European Journal of Applied Physiology
https://doi.org/10.1007/s00421-018-4020-y

ORIGINAL ARTICLE

Inert gas narcosis in scuba diving, different gases different reactions

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Received: 18 May 2018 / Accepted: 13 October 2018

Abstract

Purpose Underwater divers face several potential neurological hazards when breathing compressed gas mixtures including nitrogen narcosis which can impact diver's safety. Various human studies have clearly demonstrated brain impairment due to nitrogen narcosis in divers at 4 ATA using critical flicker fusion frequency (CFFF) as a cortical performance indicator. However, recently some authors have proposed a probable adaptive phenomenon during repetitive exposure to high nitrogen pressure in rats, where they found a reversal effect on dopamine release.

Methods Sixty experienced divers breathing Air, Trimix or Heliox, were studied during an open water dive to a depth of 6 ATA with a square profile testing CFFF measurement before (T_0), during the dive upon arriving at the bottom (6 ATA) (T_1), 20 min of bottom time (T_2), and at 5 m (1.5 ATA) (T_3).

Results CFFF results showed a slight increase in alertness and arousal during the deep dive regardless of the gas mixture breathed. The percent change in CFFF values at T_1 and T_2 differed among the three groups being lower in the air group than in the other groups. All CFFF values returned to basal values 5 min before the final ascent at 5 m (T_3), but the Trimix measurements were still slightly better than those at T_0.

Conclusions Our results highlight that nitrogen and oxygen alone and in combination can produce neuronal excitability or depression in a dose-related response.

Keywords Nitrogen narcosis · Divers' safety · Critical flicker fusion frequency · GABA receptors

Abbreviations

ATA	Atmospheres of pressure absolute 1 ATA = 1.01325 bar, 760 mmHg, 10 m H_2O
CFFF	Critical flicker fusion frequency
DCS	Decompression sickness
EAN	Enriched air nitrox
GABA receptors	Gamma aminobutyric acid receptors
HELIOX	Helium and oxygen
HPNS	High pressure nervous syndrome
TRIMIX	Mixture of nitrogen, helium and oxygen

Communicated by Jean-René Lacour.

The members of the ROAD Project Investigators are mentioned in Acknowledgments section.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00421-018-4020-y) contains supplementary material, which is available to authorized users.

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Introduction

Underwater divers face several potential neurological hazards when breathing compressed air or different gas mixtures because they are exposed to increased ambient pressure. The increased absorption of inert gases (helium and nitrogen) leads to several abnormal consequences, such as nitrogen narcosis (Grover and Grover 2014), decompression sickness (Bove 2014; Souday et al. 2016), high-pressure nervous syndrome (HPNS) risk (Kot 2012) and oxygen toxicity (4), which are related to oxygen partial pressure and the number of free oxygen radicals in the central nervous system that overwhelm the antioxidant capacity of our antioxidant metabolism.

Air is the most commonly used breathing gas during diving. However, alternative gas mixtures have recently been proposed. Enriched air nitrox (EANx) includes a lower nitrogen content, Trimix is a mixture of oxygen, helium and nitrogen, and Heliox lacks nitrogen. These alternatives hope to (a) reduce the risk of decompression sickness (DCS) (Souday et al. 2016) via lowering the inert gas content and potential bubble production during decompression, (b) reduce the density of the gas mixture with the use of helium to allow deeper dives (Mitchell and Doolette 2013) while maintaining acceptable breathing, and (c) reduce the nitrogen narcosis risk. However, Heliox increases HPNS (Kot 2012) more than Trimix at depths greater than 200 m.

The high pressure of inert gas dissolved in plasma produces unpleasant effects, and narcosis is the most important effect to address because it is easily achieved at "recreational" depths. Narcosis is defined as "a general and non-specific reversible depression of neuronal excitability, produced by a number of physical and chemical agents, usually resulting in stupor" (Sawatzky 2012).

The diver may experience an initial exciting and euphoric state, but narcosis quickly reduces mental function and physical performance, which is an unpleasant and frightening experience. Narcosis impairs intellectual capacities and judgement and creates difficulties in the monitoring of time, depth, air supply, and the location of a buddy. Limited visibility and cold water may increase narcosis phenomena, but no actual data support this idea, which suggests that the phenomena may be more prevalent than previously thought during uneventful dives (Lafère et al. 2016). Severe narcosis induces hallucinations, bizarre behaviour, loss of consciousness, and decreased motor ability, which increases the risk of an accident and diminishes the ability to cope with challenging situations.

