Hyperbaric He but not N₂ augments Ca^{2+}-dependent dopamine release from rat striatum

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Paul ML, Philp RB. Hyperbaric He but not N₂ augments Ca^{2+}-dependent dopamine release from rat striatum. Undersea Biomed Res 1989; 16(4):293–304.—Endogenous dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by high performance liquid chromatography with electrochemical detection in perfusate from continuously superfused rat brain striatal slices, and the effects of various pressures of He and N₂ were determined. He at 24 and 100 atmospheres absolute (ATA) significantly (P < 0.01 and < 0.05) increased the release of DA evoked by a 6-min exposure to 35 mM K⁺, whereas He at 48 ATA did not. Experiments conducted in a Ca^{2+}-free medium showed that only the extracellular Ca^{2+}-dependent component of release was affected by pressure. Similar increases in DA release were observed when DA reuptake and metabolism were blocked with cocaine and pargyline, although statistical significance was not achieved. N₂ did not significantly affect DA release at 12, 24, 48, or 100 ATA. The results indicate that He (= hydrostatic pressure) augments Ca^{2+}-dependent DA release and that substitution of N₂ negates this effect. The relevance of these observations to the phenomena of high pressure neurologic syndrome in divers and the anesthetic reversal of pressure effects is discussed.

- dopamine
- pressure
- helium
- HPNS
- nitrogen
- narcosis

Although the subjective and objective manifestations of inert gas narcosis (IGN) and high pressure neurologic syndrome (HPNS) are well documented, and it is generally accepted that the former is due to the presence of a narcotic gas such as N₂ in the breathing gas and the latter to hydrostatic pressure per se, the nature of the defects in central neurologic function remains unknown (1–4). The phenomenon of pressure reversal of anesthesia, first reported in tadpoles (5), and the practical success of preventing or reducing the severity of HPNS by the inclusion of N₂ along with He in the breathing gas (6) provided strong circumstantial evidence for some commonality in the sites of action involved in IGN and HPNS and contributed to the formulation of the critical volume hypothesis of anesthesia, which holds that hydrostatic pressure and anesthetics have opposing effects on cell membrane volume and consequently on neuronal function (7).
Previous efforts to study the effects of IGN and HPNS on whole-brain (8) or regional (9, 10) neurotransmitter levels have largely relied on postdecompression measurements, which may not truly reflect the situation at depth. A number of in vitro preparations have been used to study neurotransmitter events at pressure, all of which utilized exogenous, radiolabeled transmitter (11-16). Recent evidence indicates that the release of such exogenous transmitter may not parallel the release of the endogenous substance and consequently may not constitute an accurate marker for the latter (17).

Herein we report the effects of various pressures of He and N₂ on the synthesis and release of endogenous dopamine (DA) and its major metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) from continuously superfused rat striatal slices. This technique offers the advantage of sequential determinations before and during compression and while at pressure and avoids the interpretive complications associated with the use of exogenous-labeled transmitter.

MATERIALS AND METHODS

Cocaine HCl was purchased from Glaxo Laboratories (Toronto, Canada); citric acid and HPLC-grade acetonitrile were obtained from Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Preparation of brain slices

Male Sprague-Dawley rats (350–400 g) were decapitated and the left striatum was rapidly excised. The entire striatum was sectioned into 500-μm slices, using a mechanical tissue chopper (18), and transferred rapidly to a nonferrous stainless steel perfusion cuvette (see below).

The composition of the superfusion medium was (mM): NaCl, 120; KCl, 4.75; KH₂PO₄, 1.2; MgSO₄·7H₂O, 1.2; NaHCO₃, 25; CaCl₂, 2.6; glucose, 10. Depolarization of striatal slices was achieved using high K⁺ buffer in which KCl was increased to 35 mM and NaCl reduced to maintain constant osmolarity. For calcium-free experiments, CaCl₂ was omitted and EGTA (0.5 mM) was added to the buffer. All superfusion media were continuously oxygenated with 95:5 O₂:CO₂ and kept at 37°C, pH 7.4. Dissolved oxygen was measured by a Clark-style oxygen electrode, and the superfusion medium was found to be 90% saturated.

