

# Creatine Supplementation Enhances Corticomotor Excitability and Cognitive Performance during Oxygen Deprivation

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Impairment or interruption of oxygen supply compromises brain function and plays a role in neurological and neurodegenerative conditions. Creatine is a naturally occurring compound involved in the buffering, transport, and regulation of cellular energy, with the potential to replenish cellular adenosine triphosphate without oxygen. Creatine is also neuroprotective *in vitro* against anoxic/hypoxic damage. Dietary creatine supplementation has been associated with improved symptoms in neurological disorders defined by impaired neural energy provision. Here we investigate, for the first time in humans, the utility of creatine as a dietary supplement to protect against energetic insult. The aim of this study was to assess the influence of oral creatine supplementation on the neurophysiological and neuropsychological function of healthy young adults during acute oxygen deprivation. Fifteen healthy adults were supplemented with creatine and placebo treatments for 7 d, which increased brain creatine on average by 9.2%. A hypoxic gas mixture (10% oxygen) was administered for 90 min, causing global oxygen deficit and impairing a range of neuropsychological processes. Hypoxia-induced decrements in cognitive performance, specifically attentional capacity, were restored when participants were creatine supplemented, and corticomotor excitability increased. A neuromodulatory effect of creatine via increased energy availability is presumed to be a contributing factor of the restoration, perhaps by supporting the maintenance of appropriate neuronal membrane potentials. Dietary creatine monohydrate supplementation augments neural creatine, increases corticomotor excitability, and prevents the decline in attention that occurs during severe oxygen deficit. This is the first demonstration of creatine's utility as a neuroprotective supplement when cellular energy provision is compromised.

**Key words:** cognition; corticomotor excitability; creatine; dietary supplementation; hypoxia; neural metabolism

## Introduction

The brain is reliant on an uninterrupted supply of energy to maintain electrical membrane potentials, action potential propagation, and signaling activities. Impairment or interruption of neural energy supply compromises brain function and plays a role in the pathogenesis and progression of neurological and neurodegenerative conditions. Acute disruption to energy supply, such as that experienced in ischemic brain injury, can be induced experimentally via models that create an hypoxic–anoxic cellular environment. Chronic energy disruption degrades cellular structure, which indirectly impairs energy provision processes, such as

the disruption to mitochondrial structure and function that occurs in Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Beal, 2005; van den Bogaard et al., 2011; Martin, 2012). Interventions that augment the brain's local energy stores may be neuroprotective and represent a potential for therapeutic intervention.

Creatine (Cr) has been proposed as a potential therapeutic agent because it can replenish cellular ATP without a reliance on oxygen. Creatine is a critical component of the Cr kinase/phosphocreatine (CK/PCr) system, which shapes a large metabolic network in the CNS that is highly versatile and involved in many physiological functions. For a review of Cr in the CNS and the functioning of the CK/PCr energy system in neurons, we refer the reader to Turner and Gant (2014). The CK/PCr system is of greater potential relevance in disease states where there is disruption to cellular energy metabolism and diminished capacity to meet neuronal energy needs.

Creatine pretreatment offers a neuroprotective effect against anoxic and ischemic cell damage *in vitro*, providing enhanced intracellular PCr concentrations, protection against ATP depletion, delayed membrane depolarization, and reduced structural damage (Carter et al., 1995; Balestrino et al., 1999; Shen and Goldberg, 2012). Additionally, transfer of neuroprotection has

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been reported in energy-deprived rodent offspring in response to maternal Cr feeding (Wilken et al., 1998, 2000). Despite these convincing findings *in vitro*, investigation into Cr-mediated effects in the functioning human brain have been contradictory, and physiological mechanisms are poorly understood (Shefner et al., 2004; Tabrizi et al., 2005; NINDS NET-PD Investigators, 2008).

It is possible to modify brain Cr concentration with oral Cr monohydrate (CrM) supplementation (Dechent et al., 1999; Lyoo et al., 2003), similar to the practice of supplementing skeletal muscle, which enhances athletic performance during strenuous exercise (Birch et al., 1994). The aim of this study was to confirm that oral Cr supplementation would augment neural Cr stores, and to assess the influence of increased neural Cr availability on neuropsychological and neurophysiological measures. To achieve this, healthy humans were exposed to severe experimental hypoxia using a gas breathing intervention to induce acute energy disruption after dietary CrM supplementation. Magnetic resonance spectroscopy (MRS) was used to measure neural Cr availability. Computer-based neuropsychological assessments and transcranial magnetic stimulation (TMS) of primary motor cortex were used to derive measures of cognitive function and corticomotor excitability, respectively. We hypothesized that oxygen deprivation would severely impair brain function, indexed by cognition and corticomotor excitability, and Cr supplementation would offset these impairments.

## Materials and Methods

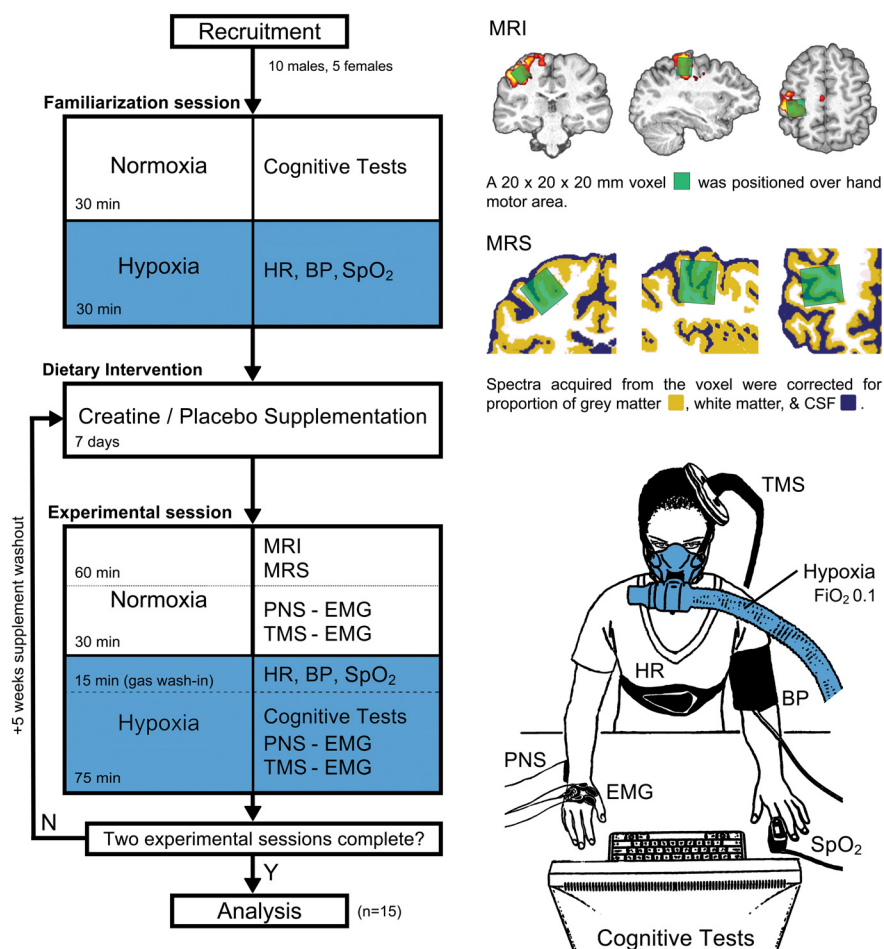
### Participants and experimental design

