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Brain connectivity is altered by extreme physical exercise during non-REM sleep and wakefulness: indications from EEG and fMRI studies

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ABSTRACT

Brain connectivity is associated to behavioral states (e.g. wake, sleep) and modified by physical activity although, to date, it is not clear which components (e.g. hypothalamus-pituitary-adrenal axis hormones, cytokines) associated to the exercise are involved.

In this pilot study, we used extreme exercise (UltraTriathlon) as a model to investigate physical-activity-related changes of brain connectivity. We studied post-race brain synchronization during wakefulness and sleep as well as possible correlations between exercise-related cytokines/hormones and synchronization features.

For wakefulness, global synchronization was evaluated by estimating from fMRI data (12 athletes) the brain global connectivity (GC). GC increased in several brain regions, mainly related to sensory-motor activity, emotional modulation and response to stress that may foster rapid exchange of information across regions, and reflect post-race internally-focused mental activity or disengagement from previous motor programs. No significant correlations between cytokines/hormones and GC were found.

For sleep (8 athletes), synchronization was evaluated by estimating the local-(cortical) and global-related (thalamo-cortical) EEG features associated to the phenomenon of Sleep Slow Oscillations (SSO) of NREM sleep. Results showed that: power of fast rhythms in the baseline preceding the SSO increased in midline and parietal regions; amplitude and duration of SSOs increased, mainly in posterior areas; sigma modulation in the SSO up state decreased. In the post race, IL-10 positively correlated with fast rhythms baseline, SSO rate and positive slope; IL-1ra and cortisol inversely correlated with SSO duration; TNF-α and C-reactive protein positively correlated with fast rhythm modulation in the SSO up state.

Sleep results suggest that: arousal during sleep, estimated by baseline fast rhythms, is increased; SSO may be sustained by cortical excitability, linked to anti-inflammatory markers (IL-10); thalamo-cortical entrainment, (sigma modulation), is impaired in athletes with higher inflammatory markers.

Key words

Physical exercise • Sleep slow oscillations • Global connectivity • Cytokines • Synchronization • Cerebral excitability • Humans

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Introduction

Brain connectivity identified by statistical dependencies between activities of different neuronal units and its study has improved the level of investigation of brain functioning as a whole (Ganzetti and Mantini, 2013). Modifications of connectivity can be observed at multiple temporal and spatial scales and are associated with the behavioral state, namely, active wakefulness, relaxation or the different stages of sleep, and with the occurrence of plastic changes due to learning tasks (Steriade, 2006, Kilgard et al., 2007). Alterations of connectivity correspond at a local level to changes in local neuronal synchronization and in turn in the amplitude and steepness of brain waves while at a global level to changes in synchronization between rhythms produced by distant structures. Synchronization measures can be derived both from electrophysiological (e.g. EEG) and hemodynamic (e.g. BOLD signal from fMRI) signals.

During restful wakefulness, the main activity involves the so called Default Mode Network (DMN), composed of efficiently wired areas serving as global hubs for different functional systems (Elton and Gao, 2015). Recently, it has been shown that resting state is altered by prior brain activity such as motor (Duff et al., 2008) or mental (working memory, Gordon et al. 2014) tasks. In general, prior load produces increases in the DMN connectivity, which have been associated with factors such as fatigue (Esposito et al., 2014) and learning consolidation (Gordon et al. 2014).

During NREM sleep, local connectivity increases correspond to the occurrence of wide and steep sleep slow oscillations (SSO, < 1 Hz), while global connectivity can be sustained by the effect of the thalamo-reticular pacing on the cortex (sigma band, 12-15Hz), which is mainly expressed in the up state of the SSO. The SSO, corresponding during NREM sleep to neurons that synchronously oscillate between a down state with synaptic stillness and an up state of intense firing (Steriade, 2006), includes thus features that can be associated either to the cortico-cortical, local, connectivity or to the thalamo-cortical, global, connectivity.

Increased functional connectivity has been linked to physical exercise in healthy individuals (Rajab et al, 2014). Physical exercise encompasses several phenomena such as hypothalamus-pituitary-adrenal axis (HPA) and immune system activations, as well as brain plasticity enhancement via BDNF expression (Erickson et al., 2012), but to date the specific contribution of these factors in modulating brain connectivity is unknown.

Concerning the immune system, physical exercise, even if sustained, induces the activation of anti-inflammatory cytokines patterns. This anti-inflammatory milieu can be promoted by catecholamines, which are known to potentiate IL-10 release (van der Poll et al., 1996), stimulate IL-6 (Steensberg et al., 2001), and, in turn, inhibit pro-inflammatory cytokines (van der Poll et al., 1996). Moreover, skeletal muscles can be per se a significant source of IL-6 (Pedersen, 2011), which turns out in anti-inflammatory effects by inhibiting the production of TNF-alpha and IL-1beta, inducing increase of IL-1ra and IL-10 (Ostrowski et al., 1999; Petersen and Pedersen, 2005), and contributing to keep cortisol at high plasmatic levels for several hours after exercise (Steensberg et al., 2003). Recent evidence suggests that pro-inflammatory cytokines, such as IL-1beta, interact negatively with BDNF synthesis in the hippocampus, thus affecting BDNF-related plastic processes, and that anti-inflammatory molecules (i.e. IL-1ra) can counteract these effects (Patterson, 2015).

Herein we present a pilot study based on the Ultra Triathlon (Ironmen) model (Menicucci et al., 2013b) to investigate changes of brain synchronization related to a strenuous and sustained, in short "extreme", physical exercise during wakefulness and sleep and to suggest biohumoral factors (endocrine and immune) influencing brain synchronization processes. Regarding wakefulness, the fMRI was used to to investigate changes in brain global connectivity (GC) after the race compared to a basal rest condition, far away from the competition. GC can be considered an index quantifying the connections between each region and the rest of the brain and it has been suggested that highly connected regions are those related to information processing and work as an information hub. Regarding sleep, we focused on the NREM sleep since it is modulated by highly active wakefulness and we compared NREM EEG (Torsvall et al, 1984), during a basal rest condition to that after the race, in order to investigate alterations of connectivity both at a local and a global level standing on the different properties previously,

described for the SSO. Also, possible correlations between exercise-related-cytokines/hormones and global GC as well as SSO features were investigated.