Superfusion system and compression chamber

The perfusion cuvette (vol 1.0 ml) was secured inside a stainless steel compression chamber (vol 250 ml). The temperature of the compression chamber and perfusion cuvette was maintained at 37° ± 0.5°C by an external heating tape and proportional temperature controller responding to a sensor located within the chamber in the cuvette support. Continuous recording of supernatant fluid temperature was provided by a thermistor probe inserted into the well of the tissue cuvette. Compression with either He or N₂ did not alter fluid temperature. Perfusion buffer was delivered to the open cuvette at 1 ml/min by an external high pressure pump and was allowed to
trickle down a stainless steel wire to maximize surface area and inert gas saturation. The tissue cuvette was designed so that the perfusion fluid entered the bottom and was withdrawn from the top. A driving pressure of at least 12 psi (1.8 ATA) was required to collect perfusate exterior to the chamber via a siphoning tube in the cuvette. Decompression of the sample was controlled by an external metering valve. Consecutive 2-ml fractions were collected continuously in chilled tubes containing 150 µg/ml reduced glutathione and 12.5 ng/ml dihydroxybenzylamine (DHBA) dissolved in 0.06 ml 2 N HCl. Final pH was 3.0. The superfusion and compression apparatus is depicted in Fig. 1.

Experimental protocol

Following a 30-min wash at ambient pressure (taken to be 1 ATA), the perfusion cuvette with striatal slices was transferred to the compression chamber. Spontaneous release of DA and its metabolite DOPAC was measured at 1.8 ATA and during compression at 10 atmospheres (atm)/min with either 100% He or N₂. Six minutes after reaching a final pressure of 12, 24, 48, or 100 ATA, evoked release was measured in response to a 6-min pulse of 35 mM KCl. At the end of decompression, slices were recovered and homogenized by sonic disruption in acetate buffer containing acetic acid, 0.5 M; Na acetate, 0.5 M; HClO₄, 0.4 M; reduced glutathione, 0.5 mM; DHBA, 25 ng/ml, at a final pH of 4.8. The sample was clarified by centrifugation at 12,000 × g for 40 min at 4°C. The supernatant was saved for high performance liquid chromatography (HPLC) analysis and the pellet was stored at -80°C for subsequent Lowry protein assay (19). Separate control experiments were conducted at 1 ATA air using identical perfusion conditions, except that sample collection was facilitated by using a closed cuvette so that the pump itself provided the flow of perfusate.
Tissue viability was evaluated by measuring lactate dehydrogenase (LDH) activity in the superfusate. Neither compression nor exposure to any pressure of He or N₂ significantly increased LDH activity over the 1 ATA control value. Approximately 2–7% of the total tissue LDH activity was released during the entire experiment. In contrast, rapid decompression (25 atm/min) from 100 ATA caused a marked increase in perfusate LDH activity (approximately 10-fold greater than pre- or postcompression values), indicating that membrane damage could be detected using this enzymatic marker.

HPLC analysis

 Dopamine and its metabolites DOPAC, homovanillic acid, and 3-methoxytyramine were quantified using HPLC with electrochemical detection (20). Superfusate and supernatant samples (20 μl) were injected without prior extraction onto an ultrasphere reverse phase ODS column (75 × 4.6 mm i.d., 3 μm particle size) protected by an Uptight precolumn (20 × 2 mm i.d., 10 μm particle size). Electrochemical detection was carried out with a glassy carbon electrode (TL-5A, Bioanalytical Systems, West Lafayette, IN) and a model LC-4B amperometric controller (BAS) set to maintain an applied potential of 0.8 V vs. an Ag/AgCl electrode (model RE-1, BAS). DA and metabolites were quantified using calibration curves prepared from pure chemicals and corrected for recoveries. The mobile phase consisting of 0.1 M citric acid, 0.05 M EDTA, 0.175 mM Na octyl sulfate, and 4.4% acetonitrile, buffered to an apparent pH of 4.4, was delivered at 1 ml/min by a Waters 510 pump. The entire system was at ambient temperature. All samples were analyzed on the day of the experiment.

