Central CO₂ Chemoreception in Cardiorespiratory Control

Cardiorespiratory and neural consequences of rats brought past their aerobic dive limit

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Panneton WM, Gan Q, Dahms TE. Cardiorespiratory and neural consequences of rats brought past their aerobic dive limit. J Appl Physiol 109: 1256–1269, 2010. First published August 12, 2010; doi:10.1152/japplphysiol.00110.2010.—The mammalian diving response is a dramatic autonomic adjustment to underwater submersion affecting heart rate, arterial blood pressure, and ventilation. The bradycardia is known to be modulated by the parasympathetic nervous system, arterial blood pressure is modulated via the sympathetic system, and still other circuits modulate the respiratory changes. In the present study, we investigate the submersion of rats brought past their aerobic dive limit, defined as the diving duration beyond which blood lactate concentration increases above resting levels. Hemodynamic measurements were made during underwater submergence with biotelemetric transmitters, and blood was drawn from cannulas previously implanted in the rats’ carotid arteries. Such prolonged submersion induces radical changes in blood chemistry; mean arterial PCO₂ rose to 62.4 Torr, while mean arterial PO₂ and pH reached nadirs of 21.8 Torr and 7.18, respectively. Despite these radical changes in blood chemistry, the rats neither attempted to gasp nor breathe while underwater. Immunohistochemistry for Fos protein done on their brains revealed numerous Fos-positive profiles. Especially noteworthy were the large number of immunopositive profiles in loci where presumptive chemoreceptors are found. Despite the activation of these presumptive chemoreceptors, the rats did not attempt to breathe. Injections of biotinylated dextran amine were made into ventral parts of the medullary dorsal horn, where central fibers of the anterior ethmoidal nerve terminate. Labeled fibers coursed caudal, ventral, and medial from the injection to neurons on the ventral surface of the medulla, where numerous Fos-labeled profiles were seen in the rats brought past their aerobic dive limit. We propose that this projection inhibits the homeostatic chemoreceptor reflex, despite the gross activation of chemoreceptors.

THE DIVING RESPONSE IS CONSIDERED an organism’s defense against asphyxia (29, 115). It is present in all mammals investigated (3, 7, 8, 14, 15, 30, 33, 35, 59, 61), including rats (65, 66, 68, 69, 71, 89, 90, 92). The diving response consists of at least three simpler reflexes involving a parasympathetically modulated bradycardia, a sympathetically modulated peripheral vasoconstriction, and an apnea [see Panneton et al. (92) for review].

Kooyman and colleagues (58, 60) defined the aerobic dive limit (ADL) in diving mammals as the diving duration beyond which blood lactate concentration increases above resting levels (62). Naturally diving mammals have several physiological adaptations that buffer against an underwater environment [see Meir et al. (75), for discussion], but most dives are shorter in duration than the ADL. Nevertheless, the diving mammals’ blood chemistry is constantly in flux while underwater, since the animal is no longer breathing. Thus variables such as partial pressures of arterial oxygen (PₐO₂), arterial carbon dioxide (PₐCO₂), arterial oxygen saturation (SₐO₂), and acidity (pH) are altered progressively (43, 75, 107) toward hypoxia, hypercapnia, and acidosis. Bert was the first to show that rats die after ~2 min underwater (see 53), suggesting their ADL must be less than this time. Thus we investigated terrestrial laboratory rats after submersion until they developed extreme hypoxemia, hypercapnia, and acidemia to compare their ADL to that of diving mammals.

Chemoreceptors in both the carotid body and the central nervous system sense changes in the chemistry of blood and neural tissue and regulate ventilation appropriate to physiological needs. It is well known that increasing levels of PₐCO₂ and hydrogen ions, as well as decreasing levels of PₐO₂, increase ventilation by increasing both respiratory rate and depth (23, 32, 41, 44, 45); this reflex is called the respiratory chemoreceptor reflex. Despite this, diving animals by necessity cannot breathe while underwater, or they will drown. This has prompted suggestions that the respiratory chemoreceptor reflex is inhibited during underwater submersion (29). This is especially pertinent in mammals as they approach their ADL.

Previous investigations in rodents have shown that innervation of the nasal mucosa is important for cardiorespiratory behaviors similar to the diving response (22, 74, 130), since numbing the nasal mucosa inhibits them. The anterior ethmoidal nerve innervates the anterior nasal mucosa, as well as tissue surrounding the nares [see Panneton et al. (91), for review] and, when stimulated electrically, induces similar cardiac and respiratory depression (24–28, 70, 112). This nerve projects densely into areas of the medullary dorsal horn (MDH) (86, 91), where the autonomic responses due to stimulation of the nasal mucosa can be inhibited (87, 99). Moreover, neuronal transport techniques show that neurons in similar parts of the MDH project to numerous nuclei in the brainstem associated with cardiorespiratory behaviors (91, 96). The diving response must be driven via neurons within the medulla and spinal cord, however, since these behaviors still are elicited in vertebrates.
after transecting their brain stems rostral to the caudal pons (22, 52, 67, 92, 98).

