protein was carried out by using an avidin–biotin immunoperoxidase protocol (16) to localize a primary antiserum raised against an N-terminal (residues 4–17) fragment of Fos (Oncogene Science). Specific staining in stimulated animals was abolished by preadsorption with the synthetic peptide immunogen at 50 μM.

Hybridization histochemical localization was carried out by using ^{35}S-labeled antisense complementary RNA probes synthesized as described elsewhere (17). The CRF probe was generated from a 1.2-kb *Eco*RI fragment of a full-length rat CRF cDNA that was subcloned into the Bluescript SK+ transcription vector (Stratagene). Labeled sense-strand transcripts failed to yield any positive signal in hypothalami of salt-loaded or control animals. The oxytocin probe was synthesized from a 190-bp *Eco*RI/*Hin*dIII digest spanning exon C and a portion of the second intron of the rat oxytocin gene, subcloned into the pSP64 transcription vector (18). Probes were labeled to specific activities of 1–3 × 10^9 dpm/μg and were applied to tissue at ≈10^7 cpm/ml.

Combined immunoperoxidase and isotopic hybridization histochemical localization of oxytocin immunoreactivity and CRF mRNA, respectively, was carried out using modifications of the constituent protocols detailed elsewhere (19).

Analysis. Densitometric data were gathered by using ICC-GRAIN software (Loats Associates, Westminster, MD) from the magnocellular and parvocellular regions of the PVH, defined using redirected sampling based on Nissl staining patterns. Values were corrected for background and read from a curve relating optical density to dpm in brain paste standards (20).

The effects of salt loading were evaluated using a two-tailed Student's *t* test. Unilateral lesion effects were analyzed by using a two-way analysis of variance, with one within-group (lesioned versus intact sides) and one between-group (salt-loaded versus tap water) variable. Bilateral lesion effects (i.e., of horizontal knife cuts and of excitotoxin lesions) were compared by using a completely randomized two-factor design. Individual pairwise comparisons were made by using Duncan's multiple range test.

RESULTS

To identify cell groups that might be involved in the transduction and/or conveyance of osmotic information to the endocrine hypothalamus, we monitored the expression of Fos, an inducible transcription factor encoded by the protooncogene c-*fos* in salt-loaded and control rats. Fos, in particular, has proven to be a sensitive and widely applicable inducible marker for neurons activated by a variety of extracellular stimuli (21). Animals (n = 7) sacrificed after 7 days of maintenance on 2% saline as a sole source of fluids displayed extensive nuclear Fos immunoreactivity in the magnocellular division of the PVH, along with the expected increase in CRF mRNA levels in the magnocellular division of the PVH and the SO to levels that were 5.1- and 9.7-fold, respectively, above the near-background expression seen in controls maintained on tap water (n = 6; $P < 0.001$; Fig. 1). Combined localization of CRF mRNA and oxytocin immunoreactivity in the magnocellular nuclei revealed these effects to be overwhelmingly localized to oxytocin neurons (Fig. 1). Salt loading also resulted in the predicted decrease in CRF mRNA levels in the parvocellular division of the nucleus to 24% of control levels ($P < 0.005$). Interestingly, this inhibitory event in the parvocellular part of the PVH was not marked by Fos induction. The other most robust sites of Fos expression that were seen consistently and uniquely in salt-loaded animals included the three contiguous cell groups

