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

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Abstract

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 submergence 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 PaCO₂ rose to 62.4 mmHg, while mean PaO₂ and pH reached nadirs of 21.8 mmHg 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.
Introduction

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., 2010, 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., 2009, 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) towards hypoxia, hypercapnia, and acidosis. Bert was the first to show that rats die after approximately 2 minutes underwater (53), suggesting their aerobic dive limit 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., 2006, 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 brainstems 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 brainstem circuits inhibiting ventilation are activated as well. These data
support ideas of the diving response being the most powerful autonomic response known, as well as fetal reflexes as a cause of the Sudden Infant Death Syndrome (64).
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A1</td>
<td>noradrenergic cell group of caudal medulla</td>
</tr>
<tr>
<td>A5</td>
<td>noradrenergic cell group in ventrolateral pons</td>
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<tr>
<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>AP</td>
<td>area postrema</td>
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<tr>
<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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<tr>
<td>Cu</td>
<td>cuneate nucleus</td>
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<tr>
<td>E5</td>
<td>ectotrigeminal nucleus</td>
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<tr>
<td>Gr</td>
<td>gracile nucleus</td>
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<tr>
<td>GiA</td>
<td>gigantocellular reticular nucleus, pars alpha</td>
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<tr>
<td>Hb</td>
<td>hemoglobin</td>
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<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>IO</td>
<td>inferior olivary nucleus</td>
</tr>
<tr>
<td>LC</td>
<td>nucleus locus coeruleus</td>
</tr>
<tr>
<td>LRt</td>
<td>lateral reticular nucleus</td>
</tr>
<tr>
<td>LRtPC</td>
<td>lateral reticular nucleus, pars parvocellularis</td>
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<tr>
<td>MDH</td>
<td>medullary dorsal horn</td>
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<tr>
<td>Me5</td>
<td>nucleus of the mesencephalic tract of the trigeminal nerve</td>
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<tr>
<td>MABP</td>
<td>mean arterial blood pressure</td>
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<tr>
<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>Pa5</td>
<td>paratrigeminal nucleus</td>
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<tr>
<td>PaCO₂</td>
<td>partial pressure arterial carbon dioxide</td>
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<tr>
<td>PaO₂</td>
<td>partial pressure arterial oxygen</td>
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<tr>
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<tr>
<td>Pr5</td>
<td>principal trigeminal nucleus</td>
</tr>
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<td>POI</td>
<td>paraolivary nucleus</td>
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<tr>
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<td>parapyramidal nucleus</td>
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<td>reticulotegmental nucleus of the pons</td>
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<td>Rtz</td>
<td>retrotroapezoid nucleus</td>
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<td>RVLM</td>
<td>pressor area of the rostral medulla</td>
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<tr>
<td>SaO₂</td>
<td>arterial oxygen saturation</td>
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<tr>
<td>Sol, NTS</td>
<td>nucleus tractus solitarii</td>
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<td>SO</td>
<td>superior olivary nucleus</td>
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<tr>
<td>SpVe</td>
<td>spinal vestibular nucleus</td>
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<tr>
<td>SSpn</td>
<td>superior salivary nucleus</td>
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<td>SpSI</td>
<td>nucleus of the spinal tract of the trigeminal nerve, interpolar part</td>
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<tr>
<td>SpSO</td>
<td>nucleus of the spinal tract of the trigeminal nerve, oral part</td>
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<tr>
<td>SpVe</td>
<td>spinal vestibular nucleus</td>
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<tr>
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<td>pyramidal tract</td>
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<tr>
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<td>tractus solitarii</td>
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<tr>
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<td>spinal tract of the trigeminal nerve</td>
</tr>
<tr>
<td>7</td>
<td>facial motor nucleus</td>
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<tr>
<td>7n</td>
<td>facial nerve root</td>
</tr>
<tr>
<td>12</td>
<td>hypoglossal motor nucleus</td>
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Materials and Methods
Aerobic Dive Limit 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 biotelemetric transmitter (Model PA-C40; Data Sciences International, DSI; St. Paul, MN USA) 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, cut, and polyethylene tubing (PE 50) 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 both prior to, 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 submergence.

