invited review

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Neuronal sensitivity to hyperoxia, hypercapnia, and inert gases at hyperbaric pressures

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Dean, Jay B., Daniel K. Mulkey, Alfredo J. Garcia III, Robert W. Putnam, and Richard A. Henderson III. Neuronal sensitivity to hyperoxia, hypercapnia, and inert gases at hyperbaric pressures. *J Appl Physiol* 95: 883–909, 2003; 10.1152/japplphysiol.00920.2002.—As ambient pressure increases, hydrostatic compression of the central nervous system, combined with increasing levels of inspired P_{O_2}, P_{CO_2}, and N_2 partial pressure, has deleterious effects on neuronal function, resulting in O_2 toxicity, CO_2 toxicity, N_2 narcosis, and high-pressure nervous syndrome. The cellular mechanisms responsible for each disorder have been difficult to study by using classic in vitro electrophysiological methods, due to the physical barrier imposed by the sealed pressure chamber and mechanical disturbances during tissue compression. Improved chamber designs and methods have made such experiments feasible in mammalian neurons, especially at ambient pressures <5 atmospheres absolute (ATA). Here we summarize these methods, the physiologically relevant test pressures, potential research applications, and results of previous research, focusing on the significance of electrophysiological studies at <5 ATA. Intracellular recordings and tissue P_{O_2} measurements in slices of rat brain demonstrate how to differentiate the neuronal effects of increased gas pressures from pressure per se. Examples also highlight the use of hyperoxia (≤3 ATA O_2) as a model for studying the cellular mechanisms of oxidative stress in the mammalian central nervous system.

anesthesia; carbon dioxide toxicity; free radicals; high-pressure nervous syndrome; membrane potential; nitrogen narcosis; oxidative stress; oxygen toxicity; polarographic oxygen electrode

MOST HUMANS ARE PHYSIOLOGICALLY adapted to live and work near sea level, where ambient pressure is ~1 atmosphere absolute (ATA).[1] Nonetheless, we exploit a continuum of barometric pressure (P_B) ranging from the near vacuum of outer space, which we survive during Space Shuttle extravehicular activity by wearing a 4.3 lb./in.^{2} absolute (psia) pressure suit (30,300 ft. pressure equivalent, 0.29 ATA) (120, 220), down to the summit of Mt. Everest at 29,029 ft., where P_B is 0.31 ATA (221), to ocean depths as great as 2,300 ft. of sea water (fsw), where P_B increases to ~70 ATA (18). These situations are the pressure extremes of our inhabitable environment, which only relatively few highly trained individuals have ever occupied.

Military personnel, medical personnel, and other humans, however, frequently encounter levels of hyperbaric pressure (i.e., >1 ATA) of lesser degrees in their normal work environments. Examples of moderate hyperbaric environments (e.g., <5 ATA) include the following: patients and medical attendants undergoing hyperbaric O_2 therapy (HBOT) (38, 203); diving with an underwater breathing apparatus for recreational, professional (oil and salvage companies), and combat purposes (89); simulated dry and wet dives for hyperbaric research and dive training (217); and working in the compressed atmosphere of a subterranean environment (117). Abnormal work environments resulting from catastrophic accidents while at sea (32, 165) or underground (155) also can result in prolonged breathing of hyperbaric gases. For example, submariners

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[1]One ATA is the P_B at sea level, which is equivalent to 760 Torr. ATG is defined as chamber pressure minus room pressure; thus at sea level (1 ATA), 2 ATA inside a pressure chamber is 1 ATG, 3 ATA is equivalent 2 ATG, and so forth. Ambient pressure increases 1 atm for every 33 fsw depth. Therefore, 1 ATA = 0 fsw, 2 ATA = 33 fsw, 3 ATA = 66 fsw, etc. The SI unit for pressure is the kilo-Pascal (kPa), where 1 ATA = 101.3 kPa. Other commonly used pressure equivalents for 1 ATA include 14.7 psia, 0.101 mega-Pascal (MPa), and 1.013 bars.

breathe a hyperbaric atmosphere while awaiting rescue inside a disabled submarine (DISSUB) (165). Although submarine accidents rarely occur, the sinking of the Russian submarine *Kursk* in the Barents Sea in August 2000, in 356 fsw (11.8 ATA), with loss of all 117 crew members, is a somber reminder of the importance of preparing for DISSUB emergencies. Furthermore, the need to prepare for similar DISSUB scenarios on land, in which men are trapped under increased pressure, was demonstrated recently when nine Pennsylvania coal miners were stranded for 77 h inside a water-filled mine. Exposure to ambient pressures up to 40 fsw [1.21 atmospheres gauge pressure (ATG), or 2.21 ATA] necessitated hyperbaric medical support by medical officers and divers from the US Navy's diving and salvage community (155). Presently, the US Navy maintains agreements with >20 countries for assistance with DISSUB events (68).

Hyperbaric environments present many physiological challenges, affecting especially the lungs, hollow viscera, and nervous system. With respect to the mammalian central nervous system (CNS; mCNS), breathing air, pure O_2, or mixtures of O_2 and inert gases (N_2, H_2, and He), and air contaminated with CO_2, at hyperbaric pressure for extended periods, can impair the normal functioning of neural networks (28, 89). Common neurological problems associated with hyperbaric environments included O_2 toxicity, which is thought to occur through increased oxidative stress, as well as N_2 narcosis (inert-gas narcosis), CO_2 toxicity, and high-pressure nervous syndrome (HPNS) (18, 43, 89, 203).[2]

In neurophysiology, one of the commonly used experimental approaches to identify how changes in gas tension affect neural function is to conduct an electrophysiological study of single neurons in an isolated tissue preparation of the rodent CNS, such as brain slices or the neonatal brain stem-spinal cord, while manipulating the P_{O_2}, P_{CO_2}, and/or N_2 partial pressure (P_{N_2}) of the perfusate at room pressure (i.e., normobaric pressure) (54, 65, 98). However, making a single-cell recording in an isolated tissue preparation of the rodent CNS, which is maintained inside a hyperbaric chamber, while increasing ambient pressure, has proven to be technically challenging due to the physical barrier imposed by the sealed pressure chamber and mechanical disruption of the recording microelectrode and neuron during tissue compression and decompression (191, 192). Consequently, relatively little is known about how hyperbaric gases and increased hydrostatic pressure affect the intracellular properties of neurons in the mCNS. Recent improvements in hyperbaric chamber designs, however, have now made intracellular recordings of mammalian neurons, maintained under hyperbaric conditions, technically feasible (58, 59, 150, 152, 153).

In 1979 and 1980, Wann and colleagues reviewed hyperbaric electrophysiological methods (215) and summarized the effects of large hydrostatic pressures (>100 ATA) on cellular excitability (213). Research at that time was based primarily on studies of large robust neurons, axons, and muscle cells in the invertebrate CNS (iCNS). Given the technical advances made in this field during the past two decades, which have increased the productivity of single-cell studies in the mCNS (58, 72–74, 150, 152–154), we have reassessed the methods used in hyperbaric electrophysiology along with their potential research applications. Unlike previous reviews of hyperbaric electrophysiology, which focused exclusively on the effects of hyperbaria exceeding 50 or 100 ATA (89, 114, 130, 213), we have emphasized the importance of studying neuronal barosensitivity and chemosensitivity to hyperbaric gases at P_B <5 ATA. The various methods used to differentiate the effects on neurons of gas partial pressures vs. pressure per se are summarized (APPENDIXES A–D). Moreover, barodependent disorders of the mCNS are summarized, presenting their clinical signs and symptoms and the pressure ranges (total pressure and gas partial pressures) over which they occur, as an aid to designing physiologically relevant in vitro studies (see OVERVIEW OF BARO-RELATED DISORDERS OF THE MCNS). Specific examples are presented to demonstrate how hyperbaric pressure per se (see CENTRAL EFFECTS OF PRESSURE PER SE) and increased gas partial pressures (see NARCOTIC AND TOXIC PROPERTIES OF GASES) can alter neuronal excitability. In particular, we emphasize the use of hyperoxia under pressure [i.e., hyperbaric O_2 (HBO_2)] not only as an in vitro model for studying CNS O_2 toxicity, but also as a new model for studying the cellular mechanisms by which acute oxidative stress [i.e., reactive O_2 species (ROS)] alters neuronal activity. Accordingly, we have critically evaluated the control levels and experimental levels of tissue P_{O_2} (Pti_{O_2}) that are used in the rat brain slice model (3, 151, 169), comparing each to levels of Pti_{O_2} that occur in the intact mCNS when breathing normobaric air, normobaric hyperoxic gas mixtures, and HBO_2 (see RESEARCH APPLICATION: HYPEROXIA AND O_2 TOXICITY).

Glossary

ATA	Atmospheres absolute pressure
ATG	Atmospheres gauge pressure
CNS	Central nervous system
DISSUB	Disabled submarine
ΔE	Change in internal energy
$F_{I_{gas\ x}}$	Fractional concentration of inspired *gas x*, where *gas x* is either O_2, CO_2, or N_2 ($F_{I_{O_2}}$, $F_{I_{CO_2}}$, $F_{I_{N_2}}$)
fsw	Feet of sea water
ΔG	Change in free energy
ΔH	Enthalpy change
HBHe	Hyperbaric helium
HBO_2	Hyperbaric oxygen
HBOT	Hyperbaric oxygen therapy

[2] Decompression sickness, commonly known as the "bends" and "chokes," is not covered here because it is a problem that occurs subsequent to decompression while diving and/or flying, and this review focuses on neurophysiological challenges that occur while breathing hyperbaric gases; i.e., gases in the compressed state.

HPLC	High-pressure liquid chromatography
HPNS	High-pressure nervous syndrome
iCNS	Invertebrate central nervous system
K	Equilibrium constant
k	Reaction rate constant
mCNS	Mammalian central nervous system
NO·	Nitric oxide radical
NOS	Nitric oxide synthase
P	Pressure
P_B	Barometric pressure
$P_{gas\ x}$	Partial pressure of a gas, where *gas x* is either O_2, CO_2, N_2, or He (P_{O_2}, P_{CO_2}, P_{N_2}, P_{He})
$PI_{gas\ x}$	Partial pressure of inspired *gas x*, where *gas x* is either O_2, CO_2, or N_2 (PI_{O_2}, PI_{CO_2}, PI_{N_2})
Pti_{O_2}	Partial pressure of oxygen in neural tissue
pH_i	Intracellular pH
pH_o	Outside or extracellular pH
psia	Pounds per square inch absolute
R	Gas constant
R_{in}	Input resistance (MΩ)
ROS	Reactive oxygen species
ΔS	Entropy change
T	Temperature, in degrees Kelvin
ΔV	Molar volume change
ΔV^*	Activation volume change for the rate-limiting step
V_m	Membrane potential (mV)

OVERVIEW OF BARO-RELATED DISORDERS OF THE MCNS

When designing a hyperbaric electrophysiological experiment, it is important to consider the ranges of pressures that are physiologically relevant, which includes *1*) the partial pressure of each gas in the inspired atmosphere and *2*) the surrounding ambient pressure, or P_B, which exerts a force against the body, resulting in hydrostatic compression of all tissues, including neural tissue. The following four sections summarize the major neurological problems encountered when humans and other animals breathe hyperbaric gases containing O_2, N_2, CO_2, He, or H_2. Our discussion will focus on the major signs and symptoms of each baro-related disorder and the pressure range over which these signs and symptoms occur.

N_2 Narcosis

Figure 1 shows the broad range of ambient pressure over which the human body can function. Extended stay at pressures exceeding ~4 ATA (~3 ATG, ~99 fsw depth) is only possible by reducing the fractional concentration of N_2 in the inspired air to avoid the narcotic effects of N_2 (N_2 narcosis). For example, nitrox is O_2-enriched air, which is made by lowering the fractional concentration of inspired N_2 (F_{IN_2}). It is breathed to avoid N_2 narcosis while the diver is at depth. The reduction in partial pressure of inspired N_2 (P_{IN_2}) when breathing nitrox also reduces the incidence of decompression sickness and the time needed for decompression during ascent. Typically, nitrox is used at depths

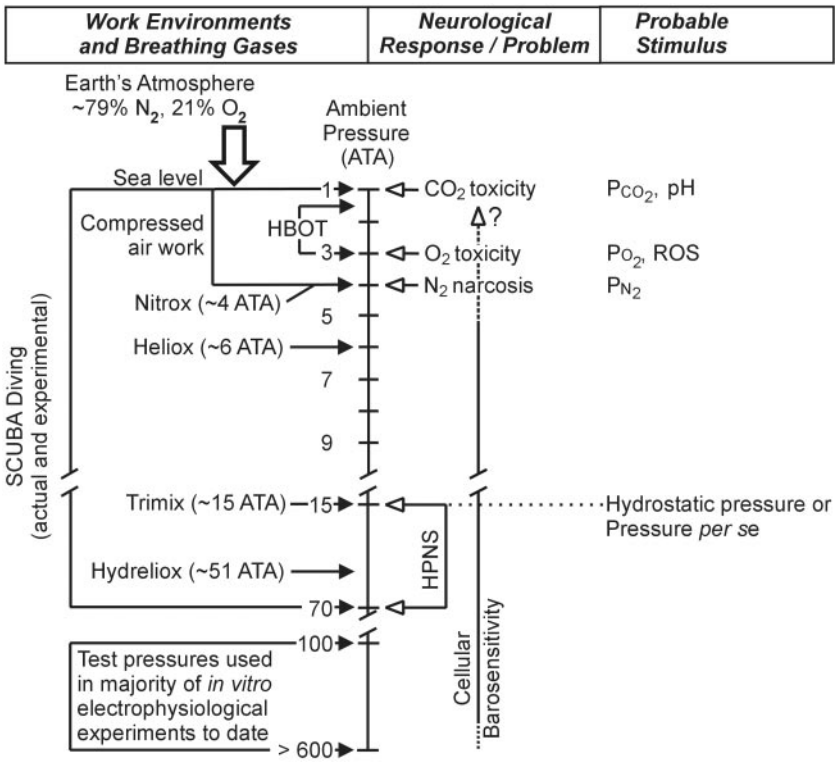

Fig. 1. Physiologically relevant ambient pressures encountered by humans breathing air and various hyperbaric gas mixtures (*left*), the neurological problems that can result (*middle*), and the proposed stimulus affecting neuronal function (*right*). Hyperbaric environments are encountered in clinical medicine [e.g., hyperbaric O_2 therapy (HBOT)], compressed air work (e.g., subterranean tunneling), diving medicine, and disabled submarine accidents. By breathing an assortment of gas mixtures, to avert the narcotic and toxic effects of N_2 and O_2, as well as the direct effects of hydrostatic compression on the mammalian central nervous system (mCNS), humans are able to inhabit hyperbaric environments ranging from >1 to ~70 atmospheres absolute pressure (ATA). At extreme hyperbaric pressures (>100 ATA), pressure becomes a tool that can be used to perturb biological systems (in vitro) by altering protein conformation, membrane fluidity, and configuration of the cytoskeleton. Humans and most mammals, however, have never occupied this range of ambient pressures. See text for further explanation of breathing gas mixtures and pressure thresholds for various neurological disorders. ROS, reactive O_2 species; SCUBA, self-contained underwater breathing apparatus; HPNS, high-pressure nervous syndrome; P_{N_2}, N_2 partial pressure.