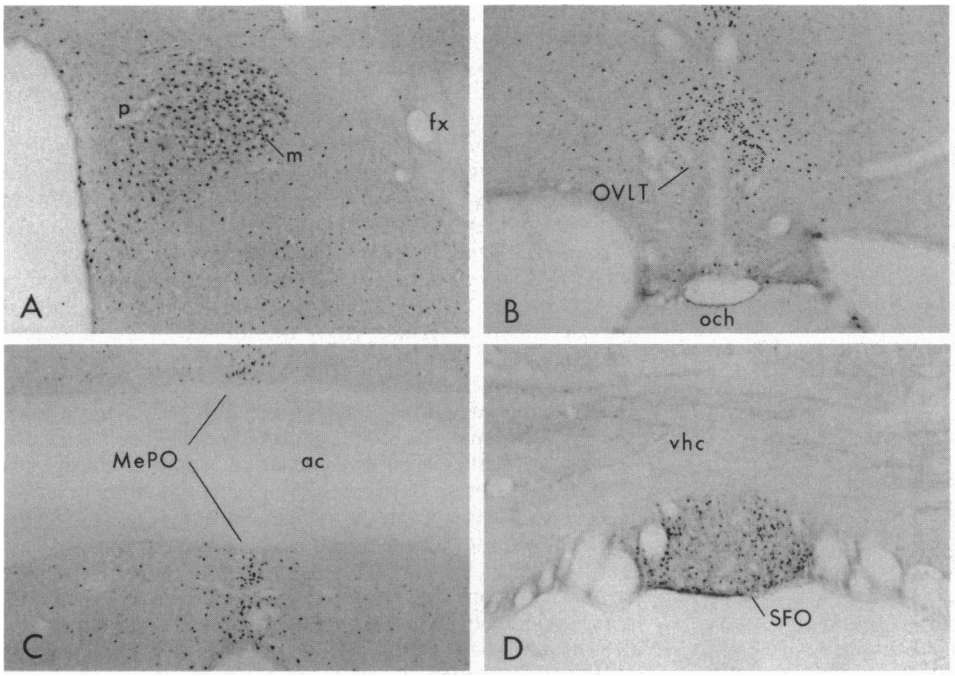

FIG. 2. Salt loading-induced Fos expression in components of the endocrine hypothalamus and lamina terminalis. Bright-field photomicrographs of immunoperoxidase staining for Fos protein in the magnocellular division (m) of the PVH (*A*) and components of the lamina terminalis, including the OVLT (*B*), MePO (*C*), and SFO (*D*), from salt-loaded animals are shown. Nuclear Fos immunoreactivity was not detected in any of these loci in controls (not shown). ac, anterior commissure; vhc, ventral hippocampal commissure; p, parvocellular division; fx, fornix; och, optic chiasm. (×50.)

lining the lamina terminalis (Fig. 2): the SFO, MePO, and OVLT. This included extensive Fos induction at the dorsal margin of the OVLT, a region whose morphological and functional similarities to the OVLT, as opposed to MePO, are uncertain. Scattered Fos-immunoreactive nuclei were also observed in the nucleus of the solitary tract, ventrolateral medulla, spinal trigeminal complex, nucleus prepositus hypoglossi, and the inferior olive, the former two of which are known to project to the PVH via largely catecholaminergic pathways (22, 23). The sites of nuclear Fos induction seen in osmotically challenged rats were similar to those reported previously (24–26), and the extrahypothalamic loci were taken as candidate afferent sources through which the effects of salt loading might be brought to bear on the endocrine hypothalamus.

To determine whether projections from lamina terminalis-associated structures and/or the medulla are in fact involved in mediating the effects of salt loading on CRF mRNA levels in parvo- and/or magnocellular neurosecretory neurons, discrete unilateral fiber transections were stereotaxically

