Muscimol infusions in the brain stem reticular formation reversibly block ingestion in the awake rat

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Received 17 July 2000; accepted in final form 4 December 2000

Chen, Zhixiong, Susan P. Travers, and Joseph B. Travers. Muscimol infusions in the brain stem reticular formation reversibly block ingestion in the awake rat. Am J Physiol Regulatory Integrative Comp Physiol 280: R1085–R1094, 2001.—Previous studies have localized a central pattern generator for mastication to the midline pontomedullary reticular formation (RF) based on cortically induced ororhythmic movements. The present study determined whether this same substrate mediated licking responses evoked by more natural stimuli. Licking in the awake rat was initiated either through an appetitive response to sucrose presented in a bottle or by intraoral (IO) infusions. Oral rejection responses also were obtained by IO infusions of quinine hydrochloride. Small volumes of the GABA<sub>A</sub> agonist muscimol bilaterally infused into the lateral medullary RF significantly reduced licking and oral rejection responses measured electromyographically from the anterior digastric and geniohyoid muscles. Other than the decrement or absence of ororhythmic activity, rats appeared normal and actively approached and probed the water bottle. The suppression was reversible and returned to baseline within 3 h. In contrast, midline infusions of muscimol did not affect licking or rejection responses. We postulate that the lateral medullary RF is an essential final common path for ingestive consummatory responses.

EXPERIMENTS WITH DECEREBRATE preparations conclusively demonstrate that the caudal brain stem contains a substrate sufficient to produce the consummatory responses of ingestion (licking and mastication), as well as the oral component of the rejection response elicited by unpalatable substances (13, 31). The location of this substrate is generally thought to include the brain stem reticular formation (RF); however, the precise site remains somewhat unclear.

Studies employing electrical stimulation of the orbital (masticatory) cortex combined with knife cuts in the lower brain stem suggest an essential role for nucleus gigantocellularis and paragigantocellularis (6, 34), reviewed by Nakamura and Katakura (33) and Travers et al. (44). Neurons that are premotor to the oromotor nuclei, however, are concentrated more laterally in the intermediate and to a lesser extent the parvocellular RF, suggesting the necessity of more lateral regions of the RF for the production of coordinated oromotor behavior (6, 18, 47). In addition, these lateral regions are the recipients of efferent projections from brain stem sensory nuclei and thus appear to be part of the circuitry through which gustatory and orotactile inputs pass to influence oromotor responses (2, 16, 22). The lateral RF is also the target of descending input from forebrain structures related to feeding (26, 32, 37, 49, 51). In addition to anatomic studies, chronic unit recording confirms the presence of orosensory and ororhythmic responsive neurons in the lateral and intermediate subdivisions of the RF (17, 41, 45).

Despite the wealth of anatomic, physiological, and lesion data, direct evidence for the necessity of any particular RF structure in producing oromotor behavior in awake preparations is lacking. Moreover, most lesion studies, aimed at identifying critical substrates underlying ororhythmic function, have not only used anesthetized preparations but have focused on mastication, with relatively little attention directed to lingual movement. Consummatory responses usually require the coordinated movement of both tongue and jaws, as well as facial musculature (43). It seems likely, although as yet unproven, that coordination of this diversely innervated oral musculature employs a common brain stem substrate (21).

In a previous study, we demonstrated that microinfusions of lidocaine into the RF reversibly suppressed licking and gaping (rejection) in the awake, freely moving rat (8). Because lidocaine blocks conductance of both cell soma and axons, this suppression could not unambiguously be attributed to loss of cell function at the site of infusion (25). Thus the present study used microinfusions of the γ-amino butyric acid<sub>A</sub> (GABA<sub>A</sub>) agonist muscimol. GABA<sub>A</sub> receptors are located on or near cell bodies, and the effect of muscimol is thus not likely to be attributable to fibers of passage. GABA<sub>A</sub> receptors are nearly ubiquitous throughout the brain, and muscimol has been used extensively in chronic preparations to produce temporary reversible lesions (25, 30). In the present study, ingestion and rejection behavior was quantified from electromyographic activity from subsets of tongue and jaw muscles in awake, freely moving rats in response to the intraoral (IO)
METHODS