Fifteen healthy participants (10 males, 5 females) with a mean age of 31 years (21–55 years) volunteered to participate. All participants were right-handed with a mean laterality quotient of +78% ( $\pm 23\%$ ). Handedness was assessed using the Edinburgh Handedness Inventory (Oldfield, 1971). Participants were screened for contraindications to TMS and magnetic resonance imaging (MRI) and for health complications related to the hypoxia intervention (see below, Hypoxia intervention) and CrM supplementation (see below, Dietary supplementation). All participants gave written informed consent. The study was conducted in accordance with the declaration of Helsinki and approved by the institutional ethics committee.

A familiarization session was conducted to collect baseline neuropsychological data and to introduce participants to the hypoxia intervention. Two experimental sessions involved the collection of neurophysiological, neuropsychological, and neuroimaging data at baseline and/or during the hypoxia intervention. Experimental sessions were completed the day after dietary supplementation was complete. See Figure 1 for a schematic representation of experimental procedures.

### Dietary supplementation

Supplementation was performed in a randomized, double-blind, crossover design. A CrM and placebo (PLA) supplementation regime was



**Figure 1.** Experimental workflow and procedures. Fifteen healthy participants were recruited to participate in the study. A familiarization session was conducted to collect the baseline neuropsychological assessment (Cognitive Tests) and introduce the participant to the hypoxia intervention (Hypoxia). Participants were then randomized into an initial supplementation regime (CrM or PLA), and supplementation was conducted for 7 d, with a 5 week washout period. Experimental sessions were conducted 24 h after supplementation, involving the collection of neuroimaging, neurophysiological, and neuropsychological data. Structural MRI was used to position an MRS volume of interest (voxel) over the hand knob of the primary motor cortex. The location was verified with functional MRI. The red–yellow contrast shows areas of increased activation during a functional finger-tapping task ( $Z > 2.3$ ,  $p < 0.05$ ). Spectroscopy data were acquired and corrected for the proportion of tissue types using neural tissue segmentation and transformed to absolute quantities using a brain-mimicking phantom containing a range of known physiological Cr concentrations. Neurophysiological data, as assessed via TMS and PNS, were recorded using surface EMG at baseline and during the hypoxia intervention to measure central and peripheral excitability levels, respectively. The hypoxia intervention was conducted for 90 min using a gas mixture with a fraction of inspired oxygen ( $F_{iO_2}$ ) of 0.1 and delivered via a one-way valve face-mask system. Cardiovascular measures [arterial oxygen saturation ( $SpO_2$ ), heart rate (HR), and blood pressure (BP)] were monitored throughout to assess autonomic system regulation to the hypoxic intervention.

conducted for 7 d and separated by a washout period of 5 weeks, shown previously to be effective in reducing tissue Cr concentrations to pre-supplementation levels (Hultman et al., 1996; McKenna et al., 1999; Preen et al., 2003). Participants were given taste-matched sachets of powder for each treatment and instructed to dissolve each dose in one glass of water and consume at four equally spaced intervals throughout the day, ensuring that consumption respective to meal times was consistent. Each dose of the test substance contained 5 g of CrM (Musashi creatine monohydrate, Nestlé Australia), equating to a daily dose of 20 g. Both supplements contained 3.75 g of a low-calorie powdered flavoring per dose (Lemon and Lime Flavored Drink Mix, Weight Watchers International).

### Hypoxia intervention

Hypoxia was induced by inhaling a gas mixture with an inspired oxygen fraction of 0.1 for 90 min. Atmospheric air was diluted with nitrogen and stored as a compressed gas mixture. Gas was delivered from an intermediate bladder via a one-way valve face-mask system. Respiration was

monitored using a pneumotachometer (MLT1000L, ADInstruments), paramagnetic O<sub>2</sub> analyzer (S-3A/I, AEI Technologies), and infrared CO<sub>2</sub> analyzer (CD-3A, AEI Technologies). Arterial oxygen saturation, heart rate, and blood pressure were continuously monitored throughout the hypoxia intervention by a pulse oximeter (VacuMed), heart rate monitor (Polar Electro), and digital automatic blood pressure monitor (Omron), respectively. A gas wash-in period of 15 min allowed for the hypoxic ventilatory response and stable reduction in arterial oxygen saturation, after which neuropsychological assessment and neurophysiological procedures were performed.

### Neuropsychological assessment

A standardized and automated computerized battery of neuropsychological tests was used to assess neuropsychological function (CNS Vital Signs; <http://www.cnsvs.com/>). The test battery comprised seven tests: verbal and visual memory, finger tapping, symbol digit coding, the Stroop test, a test of shifting attention, and a continuous performance test. A detailed description of each test is given by Gualtieri and Johnson (2006). Computer instructions on how to complete each test were given before the test along with practice sessions when necessary. Tests were initiated and supervised by an experimenter and delivered unassisted and uninterrupted using a laptop computer to ensure consistency in administration.