Blood chemistry data

Experiments commenced on each rat individually 2-3 days after the placement of the carotid cannulas. After attaching a sterile 1ml heparinized syringe to the carotid cannula, approximately 300µl of blood (baseline sample) was 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 300µl blood withdrawn and stored in ice. Both the rat and tube then were submerged underwater (29-32°C) for 90-100 sec. Blood was continuously withdrawn during this period, each syringe taking approximately 20 sec to fill and be replaced with another. The rats were removed from the tube and another 300µl of arterial blood withdrawn at approximately 30 sec, 2 min, and up to 20 min after emergence from the water. All blood samples were placed immediately on ice after collection. Although 3 rats were unconscious and not breathing after their removal from the tube, they were resuscitated within secs 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 partial pressures of arterial oxygen (PaO2), arterial carbon dioxide (PaCO2), arterial oxygen saturation (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 two hours after submersion. They then were deeply anesthetized (Sleepaway, 0.1ml/100g; 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 0.1M 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 prior to cutting frozen transverse sections (40µm) 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, Inc.) mixed in 0.1M PB with 0.3% Triton. On the following day, the sections were washed, and then incubated
for one hour in goat anti-rabbit biotinylated secondary IgG (1:500; Vector Labs), washed, and
then incubated in an ABC complex (Vectastain Elite; Vector Labs) for another hour. The Fos
antigen was visualized in the brainstem with the chromogen diaminobenzidine (DAB) enhanced
with nickel ammonium sulfate catalyzed by hydrogen peroxide. Sections were mounted serially
on gelatin-coated slides, counter-stained 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 brightfield 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 brainstem nuclei were photographed digitally (MicroImager II)
with Northern Eclipse Software (Empix, Inc.). Sections were drawn with a Nikon E600
microscope interfaced with Neurolucida software (MicroBrightField, Inc.). The figures were
standardized in Adobe Photoshop software (v.7) using levels, brightness and contrast and
aligned in Adobe Illustrator software (v.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 dorsal incision and the acromiotrapezius
muscle split along the dorsal midline. After retracting the deep neck muscles laterally, the
medulla oblongata was exposed after cutting the dura. Micropipettes (20 ~ 25 µm OD) were
filled with 10% biotinylated dextran amine (BDA; Molecular Probes; 10,000 MW) in saline and
lowered into the ventral MDH. The carrier was angled anteriorly at 24° from vertical; the
coordinates were 0.5 mm rostral to calamus scriptorius, 2.4-2.6 mm lateral to the midline and
1.4mm ventral from the dorsal surface of the brainstem. Tracers were deposited in the brain with a positive current (5mA; 7 sec on/off) via a silver wire inserted into the micropipette for 10-15 min using a constant current device (MidGuard). The micropipette was left in place for 5 min after the injections. Wounds were washed with sterile saline and closed with silk.

After survivals of 7 to 10 d, the animals were deeply anesthetized and perfused, their brains and spinal cords removed, refrigerated in fixative with 20% sucrose and sectioned frozen (vide supra). A 1:3 series was then processed for BDA. Sections were washed three times with 0.1 M PB for 10 min, and then in 0.1 M PB with 0.3% triton for at least 5 min. The sections then were incubated to visualize the immunoprecipitate. The sections finally were rinsed, mounted on gelatinized slides, air-dried, counterstained with Neutral Red, dehydrated in alcohols, defatted in xylenes, and coverslipped with Permount. Sections were examined and reconstructed as above. Varicosities on labeled fibers were considered synaptic boutons.

**Data Analysis**

Means and standard errors (M ± S.E.) were determined for both hemodynamic and blood gas measurements and compared to control values for significance (SPSS software; v. 15). Cardiovascular and blood chemistry parameters during submersion were compared to data considered control. Control data was taken from the rats in the physiological experiments when they freely roamed in their home cages prior to 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 M ± S.E. and significance was calculated as p<0.05.

