special communication

Brain stem lesion size determined by DEAD red or conjugation of neurotoxin to fluorescent beads

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Nattie, Eugene E., Joseph S. Erlichman, and Aihua Li. Brain stem lesion size determined by DEAD red or conjugation of neurotoxin to fluorescent beads. J. Appl. Physiol. 85(6): 2370–2375, 1998.—Neurotoxin microinjected into the retrotrapezoid nucleus of anesthetized rats decreases phrenic activity and eliminates the response to CO2. In unanesthetized rats, such treatment has no effect on awake, resting breathing and decreases CO2 sensitivity by 40% (M. Akilesh, M. Kamper, A. Li, and E. E. Nattie. J. Appl. Physiol. 82: 469–479, 1997). One important factor in explaining these disparate results is the actual size of the anatomic lesion. In the present study, we injected ibotenic acid into the retrotrapezoid nucleus of anesthetized rats and evaluated lesion size by using two new approaches: 1) DEAD red, a fluorescent probe that enters impaired cells through leaky membranes and binds to nucleic acids, and 2) conjugation of toxin to fluorescent beads. With the use of DEAD red, the region containing labeled dying cells was 313 ± 104 nl (n = 4), six times larger than the initial injected volume, and the physiological effects on phrenic amplitude, the CO2 response, and blood pressure began within minutes and were substantial. With conjugated toxin, in theory, neuronal damage would be limited to the region of detectable fluorescence (49 ± 10 nl; n = 4). Effects on phrenic amplitude, CO2 sensitivity, and blood pressure were absent until ~2 h postinjection. Control experiments, with 2 h of in vitro incubation of the neurotoxin-microbead conjugate and injection of the supernatant after centrifugation, showed similar results that suggest release of conjugated neurotoxin. We conclude that DEAD red provides a useful means to monitor neuronal impairment in acute studies in vivo. Conjugation of neurotoxin to microbeads may be less reliable in this regard.

ventrolateral medulla; control of breathing; central chemoreceptors; carbon dioxide sensitivity

DISRUPTION OF NEURONAL FUNCTION near to the surface of the ventrolateral medulla profoundly inhibits breathing in anesthetized animals (3, 13, 15, 27). Regions possibly affected include parapyramidal neurons (3), the retrotrapezoid nucleus (RTN) (18–21), portions of the nucleus paragigantocellularis lateralis (29), and dendrites from the more dorsal retrofacial nucleus (16, 25). The RTN, a focus of this laboratory, extends from the rostral border of the nucleus ambiguus to the rostral border of the facial nucleus; it lies within a few hundred micrometers of the ventrolateral medullary surface and is bounded laterally by the spinotrigeminal tract and medially by the pyramidal tract (7, 24, 28). RTN neurons have anatomic (7, 24, 28) and physiological connections (5) to dorsally located respiratory neurons, discharge tonically or phasically with the respiratory rhythm (5), and are responsive to increased systemic CO2. Focal acidosis (4, 11) increases phrenic nerve output, indicating the presence of central chemo-reception within this region, as well as at many other sites (2, 4, 11, 14, 17). Thyrotropin-releasing hormone or glutamate injected into the RTN produces a long-lasting stimulation of respiratory output (6, 12). Unilateral chemical or electrolytic lesions of the RTN region in anesthetized or decerebrate cats (18, 19) and in anesthetized rats (20) decrease baseline phrenic nerve output, often to apnea, and decrease markedly the ventilatory response to breathing CO2. These effects are most impressive in the anesthetized preparations but less so in the decerebrate animals, and, in unanesthetized rats, such lesions have no effect on baseline breathing and decrease the CO2 response by 40% (1). These observations suggest the presence of an important tonic drive that is of RTN origin, but the strength of this drive may be dependent on the animal’s state of arousal.

In goats chronically implanted with ventrolateral medulla surface-cooling devices (9, 21, 22), bilateral cooling of the rostral and intermediate areas caused sustained apnea under anesthesia but caused a modest attenuation of breathing in the awake state. These observations support the idea that the arousal state influences the effectiveness of RTN lesions. In contrast, Schlaffer et al. (27) produced bilateral coagulation of the intermediate areas in cats, which, when subsequently studied under unanesthetized conditions, demonstrated hyperventilation at rest when breathing air and an almost complete loss of the ventilatory response when breathing CO2. In these experiments, the lesions are likely to have been quite a bit larger than in our previous study (1) of unanesthetized rats. The anatomic size of the region affected by the cooling devices in the chronically implanted goats is not known precisely, but measures of tissue temperature suggest that substantial tissue volumes might have been affected. Part, but not all, of the variability in these responses is likely...
to be caused by differences in the size of the affected region.