Test scoring was generated from 17 primary scores based on correct responses, error responses, number of responses, and reaction times. Primary scores were used to generate nine neurocognitive domain scores to reflect basic mental functions (composite memory, verbal memory, visual memory, processing speed, executive function, psychomotor speed, reaction time, complex attention, and cognitive flexibility) and an overall neurocognitive index score. Domain scores were generated as raw scores, calculated from composite primary scores of relevant tests, and then computed to standard scores and percentiles that represent the participants' raw score relative to an age-matched normative data set of healthy individuals. Standardized domain scores and the 17 primary scores were used for statistical analysis.

Upon completion of the test battery, a six-point Likert-type rating scale was presented on the computer screen prompting the participant to rate their current level of alertness ranging from 1 (exhausted, unable to function effectively) to 6 (fully awake and excellent alertness).

Baseline neuropsychological testing was conducted during the familiarization trial when the participant was inhaling atmospheric air. During the experimental sessions, participants completed neuropsychological assessments during hypoxia, immediately following a 15 min wash-in with the hypoxic gas mixture.

### Neurophysiological procedures

**Preparation.** Peripheral nerve stimulation (PNS) and TMS were used to evaluate peripheral and corticomotor excitability levels, respectively. Each participant was seated comfortably in a chair with their arms rested on a cushion in their lap. Skin sites were prepared using standard techniques for surface electromyography (EMG) collection of the right first dorsal interosseous (FDI) muscle in a tendon-belly arrangement, with the reference electrode on the dorsal surface of the hand. Raw EMG signals were amplified and filtered (3 Hz to 1 kHz, P511AC, Grass Instruments), sampled at 2 kHz, and stored for offline analysis using custom LabView software.

**Transcranial magnetic stimulation.** A figure-of-eight coil (70 mm) was used to apply focal TMS over the hand area of the left motor cortex using a MagStim 200 magnetic stimulator (MagStim; maximum output intensity, 2.0 T). The coil was positioned over the motor cortex contralateral to the target muscle with the coil handle directed posteriorly and rotated away from the midline at an angle of ~45° to produce posterior-to-anterior current flow perpendicular to the precentral gyrus (Kaneko et al., 1996). The optimal scalp site for stimulation was defined as the position that consistently produced the greatest motor evoked potential (MEP) amplitude in the contralateral FDI muscle for a given stimulator intensity. This was determined by stimulating sites over the motor cortex in a 1 cm grid pattern. The optimal scalp site was marked, and all stimulation from this point on was conducted at this location. Stimulus-response (SR) curves of the FDI motor cortex were assessed at rest in

response to stimulation intensity increments of 5% maximum stimulator output (MSO), from 40–85% MSO (Devanne et al., 1997). Six stimuli were delivered at each intensity with the intensity order randomized. Rest motor threshold (RMT), defined as the lowest stimulator intensity evoking MEPs in the relaxed FDI with amplitudes of at least 50  $\mu$ V in four of eight trials, was evaluated separately (Rossini et al., 1994).

**Peripheral nerve stimulation.** The ulnar nerve was stimulated using surface electrodes placed 2 to 4 cm proximal to the wrist of the right arm (cathode, proximal). Electrical stimulation was delivered by a constant current Digitimer stimulator (DS7A). The optimal site for stimulation, defined as the position that consistently produced the greatest M wave amplitude in the right FDI for a given stimulator intensity, was found, and all peripheral stimulation was delivered from this site. The stimulation intensity was gradually increased until M wave amplitude did not increase further. To ensure supramaximal stimulation, the intensity was increased to 110% of maximum, and 20 supramaximal stimuli were delivered.

**Data analysis.** Peak-to-peak FDI MEP and M wave amplitude and prestimulus root mean square EMG (rmsEMG) were analyzed for all SR curve data. Background muscle activity before the stimulus was monitored, and trials were discarded when the rmsEMG calculated in the 100 ms window before the stimulus exceeded 10  $\mu$ V. The remaining MEP peak-to-peak amplitude data were ordered and trimmed to remove outliers. Motor evoked potential amplitudes were normalized to the largest remaining MEP collected within a session, and SR curves were constructed from seven stimulation intensities (one subthreshold and six suprathreshold) at each condition (normoxic baseline and hypoxia). The sum of the mean normalized MEP amplitudes at the seven stimulation intensities were determined, and the area under the SR curve was calculated at each condition ( $\Sigma$ MEP). Threshold stimulus intensity was analyzed for all RMT data. Peak-to-peak FDI M wave amplitude was analyzed for all PNS data. The relative change in  $\Sigma$ MEP, RMT, and M wave amplitude measures from baseline were calculated for both supplementation regimes and used for statistical analyses.

All neurophysiological procedures were conducted the day immediately following supplementation. Neurophysiological outcome measures ( $\Sigma$ MEP, RMT, and M wave amplitude) were collected at baseline while inhaling atmospheric air and during hypoxia following a 15 min wash-in with the hypoxic gas mixture and the neuropsychological assessment.