**Results**

All rats showed a marked drop in heart rate (HR) and an immediate but temporary increase in arterial blood pressure (ABP) to prolonged involuntary submersion (Fig. 1). Numerous brainstem 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.

**Aerobic Dive Limit Experiments**

**Cardiovascular data**

Both the bradycardia and the increase in ABP seen with underwater submersion were significantly different from control values obtained prior to submersion (Figs. 2A, 2B). Heart rate reached a nadir of 106±7 bpm in submerged rats, significantly (p<0.001) different from control values of 461±11 bpm (Fig. 2A). The bradycardia was maintained throughout the period of submergence compared to predive, but was less intense during the last 10 sec 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 sec the rats were underwater (Fig. 1B1). 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. Upon emersion, the heart beats again became arrhythmic after respirations 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).

Mean arterial blood pressure rose from 115±2 mmHg in control periods to 124±3 mmHg after approximately 30 sec underwater (Fig. 2B), but then fell significantly (p<0.05) to 98±4 mmHg as the aerobic dive limit was approached (Figs. 1A, 2B). MABP returned to control levels by two minutes 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 mmHg and peaked significantly at 62.4±3.7 mmHg at the end of the submersion period (Fig. 2C). PaO₂ dropped from 93.1±1.6 mmHg at control times to 21.8±2.2 mmHg 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 percent at the end of submersion (Fig. 2E). All these parameters approached control values within two minutes 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 at the end of submersion and continued falling to 6.99±0.02 two minutes later (Fig. 2F). Similarly BE initially fell significantly (p<0.01) to -5.7±0.6 after 20 sec submersion from a control value of 0.86±0.4, and continued to plummet rapidly to -18.2±1.6 two minutes after exit from the water (Fig. 2G). Hemoglobin concentration was never significantly different from control values.

**Fos data**

Neurons immunolabeled with Fos protein were noted in numerous cell groups in the brainstem after prolonged submersion of 90-100 sec (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, 4C-D), the medullary dorsal horn (MDH; Figs. 3D-E, 4A-D), the lateral reticular nucleus, pars parvocellularis, subnuclei of the nucleus tractus solitarii (NTS) near the level of the obex (Figs. 4D-E) as well as its subnucleus commissuralis (Figs. 3C, 4B-C), the rostroventrolateral medulla (RVLM; Figs. 3G, 4F), the lateral medulla (Figs. 4C-G), the nucleus raphe pallidus (RPa; Figs. 3H, 4C-G), the nucleus raphe magnus and adjacent nucleus gigantocellular reticular nucleus, pars alpha (Fig. 4G), the A5 area (A5), the superior salivatory nucleus (SSn; Fig. 3J) and the nucleus locus coeruleus (LC; Figs. 3L, 4H). Fos protein also was immunolabeled in the medial and spinal vestibular nuclei (Figs. 4F-G), the ventral cochlear nuclei (Figs. 3L.2) and the dorsal tegmental nucleus (Figs. 3L, 4H) in the present study. Presumptive catecholaminergic neurons were labeled in all submerged rats; these have been discussed previously (72). We also will not report herein on labeling in the Kölliker-Fuse nucleus, and the external lateral and superior lateral subnuclei of the parabrachial complex since the sections containing these nuclei were lost in three cases during the sectioning procedure. Although we did not quantify these neurons labeled with Fos in the present study, it
was our qualitative perception that the number of labeled neurons was greater than that reported previously (90).

The rats in the present study were submerged for 90-100 sec, which was approximately 70-80 sec longer than previous studies (90, 92). This prolonged submersion induced radical changes in blood chemistry (Fig. 2) and such changes presumably activated both central and peripheral chemoreceptors. Numerous brainstem cells thought either to be putative respiratory central chemoreceptors or general chemoreceptors (32, 81) were labeled with Fos after these prolonged submersions. Numerous cells on the ventral surface of the medulla were labeled (Fig. 3B; arrows) from the level of the pyramidal decussation to the caudal pons.