Most gases, except for helium and neon, exert a narcotic effect when breathed at recreational depths, but the degree of narcosis varies widely.

Critical flicker fusion frequency (CFFF) is a useful tool in the general measurement of neural integrity and is a good biomarker of higher cognitive function in specific clinical situations, such as encephalopathy (Mewborn et al. 2015). CFFF is used in various research fields (Curran et al. 2004; Kowacs et al. 2005; Torlot et al. 2013), including underwater and spaceflight activities, to measure alertness and arousal in humans using an uncomplicated, non-invasive and objective methodology (Lafère et al. 2010; Balestra et al. 2012, 2018; Hemelryck et al. 2013). CFFF is also used to assess cortical activity and alterations due to physical workload (Davranche and Pichon 2005) and drugs (Smith and Misiak 1976). Various studies (Lafère et al. 2010; Balestra et al. 2012; Hemelryck et al. 2013) have clearly demonstrated brain impairment due to nitrogen narcosis in divers at 4 ATA using CFFF as a cortical performance indicator.

Recent studies have proposed an adaptive phenomenon during repetitive exposure to high nitrogen pressure in rats and have found a reversal effect on dopamine release (Lavoute et al. 2012).

The present study investigated alertness and arousal using CFFF in expert divers at 6 ATA breathing three different gas mixtures with variable narcotic gases.

Methods

All experimental procedures were performed in accordance with the Declaration of Helsinki, and the Institutional Review Board (IRB) of Sapienza University of Rome approved all protocols (CE 2035/2015). Written informed consent was obtained from all subjects.

Subject and procedures

This observational study enrolled 60 experienced and certified divers. Each group consisted of at least 13 subjects who planned to dive with one of the three different gas mixtures. All subjects were non-smokers and performed regular physical activity (aerobic exercise 2/3 times a week).

All divers were instructed not to consume alcohol for 72 h or coffee for 6 h before the experimental dive.

Each diver performed an open water dive (Giannutri Island, east coast) during the summer at a water temperature of 15 °C at bottom wearing a scuba dry suit to a depth of 6 ATA (50 m) with a square profile and 20-min bottom time. V planner decompression software was used to calculate the decompression profile (the "V planner" is freely available at: https://v-planner.soft112.com).

Divers' alertness and vigilance were determined using CFFF measurements before (T_0), during the dive upon arriving at the bottom (6 ATA) (T_1), after 20 min of bottom time (T_2), and 5 min before the final ascent at 5 m (1.5 ATA) (T_3).

Measurements were performed three times to confirm reproducibility, and the average value was used as the individual's CFFF measurement. All data were stored in an internal micro-SD memory card and subsequently exported.

The divers in group A breathed compressed air in open circuit (OC) (21% oxygen, 79% nitrogen). Divers in group *T* breathed Trimix 21/35 in open circuit (OC) (21% oxygen, 35% helium, 44% nitrogen). Divers in group H breathed Heliox in closed circuit rebreather (CCR) (21% oxygen, 79% helium).

Groups A and *T* used an EAN 50 mixture (50% oxygen and 50% nitrogen) during ascent at decompression stops, and group H continually used the same helium/oxygen mixture (T_3).

Group A at 6 ATA (T_1 and T_2) breathed 4.74 ATA of nitrogen and 1.26 ATA of oxygen. Group *T* breathed 2.64 nitrogen and 1.26 oxygen (T_1 and T_2). Group H breathed 1.26 oxygen as a rebreather set point and no nitrogen (T_1 and T_2). Rebreather set point is a fixed partial pressure of oxygen set at the beginning of the dive and maintained constant by CCR during the depth.

Groups A and T at 1.5 ATA (T_3) breathed 0.75 ATA of nitrogen and 0.75 ATA of oxygen, and group H breathed 1.26 ATA oxygen as a rebreather set point and no nitrogen.

The number of divers in group H was less than the other groups because there are fewer Heliox-certified divers available worldwide.

Divers were requested to swim slowly and avoid effort as much as possible.

We attempted to wait for constant environmental variables, such as water temperature (15 ± 2 °C), same dive path using previously positioned markers on the seabed, and weather variables.

A team of scuba divers supported the participants during the dive and controlled the breathing rate of each participant to avoid excessive breathing workload or excessive inert gas uploading.