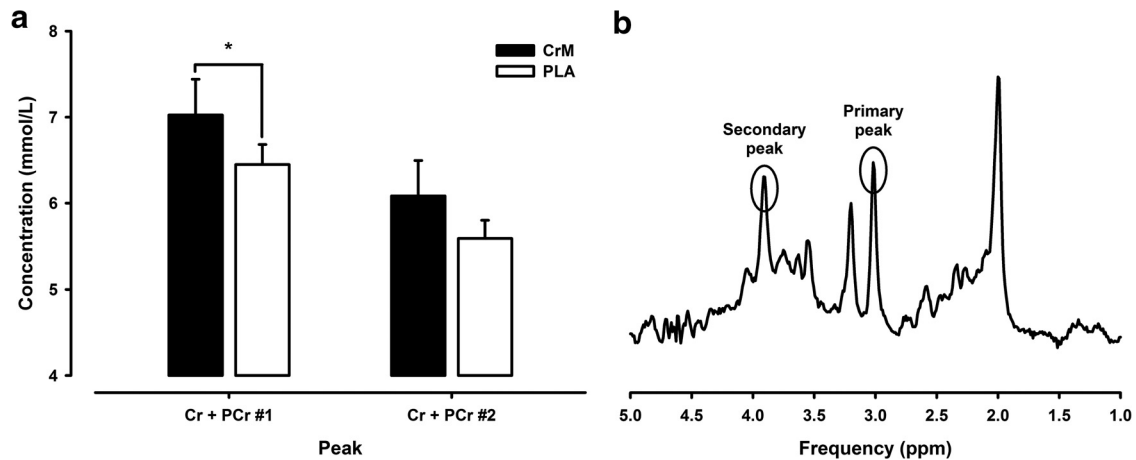
### Neuroimaging procedures

**Image acquisition.** All images were acquired using a Siemens Skyra 3 T scanner. A high-resolution T<sub>1</sub>-weighted anatomical data set (FOV, 256 mm sagittal; voxel dimensions, 1.0  $\times$  1.0  $\times$  1.0 mm) was acquired for each participant. Blood oxygenation level-dependent (BOLD) contrast images were acquired using a T<sub>2</sub>\*-weighted single-shot echoplanar sequence (TR, 3 s; voxel dimensions, 2.0  $\times$  2.0  $\times$  3.0 mm; 42 oblique axial slices with a 1 mm gap) with online analysis used to monitor real-time BOLD activation in response to a finger-tapping motor task. Single-voxel spectroscopy data were acquired using a PRESS (point-resolved spectroscopy) sequence (TR, 2 s; TE, 30 ms; 80 averages). A 20  $\times$  20  $\times$  20 mm voxel of interest was placed over the left precentral gyrus on the hand knob of the primary motor cortex to target the hand motor representation using anatomical landmarks from each participant's T<sub>1</sub>-weighted image (Yousry et al., 1997).

**Functional task.** Participants were instructed to keep their right hand relaxed (rest) when a circle was presented on a visual presentation viewed by a mirror in the head coil and to initiate and maintain a rhythmic pinch of their right index finger to thumb at the rate of ~1 Hz when a cross (active) was presented. The task cycled between rest and active conditions four times. Each condition lasted 30 s, resulting in a total functional scan of 4 min.

**Spectroscopy and image analysis.** Quantitative analysis of the spectra were performed using the Java-based Magnetic Resonance User Interface software package (<http://www.mrui.uab.es/mrui/>; Naressi et al., 2001a,b). The free induction decay signal was corrected for any non-zero DC offset. The residual water signal was suppressed by fitting and removing Gaussian peaks around the water frequency using singular





**Figure 2.** Neural creatine concentration after supplementation. *a*, Mean Cr concentration detected in the sensorimotor cortex increased after 1 week of dietary CrM supplementation (black bars) compared to PLA (white bars). Data were recorded using  $^1\text{H}$  MRS, transformed to an absolute quantity (in millimoles per liter) and corrected for the proportion of gray and white matter present in the volume of interest. Bars show the mean  $\pm$  SEM.  $*p < 0.05$ . *b*, Amplitude of the Cr + PCr peaks from  $^1\text{H}$  MRS. An example spectra acquired at 3 T from the sensorimotor cortex of one participant is shown. Resonances at 3.069 and 3.960 ppm represent the primary and secondary Cr + PCr peaks, respectively.

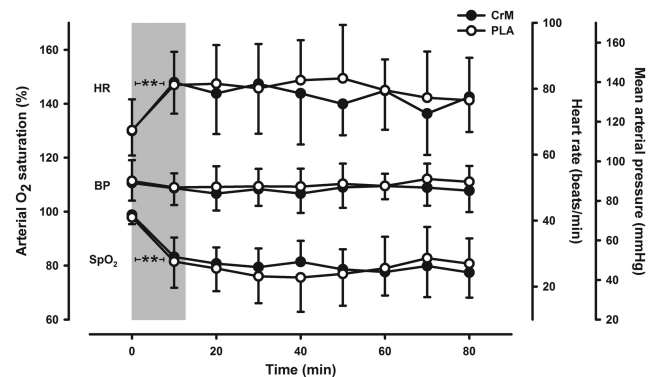
value decomposition techniques. Zero-order phase correction was applied manually to correct for peak distortion. Spectral analysis was performed in the time domain using a nonlinear least-squares-fitting optimization algorithm (Vanhamme et al., 1997). Prior knowledge constraints for peak fitting were optimized for *N*-acetylaspartate (NAA), Cr, and choline (Cho) resonances and set as follows. A relative phase of  $0^\circ$  was applied to all peaks. A fixed ratio line width, restrained to the prominent NAA peak, was applied to the remaining Cr and Cho peaks. Upper and lower frequency bounds were specified using the soft constraints function for the peak position. All peak shapes were specified as a fixed Lorentzian curve.

The participants'  $T_1$ -weighted structural images were extracted and segmented using BET (brain extraction tool; Smith, 2002) and FAST (FMRIB's automated segmentation tool; Zhang et al., 2001), respectively, both part of the FMRIB software library (www.fmrib.ox.ac.uk/fsl). The amplitude of the Cr peaks (primary peak at 3.069 ppm and secondary peak at 3.960 ppm) were corrected for the proportion of total brain tissue volume (gray and white matter) within the voxel of interest (Stagg et al., 2011). The corrected peak amplitudes were then converted to absolute concentration units. An interpolation procedure obtained as a result of scanning a brain-mimicking phantom (Woo et al., 2007) with a range of known physiological Cr concentrations and regressing them with their corresponding resonance amplitudes was used.

All neuroimaging data were collected the day immediately following supplementation. Neuroimaging measures were collected at baseline while participants were inhaling atmospheric air.