>100 fsw. Symptoms of N_2 narcosis are significantly manifested in most individuals when breathing air at P_B of ~4 ATA and then increase insidiously but rapidly with further increasing depth and are best characterized as a graded rapture, reduction of higher mental processes, and impaired neuromuscular coordination, usually resembling the symptoms of alcohol intoxication, or similarly, the early stages of anesthesia and hypoxia. With increasing depth and $P_{I_{N_2}}$, the symptoms worsen and eventually lead to unconsciousness at depths where P_B exceeds 10 ATA (18). To date, very little electrophysiological research has been conducted on the cellular mechanisms involved in N_2 narcosis (34, 35, 154).

CO_2 Toxicity

Retention of CO_2 under hyperbaric conditions, due to either increased fractional concentration of inspired CO_2 ($F_{I_{CO_2}}$) and/or decreased alveolar ventilation, or breathing a CO_2-contaminated gas mixture, impairs neurological function. It is well established that end-tidal P_{CO_2} increases in a diver due to increased metabolic CO_2 production caused by physical exertion of swimming, working, and breathing underwater, and due to reduced ventilatory efficiency (alveolar hypoventilation) caused by the added dead space of a breathing apparatus and the increased airway resistance caused by increased density of gases at increased ambient pressure (79, 147, 194). Problems associated with CO_2 retention are exacerbated in experienced divers, compared with nondivers and amateur (recreational) divers, because experienced divers typically exhibit higher levels of resting end-tidal P_{CO_2} caused by reduced ventilatory chemosensitivity to inspired CO_2 at normobaric pressure (116, 217). For example, mean end-tidal P_{CO_2} in nondivers and diving trainees was 40 Torr, whereas experienced Navy divers had a significantly higher mean end-tidal P_{CO_2} of 46 Torr. During hyperbaria and exercise, end-tidal P_{CO_2} in experienced divers often increased to >50–70 Torr, and, during severe exercise, end-tidal P_{CO_2} reached >90 Torr (116, 194, 217).

CO_2 retention at hyperbaric pressure can produce two types of neurological problems. First, retention of even modest levels of CO_2 at hyperbaric pressure presumably increases nitric oxide radical (NO·) and results in cerebral vasodilation (101), which increases delivery of perfusion-limited gases to neural tissue (108, 122). Consequently, the severity of symptoms of CNS O_2 toxicity (and N_2 narcosis) are enhanced during respiratory acidosis and/or begin at lower levels of hyperbaria (5, 42, 95, 134). Second, tissue P_{CO_2}, once reaching a sufficiently high level, either directly due to its narcotic properties, or indirectly by the ensuing intracellular acidification, induces CO_2 toxicity without warning. Normally, arterial P_{CO_2} ranges from ~35 to 45 Torr (arterial pH ~7.35–7.45), and small increases in arterial P_{CO_2} of only a few Torr stimulate both central and peripheral CO_2/H^+ chemoreceptors, activating powerful cardiorespiratory reflexes to remove CO_2 from the body for homeostasis of tissue pH (86, 156, 157, 183). In rats, going from air to 6% CO_2 in air, which stimulates breathing, causes pH of the cerebrospinal fluid to decrease from 7.396 to 7.294 and intracellular pH (pH_i) of neural tissue to decrease from 7.044 to 6.982. Breathing a higher level of CO_2 (11% in air) decreases pH of the cerebrospinal fluid even further to 7.190 and pH_i of neural tissue to 6.910 (187). Under in vitro conditions (brain stem slice), moderate levels of hypercapnic acidosis decrease pH_i by ≤0.2 pH units (control pH_i is ~7.24) (56, 78, 176, 177) and increase neuronal excitability in central CO_2-chemoreceptor areas of the mammalian brain stem (54, 78, 98). This stimulation of putative central CO_2/H^+-chemoreceptor neurons is believed to underlie the reflex stimulation of ventilation, which is interpreted as the physiological response of the respiratory control network to hypercapnic acidosis (156, 157, 183).

As inspired CO_2 increases further to 10–15% CO_2, and end-tidal P_{CO_2} exceeds 50–70 Torr, mental ability becomes increasingly impaired and is characterized by confusion, irrational behavior, drowsiness, dizziness, and impaired short-term memory (146, 217). Recovery from CO_2 toxicity is often associated with severe headache (146). In some cases, inspiring 30% CO_2 produces seizures (225). In rats, breathing 75–100% CO_2 rapidly produces ataxia followed by loss of righting reflex and pedal reflexes, and, eventually, anesthesia (45). Divers lose consciousness when end-tidal P_{CO_2} exceeds 90 Torr (194, 217), and prolonged exposure to >70% CO_2 (balance O_2) quickly leads to death in animal models due to depression of respiratory centers (53).

Despite the widespread use of extreme hypercapnia as an anesthetic and its potential danger as an environmental toxicant, very little electrophysiological research has been done on the cellular mechanism of CO_2 toxicity. The toxic effects of CO_2, however, are believed to be due, in part, to the large acidification that occurs when alveolar P_{CO_2} increases at high levels of inspired P_{CO_2} ($P_{I_{CO_2}}$). Because CO_2 is extremely soluble in lipids and water, it rapidly diffuses into the cell membrane and through the cytoplasm, where it is hydrated to form carbonic acid, which rapidly dissociates to form protons and bicarbonate ions. The effects of extreme levels of hypercapnia (i.e., supercapnia, Ref. 211) on pH_i in the intact mCNS remains unknown. In the rat brain stem slice, however, extreme hypercapnia, which also impairs neuronal excitability, decreases pH_i, on average, from 7.24 (5% CO_2) to 6.71 (33% CO_2) to 6.67 (50% CO_2) and to 6.49 (100% CO_2 at P_B ~1 ATA) in locus coeruleus neurons and results in abnormal electrical activity (56).

O_2 Toxicity

Pure O_2 is breathed at >1 ATA by using the LAR V Draeger underwater breathing apparatus on combat diving operations by Special Operations personnel (US Navy SEALs and Marine Force Reconnaissance units) to prevent N_2 narcosis at depth, to reduce the length of decompression stops during ascent, and to decrease the

risk of decompression sickness during and after ascent (36, 37). However, breathing pure O_2 or nitrox (>21% O_2) also increases the risk of CNS O_2 toxicity at greater depths (Fig. 1). Similarly, Fig. 1 shows that, on land, HBOT uses intermittent exposure to pure O_2 at >1–3 ATA to increase arterial P_{O_2}, and thus O_2 delivery to tissues, to treat a variety of clinical disorders due to diving and nondiving causes.[3] Typically, several 20- to 30-min exposures to HBO_2 are interspersed with 5- to 10-min air breaks to avert symptoms of CNS and pulmonary O_2 toxicity (38, 203). CNS O_2 toxicity manifests itself ultimately as grand mal convulsions (i.e., the "Paul Bert effect"), although other autonomic, motor, and cardiorespiratory signs and symptoms also may occur, such as bradycardia, hyperventilation, dyspnea, and altered cardiorespiratory neural reflexes (43, 51, 189, 209). Interestingly, Mulkey et al. (152) have reported that neurons in the solitary complex, an important cardiorespiratory control center in the dorsal medulla oblongata, are highly sensitive to HBO_2 and this sensitivity to oxidative stress may contribute to the cardiorespiratory perturbations that occur in CNS O_2 toxicity.

The inter- and intraindividual variations in susceptibility to O_2 seizures make it difficult to predict who is vulnerable to O_2 toxicity and when O_2 seizures will occur (43). O_2 seizures per se resulting from acute exposure to hyperbaric hyperoxia are not believed to be harmful. If, however, the subject is not removed immediately from the hyperoxic environment, then continued exposure to HBO_2 can result, first, in permanent neurological damage and paralysis (i.e., the "John Bean effect") and eventually death with prolonged exposure (12). Presently, CNS O_2 toxicity is the limiting factor in protocols employed for closed-circuit diving operations by combat divers (37) and for HBOT (38).

The pressure threshold for onset of seizures can be lowered to <3 ATA O_2, however, by coexisting conditions, such as immersion (36), exercise and increased metabolic rate (5, 43, 116), and hypoventilation and respiratory acidosis (5, 42, 95, 223). For example, moderate CO_2 retention (respiratory acidosis) lowers the threshold for O_2 toxicity, increases the severity of seizures, and decreases survival time in human divers and animals (5, 15, 42, 134). One mechanism for this increased sensitivity to hyperbaric hyperoxia is due to CO_2-induced cerebral vasodilation, which increases neural Pti_{O_2} (101). During exposure to HBO_2, cerebral blood flow initially is reduced by hyperoxia due to interruption of NO· release from *S*-nitrosohemoglobin (193) and increased production of superoxide, which reacts with NO· to effectively decrease biologically active NO·, thereby causing vasoconstriction (61–63). CO_2 retention, however, presumably increases NO· production (101), causing cerebral vasodilation, which antagonizes O_2-induced cerebral vasoconstriction and increases blood flow through neural tissues (13, 122). This, in turn, increases delivery of O_2 to neural tissue (108, 122), thereby increasing Pti_{O_2} at any given level of inspired P_{O_2} (PI_{O_2}) (see below, Fig. 4: #2, open squares vs. #1, open circles), resulting in increased production of ROS (60, 61, 63, 70, 107, 205). Tissue P_{CO_2} increases during HBO_2 by one or more of the following mechanisms: *1*) CO_2-carrying capacity of venous hemoglobin is decreased because venous hemoglobin is fully saturated with O_2, which leads to increased tissue and venous P_{CO_2} (i.e., Haldane effect); *2*) alveolar hypoventilation and CO_2 retention (see *CO_2 Toxicity* above); and *3*) breathing CO_2-contaminated gases. In addition, and independently of the increased cerebral blood flow, it is possible that there is increased production of ROS in the presence of H^+ and CO_2 (174, 188, 218) that renders neurons more sensitive to hyperoxia (152). In contrast to moderate hypercapnia, which enhances CNS O_2 toxicity, severe hypercapnia that produces anesthesia depresses development of CNS O_2 toxicity in newborn rats by an unknown mechanism (15, 211). Obviously, the interactions among CO_2, pH_i, and ROS during hyperbaric hyperoxia require additional study. The sensitivity of single neurons to HBO_2 will be considered in more detail below (see RESEARCH APPLICATION: HYPEROXIA AND O_2 TOXICITY) and in the companion paper (152).

He, H_2, and O_2 Mixtures: HPNS

Figure 1 also includes heliox, which is a He-O_2 gas mixture that is breathed at depths >150–200 fsw; specifically, 79% N_2 in air is replaced with He to avert N_2 narcosis, and the fractional concentration of inspired O_2 (FI_{O_2}) is lowered to prevent CNS O_2 toxicity (18). Breathing heliox also greatly ameliorates the increasing airway resistance at those depths due to greater gas density. However, diving at depths greater than ~15 ATA can also result in HPNS, which is due to the effects of pressure per se and not to increased He partial pressure (P_{He}), P_{O_2}, or P_{CO_2} (18). Signs and symptoms of HPNS include muscular tremors, EEG changes, loss of coordination, nausea, respiratory difficulties, memory deficit, and seizures (in animals), which typically begin at ~15–16 ATA or higher (18, 89). It is likely that seizures would also occur in humans if they were subjected to high enough pressures while breathing heliox; however, this is neither practical nor ethical for obvious reasons. At even greater depths (>15 ATA), a small amount of N_2 is added back to the breathing gas mixture to produce trimix (O_2-He-N_2; Fig. 1), which increases the depth and pressure a diver can reach before onset of symptoms of HPNS (18, 29). Similarly, H_2 is substituted for N_2 to produce a gas mixture of O_2-H_2-He, referred to as hydreliox (Fig. 1),

[3] Clinical administration of O_2 at P_B >1 ATA is called HBOT and it is used clinically to help "resolve certain recalcitrant, expensive, or otherwise hopeless medical problems" (38). Some of the accepted uses of HBOT for acute medical emergencies include carbon monoxide poisoning, necrotizing fasciitis, air embolism, decompression sickness, gas gangrene, and radiation necrosis (38). In addition, HBOT has been used to treat a variety of neurological problems, such as traumatic brain injury (38, 158). The application of HBOT to neurological problems is still in its infancy, however, and debated because of the lack of relevant cellular and clinical research on these potentially important applications of hyperbaric medicine.

which is also used for deep dives to delay onset of signs and symptoms of HPNS (1, 18, 29). H_2, compared with N_2, is even less dense and easier than He to breathe at pressure, yet it also affords protection from HPNS. It is well known that anesthetic gases (e.g., hyperbaric N_2 or H_2) and hydrostatic pressure have antagonistic actions on neurological function, such that, when combined, each reduces the deleterious effects of the other (29, 113, 114). The basis for this interaction between anesthetics and pressure is unclear and is one of the areas of hyperbaric electrophysiology research requiring additional study (27, 113). The sensitivity of single neurons to pressure per se will be considered in more detail in the next section and in the companion paper (153).

CENTRAL EFFECTS OF PRESSURE PER SE

Hydrostatic Compression of the Brain

We live in a sea of gas, and the weight of the Earth's atmosphere exerts a physical pressure at sea level equal to 1 ATA, to which our bodies are physiologically adapted. As we descend beneath the ocean surface, pressure continues to increase an additional 1 atm each additional 33 fsw (Fig. 1). Thus a diver at 132 fsw is exposed to 4 atm of sea water plus 1 atm of air, or 5 ATA of total pressure. Similarly, a person inside a pressure chamber (145, 203, 217), caisson, or subterranean tunnel (117) experiences an equivalent depth of sea water when the density of gas inside the sealed pressure chamber is increased.

In a hyperbaric environment, soft tissues of the body behave as a fluid and rapidly transmit any pressure-applied force against the surface of the body to the adjacent fluid compartments, thereby removing any pressure differential across structures. This results in hydrostatic compression of the cerebral spinal fluid, cerebral circulation, and extracellular and intracellular fluid compartments of the mCNS (89, 109). The best known example of neuronal barosensitivity is HPNS, which was already discussed above (18, 89). The threshold pressure for HPNS is variable and affected by the rate of compression (18). Details regarding the cellular mechanism(s) underlying HPNS are unclear (114), but it appears that both synaptic (74, 76, 85, 228, 229) and intrinsic membrane properties are involved (72, 73, 191, 192, 216). Several excellent reviews on HPNS exist elsewhere (18, 100, 104, 114). When studying in vitro neuronal activity as a model of HPNS, it is important to remember the range of ambient pressures over which symptoms of HPNS occur (~15 to ~70 ATA, Fig. 1). For example, intracellular and extracellular recordings of neurons and axons in invertebrates and terrestrial mammals have shown that hydrostatic compression, ranging from 100 to 200 ATA and higher, alters neural activity (72–74, 76, 114, 130, 191, 192, 197, 198, 216). As a test for studying barosensitivity, particularly in the mCNS, the use of extremely large levels of pressure is of questionable importance, because terrestrial mammals are never exposed to these levels of hydrostatic pressure. As cautioned by Halsey (89): ". . . much of the work with excitable cells has been carried out over the maximum tolerated pressure range, which in some cases extend up to 1,000 ATA. This is clearly appropriate in terms of the studies per se, but it is difficult to relate such work to the intact mammalian CNS, which would not be expected to function above 100 ATA . . ." Thus, although the use of test pressures ≥100 ATA will likely increase the magnitude of the cell's pressure response, if present, it is also important to report what happens over the pressure range for which the symptoms of HPNS are known to occur for the species being used.