Critical flicker fusion frequency (CFFF) apparatus

The device used was designed and built as part of the ROAD project (Robotic for Assisted Diving), which was funded by the Italian National Scientific Research Program. The project develops tools and methods to increase safety in professional and recreational underwater diving using robotic devices and mechatronic structures to assist divers to identify and handle dangerous situations. We designed and produced an underwater waterproof (up to 60 m depth) portable CFFF device that was light enough to be carried by divers. The device included a LiPo battery pack to power the entire system (Conte et al. 2016).

The Flicker Fusion Detector evaluates the psychophysical state of the diver via monitoring responses to a particular visual stimulation, the blinking of an LED. A test was performed using flashing blue LEDs to identify the Critical Flicker Frequency. The diver stops the test when the light is no longer steady, but intermittent. The frequency at which the test is stopped is the Critical Flicker Frequency.

Test results obtained during the dive were stored in an internal micro-SD memory card. Each result was tagged using the updated date and time.

We tested the device under dry hyperbaric conditions at 6 ATA pressure (50 m) in the Sapienza University Hyperbaric facilities (Drass Galeazzi, Bergamo, Italy) to assess the full functionality and usability of the proposed device prior to use in medical studies.

No malfunctions were detected, and the performance of the device was optimal.

Any diver who could not perform the programmed dive profile or use the CFFF device was excluded from our evaluation.

We enrolled 24 subjects in group A, 21 subjects in group *T* and 15 subjects in group H.

The primary endpoint was differences in the alertness and arousal of expert divers at 6 ATA seawater while breathing different gas mixtures.

Statistical analysis

Preliminary data calculated that a sample size of 11 subjects was needed to detect a 1.3% difference in the variable means, with a probability power of 80% and an alpha error of 0.05 (PS Size Calculations software version 3.0; http://biostat.mc.vanderbilt.edu).

The Kolmogorov–Smirnov test was used to assess normal data distribution. We used one-way analysis of variance (ANOVA) on ranks for repeated measurements with Greenhouse–Geisser or Huynh–Feldt corrections to establish differences between the four data collection points and the post hoc Bonferroni or Tukey tests for normally or non-normally distributed data.

The percent change from baseline (considered as 100%) was calculated for each group of subjects at different time points.

The percent change at each data collection point was compared among groups using a paired *t* test and corrected with the Welch's test for inequalities in variance for normally distributed data, otherwise the Mann–Whitney test was used. Linear regressions were used to analyze gas mixtures differences at different time points. Analysis of covariance (ANCOVA) has been performed to discriminate between percentage change from baseline and the different time points using as a covariate the gas mixture group, considering that each group has different nitrogen concentration (Levene's tests and homogeneity of regression slopes).

Table 1 Demographic and dive experience data

Variables	Air	Heliox	Trimix	*p* value
Age, y, mean, (SD)	44.9 (5.8)	43.9 (5.7)	46.8 (4.6)	0.403
BMI, kg/m, mean (SD)	24.0 (2.3)	25.2 (1.5)	25.0 (2.7)	0.262
Dives/year, median (IQR)	50.0 (30.0–80.0)	75.0 (50.0–100.0)	55.0 (37.5–100.0)	0.653

Air mixture of 21% oxygen 79% nitrogen, *Trimix* mixture of 21% oxygen 35% helium, 44% nitrogen; *Heliox* mixture of 21% oxygen 79% helium, *BMI*, body mass index, *SD* standard deviation, *IQR* 25–74% interquartile range

Table 2 Results are presented as values of variables before diving (baseline), arrival at the bottom (T_1), 20 min at bottom time (T_2), and at 5 m

Groups, CFFF	Baseline	T_1	T_2	T_3	ANOVA *p* value
AIR, median (IQR) 22 subjects	38.50 (37.5–40.3)	‡41.00 (39.0–43.0)	‡41.00 (38.8–43.3)	40.00 (38.0–41.0)	<0.001
TRIMIX, mean ± (SD) 18 subjects	39.08 (1.29)	†42.44 (1.65)	†41.94 (2.75)	†41.11 (1.61)	<0.001
HELIOX, median (IQR) 11 subjects	38.00 (37.0–40.0)	‡42.00 (42.0–44.0)	‡43.00 (42.0–43.0)	42.00 (41.0–43.0)	<0.001