Materials and Methods

Experimental protocol

Twenty male volunteers were enrolled from a cohort of participants to an official Ironman Triathlon competition. The competition is composed by three sport activities: 3.8 Km swimming, 180 Km cycling and 42.2 Km running, performed consecutively. Participants were recruited on the basis of age (35 to 47 years), of experience in Ironman races (from 5 to 10 years), and according to a common weekly training plan (i.e. performing at least one of the segments of the Ironman Triathlon nearly each day). All participants had a license from their national triathlon federation for competing, thus we did not perform any preliminary medical evaluation. Further inclusion criteria were: not taking any medication for at least 1 year, no personal or family history of sleep disorders, and no medical, neurological or psychiatric disorders, as assessed by semi-structured interviews. In order to avoid the variability related to the different phases of the menstrual cycle we chose to restrict our study to males since EEG fast sigma activity is influenced by reproductive hormones (Carrier et al., 2001).

The experimental protocol consisted of the characterization of cytokine patterns (blood markers), brain Global Connectivity (by means of fMRI recording) during wakefulness, and brain activity during NREM sleep (by means of EEG recording) of athletes both in basal condition and after the race. Regarding the basal condition, participants were asked to come to our laboratories four months after the race, during the winter, a period away from the championships and of reduced workouts.

All participants underwent a characterization of cytokine patterns whereas, due to logistic limitations, athletes included in the fMRI study (n= 12) did not undergo the sleep study and vice-versa.

The experimental procedures conformed to the World Medical Association Declaration of Helsinki and all participants signed an informed consent approved by the Azienda Ospedaliero-Universitaria Pisana Ethical Committee (ID 2805).

Analysis of blood markers

The athletes underwent a blood sampling within 10 minutes from the end of the Ironman competition. From blood samples, hormonal and inflammatory markers were evaluated. Plasma concentrations of cytokines (TGF-beta, TNF-alpha, IL-1beta, IL-6, IL-1RA, MCP-1, IL-2, IL-8, IL-10) and C-reactive protein (CRP) were determined quantitatively by ELISA (Bender Medsystems, Austria, R&D Systems, Italy and Invitrogen, USA, Diagnostics Biochem Canada Inc, Canada).

Inflammation markers set was defined based on previously published data about inflammatory response to the Ironman race (Menicucci et al., 2013b); moreover IL-2 and TGF-beta, although not previously investigated in the Ironman model, were included due to their known role as sleep modulators (Marshall and Born, 2002). Owing to its known interaction with cytokine production, we also measured serum cortisol by means of a fluorescent polarization immunoassay method using the TDx system (Abbott Diagnostics, USA).

MRI study

Each participant underwent two MRI scans: one scan was conducted in the basal condition (see the Experimental protocol section), the other one was performed post-race, within 3 hours from the end of the competition due to the duration and preparation times of the MRI study (see below). In both conditions, a single fMRI sequence of six minutes was acquired (3T GE; TR/TE 2500/30; 30 axial slices; matrix: 128x128). Participants were required to rest with their eyes closed during fMRI and were asked not to sleep before or during the scan. In order to prevent them from falling asleep they were asked to perform a finger to thumb opposition task with their right hand (12s on 48 s off). On and off periods were given through interphone. The MRI study was completed by a high resolution T1 anatomical scan; moreover, clinical scans to be referred by a board of neuroradiologists were taken (namely a T1-Flair acquisition and a T2).

Functional MRI data were analyzed for the Global Connectivity (GC) index estimation (Cole et al., 2010). GC was calculated on the MRI time series after the following preprocessing steps: spatial and time registration, low-pass filtering ($f < 0.1$ Hz) and 6 mm smoothing. Through the use of the option-

ertts of the program 3dDeconvolve of the AFNI distribution we regressed out from the time-series nine regressors of no interest. Namely, six time-series describing three rigid body translations and three rigid body rotations of the head across time were obtained from a volume registration algorithm; one time-series describing the BOLD time course of cerebrovascular fluid was obtained with a 4-mm radius sphere ROI placed in the left ventricle (Tailarach Coordinates -8; -18; 21); one time series describing the BOLD time course in the white matter was obtained with a 4-mm radius sphere placed in the posterior part of the corpus callosus (Tailarach Coordinates -2; -34; 17); one describing the task related BOLD changes was obtained with a 4-mm radius sphere placed in the left primary motor cortex (Tailarach Coordinates -44; -8; 38). In line with previous studies (see Danti et al., 2009) this latter regressor was introduced to control for the effect of task and task-related connectivity, regressing it out from the GC model. These time series were used in a multiple regression model applied to each voxel. A time-dependent regressor was also included to linearly detrended voxel time series and time series were consequently obtained as the residuals of the model. The whole analysis was performed by means of AFNI package, and in particular, for the GC estimation the program 3dTcorrMap of the AFNI package was used. The program computes the Pearson correlation coefficient between each voxel and all other voxels, and combines this set of values in order to obtain the global index of connectivity.

Sleep study

EEG recordings were carried out during the first sleep cycle of the night after the race, and of a night in the basal period (see the Experimental protocol section), by means of a 32-EEG channels monopolar amplifier (GEM100, EB Neuro, Florence, IT). EEG signals were acquired with a sampling rate of 500 Hz, electrodes were referenced to the Cz potential and their impedance was kept below 20KOhm. For the analysis, EEG signals were offline re-referenced to the average mastoids potential.

EEG epochs with artifacts were detected on the basis of an automated threshold-crossing detection algorithm (Piarulli et al., 2010) and a posteriori verified by visual inspection. All time segments containing artifacts in at least one channel were discarded. On average, 90% of recording time was free from artifacts. The artifact-free EEG segments were scored according to the AASM criteria (Iber et al., 2007) and analyzed with two aims: 1) the characterization of the whole NREM sleep recording in the frequency domain, and 2) the detection and characterization of SSOs.

For the characterization of NREM sleep recording in the frequency domain we analyzed the spectral power distribution of baseline epochs before each detected SSO not preceded by another SSO within 4 s (time distances between events measured using negative peaks time locations). The baseline epoch was defined as the EEG segment preceding each SSO event, starting from 2s before the negative peak and ending 1s before the peak itself. This ending point occurs before the early positive deflection as shown in a previous study (Menicucci et al., 2013a), and thus the epoch was appropriate for estimating baseline activities of the sleeping brain without contamination of phasic events such as K-complexes. The presence of any kind of artifact in the baseline was excluded thanks to the annotation marks placed during the manual sleep staging. For each baseline epoch we computed the time-frequency power spectrum (spectrogram) with a time-frequency step of 66ms-6Hz via the Fast Fourier Transform applied on Hamming-weighted sliding-windows. Each window was of 166 ms with a 60% overlap between contiguous windows (the signal corresponding to each 166 ms window was zero-padded in order to obtain a frequency resolution of 1 Hz). Then, from the spectrogram of each baseline epoch, we estimated the activity in the five classical sleep frequency bands (delta, 1-4 Hz; theta, 4-9 Hz; sigma, 9-15 Hz; beta, 15-30 Hz; gamma, >30 Hz) by averaging the related bins.

