Fos-like immunoreactivity in the brain stem following oral quinine stimulation in decerebrate rats

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Travers, Joseph B., Kevin Urbanek, and Harvey J. Grill. Fos-like immunoreactivity in the brain stem following oral quinine stimulation in decerebrate rats. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R384–R394, 1999.—The present study compared the distribution of Fos-like immunoreactivity (FLI) following intraoral stimulation with quinine monohydrochloride (QHCl) in awake intact rats to the pattern obtained in chronic supracollicular decerebrate (CD) rats. Because the behavioral rejection response to QHCl is evident in the CD rat, it was hypothesized that the pattern of FLI in the lower brain stem should be similar in both groups. Overall, the distribution of FLI in the brain stem was quite similar in both intact and CD groups, and QHCl stimulation increased FLI in the rostral (gustatory) nucleus of the solitary tract, the parabrachial nucleus (PBN), and the lateral reticular formation (RF) compared with an unstimulated control group. The CD group differed from the intact group, however, with a trend toward less FLI in the RF and a shift in the pattern of label away from the external subdivision of the PBN. CD rats also had increased FLI in the caudal nucleus of the solitary tract, with or without intraoral infusions. The distribution of QHCl-induced FLI in the brain stem of intact rats thus indicates both local sensorimotor processing as well as the influence of forebrain structures.

Lesion-behavioral studies have established that the lower brain stem contains the circuitry essential to produce the rejection response to aversive gustatory stimuli (9). Neuroanatomic studies using conventional anterograde and retrograde tracers (1, 35, 40), as well as studies using the transsynaptic transport of virus (6, 39), all indicate that the sensorimotor components of the oral rejection response are mediated through multisynaptic connections. One likely pathway includes projections from the rostral (taste) nucleus of the solitary tract (rNST) to the subjacent lateral parvocellular (PCRt) and intermediate (IRt) zones of the medullary reticular formation (RF). These regions of the medullary RF also receive projections from gustatory responsive sites in the “waist” area of the parabrachial nucleus (PBN) (21). Both the PCRt and IRt project to the oral motor nuclei (i.e., hypoglossal, motor trigeminal, facial nuclei), and numerous studies in the rat implicate these regions as fundamental to the generation of rhythmic oromotor behavior (reviewed in Refs. 26 and 37, see also Ref. 35).

Functional neuroanatomic studies using the expression of the immediate-early gene c-fos conform with the view that specific regions of the medulla are involved with the production of the rejection response. Thus awake rats given infusions of quinine monohydrochloride (QHCl) display stereotypic rejection behavior (9) and show enhanced Fos-like immunoreactivity (FLI) in the rNST, in areas from which physiological responses to gustatory stimulation can be obtained (13). After stimulation with QHCl, rats also show a specific pattern of FLI in the medullary RF (5), again in areas with physiological activity correlated with the oral rejection response (20). In the PBN, stimulation with QHCl increases FLI label, most notably in the external region, including the external lateral and external medial subnucleus (45).

These regions of the rNST, RF, and PBN all constitute probable substrates in the production of the rejection response to gustatory stimuli. If the pattern of FLI seen in these regions reflects local brain stem pathways mediating the oral rejection response to QHCl, then similar patterns ought to be evident in chronic decerebrate (CD) rats. Alternatively, increased FLI in the brain stem in response to QHCl could reflect the influence of descending projections (29, 33, 46). The present report demonstrates that overall the distribution of FLI in the rNST, RF, and PBN were quite similar in both intact and CD groups. Differences, however, were also evident and intact and CD preparations varied in the pattern or density of FLI in the mediolateral subdivision of the caudal nucleus of the solitary tract (cNST), the external subdivision of the PBN and the PCRt.