There is evidence that lower levels of hyperbaria also affect excitability of neurons and synapses. Excitable cells in invertebrates are barosensitive to ambient pressures ranging from 4 to 10 ATA by using either true hydrostatic compression or He compression (34, 47, 48, 192). Similarly, neurons in the rat brain stem are barosensitive to even smaller pressures of only 2.4–4 ATA He (58, 150, 153), and brief hydraulic pressures ranging from 1.17 to 4.0 ATA can alter neuronal excitability in dorsal root ganglion neurons (90, 138, 181). For example, Fig. 2*A* shows the integrated firing rate response of a neuron in the solitary complex in the caudal-dorsal medulla oblongata in a 300-μm-thick submerged slice before, during, and after compression from 1 to 3 ATA He (P_B). Notice that input resistance (R_{in}) decreased during He compression (*inset*, superimposed traces) concomitant with the increased firing rate. Typically, membrane potential (V_m) depolarized by ≤3 mV (not shown) (58, 150, 153). This indicates that 3 ATA pressure caused an increase in membrane conductance (see Fig. 2 legend for further explanation). Mulkey et al. (153) have shown that the increased firing rate response to hyperbaria is retained during chemical synaptic blockade, which indicates that it is an intrinsic membrane property of certain neurons in the solitary complex. Similarly, McCarter et al. (138) reported that a brief, graded hydraulic pressure pulse, ranging from 0.17 to 3 ATG, depolarized V_m and increased inward current in a graded fashion in dorsal root ganglion neurons.

Figure 2*B* shows a similar response in another neuron in the solitary complex that was stimulated and displayed increased membrane conductance (decreased R_{in}) during compression to a much higher pressure, 20 ATA He. To our knowledge, this is the first example of an intracellular recording of a mammalian neuron that was maintained continuously during cyclical compression and decompression to this high level of pressure (compare Refs. 191, 192, 216). The significance of this experiment is that it demonstrates that the electrical activity of individual neurons in the mCNS can be studied over the lower range of ambient pressures at which symptoms of HPNS are first detected (Fig. 1).

The above studies indicate that neurons are sensitive to much lower levels of hyperbaric pressure than previously believed (50, 114, 130). The importance of barosensitivity to ambient pressures <5 ATA remains to be determined; however, it may, like temperature,

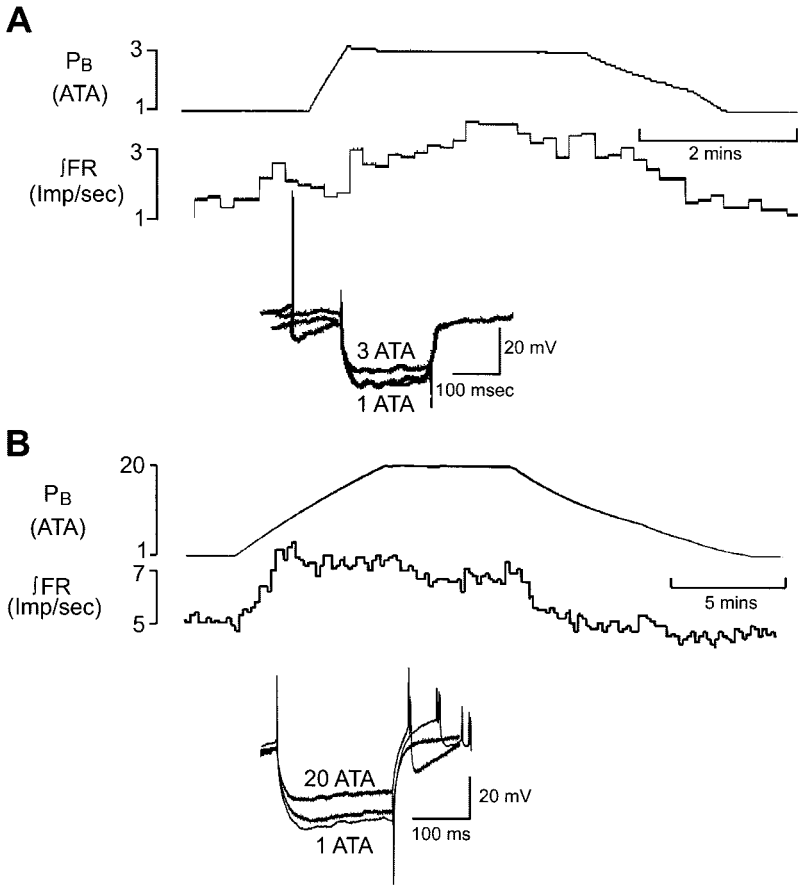

Fig. 2. Examples of 2 barosensitive neurons in the solitary complex. Intracellular recordings from 2 different neurons showing that 3 ATA (*A*) and 20 ATA of helium (He) (*B*) decrease input resistance and stimulate neuronal firing rate in a reversible manner. In these 2 experiments, input resistance was measured by using a constant hyperpolarizing current pulse (superimposed voltage traces in *A* and *B*). Based on Ohm's law (voltage = current × resistance), the change in membrane potential (V_m) during hyperpolarizing current injection through the microelectrode is proportional to the change in input resistance and inversely proportional to the membrane conductance. This, the decrease in input resistance measured during exposure to hyperbaric pressure, is indicative of an increase in membrane conductance. Action potentials are truncated in *B* in *inset* of V_m traces. Data are unpublished examples from the study by Mulkey et al. (153). PB, barometric pressure; ∫FR, integrated firing rate; Imp/sec, impulses/second.

act as another environmental stimulus that determines how the organism responds and adapts to its environment (114, 150, 153). To speculate further, neuronal barosensitivity to relatively low levels of hyperbaric pressure may be the early stage of a pressure continuum that is eventually exhibited as HPNS at higher pressures. What is interesting, however, is that not all neurons in the mCNS are equally sensitive to moderate levels of hyperbaria (82, 153). For example, initial reports indicate that hippocampal neurons do not respond to 3 ATA He or hydraulic pressure (82, 90). Similarly, not all neurons in the solitary complex are sensitive to 2–4 ATA He (153). This suggests that neuronal barosensitivity to low levels of hyperbaria is not a generalized phenomenon, but represents a specific physiological response of certain populations of neurons in the mCNS.

Intracranial Hypertension and Increased Intraocular Pressure

Another source of neuronal barosensitivity, which occurs on a much smaller pressure scale and independently of changes in PB, is compression of neural tissue that occurs whenever the mass of tissue inside the rigid, closed intracranial compartment is increased by tissue edema and bleeding subsequent to head injury or by increased production or decreased removal of cerebral spinal fluid (4, 33, 140). Typically, normal intracranial pressure ranges from 0 to 10 or 15 mmHg in humans (139) and from 8 to 10 mmHg in conscious rats (111). Intracranial hypertension is considered as a sustained elevation in intracranial pressure exceeding 15 or 20 mmHg and going as high as ≥100 mmHg (139, 140). Historically, neurological symptoms associated with traumatic brain injury have been attributed to cerebral ischemia and the resulting tissue hypoxia and acidosis as intracranial pressure approaches cerebral arterial blood pressure. However, it is likely that increased tissue pressure also contributes to neurological dysfunction in these cases in a manner that remains to be determined. In addition, the initial traumatic brain injury itself, which is a fluid-percussive injury, produces a brief, transient period of hydrostatic compression ranging from 1.4 to 2.1 ATG that results in protracted dysfunction of electrical signaling and cellular metabolism (64, 181). For the sake of completeness, retinal cells, which are considered as part of the mCNS, are exposed normally to intraocular pressures ranging from ~14 to 16 mmHg, depending on the person's age, race, and gender (123, 173). Intraocular hypertension and glaucoma are associated with intraocular pressures ≥21 mmHg, which produce long-term destruction of nerve cells and their function (123). However, the effects of increased intraocular pressure on the excitability and electrical signaling of retinal cells are unknown.

Thermodynamic Reactions vs. Mechanical Forces

The preceding discussion indicates that neuronal barosensitivity occurs over a very large range of pressure (89, 90, 114, 130, 138, 150, 153, 192). It has been suggested that, depending on the level of hyperbaric pressure, different cellular mechanisms may be responsible for mediating cellular barosensitivity (41, 94, 129, 131, 136). At extremely high levels of compression (e.g., >100 ATA), hydrostatic pressure is a thermodynamic intensity parameter, similar to temperature, which determines various thermodynamic equilibria, and thus could affect various cellular processes. It can be analyzed theoretically, based on the way in which pressure (P) and the molar volume change (ΔV) combine to change the free energy (ΔG) of a reaction (129–131); i.e.

$$\Delta G = \Delta H - T \cdot \Delta S \quad (1)$$

$$= (\Delta E + P \cdot \Delta V) - T \cdot \Delta S \quad (2)$$

where ΔH is the enthalpy change and is equal to (ΔE + $P \cdot \Delta V$), ΔE is the change in internal energy, T is the temperature in degrees Kelvin, and ΔS is the entropy change. At constant T, the equilibrium constant (K), ΔV, and P are related by the following expression

$$\left(\frac{d \ln K}{d P}\right)_T = \frac{-\Delta V}{R \cdot T} \quad (3)$$

where R is the gas constant. Thus, for a reaction involving a large ΔV, pressure changes can have a large effect on the equilibrium of that reaction.

Pressure can also alter the rate of a chemical reaction by changing the activation volume for the rate-limiting step (ΔV^*) (50). At constant temperature, the reaction rate constant (k), activation volume, and ambient pressure are related by the following expression

$$\left(\frac{d \ln k}{d P}\right)_T = \frac{-\Delta V^*}{R \cdot T} \quad (4)$$

For a reaction involving a large ΔV^* , pressure changes can have a large effect on the rate of that reaction. Stated another way, Macdonald and Fraser (129, 131) have proposed that, even for reactions with relatively small changes in molar volume and activation volume, very large hydrostatic pressures can significantly alter the equilibrium and rate of the reaction, respectively. For this reason, as indicated in Fig. 1, neural tissue has been exposed to extremely high pressures, on the order of hundreds of atmospheres (100 to >600 ATA), as a means of altering cellular functions through changes in protein structure (94) and enzymatic activity (103). In addition, extremely high pressures can alter membrane fluidity (128) and cause volume changes in cell membranes by bulk compression of lipid bilayers, thereby decreasing the intermolecular distances between acyl chains. Extremely high pressures can also change the hydration of lipid bilayers and proteins and the crystalline-to-liquid-crystalline phase transitions in lipid bilayers (127, 128).

Conversely, at lower levels of hydrostatic pressure, Macdonald and Fraser (129, 131) have proposed that thermodynamic and kinetic effects described by *Eqs. 1–4* would be too small to be of physiological significance, based on conventional solute-solvent interactions, because the ΔV and ΔV^* terms would have to be astoundingly large for any significant change to occur in ΔG or k. Traditional dogma maintains that hydrostatic pressures <15 ATA have no significant effects on neuronal function (18, 192, 197). However, as proposed in Fig. 1 ("Cellular Barosensitivity") and illustrated in Fig. 2 and elsewhere (90, 138, 150, 153, 192), neuronal barosensitivity, as defined by changes in firing rate, V_m, and R_{in}, occurs at 2.5–4 ATA and possibly as low as 100 mmHg (~1.13 ATA or ~0.13 ATG) (2). Because relatively few studies have attempted to differentiate the effects of hydrostatic pressure from gas partial pressure in the mCNS, especially at P_B <5 ATA (89), we would argue that it is premature to conclude that small changes in physical pressure do not alter neuronal function in the mCNS.

In fact, Macdonald and Fraser (131) have proposed that many nonneuronal cells respond to micropressures, which they define as ≤20 kPa (0.2 ATG or 1.2 ATA), by a mechanical process that they hypothesize to be localized shear and strain forces resulting from the differential compression of various adjoining cellular components, such as lipid bilayers, membrane-bound proteins, cytoskeletal proteins, and extracellular matrix. However, they go on to say that ". . . at present, there are no distinctive candidates [cellular structures] and a practical, specific working hypothesis is lacking" (131). If true, however, these same mechanical forces could be a significant factor at small-to-moderate hydrostatic pressures (e.g., 2–5 ATA) in neurons, which, in turn, would alter protein configurations (e.g., ion channels, postsynaptic receptors, etc.) and thus neuronal excitability. It remains to be determined, however, at what range of hydrostatic pressures are shear and strain involved, if at all, in altering the function of cells and tissues, and ". . . at what pressure does the putative 'micro-pressure' effect saturate and orthodox high pressure thermodynamics take over" (131).

NARCOTIC AND TOXIC PROPERTIES OF GASES

Several of the neurological problems caused by breathing hyperbaric gases are related to the narcotic properties of the gas, and not pressure per se, which we will consider now. The ambient pressure at which first signs and symptoms occur for a specific gas-related disorder depends on the composition of the inspired gas mixture. In general, we can state that the sensitivity of the mCNS for a given gas, as defined by P_B and fractional concentration of gas, increases in the following order (Fig. 1): CO_2 toxicity (a problem at any level of P_B, depending on F_{ICO_2} and thus P_{ICO_2}), O_2 toxicity (~3 ATA breathing 100% O_2), N_2 narcosis (~4 ATA breathing air; i.e., 79% N_2), and He narcosis [usually ~100 ATA or higher as determined in various nonmammalian tissue models (200)]. Figure 1 shows the threshold

P_B at which the symptoms or neural responses typically occur when the requisite gas mixture is breathed, but these values serve as guidelines only. The threshold ambient pressure at which each neurological problem develops is not absolute and can vary considerably in either direction for an individual and between individuals, due to preexisting physiological conditions that affect cerebral blood flow, metabolic rate, body temperature, and acid-base status (18, 43).

We can state, in general, that the narcotic potency of a gas is directly dependent on its *1*) partial pressure in the inspired gas mixture, blood, and neural tissues, which increases with increasing P_B, and *2*) its lipid solubility in cell membranes, which determines the level to which a gas accumulates in the cell membrane and cytoplasm at any partial pressure (18, 43). Figure 3 shows the solubility of CO_2, O_2, and two inert gases (He, N_2) in a monolayer of egg phospholipid as a function of increasing gas pressure, as reported by Bennett et al. (19). All gases, excluding He, cause a linear increase, at differing rates (CO_2 >>> O_2 > N_2), in monolayer film pressure as gas pressure increases (increased film pressure corresponds to increased membrane volume). Comparing Figs. 1 and 3, we can see that CO_2, which has the highest solubility in cell membranes in this example (Fig. 3), likewise has significant effects on the mCNS at the lowest ambient pressure (Fig. 1) (43). Conversely, O_2 and N_2 have lipid solubilities, and, therefore, narcotic effects, which are intermediate to CO_2 and He and are manifest only at hyperbaric pressure. He is essentially insoluble in lipid bilayers and has no noticeable narcotic effects over the range of ambient pressure that most mammals encounter (28). This is why He is used as a compression medium for many in vitro electrophysiology studies (Fig. 2) (e.g., Refs. 58, 75, 191; also see in APPENDIX A, *Compression media: HBHe vs. hydrostatic compression*).