Surgical preparation. Adult male Sprague-Dawley rats (270–450 g) were maintained on a normal 12:12-h light-dark cycle and trained to lick from a bottle containing 0.3 M sucrose for 3–4 days. After training, rats were anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg ip). Supplemental doses of Nembutal (5 mg) were given to maintain a surgical level of anesthesia characterized by hindlimb areflexia. Body temperature was maintained throughout the surgery at 37°C with a body warmer. Animals were fitted with two IO cannulas to allow delivery of taste solutions into the oral cavity (12). The cannulas were inserted into oral mucosa and exited through an incision on the skull. Bipolar electromyogram (EMG) electrodes, consisting of a pair of twisted fine wires (67 μm NiCr) insulated except for 0.5 mm at the tip, were implanted via a 26-gauge needle into anterior digastric (AD) and geniohyoid (Gen) muscles (48). Leads from the EMG electrodes were guided subcutaneously to the top of the head and attached into an Amphenol connector (46).

After the IO cannulas and EMG electrodes were implanted, the head of the rat was fixed in a conventional stereotaxic instrument equipped with blunt ear bars, and the skull was made horizontal with respect to bregma and lambda. After removal of a small portion of bone 4 mm posterior to lambda, the dura was cut and removed. A 26-gauge stainless steel guide cannula (24 mm) was positioned after the anterior pole of the nucleus of the solitary tract (NST) was located and identified on the basis of recording electrophysiological responses to gustatory stimulation of the anterior tongue using a tungsten electrode (15). After this landmark was located, the guide cannula was positioned over various locations along the rostrocaudal extent of the medulla. An additional set of targets included the NST (n = 4). In many instances, recording from a fine-wire extending through the guide cannula (subsequently removed) ensured accurate placement because various landmarks could be discerned, e.g., fourth ventricle, brain stem surface, NST, etc. We found that the added effort of electrophysiological guidance usually led to more accurate placement than relying on stereotaxic coordinates alone; however, all locations were subsequently verified histologically. In three instances, the guide cannulas were implanted into the RF at an angle (either across the midline or posteriorly directed) to avoid the overlying NST.

The guide cannulas, as well as the IO cannulas and Amphenol strip connector, were secured to the skull with dental acrylic. A 33-gauge stainless steel tube was used as a stylet (obturator). The incision was closed with suture, and rats were given penicillin-G procaine (30,000 U im daily) for 3–4 days postoperatively. During the recovery period rats were fed a mixture of powdered rat chow and Crisco to enhance weight gain. All procedures were approved by the Institutional Animal Care and Use Committee.

Adaptation and stimulation. After 2 days of recovery, rats were adapted to the testing chamber and to IO stimulation with distilled water. On the last day of adaptation and on subsequent test days, rats received three to six blocks of IO taste stimuli. Each block consisted of a 50-μl IO infusion of 0.1 M sucrose, 0.003 M quinine monohydrochloride (QHCl), and 0.1 M NaCl. Each taste stimulus was followed by three (or 6 following QHCl) infusions of water. After the water rinses to NaCl, the rat was given access to a bottle containing 0.3 M sucrose for 10 s. Each block lasted ~15 min, and consecutive blocks were separated by 30 min.

Recording and drug infusion. There were four main groups of rats. One group with bilateral guide cannulas in the lateral RF (n = 15) received the “standard” dose of muscimol (0.06 nmol in 100 nl volume or 0.06 nmol/100 nl). A second group with bilateral cannulas (n = 7), received a “low” dose of muscimol (0.03 nmol/50 nl). Two rats in the low-dose group also received bilateral infusions of muscimol intermediate to the low and high dose (0.045 nmol/75 nl) and two rats in the bilateral standard dose group also received a “very high” dose (X = 0.22 nmol/150 nl). One additional rat received only the very high dose of muscimol. The third group (n = 7) had midline cannulas and received 1.5× the standard dose of muscimol used for the bilaterally infused group (range 0.06–0.24 nmol, X = 0.096 nmol; X = 200 nl). In two cases, both bilateral and midline guide cannulas were implanted in the same preparation. The fourth group had bilateral cannulas implanted in the rostral nucleus of the solitary tract (n = 4) and received the low dose of muscimol (0.03 nmol/50 nl). To minimize damage and reconstruct infusion sites, the number of infusions per animal was kept to a minimum (X = 2) at any given site (40). Test days were separated by rest days.