Our goal in the present experiment was to evaluate two methods for more precise estimation of the size of the toxin-induced lesions. The first method involves the conjugation of neurotoxin to fluorescent microbeads with carboxyl groups attached (Polysciences), a modification of an approach previously used for carbachol (26). In the present study, bead location was observed anatomically with 2D imaging, in theory, define the affected tissue volume. The second method uses a separate injection of a fluorescent dye, ethidium homodimer-1 (DEAD red; Molecular Probes), which enters cells through impaired membranes and binds to nucleic acids. Here, the dye labels dying and recently dead cells and allows a description of the affected region at the neuronal level.

METHODS

General protocol. There were three experimental groups, according to the method used: 1) ibotenic acid conjugated to fluorescent beads (50 nl; n = 5), 2) ibotenic acid (50 nl, 100 mM; n = 6) mixed with artificial cerebrospinal fluid (aCSF) and injected with 1.0 µl of DEAD red, and 3) a control group (n = 5) to evaluate the effect of injection of the supernatant after centrifuging the aCSF plus conjugated ibotenic acid. This was done immediately after the initial wash (n = 2) and after 2 h of incubation of the conjugated beads and ibotenic acid at 37°C to simulate the period in vivo during which measurements were made (n = 3). This second control tested for any leaching or loss of ibotenic acid from the beads. All groups had integrated phrenic nerve amplitude (PNA) and frequency measurements made before, during, and after neurotoxin administration, which was made unilaterally into the RTN region. The PNA and frequency responses to systemic CO2 administration were evaluated before and during the response to the neurotoxin. Because frequency was unaffected by any treatment in these vagotomized rats, the results will be reported in terms of PNA. As reported in a separate paper (8), another group of four rats received DEAD red only, and they were sacrificed at 30–60 min (n = 2) or 120 min (n = 2) to monitor the spread of DEAD red in the brain stem extracellular space without neurotoxin.

Surgery. The experimental protocol was approved by the Dartmouth College Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (290–410 g) were initially anesthetized with 2.0% halothane in O2. The trachea was then cannulated, and catheters were placed in the femoral artery and vein. While blood pressure was monitored, a mixture of urethane (550 mg/kg) and alphachloralose (60 mg/kg) was injected into the femoral vein over several minutes, while the inspired halothane concentration was slowly decreased. Gallamine triethiodide (3 mg/kg) was used to paralyze the rats, and supplemental doses were administered as needed. The rats were artificially ventilated (model 683 rodent ventilator, Harvard Instruments) with 100% O2. Rectal temperature was maintained at 37°C using a feedback system, and end-tidal PCO2 (PetCO2) was monitored with a CO2 analyzer (Lifespan 100, ETCO2 Monitor, Biochem) and was maintained at any set value by manually controlling the ventilator rate and/or tidal volume. Bilateral thoracotomies were performed, and a positive end-expiratory pressure of 3–5 cmH2O was maintained during the experiment. Bilateral vagotomy was performed before the ventral medullary surface was exposed. The phrenic nerve was isolated and then cut, and the central end was placed on bipolar electrodes and covered with Wacker Sil-gel (an insulating medium). PNA was amplified (BMA 831 amplifiers, Charles Ward), integrated (Paynter filter in an MA-821 moving averager, Charles Ward), and displayed on a storage oscilloscope. Integrated PNA, arterial blood pressure, and PetCO2 were recorded on a strip-chart recorder (MF-E 1420). An on-line program, using a 12-bit processor and a 150- to 200-Hz sampling rate, was used to calculate the amplitude of the integrated PNA, phrenic burst frequency, blood pressure, and PetCO2. Blood pressure and the frequency and integrated amplitude of phrenic discharge were continuously monitored during the experiment to evaluate the depth of anesthesia. An increase in respiratory frequency or blood pressure that could not be attributed to an experimental perturbation or that occurred in response to a noxious stimulus (e.g., a pinch to the hindpaw) were viewed as signs that the animal needed additional anesthesia. This was given in the form of one-fourth to one-third of the initial dose.