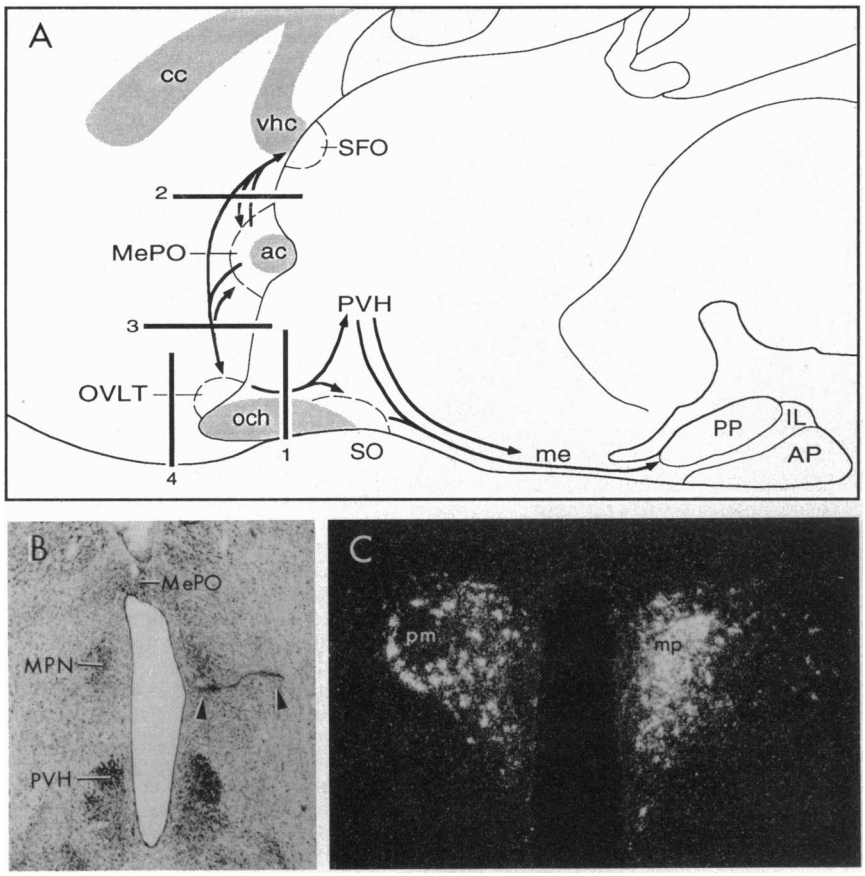

FIG. 3. (*A*) Schematic sagittal drawing to show connections among lamina terminalis structures and the endocrine hypothalamus. Numbered lines represent the position of knife cuts employed to clarify the relationships of lamina terminalis components to salt-loading effects. Cut 1 was a unilateral cut designed to eliminate all lamina terminalis influences on the PVH and SO. A horizontal section showing the location of such cuts and a frontal section illustrating their impact on CRF mRNA in the PVH are shown in *B* and *C*, respectively. (*C*) The contralateral (left) side shows elevated magnocellular (pm) and reduced parvocellular (mp) expression of CRF mRNA characteristic of salt-loaded animals; these effects are completely normalized on the side of the transection (right). Analogous unilateral cuts immediately rostral to the OVLT (cut 4) or cuts in the horizontal plane designed to eliminate influences of the SFO (cut 2) or of the SFO and MePO (cut 3) had no effect on CRF mRNA response to salt loading in either cell type (see Fig. 4). ac, anterior commissure; AP, anterior pituitary; cc, corpus callosum; IL, intermediate lobe; me, median eminence; MPN, medial preoptic nucleus; PP, posterior pituitary; vhc, ventral hippocampal commissure; och, optic chiasm. (*B*, ×10; *C*, ×35.)

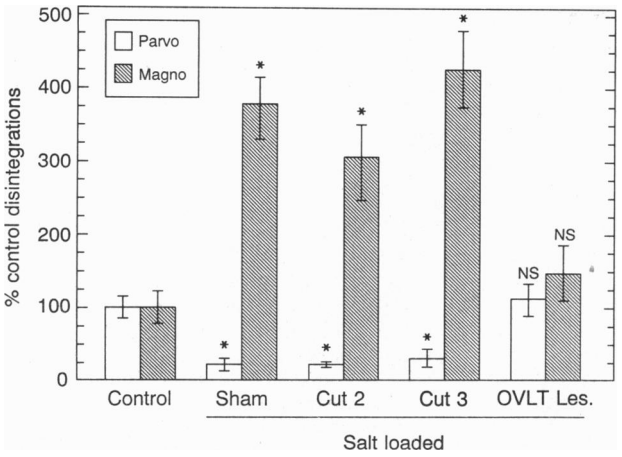

FIG. 4. Effects of lamina terminalis ablations on CRF mRNA in the parvocellular (open bars) and magnocellular (hatched bars) divisions of the PVH. Values are the mean ± SEM, expressed as a percentage of those of sham-operated (Control) animals maintained on tap water. Salt loading resulted in a reliable down- and up-regulation of CRF mRNA in the parvocellular and magnocellular compartments of the PVH in sham-operated animals (Sham). Similar effects were seen in animals bearing horizontal cuts between the SFO and MePO (Cut 2; see Fig. 3) or between MePO and the OVLT (Cut 3). Excitotoxin lesions centered in the OVLT (OVLT Les.), however, eliminated the effects of salt loading in both neuroendocrine cell types. *, $P < 0.01$; NS, $P > 0.10$, versus control group ($n = 5$–8 rats per group).

administered to rats with the intent of isolating the PVH on one side of the brain from these influences. For lamina terminalis projections, cuts were placed in the coronal plane immediately caudal to the OVLT (Fig. 3). Upon recovery of preoperative body weight (typically 1–3 days), lesioned rats were exposed to 7 days of maintenance on 2% saline or tap water. Animals with well-placed transections ($n = 5$) and exposed to salt displayed bilaterally asymmetric patterns of CRF mRNA expression (Fig. 3). On the side contralateral to the lesion, the oxytocin-like pattern expected of salt-loaded animals was evident, while on the ipsilateral side the pattern and relative levels of CRF transcripts in both the magnocellular and parvocellular divisions of the nucleus were not distinguishable from those on either side of the brains of control rats bearing similar lesions and maintained on tap water ($P > 0.10$). Salt loading-induced Fos immunoreactivity was also markedly reduced in the PVH and SO on the side ipsilateral to well-placed transections, though we did not attempt to quantify this effect.