METHODS

Surgical procedures. Adult, male Sprague-Dawley rats (n = 14) weighing between 325 and 375 g were used in these experiments. Under anesthesia (9 mg/kg ketamine and 1.5 mg/kg xylazine), all rats were implanted with intraoral cannulas (9). Eight rats were decerebrated at the supracollicular level using a two-stage procedure described by Grill and Norgren (9). Six other rats were anesthetized (as previously described), fitted with intraoral cannulas, and served as intact controls. These procedures were conducted in the laboratory at the University of Pennsylvania (Grill) and conformed with University Animal Care and Use standards.

Testing procedures. After surgery, CD and control rats were maintained on an identical feeding regimen before testing. Both groups were fed a liquid diet by gavage three times a day. Each gavage consisted of 50% water and sweetened condensed milk (Borden Eagle Brand) and supplemented with Polyvisol vitamins and iron. The interval between gavages was at least 2 h, and rats were fed between 9 AM and 6 PM. Rats were maintained on a normal light-dark cycle.
Before testing, rats were adapted to a test chamber and the infusion of water through the intraoral cannula. At a minimum of 4 wk after surgery, four CD rats and three intact rats received stimulation with 7.2 ml of 0.003 M QHCl over a 29-min period. On the day of testing, rats received an early morning meal (~9 AM) and were tested 3 h later. The 7.2 ml of QHCl were delivered in 144 discrete infusions, each infusion delivered 50 µl over 2 s and 10 s separated each infusion. The other seven animals served as unstimulated controls. Rats were videotaped during the test session for subsequent analysis of gape responses. Collars on the animals prevented other behaviors, e.g., face washing, from being scored.

Animals were perfused through the heart 30 min after the period of stimulation or (control) no stimulation following the protocol developed by Hoffman et al. (17). Rats were deeply anesthetized with Nembutal (150 mg/kg) and perfused transcardially with 250–400 ml of 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 5–10 min followed by 500–600 ml of a 4% paraformaldehyde solution (pH 6.8) for 0.5–1 h. The brain was removed and postfixed in a 20% sucrose paraformaldehyde solution overnight. Brains were blocked into a rostral and caudal portion. The caudal portion, ending caudal 2–3 mm rostral to the transection, was sectioned sagittally and stained with cresyl violet to evaluate the completeness of the transection.

Immunohistochemistry. The caudal brain stem was sectioned at 40 µm into four parallel series for Fos immunohistochemistry following procedures described in detail by Di-Nardo and Travers (5). Briefly, all tissue was incubated in 10% sheep serum in PBS for 1 h at room temperature. After the tissue was rinsed in PBS for 30 min, it was incubated in rabbit polyclonal antibody. Most of the brains were processed using antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) diluted to a concentration of 1:6,000 in PBS containing 0.4% Triton X-100 for ~66 h at 4°C. The remaining brains were treated with antibody from Oncogene at a dilution of 1:24,000. With use of tissue from the same brain, this dilution was chosen to empirically match the level of staining from the two antibodies. Several sections were separated prior to the primary antibody incubation and placed only into the PBS-0.4% Triton X-100 solution to serve as a negative control. No FLI was observed in the negative control tissue. After primary antibody incubation, the sections were rinsed in PBS for 30 min. The tissue was then incubated in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted to 1:600 in PBS containing 0.1% bovine serum albumin (BSA) for 1 h. After the tissue was rinsed in phosphate buffer (PB) for 15 min, it was incubated in a PB solution containing 0.05% 3,3′-diaminobenzidine-HCl (0.5 mg/ml) and 0.02% nickel ammonium sulfate for 15 min. At this time, 10 µl of 30% H2O2 were added to the solution for 3–5 min. The tissue went through final rinses in 0.1 M and 0.05 M PB. After mounting onto gelatin-coated slides, sections were air dried overnight, dehydrated through ascending concentrations of alcohols, cleared with Hemo-De, and coverslipped with Permount.