Injections. The aCSF contained the following salts (in mM): 126 NaCl, 3.0 KCl, 2.1 MgCl2, 2.2 CaCl2, and 26.2 NaHCO3. In addition, glucose was added to a final concentration of 5 mM. Neurotoxin was covalently bound to carboxylated fluorescent microbeads (0.5-µm diameter) by using a carbodiimide reaction (Polysciences). The initial neurotoxin concentration that was used for the reaction with an ~20% mixture of beads was 10 mM. We do not know the final “effective” neurotoxin concentration after binding. One goal of the study was to test, in vivo, the effectiveness of this dose. The microinjections were made by using glass pipettes with a tip diameter of ~20–30 µm. Microinjections were made just below the ventral medullary surface, so that they were located between this surface and the ventral aspect of the facial nucleus. Injections were made via a Picospritzer over a 3-s period. Injection volume was monitored by microscopic observation of the movement of the meniscus of the air-fluid interface. For application of DEAD red, just before injection of neurotoxin, double-barreled pipettes were used.

Distribution of cells labeled with DEAD red and anatomic analysis. To determine the pattern of cells with impaired membrane function produced by neurotoxin injection into the RTN, the fluorescent, nucleic acid stain DEAD red was microinjected into the RTN. DEAD red is a cell-impermeant dye that can only diffuse across leaky or otherwise compromised cell membranes (10). Approximately 1 µl of aCSF containing DEAD red (1:50 dilution) was slowly microinjected into the RTN over a period of 10 min. The large volume was to ensure an adequate concentration of the dye in the extracellular space in the region of the RTN exposed to the neurotoxins. In preliminary experiments, the microinjection of DEAD red alone into the RTN had no noticeable effect on baseline PNA. To have dye present during the period of cell disruption, we injected DEAD red into the RTN just before injection of the neurotoxin. At the end of the study period, the brain was fixed in situ by serially perfusing 0.1 M PBS, pH 7.3, through the left ventricle of the heart followed by perfusion with PBS containing 4% paraformaldehyde. After the brain was fixed, it was removed, frozen in dry ice, and sectioned at a 50-µm thickness with a cryostat (Cryocut 1800) maintained at −20°C. Tissue sections were mounted on glass slides, cleared in 100% DMSO, and viewed under epifluorescence by using a long-pass fluorescent isothiocyanate filter set. Processed tissue sections were mounted on glass slides and viewed with a Nikon Optiphot 2-UD microscope. Emitted fluorescence was passed through appropriate filters and directed to a digitizing charge-coupled device camera (SenSys 1400, Photometrics),
and images were processed by using software (Axon Imaging Workbench, Axon Instruments; Image Pro). The volume of tissue that contained DEAD red-positive cells and the cell diameter were measured from the digitized images by using a micrometer. The area containing DEAD red-positive cells was measured in each cross section. The volume of tissue containing DEAD red-positive cells was calculated from the sum of these areas. [See Fig. 5 for an example of a series of such cross sections (left), with traditional anatomical landmarks (right).] For evaluation of microbead location, fluorescence was evaluated by using an Olympus fluorescence microscope. Alternate sections were stained by using cresyl violet. Computer-image analysis (Image Pro) was used to superimpose the fluorescent and stained images of adjacent brain stem sections. The volume of injection was calculated as follows: the total number of sections that contained fluorescent beads were counted and multiplied by the section thickness (50 µm) to determine the rostral-caudal length of the injection. The section with the largest cross-sectional area of beads was taken as the center of injection. This area was measured by using a computer image-analysis system (Image Pro). The injection volume was calculated by using the measured area and height and a simple geometric model of two adjoining circular cones. In the stained slides, the area corresponding to the location of maximal fluorescent beads as well as the adjacent areas were examined in detail, looking for gliosis, swelling, and neuronal destruction. Video camera images of these areas were digitized with computer software (Image Pro).