Expression of results and statistical analysis

Release profiles of DA and DOPAC over the time course of the experiments were expressed as picomoles per milligram protein per minute. Comparison of total evoked release, however, required a different treatment.

A preliminary study of the striatal content of DA and metabolites in 7 normal control rats indicated that considerable intersubject variation occurred as previously reported (21). The mean total content (pmol per mg protein) of the left lobes was 976.3 ± 187.06 (SD) and of the right, 901.4 ± 187.35 (SD). The range for a single lobe was 634.7 to 1316.2. During the course of the perfusion experiments it was also observed with the amount of DA and DOPAC released correlated with the residual amounts remaining in the tissue at the conclusion of the experiment (r = 0.77, n = 8). Therefore, for purposes of statistical comparison of evoked release, the results, expressed as picomole per milligram protein, are presented as the fractional release calculated according to the following formula:

\[
\text{Fractional (\% release)} = \frac{\text{Total Evoked DA + DOPAC}}{\text{Total Evoked and Residual DA + DOPAC}} \times 100
\]

Unless otherwise stated, data were compared by an overall test of significance using an F ratio derived from one-way analysis of variance (ANOVA). Where the null hypothesis was rejected, a post hoc Newman-Keuls multiple comparison test was performed.
RESULTS

DA and DOPAC release at 1 ATA air

Release profiles from control experiments (1 ATA air) are summarized in Fig. 2 (n = 7). Mean rates of spontaneous DA and DOPAC release, measured over the first 30 min of perfusion, were 0.45 ± 0.75 pmol · mg⁻¹ protein · min⁻¹, respectively. Exposure to a 6-min pulse of 35 mM KCl resulted in a rapid increase in release of both DA and DOPAC with peaks of 10.42 ± 1.31 and 16.75 ± 1.25 pmol · mg⁻¹ protein · min⁻¹, respectively.

The dependence of DA and DOPAC release on extracellular Ca²⁺ was investigated in a series of experiments (n = 4) in which Ca²⁺ was omitted from and EGTA added to the perfusion fluid. These data are shown in Fig. 2. Spontaneous release rates were not different from control: DA, 0.16 ± 0.12 and DOPAC, 5.55 ± 0.79 pmol · mg⁻¹ protein · min⁻¹. However, a significant component of evoked release was Ca²⁺ independent. In the presence of external Ca²⁺ (2.6 mM), fractional release was 13.7% ± 1.08 (SEM) and in its absence it was 5.7% ± 0.56 (SEM) (P < 0.01 Student’s t test). Thus, 42% of total evoked release was Ca²⁺ independent.

Experiments conducted in the presence of α-methyl-p-tyrosine (α-MpT), a competitive inhibitor of tyrosine hydroxylase, revealed a significant dose-dependent inhibition of evoked DA release with 0.05 mM causing 43.4% inhibition (P < 0.01) and 0.5 mM producing 74.5% inhibition (P < 0.01). Spontaneous DA release was completely inhibited by 0.5 mM α-MpT, whereas spontaneous DOPAC release was not different from control (5.64 ± 0.38 vs. 6.75 ± 0.75 pmol · mg⁻¹ protein · min⁻¹).