The narcotic effects of a gas were first thought to be related to their ability to expand the hydrophobic region of the cell membrane (141). This presumption, known as the "critical volume hypothesis of anesthesia (and narcosis)," has been revised. It is now thought that the central effects of a gas are mediated by the direct and indirect actions it has on membrane-bound proteins and cytosolic proteins, which perturb ionic conductances and neurotransmission (80). Gases such as O_2 and CO_2 also have additional effects on neurons because they react biochemically in the extracellular space, the membrane, and intracellular space to form reactive chemical species, which are important metabolic signals in numerous processes and which, at high concentrations, are toxic to cells. For example, O_2 forms a variety of highly reactive products (e.g., superoxide, hydroxyl radicals, NO·, hydrogen peroxide, and peroxynitrite), which can either modulate or disrupt normal cell function (60, 61, 63, 67, 71, 107, 205). Examples will be shown below of how HBO_2 stimulates neuronal firing rate in neurons of the solitary complex and CA1 hippocampus. Recent work by our laboratory has shown that this excitatory effect of acute exposure to hyperoxia is most likely mediated by an increased production of ROS (152). Moreover, as already stated, CO_2 forms H ions, which likewise either modulate or disrupt normal cell function, depending on its concentration (56, 137). Molecular O_2 and CO_2 will also presumably have narcotic effects on the mCNS (95), as predicted by data shown in Fig. 3 (19). The narcotic effects of O_2 and CO_2, however, will be difficult to discriminate from the effects of their highly reactive secondary reaction products.

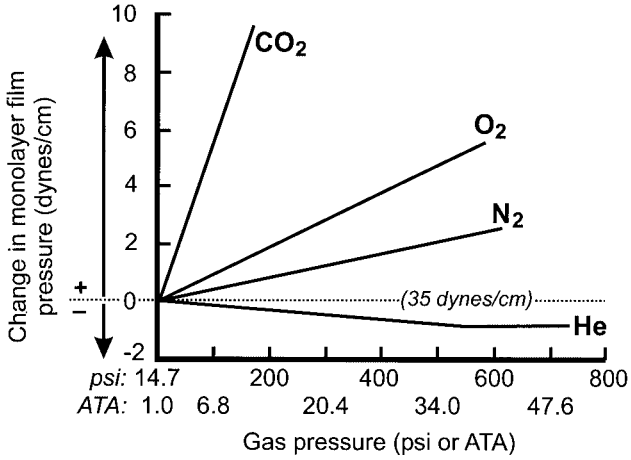

Fig. 3. Relative solubilities of gases in artificial lipid bilayers. All gases, excluding He, cause a linear increase in monolayer film pressure, which corresponds to increased membrane volume, as gas pressure increases. psi, Pounds per square inch. [From Bennett et al. (19). Reprinted with permission from Elsevier].

RESEARCH APPLICATION: HYPEROXIA AND O_2 TOXICITY

The remainder of this review article, exclusive of APPENDIXES A–D, focuses on the use of hyperbaric electrophysiology to study the effects of increased Pti_{O_2} (hyperoxia) on the mCNS and what the appropriate control and test levels of O_2 are in the rat brain slice model.

Hyperoxia as a Model of Oxidative Stress

Oxidative conditions, produced by a variety of mechanisms (e.g., an increase in exogenous or endogenous oxidizing agent or a decrease in endogenous antioxidant), have wide-ranging effects on the mCNS. Oxidation-reduction (redox) reactions are involved in normal signal transduction mechanisms (67, 71), such as O_2 sensing (121, 125, 126, 172) and modulation of neuronal electrical activity (44, 152, 170). However, at high levels, oxidative stress has been implicated as a causative agent, at least in part, in various neurological disorders and diseases (67, 175).

O_2 toxicity of the mCNS is one of the best known examples of how acute exposure to an oxidative environment can disrupt neurological function (9, 43). Normally, when animals and humans breathe normobaric air, neural Pti_{O_2} is surprisingly low, ranging from 1–3 Torr up to ~30–34 Torr, depending on the area of the mCNS (71, 135, 151). Thus, for the purpose of this

review, we will define hyperoxia as any level of inspired P_{O_2} (in vivo) or perfusate P_{O_2} (in vitro) that results in a neural Pti_{O_2} >34 Torr. Depending on the level of Pti_{O_2} in the brain and the duration of exposure, the neurological response to hyperoxia is quite variable. However, in each instance, the effects of hyperoxia on the mCNS are attributed to the oxidative effects of ROS (60–63, 70, 107, 205). For example, the mCNS response to hyperoxia can range from a moderate, but reversible change in neural activity (22, 51, 142, 150, 152), possibly with therapeutic benefits for improved neurological function (33, 38, 158, 203), to violent and reversible seizures at higher levels of Pti_{O_2} (5, 60, 63), to irreversible motor deficits (and even death) at the highest dosages of hyperoxia (9, 12). The use of graded levels of hyperoxia at room pressure (normobaric hyperoxia) and increased ambient pressure (hyperbaric hyperoxia, or HBO_2), therefore, is a useful model for studying the wide-ranging effects of oxidative stress on neurological function, which complements more traditional in vitro models of oxidative stress used at normobaric pressure (e.g., Refs. 169, 170). The utility of employing hyperoxia as an oxidative stimulus is that the natural substrate, molecular O_2 , is supplied to the cell, which, in turn, reacts in one or more biochemical pathways to produce various free radicals (9, 60, 61, 63, 67, 70, 71, 169, 170). Thus, by using graded levels of hyperoxia, one can dissect out specific biochemical steps in redox modulation of neuronal activity and the steps that lead to onset of oxidative damage in brain cells.

The hyperoxic brain slice model, however, has rarely been used to study the effects of oxidative stress on neuronal activity. This is because most in vitro tissue preparations of the mCNS, including the brain slice, brain stem spinal cord, and many cell cultures use a control level of O_2 that is already hyperoxic (i.e., 95% O_2), which leaves little room for increasing Pti_{O_2} further at normobaric pressure. Consequently, the only means of investigating the effects of further hyperoxia is to do so at hyperbaric pressure by using HBO_2 (58, 118, 150–152).

Neural Tissue P_{O_2} in the Intact mCNS During Normoxia and Hyperoxia

The likelihood of onset of O_2-induced seizures (see above, *O_2 Toxicity*) is dependent on the level of hyperoxia. The latency to seizure becomes shorter with increasing $F_{I_{O_2}}$ and/or increasing P_B. In rats, seizures occur after breathing 3 ATA O_2 for 3–5 h, 5 ATA O_2 for 5–90 min, and 6 ATA O_2 for ≤20 min (189). Consecutive exposures to 3–5 ATA O_2 for 1 h/day produce paralysis in rats and frequently death, after four to seven exposures (7). Blood-gas measurements in humans show that arterial P_{O_2} increases from ~90 Torr while normobaric air is breathed to a maximum of 1,900–2,000 Torr while 100% O_2 at 3.5 ATA is breathed (122). Polarographic measurements of Pti_{O_2} in animals breathing normobaric air indicate that O_2 levels in the mCNS range from <10 to 34 Torr (108, 151), but that they increase tremendously when the animal breathes normobaric hyperoxia (39) and HBO_2 (108, 151, 210). For example, as shown in Fig. 4, cortical Pti_{O_2} increases on average (±SE) from 34 ± 4 Torr in a rat breathing normobaric air (defined by the intersection of *points a* and *b*, open symbols) to 452 ± 68 Torr when breathing 100% O_2 at 3 ATA, and to 917 ± 123 Torr while breathing 5 ATA O_2 (#1, open circles) (108). Cortical Pti_{O_2} increases even further, for each level of $P_{I_{O_2}}$, when CO_2 is added to the inspired gas because of CO_2-induced vasodilation of cerebral arteries and, therefore, increased cerebral blood flow. This effect of CO_2 counteracts the vasoconstriction caused by hyperoxia, as discussed above. For example, also shown in Fig. 4 (#2, open squares), cortical Pti_{O_2} increases to 791 ± 51 Torr and 1,540 ± 94 Torr when 95% O_2 + 5% CO_2 at P_B of 3 and 5 ATA, respectively, is breathed.

Neural Pti_{O_2} during normobaric normoxia, normobaric hyperoxia, and HBO_2 is not homogeneous, but varies regionally within the intact mCNS. When normobaric air is breathed, regional Pti_{O_2} is variable [see Table 1 in Mulkey et al. (151) and Erecinska and Silver (71)] with gray matter averaging 13 Torr, whereas white matter is lower and cerebral spinal fluid is higher (39, 52). During HBO_2, regional differences in Pti_{O_2}, similarly, are attributed to differences in neuronal activity, metabolic rate, and cerebral blood flow (71, 162, 204, 206–208). Changes in regional cerebral blood flow during HBO_2 are complex and depend on the pressure and duration of exposure (i.e., dose of hyperoxia). Breathing 3–4 ATA O_2 initially decreases the effective NO· concentration (NO· is a potent cerebral vasodilator) and decreases blood flow by 26–43% in deep brain nuclei (61, 62). The mechanism for this vasoconstrictor effect of hyperoxia was discussed above (*O_2 Toxicity*). Cerebral vasoconstriction provides protection to the mCNS when HBO_2 is breathed, at least initially, by blunting the magnitude of the increase in Pti_{O_2} and, therefore, ROS (61, 62). Longer exposures and/or larger levels of HBO_2 (5–6 ATA O_2), conversely, increase NO· production and augment regional blood flow (63). The mechanism for increased NO· production during prolonged hyperoxia is uncertain. It has been proposed that it results from increased NO· synthesis in the presence of excess molecular O_2, increased nitric oxide synthase (NOS) activity, and increased brain L-arginine levels (63). Regardless of the mechanism, the escape of cerebral blood flow from O_2-induced vasoconstriction, which results in a large increase in regional Pti_{O_2} (I. Demchenko and C.A. Piantadosi, personal communication), is believed to be an important factor in the pathogenesis of CNS O_2 toxicity, because it always precedes onset of increased EEG spiking activity (63).

Other investigators report, however, that the increase in Pti_{O_2} remains sustained throughout the duration of HBO_2 (108, 162) or exhibits a secondary

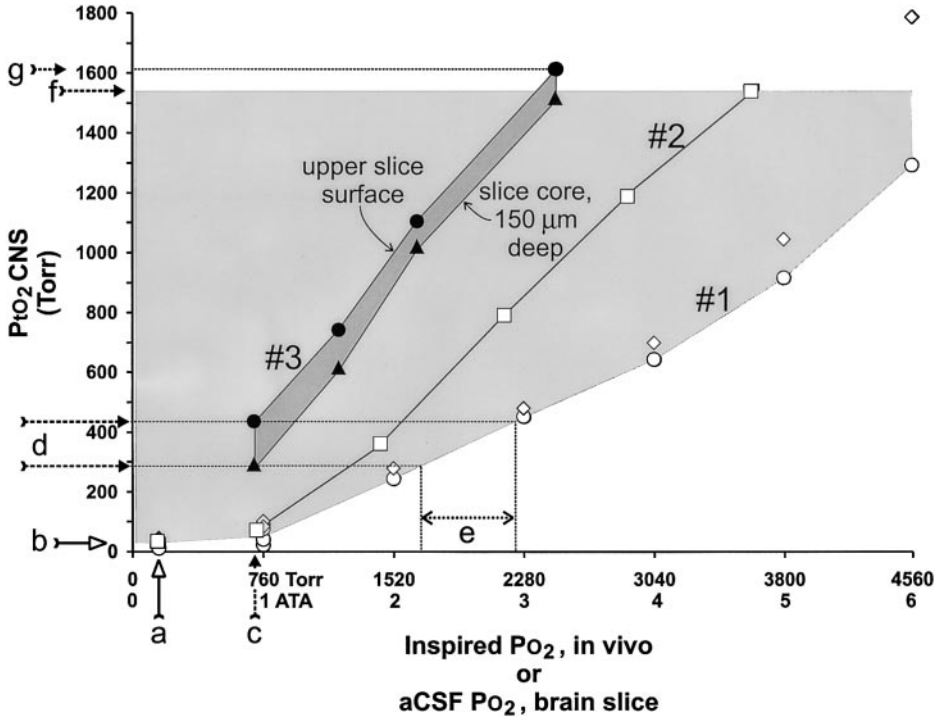

Fig. 4. Comparison of tissue P_{O_2} (Pti_{O_2}) measured in the intact mCNS and in rat brain stem slices. The use of 95% O_2 at ~1 ATA for brain slice experiments yields a control level of Pti_{O_2} that is too high compared with values measured in the intact mCNS. For instance, the first two traces (#1 and #2; light shaded region) show the relationship between inspired P_{O_2} ($P_{I_{O_2}}$) vs. Pti_{O_2} measured in the intact rat CNS when either normobaric air or pure O_2 is breathed at 1–6 ATA (○) or 5% CO_2 in O_2 (□) at 1–6 ATA (108). The P_{O_2} measured in cerebral spinal fluid when pure O_2 is breathed at 1–6 ATA is also shown (◇) near #1. Measurements of Pti_{O_2} (in vivo) in rats range from <10 to 34 Torr (*point b* on *y*-axis) while air was breathed (*point a* on the *x*-axis; i.e., normobaric normoxia) to ~1,600 Torr when 5% CO_2 in O_2 is breathed at 5 ATA ambient pressure (*point f* on the *y*-axis). For comparison, the Pti_{O_2} measured in a 300-μm-thick brain slice at the surface (●) and center (▲, 150-μm depth) at normobaric pressure and during various levels of hyperbaric O_2 (HBO_2) are also shown in the dark shaded area (#3; data are replotted from Ref. 151). The slice was submerged in perfusate, equilibrated at P_B OF ~1 ATA with 95% O_2 at ~37°C (P_{O_2} ~720 Torr, *point c* on *x*-axis). The level of Pti_{O_2} measured at the surface under control conditions is, on average, (means ± SE) 436 ± 21 Torr and at the center of the slice is 291 ± 20 Torr (*region d* on *y*-axis). Notice that this is equivalent to Pti_{O_2} measured in vivo while ~2.2–2.9 ATA HBO_2 is breathed (*region e* on *x*-axis), but without the physical pressure component. Thus, control conditions in brain slice, including the core of the slice, are extremely hyperoxic compared with the intact mCNS. aCSF, artificial cerebrospinal fluid.

decrease in neural Pti_{O_2} that remains elevated above normoxic control level (i.e., transient increase in Pti_{O_2} that partially recovers during hyperoxia) (9, 17, 99, 162, 210). This secondary drop in Pti_{O_2} could merely be an artifact caused by O_2 poisoning of the polarographic electrode; however, it has also been attributed to changes in body temperature during compression (17), O_2-induced vasconstriction of cerebral blood vessels, increased metabolic rate, and development of diffusion impairment caused by pulmonary O_2 toxicity when present (9, 206–208, 210).

Cellular Mechanism: O_2-induced Free Radicals

O_2 toxicity is believed to occur when the body's antioxidant defenses are overwhelmed by increased production of free radicals during the high levels of O_2 reported above. Included in this list are superoxide, hydrogen peroxide, hydroxyl radicals, and peroxynitrite at high levels of Pti_{O_2} (60–63, 70, 160, 171, 205). Excessive production and accumulation of ROS during HBO_2 is thought to alter cellular components, and thus various ionic conductances that regulate cell excitability (58, 150, 152). Similarly, ROS are reported to target certain neurotransmitter and neuromodulator systems, and thus to alter chemical synaptic transmission (25, 40, 227). CNS O_2 toxicity is thought to involve, or be influenced by, glutamate, GABA, and NO· signaling pathways. MK-801 antagonism of NMDA glutamate receptors (40) and inhibition of NOS have been shown to alter HBO_2-induced seizure activity (25, 60, 70, 227), and the actions of O_2 have been shown to decrease inhibitory (GABAergic) synaptic conductance (48). Gap junctional conductance through electrical synapses is also reduced by increased production of ROS (143, 212), but the effects of HBO_2 on electrical coupling in mammalian neurons have not been investigated (56, 98). This will be important to determine, however, because recent research has shown that CO_2/H^+-chemosensitive neurons, which are coupled via gap junctions (56, 57, 98, 190), are highly sensitive to HBO_2 and other chemical oxidants (152).