The SSO detection was performed taking advantage of the Likeness Method, a previously published and validated SSO detection algorithm (Menicucci et al., 2009; Piarulli et al., 2010). According to the Likeness Method, we first classified as a classical SSO each wave consisting of (a) two zero-crossings separated by 0.3-1.0 s, the first one having a negative slope; (b) a negative peak between the two zero-crossings with a voltage less than 80 microV; (c) a negative-to-positive peak amplitude of at least 140 microV. Then, detected SSO waves were grouped into events depending on the time-distance between their negative peaks occurrence (events are created by waves in a 400 ms interval

centered on the first detected classical wave negative peak). As a further step, events were enriched by clustering full-fledged SSOs with concurrent similar waves, even if sub-threshold with respect to standard criteria. These detection criteria naturally include all K-complexes (Massimini et al., 2004).

SSO characterization was performed estimating the *SSO event rate*, that is the number of SSO events per time unit, the *SSO rate*, that is the number of SSO wave per time unit per each electrode, and the mean event *extent of propagation*, corresponding to the average over the events of the number of detected SSOs within each event (Menicucci et al., 2009). In addition, SSO shape analysis was based on five morphological features: 1) negative peak amplitude (*N amp*); 2) time interval between the first zero crossing and the negative peak (*ZN time*) 3) time interval between the negative and the positive peak (*NP time*), and two steepness features, 4) slope of the signal between the first zero crossing and the negative peak (*slope 1*) and 5) slope between the negative peak and the second zero crossing (*slope 2*). The analysis of fast rhythms modulation in the SSO up state gives an estimate of the change of EEG activity passing from the down state to the up state in sigma (9-15 Hz), beta (15-30 Hz) and gamma (>30 Hz) bands, *mod s*, *mod b* and *mod g*, respectively. Power estimates are calculated by using a Hamming windowed FFT with a window length of 500ms and the modulations are defined as the difference between the positive peak power and the negative peak power of each band (Gemignani et al., 2012).

Statistical analysis

We studied individual post-race versus basal feature changes by means of two-tails paired t-tests applied on subject-based averages of both the fMRI- and the NREM sleep-related variables. In particular, global connectivity changes between basal and post-race conditions were studied applying the following steps: 1) the maps of global connectivity of each subject were projected to standardized Talairach space (Talairach and Tournoux, 1988) and 2) a paired t-test between conditions was performed (with the 3dttest++ program of AFNI package) on the whole brain. Clusters with $p < 0.001$ corresponding to a False Discovery Rate (FDR) correction of $q < 0.026$ were considered significant. The robustness of significant findings was ensured by the application of a volumetric threshold on the cluster (i.e. only those clusters with a volume > 150 microliters were considered as significant). The threshold was determined in order to obtain a family wise error (FWE) of 0.05 for an uncorrected threshold of 0.001 (MonteCarlo Simulation, program 3dClustSym of the AFNI package).

Besides, we performed the analysis of correlations between feature values measured in the post-race condition resting on the hypothesis that the process of adaptation to stress load involves the regulation of systems by the action of coordinated controls. For each pair of features, the Pearson correlation coefficients calculated over the subjects was considered significant according to the threshold $|r|>0.60$ ($p<0.01$). The resulting list of significant correlations was organized in a graph.

Results

All volunteers completed the race in a time between 10 and 13 hours. Changes of cytokines and hormones blood concentration from basal condition to post-race are shown in Table 1. fMRI and sleep groups showed analogous levels of cytokines and hormones and the statistical test comparing conditions provided the same results for the two groups, thus in Table 1 a unique p-value has been indicated. Post-race plasma concentrations of IL-6, IL-1RA, CRP and cortisol significantly increased with respect to basal condition. Specifically, all participants showed IL-1RA increase, 17 out of 20 participants (10 enrolled in the fMRI study and 7 in the sleep one) had IL-6, CRP and cortisol increase. TGF-beta, TNF-alpha and IL-1beta significantly decreased in all subjects, whereas MCP-1, IL-2, IL-8 and IL-10 had small and scattered post-race changes compared to basal levels.

Global connectivity increase unveils a brain network affected by strenuous physical exercise

Global Connectivity (GC) increased after the ironmen competition as compared to the baseline in several cortical regions. Namely an increased GC was found in the dorsolateral and orbitofrontal cortices, in the temporal lobes, in the limbic lobe (including the anterior cingulate cortex – ACC) and in the visual areas. Moreover, increased GC was found in the right amygdala, in bilateral hippocampus, in

Tab. 1. - Blood concentration of inflammatory markers in the basal and post-race conditions.

		TGF-B	IL-6	MCP-1	TNF-α	IL-1B	IL-1RA	IL-2	IL-8	IL-10	CRP	cortisol
Basal	fMRI	72±39	0.6±0.6	304±86	11.3±1.6	1.7±2.2	190±103	0.3±1	0.2±0.2	1.9±1	0.15±0.19	72±21
	sleep	76±21	0.6±0.3	266±30	11.6±0.6	2.4±1.1	211±52	0.4±1	0.3±0.2	1.65±0.3	0.12±0.06	81±20
post race	fMRI	37±19	3.3±1.7	365±220	9.7±1.5	0.4±0.1	733±424	0.3±1.	4±6	1.6±1	0.9±1.2	490±240
	sleep	38±21	3.1±1.9	328±195	9.5±1	0.4±0.07	614±317	0.5±1.	5±7	1.7±1.5	0.53±0.35	417±230
post race vs basal		-	+	~	-	-	+	~	~	~	+	+
p-value (*)		<0.05	<0.01	ns	<0.05	<0.05	<0.01	ns	ns	ns	<0.05	<0.01

(*) the statistical test comparing conditions provided the same results for the two groups, thus a unique p-value has been indicated.