**Experimental protocol.** All animals were first tested for their responsiveness to inspired CO₂. Baseline P ETCO₂ was set at 28 Torr, slightly above the apneic threshold, while the animal was ventilated with 100% O₂. To determine the ventilatory response to CO₂, the P ETCO₂ was increased in 7 Torr steps by adding controlled amounts of 100% CO₂ into the inspired air, while maintaining ventilator frequency and tidal volume constant. Responses were measured when PNA had stabilized (~3–5 min at any CO₂ level). When the animal recovered from the CO₂ challenge and showed stable baseline PNA, neurotoxin was injected. After 2 h, the animal was exposed to hypercapnia a second time. In experiments in which DEAD red was used, it was microinjected into the RTN before neurotoxin was injected.

**RESULTS**

Figure 1 summarizes the baseline integrated PNA responses in the three groups. A comparison of responses to injection of ibotenic acid when mixed with aCSF (mixed) vs. conjugated to microbeads (bound) shows greater effects that are manifest more quickly in the mixed protocol. The effect on PNA when the ibotenic acid is conjugated to microbeads does not become apparent until after 120 min. Ibotenic acid mixed with aCSF decreased PNA within minutes of the injection. Figure 2 shows that the response to hypercapnia was virtually absent after each treatment; the measurements were obtained at 2 h after injection. Mean arterial blood pressure (Fig. 3) was unchanged statistically in control and ibotenic acid-conjugated groups (although it did tend to decrease at 120 min in the ibotenic acid-conjugated group) but decreased significantly in the group that received ibotenic acid mixed with aCSF.

It is important to emphasize the results from controls for conjugation of ibotenic acid to microbeads. As an in vitro simulation of the in vivo experiment, we washed the ibotenic acid-microbead conjugate with aCSF before (n = 2) and after (n = 3) incubation at 37°C for 2 h.

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**Fig. 1.** Integrated phrenic amplitude (means ± SE) expressed as %baseline just before (t = 0) and after injection into retrotrapezoid nucleus (RTN) of ibotenic acid (IA) covalently bound to fluorescent microbeads ( ); 50 nl; n = 5); IA mixed with artificial cerebrospinal fluid (aCSF) containing fluorescent microbeads ( ); 50 nl; 100 mM; n = 6); supernatant from the aCSF-conjugated IA preparation (control; ; 50 nl; n = 2).

**Fig. 2.** Responses to increased CO₂ are shown for same 3 groups as in Fig. 1. Phrenic nerve amplitude responses here are normalized to maximal response obtained to 9% end-tidal CO₂ observed in each animal at beginning of experiment.

**Fig. 3.** Responses of mean arterial blood pressure are shown for same 3 groups of animals as in Fig. 1.
The supernatant obtained before incubation had no effect on PNA, CO2 sensitivity, or blood pressure for 2–3 h after injection (see Control in Figs. 1–3). The supernatant obtained after 2 h of incubation decreased PNA by 10% after 2 h, decreased mean arterial blood pressure from 136 ± 6 to 119 ± 13 mmHg after 2 h, and eliminated the CO2 response after 2 h (data not shown). These results suggest that some ibotenic acid may be released from the microbeads during this 2-h period, and this released ibotenic acid could account for the delayed effects observed in the ibotenic acid microbead conjugate group.

The locations of the centers of the injections of neurotoxin in the three groups are shown in Fig. 4. The data for ibotenic acid mixed with aCSF were obtained from analysis of fluorescent beads added to the mixture.

The fluorescent dye DEAD red was used to define the region containing cells with faulty membrane function in the group injected with ibotenic acid mixed with aCSF. As reported previously (8), injection of DEAD red alone, without neurotoxin, showed a volume of dye distribution of 211 ± 45 (SE) nl at 30–60 min and 55 ± 12 nl at 120 min. These values are lower than the 1.0 µl injected, a finding interpreted to indicate rapid clearance of the injected DEAD red (8). Figure 5 shows [on the left side of a series of cross sections of the medulla, modified from the atlas of Paxinos and Watson (23)] the actual area containing neurons positive for DEAD red in a single animal. On the right side, landmark structures are shown. In the ibotenic acid mixed group, DEAD red-stained cells were found in a tissue distribution of 313 ± 104 nl (average diameter of tissue containing dying cells, 672 µm; length of tissue, 838 µm; n = 4). A nonrandom sample of 372 stained cells had a diameter of 17.3 ± 10 (SD) µm. In the ibotenic acid-conjugate group, the volume of tissue containing fluorescence was 49 ± 10 nl (average diameter of tissue containing fluorescence, 531 µm; length of tissue, 588 µm; n = 4).
DISCUSSION