Electrophysiological and Neuroanatomic Responses to HBO_2

The effects of Pti_{O_2} and free radicals on the mCNS have been studied by using a variety of electrophysiological and neuroanatomic techniques. Cortical EEG activity in animals increases before the onset of seizure (14, 60, 63, 189), typically preceded by increased Pti_{O_2} (210) (I. Demchenko and C. A. Piantadosi, personal communication) and increased ROS production (160, 171). Important sites in the mCNS involved in the pathogenesis of O_2 seizures have been identified by using c-*fos* expression (6), increased 2-deoxyglucose uptake (204, 206–208), and changes in neuronal morphology indicative of lesions and tissue necrosis after extended exposures to hyperoxia (8–10). In the latter case, lesions and tissue necrosis occurred after repeated exposure to HBO_2 on consecutive days, which ultimately produced symptoms associated with the John Bean effect, i.e., paralysis. According to these criteria, many areas in the mCNS are affected by HBO_2 and, therefore, are involved in the onset of O_2-induced seizures and paralysis [Table I in Balentine and Gutsche (10)]. Areas that were consistently affected by HBO_2 include the globus pallidus, substantia nigra, superior olivary nucleus, ventral cochlear nucleus, and spinal cord gray matter (8, 10, 162). Several of these regions also endure the highest level of Pti_{O_2} during HBO_2 (162, 210). However, because prolonged exposures to HBO_2 were used to identify these O_2-sensitive regions, which resulted in permanent neurological deficits, these experiments do not rule out other regions of the mCNS as being involved in O_2 sensitivity during acute exposure to HBO_2. The advent of more sensitive assays of neuronal function, such as single-cell electrophysiology [see below, Figs. 6 and 7, and in the companion paper (152)], have shown that neuronal electrical activity in other regions of the mCNS (brain stem, hippocampus) is affected by acute exposure to HBO_2, and, moreover, that not all neurons in these areas are equally sensitive to HBO_2 and O_2 free radicals (58, 149, 152).

Overall, electrophysiological data at the single-cell level are sparse concerning how HBO_2 affects neurons. Refinements in hyperbaric chamber designs, however, have improved the success rate of intracellular experiments in rat brain slices (APPENDIXES A–D). For example, Fig. 5*A* shows the standard protocol used in our laboratory for testing neuronal sensitivity to pressure per se vs. increased Pti_{O_2} (HBO_2). By carefully adjusting the relative levels of O_2 and CO_2 in the high-pressure sample cylinders (APPENDIX B), a range of hyperbaric hyperoxia can be tested (58, 152). With the use of this protocol, acute exposure to HBO_2 typically increases firing rate in some neurons (26, 150, 152). For example, as shown in Fig. 6*A*, increasing P_B to 3 ATA with 100% He caused a small, but nonsignificant, increase in spontaneous firing rate, which is interpreted as being a baro-insensitive response. Isobaric exposure to 5 min of HBO_2 caused a significant increase in firing rate that was reversible on lowering P_{O_2} and repeatable during a second bout of HBO_2 (152). Neuronal stimulation by acute exposure to HBO_2 is not a generalized property of all solitary complex neurons; in fact, it occurs mostly in CO_2/H^+-chemosensitive neurons (152), which are thought to function as central cardiorespiratory chemoreceptors (54, 56, 98, 156, 157, 183). The excitatory firing rate response to HBO_2 in the solitary complex is reduced or abolished by antioxidants and mimicked by using prooxidants at normobaric pressure (152). These findings indicate that acute exposure to HBO_2 stimulates certain dorsal medullary neurons by increased production of free radicals and/or their reactive nonradical derivatives. Moreover, the firing rate response to HBO_2 may be enhanced by hypercapnic acidosis, suggesting that intracellular acidosis may augment neuronal sensitivity to HBO_2 and ROS in some neurons (152); however, further work is needed to continue testing this latter hypothesis.

Figure 6*B* shows the same experiment repeated in a CA1 neuron in a rat hippocampal slice. In this case, hyperbaric He (HBHe; 3 ATA) also had no significant effect on firing rate (not shown), but acute exposure (12–15 min) to HBO_2 caused a large stimulation in firing rate that was also reversible and repeatable. In both solitary complex and hippocampal neurons, HBO_2 typically increases R_{in}, as shown in Fig. 6*B* (58, 82, 152). In contrast to the solitary complex, our preliminary findings suggest that a greater proportion of neurons in the hippocampus are stimulated by hyperoxia (82).

Fig. 5*B* shows a variation of the above HBO_2 protocol. In this protocol, the brain slice is maintained at the interface of perfusate and gas rather than completely submerged in perfusate (APPENDIX B, *Fluid-gas interface brain slice preparation*). In this example, P_{O_2} and extracellular pH (pH_o) were measured in the tissue bath while a small stream of 95% O_2 + 5% CO_2 was continuously blown across the surface of the brain slice (59). As 100% He flows into the hyperbaric chamber, P_B increases, driving O_2 (increases P_{O_2}) and CO_2 (decreases pH_o) into the perfusate, as predicted by Henry's Gas Law. After decompression, bath P_{O_2} and pH_o return to their control levels. Notice that this effect of He compression on gas tensions in the perfusate is very different from the situation in the submerged slice in which no changes in P_{O_2} and pH_o occurred during He compression due to the absence of any O_2 and CO_2 in the overlying atmosphere (Fig. 5*A*). When the test gas line was shut off at normobaric pressure (Fig. 5*B*, see asterisk), thereby removing the primary source of O_2 and CO_2 in the interface slice (54, 65), P_{O_2} decreased and pH_o increased (i.e., P_{CO_2} decreased), and, during the ensuing He compression and decompression, there were no significant changes in P_{O_2} and pH_o. The utility of this alternative protocol for inducing hyperoxia and the precautions required when using it are discussed in APPENDIX B and elsewhere (59).

Figure 7 shows an example of a neuron in the solitary complex that was exposed to hypercapnic HBO_2 during He compression by using the fluid-gas interface

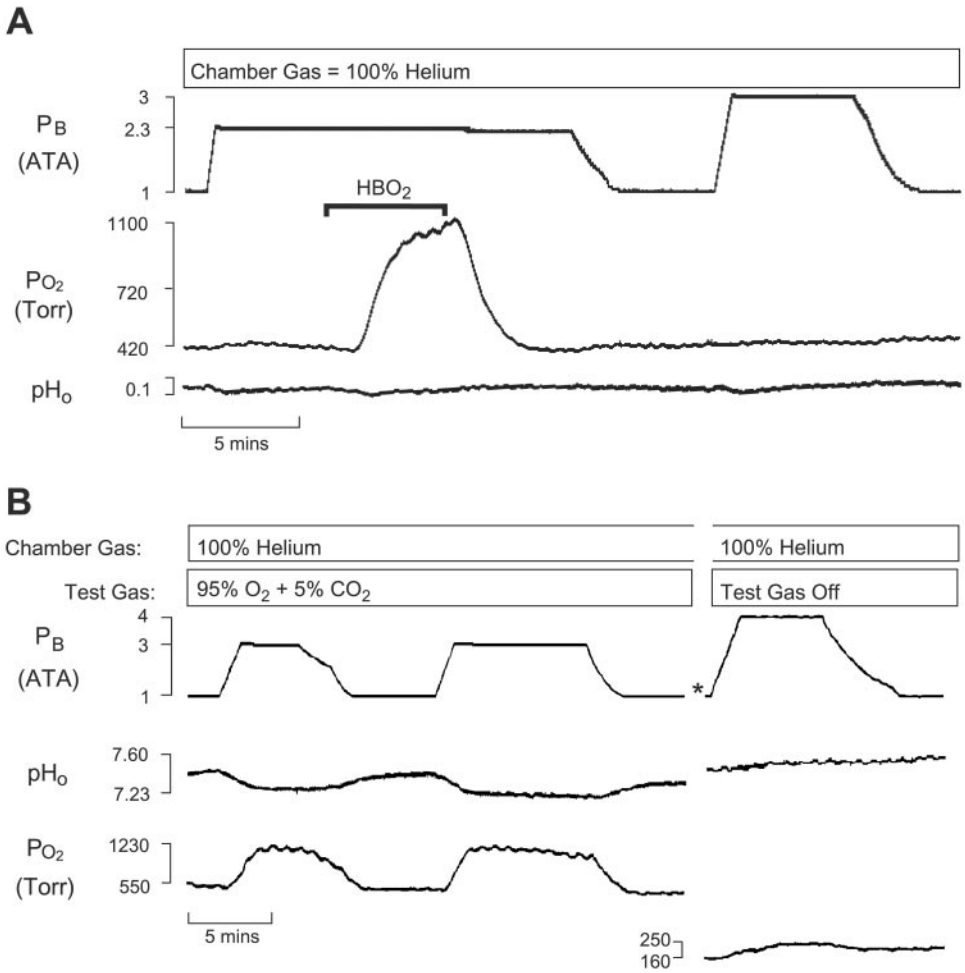

Fig. 5. Two ways to manipulate gas partial pressure and ambient pressure in a rat brain slice by using a cylinder gas compression chamber. *A*: in the submerged brain slice, the levels of O_2 and CO_2 are set in the perfusate at either normobaric pressure or hyperbaric pressure. In the latter case, high-pressure sample cylinders are used (APPENDIX B). Notice that P_{O_2} and extracellular pH (pH_o) remain unchanged as P_B increases during He pressurization. In each case, temperature measured in the bath immediately beneath the brain slice did not change during He compression and decompression (58). Data shown in *A* are redrawn from Dean and Mulkey (58). *B*: the interface brain slice is maintained at the fluid-gas interface of the perfusate and warmed and humidified 95% O_2 + 5% CO_2 atmosphere (Test Gas), which is passed over the slice surface at ~240 ml/min (59). Perfusate (~37°C) is also preequilibrated at room pressure (P_B ~1 ATA) with 95% O_2 + 5% CO_2, before being delivered to the slice with a HPLC pump. The overlying atmosphere of chamber gas is 100% He. When P_B is increased by flowing additional He into the chamber, bath P_{O_2} increases and bath pH decreases (i.e., a rise in P_{CO_2}) as O_2 and CO_2 supplied by the test gas line are driven into the bath and slice. The rise in P_{O_2} and drop in pH_o are reversed with decompression. When the test gas line is shut off while at normobaric pressure (*), thereby removing the primary sources of O_2 and CO_2 in the interface slice (54, 65), P_{O_2} decreases and pH_o increases (i.e., P_{CO_2} decreases) and does not change during the ensuing compression and decompression with He. Data shown in *B* are previously unpublished examples from earlier work by the authors (58, 59). Examples of intracellular recordings made in brain stem and hippocampal neurons using these 2 protocols are shown in Figs. 2*B*, 6*B* (submerged slice), and 7*B* (interface slice). Additional examples of Pti_{O_2} measurements made in a submerged brain stem slice using the first protocol are shown in Figs. 4 and 8.

slice protocol presented in Fig. 5*B*. As P_B was increased with He, tissue P_{O_2} increased and pH_o decreased as O_2 and CO_2 supplied from the test gas line were driven into the brain slice, which, in turn, stimulated firing rate. We have reported that certain neurons in the solitary complex are stimulated by the combination of CO_2-induced intracellular acidification and HBO_2 (152). A second compression, this time using air, also stimulated firing rate, but to a lesser extent than did HBHe, which could possibly be due to the narcotic effects of increased P_{N_2} superimposed on the hypercapnic hyperoxic stimulus (154). Finally, a third test, but this time using hypobaric (i.e., $P_B \ll 1$ ATA) air, caused a decrease in firing rate. Although we have yet to measure tissue P_{O_2} and pH_o at hypobaric pressure, we would anticipate that, as P_B decreases, P_{O_2} is reduced and pH_o is increased. This experiment is an interesting example that illustrates how different gas mixtures administered at equivalent total pressures have different effects on neuronal activity (HBHe vs.

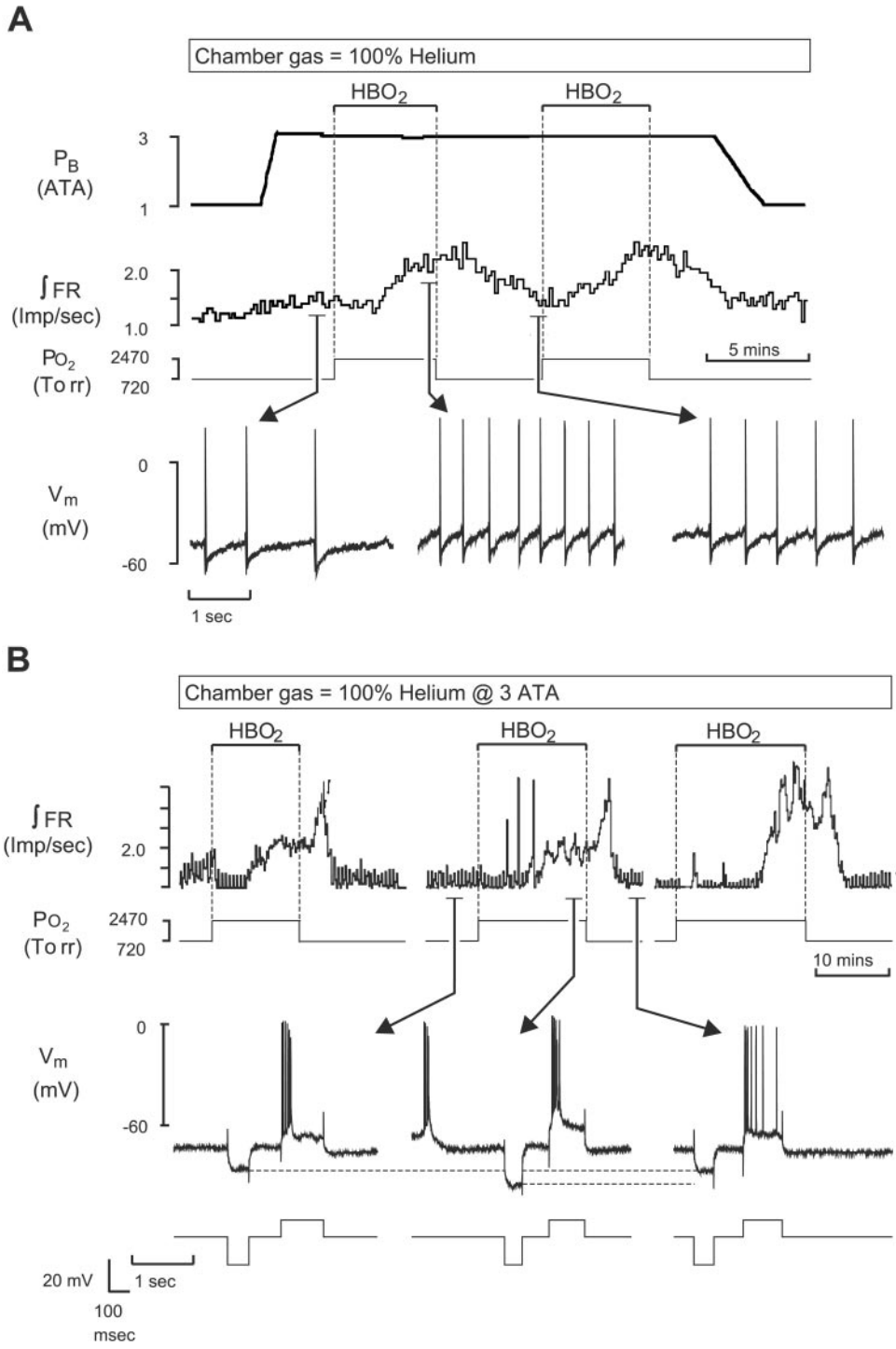

Fig. 6. Intracellular recordings in 300- to 400-μm brain slices prepared from adult rat of a brain stem solitary complex neuron (*A*) and a CA1 hippocampal neuron (*B*) during repeated exposures to short bouts of HBO_2. P_B is increased by using 100% He, and perfusate P_{O_2} is raised by using a high-pressure sample cylinder, as demonstrated in Fig. 5*A*. Spontaneous action potentials are counted, integrated (∫FR; impulses/s), and averaged into 10-s bins by using a window discriminator and rate meter. The solitary complex is an important cardiorespiratory control area in the dorsal brain stem, and it is hypothesized that the excitatory effect of acute HBO_2 in the solitary complex contributes more to the cardiorespiratory symptoms of O_2 toxicity rather than the onset of seizures (150, 152). The hippocampus has been implicated in seizure activity during O_2 toxicity (118, 196). Both cells showed an increased input resistance (*A*, not shown; *B*, see high-gain voltage traces) during HBO_2 and an increase in spontaneous firing (82, 150, 152). In these 2 examples, both cells were determined to be baro-insensitive during compression with hyperbaric He. Data in *A* are an unpublished example from the study by Mulkey et al. (152), and data in *B* are from a preliminary work of Garcia et al. (82).

hyperbaric air). Furthermore, it demonstrates the technical feasibility, for the first time, of maintaining an intracellular recording during decompression to hypobaric pressures, which will be useful for studying both neuronal barosensitivity (153) and the effects of hypobaric hypoxia (97, 148).