### Statistical analyses

A two-way repeated-measures (RM) ANOVA was used to assess the effect of supplementation and hypoxia on oxygen saturation, heart rate, and mean arterial blood pressure. Within-subjects factors were defined: treatment (CrM and PLA) and time (nine levels at 10 min intervals). Paired-samples *t* tests were used to assess the effect of supplementation on sensorimotor cortex Cr concentration, gray matter, white matter, and cerebrospinal fluid (CSF) proportions in the MRS voxel of interest, neuropsychological and neurophysiological outcome variables collected during hypoxia. One-sample *t* tests were used to assess the effect of hypoxia on neuropsychological and neurophysiological measures. Baseline values for neuropsychological hypoxia comparisons use between-sessions data (from the familiarization trial), whereas relative neurophysiological measures ( $\Sigma$ MEP, RMT, and M wave amplitude) use within-session data from 0 min. Two data sets were contaminated by electrical interference and excluded from neurophysiological analyses. Linear regression analyses were performed to examine the association between the supplementation bias with CrM in neuropsychological and neurophysiological measures from baseline. This analysis was also used



**Figure 3.** Autonomic regulation during hypoxia. Arterial oxygen saturation ( $\text{SpO}_2$ ) was reduced by 19% during hypoxia. A compensatory 12% increase, on average, in heart rate (HR) occurred, with no changes in blood pressure (BP) detected. Gray shading represents wash-in stabilization period followed by 80 min of hypoxia for CrM (black circles) and PLA (white circles) treatments. Data are mean  $\pm$  SD;  $n = 12$  ( $n = 11$  for BP).  $**p < 0.01$  for 0 min versus subsequent observations.

to assess the association between the bias in Cr accrued during CrM and the neurocognitive index from baseline.

## Results

### Effectiveness of creatine supplementation

First we examined whether 1 week of dietary CrM supplementation was effective in augmenting total neural Cr concentration within the hand knob of the left precentral gyrus. Using MRS, we detected an increase in the amplitude of the primary MRS-induced Cr + PCr peak at 3.069 ppm (Fig. 2; paired-samples *t* test,  $t_{(14)} = 1.83$ ;  $p = 0.04$ ). This shows that 7 d of oral CrM supplementation is able to increase the amount of Cr stored in the brain ( $7.03 \pm 1.62$  mmol/L) compared to PLA supplementation ( $6.44 \pm 0.90$  mmol/L). Both the primary and secondary Cr + PCr peaks exhibited a similar bias between treatments, but no statistical difference was detected in the secondary peak at 3.960 ppm (paired-samples *t* test,  $t_{(14)} = 1.20$ ;  $p = 0.13$ ). The secondary peak is typically smaller than the primary peak, so less signal is detected during data acquisition, resulting in a lower signal-to-noise ratio. The density of gray matter within the region of inter-

**Table 1. Neuropsychological responses to hypoxia**

|                              | Baseline, raw scores | Creatine, raw scores | Placebo, raw scores | Effect of hypoxia <sup>a</sup> |                    | Effect of supplementation <sup>b</sup> |                    |
|------------------------------|----------------------|----------------------|---------------------|--------------------------------|--------------------|--|--------------------|
|                              |                      |                      |                     | <i>t</i>                       | <i>p</i>           | <i>t</i>                               | <i>p</i>           |
| Alertness rating scale       | 4.1 ± 0.8            | 2.7 ± 0.9            | 2.6 ± 1.0           | −4.1                           | < 0.001*           | 0.1                                    | 0.464              |
| <i>Complex attention</i>     | 93.7 ± 16.8          | 86.4 ± 22.7          | 70.7 ± 51.5         | −2.0                           | <b>0.031*</b>      | 1.8                                    | <b>0.049*</b>      |
| ST, incongruent CE           | 1.9 ± 1.8            | 2.1 ± 1.8            | 2.0 ± 1.5           | 1.5                            | 0.079 <sup>†</sup> | 0.9                                    | 0.195              |
| SAT, errors                  | 5.4 ± 3.8            | 6.4 ± 4.4            | 8.3 ± 6.9           | 2.4                            | <b>0.017*</b>      | −0.8                                   | 0.230              |
| CPT, CE                      | 0.7 ± 1.2            | 1.1 ± 1.7            | 3.0 ± 5.6           | 2.5                            | <b>0.013*</b>      | −2.2                                   | <b>0.020*</b>      |
| CPT, omission errors         | 0.1 ± 0.3            | 0.9 ± 1.9            | 1.8 ± 4.6           | 1.4                            | 0.087 <sup>†</sup> | −1.1                                   | 0.140              |
| <i>Executive function</i>    | 100.5 ± 17.9         | 100.9 ± 17.9         | 91.9 ± 28.9         | −1.2                           | 0.118              | 1.5                                    | 0.082 <sup>†</sup> |
| SAT, correct                 | 55.5 ± 9.0           | 56.6 ± 8.4           | 52.9 ± 12.4         | −0.8                           | 0.226              | 1.3                                    | 0.110              |
| SAT, errors                  | 5.4 ± 3.8            | 6.4 ± 4.4            | 8.3 ± 6.9           | 2.4                            | <b>0.017*</b>      | −0.8                                   | 0.230              |
| <i>Cognitive flexibility</i> | 98.8 ± 18.2          | 98.9 ± 19.3          | 88.9 ± 31.7         | −1.4                           | 0.092 <sup>†</sup> | 1.6                                    | 0.072 <sup>†</sup> |
| SAT, correct                 | 55.5 ± 9.0           | 56.6 ± 8.4           | 52.9 ± 12.4         | −0.8                           | 0.226              | 1.3                                    | 0.110              |
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| <i>Neurocognitive index</i>  | 104.2 ± 10.6         | 99.7 ± 14.3          | 92.2 ± 23.0         | −2.2                           | <b>0.022*</b>      | 1.6                                    | 0.071 <sup>†</sup> |
| Composite memory             | 106.0 ± 13.1         | 97.8 ± 21.2          | 96.1 ± 16.7         | −2.1                           | <b>0.029*</b>      | 0.3                                    | 0.400              |
| Psychomotor speed            | 118.8 ± 18.5         | 114.5 ± 23.0         | 112.0 ± 22.9        | −2.0                           | <b>0.033*</b>      | 0.6                                    | 0.279              |
| Reaction time                | 103.2 ± 10.6         | 100.7 ± 12.6         | 98.9 ± 13.8         | −0.7                           | 0.259              | 0.4                                    | 0.332              |
| Complex attention            | 93.7 ± 16.8          | 86.4 ± 22.7          | 70.7 ± 51.5         | −2.0                           | <b>0.031*</b>      | 1.8                                    | <b>0.049*</b>      |
| Cognitive flexibility        | 98.8 ± 18.2          | 98.9 ± 19.3          | 88.9 ± 31.7         | −1.4                           | 0.092 <sup>†</sup> | 1.6                                    | 0.072 <sup>†</sup> |

A range of composite domain (italic type) and standard neurophysiological scores were reduced by hypoxia during PLA (effect of hypoxia). CrM standard scores tended to be higher during hypoxia than PLA (effect of supplementation), suggesting that these processes were robust to the effects of hypoxia. Note that for standard neurophysiological scores, higher error scores indicate worse performance, and higher correct response scores indicate better performance. For composite domain scores, higher scores indicate better performance. The alertness rating was measured using a six-point rating scale and was reduced with hypoxia and not corrected by CrM. ST, Stroop test; SAT, shifting attention test; CPT, continuous performance test; CE, commission errors. Descriptive data are mean ± SD.