Fig. 1. Distribution of Fos-like immunoreactivity (FLI) at 2 levels of medulla from 4 preparations. A and B: intact and stimulated with quinine monohydrochloride (QHCl). C and D: chronic decerebrate (CD) and stimulated with QHCl. E and F: intact with no stimulation. G and H: CD with no stimulation. Each dot represents one positively stained neuron. Arrow in G points to prepositus hypoglossal nucleus. rNST, rostral nucleus of solitary tract; sV, spinal trigeminal nucleus; PCRt, parvocellular reticular formation; IRT, intermediate reticular formation; NST, nucleus of solitary tract; MdD, dorsal subdivision of medullary reticular formation; MdV, ventral subdivision of medullary reticular formation; GI, nucleus gigantocellularis; mXII, hypoglossal nucleus.
Tissue from “positive control” animals was reacted in tandem with tissue from each rat to monitor the quality of the immunohistochemical reactions. Some positive control tissue came from anesthetized preparations (Nembutal, 50 mg/kg ip) in which the chemical irritant capsaicin (0.001 M) was injected ipsilaterally into either the oral cavity or swabbed over the cornea. Other positive controls came from awake animals injected intraperitoneally with the endotoxin lipopolysaccharide (40 µg/0.2 ml). Forty-five minutes later, these rats were perfused using the same procedure as previously described. These manipulations produce reliable FLI in the brain stem (5, 13).

Data analysis. FLI was quantified at two levels of the nucleus of the solitary tract (NST) and medullary RF and two levels of the PBN. Representative levels in the medulla were chosen based on previous studies in intact preparations (5, 13). Thus a rostral medullary level just caudal to the caudalmost dorsal cochlear nucleus corresponds to “level 4” as described by DiNardo and Travers (5) and the “mid” level as described by Harrer and Travers (13). The caudal medullary level, just rostral to the area postrema corresponded to level 1 in the study by DiNardo and Travers (5). The rostral level in the PBN was at a level that included the external medial subnucleus; the more caudal level was through the classical waist region and included the central medial and ventrolateral subnuclei. The criterion for a labeled neuron was a uniform brown or brown-to-black reaction product within the cell nucleus. Sections were drawn using a computer-aided camera lucida (Microbrightfield), and nuclei showing FLI were plotted at ×400. Most of the statistical analyses used either a two- or three-way ANOVA with condition (CD vs. intact group) and stimulus (QHCl vs. no stimulation) serving as between animal factors and different anatomic structures serving as within animal factors.

RESULTS

The transection was judged to be complete in all cases. The distribution of FLI at two medullary levels for representative CD and intact cases after QHCl or no stimulation are depicted in Fig. 1. Stimulation with QHCl increased FLI in the NST and medullary RF in both groups. The increased FLI in the rostral (gustatory) NST following stimulation with QHCl in a CD case is contrasted with the absence of such label in a nonstimulated CD case in the photomicrographs of Fig. 2, A and C. A quantification of the amount of FLI in each of four rNST subdivisions across the four conditions is shown in Fig. 3. After stimulation with QHCl,
most of the FLI appeared in the central subdivision for both the CD (Fig. 3A) and intact (Fig. 3B) groups. This was verified with a three-way ANOVA that revealed significant stimulus (P < 0.001), subdivision (P < 0.001), and stimulus times subdivision interactions (P < 0.001) but with no difference between CD and intact conditions. The central and ventral subdivisions in the CD group had significantly more FLI following QHCl stimulation than the unstimulated group using (unadjusted) t-tests (central P = 0.002, ventral P = 0.003). For the intact group, the central subdivision had significantly more FLI after QHCl stimulation (P = 0.004) but the ventral subdivision was only marginally different (P = 0.085).

In the CD group there was more FLI in the cNST regardless of stimulus condition. Although the case depicted in Fig. 1 suggests that stimulation with QHCl in intact animals may have increased FLI in the cNST, this was not consistent across animals (Fig. 4). A two-way ANOVA for the total amount of FLI in the NST at this level showed no stimulus effect but a significant difference between CD and intact animals (P < 0.04). The interaction between stimulus and group was not significant. The high levels of FLI in the cNST of the CD regardless of stimulus condition (Fig. 1, D and H) are also evident in the photomicrographs in Fig. 2, B and D.