EMG recording procedures were similar to those described previously (9, 48). Briefly, on the test day, rats were placed in the Plexiglas chamber for 1 h prior to testing. The Amphenol connector on the head was mated to a cable that connected the EMG electrodes to conventional AC amplifiers. The raw EMG signals were recorded on a digital recorder (VR-100, Instrutech). Raw and integrated EMG records were monitored online using Modular Instruments software and hardware connected to a thermal printer. EMG activity in response to each stimulus was digitized and saved as a computer file for off-line analysis. After EMG responses were obtained to two blocks of stimuli, the obturators were removed and two preloaded drug infusers were inserted into the brain via guide cannulas. The infusers contained either the GABA<sub>A</sub> receptor agonist muscimol dissolved in saline or the saline vehicle. Infusers were constructed from 33-gauge stainless steel tubing (extending 0.5–1.0 mm beyond the guide cannula) and were fit to PE-10 tubing that was connected to 10-μl syringes driven by a micropump (KD Scientific). A volume of 50–200 nl of the drug or saline solution was infused at the rate of 190 nl/min either bilaterally into the lateral RF (or NST) or a single larger volume of 100–400 nl into the medial core of the RF. Infusers were kept in the brain for 30 s after the infusion and then removed. Two to four blocks of taste stimuli were given following infusions of either muscimol or vehicle.

At the end of the experiment, Fluorogold (2%) was injected to mark the infusion sites. After injection of a lethal dose of Nembutal (150 mg/kg), the rat was perfused transcardially with 0.9% saline followed by 10% Formalin. The brain was removed, sectioned (50 μm) into two series, and mounted. The centers of the injection sites were identified under a fluorescent microscope.

Data analysis. Rectified and integrated EMG activity (Payntor filter, 0.02-s time constant, Bak Electronics) was analyzed off-line with Modular Instruments XYZ Spreadsheet software. For each stimulus trial, the mean amplitude and rate of EMG activity were determined. To compare across animals, EMG amplitudes and rates were normalized to their respective responses obtained from the first stimulus block. These normalized responses were used in ANOVAs to test for block, stimulus, and location effects across animals. Oral rejection responses to QHCl (gapes) were identifiable in
the EMG records and treated separately from licks. Gapes were characterized by larger amplitude contractions compared with licks and occurred at a slower rate (12, 48).

Although data were collected for IO stimulation following both 0.1 M sucrose and 0.1 M NaCl, preliminary analyses revealed virtually identical effects and so only the 0.1 M sucrose response data are presented.

RESULTS

Suppression of oromotor EMG. In 7 of 23 cases, infusing muscimol into the lateral medullary RF completely eliminated ingestion and rejection responses (Fig. 1). In other cases, EMG responses were attenuated but not eliminated (Fig. 2). These effects were both dose and location dependent.

Infusions of the standard dose of muscimol into the lateral RF (100 nl/0.06 nmol) significantly reduced the mean amplitude of both the AD and Gen contractions during licking and gaping (rejection). Figure 3 shows mean EMG amplitudes as a function of stimulus block following IO sucrose (Fig. 3A), bottle licking (Fig. 3B), and QHCl (Fig. 3C). The reduction in blocks 3 and 4 (muscimol infused just prior to block 3) began to recover by block 5, ~1.5 h postinfusion. An ANOVA for nine cases for which there was complete data for five stimulus blocks showed a highly significant block effect for AD amplitude ($P < 0.001$) but no effect for stimulus ($P = 0.67$), and no stimulus × block interaction ($P = 0.861$). An ANOVA for Gen amplitude produced similar results (block $P < 0.001$; stimulus $P = 0.659$; stimulus × block interaction $P = 0.653$). Infusions of saline into the lateral RF were ineffective in altering AD electromyographic activity (Fig. 3, A-C, △; ANOVA, block $P = 0.926$; stimulus $P = 0.486$; stimulus × block interaction $P = 0.125$).