<sup>a</sup>Comparisons with baseline used one-sample *t* tests of normalized PLA scores compared to baseline (0) to assess the effects of hypoxia.

<sup>b</sup>Between-treatment comparisons used paired *t* tests of normalized scores for CrM compared to PLA to assess the effects of supplementation.

\**p* < 0.05; <sup>†</sup>*p* ≥ 0.05 and < 0.1. Bold type highlights statistically significant comparisons.

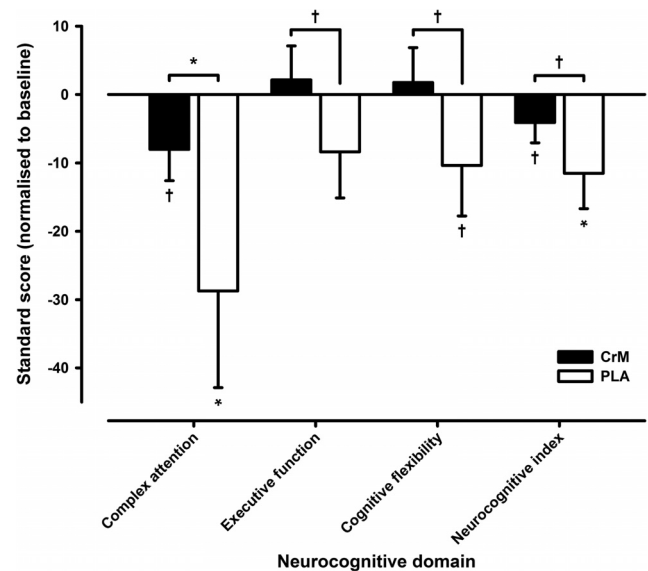
est (CrM, 0.34 ± 0.05; PLA, 0.37 ± 0.06) was similar for both treatments (paired-samples *t* test,  $t_{(14)} = -1.94$ ;  $p = 0.07$ ). This trend ( $p = 0.07$ ) was not associated with supplementation-induced changes in CrM concentration (linear regression;  $R^2 = 0.06$ ;  $F_{(1,14)} = 0.80$ ;  $p = 0.39$ ). There were also no changes in white matter (CrM, 0.55 ± 0.09; PLA, 0.51 ± 0.09) and CSF (CrM, 0.11 ± 0.05; PLA, 0.13 ± 0.04) between the supplementation treatments (paired-samples *t* tests; white matter,  $t_{(14)} = 1.75$ ,  $p = 0.10$ ; CSF,  $t_{(14)} = -1.14$ ,  $p = 0.27$ ).

### Assessment of global oxygen deficit

We monitored cardiovascular responses (Fig. 3) and ventilatory adjustments during the hypoxic insult to confirm that the gas mixture (FiO<sub>2</sub> = 0.10) induced the expected oxygen deficit. Arterial oxygen saturation was significantly reduced (RM ANOVA;  $F_{(8,88)} = 18.42$ ;  $p < 0.01$ ), and heart rate displayed the expected compensatory increase (RM ANOVA;  $F_{(8,88)} = 11.29$ ;  $p < 0.01$ ). Importantly, these responses were identical between nutritional treatments, suggesting that the disruption to energy metabolism was comparable for CrM and PLA treatments.

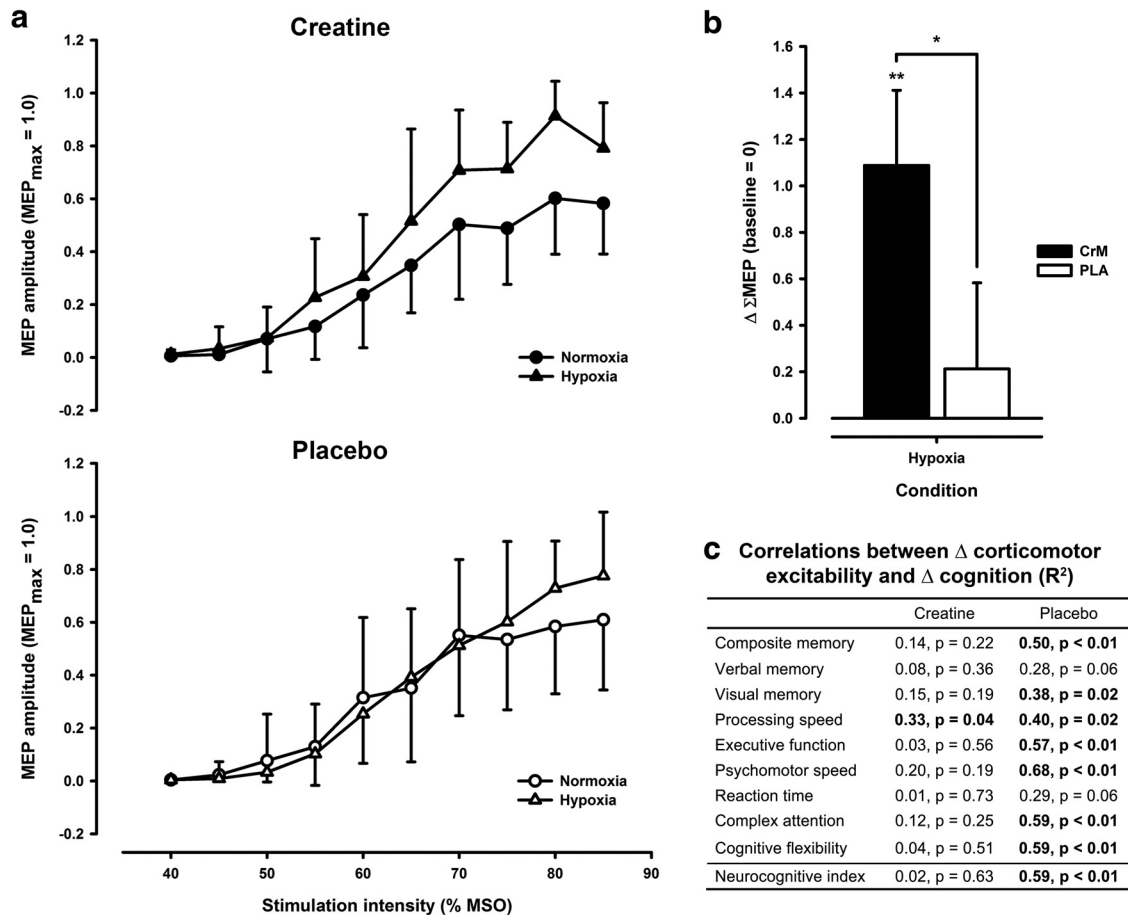
### Assessment of neuropsychological function