In the present investigation, we submerged rats underwater past their ADL, while extracting arterial blood to measure blood chemistry. We also processed their brains immunohistochemically to visualize Fos protein induced with activation of neurons (6, 21) and compare these data with those derived from brains immunohistochemically stained for antibodies against Neun, which labels neurons. We finally describe a pathway from the ventral MDH, known to be important for the cardiorespiratory depression after nasal stimulation, to the ventral surface of the caudal medulla, where numerous chemoreceptors are known to reside (76, 77, 114). We show that the rats did not breathe while underwater, despite increases in multiple stimuli in blood chemistry, which usually induce hyperventilation. These experiments suggest numerous chemoreceptors are activated during prolonged underwater submersion and also suggest that brain stem circuits initiating ventilation are activated as well. These data support ideas of the diving response both being the most powerful autonomic response known, as well as fetal reflexes as a cause of the sudden infant death syndrome (64).

Glossary

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>A5</td>
<td>Noradrenergic cell group in ventrolateral pons</td>
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<td>ABP</td>
<td>Arterial blood pressure</td>
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<tr>
<td>Amb</td>
<td>Nucleus ambiguus</td>
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<tr>
<td>bc</td>
<td>Brachium conjunctivum</td>
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<td>BE</td>
<td>Base excess</td>
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<tr>
<td>CPA</td>
<td>Caudal pressor area of the caudal medulla</td>
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<td>Cu</td>
<td>Cuneate nucleus</td>
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<td>E5</td>
<td>Ectotrigeminal nucleus</td>
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<tr>
<td>GiA</td>
<td>Gigatocellular reticular nucleus, pars alpha</td>
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<td>Gracile nucleus</td>
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<td>Hb</td>
<td>Hemoglobin</td>
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<td>Heart rate</td>
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<tr>
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<td>Inferior olivary nucleus</td>
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<tr>
<td>LC</td>
<td>Locus nucius coeruleus</td>
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<tr>
<td>Lrt</td>
<td>Lateral reticular nucleus</td>
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<tr>
<td>LriPC</td>
<td>Lateral reticular nucleus, pars parvocellularis</td>
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<tr>
<td>MABP</td>
<td>Mean arterial blood pressure</td>
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<tr>
<td>MDH</td>
<td>Medullary dorsal horn</td>
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<td>m5</td>
<td>Trigeminal motor root</td>
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<td>Mo5</td>
<td>Motor trigeminal nucleus</td>
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<td>MVe</td>
<td>Medial vestibular nucleus</td>
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<td>PaCO2</td>
<td>Partial pressure of arterial carbon dioxide</td>
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<tr>
<td>PaO2</td>
<td>Partial pressure of arterial oxygen</td>
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<tr>
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<tr>
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<td>Principal trigeminal nucleus</td>
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<td>Arterial oxygen saturation</td>
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<td>sol</td>
<td>Tractus solitarii</td>
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<td>Sol, NTS</td>
<td>Nucleus tractus solitarii</td>
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MATERIALS AND METHODS

ADL Experiments

Cardiovascular data. Thirteen adult (~275–325 g) Sprague-Dawley male rats were obtained commercially (Harlan, Indianapolis, IN) and used in this study. All protocols were approved by the Animal Care Committee of Saint Louis University and followed the guidelines of the National Institutes of Health Guide for Care and Handling of Laboratory Animals.

Eight rats were anesthetized with ketamine-xylazine (60:40 mg/kg ip), and the catheter of a bioelectric transmitter (model PA-C40; Data Sciences International, DSI, St. Paul, MN) was inserted through their femoral arteries while the transmitter itself was implanted in their abdominal cavities. The rats healed for 5–7 days and then were anesthetized again and their anterior neck dissected. The right carotid artery was located and cut, and polyethylene tubing (PE 50) was inserted caudally to the level of the aortic arch. The distal end of the tubing was routed subcutaneously and exited on the dorsal cranium. After 2–3 more days of postoperative recovery, cardiovascular data were obtained from all rats before, during, and after experimental submersion. The transmitter’s broadcast was received with a radio receiver (model RLA3000, DSI), relayed to a Calibrated Pressure Analog Adaptor (model R11CPA, DSI), and transferred through an A/D interface (1401 plus, Cambridge Electronic Design, CED, Cambridge UK), stored in the computer, and analyzed using Spike 2 software (CED). Systolic, diastolic, and mean arterial blood pressure (MABP) were calculated, and heart rate (HR) determined by counting peaks of systolic pressure. We assumed the rats made no attempt to breathe while underwater, since none drowned during submersion.