Pressure experiments

The effects of various pressures of He and N₂ on the spontaneous and evoked release of DA and DOPAC were examined. Compression per se at 10 atm/min had no significant effect on spontaneous release regardless of the final pressure (12, 24,

![Graph](image)

Fig. 2. Release of endogenous DA (solid circles) and DOPAC (solid squares) from striatal slices at 1 ATA air in the presence (+) n = 7 and absence (−) n = 4 of extracellular Ca²⁺. Evoked release was measured in response to a 6-min pulse of 35 mM KCl (open bar). Each point represents the mean ± SEM.
48, or 100 ATA) or compression gas (Table 1). For reasons unknown, the 12 ATA
He group had a higher level of basal spontaneous release before compression. Temp-
oral profiles of evoked DA and DOPAC at high pressures of He or N₂ were notably
different from 1 ATA controls. Figures 3 and 4 are examples of such profiles at 100
ATA He and N₂, respectively. At 1 ATA, the release of DA increased continuously
throughout the exposure to K⁺, whereas at 100 ATA of either He or N₂, peak release
occurred almost instantly upon exposure to K⁺. Similar profiles were observed with
both gases at lower pressures. Peak-evoked DA at 100 ATA He was 3.6-fold greater

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<td>24 ATA N₂</td>
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<td>48 ATA He</td>
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*P < 0.01 compared to control

Fig. 3. Endogenous DA (solid circles) and DOPAC (solid squares) release at 100 ATA He in the
presence (+) n = 6 and absence (-) n = 3 of extracellular Ca²⁺. Chamber pressure (dotted line) was
increased from 1.8 ATA at a rate of 10 atm/min. Evoked release was measured at 100 ATA in response to
a 6-min pulse of 35 mM KCl (open bar). Each point is the x ± SEM.
HYPERBARIC He AND N₂ AND DOPAMINE RELEASE

Fig. 4. Endogenous DA (solid circles) and DOPAC (solid squares) release at 100 ATA N₂ in the presence (+) n = 6 and absence (-) n = 3 of extracellular Ca²⁺. Chamber pressure (dotted line) was increased from 1.8 ATA at a rate of 10 atm/min. Evoked release was measured at 100 ATA in response to a 6-min pulse of 35 mM KCl (open bar). Each point is the ± SEM.

than at 1 ATA air (38.05 ± 7.03 vs. 10.42 ± 1.31 pmol/mg protein/min, P < 0.001) whereas with 100 ATA N₂ it was not significantly different from control (16.58 ± 2.53 vs. 10.42 ± 1.31 pmol/mg protein/min). Peak-evoked DOPAC (pmol/mg protein/min) at 100 ATA He (20.37 ± 2.38) or N₂ (15.90 ± 1.36) was not significantly different from 1 ATA control (16.82 ± 1.19).

To determine whether the Ca²⁺-independent component of evoked release was affected by pressure, a separate series of experiments was conducted whereby K⁺-evoked release was initiated in Ca²⁺-free, 0.5 mM EGTA superfusion buffer. As indicated in Figs. 3 and 4, a Ca²⁺-independent component of evoked release was consistently observed at all gas-pressure combinations, but in no case was this statistically different from 1 ATA Ca²⁺-free control.

The total amounts of DA and DOPAC released during a 6-min exposure to 35 mM K⁺ are compared as total fractional release (as defined in Methods) in Fig. 5. In the presence of Ca²⁺, significant enhancement of fractional release was observed with He at 24 (P < 0.01) and 100 ATA (P < 0.05) but not at 12 or 48 ATA. N₂ did not differ significantly from control values at any pressure. The Ca²⁺-independent component of fractional release was unaffected by either gas at any pressure.

Effects of blockade of reuptake and metabolism of DA

A series of experiments at 24, 48, and 100 ATA He and N₂ and 1 ATA air was conducted in the presence of 0.05 mM cocaine, an inhibitor of amine reuptake, and 0.05 mM pargyline, a nonspecific inhibitor of monoamine oxidase enzyme. Addition of these drugs to the perfusion media at 1 ATA air resulted in complete abolition of detectable DOPAC. In these blocked preparations, spontaneous DA release was 0.49 ± 0.24 pmol/mg protein/min, and exposure to 35 mM KCl caused a rapid increase in DA release reaching a peak of 29.79 ± 3.33 pmol/mg protein/min. Total fractional release was 13.9% ± 1.28 (n = 6) (Fig. 6).
M. L. Paul and R. B. Philp