Similarly sized cuts ($n = 5$) placed immediately rostral to the OVLT were without effect on salt loading-induced alterations in CRF mRNA levels in either the magnocellular or parvocellular systems. Also ineffective were unilateral transections of ascending catecholaminergic inputs from the medulla ($n = 5$), a potential route by which osmotic inputs from the hepatic and/or splanchnic circulations might reach the hypothalamus (27, 28). Collectively, these data implicate lamina terminalis outputs as mediating osmotic influences on CRF expression in both neuroendocrine venues.

To evaluate the possibility of a more precise localization of function among lamina terminalis-associated structures, a series of surgical maneuvers were carried out to isolate any critical component(s). Knife cuts placed in the horizontal plane, which severed the so-called ventral stalk of the SFO ($n = 6$), or horizontal cuts placed between the OVLT and the MePO ($n = 8$; Figs. 3 and 4) left the relative levels of CRF mRNA in the magnocellular and parvocellular divisions of the PVH seen in response to salt loading indistinguishable from those of sham-operated controls ($P > 0.10$). By contrast, lesions produced by microinjection of ibotenic acid centered in the OVLT ($n = 6$) yielded patterns and relative levels of CRF expression in both neuroendocrine cell types of salt-loaded animals that did not differ significantly from those seen in sham-operated controls maintained on tap water ($P > 0.10$; Figs. 4 and 5). Similar lesions in animals maintained on tap water ($n = 5$) did not significantly affect CRF mRNA levels in either the magnocellular or the parvocellular systems ($P > 0.10$ versus five sham-operated animals fed tap water). In addition to extensive (>80%) damage of the OVLT proper, tissue consistently compromised in the lesioned groups included variable amounts of the most ventral