Blood chemistry data. Experiments commenced on each rat individually 2–3 days after the placement of the carotid cannulas. After a sterile 1-ml heparinized syringe was attached to the carotid cannula, ~300 µl of blood (baseline sample) were withdrawn from the rat’s carotid artery and placed on ice. The cannula then was flushed with sterile heparinized saline and readied for the next sample. The rats then were placed in a Plexiglas restrainer tube, the syringe assembly reattached, and another 300 µl of arterial blood were withdrawn and stored in ice. Both the rat and tube were submerged underwater (29–32°C) for ~90–100 s. Blood was continuously withdrawn during this period, with each syringe taking ~20 s to fill and be replaced with another. The rats were removed from the tube, and another 300 µl of arterial blood were withdrawn at ~30 s, 2 min, and up to 20 min after emergence from the water. All blood samples were placed immediately on ice after collection. Although three rats were unconscious and not breathing after their removal from the tube, they were resuscitated within seconds by gently protracting the tongue. The cold blood samples were taken immediately to a blood-gas analyzer (ABL700 Radiometer, Westlake, OH) and an oximeter (OSM3, Radiometer, Westlake, OH) for measurement of PaO2, PaCO2, SaO2, acidity (pH), hemoglobin (Hb), and calculation of base excess (BE).
Immunohistochemical data. The eight rats from the blood chemistry experiments remained in their home cage for 2 h after submersion. They were deeply anesthetized (Sleepaway, 0.1 ml/100 g ip) and perfused through the heart with a peristaltic pump (Masterflex), first with a saline-procaine solution, followed immediately by a fixative of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.3). Brains and spinal cords were removed and refrigerated in the fixative with 20% sucrose at 4°C. The brains were blocked in the coronal plane using a precision brain slicer before frozen transverse sections (40 μm) were cut with a microtome.

Every third section was processed immunohistochemically overnight with antibodies against Fos (rabbit polyclonal IgG for c-fos p62; 1:20,000; Santa Cruz Biotechnology) mixed in 0.1 M PB with 0.3% Triton. On the following day, the sections were washed and then incubated for 1 h in goat anti-rabbit biotinylated secondary IgG (1:500; Vector Laboratories), washed, and then incubated in an ABC complex (Vectastain Elite; Vector Laboratories) for another hour. The Fos antigen was visualized in the brain stem with the chromogen diaminobenzidine enhanced with nickel ammonium sulfate catalyzed by hydrogen peroxide. Sections were mounted serially on gelatin-coated slides, counterstained with Neutral Red, dehydrated in alcohol, defatted in xylene, and coverslipped with Permount. Fos-positive neurons appeared as cells with black-labeled nuclei and were visualized with bright-field optics (Nikon E800). Two cases usually were processed simultaneously to minimize procedural differences in immunohistochemistry (i.e., slight differences in reaction times and/or dilution factors of the primary or secondary antibodies).

Neurons were considered Fos-positive if their nuclei contained the black immunoprecipitate. Fos-positive neurons in selected brain stem nuclei were photographed digitally (MicroImager II) with Northern Eclipse Software (Empix). Sections were drawn with a Nikon E600 microscope interfaced with Neurolucida software (MicroBrightField). The figures were standardized in Adobe Photoshop software (version 7) using levels, brightness, and contrast and aligned in Adobe Illustrator software (version 11). All nomenclature and abbreviations are from a stereotaxic rat atlas (102).

Tract-tracing Experiments

Five animals were anesthetized (vide supra) and secured prone in a flat skull position in a stereotaxic device. The skull was exposed via a frontal sinusotomy (Neurotron software (version 11)). All nomenclature and abbreviations are from the rat brain atlas (102). Using levels, brightness, and contrast and aligned in Adobe Illustrator (version 7) and using the fixative and peroxidase methods as described in the Materials and Methods section.

Data Analysis

Means and standard errors (means ± SE) were determined for both hemodynamic and blood-gas measurements and compared with control values for significance (SPSS software, version 15). Cardiovascular and blood chemistry parameters during submersion were compared with data considered control. Control data were taken from the rats in the physiological experiments when they freely roamed in their home cages before submersion. A repeated one-way ANOVA revealed significant differences in these parameters from control values. This strict statistical analysis amplified the findings presented herein. Data are presented as means ± SE, and significance was calculated as P < 0.05.

RESULTS

All rats showed a marked drop in HR and an immediate but temporary increase in arterial blood pressure (ABP) to prolonged involuntary submersion (Fig. 1). Numerous brain stem neurons also were immunolabeled with Fos after this prolonged submergence. Tract tracing experiments showed projections from the MDH to the caudal superficial ventral medulla, where neurons immunolabeled with Fos after prolonged submergence also were found.

ADL Experiments

Cardiovascular data. Both the bradycardia and the increase in ABP seen with underwater submersion were significantly different from control values obtained before submersion (Fig. 2, A and B). HR reached a nadir of 106 ± 7 beats/min in submerged rats, significantly (P < 0.001) different from control values of 461 ± 11 beats/min (Fig. 2A). The bradycardia was maintained throughout the period of submergence compared with predive, but was less intense during the last 10 s underwater; HR rose rapidly after the rat was taken from the water (Fig. 2A). There were numerous arrhythmias in these untrained rats for approximately the first 70 s that the rats were underwater (Fig. 1B). A rhythmic cardiac cycle then appeared as the rat became more hypoxic, hypercapnic, and acidotic (Fig. 1B2); some rats apparently became unconscious during this time, since they stopped moving in the restrainer tube. On emersion, the heartbeats again became arrhythmic after respiration commenced and the rat awoke (Fig. 1B3) before settling into a normal cardiac rhythm (Figs. 1A), returning to control values 2 min after emersion (Fig. 2A).

MABP rose from 115 ± 2 mmHg in control periods to 124 ± 3 mmHg after ~30 s underwater (Fig. 2B), but then fell significantly (P < 0.05) to 98 ± 4 mmHg as the ADL was approached (Figs. 1A and 2B). MABP returned to control levels by 2 min after emersion from the water (Fig. 2B).