Fig. 5. Effects of various pressures of He and N₂ on evoked release of DA and DOPAC in the presence (+) and absence (−) of extracellular Ca²⁺. Fractional release (%) was calculated according to the formula:

\[ \text{Fractional release} = \frac{\text{Evoked DA} + \text{DOPAC}}{\text{Total Evoked and Residual DA} + \text{DOPAC} \times 100} \]  

Shown are the \( \bar{x} \pm \text{SEM} \). \( n \) values for each experimental group are indicated above the bar. For Ca²⁺-free experiments \( n = 4 \) for 1 ATA control and \( n = 3 \) for each pressure group. Evoked release was measured during 6-min exposure to 35 mM KCl. \(*P < 0.05\) and \(**P < 0.01\) compared to 1 ATA air control using one-way ANOVA.

A separate series of experiments (\( n = 3 \)) was conducted in the absence of extracellular Ca²⁺, and fractional release (4.9% ± 1.71) was significantly less than the value obtained in the presence of Ca²⁺ (\( P < 0.01\), Student’s \( t \) test).

Results of these experiments with blockade of reuptake and metabolism are summarized in Fig. 6. Helium at 24 and 100 ATA (but not 48 ATA) seemed to augment total fractional release as it did in the untreated preparations. However, in no instance were the data obtained in the presence of pargyline and cocaine significantly different from 1 ATA control. It was noted that the variability between preparations exposed to pressure in the presence of these drugs was nearly twice that seen in experiments conducted in their absence.

DISCUSSION

Other investigators (17, 22) have reported that the spontaneous (unevoked) release of DA is independent of extracellular Ca²⁺ and that the release rate of the principal metabolite DOPAC is 15 times that of DA. Our control (1 ATA) results essentially confirmed these observations, and no other metabolite of DA was detected despite the capability of the chromatographic analysis to do so. Similarly we have also confirmed that DA release evoked by K⁺ consisted of components that were both dependent on and independent of extracellular Ca²⁺. While our Ca²⁺-dependent component seemed smaller (58% of total) than previously reported, other workers have shown that an increase in the strength of the depolarizing stimulus (K⁺) from...
HYPERBARIC He AND N₂ AND DOPAMINE RELEASE

![Graph showing evoked release and percent total content vs. pressure (ATA)].

Fig. 6. Effects of various pressures of He and N₂ on evoked DA release from striatal slices continuously treated with cocaine and pargyline (0.05 mM). DA release was expressed as a fraction of total content as defined in Methods. Shown are the \( \bar{x} \pm \text{SEM} \). \( n \) values are indicated above each bar.

30 to 50 mM resulted in a marked increase in Ca\(^{2+}\)-dependent release (23). We chose a lower stimulus strength (35 mM) to avoid overwhelming subtle differences induced by the experimental conditions. We have also confirmed that the DA released in response to K\(^+\) is largely synthesized de novo. High K\(^+\) depolarization induces the phosphorylation and activation of the rate-limiting enzyme tyrosine hydroxylase, and this is a Ca\(^{2+}\)-dependent phenomenon. The newly synthesized DA resulting from this activation is preferentially released (17, 24–26). In our study, the tyrosine hydroxylase inhibitor \( \alpha \)-MPT reduced evoked DA release by 75%. Our findings thus support previously reported observations and confirm the suitability of the preparation for studies on the effects of pressure.

Our results indicate that the evoked release of endogenous DA was significantly enhanced by high pressures of He but not of N₂, suggesting that the narcotic gas N₂ at least partially negated the pressure effect (remembering that the hydrostatic pressure component is present when either gas is used). The profile of evoked DA release was also modified by pressure, occurring as a rapid burst under pressure in contrast to a more gradual release at 1 ATA. This was observed both with He and with N₂. Thus the antagonism of the pressure effect by N₂ was not absolute. Pressure apparently rendered the nerve terminals more reactive to the depolarizing stimulus.