Friedman repeated measurements analysis of variance (F-ANOVA) was used to compare data at the different time points for each group and Bonferroni's (†) or Tukey's (‡) tests were used for post-hoc comparison among variables

Air: $p < 0.05$: ‡ vs baseline; Trimix: $p < 0.001$: †vs baseline; Heliox: $p < 0.05$: ‡vs baseline

Data are expressed as mean ± standard deviation (SD) or median and 25–75% interquartile range (IQR)

Data are expressed as the means ± standard deviation (SD) when normally distributed or medians and 25–75% interquartile ranges (IQR) when data failed the normality test. A *p* value less than 0.05 was considered statistically significant. Data were analyzed using the software program MedCalc, version 11.5.

Results

Fifty-five of the 60 enrolled subjects completed the study. Five divers did not complete the dive protocol because of technical problems with the rebreather apparatus, and 2 of those experienced also ear compensation problems. Four female divers were excluded because the discrepancy between genders (51 males and 4 females) could influence data and give confounding results. All data are presented as "Online Resource" (ESM_1.pdf; ESM_2.pdf). All divers performed their dives at the same sites with equal external conditions in terms of water temperature and visibility. Table 1 shows the demographic data and diving experience. There were no statistically significant differences between the three groups in demographic data, diving experience or initial CFFF data. Ages ranged from 35 to 56 years (mean ± SD).

Baseline CFFF values in the three groups were similar, and these values increased in all groups after arriving at the bottom (T_1 and T_2) (Table 2). CFFF values in the Air and Heliox groups recovered at 5 m (T_3) and matched the values measured at baseline. However, CFFF values in the Trimix group remained higher than baseline (Fig. 1).

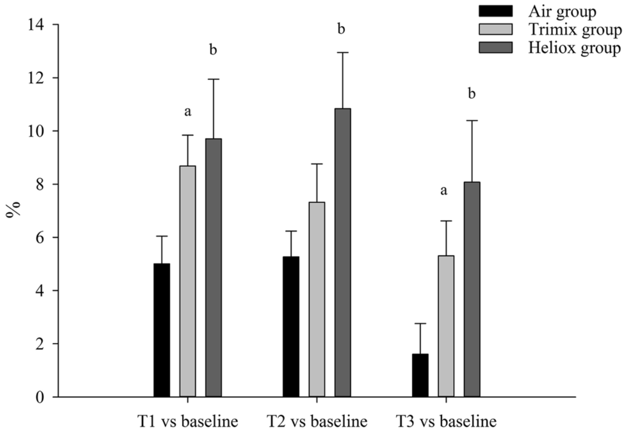

Fig. 1 Percentage changes comparison among the three groups of subjects breathing Air, Trimix and Heliox mixtures at the different time points. [a]*t* test for comparison between Air and Trimix at T_1 vs baseline: $p=0.0233$ and T_3 vs baseline: $p=0.0404$; [b]Air and Heliox at T_1 vs baseline: $p=0.0366$, T_2 vs baseline: $p=0.0095$ and T_3 vs baseline: $p=0.0290$. Mann–Witney's test was used to compare Air and Heliox at T_3 vs baseline

The increase in the CFFF values at T_1 and T_2 differed among the three groups. We calculated the percent change in values at each time point from baseline and compared these changes among the groups to evaluate differences in the CFFF increment. *T* tests demonstrated that the percentage increase in the Air group at T_1 was significantly lower than the other groups ($p < 0.023$ vs Trimix; $p < 0.037$ vs Heliox) and remained lower until the end of the study (Fig. 1).

Linear regression analysis of gas mixtures percentage changes from baseline measured at the three time points shows a significant inverse coefficients of determination (R^2) in air (0.76) and Trimix (0.96) groups but Heliox (0.35).

Analysis of covariance shows homogeneity for regression of percentage changes among groups but difference in the intercepts ($p = 0.005$) (Fig. 2); it is, therefore, possible to express a single general regression line $Y = -1.22 * X + 109.17$.

Discussion

Our observational study in a population of expert divers demonstrated a slight increase in alertness and arousal during a deep dive, as measured using the CFFF, regardless of the gas mixture breathed.

These results suggest a competitive effect between oxygen and nitrogen in our selected diver population. The only difference among the three groups was the different proportion of oxygen and nitrogen present in their breathing mixtures. The three groups breathed the same oxygen quantity (1.26 ATA) but different nitrogen partial pressures; specifically, the nitrogen partial pressure was higher in the air group (4.7 ATA), lower in the nitrox group (2.7 ATA) and absent in the heliox group.