Blood chemistry data. Partial pressures of carbon dioxide, oxygen, and oxygen saturation were measured throughout the experiments. Initially, Paco2 was 30.3 ± 0.6 Torr and peaked significantly at 62.4 ± 3.7 Torr at the end of the submersion period (Fig. 2C). Pao2 dropped from 93.1 ± 1.6 Torr at control times to 21.8 ± 2.3 Torr at the end of submersion (Fig. 2D), which also was significant (P < 0.001). Oxygen saturation also dropped significantly (P < 0.001), changing from 93.7 ± 0.4 to 17.6 ± 2.8% at the end of submersion (Fig. 2E). All of these parameters approached control values within 2 min after emerging from the water. Blood acidity, however, continued to rise after the rat emerged from the water. pH fell significantly (P < 0.001) from control levels of 7.51 ± 0.01 to 7.18 ± 0.02
Fig. 1. Traces illustrating the typical cardiovascular behavior of a submerged rat brought toward its dive limit. A: trace illustrates changes in cardiovascular dynamics, as well as arterial blood chemistry over a 250-s experiment. Downward-pointing arrow on bottom indicates submersion, while the upward-pointing arrow to the right marks exit from the water. There was an immediate bradycardia and increase in arterial blood pressure (ABP) on submersion highlighted by numerous arrhythmias (the 40-s period marked 1 in A is seen on an expanded time scale in B1). After ~70 s of submersion, ABP fell in all rats, and the arrhythmias ceased (the 40-s period marked 2 in A is shown at expanded time scale in B2). This behavior continued until the rat exited the water and started to breathe again. Heart rate gradually accelerated with respiratory activity, but arrhythmias returned temporarily (the 40-s period marked 3 in A is shown at expanded time scale in B3). Note that the rat became extremely hypoxic, hypercapnic, and acidic during submersion. PaO2, arterial PO2; PaCO2, arterial PCO2.
at the end of submersion and continued falling to $6.99 \pm 0.02$ 2 min later (Fig. 2F). Similarly, BE initially fell significantly ($P < 0.01$) to $-5.7 \pm 0.6$ after 20-s submersion from a control value of $0.86 \pm 0.4$, and continued to plummet rapidly to $-18.2 \pm 1.6$ 2 min after exit from the water (Fig. 2G). Hb concentration was never significantly different from control values.

*Fos data.* Neurons immunolabeled with Fos protein were noted in numerous cell groups in the brain stem after prolonged submersion of 90 –100 s (Fig. 3). Many of these nuclei were similar to those labeled during voluntary diving for shorter time periods (90). These nuclei included the caudal pressor area (CPA; Figs. 3A and 4, C and D), the MDH (Figs. 3, D–E,

Fig. 2. Line graphs illustrating the mean changes in hemodynamics and blood chemistry during prolonged underwater submersion. A: there was a dramatic bradycardia with submersion, which persisted while the rat was underwater. B: there also was a slight increase in mean ABP (MABP) initially, but pressure fell during later stages of submersion before rising to control levels after the rat emerged and started breathing. PaCO$_2$ rose dramatically (C), while PaO$_2$ (D) and arterial oxygen saturation (SaO$_2$; E) fell sharply in the rats while underwater, reaching levels at which most mammals are induced to breathe. The blood also quickly became very acidic (F) with great loss of base excess (BE; G). H: hemoglobin (Hb) fluctuated nonsignificantly during the period of underwater submersion and afterwards. The first point represents data from rats free in their home cage, while the second data point is after their enclosure in the restraining tube. Dashed lines mark time either just before submersion or out of the water. The four middle data points represent time underwater; each slash marks $\sim 20$ s. Points without error bars represent very small standard errors. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. 
and 4, A–D), the lateral reticular nucleus, pars parvocellularis, subnuclei of the nucleus tractus solitarii (NTS) near the level of the obex (Fig. 4, D–E), as well as its subnucleus commissuralis (Figs. 3C and 4, B and C), the rostroventrolateral medulla (RVLM; Figs. 3G and 4F), the lateral medulla (Fig. 4, C–F), the nucleus raphe pallidus (Figs. 3H and 4, C–G), the nucleus raphe magnus and adjacent nucleus gigantocellular reticular nucleus, pars alpha (GiA; Fig. 4G), the A5 area (A5), the