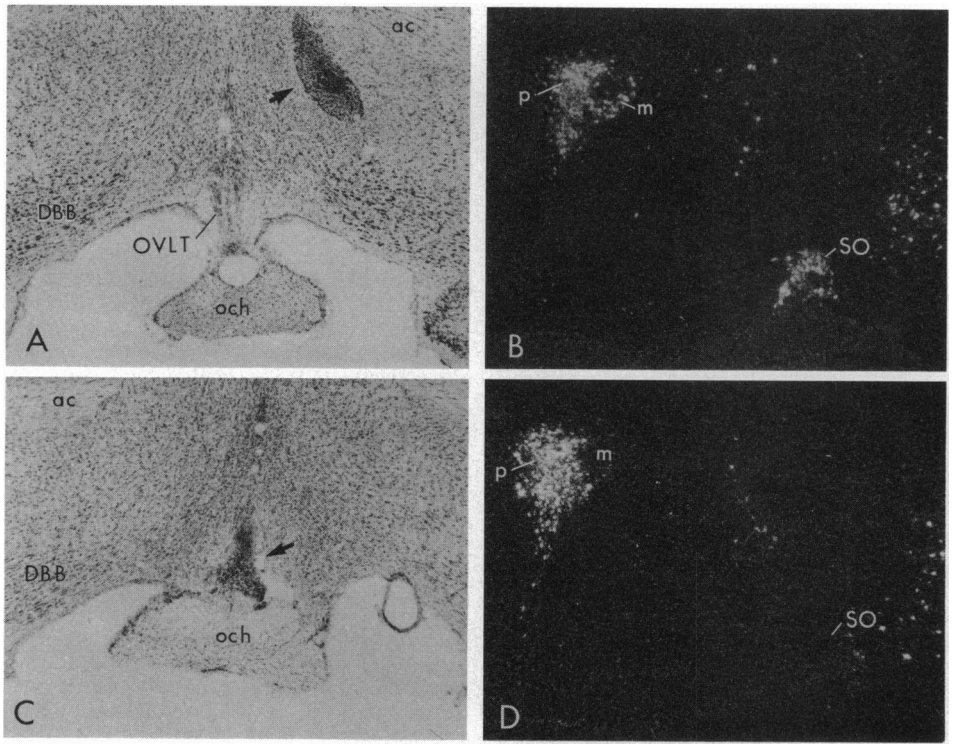

FIG. 5. Excitotoxin lesions of the ventral lamina terminalis abolish the effects of salt loading on CRF mRNA in magno- and parvocellular neurosecretory neurons. Bright-field photomicrographs of ibotenic acid lesions that spared (*A*) or were centered in (*C*) the OVLT are shown. Corresponding dark-field photomicrographs of the resulting distribution and strength of the hypothalamic CRF mRNA signal that were seen in these salt-loaded animals are shown in *B* and *D*, respectively. The animal bearing the misplaced lesion displays characteristic salt loading-induced patterns of CRF expression in both the magnocellular and parvocellular neurosecretory systems. The lesion depicted in *C* was the smallest of those that normalized both responses; typically, these spread to involve adjoining portions of the preoptic region, especially the most ventral aspects of MePO, to varying extents. DBB, nucleus of the diagonal band; och, optic chiasm; ac, anterior commissure; p, parvocellular division; m, magnocellular compartments. (*A* and *C*, ×35; *B* and *D*, ×20.)

extensions of MePO, and periventricular tissue surrounding the anterior third ventricle. The latter, at least, did not appear to be a determinant of the lesion effects, as ibotenic acid injections directly into the third ventricle, which resulted in more extensive damage to the periventricular region, consistently failed to qualitatively alter the distinctive pattern of CRF mRNA expression induced by salt loading.

DISCUSSION

The results suggest that the integrity of the ventral portion of the lamina terminalis is both necessary and sufficient to support the effects of chronic hyperosmolality on CRF mRNA levels in the magnocellular and parvocellular neurosecretory systems. Each of the components of the lamina terminalis has been demonstrated to project to the PVH and SO, and to one another (12–15). Portions of the SFO and OVLT lack a blood–brain barrier to circulating macromolecules, and this, coupled with their expansive capillary surface area and perivascular spaces, makes them well-equipped to sample the ionic milieu as well (29). Moreover, individual neurons in the OVLT region can be depolarized in response to hyperosmotic solutions, and lesions of this area disrupt osmotically driven responses of the magnocellular neurosecretory system (30). On the basis of these findings and the limited extent of salt loading-induced Fos expression in the brain, it seems likely that cells in and/or immediately proximal to the OVLT serve both to monitor the osmotic composition of the blood and transmit this information to at least two compartments of the endocrine hypothalamus. We cannot, however, formally exclude the possibility that the OVLT region may merely serve as an obligate relay for sensors that either do not manifest a capacity for Fos induction or whose ability to do so might adapt during prolonged salt loading. While our excitotoxin lesion data suggest the OVLT to be the critical focus mediating the effects of salt loading on hypothalamic CRF expression, we cannot rule out the possibility that immediately contiguous aspects of the preoptic region may also be involved.

How an ostensibly common afferent source might serve to effect changes in CRF mRNA expression that are opposite in sign in distinct neuroendocrine cell types is not clear. Signal transduction mechanisms unique to magno- and parvocellular neurons that respond differentially to a common afferent represent one possible explanation. Alternatively, differences in the complement and/or sensitivity to neurotransmitters resident within ventral lamina terminalis neurons may be involved. Electrophysiological studies have provided evidence for both excitatory (glutamatergic) and inhibitory (GABAergic) components to the response of identified neuroendocrine neurons to stimulation of the OVLT (31, 32). In support of the latter, we have noted extensive expression of mRNAs encoding the γ-aminobutyric acid synthetic enzyme, glutamate decarboxylase, in lamina terminalis cells identified as projecting to the PVH (33). It remains to be determined how excitatory and inhibitory transmitter mechanisms within lamina terminalis projections to the hypothalamus may be involved in the differential effects of salt loading on CRF expression in the magno- and parvocellular neurosecretory systems.

Our observations and approach hold relevance to the long-standing search for the central osmoreceptors driving the expression and secretion of the neurohypophyseal hormones oxytocin and, particularly, vasopressin. The seminal studies of Jewell and Verney (34) localized these to the anterior hypothalamus, and a substantial body of evidence has since implicated aspects of the lamina terminalis and the anteroventral third ventricular region as participating in the osmotic control of vasopressin secretion (15, 35). Although the dependence on the OVLT region of osmotically mediated alterations in CRF mRNA expression in the magnocellular nuclei need not necessarily generalize to vasopressin, it is worthy of note that the expression and secretion of both oxytocin and vasopressin are enhanced by salt loading (36, 37). At least, the list of candidate substrates revealed by patterns of Fos induction should be similar.

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