It is important to distinguish the effect of these two gases on brain function in analyses of these complex phenomena.

Nitrogen

Narcosis in divers has been a fascinating topic for many years. Albert Behnke suggested in the early 1900s that narcosis may be the result of the increased partial pressure of nitrogen and suggested that non-nitrogen-containing gas mixtures should be used at greater depths (Grover and Grover 2014). An excitement and euphoric phase precede the brain sedative effect, and some authors suggested that diving is addictive because many divers enjoy deep diving using air (Sawatzky 2012).

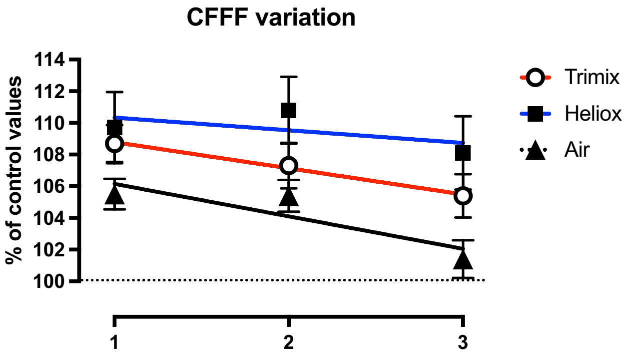

Fig. 2 Linear regressions of gas mixtures percentage changes from baseline measured at the three data points. Coefficients of determination (R^2) are 0.76 for Air, 0.96 for Trimix and 0.35 for Heliox. Levene's tests: $p = 0.184$; Homogeneity of regression slopes: $p = 0.193$; Intercept: $p = 0.005$

This issue is generally explained as a psychological addiction similar to all pleasurable experiences. However, this issue must be investigated because more sensitive divers develop symptoms at only 4 ATA, and other divers may not be affected until depths of 7 ATA (Grover and Grover 2014). Hyperbaric workers in hyperbaric medical facilities and underwater environments, such as shallow saturations, subjectively report an increased tolerance to the putative narcotic effects, but recreational scuba divers may undergo a completely different experience, as recently published (Lafère et al. 2016; Germonpré et al. 2017).

The mechanism of nitrogen narcosis, described as "rapture of the deep" by Jacques–Yves Cousteau or anaesthetic gas effects on the nervous system, correlates with the solubility of nitrogen in lipids, which suggests a parallel between lipid affinity and narcotic potency (Brubakk et al. 2014). The observation of the pressure reversal effect of general anaesthesia and the critical volume hypothesis for different anaesthetics, including inert gases, supports the lipid theory (Miller et al. 1973).

However, Abraini et al. (2003) demonstrated that inert gases, such as inhaled anaesthetics, bind directly to the modulatory site of a protein receptor and act as allosteric modulators. Their findings are consistent with a direct mechanism of action on $GABA_A$ receptors, which was confirmed in a previous study (David et al. 2001).

Pressure-dependent conformational changes occur due to gas-protein binding, particularly at *N*-methyl-D-aspartate (NMDA) and $GABA_A$ (Gamma amino butyric acid a) receptors in the substantia nigra pars compacta (Rostain et al. 2011; Rostain and Lavoute 2016), which further supports the protein-binding theory of narcosis. Other pathways are likely involved in the nitrogen narcosis effects, such as reduced release of glutamate and other amino acids, which partially explain the depletion in extracellular concentrations of dopamine (Vallee et al. 2009a, b). Recurrent exposure to hyperbaric nitrogen suppresses gas-induced decreases in striatal dopamine levels, and dopamine levels increase. Lavoute et al. (2012) demonstrated that rats submitted to several exposures exhibited a desensitization of $GABA_A$ receptors on dopaminergic cells and GABA interneurons of the central nervous system, which increased striatal dopamine release. This response was unlike to occur during the first exposure to nitrogen at 31 ATA pressure. This result suggests a mechanism for tolerance (Brebeck et al. 2017; Germonpré et al. 2017) that is analogous to benzodiazepine (Gravielle 2016) and alcohol

(Blednov et al. 2017) tolerance that develops following repeated consumption of these substances.

Numerous in vivo and in vitro experiments have demonstrated that chronic benzodiazepine exposure induced alterations in $GABA_A$ receptors that may induce tolerance (Gravielle 2016).