Fig. 3. Photomicrographs illustrating the activation of numerous brain stem neurons by prolonged underwater submersion. Numerous neurons were immunolabeled for Fos in the caudal pressor area (CPA; circled; A) and pars commissuralis of nucleus tractus solitarii (NTS; C). The rostral medullary dorsal horn showed abundant Fos label in both its laminar (D) and alaminar parts (E); these areas closely match the terminal fields of the anterior ethmoidal nerve (outlined). The rostral ventrolateral medulla (RVLM), where many neurons modulating ABP lie, showed intense immunolabeling (G); the boxed area shows the area we defined as the RVLM. Immunolabeling of cells on the ventral surface of the medulla, presumptive chemoreceptors, were robustly labeled for Fos throughout the medulla (B); the parapyramidal nucleus (PP) is shown here. Other cells on the brain stem surface also were activated by the prolonged hypoxia, hypercapnia, and academia, including the ectotrigeminal nucleus (E5; large arrows; F), the raphe pallidus (RPa; H), and a layer of cells covering the medial vestibular (MVe; I.1) and ventral cochlear nuclei (VCn; I.2). Similar cells were also noted in the dorsal half of the epithelium lining the central canal (C). Note that this epipia on the MVe and VCn looks similar to that seen on the ventral medullary surface (see Fig. 6). Neurons in the superior salivatory nucleus (SSn; J) were immunolabeled with Fos after these prolonged breath holds. Neurons in the caudal pons, including those of the A5 area and others surrounding the trigeminal nerve root (m5; K) and the locus coeruleus (LC; L), were well-labeled with Fos. Also note numerous small neurons labeled in the reticular formation just dorsal to the reticulotegmental nucleus (L; arrows). See Glossary for definitions of acronyms.
The mammalian diving response is a dramatic perturbation of normal autonomic function and may be the most powerful autonomic response known. Our laboratory has previously documented the cardiovascular dynamics of this response in the rat (92), as well as some of the neural pathways that involve central chemoreceptors or general chemoreceptors (32, 81). We thus compared the Fos data to laboratory reference sections immunostained with antibodies against Neun, a protein that marks neurons. Indeed, the thickened epi-pia covering most of the fourth ventricle and cochlear nuclei showed no immunostaining with Neun (Fig. 5A; arrows), and none of the smaller profiles on the ventral medullary surface was stained. What was seen, however, were neurons immunostained with Neun within the epi-pia. These more often were isolated (Fig. 5C; arrows), but sometimes were clumped, especially close to exits of cranial motor nerves, particularly the trigeminal (Fig. 3K), glossopharyngeal/vagus, and hypoglossal (Fig. 5H; arrows) nerves. Moreover, this distribution of Neun-stained profiles mimicked that of the larger profiles immunostained with Fos (Fig. 5, F and K; arrows), suggesting that these larger profiles were chemoreceptive neurons, probably respiratory, while the smaller immunoreactive profiles were general chemoreceptors. While there was no immunostaining of neurons with Neun in the thickened glia layer over the cochlear nucleus or lining the fourth ventricle, there were several neurons immunostained against both Fos and Neun juxtaposed to this membrane in the NTS and LC. Neurons in the retrotrapezoid nucleus (Rtz) were well labeled in these animals brought past their ADL [compare the immunoreactivity of these neurons immunostained for Neun (Fig. 5B) with those immunostained for Fos (Fig. 5E)] as were those in the paraventricular (POI; Figs. 5, G and J) and parapyramidal (Fig. 5, H and K) nuclei (PPy).

**Tract Tracing Experiments**

Numerous labeled fibers of small diameter emerged medi ally from the injection of BDA in the ventral MDH (Fig. 5I, inset); such injections were targeted in the area innervated by the central processes of the anterior ethmoidal nerve (86, 91). These small fibers were directed along the ventral surface of the medulla at the level of the caudal pole of the inferior olivary complex and lateral reticular nuclei (Fig. 5I), where they contained numerous swellings. These swellings were found both in the thickened epi-pia membrane, as well as juxtaposed to triangular-shaped neurons close to the ventral surface (Fig. 5, L1 and L2). The rostrocaudal length of this projection was only ~500 μm. A line drawing on one such section is shown (Fig. 5M).

**DISCUSSION**

The mammalian diving response is a dramatic perturbation of normal autonomic function and may be the most powerful autonomic response known. Our laboratory has previously documented the cardiovascular dynamics of this response in the rat (92), as well as some of the neural pathways that involve central chemoreceptors or general chemoreceptors (32, 81). We thus compared the Fos data to laboratory reference sections immunostained with antibodies against Neun, a protein that marks neurons. Indeed, the thickened epi-pia covering most of the fourth ventricle and cochlear nuclei showed no immunostaining with Neun (Fig. 5A; arrows), and none of the smaller profiles on the ventral medullary surface was stained. What was seen, however, were neurons immunostained with Neun within the epi-pia. These more often were isolated (Fig. 5C; arrows), but sometimes were clumped, especially close to exits of cranial motor nerves, particularly the trigeminal (Fig. 3K), glossopharyngeal/vagus, and hypoglossal (Fig. 5H; arrows) nerves. Moreover, this distribution of Neun-stained profiles mimicked that of the larger profiles immunostained with Fos (Fig. 5, F and K; arrows), suggesting that these larger profiles were chemoreceptive neurons, probably respiratory, while the smaller immunoreactive profiles were general chemoreceptors. While there was no immunostaining of neurons with Neun in the thickened glia layer over the cochlear nucleus or lining the fourth ventricle, there were several neurons immunostained against both Fos and Neun juxtaposed to this membrane in the NTS and LC. Neurons in the retrotrapezoid nucleus (Rtz) were well labeled in these animals brought past their ADL [compare the immunoreactivity of these neurons immunostained for Neun (Fig. 5B) with those immunostained for Fos (Fig. 5E)] as were those in the paraventricular (POI; Figs. 5, G and J) and parapyramidal (Fig. 5, H and K) nuclei (PPy).