The preceding electrophysiological examples do not differentiate between the presynaptic and postsynaptic effects of hyperoxia and ROS on mammalian neurons (152). It is also possible that O_2-induced seizures occur, in part, through an imbalance of excitatory and inhibitory synaptic transmission, involving disinhibition and/or activation of excitatory neurotransmission. Studies in the iCNS suggest that the excitatory effects of HBO_2 are attributed to decreased presynaptic release of glutamate (46, 47) and GABA (48). However, a portion of this effect may be due to the effect of pressure per se that occurs independently of increased Pti_{O_2} (47). In addition, Garcia et al. (83) have shown that 3.0 ATA O_2 increases the amplitude of the population

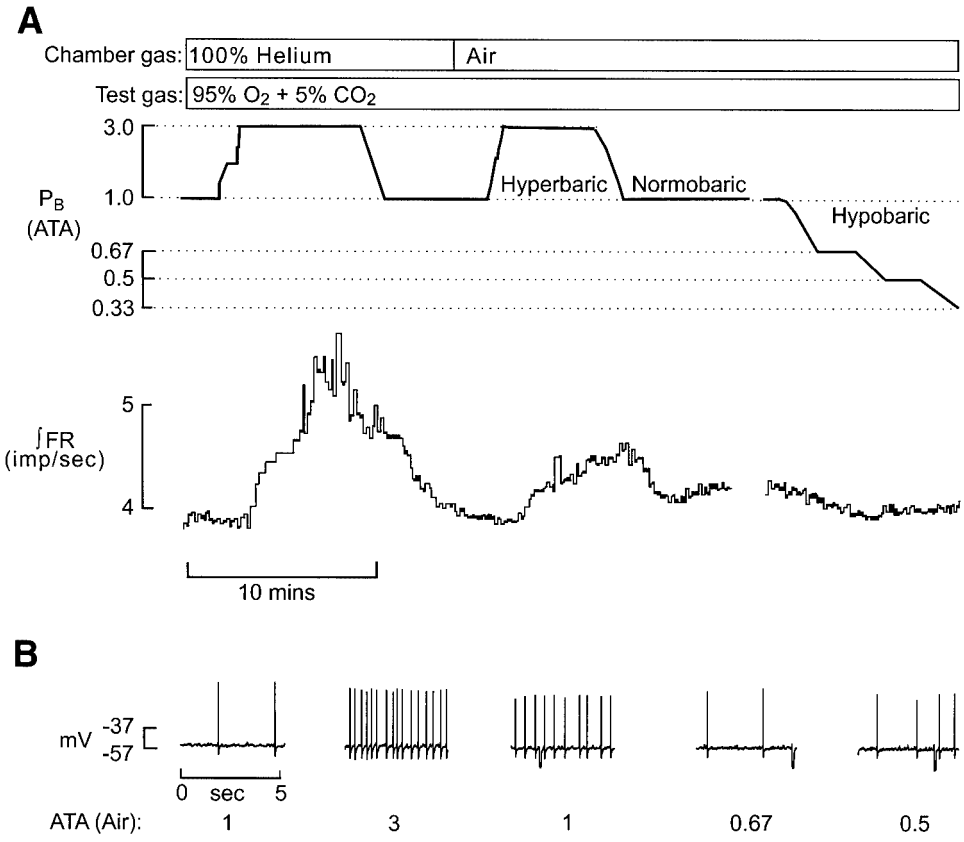

Fig. 7. Administering hyperoxia and hypercapnic acidosis during compression with He or air and during decompression with air <1 ATA (i.e., hypobaric pressure). *A*: intracellular recording of a solitary complex neuron maintained in an interface brain slice during exposure to hyperbaric He, hyperbaric air, and hypobaric air. As shown in Fig. 5*B*, tissue P_{O_2} and P_{CO_2} would increase during compression and decrease during decompression due to changes in P_B affecting the amounts of O_2 and CO_2 being supplied to the brain slice by the test gas blowing across the surface of the slice. *B*: representative action potentials at different pressures used in *A*. Unpublished data are from a preliminary report by Mulkey et al. (154).

spike in the rat CA1 hippocampus evoked by Schaffer collateral stimulation and frequently induces secondary spikes. Similarly, King and Parmentier (118) demonstrated that the prevolley potential and the slope of the field excitatory postsynaptic potential both increased during HBO_2. However, input-output analysis showed that overall synaptic efficiency was decreased. Taken together, these observations demonstrate that HBO_2 affects network activity at multiple levels, yet, ultimately, the net effect is an overall increase in network excitability (83, 118).

Neural Tissue P_{O_2}: In Vitro Studies

What range of Pti_{O_2} should be used to mimic the conditions that occur in vivo during normoxia and hyperoxia? We propose that neuronal sensitivity to hyperoxia should be studied over a broad range of Pti_{O_2}, which includes both normobaric pressure and hyperbaric pressure, for two reasons. First, the O_2 tension that a single neuron is exposed to in vivo, at any level of $P_{I_{O_2}}$, is highly variable, as discussed above. Second, employing a broad range of test P_{O_2} values enables neuronal responses to be studied at subthreshold levels of hyperoxia (nonseizure), threshold levels of hyperoxia (onset of seizures: the Paul Bert effect), and suprathreshold and lethal levels of extreme hyperoxia (sustained seizures, paralysis, and death: the John Bean effect). O_2 -induced seizures are a violent neurophysiological end point. It is likely that many neurophysiological events precede this violent end point and occur at subthreshold levels of hyperoxia without any outward expression of clinical signs and symptoms. If the neurophysiological events that precede O_2-induced seizures can be identified, they will provide insight as to the neural mechanisms that are responsible for the intra- and interindividual variability of mCNS O_2 toxicity (43, 105), as well as ways to prevent onset of seizures. In this context, it is highly relevant that normobaric hyperoxia (i.e., $P_{I_{O_2}}$ >150 but ≤760 Torr), which was once thought to be innocuous to neurological function, is now known to alter neural activity and to stimulate certain functional networks (22, 88, 121, 144, 186, 226). Thus hyperoxia at presumably nontoxic levels is an environmental modulator of neuronal activity (44, 71, 135). For example, in respiratory control, the well-known paradox of hyperoxic hyperventilation at normobaric and hyperbaric pressures is thought to be due, in part, to increased excitability of neurons in brain stem respiratory centers (51, 142). In support of this hypothesis, we have shown that HBO_2 stimulates putative central CO_2/H^+-chemoreceptor neurons in the dorsal brain stem (152), which is one of several sites of central chemoreception for breathing (156, 157, 183). Because hyperoxic hyperventilation serves no adaptive value to the animal in terms of O_2 homeostasis, it is more likely that the paradoxical rise in ventilation represents the high sensitivity of the mammalian brain stem respiratory centers, in particular, the central chemoreceptors to oxidative environments (152).

In in vitro tissue preparations of the mCNS, which lack blood flow, Pti_{O_2} is affected by several factors, including region (gray vs. white matter), position of the tissue in the bath (submerged vs. fluid-gas interface), flow rate of oxygenated perfusate over the tissue, tissue temperature, tissue thickness, metabolic rate, and age of the animal (31, 110, 151, 161). Measurements of Pti_{O_2} in metabolically active brain slices maintained under control conditions (95% O_2 at P_B ~1 ATA) and during HBO_2 (~98% O_2 at P_B ~2.4–3.0 ATA) reveal several features about the brain slice model of mCNS O_2 toxicity (151). First, a significant gradient of Pti_{O_2} occurs in the brain slice that depends on the level of P_{O_2} in the perfusate and the tissue metabolic rate. Figure 8 shows that, as the polarographic electrode is advanced in 50-μm intervals toward the submerged brain stem slice, the P_{O_2} in the overlying perfusate begins to decrease as O_2 is consumed by cells in the upper layers of the slice. At normobaric pressure, at the upper tissue surface, Pti_{O_2} averages ~436 Torr, and, at the middle of the 300-μm slice (150 μm deep), Pti_{O_2} averages ~291 Torr (151). As the polarographic electrode passes through the bottom of the slice and downward, the P_{O_2} gradient due to O_2 consumption by cells in the bottom layers of the slice is evident.

During exposure to graded levels of HBO_2, Pti_{O_2} in the slice increases significantly (Fig. 8, *top* P_{O_2} trace), encompassing a range of values circumscribed by the dark gray area in Fig. 4 (#3, solid symbols; data come from experiments as illustrated in Fig. 8). The magnitude of the Pti_{O_2} gradient from the surface to the center of the slice [see Fig. 7 in Mulkey et al. (151)] is decreased compared with the gradient at 1 ATA, which has been attributed to decreased O_2 consumption during extended exposures to HBO_2 (>10 min). The range of Pti_{O_2} that is produced in a 300-μm brain slice exposed to 2.4–3.4 ATA of O_2 is similar to Pti_{O_2} measured in the intact mCNS of an animal breathing 100% O_2 (open circles) at P_B of 5.4 to >6 ATA and 95% O_2 + 5% CO_2 (open squares) at P_B 3.8 to >5 ATA (range *d*–*g* on *y*-axis in Fig. 4). Thus we have a good in vitro model for studying CNS O_2 toxicity. As was shown above in Fig. 6 and elsewhere (82, 152), acute exposure (≤10 min) to this level of extreme hyperoxia stimulates neuronal activity in the brain stem and hippocampus in a reversible and repeatable fashion. The control condition, however, which will be discussed next, is not normoxic; it is hyperoxic and needs to be reevaluated, given the deleterious effects that oxidative stress has on certain neurons (67, 107).

Choosing a Control Level of Pti_{O_2}

> . . .oxygen pressure in the mammalian CNS is maintained at a level which is sufficiently high to ensure undisturbed function of brain cells and sufficiently low to minimize generation of free radicals.—Erecinska and Silver (71)

The above discussion indicates that hyperoxia and increased levels of ROS can alter neuronal excitability and synaptic transmission. It becomes important, therefore, to reassess the control level of Pti_{O_2} used in most in vitro electrophysiology studies, for it has been stated that ". . . it is a standard Krebs bicarbonate buffer, pregassed with 5% CO_2 and 95% O_2 . . . maybe we use too much O_2. I don't know." (3). The use of 95% O_2 at P_B of ~1 ATA produces a P_{O_2} in the perfusate of ~720 Torr (24, 81, 110, 151), which, in turn, produces a Pti_{O_2} in a submerged 300-μm slice ranging from ~436 Torr (surface) to 291 Torr (center) (151). Stated another way, the core of a 300-μm brain slice, where Pti_{O_2} is lowest, is one order of magnitude or more above the normoxic level in the intact mCNS! As shown in Fig. 4

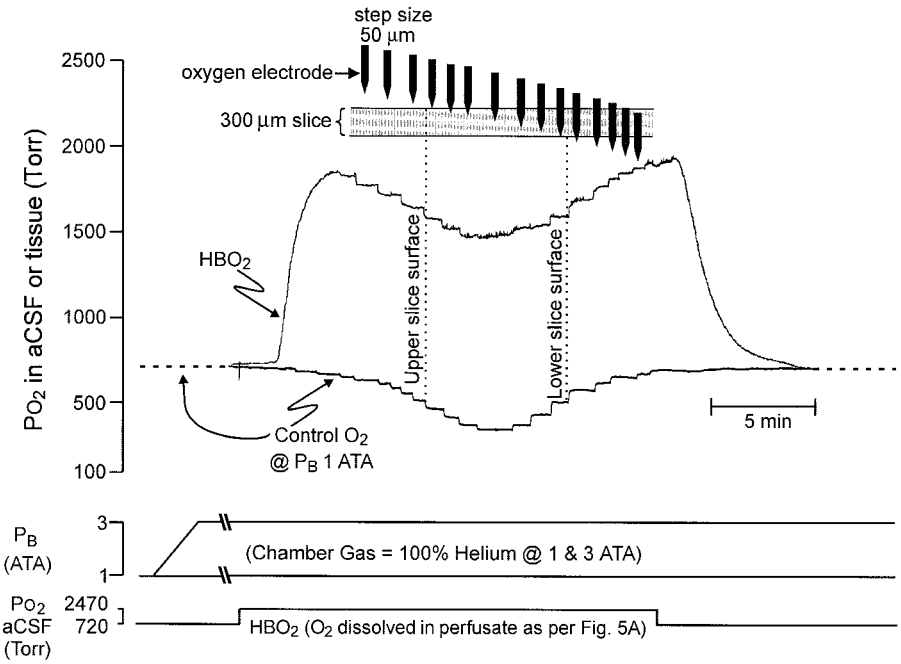

Fig. 8. Brain stem slice P_{O_2} profiles measured at 50-μm intervals at normobaric pressure (Control O_2 at P_B 1 ATA) and during 3 ATA O_2 (HBO_2) in a 300-μm-thick slice (~37°C). See text and APPENDIX B for an explanation of how high-pressure sample cylinder is used to prepare HBO_2 perfusate. These data are unpublished examples from the study by Mulkey et al. (151).