The major findings of this study show that the conjugation of ibotenic acid to fluorescent microbeads, and subsequent injection into the RTN, produces minimal physiological effects that could be explained by release of the ibotenic acid from the conjugate. Thus the attractive idea that the location of the fluorescent beads in this procedure might mark the entire extent of neurotoxin spread does not appear to be tenable. In contrast, DEAD red injected into the RTN just before injection of neurotoxin appears to be a potentially useful way to estimate the size of the anatomical region that contains neurons affected by the toxin.

A fundamental problem in the use of neurotoxins to produce chemical lesions to study function of specific brain locations is the evaluation of the exact region affected by the neurotoxin. The cell and glial changes that reflect tissue damage and neuronal death, as defined by using neuropathological criteria, take days to develop. Cell counts depend on an absence of cells and that the dead neurons have been removed by scavenger mechanisms (1). In experiments of a more acute nature, it has proven difficult to demonstrate the exact region affected. Dyes such as Sky blue have been injected along with the neurotoxin to mark the region that the injection covers, but this approach assumes that visible Sky blue and the effective neurotoxin concentration are related. We have previously used fluorescent microbeads mixed in with the neurotoxin injections (18–20), as done in part of this study, but the location of the fluorescent microbeads shows only the location and size of the microbead injection; it does not show the region within which the neurotoxin can spread.

DEAD red, a fluorescent, nucleic acid stain, is a cell-impermeant dye that can only diffuse across leaky or otherwise compromised cell membranes and then bind to nucleic acids, thereby labeling the cell (10). We injected a large volume of DEAD red at the time of the neurotoxin injection (1.0 µl). We reasoned that this would be more than sufficient to show the distribution of dying cells that would accompany a 50-nl injection of the neurotoxin. In control rats (with injections of DEAD red alone to examine the distribution of the dye in the absence of neurotoxin (8)), we estimated the volume affected as a cylinder by measuring the number of sections that contained fluorescence and used the average cross-sectional area of fluorescence. At 30–60 min, this volume was 211 nl; at 2 h, the volume was 55 nl. Both volumes were considerably less than the 1.0-µl volume that was injected. Most of the initial DEAD red injection had disappeared, probably cleared from the brain, by bulk flow of brain interstitial and cerebrospinal fluid. Thus the rate of clearance of the injected DEAD red must be taken into account when using this substance to mark the tissue distribution of a neurotoxin. The initial or subsequent dye injections must be timed to coincide with the period when damaged neurons that are present with leaky membranes allow the dye to enter, be trapped, and escape from brain clearance processes.

In the experiment with simultaneous injection of 1.0 µl of DEAD red and 50 nl of ibotenic acid, we measured the length and diameter of the tissue cylinder that contained cells labeled with DEAD red. This volume of 313 nl, with an average length of 838 µm and average diameter of 643 µm, was six times larger than the 50-nl injection, which, as a sphere, would have a diameter of 457 µm. This volume is also larger than that measured when DEAD red was injected alone (see above). This indicates that the dye entered some cells and was bound to their nucleic acids before it could be cleared from the brain extracellular fluid. This volume still could be an underestimate, however, as dye may have been cleared before development of a sufficient level of membrane disruption to allow the dye to enter the cell.

The tissue volume of the RTN itself has been estimated to be ~1,200 nl in the rat (12). This 50-nl injection of ibotenic acid with DEAD red affected ~25% of this RTN volume, if one assumes that all labeled neurons were only within the RTN. Some were surely outside of the confines of the RTN, so this 25% RTN damage estimate is probably on the high side. For comparison, in unanesthetized rats that had been given neurotoxin injections (50 mM, 24 nl) while under anesthesia and were then evaluated for 3 wk before they were sacrificed, the estimated RTN volume affected, by using cell counts as the indicator, was 35% (1). In contrast, the injections of toxin conjugated to fluorescent beads were measured in the tissue to take up a volume of 49 nl or 4% of the RTN volume. However, it appears likely that some ibotenic acid was released from the conjugate. Unfortunately, we did not use DEAD red in these experiments, and thus we do not know the size of the affected region.

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