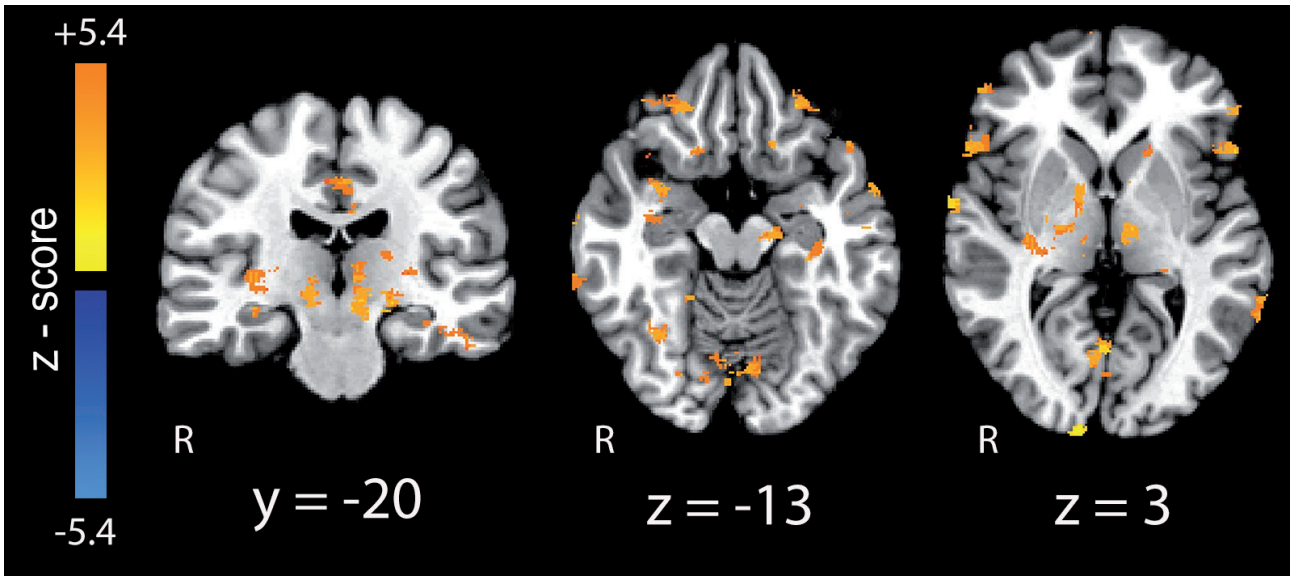

Fig. 1. - Brain regions where global connectivity is higher in post-race (warm colors) as compared to basal acquisitions. All the regions are significant at a p < 0.001, FDR corrected.

bilateral thalamus and in the basal ganglia (Figure 1, Table 2). No region of reduced GC was found.

Macrostructure of the first sleep cycle is preserved following strenuous physical exercise

Table 3 shows parameters describing the first cycle of sleep in the time domain. The time spent in N2, N3 and REM stages, as well as the relative percentages did not change from the basal condition to the post-race condition. REM latency in the basal and post-race conditions was similar and within the normality range. Also the parameters describing sleep continuity – stage shifts frequency, wake time after sleep onset (WASO), arousal frequency – were not significantly different when comparing the basal to the post-race condition.

Despite the lack of differences in the first cycle sleep macrostructure, the post-race condition as compared to the basal one showed power spectral distributions in the baseline epochs before each detected SSO characterized by a general decrease of delta activity ($p<0.05$) together with a shift towards faster rhythms: an increase of theta power in the midline structures and of fast rhythms in midline and parietal areas (Figure 2).

SSOs showed no significant differences in the detection rate and topology between basal and

Tab. 2. - Brain areas with different global connectivity in the post-race compared to basal acquisitions.

Area	BA	H	center of mass				peak			Vol	tscores	p-values	z-scores
Frontal lobe			x	y	z		x	y	z	(microl)			
MFG	10	R	3,5	60,5	20,6		-1	63	19	163	4,5	0,001	3,1
MFG	10	L	-42	41,3	19,4		-41	42	19	259	5,19	0,000	3,4
IFG	10	R	49,6	41,4	0,1		52	42	5	192	4,48	0,001	3,1
IFG	47	L	-42,1	36,9	-7,8		-44	33	-9	270	4,47	0,001	3,1
OFC	11	L	-25,2	39,4	-11,9		-24	36	-13	247	5,02	0,000	3,4
OFC	11	R	24,3	35,9	-13,4		23	35	-16	314	9,34	0,000	4,7
IFG	45	R	52,9	19,8	1,3		56	17	1	498	7,38	0,000	4,2
IFG	47	L	-42,8	19,6	-7,2		-46	15	-6	520	5,99	0,000	3,7
SubcalGyr	47	R	17	19	-9,1		17	23	-9	425	4,56	0,001	3,1
SubcalGyr	47	L	-15,8	17,3	-6,6		-19	17	-7	569	5,49	0,000	3,6
Temporal lobe													
amygdala		R	30,6	-0,3	-9		34	0	-7	278	6	0,000	3,7
hip/parahipGyr		L	-29,6	-7,6	-6,4		-29	-11	-9	398	4,93	0,000	3,3
STG	22	L	-50,8	-9,2	-8,4		-54	-12	-9	208	5,11	0,000	3,4
ITG	21	L	-51,1	-13,2	-18,7		-53	-13	-19	874	5,99	0,000	3,7
hip		R	32,8	-13,8	-10,5		32	-18	-8	153	5,55	0,000	3,6
FGyr	20	L	-39,7	-41	-19,4		-39	-41	-22	1064	5,34	0,000	3,5
FGyr	20	R	33,8	-43,2	-19,4		33	-39	-21	755	4,99	0,000	3,3
MTG	21/22	L	-63,6	-43	1,8		-66	-42	0	518	4,92	0,000	3,3
AngGyr	39	L	-46,2	-66,8	26,5		-45	-72	32	439	7,56	0,000	4,2
Limbic lobe													
ACC	32	L	-1	29,3	26		1	30	25	165	5,43	0,000	3,5
ACC	24	L	-1,9	-18,1	37,6		-3	-21	41	274	4,67	0,001	3,2
PCC/ precuneus	7	R	2,6	-62,5	58,7		2	-67	60	280	5,66	0,000	3,6
occipital lobe													
lingual gyrus	18	R	13,7	-56,4	-5,3		12	-63	-6	161	4,61	0,001	3,2
lingual gyrus	18	R	1,7	-69,2	-5,5		1	-68	0	2132	4,6	0,001	3,2
cuneus	17	R	3,9	-87,2	15		0	-90	13	263	4,85	0,001	3,3
cuneus	19	R	19,9	-88	26,4		20	-88	29	211	5,77	0,000	3,7
cuneus	17	L	-17,6	-94,8	12,9		-18	-96	14	351	6,51	0,000	3,9
cuneus	17	R	12,3	-97,2	3,4		12	-97	3	248	6,9	0,000	4,0
cuneus	19	L	-3,7	-97,2	7,1		-3	-94	9	203	5,15	0,000	3,4
Basal Ganglia & thalamus													
putamen		R	14,8	-0,2	4,5		19	1	8	745	6,29	0,000	3,8
thalamus	VLN	L	-10,4	-13,9	4,9		-14	-16	1	708	5,06	0,000	3,4
thalamus	VPMN	R	15,1	-16,4	-1,4		17	-14	-1	640	5,47	0,000	3,5
thalamus		L	-11,4	-18,3	-7,7		-9	-23	-5	816	5,07	0,000	3,4
putamen		R	29,2	-21,5	3,5		33	-18	4	427	6,89	0,000	4,0
thalamus	LPN	L	-17,7	-22,9	13		-19	-23	14	182	5,58	0,000	3,6
cerebellum		L	-14,7	-56,6	-21,8		-13	-60	-24	393	4,43	0,001	3,1
cerebellum		R	25,9	-71,4	-18,1		27	-73	-18	374	5,42	0,000	3,5