Many of these cells were located in a thickened ‘epi-pia’ (128), which was most prominent along the ventral medullary surface from the lateral border of the pyramidal tract medially to the medial border of the spinal trigeminal tract laterally (Fig. 4, arrows), lining the fourth ventricle (Figs. 3I.1;4E-H), and over the cochlear nuclei (Fig. 3I.2). These immunoreactive profiles were generally of two sizes and we questioned whether all were neurons. We thus compared the Fos data to laboratory reference sections immunostained with antibodies against Neun, a protein which 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 were 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), glosso-pharyngeal/vagus, and hypoglossal (Fig. 5H; arrows) nerves. Moreover, this distribution of Neun-stained profiles mimicked that of the larger profiles immunostained with Fos (Figs. 5F, 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 were well-labeled in these animals brought past their aerobic dive limit [compare the immunoreactivity of these neurons immunostained for Neun (Fig. 5B) with those immunostained for Fos (Fig. 5E)] as were those in the paraolivary (Figs. 5G, J) and parapyramidal (Figs. 5H, K) nuclei.

**Tract tracing experiments**

Numerous labeled fibers of small diameter emerged medially from the injection of BDA in the ventral MDH (Fig. 5I; insert); 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 (Figs. 5L.1, L.2). The rostrocaudal length of this projection was only approximately 500µm. 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. We previously have documented the cardiovascular dynamics of this response in the rat (92), as well as some of the neural pathways which 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 putative central chemoreceptors and illustrate a neural pathway which 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 twenty-four hours after carotid occlusion in normocapnic rats, but is after 15 minutes of hypercapnia (16). Moreover, brainstem 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 brainstem through the thalamus (22), colliculi (52, 67, 92) or pons (98), suggesting the neural circuits driving this response are intrinsic to the brainstem and were not compromised by unilateral carotid occlusion.

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

Aerobic dive limit

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 the cardiovascular adjustments result in their profound diving response to underwater submersion. The massive peripheral vasoconstriction reduces or eliminates perfusion of non-essential organs, saving oxygen in the blood for the heart and brain.
The heart slows considerably, reducing cardiac output and inducing profound reflex peripheral vasoconstriction to maintain central arterial pressure (54). It is thought diving mammals generally utilize these cardiovascular adjustments for maintenance of levels of gases in their blood necessary for aerobic metabolism. Indeed, the dives of aquatic mammals generally are shorter in duration than the ADL, the animal surfacing from the water before anaerobic biochemical pathways are needed. However, prolonged underwater submersion activates anaerobic pathways, resulting in accumulations of its waste product, lactic acid (60, 62, 104). The result of prolonged underwater submersion is greatly lowered PaO₂ and pH, and much higher PaCO₂ and lactic acid.

Blood chemistry is difficult to measure in feral underwater mammals, but despite this Kooyman et al. (62) first documented the blood chemistry of diving seals and coined the term ‘aerobic dive limit’ (58, 60). This term marks the shift from aerobic to anaerobic metabolism and suggests the progressive release of lactic acid from cells. The ADL has been measured in only a few aquatic species, but has been calculated in more (60, 62, 103, 104), and to our knowledge that of terrestrial mammals has never been measured or calculated. Nevertheless, we report herein on the cardiovascular changes, blood chemistry, and activation of neurons of rats brought beyond their aerobic dive limit. However, the time needed for such a transformation of metabolism in the rat may have been accomplished with less than 100 sec of submersion herein. A detailed analysis of blood chemistry over different times is needed to define the ADL in the rat. For those studying hypoxemic tolerance of bodily tissues or regulation of tissue oxygen delivery and consumption, this rat model may circumnavigate the problems of variable depth, duration, and behavioral strategies seen in pinniped divers.

**Cardiovascular consequences of prolonged underwater submersion**

There was an immediate drop of heart rate upon submersion of 77%, comparable to that previously reported by us 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 arterial blood pressure (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.

Mean arterial blood pressure rose early in the submersion period, similar to our 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 arterial blood pressure.