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**DISCUSSION**

The mammalian diving response is a dramatic perturbation of normal autonomic function and may be the most powerful autonomic response known. Our laboratory has previously documented the cardiovascular dynamics of this response in the rat (92), as well as some of the neural pathways that
mediate these responses (70, 72, 73, 86–88, 90, 91, 93, 94, 96–99, 130). While the homeostatic baroreceptor reflex apparently is inhibited during diving (73), less is known about the inhibition of the respiratory chemoreceptor reflex. We present data herein for the first time on activation of presumptive central chemoreceptors and illustrate a neural pathway that may initiate inhibition of the homeostatic chemoreceptor reflex.

Technical Considerations

Occlusion of a carotid artery with the cannula for blood sampling may have disrupted hemispheric cerebral blood flow (16) and biased our results. However, the rats recovered for 2–3 days postoperatively, and cerebral blood flow is not significantly different 24 h after carotid occlusion in normocapnic rats, but is after 15 min of hypercapnia (16). Moreover, brain stem tissues are mostly perfused by the vertebral arteries, which branch from the subclavian arteries. Several studies have shown the diving response persists after transecting the brain stem through the thalamus (22), colliculi (52, 67, 92), or pons (98), suggesting the neural circuits driving this response are intrinsic to the brain stem and were not compromised by unilateral carotid occlusion.

Approximately 2 ml of blood were withdrawn from these rats over the experimental period of 3–4 min, inducing a hypovolemia of <10% (estimating the total blood volume of our rats to be ~24 ml). The volume withdrawn was considerably less than other studies investigating hemorrhage, where 30–40% of volume is commonly withdrawn (see Ref. 12 for references). Nevertheless, hypovolemia may have contributed to the slight increase in HR and decrease in MABP toward the end of the submersion period.

ADL. All cells in a mammalian organism’s body need oxygen to survive. Such oxygen is derived from inspired air and exchanged for carbon dioxide, the waste product of aerobic metabolism, in the lungs. Terrestrial animals mostly utilize aerobic metabolic pathways, maintaining levels of carbon dioxide, oxygen, and pH in the blood within tight limits by adjusting ventilation. The metabolism of aquatic mammals, such as seals is similar, but these animals routinely perform underwater breath holds of unusual length, denying the replenishment of oxygen for their cells. These apneic periods, combined with massive cardiac parasympathetic outflow of the diving response, may result in the numerous arrhythmias seen in the present study. Some of the bradycardia from these restrained rats also may be the result of extreme fear, which also induces significant bradycardia and increases in ABP (11, 36, 119–121, 131). However, the bradycardia noted herein was locked tightly to the time submerged, and we speculate it was the result of underwater submersion rather than fear.

MABP rose early in the submersion period, similar to our laboratory’s previous observations (92). However, it fell precipitously as normal blood gases eroded, before it again stabilized. The etiology of this drop in arterial pressure is unknown, but could have been the result of either a fall in peripheral resistance or of increased vasoconstriction near the opening of the cannula of the biotelemetric transmitter measuring ABP.

Changes in blood chemistry. It is well known that hypercapnia, hypoxia, and acidity induce increases in ventilation. The acute hypercapnia and hypoxia seen in the submerged rats in the present study was dramatic: PaCO₂ rose to 79.2 Torr in one case, and PaO₂ dropped to 15.7 Torr in another. Studies have shown that small increases in PaCO₂ induce vigorous ventilation (55, 80), while decreases in PaO₂ (105) and pH also increase ventilation. pH dropped from 7.51 in control periods to 7.18 at the end of submersion, comparable to that previously reported by our laboratory in rats (92). This bradycardia lasted throughout the period of submersion, similar to that seen in aquatic animals (3, 15, 30, 59, 61). It should be noted, however, that there was a slight increase in both HR and MABP upon entering the restrictive tube, as well as numerous cardiac arrhythmias, much different than the regular cardiac rhythms seen after trained rats voluntarily dive without the tight quarters of the restraining tube (92). Such tubes usually induce restraint stress, resulting in a tachycardia, increases in arterial pressure, and arrhythmias (57, 79, 116, 117). The sympathetic input from this stressor, combined with massive cardiac parasympathetic outflow of the diving response, may result in the numerous arrhythmias seen in the present study. Some of the bradycardia from these restrained rats also may be the result of extreme fear, which also induces significant bradycardia and increases in ABP (11, 36, 119–121, 131). However, the bradycardia noted herein was locked tightly to the time submerged, and we speculate it was the result of underwater submersion rather than fear.