* All the areas are significant at a $p < 0.001$ corrected. MFG, middle frontal gyrus; IFG, inferior frontal gyrus; OFC, orbitofrontal cortex; SubcalGyr, Subcalcarine gyrus, hip, hippocampus; parahipGyr, parahippocampal gyrus; STG, superior temporal gyrus, ITG, inferior temporal gyrus, Fgyr, frontal gyrus; MTG, medio temporal gyrus, ACC, anterior cingulate cortex, PCC, posterior cingulate cortex.

Tab. 3. - Sleep macrostructure of the first cycle in the basal and post-race conditions.

	basal	post-race	
N2 time (min)	51±27	42±11	ns
N3 time (min)	33±28	42±13	ns
REM time (min)	7±5	6±5	ns
REM latency (min)	74±31	78±14	ns
WASO time (min)	0.85±2	0.72±2	ns
Arousal frequency (1/min)	0.1±0.03	0.1±0.04	ns
Stage shifts frequency (1/min)	0.06±0.01	0.05±0.02	ns

post-race conditions (Figure 3); however, the SSOs rate showed a tendency to increase in the post-race condition (up to 20%) at the fronto-central sites. The amplitude (N amp) and the speed of transition from down to up state (slope 2) increased in the post-race condition, in particular when considering the posterior areas ($p<0.05$). Also, the total time of down to up state transition (NP time) increased, but with greater increases ($p<0.05$) in frontal and central areas (Figure 4). The modulation of fast rhythms (*mod s*, *mod b* and *mod g*) in the SSO up state was similar between conditions, even though sigma exhibited a moderate, still non-statistically significant, decrease in the post-race condition.

Inter-subject variability of inflammatory response and of SSO features are associated in the post-race condition

The search of a brain-immune systems integrated response induced by sustained physical exercise was based on correlation analysis that took advantage of the inter-subject variability. The study of inter-subject associations between features revealed several statistically significant ($p<0.01$) correlations that resulted in two clusters (Figure 5). In the first cluster, subjects with higher SSO detection rate had also higher fast rhythms activities that, in turn, were correlated with the post-race levels of IL-10. The same cluster exhibited a positive correlation between the positive slope of SSO and the level of IL-10. In the second cluster, the features related to the temporal width of SSO were inversely correlated with the anti-inflammatory markers IL-1ra and cortisol, while the magnitudes of fast rhythms modulation in the SSO up state were positively correlated with the post-race levels of TNF-alpha and C-reactive protein.

Discussion

Results of this pilot study suggest that extreme physical exercise is able to modify the brain connectivity during the following hours both in wakefulness and sleep conditions. Herein we interpret most of the observed effects as mediated by the increased cerebral excitability following exercise.

Considering the fMRI study, it is worth underlining that global connectivity (GC) can be considered a global index of synchronization of the brain, and that alterations of GC have been found in various psychopathological conditions that are supposed to widely affect the brain itself (Geisler et al., 2015; Wozniak et al., 2013). Moreover, in the experimental model of extreme exercise, the increased GC could be sustained by the high levels of noradrenaline associated to the stressful condition induced by exercise (Metzger et al., 2015). Catecholamines were not measured in the present work, however their levels have been evaluated and resulted strongly increased after Ironman competitions (Menicucci et al., 2013b). Specifically, we identified GC increases in several cortical and subcortical regions including bilateral hippocampus, orbitofrontal cortex and basal ganglia, namely in regions related to sensory-motor (lingual gyrus, fusiform gyrus, superior temporal sulcus, basal ganglia), cognitive, and emotional functions as well as to stress response modulation. Increased GC was found also in regions typically involved in the control and modulation of emotions – amygdala (Bennet et al., 2015; Zhang et al., 2015) and orbitofrontal cortex (Ray and Zald, 2012) – cognitive control of stressful situations – inferior frontal gyrus – and regulation of sympathetic / para-sympathetic balance – subgenual cingulate cortex (Greicius et al., 2007). This pattern suggests an