Pressure augmentation of release was not detected at all pressures tested, being observed at 24 and 100 ATA of He but not at 48 ATA. At present there is no explanation for this bimodal response, but the consistency of the observation argues against it being experimental artifact. Furthermore, previous studies in this laboratory (27) have shown that equal pressures of N₂ are more inhibitory to platelet aggregation.
than He until pressures greater than 45 ATA, when He became more inhibitory than N₂. Thus a reversal of the response has also been observed in a completely different preparation.

Dopamine release is modulated by numerous other neurotransmitters, such as acetylcholine (ACh) and γ-aminobutyric acid (GABA) (28) and cholecystokinin (29), which also could be influenced by pressure. The lack of effect of pressure on DA release noted at 48 ATA could be explained on the basis of predominant activity of one of these neuromodulating systems. More experiments are required to examine this hypothesis.

All the effects of pressure were restricted to that component of evoked release that was dependent on extracellular Ca²⁺. Moreover, our results suggest that pressure affected synthesis and release of DA rather than reuptake and metabolism. Not only was most of the released DA synthesized de novo, but when DA release was increased (by pressure) there was no concomitant decrease in DOPAC release, both tending to increase in parallel. When reuptake and metabolism of DA were blocked by cocaine and pargyline, respectively, DA release was still increased by pressure, although statistical significance was not achieved with these blocking experiments. We have consistently observed greater variability in results when drugs were employed at pressure as compared to similar experiments in the absence of drugs. Others (30, 31) have reported pressure-induced reduction in ligand-receptor binding for ACh and its isolated receptor. Thus, pressure-induced changes in affinity of a receptor for its ligand could account for greater variability in drug effects under pressure observed in this study. This possibility needs to be explored further.

Existing literature suggests that the observed effects of pressure on neurotransmitter release are strongly influenced by the preparation employed. Two other studies have presented evidence of increased DA release under pressure. One investigated the release of exogenous [³H]DA from rat striatal slices (16) and the other used differential pulse voltametry in vivo and reported a 30% increase in the release of DA from brains of unrestrained hamsters exposed to 80 ATA of He (32). In contrast, a study of the release of [³H]DA from synaptosomes reported depression of release by 68 ATA of He (14). Thus, investigations utilizing intact synapses are consistent with our findings whereas the one using synaptosomes reported contrary results. For some years there has been controversy concerning whether a newly synthesized neurotransmitter can be secreted directly into the synaptic cleft without first being incorporated into synaptic vesicles (33, 34). Although this controversy is as yet unresolved, the hypothesis could serve to reconcile our findings of pressure-induced increase in the release of newly synthesized DA with the evidence of others showing pressure inhibition of vesicular release of [³H]DA.

It was noted (14) that the depression of DA release by pressure was restricted to the Ca²⁺-dependent component of release. Another investigation (11) reported that the release of [³H]GABA from isolated frog spinal cord was potentiated by pressure and it was the Ca²⁺-dependent component that was affected. A pressure-induced increase in intracellular Ca²⁺ thus may be a universal phenomenon related to the behavioral effects of pressure. Studies of single-celled organisms (35) and mammalian myocardial cells (36) have revealed such increases. Pressure could act to cause a rise in intracellular Ca²⁺ at several sites, but further investigations are required to determine which of these are responsible for the changes in neurotransmitter release. To our knowledge this is the first report to indicate that the pressure-induced increase
HYPERBARIC He AND N₂ AND DOPAMINE RELEASE

in DA release is linked to an increase in de novo synthesis of DA, a process also influenced by intracellular Ca²⁺ levels.

The complex neurologic phenomena of HPNS obviously cannot be explained solely on the basis of increased synthesis and release of DA. Undoubtedly, as has been suggested (4), HPNS probably results from pressure perturbation of several neurotransmitter systems. Our results suggest that changes in intracellular Ca²⁺ could be the common denominator underlying the effects of pressure on central neurotransmission.

In summary, we have shown that pressure per se causes an increase in the K⁺-evoked synthesis and Ca²⁺-dependent release of DA, and that the substitution of N₂ for He prevents or reduces this increase, which is indicative of pressure-anesthetic antagonism.

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