The disruption to cognitive function caused by hypoxia was accessed in a range of neuropsychological tests. Cognitive domains degraded by hypoxia were complex attention (one-sample *t* test,  $t_{(14)} = -2.04$ ;  $p = 0.03$ ), composite memory (one-sample *t* test,  $t_{(14)} = -2.07$ ;  $p = 0.03$ ), psychomotor speed (one-sample *t* test,  $t_{(14)} = -2.01$ ;  $p = 0.03$ ), and the overall neurocognitive index (one-sample *t* test,  $t_{(14)} = -2.21$ ;  $p = 0.02$ ). A trend for decreases in performance were found in visual memory (one-sample *t* test,  $t_{(14)} = -1.36$ ;  $p = 0.098$ ) and cognitive flexibility (one-sample *t* test,  $t_{(14)} = -1.40$ ;  $p = 0.09$ ). Hypoxia also abolished any task learning effect that is observed when cognitive tests are repeated in normoxic conditions. Verbal memory, processing speed, executive function, and reaction time tests were robust to hypoxia.



**Figure 4.** Changes in cognitive domain scores with hypoxia. Hypoxia degraded performance significantly in four neurocognitive domains with PLA (white bars), but these decrements were lessened or prevented with CrM (black bars). Standard scores are raw scores relative to an age-matched normative data set of healthy individuals. Bars show the mean ± SEM. \* $p < 0.05$ ; <sup>†</sup> $p \geq 0.05$  and < 0.1.

Creatine supplementation improved performance in a number of cognitive scores, effectively offsetting the decrements that were observed with hypoxia (Table 1). Creatine was particularly efficacious at improving tasks involving complex attention (paired-samples *t* test,  $t_{(14)} = 1.78$ ;  $p = 0.049$ ). Trends for improvements were observed in executive function (paired-samples *t* test,  $t_{(14)} = 1.47$ ;  $p = 0.08$ ), cognitive flexibility (paired-samples *t* test,  $t_{(14)} = 1.55$ ;  $p = 0.07$ ), and neurocognitive index (paired-samples *t* test,  $t_{(14)} = 1.56$ ;  $p = 0.07$ ; Fig. 4). Because cognitive



**Figure 5.** Corticomotor excitability and correlations with cognition during hypoxia. *a*, Stimulus–response curves for CrM and PLA treatments during normoxic (circles) and hypoxic (triangles) conditions. Data are mean  $\pm$  SD; *n* = 13. *b*, Compared to normoxia, corticomotor excitability increased during hypoxia with CrM (black fill), but not PLA (white fill). Bars show the mean  $\pm$  SEM; *n* = 13. \**p* < 0.05; \*\**p* < 0.01. *c*, Correlations between the change in corticomotor excitability and the change in cognitive performance from baseline. Decreases in cognitive performance were prevalent in most variables with PLA. Bold type highlights statistically significant correlations.

domain standard scores were higher when supplemented with CrM under hypoxia compared to those recorded when supplemented with PLA under hypoxia, these results suggest that supplementing with CrM may counteract the detrimental effect of hypoxia on a range of cognitive functions.

#### Assessment of neurophysiological function

Hypoxia led to an increase in corticomotor excitability (assessed via the area under the SR curve;  $\Sigma$ MEP) that was greater with CrM supplementation ( $1.09 \pm 1.17$ ; paired-samples *t* test,  $t_{(12)} = 1.85$ ; *p* = 0.04) compared to PLA supplementation ( $0.21 \pm 1.33$ ; one-sample *t* test,  $t_{(12)} = 0.57$ ; *p* = 0.29; Fig. 5*a*). It seems unlikely that the earliest excitable components contributing to the MEP were involved in this response, as measures of RMT were unaltered by hypoxia (one-sample *t* test,  $t_{(12)} = 0.93$ ; *p* = 0.19) or CrM supplementation (paired-samples *t* test,  $t_{(12)} = -0.78$ ; *p* = 0.22). Also, the maximal M wave amplitude was robust to hypoxia (one-sample *t* test,  $t_{(12)} = 0.78$ ; *p* = 0.23) and CrM supplementation (paired-samples *t* test,  $t_{(12)} = 0.83$ ; *p* = 0.21), suggesting that neuromuscular transmission was consistent throughout, and membrane excitability was unaltered by respiratory alkalosis (Higashi et al., 1972).

Importantly, there was a significant and strong ( $r = 0.8$ ) positive correlation between changes in excitability and overall neurocognitive index scores with PLA (linear regression;  $R^2 = 0.59$ ;  $F_{(1,12)} = 15.98$ ; *p* = 0.002), but not CrM (linear regression;  $R^2 =$

0.02;  $F_{(1,12)} = 0.24$ ; *p* = 0.63). Lower levels of corticomotor excitability were associated with reduced cognition with PLA, but this relationship was abolished with CrM supplementation (Fig. 5*c*). The quantity of neural Cr accrued during supplementation (from CrM MRS data) did not predict the extent to which neurocognitive deficits were corrected during hypoxia (linear regression;  $R^2 = 0.002$ ;  $F_{(1,12)} = 0.02$ ; *p* = 0.88).