(#3), there is a range of control Pti_{O_2} in the brain slice that is equivalent to the level of Pti_{O_2} that occurs in vivo when ~2.2–2.9 ATA O_2 is breathed (#1; compare *range d* on *y*-axis to *range e* on *x*-axis in Fig. 4). Thus the standard control conditions used for most in vitro experiments (brain slice and brain stem-spinal cord preparations) mimics the Pti_{O_2} that occurs during HBO_2 (2.2–2.9 ATA), excluding the physical pressure component. Regardless, with few exceptions (71, 151, 186, 226), this high level of Pti_{O_2} is accepted by most investigators as the appropriate level of O_2 for maintaining thick and thin preparations of the mCNS (3, 31, 110, 161).

What effects are 95% O_2 at normobaric pressure having on the isolated mCNS (3), and, moreover, are neuronal responses observed during HBO_2 (e.g., Fig. 6) blunted by the hyperoxic control condition that is used during slice preparation and incubation (58, 150, 152)? It is interesting that very few investigators have ever questioned the possibility that too high a control Pti_{O_2} is used for brain slice (and isolated brain stem-spinal cord) experiments, especially because the optimum Pti_{O_2} for cell growth and viability has been determined experimentally for neuronal cultures; for example, 9% O_2 at normobaric pressure produces Pti_{O_2} of 68 Torr, which results in maximum cell survival and synthesis of neurofilament in cultures of neonatal rat cerebral cortex (30, 112). Historically, 95% O_2 was used in brain slices to produce an adequate partial pressure gradient to deliver O_2 to the core of the slice to avoid tissue hypoxia and anoxia (3). Whereas an anoxic core was a concern for brain slices that ranged in thickness from >300 up to 1,000 μm (24, 110), in recent years, the trend has been for brain slices to be cut thinner, typically at 100- to 300-μm intervals (20, 69). In addition, because of widespread use in brain slices of infrared video microscopy for patch clamping and fluorescence microscopy, the tendency in recent years has been to study neurons exclusively in the outermost cell layers (55, 69, 78, 176), where Pti_{O_2} is highest (24, 81, 110, 151). Despite the gradual reduction in thickness of the slice preparation during the past decade, electrophysiologists have continued to employ 95% O_2 as the control level of P_{O_2} with disregard for what effect increased levels of ROS might be having on baseline neuronal activity, responsiveness to the test stimulus, and slice viability in general.

In brain slice electrophysiology, very little is known about how normobaric O_2 in the range of >15% up through ≤95% affects excitability of mammalian neurons. Typically, ≤15% O_2 is used to study the effects of hypoxia and anoxia in the brain slice model [e.g., see Refs. 65 and 110 and Table 1 in Mulkey et al. (152)]. When a lower level of control O_2 was studied in slices, it was reported that 95–100% O_2, compared with 21% O_2 (control), impaired neuronal thermosensitivity in hypothalamic neurons (186), altered V_m and firing patterns in hippocampal neurons (23), and attenuated O_2 sensitivity of the peripheral chemoreceptors for cardiorespiratory control (121, 144). All of these authors proposed that the changes in neuronal activity observed in 95–100% O_2 were due to the effects of increased production of ROS during normobaric hyperoxia, which were decreased in 21% O_2. This hypothesis is supported by reports (119, 168) that the amount of tissue damage resulting from lipid peroxidation was significantly increased in brain slices incubated in 95% O_2 compared with 21% O_2. Similarly, there were greater levels of F_2-isoprostanes and F_4-neuroprostanes in hippocampal slices maintained for up to 7 h in perfusate equilibrated with 95% O_2, compared with control slices analyzed before incubation in 95% O_2 (J. Fessel, personal communication; Ref. 77). Isoprostane-like compounds are reaction products derived from oxidation of docosahexaenoic acid, a component in neuronal membranes, and are used as a marker for increased oxidative stress. It is not clear, however, that 21% O_2 is a better level to use in all cases (81), and a recent brain slice study in the hippocampus suggests that the optimal control Pti_{O_2}, as determined by electrophysiological criteria, may, in fact, lie somewhere between 21 and 50% O_2 (219).

Given the effect that oxidative stress has on neuronal activity (82, 118, 150, 152, 169, 170), future studies are needed to determine the effects of normobaric hyperoxia on brain slice metabolism and electrophysiology. Because of the characteristic Pti_{O_2} gradient that occurs in the slice (Fig. 8), due to long diffusion distances and regional differences in O_2 consumption, neurons within the slice will always be exposed to a range of Pti_{O_2}, regardless of the level of P_{O_2} in the perfusate. However, we are proposing that the range of O_2 used during slice preparation, incubation, and recording can be adjusted down toward a level that is intermediate to tissue hypoxia and hyperoxia. The use of lower control P_{O_2} and/or addition of a suitable antioxidant to the medium for certain types of experiments may likewise enhance slice viability and neuronal activity as already demonstrated for cell culture preparations (30, 112), although to do so will be problematic for studies of the effects of hyperoxia. In thicker preparations like the neonatal brain stem-spinal cord, it may be advantageous to include antioxidants to reduce the effects of oxidative damage to the outermost cell layers where Pti_{O_2} is highest. Alternatively, the perfused version of this in vitro preparation can be used, the so-called rat "working heart-brain stem preparation," in which the vascular system is perfused with oxygenated buffer. However, it will be important to lower the level of P_{O_2} in the perfusate, which also is hyperoxic (95% O_2) compared with the intact mCNS, producing an average Pti_{O_2} of 294 Torr (222).

PERSPECTIVE

Hyperbaric gases (O_2, CO_2, and N_2) and pressure per se (HBHe and hydrostatic pressure) are powerful modulators of neuronal function at ambient pressures <5 ATA. We have discussed and demonstrated that the ability to measure V_m, membrane conductance, firing rate, and synaptic potentials at hyperbaric pressure is a valuable research tool for studying the underlying

neurophysiology of baro-dependent disorders of the mCNS that are encountered at P_B <5 ATA in hyperbaric medicine, diving medicine, and submarine medicine. Examples that were demonstrated or discussed included O_2 toxicity, N_2 narcosis, and CO_2 toxicity. In addition, the maximum pressure range to date, at which it has been feasible to maintain a stable intracellular recording of a mammalian neuron, during cyclical He compression and decompression, is 1–20 ATA. Thus it is also possible to study the early neuronal mechanisms of barosensitivity that precede and/or participate in onset of HPNS. The utility of hyperbaric electrophysiology in studying other baro-dependent problems of the mCNS, such as decompression sickness, remains to be determined.

We would also propose that the utility of hyperbaric electrophysiology is not limited to just these specialized fields of environmental physiology and hyperbaric medicine. In addition, hyperbaric electrophysiology is a powerful research tool that can be used for studying the neurophysiological consequences, at the level of the single neuron, of oxidative stress (9, 43, 106, 150, 152). For example, because of the almost universal use of 95% O_2 as the control level of O_2 in brain slice experiments, which leaves essentially no room for elevating Pti_{O_2} by conventional slice methods, the only means of identifying the effects of hyperoxia on mammalian neurons in slices was to do so by using hyperbaric electrophysiology. Without this novel research tool, it is unlikely that the effects of hyperoxia on the membrane properties of putative central CO_2/H^+-chemoreceptor neurons would have been discovered (152). Moreover, hyperbaric electrophysiology can be used to study cellular mechanisms of anesthesia; for example, hyperbaria can be used either to study the relative narcotic potencies of various gases on neuronal excitability (28), or as a tool to study pressure reversal of anesthesia (113, 115, 167). Hyperbaric electrophysiology can also be used to study other neurological questions that are associated with increased neural tissue pressure, such as intracranial hypertension that occurs during adaptation to inertial force environments (224), space adaptation syndrome (109), and high-altitude sickness (97). Similarly, studying how increased intracranial pressure alone and in conjunction with other cellular insults (e.g., anoxia and hypoxia, acidosis, osmotic imbalance, etc.) affects neuronal activity will be a useful model for studying cellular responses to traumatic brain injuries (4, 64, 140, 181).

APPENDIXES A–D: HYPERBARIC METHODS FOR IN VITRO ELECTROPHYSIOLOGY AND MICROSCOPY

The preceding review of disorders of the mCNS caused by breathing hyperbaric gases (see OVERVIEW OF BARO-RELATED DISORDERS OF THE MCNS) indicates that neuronal function should be studied over a broad range of ambient pressures, ranging from 1 up through 4 ATA for CO_2 toxicity and O_2 toxicity, to ≥4 ATA for N_2 narcosis, and from ≤15 to ~70 ATA for HPNS (Fig. 1). Moreover, two conditions must be considered as possibly causing neuronal dysfunction: increased gas partial pressures (P_{O_2}, P_{N_2}, and P_{CO_2}; see NARCOTIC AND TOXIC PROPERTIES OF GASES) and increased hydrostatic pressure or "pressure per se" (see CENTRAL EFFECTS OF PRESSURE PER SE). The following sections (APPENDIX A) describe methods for differentiating the effects of pressure per se (barosensitivity) from those of increased gas partial pressure (chemosensitivity and narcosis) on neurons (in vitro). The variations used in these methods and their inherent complexity warrant a brief summary of their key features and differences for those unfamiliar with hyperbaric cell physiology.

Appendix A: Testing Neuronal Barosensitivity

Hydrostatic compression chambers. There are two general methods of compression used to study neuronal barosensitivity. The first is hydrostatic compression of the submerged tissue preparation by using a liquid inert medium of mineral oil, paraffin, or perfusate. Hyperbaric chambers designed for hydrostatic compression typically have a smaller internal volume (milliliters) compared with hyperbaric chambers designed for cylinder gas compression (tens of liters; see next section). The small internal volume of a hydrostatic compression chamber is beneficial because it enables the investigator to rapidly increase ambient pressure when studying the biophysical effects of an extreme level of hyperbaria (184). Hydrostatic compression chambers have typically been used to impose extremely high levels of hydrostatic pressure, usually >100 ATA, on nonmammalian neurons and axons (214, 215) and large nonneuronal cells (91, 184). The tissue preparation is submerged in a static bath, and a pneumatic pump delivers compression medium to the interior of the chamber, completely displacing any gas overlying the tissue bath. Cam-driven microdrives are used to manipulate the recording microelectrode, rather than electric microdrives, because the latter are incompatible with aqueous compression medium (91, 166, 215). Typically, adiabatic temperature changes occur during rapid compression and decompression that require several minutes to dissipate, which often confounds electrophysiological data collected during the act of compressing and decompressing the chamber (91, 215). In addition, because most chamber designs use a static bath, the composition of the tissue bath cannot be manipulated by the investigator, and the small internal volume of the chamber, which facilitates rapid compression and decompression, makes it difficult to position and manipulate the recording micropipette (184).

Cylinder gas compression chambers: HBHe. The second method of compressing the tissue preparation uses cylinder gases, typically pure He. It has been the preferred method for working at moderate levels of hyperbaria and for working with in vitro preparations of the mCNS (58, 75, 191). It is the method that was used in all of the examples presented in this review article. The larger internal volume of the hyperbaric chamber and use of gaseous compression medium allow the electrophysiology equipment (microdrives, stimulating electrode, etc.) to be placed directly inside the chamber, with the brain slice for easy positioning and manipulation, when the chamber door is open at room pressure, or by remote control after the chamber door is sealed (58, 59). In addition, this style of chamber can be used to hold a custom-built, fixed-stage epifluorescence microscope, equipped with Hoffman modulation contrast optics and video-imaging capabilities, to visualize neurons in a brain slice for patch-clamp electrophysiology and/or ratiometric fluorescence imaging (see APPENDIX D) (84).

The tissue preparation is either superfused (i.e., maintained at a fluid-gas interface) or submerged in perfusate that is continuously delivered via a high-pressure liquid

chromatography (HPLC) pump. Pure He flows into the pressure chamber, which, in turn, compresses the tissue bath and neural tissue. At low levels of hyperbaria, He is believed to effectively mimic the effects of hydrostatic compression (58, 192, 197, 198). Because He has the lowest solubility in lipid membranes of any of the gases (Fig. 3), it has no known narcotic or toxic effects on cells at low levels of hyperbaria (28, 215). Thus, despite increasing the P_{He} in the tissue during compression, any neuronal response to HBHe, at moderate pressures, is attributed to the effects of pressure per se rather than the effects of increased P_{He} (58, 72, 73, 150, 153, 197, 215); see below, *Compression media: HBHe vs. hydrostatic compression*.

As in most brain slice experiments (3), 95% O_2 + 5% CO_2 gas mixture is used to aerate a bicarbonate-buffered perfusate at normobaric pressure before it is pumped into the hyperbaric chamber. The use of 95% O_2 at P_B ~1 ATA establishes a large O_2 diffusion gradient from the perfusate into the avascular brain slice, resulting in a characteristic Pti_{O_2} profile that is hyperoxic at all depths in a ≤300-μm brain slice (151) (Figs. 4 and 8). Similarly, a qualitatively similar but larger Pti_{O_2} gradient profile is measured in the isolated neonatal rat brain stem-spinal cord (31, 161), which has also been used in hyperbaric studies (197, 198). The potential problem arising from using a hyperoxic control medium for studies of mCNS O_2 toxicity, and for in vitro studies in general, was discussed above (*Neural Tissue P_{O_2}: In Vitro Studies* and *Choosing a Control Level of Pti_{O_2}*).

At normobaric pressure, medium P_{O_2} and P_{CO_2} and pH_o remain constant as perfusate is pumped into the unpressurized hyperbaric chamber, because the solution has no opportunity to degas before reaching the tissue bath (58, 75, 191). Once at the tissue bath, however, gases dissolved in the perfusate diffuse into the overlying He atmosphere (151). The continuous flow conditions at the tissue slice prevent any significant changes in tissue P_{O_2}, P_{CO_2}, and pH_o while at normobaric pressure (Fig. 5*A*). A common misconception is that, once He compression commences, P_{O_2} and P_{CO_2} in the perfusate and tissue will increase as P_B increases. However, as shown above in Fig. 5*A*, this is not the case for two reasons. First, the hyperbaric chamber contains no source of O_2 and CO_2 in the overlying atmosphere to be driven into the tissue (58, 75, 197, 198); the amount of O_2 lost from the perfusate to the overlying atmosphere is infinitely small compared with the volume of the hyperbaric chamber and the frequency with which it is flushed with He (59). Second, the solubility coefficients for O_2 and CO_2 and the negative log of the dissociation coefficient of a bicarbonate buffered solution are essentially unchanged when P_B is increased to hyperbaric pressures ≪100 ATA. Consequently, there are virtually no changes in dissolved gas pressures and pH of the perfusate during compression and decompression (159, 199, 200). Thus bath and tissue P_{O_2} and P_{CO_2} (and pH_o) remain at control levels during He compression (Fig. 5*A*). In addition, bath and tissue temperatures can be tightly regulated by using a servo-controlled temperature regulator, despite fluctuations in atmospheric temperature during He or air compression and decompression (58).

Other variations of the cylinder gas compression protocol exist (34, 96, 191). One variation to the above method that is worth elaborating interposes a physical diffusion barrier between the overlying chamber atmosphere and the tissue bath by using either mineral oil (34) or a Plexiglas lid (96, 124). In this case, air rather than He was used to compress the tissue bath. Because neither P_{O_2} nor P_{N_2} increases in the perfusate, this approach provides true hydrostatic compression of the tissue preparation without any accompanying change in P_{O_2} or P_{N_2} (34, 96, 124). This same approach could also be used to study the effects of hydrostatic compression vs. He compression to test for any possible narcotic effects of HBHe at higher pressures, which are discussed next.