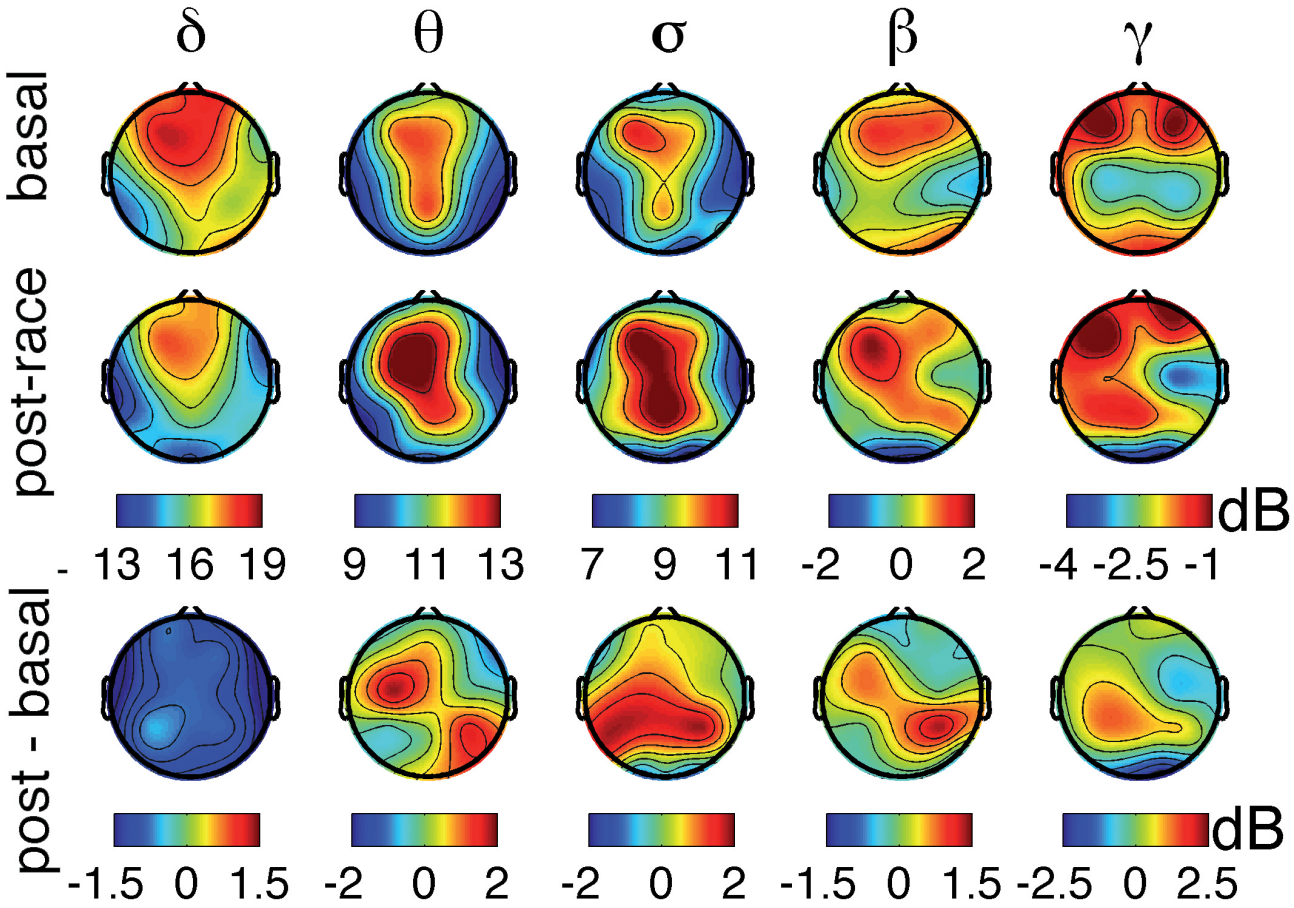

Fig. 2. - Maps of power band density measured in the baseline epochs before spontaneous SSO events in basal and post-race conditions. Maps of between conditions differences (post-race minus basal) are shown.

enhanced central control of the cardiovascular and emotional-cognitive functions, probably necessary for the psychophysical recovery of athletes after the intense physical effort; an increased GC was found also in the anterior and posterior cingulate regions.

Of note, a part of the cortical regions characterized by increased GC are part of the Default Mode Network (DMN) and, in general, these areas may be part of a hub system that 'wires' distant areas of the brain fostering rapid exchange of information across regions (Anterior Cingulate – ACC; Middle Frontal Gyrus – MFG; Precuneus-Posterior Cingulate) (Power et al., 2013; Elton and Gao, 2015). Changes in the DMN connectivity are widely described in stressful conditions (Dedovic et al., 2009), and have been related to the prior brain state (e.g. motor or mental activity). Indeed, the increased connectivity within the DMN following the intense physical exercise could reflect post-race internally focused mental activity such as the rehearsal of the race into memory or, alternatively, could represent a mechanism facilitating the active disengagement from the previous activity (Tailby et al., 2015).

Considering the sleep study, it is worth underlying that during NREM several processes of homeostatic regulation occur and that the brain activity during this stage could play an role in the recovery after the extreme physical exercise and in particular in preventing possible long-term effects of increased cerebral excitability.

The study of NREM sleep in the post-race condition indicates a general decrease of delta activity with a shift to faster rhythms in midline and parietal areas when considering baseline epochs before each detected SSO. We recall that baseline windows are special time frames of NREM sleep free of any underlying bistability process and allow estimating cortical rhythms ruling out the grouping effect of SSO on fast rhythms (Menicucci et al., 2015). In addition, in the post-race condition we found

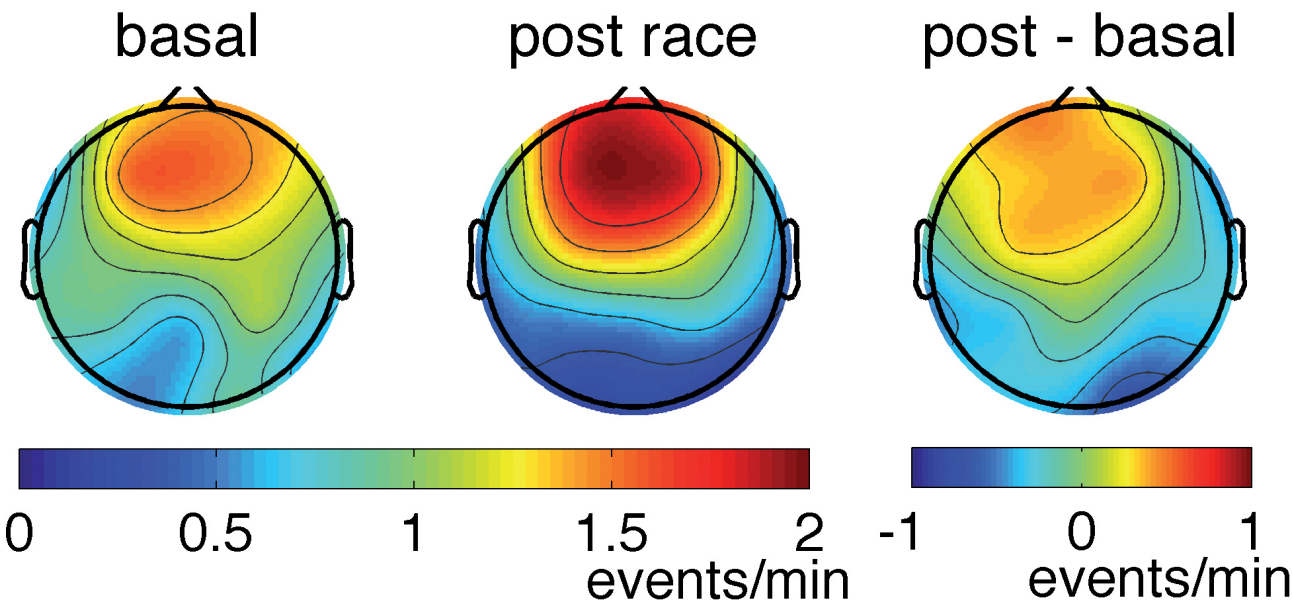

Fig. 3. - SSO detection maps of the first sleep cycle recorded in basal and post-race conditions. Each map represents the frequency (events/min) of SSO detections for each electrode. The map of between conditions differences (post-race minus basal) is shown.