#### Discussion

In this study we used a simple dietary supplementation strategy to augment neural Cr stores, increase corticomotor excitability, and prevent the decline in cognition, and particularly attentional capacity, that accompanies severe oxygen deficit. These results support the idea that Cr may be neuroprotective, a finding that has only been reported previously *in vitro*.

Dietary CrM supplementation may have influenced similar cellular processes to those reported *in vitro*: delaying hypoxia-induced membrane depolarization, effectively maintaining neuronal integrity, and better sustaining basic neuronal function. When Cr is administered before oxidative stress, impaired protein synthesis and axonal damage are prevented, promoting greater amplitude and duration of neural activity. This is, in turn, protective against synaptic transmission failure and delays the onset of anoxic depolarization that ultimately reduces neuronal death (Kass and Lipton, 1982; Lipton and Whittingham, 1982; Balestrino, 1995; Carter et al., 1995; Wilken et al., 1998, 2000;

Balestrino et al., 1999; Shen and Goldberg, 2012). In the present study, the supplemented CrM could have provided a more direct and abundant pool of energy as PCr, and more efficient coupling between ATP-generating and ATP-consuming sites within the cell. Creatine stores may have enhanced anaerobic energy provision by prolonging the transfer of high-energy phosphates to areas of the neuron that require additional energy when oxidative glycolysis is compromised by hypoxia. These processes can slow the rate of fall in ATP levels that is typically experienced during oxygen deprivation. Surprisingly however, the magnitude of Cr stored by supplementation did not predict the extent to which neurocognitive deficits were corrected during hypoxia. This finding might suggest possible nonenergetic, neuromodulatory roles for Cr as proposed by Rambo et al. (2012), Royes et al. (2008), and Souza et al. (2012). Similar outcomes have been reported in skeletal muscle, where the same CrM supplementation regimen results in variable intramuscular Cr concentrations (Greenhaff et al., 1994; Lukaszuk et al., 2002, 2005) but consistent performance improvements (Birch et al., 1994).

Dietary CrM supplementation restored the cognitive decline associated with hypoxia-induced oxygen deprivation. The hypoxia intervention had a profound negative effect on a range of cognitive functions, consistent with previous work that has examined cognitive performance at high altitudes (Virués-Ortega et al., 2004; de Aquino Lemos et al., 2012; Asmaro et al., 2013). The neurocognitive index score that represents overall cognitive function was reduced by 12% with hypoxia, indicative of a drop in performance from the 60th percentile to the 45th percentile when compared to an age-matched normative data set. These are large decrements that highlight the importance of an uninterrupted energy supply for maintaining basic neuronal function to sustain complex cognitive processes. Enhanced Cr availability was able to restore or partially correct several cognitive deficiencies, with complex attentional processes most improved by CrM (21% difference compared to PLA), perhaps via more efficient anaerobic energy delivery during the most aerobically stressful mental tasks.

These new findings also demonstrate that increased neural Cr availability has a neuromodulatory influence upon cortical excitability during acute hypoxia. Hypoxia-induced changes in excitability were associated with widespread decrements in cognition in the PLA condition, but not with CrM supplementation. The enhanced energy-buffering capacity that is afforded by augmented Cr stores may have prevented hypoxia-induced disruptions to the maintenance of cellular membrane potential. These disruptions are evident during complete oxygen deprivation, where maintenance of neuronal resting membrane potential at an adequate level necessary for normal neuronal function is not possible (Shen and Goldberg, 2012). Modulation of  $\text{Na}^+/\text{K}^+$ -ATPase activity by intracellular Cr has been proposed as a potential mechanistic pathway for this effect (Royes et al., 2008; Rambo et al., 2012, 2013; Souza et al., 2012). The action that Cr may have on  $\text{Na}^+/\text{K}^+$ -ATPase function and how this directly influences neural excitability levels is not entirely understood and warrants further investigation.

Neural excitability did not appear to be effected by the hypoxia intervention alone, as we hypothesized based on existing *in vitro* work examining anoxic depolarization. Neither did baseline corticomotor excitability change with CrM supplementation alone. However, when oxygen delivery was disrupted in the presence of enhanced neural Cr levels, corticomotor excitability increased by 70% and was associated with improved cognition. This neuromodulatory response may overcome neuronal defi-

ciencies caused by hypoxia, but might only be possible when cells are in a high-energy state. The level of the neural axis modified by CrM supplementation in the presence of hypoxia is difficult to determine definitively with noninvasive studies in humans. Our study suggests the increased excitability was mediated, at least in part, via supraspinal circuits.

Creatine concentration increased by 9.2% within the measured volume of interest after 1 week of dietary CrM supplementation, and MEPs were obtained from stimulation of this region. Although we cannot know about Cr concentrations in other brain regions, previous studies have reported widespread and systemic increases in Cr storage following dietary CrM supplementation. Regions examined previously include gray matter of the frontal (Lyo et al., 2003) and parietal cortices, white matter tracts of the parietooccipital lobe, deep gray matter in the thalamus, and the central cerebellum (Dechent et al., 1999).

This is the first *in vivo* investigation into the involvement of CrM supplementation in brain function during oxygen deprivation in humans. Dietary CrM supplementation augments the storage of Cr in the brain. Enhanced availability of neural Cr prevents deterioration to cognitive functions associated with attention and increases corticomotor excitability during oxygen deprivation.

These findings provide insight into the importance of the CK/PCr energy system in the human brain and demonstrate that 7 d of CrM supplementation has potential utility as an ergogenic aid and neuroprotective supplement when cellular energy provision is compromised. Because impaired energy metabolism and failure to maintain adequate membrane potential play a critical role in the pathogenesis and progression of a range of neurological and neurodegenerative conditions, this work also provides evidence to support the study of CrM as a therapeutic supplement.

## Notes

Supplemental data for additional cognitive, spectroscopic, and ventilatory parameters are available at <http://lab.gant.kiwi>. This material has not been peer reviewed.

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