The effect of muscimol infusions on lick or gape rate (licks/s) was less profound than the effects on amplitude. For example, in Fig. 2 the reduction in EMG amplitude during IO licking following muscimol was

Fig. 1. Integrated electromyogram (EMG) activity from the anterior digastric (AD) muscle following intraoral (IO) infusion of sucrose before (A), immediately after (B), and 2.5 h after (C) a bilateral infusion of muscimol (0.06 nmol/100 nl) into the lateral medullary reticular formation (RF). The responses to QHCl (D-F) and bottle licking (G-I) before, immediately after, and 2.5 h later also are shown.

Fig. 2. Integrated EMG activity from the anterior digastric muscle following IO infusion of sucrose before (A), immediately after (B), and 2 h after (C) a bilateral infusion of muscimol (80 nl, 0.048 nmol) into the lateral medullary RF. The responses to QHCl (D-F) and bottle licking (G-I) before, immediately after, and 2 h later also are shown. Responses in $E$ have been amplified by a factor of 2 compared with the other panels.
54%, compared with a reduction in lick rate of just 19%. The contrast between amplitude and rate effects was quantified for those cases that showed an attenuation but not elimination of the response (Fig. 4). This analysis included all standard dose cases with partial suppression, as well as one additional case each at the intermediate and low dose. Despite a clear effect on amplitude, effects on rate appear negligible. An ANOVA demonstrated a significant block effect for amplitude ($P < 0.005$) but not for response rate ($P = 0.234$).

During the oromotor suppression following muscimol infusions, the rats otherwise appeared normal. The most compelling evidence for this was observed during bottle licking. Even in those cases showing complete suppression following IO sucrose and QHCl stimulation, the rats still approached the bottle, frequently rising to a position on two legs, and grasped the spout with their forepaws. They showed a clear appetitive response and the ability to find and orient to the bottle and complete all but the consummatory response(s) of ingestion. In a previous study using lidocaine (8), we noted that infusions more ventral than those of the present study could cause disruption of the respiratory rhythm and that sites dorsal in the vestibular nuclei caused some imbalance. In the present study we did observe postural imbalance in two cases directed dorsally into the NST. Presumably, this occurred because of spread into the immediately adjacent vestibular nuclei.

Unlike infusions placed more laterally, infusions into the midline RF did not significantly alter the amplitude of licks or gapes (Fig. 3D). An ANOVA showed no stimulus effect ($P = 0.664$), block effect ($P = 0.444$) or stimulus × block interaction ($P = 0.775$). A second ANOVA for lick/gape rate revealed no significant block ($P = 0.127$), stimulus ($P = 0.16$), or stimulus × block interaction ($P = 0.833$).
**Location of effective sites.** The effect of infusion location on suppressing oromotor responses is summarized on a horizontal schematic of the brain stem in Fig. 5 and again on coronal sections in Fig. 6. Cases were divided into three groups based on their rostrocaudal location. *Level I* was anterior to the rostral pole of the NST (rNST), *level II* was between the rNST and the junction of the NST with the fourth ventricle, and *level III* was caudal to the junction. Summarized for each level are the mean percent reductions of the amplitude collapsed over the three stimulus conditions (IO licking, bottle licking, and QHCl stimulation) as a function of stimulus block. Two ANOVAs were performed. The first ANOVA compared the lateral cases in *level II* with midline cases (predominantly also in *level II*) as a function of stimulus and block. Mediolateral location was highly significant ($P < 0.001$) as was block ($P < 0.001$), but there was no effect of stimulus condition ($P = 0.596$). The interaction between block and location was also significant ($P < 0.001$), but there was no stimulus × block interaction ($P = 0.724$). The lack of a stimulus effect in the ANOVA justified the use of plotting the mean of the three stimulus conditions in Figs. 5 and 6. The second ANOVA compared the eight lateral cases in *level II* with the five cases in *level I*. As with the previous ANOVA there was a location ($P < 0.001$), block ($P < 0.001$), and location × block ($P < 0.001$) effect, but no stimulus ($P = 0.927$) or stimulus × block interaction ($P = 0.787$). A modest mean reduction in blocks 3 and 4 was noted for the two cases in *level III* at the standard dose but the small “n” precluded any statistical treatment. Two cases using the low dose at *level III* (not shown) had no effect.