Oxygen

Experimental studies have demonstrated that the facilitating effects of normobaric hyperoxia on nerve conduction are likely related to an enhanced production of reactive oxygen species (Brerro-Saby et al. 2010). Several reports have demonstrated that short-term normobaric oxygen (NBO) (maximum 1.0 ATA O2) positively influenced cognitive abilities, such as memory, visuospatial and verbal abilities (Moss and Scholey 1996; Moss et al. 1998; Scholey et al. 1998, 1999; Chung et al. 2006) and functional magnetic resonance (fMRI) studies have demonstrated that normobaric hyperoxia (NBO, 0.3 ATA O2) during verbal or visual tasks increases the activation of brain areas associated with cognitive processing (Choi et al. 2010). Kot et al. (2015) examined the effect of oxygen on neuronal excitability using CFFF in a hyperbaric environment and demonstrated a dose-dependent effect. These authors concluded that neuronal excitability and oxygen poisoning transduction pathways were intertwined. Some authors (Smith and Paton 1976; Hesser et al. 1978) suggested a narcotic effect of oxygen, they tested oxygen pressures higher than 3.0 ATA. Notably, oxygen also interacts with GABA neurotransmission and influences GABA levels and activity to inhibit or differentially modulate GABAergic function (Balestra et al. 2012).

Cerebral vasoconstriction is a well-recognized mechanism that protects the brain from oxygen excess (Winklewski et al. 2013), even if the impact of this effect is not greater than 15–20%.

However, other authors investigated these phenomena using single photon emission computed tomography (SPECT) and demonstrated that the regional cerebral blood flow (rCBF) distribution increased brain perfusion primarily in the left hemisphere during hyperbaric hyperoxic vasoconstriction with a relative CBF increase in the neural networks involving dorsal and ventral attention pathways. These results suggest a beneficial effect of HBO on cognition performance (Micarelli et al. 2013).

Cerebral flow redistribution is highly relevant to molecular changes because increased NO production due to the auto-oxidation of several reactive oxygen species directly affect the conductance of various ions that regulate cell excitability and disrupts chemical synaptic transmission (Manning 2016). NO plays an important role in experimental neurotoxicity induced in primary rat cortical cell cultures (Huang et al. 2000), and it may play a role in increasing neuronal excitability, which affects the glutamate/GABA balance, as proposed by Kot et al. (2015).

This increased neuronal excitability was more evident in the Heliox group, in which the divers breathed only oxygen and helium, than in the Trimix group, in which nitrogen was present at a low partial pressure, and the air group, in which the nitrogen partial pressure was 4.7 ATA.

The CFFF results in all divers revealed a better alertness at 6 ATA (T_1 and T_2) than at the surface more evident in the Heliox group, in which the divers breathed only oxygen and helium, than in the Trimix group, in which nitrogen was present at a low partial pressure, and in the air group, in which the nitrogen partial pressure was 4.7 ATA (Fig. 1) suggesting a prominent role of breathing nitrogen concentration. This clearly demonstrates that the partial pressure of Nitrogen counteracts the oxygen effect.

The nitrogen concentration, clearly prevalent on the air group, yields to alertness reduction independently from the heterogeneity of groups (Fig. 2) and confirms having a crucial role for the narcotic effect.

The CFFF values in the air group recovered at 5 m (T_3) and matched the values measured at baseline, but values in the Trimix group remained higher than baseline (ESM_2.pdf). The different exposures to nitrogen during the entire dive may explain these data. Two groups breathed the same mixture of oxygen and nitrogen during ascent to 5 m, but the air group breathed almost twice the dose of nitrogen at 6 ATA. Therefore, the narcotic effects dissipated more slowly than in the trimix group, in which the activated effect of oxygen remained evident at T_3, which confirms a previously described result (Balestra et al. 2012).

The present study identified the following important points:

(a) The CFFF used in this underwater research protocol is an extremely useful tool in underwater environments. CFFF is a recognized method of assessing the neuronal excitability that influences attention and alertness (Kahlbrock et al. 2012; Lu et al. 2012) and was used to assess nitrogen narcosis in various hyperbaric conditions at approximately 4 ATA depth in air or Nitrox. We improved this technology by producing a device that is self-managed by the diver when the diver notices the change from fusion to flicker without the need of an external operator. The device stored the results in a dedicated internal micro-SD memory card for subsequent exportation. A team of scuba divers supported the participants during the dive but did not manage the CFFF data. The blue LED light was clearly visible at 6 ATA, and none of the divers had difficulties using the device.