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measurements of tensions of oxygen, carbon dioxide, and pH are difficult to compare to results seen in the rats herein. Moreover, measurements of variables in blood chemistry of naturally diving feral mammals is biased by parameters such as compression hyperoxia, when the animals dive to great underwater depths, dive duration, or the “intent” of the dive (e.g., foraging or drift dives). Mean PaO₂ and SaO₂ values after submersion in the present study were 21.8 ± 2.2 Torr and 17.6 ± 2.8%, respectively, which are comparable to the minimum means of 22 ± 15 Torr and 28 ± 25.5% seen in diving elephant seals (75). However, the caveats in such comparisons are that the elephant seals dove underwater for variable times to different depths, they apparently never reached their ADL, and oxygen saturations were calculated at pHs of either 7.4 or 7.3, depending on length of the dive.
The arterial pH of the submerged rats was 7.18 ± 0.02 at the end of submersion, but continued falling to 6.99 ± 0.02 2 min later. These measurements of acid-base balance dropped after emersion, creating a combined respiratory and metabolic acidosis, probably due to a massive release of lactate produced by anaerobic metabolism during the later stages of submersion and subsequent to release of the sympathovasconstrictor. Nevertheless, the rats in the present study did not breathe while underwater, despite all of these stimulants. Studies of cellular or tissue metabolism in extreme hypoxia, hypercapnia, and pH states may find this rat model advantageous, since the study is easily controlled in the laboratory.

Spleens store numerous red blood cells and thus act as a reservoir of bound oxygen. Indeed, the spleens of naturally diving mammals are proportionately larger than those of terrestrial animals. Splenic contraction is prominent during dives in many species (10, 31, 113, 123, 124), prompting suggestions that such contractions liberate more Hb-bound oxygen into the circulation and thus curb the oxygen hunger of cells during underwater submersion. There were variable fluxes in hematocrit of the submerged rats in the present study, but none was significantly different from control values. Moreover, the Hb concentration did not change from baseline, suggesting that rats’ spleens either are not large reservoirs or fail to contract during underwater submersion.

Fos studies. The principal chemoreceptors regulating respiration are found in the carotid body and the central nervous system. It is thought that glomus cells in the peripheral carotid body mainly modulate ventilation to hypoxic conditions (40, 63, 106), while central chemoreceptors induce ventilation in response to hypercapnic and acidicotic conditions (32, 81). The carotid sinus nerve carries impulses monitoring changes in blood gases and pH and has its major projection into the caudal NTS of the medulla (1, 34, 50, 95). Physiological and Fos studies show that neurons in the commissural subnucleus of the NTS are especially sensitive to hypoxia in the peripheral blood (4, 42, 48, 100, 101, 118). It is thus of interest that considerably more neurons were labeled in subnuclei of the NTS near the calamus scriptorius in the present study vs. those labeled in voluntary diving behavior (90). The average time of submergence of voluntary dives (~20 s) seen in our laboratory’s previous studies (72, 90, 92) coincides with periods in which blood gases had changed little (Fig. 2), but the more prolonged dives reported herein should have vigorously stimulated peripheral chemoreceptors and induced formation of the Fos protein in the caudal NTS.

Cells on the ventral surface of the medulla originally were proposed as the locus of central chemoreceptors, whose activation induced increases in ventilation (76, 77, 114). This view has been expanded widely to include neurons in the raphe (83, 109, 110), the LC (19, 85), the NTS (19, 20), parapyramidal/paraventricular areas (2, 5, 78, 108), the Rtz (32, 44, 45, 80, 82), and many other neurons in between (13, 56, 80). Although these neurons are all responsive to chemical stimuli, it is uncertain if all directly influence the respiratory network (46, 111).

We observed the thickened glia on the ventral surface of the medulla described previously (84, 125, 126) and noted that nuclei immunostained for Fos were especially aggregated near the exits of the hypoglossal nerve medially (included in yellow ovals, Fig. 6), but also near the ventrolateral exit of the glossopharyngeal/vagus nerves (red oval in Fig. 6). Numerous blood vessels also penetrated the pial surface ventrolaterally near the penetration of the IX-X cranial nerves, as well as along the midline raphe, where numerous Fos-labeled nuclei were located (blue markers in Fig. 6). The nuclei immunostained for Fos protein in the superficial ventral medulla after prolonged submersion were of two sizes: the smaller nuclei mostly were embedded in the epi-pia layer, while the larger nuclei Compared favorably qualitatively, quantitatively, and in location with the superficial neurons noted in our Neun immunostained material. While the cells with small nuclei obviously were activated by prolonged underwater submersion and are potential chemoreceptors, they apparently are not neurons and thus potentially peripheral to the respiratory network. These “general chemoreceptors” in their thick pia matrix may, however, still be important in modulating respiratory output (49). The fact that numerous swellings from fibers labeled after BDA injections of the ventral MDH were found in this thickened epi-pia layer suggests that these fibers may modulate the activity of these putative chemoreceptors and inhibit ventilation. The carotid body similarly is composed of numerous glomus cells, which sense changes in blood chemistry, yet are not considered neurons. However, glomus cells depolarize primary afferent fibers in the carotid sinus nerve. It is possible that these smaller cells in the epi-pia on the ventral surface of the medulla perform similar duties. Moreover, presumptive neurons, possibly respiratory chemoreceptors (41), near the ventral surface, were juxtaposed by swellings from the MDH injections. We suggest these neurons directly modulate the respiratory network. In this regard, it is of interest that application of a local anesthetic to similar areas in the cat induces an apnea (125–127).