changes related to the SSO that is a slight increase of the rate, the peak-to-peak amplitude and the temporal width of its negative deflection (that corresponds to the cellular down state), as well as a small (not statistically significant) decrease of sigma modulation in the up state. It is worth mentioning that SSO morphology depends on the levels of GABAergic inhibition (Sanchez-Vives et al. 2010). In particular, down state duration and upward and downward transition slopes increase with the blockade of inhibition of $GABA_A$ receptors. In this light, observed shift towards EEG fast rhythms and the increased temporal width of the SSO negative peak can be caused by a decrease of inhibition, i.e. an enhancement in the excitation/inhibition ratio caused by a higher excitability. Moreover, the increased rate of SSO may be interpreted as a reactive response to the increased cortical excitability, in order to impede integration (Massimini et al., 2005) and thus to preserve sleep (Menicucci et al., 2013a, Laurino et al., 2014).

The intense physical exercise also induced a general increase in the blood concentrations of anti-inflammatory cytokines. The inhibition of inflammation associated to the increased arousal (higher cerebral excitability) and reactivity of heart and muscular functions may represent an allostatic acute response to the extreme physical exercise, essential to sustain the high functional requirements of intense motor activity (Menicucci et al., 2013b).

Notably, although based on different studies, areas with increased GC are superimposable with those identified as modulated by cytokines. For example, studies with psychological stressors showed that increased levels of IL-6 in the cortex and in the hippocampus could play a role in stress-induced long-term enhancement of central excitability by decreasing $GABA_A$ receptors-dependent cortical inhibition (Garcia-Oscos et al., 2012). Thus, in spite of the lack of significant associations between GC patterns and cytokines concentrations in our study, we cannot exclude that the increased GC could also be sustained by cytokine-related increased cerebral excitability.

In fact, the results concerning the significant correlations between cytokines and specific SSO features support the potential inflammatory/hormonal effects on sleep induced by extreme physical exercise**.** The inverse correlation between levels of IL-1ra and cortisol, which are known to attenuate NREM sleep (Krueger et al., 2001) and temporally-related features of SSO confirms the inhibitory effect of these anti-inflammatory molecules on sleep also after extreme exercise. On the other side, the positive correlation between fast rhythms modulation in the SSO upstate (worth of note is that sigma activity is a functional marker of

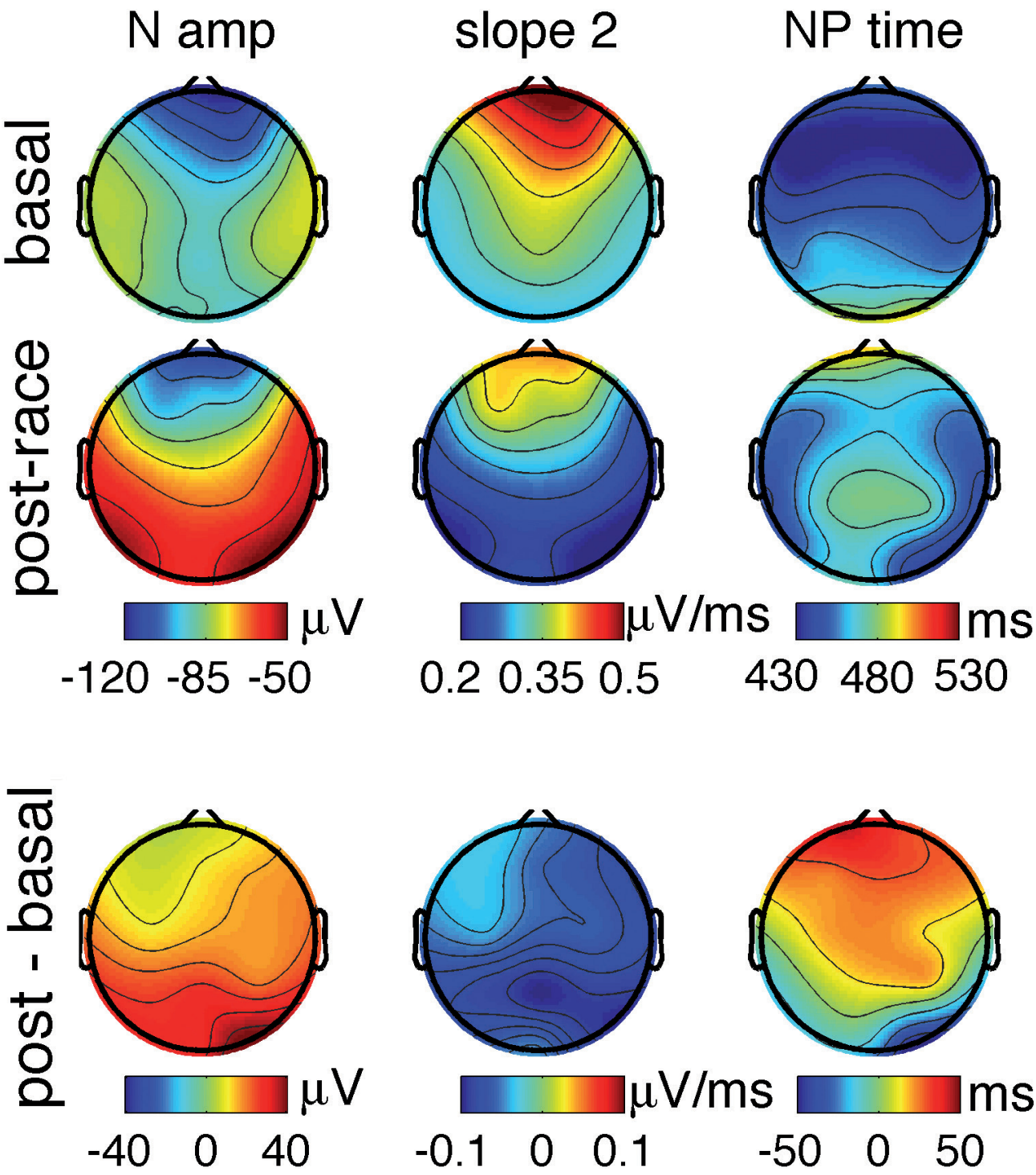

Fig. 4. - Maps of SSO features (N amp, slope 2, NP time) of the first sleep cycle recorded in basal and post-race conditions. Maps of between conditions differences (post-race minus basal) are shown.