The location of the cases shown in Fig. 5 are further illustrated in the coronal plane in Fig. 6. Figure 6 also includes additional cases tested with different doses to illustrate dose-response effects. Five cases at a lower dose (0.03 nmol/50 nl) are plotted in *level II* as are two cases at an intermediate dose (0.045 nmol/75 nl). Three cases at doses higher than the standard (0.1 nmol/175 nl) are also depicted in *level I*. A clear dose-response function was apparent at *level II* (see inset, Fig. 6B). A comparison between the low dose (0.03 nmol/50 nl) and the standard dose at *level II* showed a dose ($P < 0.018$), block ($P < 0.001$), and dose × block interaction ($P < 0.001$), but no stimulus ($P < 0.325$) or stimulus × dose interaction ($P < 0.821$). Despite the lack of mean effect at the low dose, two of five cases showed robust suppression similar to that seen with the higher doses. Across the three stimuli (IO sucrose, IO QHCl, and bottle licking), these two cases showed somewhat greater suppression to QHCl (71 and 68%) compared...
Fig. 6. Infusion sites are plotted in coronal sections, together with dose-response functions to the right of each section. EMG from the AD was normalized to the response in stimulus block 1, and a mean across the 3 stimulus conditions was calculated for each animal. The 8 lateral infusion cases at the standard dose (0.06 nmol/100 nl) at level II (○) differed significantly from the 5 low-dose (0.03 nmol/50 nl) cases at level II (△) and the 5 standard-dose cases at level I (*P < 0.001). Gi, nucleus gigantocellularis; io, inferior olive; Ir, intermediate zone of medullary RF; mVII, facial nucleus; mXII, hypoglossal nucleus; nst, nucleus of the solitary tract; PCr, parvocellular RF. Case numbers are inside of or adjacent to symbols.
Control infusions of muscimol into the overlying NST had no observable effect on oromotor behavior. Volumes of 50 nl were deliberately chosen for these control infusions because it was reasoned that only a fraction of the infusion into the deeper RF sites might have traveled up the cannula track into this structure. Moreover, in two instances, 50 nl of muscimol into the lateral RF were highly effective in attenuating the amplitude of the EMG responses, thus indicating the potential efficacy of this dose. The NST as the effective site of action of muscimol following RF infusions was also ruled out based on three infusions into the RF from an angle across the midline or from a posteriorly directed guide cannula, approaches that completely avoided the overlying NST. Although other studies making nonreversible (electrolytic) lesions in the NST have demonstrated somewhat diminished licking to water in a dehydrated state, which could be interpreted as evidence of a motor dysfunction (38), in no instances was licking totally eliminated (4, 11, 38). In summary, it appears unlikely that muscimol spread into the gustatory-responsive NST could account for the lack of oromotor responses observed in the present study.

**Location of a central pattern generator for ororhythmic function.** Earlier studies directed at determining an essential substrate for the generation of rhythmic oromotor activity used electrical stimulation of orbital (masticatory) cortex to elicit rhythmic trigeminal activity (6, 34). By combining this electrically evoked activity with brain stem transections, a minimal substrate was deduced that appeared to require the midline RF, i.e., nucleus gigantocellularis and paragigantocellularis. However, the recognition that most preoromotor neurons were more laterally located in the RF led to their inclusion in schematics of brain stem circuitry necessary to produce ororhythmic activity (6, 33, 44). The present results indicate that a critical substrate for both appetitively and intraorally induced licking includes the lateral medullary RF and that the midline RF is not likely a necessary structure for this behavior.