Compression Media: HBHe vs. Hydrostatic Compression. What is the evidence that HBHe can be used to mimic the effects of hydrostatic compression on the mCNS (Fig. 2)? All inert gases possess narcotic actions that are directly related to their lipid solubility (19, 28) (Fig. 3). However, the narcotic potency of He in excitable tissues, when it is detected, is reported to be only weak and of little consequence and is usually observed only at the highest levels of hyperbaria; e.g., ≫100 ATA (163). For example, as is the case with other narcotic gases, HBHe (101–150 ATA) can partially reverse the deleterious effects of increased hydrostatic pressure on animal behavior and motor activity (11, 66). Less common, under certain in vitro conditions (28), effects of only 6–8 ATA He on cellular processes have been reported (87, 182). Thom and Marquis (202) have speculated that the cellular effects of HBHe, like the other narcotic gases, are caused in part by gas molecules dissolving in the hydrophobic regions of the cell membrane, causing distortion of the membrane and possibly disturbing hydrophobic interactions in proteins through noncovalent interactions.

HBHe potentially has other effects that are unrelated to its weak narcotic nature. For example, Thom (201) reported that low levels of HBHe (≥2 ATA) caused increased production of superoxide radicals in two different free-radical generating systems in vitro. A similar effect of HBHe in the mCNS could potentially alter neuronal activity; however, Mulkey et al. (153) found that neuronal barosensitivity to HBHe (≤4 ATA), in solitary complex neurons, was maintained in the presence of the antioxidants ascorbate (D. K. Mulkey and J. B. Dean, unpublished observations) and Trolox C (153). This suggests that, at least in solitary complex neurons, barosensitivity to HBHe (Fig. 2) is unrelated to increased production of ROS.

In general, the effects of HBHe on cells, tissues, and intact organisms are comparable to the effects of hydrostatic pressure over a range of mechanically tolerable pressures (28, 141, 215). The few studies that have compared the effects of both types of compression media on electrophysiological properties of neurons and axons confirm this assertion. Wann et al. (215) reported that the effects of HBHe (~205 ATA) applied at a rate of 6.8 ATA/s exert an effect on *Helix* neurons that is indistinguishable from that observed during hydrostatic compression (214); both compression media increased the duration of the action potential by slowing the peak rates of depolarization and repolarization, decreased R_{in}, and depolarized V_m. These effects on the action potential were due primarily to decreased kinetics of Na^+ and K^+ activation and inactivation rather than a change in the magnitude of each early and late current (92). Interestingly, we saw the same effects on the action potential in solitary complex neurons, but at much lower pressures of only 2.5–4 ATA He; however, we have not compared these experiments to the effects of hydrostatic compression (D. K. Mulkey and J. B. Dean, unpublished observations). HBHe and hydrostatic pressure (35–137 ATA) also had identical effects on evoked synaptic extracellular responses in the isolated rat superior cervical ganglion; both compression media had no effect on the amplitudes of the preganglionic and postganglionic action potentials, depressed synaptic transmission, and antagonized the partial conduction block produced by halothane, methoxyfluorane, and ethyl alcohol (115). Bennett (16) reported that 11 ATA He (with normoxia) had no effect on either the brain stem auditory evoked response or spontaneous electri-

cal activity in the cortex in the cat, and Roth (179) reported that 68 ATA He had no effect on the stimulus-response curve for an isolated frog sciatic nerve.

Comparison of the effects of He compression vs. hydrostatic compression has also been made in intact animals and was summarized by Brauer et al. (28). Based on quantitative measurements of abnormal behaviors and motor deficits induced during hyperbaria (50 to ~200 ATA), in aquatic animals (newts) and liquid-breathing mammals (mice and dogs breathing fluorocarbon), both of which lend themselves to hydrostatic compression in the presence and absence of He, they concluded ". . . that differences between the two modes of compression were not observed." Thus, based on evidence from electrophysiological studies of excitable cells and tissues, and physiological and behavioral studies in intact animals, we would conclude that using He to compress the tissue bath and brain slice effectively mimics hydrostatic compression at P_B <5 ATA (and higher), and that any changes in neuronal excitability during exposure to HBHe, such as seen in the examples shown in Fig. 2, reflect the effects of pressure per se rather than increased P_{He} (58, 82, 150, 153).

Appendix B: Testing Neuronal Chemosensitivity to Hyperbaric O_2, CO_2, and N_2

Submerged tissue preparations: high-pressure sample cylinder. The above methods enable testing of neuronal barosensitivity at controlled levels of tissue P_{O_2}, P_{CO_2}, and pH. Once at hyperbaric pressure, tissue P_{O_2}, P_{CO_2}, and/or P_{N_2} can be raised independently of any additional increase in P_B (i.e., under isobaric conditions) by using a high-pressure sample cylinder (58, 82, 93, 96, 151, 197). Each high-pressure cylinder contains perfusate that is equilibrated with a gas mixture containing O_2, CO_2, and/or N_2, whose total pressure is equivalent to, or just slightly greater than, the total pressure in the hyperbaric chamber. For example, the protocol used in our laboratory for testing neuronal responsiveness to HBO_2 is isobaric replacement of the control perfusate [P_{O_2} ~420 Torr (151)] while at hyperbaric pressure with perfusate supplied from a high-pressure sample cylinder that has been pressurized by using a high-dose O_2 gas mixture [P_{O_2} ~1,100 Torr (151)]. In this case, the F_{CO_2} is also reduced proportionately to maintain P_{CO_2}, and thus pH_o, the same as in control perfusate (58, 82, 150–152). With the use of the protocol shown in Fig. 5*A*, the effects of HBO_2 vs. pressure per se (i.e., HBHe) on neuronal activity have been tested (58, 82, 152). Two examples of this method were shown above in Fig. 6. In addition, P_{CO_2} can be increased with P_{O_2} to study the effects of HBO_2 plus hypercapnic acidosis on neuronal activity (152).

A comparatively simpler method to study the effects of a hyperbaric gas, which does not involve the use of a high-pressure sample cylinder or changing the perfusate, is to compress the hyperbaric chamber with the gas of interest. This approach is particularly well suited for studying the effects of hyperbaric air or N_2 on neuronal function, but not HBO_2, due to the risk of a chamber fire when electrical equipment is operated in a pressurized hyperoxic atmosphere (59, 185). Pressurizing the chamber with air (21% O_2 + 79% N_2) or pure N_2 compresses the bath and tissue, while increasing the bath and tissue P_{N_2}, for investigating cellular mechanisms of N_2 narcosis (34, 35, 154); as usual, O_2 (95%) and CO_2 (5%), at normobaric pressure, are dissolved in the perfusate and pumped into the chamber by using the HPLC pump.

In addition, Imbert et al. (102) have designed an elaborate system for saturating perfusate with different gases, including H_2, to study the effects of hyperbaric gas mixtures on various in vitro tissue preparations. This system has yet to be used for electrophysiology studies, however.

Fluid-gas interface brain slice preparation. An alternative to the submerged slice preparation is the fluid-gas interface slice preparation in which either a control (95% O_2 + 5% CO_2) or test gas mixture is blown over the surface of the brain slice maintained at the interface between perfusate and an atmosphere of control and test gas mixture (59, 154). Without changing the source of perfusate, which also is aerated with 95% O_2 + 5% CO_2, tissue gas tensions are manipulated rapidly by changing the composition of the gas mixture flowing through the small test gas line. This approach has been used at normobaric pressure to study the effects of anoxia (0% O_2 + 5% CO_2 + 95% N_2), hypoxia (≤15% O_2 + 5% CO_2 + balance N_2), and hypercapnic acidosis (7–15% CO_2 + balance O_2) in brain slices (54, 65). Similarly, at hyperbaric pressure, blowing 95% O_2 + 5% CO_2 gas across the upper surface of the interface slice, while compressing the hyperbaric chamber with 100% He, increases bath and tissue P_{O_2} and P_{CO_2} and decreases pH_o (59). Figure 5*B* shows an example of P_{O_2} and pH_o measured in the tissue bath while 95% O_2 + 5% CO_2 were continuously blown across the brain slice surface at normobaric and hyperbaric pressures. Notice that both P_{O_2} and P_{CO_2} will increase during He compression as O_2 and CO_2 supplied by the test gas line are driven into the slice and perfusate. Using pure O_2 as the test gas, rather than 95% O_2 + 5% CO_2, while pressurizing the chamber with He would allow a rapid HBO_2 test without any change in pH_o. However, certain precautions must be taken, which are summarized elsewhere (59), to prevent the slow build-up atmospheric P_{O_2} being supplied by the small-diameter test gas line, which would increase the risk of a chamber fire.

Appendix C: Comparison of Electrophysiology Recording Techniques

> Pressure, like temperature, is one of the fundamental physical variables which constrain living entities. Because it is difficult to manipulate pressure as an experimental variable, however, it has not been a subject of casual study; indeed, very powerful motivation is required to pursue studies of pressure effects.—Kendig, Grossman, and Heinemann (114).

Not all electrophysiological recording methods have been equally successful when used with isolated tissue preparations during compression and decompression. The relative success of each type of electrophysiological recording in the past, however, may have been determined more by deficiencies in hyperbaric chamber designs, which limited the investigator's accessibility to the microelectrode at normobaric pressure, rather than any inherent limitation of the electrophysiological recording method per se (58, 59). The following three sections compare and contrast the various types of electrophysiological recordings and their relative merits and problems as reported in the literature to date.

Extracellular recording. Before the mid-1980s, hyperbaric electrophysiology studies employed nonmammalian and nonneuronal in vitro models exclusively. Pioneering work by several independent laboratories in the late 1980s and 1990s, however, demonstrated that in vitro preparations of the mCNS, such as the rat hippocampus and cerebellum brain slices (72–74, 76, 191, 192, 216) and neonatal rat brain stem-spinal cord (197, 198), could be manipulated under hyperbaric conditions to study HPNS. At that time, making intracellular recordings of mammalian neurons proved to be technically challenging due to the comparatively small size of the neurons, inaccessibility of the tissue preparation and

microelectrode once the pressure vessel was sealed, and the challenge of maintaining mechanical stability of the electrophysiological recording while "diving" and "surfacing" (191, 192). Consequently, the majority of in vitro hyperbaric studies of the mCNS have selected more robust electrophysiological techniques, such as extracellular recording of evoked population spikes in the hippocampus (76, 118, 191, 192, 229), macropatch clamp recordings in the cerebellar cortex (72–74), and cervical nerve recordings of respiratory-related neural activity (197, 198). Compared with intracellular recordings, extracellular recordings are easier to initiate once the chamber is sealed and to maintain during the ensuing compression and decompression periods.

Intracellular recording. Intracellular recordings provide additional mechanistic information about neuronal function by quantifying changes in a variety of cellular properties, including V_m and currents, membrane conductance and R_{in}, integrated firing rate, evoked repetitive firing properties, and voltage waveforms (action potential, afterhyperpolarizing potential, chemical and electrical synaptic potentials). In addition, intracellular dye injection allows recovery of the physiologically identified cell for subsequent morphological and immunohistochemical analyses. Intracellular recordings, however, are extremely sensitive to mechanical disturbances. The majority of successful hyperbaric studies that used the intracellular recording technique have been conducted in robust in vitro preparations, such as large nonneuronal cells (49, 93, 96) and large invertebrate neurons and axons (34, 166), which, compared with mammalian neurons, are relatively less sensitive to mechanical disturbances.

Mammalian neurons, therefore, are more difficult to record intracellularly during manipulation of P_B (191, 192, 216). Only two laboratories have studied the intracellular properties of neurons in rat brain slices, focusing on cellular mechanisms of HPNS (191, 192, 216), barosensitivity to moderate pressures (150, 153), and O_2 toxicity (150, 152). Areas studied include the solitary complex in the dorsocaudal medulla oblongata (58, 150, 152, 153) and the CA1 cell layer of the hippocampus (82, 191, 192, 216) (e.g., Figs. 2, 6, and 7). Successful intracellular recording of a mammalian neuron requires a sharp-tipped microelectrode, which can easily plug or break as it tracks through the tissue and, therefore, must be frequently replaced. If the pressure chamber is too difficult to open and close, then changing the microelectrode becomes problematic and an impediment to the experiment (191, 216). The requirement for an operator-friendly door was fulfilled recently in a hyperbaric chamber design that combined a commercially available door with the best features from earlier chamber designs, which collectively, optimized mechanical stability of the microelectrode and tissue preparation (58, 59).[4] Using this style of hyperbaric chamber, intracellular recordings have been made with routine success from mammalian neurons during exposure to 2–4 ATA of He, HBO_2, hyperbaric air, and hypobaric air, as shown here and elsewhere (58, 150, 152, 153). Continuous recordings also have been maintained while cycling P_B to and from 20 ATA of He (Fig. 2*B*).

Patch-clamp recording. Patch-clamp recordings of whole cell and single-channel ionic currents have been made at extremely high hydrostatic pressures in nonmammalian cells to study HPNS and the effects of large hyperbaric pressures (91, 130, 132, 184), and during exposure to HBO_2 (133). This technique involves visualizing and patching onto a dissociated cell maintained in a removable bath that is attached to the stage of an inverted microscope. Once the tight seal has formed, the head stage, pipette, cell, and bath are manually transferred to a hyperbaric pressure chamber and compressed hydrostatically (see *Hydrostatic compression chambers* in APPENDIX A). Recently, Heinemann's group (184) demonstrated the feasibility of conducting dual-electrode voltage-clamp experiments in oocytes during hydrostatic compressions up to 600 ATA. To our knowledge, tight-seal patch-clamp recordings have not been attempted with mammalian neurons under hyperbaric conditions by using a cylinder gas-compression chamber (i.e., HBHe); however, we anticipate that these types of recordings will also be feasible given recent improvements in hyperbaric chamber design (58, 59).

Appendix D: Hyperbaric Microscopy

The application of video microscopy (55, 195) and epifluorescence microscopy (78, 178, 183) during the past decade have greatly enhanced the power of brain slice studies conducted at normobaric pressure. Measurement of proposed signaling ions and molecules in a brain slice using fluorescent probes and ratiometric fluorescence microscopy, done simultaneously with measurements of neuronal output (i.e., V_m and firing rate) by using single-cell electrophysiology, enables investigators to determine the relationship between gas-induced stimuli and changes in neuronal function (e.g., Ref. 78).

While light and epifluorescence microscopy have been used in hyperbaric research to a limited extent, neither technique has been used in conjunction with electrophysiology. Typically, a small-volume hydrostatic compression chamber, referred to as an optical pressure chamber, is adapted to the stage of an inverted microscope. The in vitro tissue preparation is illuminated through a window in the top of the chamber and visualized through a small aperture in the bottom of the chamber, which is sealed by a glass coverslip, using a high numerical aperture objective (21, 164, 180). This design, however, prohibits introducing a recording microelectrode into the tissue inside the optical pressure chamber. To overcome this problem, our laboratory (84) recently adapted the optical components required for conducting video microscopy and epifluorescence microscopy to the interior of a hyperbaric chamber. Presently, the hyperbaric microscope is being used to study the effects of HBO_2 on baseline pH_i and pH_i recovery mechanisms in mammalian neurons. Using this new research tool, we anticipate that it also will be possible in the future to conduct electrophysiology in visualized neurons during exposure to hyperbaric gases, an approach that is now used routinely at normobaric pressure (e.g., Refs. 55, 78, 98).

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[4] We recently determined that the single-bolt closure on the old Bethlehem Hyperbaric Research Chamber (model NB-878, circa 1966) also works well. This chamber has a maximum working pressure of 10 ATG.

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