thalamo-cortical entrainment) and post-race levels of TNF-alpha and C-reactive protein as well as the positive correlation of SSO detection rate and slope with IL10 levels, suggest a modulation of thalamo-cortical entrainment by inflammatory cytokines which could facilitate the induction of neuronal bistability (Menicucci et al., 2015). In summary, the peculiar biochemical pattern occurring after an

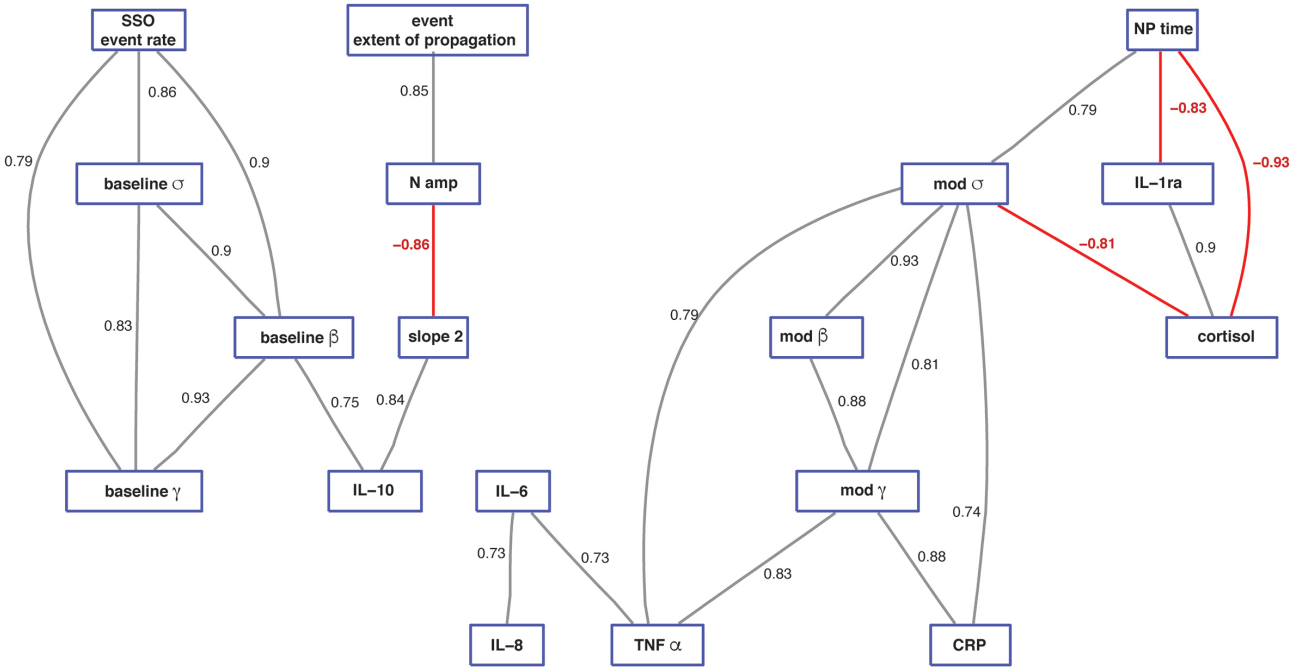

Fig. 5. - Graphical representation of integrated sleep-inflammatory profiles based on correlations between post-race feature levels. The lines between features indicate the significant links; the color of the line provides indication of direct (black) or inverse (red) proportionality; the numerical value is the related correlation coefficient.

"extreme" physical exercise appears to influence NREM sleep dichotomically, that is, on the one hand, causing a general increase of fast rhythm activities, typical of wakefulness, and, on the other hand, facilitating the induction of neuronal bistability (SSO), although with morphological characteristics compatible with a diminished local synchronization possibly related to a diminished GABAergic inhibition.

Limitations

As a pilot study, the first important limitation is the relatively low number of participants: future studies including more subjects would allow gaining more power for statistical analysis.

Also, as far as the fMRI study is concerned, we used a finger-to-thumb opposition task in our connectivity study including the time course of the left motor area as a regressor of no-interest. Although, resting state functional connectivity was not directly investigated, this method can be considered an acceptable compromise in the experimental design since it allows task-free connectivity analysis and helps subjects not to fall asleep (Danti et al., 2010; Sani et al., 2010).

Finally, we decided to use two different techniques for evaluating extreme exercise-related changes in brain activity, evaluating wake activity through fMRI and global connectivity (GC) and sleep activity with EEG. The choice of using EEG is almost mandatory given the fact that sleep staging itself is based on EEG recordings. Moreover, we opted for the EEG in the sleep study since we were specifically interested in the spontaneous SSO expression. Actually, SSOs, whose full-fledged expression corresponds to the K-complex, can be evoked as it would have happened by the noise within a MRI scan. On the contrary, the study of brain connectivity in the wakefulness after the competition would have been arduous with EEG as volunteers usually showed immediate drowsiness in the silent environment of EEG recording setting. We thus decided to use fMRI for wake analysis because of the possibility to evaluate deep brain structures including amygdala, basal ganglia and hippocampus and because of the better spatial resolution of this methodology.

We acknowledge that further studies should use the same methodologies in order to make clearer the relationship between wake and sleep changes. In any case we think that sleep and wake activity and reaction to acute physical stress are so different that the search for possible commonalities may be misleading.

Conclusions

The present study extends the identification of those cortical networks whose activity is modulated by physical exercise from areas directly related to movement, such as the somatosensory ones (Rajab et al, 2014), to networks involved in memory, emotion and motivation. Data suggest that alteration of excitation/inhibition ratio could sustain these changes. These functional alterations also affect sleep and cytokine levels seem to parametrically regulate sleep rhythms expression. The SSO is a candidate to play a homeostatic role in restoring physiological brain functioning.

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