However, many of the sites in the current study that were effective in attenuating or eliminating oromotor activity overlapped with sites producing diminutions of cortically elicited rhythmic trigeminal activity following lidocaine infusions into the guinea pig RF (7). In contrast to the present results, the guinea pig study found effective sites rostral to, and coincident with the facial nucleus (see Fig. 3 in Ref. 7). The use of lidocaine in this study, however, prevents differentiating cell bodies from axonal projections (fibers of passage) as the critical substrate. In fact, many neurons in the lateral RF project rostrally to the motor trigeminal nucleus (7, 47), making these axons vulnerable to lidocaine infusions. More recent studies using a brain stem slice preparation in rat have also implicated the RF rostral to the facial nucleus as a minimal substrate for generating ororhythmic activity (23, 27, 42). Rhythmic activity from the exiting trigeminal nerve could be elicited in response to excitatory amino acids added to the bath solution containing the isolated brain stem. In
contrast to these in vitro and electrical brain stimulation studies, our results in awake preparations responding to natural stimuli suggest that RF areas rostral to the NST and caudal to the motor trigeminal nucleus, i.e., the region coincident with the facial nucleus, may not be an essential substrate for generating licking responses.

Overall, it is not easy to reconcile data on the origin of oromotor control systems. Licking and gaping were concomitantly affected by the muscimol infusions. These data are consistent with recent chronic unit recording data that showed a high degree of overlap between RF cells rhythmically active during licking and gaping (45). In the present study, we further observed that the effects of muscimol on AD and Gen activity were highly correlated; a Pearson product correlation for normalized EMG activity between these muscles across stimulus blocks was 0.65 (P < 0.001). By inference, we conclude that the two opposing functions of ingestion and rejection, both of which require the coordinated activity of the tongue and jaws, share a common spatial substrate in the lower brain stem. Indeed, anatomic studies confirm the spatial overlap of interneurons projecting to multiple oromotor nuclei (18, 47), and this has been demonstrated at the single cell level (1, 24, 35).

Interestingly, lick (and gape) rate was less affected than was amplitude by the muscimol infusions. A similar observation of an amplitude reduction independent of frequency (rate) was made with regard to phrenic nerve discharge following muscimol infusions into the A5 region (29). This independence of amplitude and frequency suggested that respiratory rhythm is controlled by different structures (29), and a similar view has been expressed with respect to the masticatory rhythm (28). We do not think, however, that the medial core of the medullary RF is the likely source for generating the lick frequency. Although previous studies have demonstrated that microstimulation of the medial RF could influence the timing (onset) of oromotor activity of cortical origin (5), infusions of muscimol into the midline RF had virtually no effect on lick rate. Hence, such a pathway may contribute to the generation of licking but is not essential to it.

Other brain stem structures with projections to the lateral RF could contribute to generating or modulating the lick rate. These include orosensory structures and other reticular regions (10, 37). The production of a modal (i.e., 7 Hz), but by no means inflexible lick rate (50), as well as the production of the different oromotor patterns of ingestion and rejection by a common substrate undoubtedly require neuromodulatory inputs from multiple structures. Moreover, the lateral RF is a likely target for descending pathways from hypothalamic and other forebrain structures sensitive to metabolic signals (36). Because decerebrate rats lick following IO stimulation but rely on their forebrains to make an appetitive response, we conclude that both local brain stem and forebrain descending pathways converge on a common substrate that coordinates the action of several oromotor nuclei necessary to affect the consummatory response.

Perspectives

Interceptive signals that convey energy and electrolyte status are processed in both caudal and forebrain structures, e.g., the caudal NST and hypothalamus. Ultimately, regulatory information processed in either area must engage substrates that control consummatory components of ingestion. The lateral medullary RF at the level of the rNST appears to be a critical substrate for producing oral consummatory responses initiated appetitively or by direct sensory stimulation. It appears that this region of the RF is specific to oromotor function to the extent that locomotor, postural, and respiratory function appeared essentially normal when oromotor function was absent. Because both licking and gaping (rejection) were similarly affected by reversible lesions in the same area, they appear to share a common spatial substrate. However, it is unknown how a common interneuronal substrate for oromotor function orchestrates the rate, amplitude, and phase differences of the oromotor patterns that characterize the two different behaviors of ingestion and rejection. Moreover, the interface between the RF and critical regulatory structures in both the brain stem and forebrain remains poorly understood.
REFERENCES


