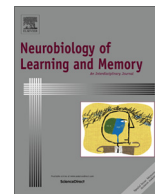




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Experience-dependent upregulation of multiple plasticity factors in the hippocampus during early REM sleep

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ABSTRACT

Sleep is beneficial to learning, but the underlying mechanisms remain controversial. The synaptic homeostasis hypothesis (SHY) proposes that the cognitive function of sleep is related to a generalized rescaling of synaptic weights to intermediate levels, due to a passive downregulation of plasticity mechanisms. A competing hypothesis proposes that the active upscaling and downscaling of synaptic weights during sleep embosses memories in circuits respectively activated or deactivated during prior waking experience, leading to memory changes beyond rescaling. Both theories have empirical support but the experimental designs underlying the conflicting studies are not congruent, therefore a consensus is yet to be reached. To advance this issue, we used real-time PCR and electrophysiological recordings to assess gene expression related to synaptic plasticity in the hippocampus and primary somatosensory cortex of rats exposed to novel objects, then kept awake (WK) for 60 min and finally killed after a 30 min period rich in WK, slow-wave sleep (SWS) or rapid-eye-movement sleep (REM). Animals similarly treated but not exposed to novel objects were used as controls. We found that the mRNA levels of *Arc*, *Egr1*, *Fos*, *Ppp2ca* and *Ppp2r2d* were significantly increased in the hippocampus of exposed animals allowed to enter REM, in comparison with control animals. Experience-dependent changes during sleep were not significant in the hippocampus for *Bdnf*, *Camk4*, *Creb1*, and *Nr4a1*, and no differences were detected between exposed and control SWS groups for any of the genes tested. No significant changes in gene expression were detected in the primary somatosensory cortex during sleep, in contrast with previous studies using longer post-stimulation intervals (>180 min). The experience-dependent induction of multiple plasticity-related genes in the hippocampus during early REM adds experimental support to the synaptic embossing theory.

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1. Introduction

Mammals spend a considerable amount of their lives sleeping. In addition to the importance of sleep for body repose, sleep is crucial for the reactivation, consolidation and restructuring of daytime memories (Rasch & Born, 2013; Stickgold & Walker, 2013). Memory formation has long been conceived as a process by which neuronal

activity reverberating in specific circuits eventually triggers enduring synaptic changes (Hebb, 1949). This process has been proposed to promote the gradual redistribution of memory representations toward sites for long-term storage, as well as the synaptic changes necessary to stabilize memories (Diekelmann & Born, 2010; Ribeiro & Nicolelis, 2004). Neurophysiological studies strongly support the notion of experience-dependent memory reverberation. In rats, hippocampal place cells are reactivated during slow wave sleep (SWS) and rapid-eye-movement sleep (REM) in a manner that recapitulates the neuronal firing sequences that occur during the preceding waking (WK) period (Pavlides & Winson, 1989; Wilson & McNaughton, 1994; Skaggs & McNaughton, 1996; Louie & Wilson, 2001; Lee & Wilson, 2002). Similar results were obtained in the cerebral cortex (Ji & Wilson, 2007; Peyrache et al., 2009; Qin et al., 1997; Ribeiro et al., 2004, 2007).

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While memory reverberation is well established at the electrophysiological level, the molecular correlates of the phenomenon remain contentious (Ribeiro, 2012; Tononi & Cirelli, 2014). In rodents, the expression levels of immediate early genes (IEG) related to synaptic plasticity, such as *Egr1*, *Arc* and *Fos* (Alberini, 2009; Bliss & Collingridge, 2013; Bozon et al., 2003; Chowdhury et al., 2006; Messaoudi et al., 2007; Racaniello et al., 2010; Shepherd et al., 2006; Smith-Hicks et al., 2010; Tzingounis & Nicoll, 2006; Waung et al., 2008), were observed to decrease from WK to sleep (Cirelli & Tononi, 2000; Pompeiano, Cirelli, & Tononi, 1994). Sleep has also been implicated in the decrease of gene expression related to energy metabolism (e.g. glucose type I transporter *Glut1*), growth (e.g. *Bdnf*), vesicle fusion (synaptotagmin IV) and many other metabolic processes (Cirelli, Gutierrez, & Tononi, 2004; Cirelli & Tononi, 2000). Another study detected a downregulation of GluR1-containing AMPA receptor (AMPA) levels during sleep, as well as a decrease in the phosphorylation of AMPARs, CamKII and GSK3beta (Vyazovskiy & et al., 2008). In *Drosophila*, sleep has been associated with a decrease in the number and size of synapses (Bushey, Tononi, & Cirelli, 2011). These findings support the Synaptic Homeostasis Hypothesis (SHY), which postulates that wakefulness and sleep are respectively associated with a net increase and decrease in synaptic strength (Tononi & Cirelli, 2003, 2012). During sleep most synapses would be down-selected, allowing for overall synaptic pruning with the relative enhancement of the strongest memories by synaptic down-selection of the weaker memories (Tononi & Cirelli, 2013, 2014).