**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;
\( \text{PaCO}_2 \) rose to 79.2 mmHg in one case and \( \text{PaO}_2 \) dropped to 15.7 mmHg in another. Studies
have shown that small increases in \( \text{PaCO}_2 \) induce vigorous ventilation (55, 80) while decreases
in \( \text{PaO}_2 \) (105) and pH also increase ventilation. pH dropped from 7.51 in control periods to 7.18
at the end of submersion while BE went from 0.86 mmol/L to -4.3 mmol/L in the present study.
Both pH and BE however, continued to drop after emersion from the water to 6.99 mmol/L and
-18.24 mmol/L, respectively. Documenting the progressive changes of blood chemistry in
diving aquatic mammals has been relatively rare due to the difficulty of such measurements.
Nevertheless, initial studies (43, 60, 62, 107) showed changes in several metabolites in blood but 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 PaO\(_2\) and SaO\(_2\) values after submersion in the present study were 21.8±2.2 mmHg and 17.6±2.8 percent, respectively, which are comparable to the minimum means of 22±15 mmHg and 28±25.5 percent 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 two minutes 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 sympatho-vasoconstriction. Nevertheless, the rats in the present study did not breathe while underwater despite all 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 hemoglobin-bound oxygen into the circulation, thereby curbing the oxygen hunger of cells during underwater submersion. There were variable fluxes in hematocrit of the submerged rats in the present study, but none were significantly different from control values. Moreover, the hemoglobin
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 hypercarbic and acidotic 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 versus those
labeled in voluntary diving behavior (90). The average time of submergence of voluntary dives
(~20 sec) seen in our previous studies (72, 90, 92) coincides to periods where 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 locus coeruleus (19,
85), the NTS (19, 20), parapyramidal/paraolivary areas (2, 5, 78, 108), the retrotrapezoid
nucleus (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
cells 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 brainstem nuclei were labeled with Fos protein herein, including the CPA,
periobex NTS, MDH, RVLM, LC, SSn, lateral medulla, and A5 area. While many of 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 neuroanatomical studies cannot describe the function of any of these neurons labeled with Fos after prolonged submersion, there was a dearth of labeled nuclei in brainstem 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 showed 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, the parapyramidal and retrotrapezoid nuclei. All 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, heart rate, 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 be a major factor dooming children in sudden infant death syndrome. Nevertheless, our data gives new emphasis to the diving response as “the master switch of life” (115). This data supports our contention that the diving response is the most powerful autonomic response known.

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**Figure Legends**

**Figure 1.** Traces illustrating the typical cardiovascular behavior of a submerged rat brought towards its dive limit. Top trace (A) illustrates changes in cardiovascular dynamics as well as arterial blood chemistry over a 250 sec experiment. Upward-pointing arrow on bottom indicates submersion while the downward-pointing arrow to the right marks exit from the water. There was an immediate bradycardia and increase in arterial blood pressure (ABP) upon submersion highlighted by numerous arrhythmias (the forty sec period marked 1 in A is seen on an expanded time scale in B1). After approximately 70 sec of submersion, ABP fell in all rats and the arrhythmias ceased (the 40 sec 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 sec 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.

**Figure 2.** Line graphs illustrating the mean changes in hemodynamics and blood chemistry during prolonged underwater submersion. There was a dramatic bradycardia with submersion, which persisted while the rat was underwater (A). There also was a slight increase in mean arterial blood pressure initially, but pressure fell during later stages of submersion before rising to control levels after the rat emerged and started breathing (B). Partial pressures of arterial carbon dioxide rose dramatically (C), while PaO2 (D) and SaO2 (E) fell sharply in the rats while underwater, reaching levels where most mammals are induced to breathe. The blood also quickly became very acidic (F) with great loss of base excess (G). Hemoglobin fluctuated non-significantly during the period of underwater submersion and afterwards. First point represents data from rats free in their home cage while the second data point is after their enclosure in the restraining tube. Broken lines mark time either just prior to submersion or out of the water. The four middle data points represent time underwater; each slash marks...
approximately 20 sec. Points without error bars represent very small standard errors. * = p ≤ 0.05, ** = p ≤ 0.01, *** p ≤ 0.001.