Increased FLI in the PCRt and IRt divisions of the medullary RF following QHCl stimulation are quanti-
Fig. 4. Mean number of FLI neurons in caudal NST (level just rostral to area postrema) in both CD and intact animals in response to QHCl or NS. SE is shown as well as responses from each individual case.

The distribution of FLI in the rostral PBN following QHCl stimulation for an intact and CD case are illustrated in the photomicrographs of Fig. 6. Quantification of FLI at the rostral level, as well as a more caudal level of the PBN are shown in Figs. 7 and 8, respectively. In the rostral PBN (Fig. 7), a two-way ANOVA showed a stimulus effect (P < 0.018) but no difference between CD and intact animals or no interaction. Nevertheless, the pattern of FLI changed as a result of the decerebration. This was determined by calculating the percentage of the total FLI in the rostral PBN accounted for by FLI in the external subnucleus (external medial plus external lateral). In the intact group nearly 60% of the total FLI was in the external region after QHCl stimulation compared with ~12% in the unstimulated condition (Fig 7B). In contrast, for the CD group, both stimulated and unstimulated rats had only 35% of the total PBN FLI in the external medial subnucleus. The significance of these differences between CD and intact rats is supported by a two-way ANOVA that showed a significant stimulus condition (P = 0.022) and a significant stimulus times group condition (P = 0.021). QHCl stimulation also increased FLI in the caudal PBN in the gustatory waist region, composed of the central medial and ventrolateral subdivisions (Fig. 8). This effect was quite variable, and a three-way ANOVA revealed only a weak stimulus effect (P = 0.07) with no difference between intact and CD animals or between the two subdivisions.

Although the focus of this study was on FLI in those structures thought to be involved in the oral rejection response pathway, FLI in several other structures was prominent in the CD group regardless of stimulus condition. FLI, for example, was seen in the prepositus nucleus in seven of eight CD cases (e.g., Figs. 1, D, and 2, B) but in none of the intact cases. Likewise, robust FLI appeared in the nucleus raphe obscurus in all four of the nonstimulated CD rats compared with one (weak) case of label in a CD QHCl case. Weak label was also evident in raphe obscurus in one intact QHCl case. In addition, more FLI was evident along the border of the inferior olive in CD cases compared with intact cases.

**DISCUSSION**

FLI in NST. The results of the present study reinforce the view that specific regions of the lower brain stem mediate the oral rejection response to gustatory stimuli. By and large the distribution of FLI in response to QHCl stimulation in CD rats was remarkably similar to that obtained from the intact rats. Thus, in both groups, stimulation with QHCl produced a distinct cluster of FLI in the central subdivision of the rNST, a pattern that compared favorably to a previous report in intact rats (13; replotted in Fig. 3). Overall, the distribution of label between the two studies is similar with the exception of relatively more label in both the stimulated and unstimulated ventral subdivision in the previous report. This increase may reflect methodological differences between the two studies. Hanner and Travers (13) used acrolein in the perfusate and adapted the animal for shorter periods to the observation chamber prior to testing. Both of these procedures may increase FLI. Acrolein in the perfusate increases FLI detection (17) and more recent studies by H. Hu and S. Travers report less label in the ventral subdivision of unstimulated controls adapted for longer periods (personal communication). Thus, despite the fact that single rNST neurons appeared less responsive to taste stimuli (30–64%) in both acute (14, 41) and CD (24) preparations, this decrease is apparently not sufficient to diminish the pattern or absolute number of rNST cells expressing FLI in response to QHCl.