Neurons in many brain stem nuclei were labeled with Fos protein herein, including the CPA, peribulbar NTS, MDH, RVLM, LC, SSn, lateral medulla, and A5 area. While many of

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**Fig. 5.** Photomicrographs of sections illustrating putative chemoreceptors and projections from the medullary dorsal horn to the ventral surface of the medulla. A: immunostaining for Neun, a neuronal marker, is shown through the ventral cochlear (VC) nucleus. Compare A to Fig. 3I; note that there are no cells labeled with Neun, suggesting no neurons in this superficial layer. Numerous brain stem neurons were labeled showed Fos immunoreactivity in rats brought close to their aerobic dive limit, suggesting many of them were chemoreceptors. The retrotrospadix nucleus (Rtz) contained numerous Fos-labeled neurons after underwater submersion (E: surrounded by oval; arrows point to Fos-labeled neurons); their distribution was similar to neurons labeled with Neun (B: surrounded by oval; arrows point to Neun-labeled neurons). Numerous cells or on near the ventral surface of the medulla were labeled with Fos antibodies (F and K; arrows), but comparing these sections to others immunostained with Neun (C) suggests that many of the smaller labeled profiles are not neurons. We considered only the larger profiles stained with antibodies against Fos as neurons; numerous such profiles are seen in the parapyramidal nucleus (PPy; arrows; D and K), the ventral surface of the medulla (F; arrows), and the paraventricular nucleus (POAI; oval; J). Compare the distribution of these profiles to those in Neun-stained material (G and H). injections of biotinylated dextran amine (BDA) into the ventral MDH (I, inset) resulted in numerous fibers with swellings coursing in along the caudal ventral medullary surface (I) bilaterally. These small fibers displayed numerous swellings both ipsilateral (L1) and contralateral (L2) to the injection. Arrows in I point to two neurons, possibly respiratory chemoreceptors, shown at higher magnification in L1. Note that many of the fibers course within a gelatinous-appearing “epi-pia”, covering the neuropil (L2). A line drawing illustrating these fibers with varicosities is shown in M. See Glossary for definitions of acronyms.
these also may influence the respiratory network, we speculate most may be more important in the sensory and cardiovascular pathways driving the diving response (70, 72, 73, 86, 87, 90–94, 96, 97, 130). Moreover, these same nuclei were labeled after voluntary diving for shorter periods (90).

Several studies have described gap junctions in central chemoreceptors (17, 122). Such coupling provides fast transmission of a message but provides little access to modulation. It also suggests a minimal synaptic input may influence a large matrix of cells. Thus it is significant that the projection described herein is only to the most caudal aspect of the ventral medulla. It also is significant that neurons coupled by gap junctions are more prominent in fetal and neonatal brains (18, 51). An inhibitory signal from the MDH to such caudal medullary chemoreceptors potentially could inhibit the chemoreceptor reflex in diving mammals and prevent them from breathing.

The diving response is a physiological stressor to voluntary diving rats, but rats dunked underwater involuntarily also may feel anxiety stress (9, 92). Although these neuroanatomic studies cannot describe the function of any of these neurons labeled with Fos after prolonged submersion, there was a dearth of labeled nuclei in brain stem areas where respiratory-related neurons are described to lie. However, numerous neurons in all nuclei where presumptive chemoreceptors are found were reactive to Fos antibodies in the rats exceeding their ADL described herein. We monitored the blood chemistry during these prolonged dives and show that the gross alterations of it should have activated numerous respiratory chemoreceptors. Nevertheless, the rats neither gasped nor breathed.

Summary

There has been much debate concerning the stimulus, location, and projections of respiratory chemoreceptors. The incongruity of ideas no doubt rests in part on the multiple approaches used to analyze the putative receptors and a clear interpretation of how the respiratory network works. We have shown herein that despite radical changes in arterial levels of partial pressures of oxygen and carbon dioxide, as well as significant changes in pH, the rats did not breathe while underwater. Immunohistochemistry against Fos suggests numerous neurons in the brain were activated, including those in areas like the commissural subnucleus of the NTS, the ventral surface of the medulla, the midline raphe, and the PPy and Rtz. All of these areas are known loci where either peripheral chemoreceptors project or central chemoreceptors lie.

The mammalian diving response is a complex configuration of numerous independent reflexes regulating respiration, HR, and blood pressure and initiated by a somatic stimulus. Aquatic
mammals develop more intense diving responses as they mature, providing credence to such statements as “practice makes perfect” or “use it or lose it.” Humans are mammals and also possess a diving response (31, 33, 35, 113), albeit the response in adults is not as profound as in aquatic mammals. However, the diving response is brisk in human neonates (38). Numerous incidents of “cold-water drowning” have been documented in humans (37, 39, 47, 129), where humans lie submerged underwater for extended periods, yet recover with minimal functional loss. We speculate this is similar to our rats brought past their ADL. We feel, however, that the cardiovascular responses occur merely to buffer the deficit of breathing. Our data also support the hypothesis that the diving response, or similar cardiorespiratory responses elicited by nasal CO2 (130), could support the hypothesis that the diving response, or similar cardiorespiratory responses elicited by nasal CO2 (130), could be a major factor dooming children in sudden infant death syndrome. Nevertheless, our data give new emphasis to the diving response as “the master switch of life” (115). These data support our contention that the diving response is the most profound autonomic response known.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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