**Figure 3.** Photomicrographs illustrating the activation of numerous brainstem neurons by prolonged underwater submersion. Numerous neurons were immunolabeled for Fos in the caudal pressor area (A; circled) and pars commissuralis of nucleus tractus solitarii (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, where many neurons modulating arterial blood pressure 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). Other cells on the brainstem surface also were activated by the prolonged hypoxia, hypercapnia and acidemia including the ectotrigeminal nucleus (F; large arrows), the raphe pallidus (H), and a layer of cells covering the medial vestibular (I.1) and ventral cochlear nuclei (I.2). Similar cells were also noted in the dorsal half of the epithelium lining the central canal (C). Note that this epi-pia on the MVe and VCn looks similar to that seen on the ventral medullary surface (see Fig. 6). Neurons in the superior salivatory nucleus (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 (K) and the locus coeruleus (L), were well-labeled with Fos. Also note numerous small neurons labeled in the reticular formation just dorsal to the reticulotegmental nucleus (L; arrows).

**Figure 4.** Line drawings illustrating the position of neurons and cells immunolabeled with Fos after prolonged underwater submersion. All labeled cells were drawn in these sections. Arrows point to numerous labeled cells on the ventral surface of the medullas well as some on dorsal
and lateral surfaces. These include cells with both large (presumptive neurons) and small nuclei.

**Figure 5.** Photomicrographs of sections illustrating putative chemoreceptors and projections from the medullary dorsal horn to the ventral surface of the medulla. Immunostaining for Neun, a neuronal marker, is shown in A through the ventral cochlear nucleus. Compare Fig. 5A to Fig. 3I.1; note that there are no cells labeled with Neun, suggesting no neurons are in this superficial layer. Numerous brainstem neurons were labeled showed Fos immunoreactivity in rats brought close to their aerobic dive limit, suggesting many of them were chemoreceptors. The retrotrapezoid nucleus 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 on or near the ventral surface of the medulla were labeled with Fos antibodies (Figs. F, 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 (D, K; arrows), the ventral surface of the medulla (F; arrows), and the paraolivary nucleus (J; oval). Compare the distribution of these profiles to those in Neun stained material (Figs. G, H). Injections of BDA into the ventral MDH (I; insert) resulted in numerous fibers with swellings coursing in along the caudal ventral medullary surface (I) bilaterally. These small fibers displayed numerous swellings both ipsilateral (L.1) and contralateral (L.2) to the injection. Arrows in I point to two neurons, possibly respiratory chemoreceptors, shown at higher magnification in L.1. Note that many of the fibers coursed within a gelatinous-appearing ‘epi-pia’ covering the neuropil (L.2). A line drawing illustrating these fibers with varicosities is shown in M.
Figure 6. A montage of serial sections through the medulla from rostral (top) to caudal (bottom) showing the numerous cells labeled with Fos on its ventral surface after prolonged underwater submersion. Neurons in both the raphe nuclei (blue squares) and cells on the ventral surface of the medulla (green dots and red asterisks) are suggested to be putative chemoreceptors. While the present study cannot determine if any of these profiles represent chemoreceptors, we did notice that the Fos immunolabeled cells on the ventral surface were of two sizes. Thus, cells labeled with green dots on the medulla’s ventral surface represent the smaller immunolabeled profiles while those marked by red asterisks denote large stained profiles seen in our material. The red asterisks also were similar in size, number and distribution to neurons stained with Neun, implicating them as neurons, while the green dots were embedded in a thickened epi-pia layer (see text for details). The yellow ovals on the left side of the figure represent the original outlines proposed by Mitchell, Loeschcke and Schläfke (see text) which generally include the exit of the hypoglossal nerve, while the red oval marks an abundance of such profiles near the exit of the glossopharyngeal and vagus nerves. The most rostral black outline represents the level of the caudal facial nucleus while the more caudal black line represents the level of the obex.