The distinct clustering of FLI cells in the rNST in response to QHCl stimulation in intact as well as CD rats is consistent with the view of a limited chemotopy...
within the rNST (13). In this study, QHCl stimulation produced FLI concentrated in the medial part of the central subdivision of the rNST, in contrast to sucrose stimulation that increased FLI more evenly distributed across the central subdivision (RC). Likewise, stimulation with water increased FLI in the rNST slightly but not in the pattern produced by QHCl. As argued in this report, there are tactile sensitive neurons in the rNST that could be responding to the tactile components of fluid stimulation; however, these neurons tend to be located in the lateral subdivision of rNST, a region not heavily invested with FLI following intraoral QHCl (or other fluid) stimulation.

Thus the tactile component of QHCl stimulation is an unlikely explanation for FLI in the rNST, a view further supported by a recent paper describing the effects of glossopharyngeal nerve transection on QHCl-evoked FLI (22). Removal of the glossopharyngeal nerve, which dramatically attenuates the gape response compared with chorda tympani nerve cuts (10, 38), had a correspondingly greater effect on the magnitude and pattern of QHCl-elicited FLI in RC of rNST compared with cutting the chorda tympani nerve (22). Thus cutting the nerve that carries the predominant QHCl sensitivity reduces FLI within the rNST subdivision in which the nerve terminates. In contrast to RC, which is the site of most gustatory nerve input, the ventral subdivision of the rNST is the primary NST source of output to the brain stem RF (12). Thus the significant increase of FLI in the ventral subdivision of
QHCl-stimulated rats may reflect output from the rNST to the medullary RF, a region implicated in orchestrating the sensorimotor response of gustatory-induced oromotor responses (discussed in Refs. 7 and 37).

FLI in RF. Because the PCPr and IRt receive input from the rNST and are major sources of premotor neurons to the oral motor nuclei (18, 35, 40), these RF regions may play a major role in coordinating the orosensory-motor responses of both ingestion (licking and chewing) and rejection responses to gustatory stimuli (reviewed in Ref. 37). In the present study, QHCl stimulation increased FLI in the (combined) PCPr and IRt of CD rats, an increase that may reflect activation of interneurons involved in the production of oromotor responses. Despite this increase, the (total) count of FLI in the PCPr and IRt subdivisions following QHCl stimulation was 37% less in the CD group compared with the intact QHCl-stimulated group. This (albeit nonstatistically significant) decrease in FLI in the RF of the CD group was more pronounced compared with the (intact) QHCl-stimulated animals from a previous study (5), which is replotted in Fig. 5. Compared with this study, FLI decreased by 64% in these (combined) RF regions. In sum, there was a trend toward lower levels of FLI in the RF of CD rats.
following QHCl stimulation. These lower levels, may reflect the absence of descending input in the CD. Projections to the medullary RF come from numerous forebrain sources associated with ingestion or oromotor function (reviewed in Refs. 26 and 37). The decrease in FLI in the RF in the CD group, however, was not the result of fewer gape responses. Despite the 37% decrease in FLI in the PCRT and IRt following QHCl stimulation in the CD compared with the intact group, the mean number of gapes/s for the CD group (0.418, range 0.24–0.623) was quite similar to the intact (0.427, range 0.31–1.169).

In contrast to rats receiving QHCl stimulation, rats receiving no stimulation remained awake but generally passive in the test chamber, with one important exception. Specifically, one of the CD nonstimulated animals was not included in the (statistical) analysis of FLI in the RF. This animal showed abnormal baseline behavior with nearly continuous mouth movements throughout the test session, despite the absence of oral infusions. As can be seen in Fig. 9, for this rat there was FLI...
in the medullary RF, but very little in the rNST. We interpret this pattern as reflecting activation of a brain stem substrate involved in producing rhythmic oral movements. In this case, the initiating stimulus for oral activity is unknown but does not involve overt gustatory activation and consequent increased FLI activity in the rNST. Additional evidence for a role of the lateral medullary RF in oromotor behavior is the similarity in the distribution of FLI following stimulation with QHCl with patterns produced by other aversive stimulus procedures. Similar patterns of FLI in the RF are produced by vomiting or fictive vomiting in decerebrate cats (25) or in awake rats that received the oral stimulus capsaicin (4). In anesthetized rats that received capsaicin applied to the tongue, relatively little label was observed in this region of the RF despite extensive labeling in the nucleus caudalis (2). Thus an active oromotor system may be a prerequisite for observing FLI in the medullary RF.