An increase in the sensitivity of the CFFF device would allow better appraisals of the small modifications we registered in the present study.

(b) The team of scuba divers supporting the participants helped ensure that all of the divers had a calm immersion in terms of physical demands (moderate muscular exercise and breathing frequency never exceeded 20 breaths/min), which decreased the risk of elevated arterial partial pressure of CO_2 and its well-studied consequences, such as impaired motor and planning performance at rest and during exercise, which are partially corrected by hyperoxia (Freiberger et al. 2016).
(c) We used three different gas mixtures at 6 ATA to obtain clear data on the effects of gas components. The air group breathed 4.74 ATA of nitrogen (PiN_2), the Trimix group breathed 2.7 ATA PiN_2 and the Heliox group was the control group (0 ATA of nitrogen) and breathed a constant 1.2 ATA of oxygen.
(d) We planned a deep profile dive, which is typical for skilled divers. It is worth noting that air tanks are used by many expert divers worldwide, as this gas is cheap and commonly available.
(e) We included only expert divers who have previously been repetitively exposed to hyperbaric nitrogen.
(f) The study was performed in an open-water field to simulate a real dive at a low but standard temperature that is experienced in a deep-sea dive. Our focus was to prevent bias from environmental and procedural conditions.

Limitations of our study

1. The present study was observational, and we did not randomize divers who voluntarily chose the mixture breathed based on their experience.
2. We did not compare the results of expert divers with a non-expert group because of ethical matters. Non-expert divers cannot exceed 4 ATA. Therefore, the non-expert dive response to this effect is questionable.
3. We did not evaluate the CFFF response of all groups of divers at the normoxic level of 0.2 ATA on surface at the end of their dive.
4. Due to helium features, the decompression profile of Heliox group is quite different from the other two groups so that the T3 results are not comparable to the others.
5. We did not measure blood carbon dioxide (CO_2) concentrations. Therefore, we cannot exclude that an increase in CO_2 occurred. Nevertheless, we carefully monitored that the divers performed the dive with reduced physical demands.
6. The measurement of CFFF was performed from fusion to flickering, previous studies used the inverse method.

Conclusions

In conclusion, our results demonstrated that the net effect on the brains of expert divers of breathing different mixtures of oxygen and nitrogen, as measured using CFFF at 6 ATA, was an increase in alertness and vigilance. We speculate that this result is due to a balance in the different pharmacokinetic interactions on GABA receptors. Oxygen exhibits activating effects, and nitrogen exhibits inhibitory effects on the synthesis, release and recapture of neurotransmitters such as glutamate, dopamine and γ-aminobutyric acid (GABA), as recently proposed in different studies (Rostain et al. 2011; Balestra et al. 2018).

These results support the idea that compressed nitrogen is a "co-agonist" of $GABA_A$ receptors that decrease glutamate release without changing NMDA receptor activities.

Our results and the conclusions of other researchers highlight the complicated network characterizing the neural circuits on which nitrogen and oxygen alone and in combination can produce neuronal excitability or depression in a dose-related response.

Acknowledgements ROAD Project Investigators include: Scaratozzo D, Gala F, Screpanti L, Argentario Diving, Nicolini S, Mesa S.

Author contribution statement RM, DBP, PP, MM, BC: conceived and designed research; GC, DBRA, ML, ROAD project investigators: designed and constructed the CFFF device; GC, RM, DBP, PP, ML, FS, BC, ROAD project investigators: supervised the protocol execution; ML,PP,DBP, FS, ROAD project investigators: collected and assembled the data; DBRA, ML, MM, GC: performed statistical analysis; RM, DBRA, MM, BC: drafted the manuscript. All the authors have read and approved the final manuscript.

Funding This study was funded by PRIN (Italian National Scientific Research Program) 2012.

Compliance with ethical standards

Conflict of interest No conflicts of interest, financial or otherwise are declared by the authors.

Ethical approval All experimental procedures were performed in accordance with the Declaration of Helsinki, and the Institutional Review Board (IRB) of Sapienza University of Rome approved all protocols (CE 2035/2015).

Informed consent Written informed consent was obtained from all subjects.

Data availability All data are available on the supplementary material.

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