FLI in PBN. The dramatic increase in FLI in the external region of the intact preparation following QHCl stimulation (45) was not as obvious in the CD. Like the waist region, the external region receives afferent input from the rNST (15) and responds to gustatory stimulation (11). Unlike the waist region, however, recent work in our laboratory has shown that the external region does not project to the PCRt (21) and thus is not likely to play a direct role in brain stem-mediated responses. The function of the external region in the intact animal may depend on forebrain influences. For example, increases in FLI are evident in the external region following a conditioned taste aversion (45), a response contingent on an intact forebrain (8). Significant FLI was also observed in the waist area following QHCl stimulation in both decerebrate and intact preparations. A descending projection from the waist area to the PCRt implicates this region in local brain stem consummatory function (15, 21, 23, 30). The stimulation-induced pattern of FLI in the PBN of CD rats further suggests that transecting the ascending axons of waist area neurons is not sufficient for retrograde degeneration of these cells.

Release of inhibition after decerebration. Despite the similar patterns of QHCl-induced FLI in the CD and intact rat, several differences were also apparent. In particular, there was clearly an increase in FLI in the CNST of CD rats compared with the intact group regardless of stimulus condition. This increase was primarily in the medial subnucleus of the NST (mNST) at the level of the rostral area postrema and extending just rostral to it. This region of the NST receives input from the vagus nerve (27) and is implicated in visceral, particularly gastric function. For example, increased FLI in this area results from a conditioned taste aversion to sucrose (19), following gastric stimulation including distension (36, 44) and stimulation with cholecystokinin (28). Similarly, stimulation with sucrose and its attendant postigestional effects increases FLI in mNST but stimulation with sodium saccharin does not (13).

Increased FLI in the caudal mNST in the absence of specific oral or gastric stimulation in CD rats suggests
the removal of inhibitory input. Forebrain inputs to this region include projections from the cortex, the central nucleus of the amygdala (CNA), the bed nucleus of the stria terminalis (BST), and the paraventricular nucleus (PVN) (33, 42). Of particular interest is the laterality of these projections, i.e., projections from the cortex and PVN appear bilateral, compared with primarily ipsilateral projections from the CNA and BST (42). Because increased FLI in the caudal mNST after a conditioned taste aversion is absent following either a hemidecerebration (32) or a lesion of the ipsilateral CNA (31), the increased FLI in the caudal mNST seen in the present study with complete decerebrations favors either a cortical or paraventricular site as the source of the inhibitory input. That is, removal of ipsilateral descending projections from the CNA decreased FLI in the mNST, whereas bilateral decerebration increased FLI in mNST. Likewise, projections from the (bilateral) BST appear primarily excitatory (16) and are thus not a likely origin for the descending inhibition. Evidence for a cortical origin of descending inhibitory input to the NST that might be “released” following a bilateral decerebra- tion comes from studies directed at more rostrally located, gustatory-responsive cells in the NST. Either anesthetizing the cortex (3) or stimulating it (34) can produce inhibitory responses. Similarly, the bilateral projection from the PVN could also be a source of inhibitory input to the NST (discussed in Ref. 43).

In conclusion, a lower brain stem substrate mediating the rejection response to QHCl involves topographically segregated neurons in the rostral, gustatory responsive nucleus of the solitary tract, neurons in the caudal mNST, and neurons in the paraventricular and intermediate zones of the medullary RF. Increased levels of FLI in both intact and decerebrate preparations in each of these regions following intraoral stimulation with QHCl are indicative of local sensorimotor circuitry. In the CD rat, decreases in the magnitude of FLI in the RF and an altered pattern of label in the PBN suggest the absence of descending input to the brain stem circuitry.

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