An alternative view proposes that synaptic downscaling does occur in most circuits during sleep, but pathways tagged by WK experience actually undergo increased synaptic plasticity during sleep, leading to an “embossing” of the mnemonic traces (Diekelmann & Born, 2010; Poe, Walsh, & Bjorness, 2010; Ribeiro & Nicolelis, 2004). This theory is rooted in evidence that *Egr1* and *Arc* levels decrease during SWS but are upregulated during REM following exposure to novel stimuli or behavioral training in rats (Ribeiro et al., 1999, 2002, 2007). A study of two-way active-avoidance learning in rats showed an increase in the density of pontine waves during post-training REM, in positive correlation with increased levels of *Bdnf*, *Arc*, and phosphorylated *Creb* in the dorsal hippocampus (Ulloor & Datta, 2005). Increased potentiation of cortical electrophysiological responses during sleep was observed in adult mice (Aton & et al., 2014) and cats (Chauvette, Seigneure, & Timofeev, 2012). In kittens, the early development of the visual cortex depends on plasticity mechanisms triggered during sleep (Jha et al., 2005; Seibt et al., 2012), including an upregulation of *Arc* and *BDNF* translation (Jha et al., 2005; Seibt et al., 2012). In the rat dorsal hippocampus, REM deprivation has been shown to decrease long-term potentiation (LTP), synaptic transmission, glutamate receptor protein levels, and ERK/MAPK activation (Ravassard & et al., 2009). A recent study has shown that REM rebound in sleep deprived rats upregulates mRNA and protein levels of *Egr-1* (Zif-268), *c-Fos*, *Arc* and *BDNF* in the hippocampus (Ravassard & et al., 2014). In mice, post-learning sleep leads to an increase in the number of post-synaptic dendritic spines in pyramidal neurons of the motor cortex (Yang & et al., 2014). In *Drosophila*, exposure to social enrichment leads to increased sleep (Ganguly-Fitzgerald, Donlea, & Shaw, 2006), but sleep rebound is absent in a strain with mutated protein phosphatase 2A (PP2A), which is associated with long-term depression (LTD) in the hippocampus (Norman et al., 2000; Thiels et al., 2000).

Both SHY and the synaptic embossing theory have empirical bases, but the experimental designs of the conflicting studies are not congruent, and therefore a consensus is yet to be established (Diekelmann & Born, 2010; Frank & Cantera, 2014; Tononi & Cirelli, 2014). To properly compare these theories it is necessary to investigate plasticity factors in animals with and without pre-

exposure to novel stimulation. It is also essential to distinguish the contributions of SWS and REM, because the former is much more abundant than the latter (Gervasoni & et al., 2004), and thus tends to dominate unsorted sleep periods. To address these critical aspects underlying the cognitive role of sleep, we set out to examine whether pre-exposure to novel sensorimotor experience influences, during subsequent SWS and REM, the neocortical and hippocampal expression of genes related to synaptic plasticity: *Arc*, *Bdnf*, *Creb1*, *Egr1*, *Fos*, *Nr4a1*, *Camk4*, *Ppp2ca*, and *Ppp2r2d*. Some of these genes, such as *Arc* and *Egr1*, have been proposed to play a key role in LTP (Alberini, 2009; Bliss & Collingridge, 2013; Bozon et al., 2003; Chowdhury et al., 2006; Messaoudi et al., 2007; Racaniello et al., 2010; Shepherd et al., 2006; Smith-Hicks et al., 2010; Tzingounis & Nicoll, 2006; Waung et al., 2008 but see Korb et al., 2013; Okuno et al., 2012). Other genes are thought to be essential for LTD, such as *Ppp2ca*, and *Ppp2r2d*, which code for PP2A subunits (Lambrecht & et al., 2013). The list includes genes previously shown to be transcriptionally and/or translationally up-regulated during sleep in an experience-dependent manner (*Arc*, *Egr1*, *Bdnf*, *Creb1*; Ribeiro et al., 1999, 2002, 2007; Ulloor & Datta, 2005), as well as genes for which similar regulation was not observed (*Nr4a1*, same as *NGFI-B*; Ribeiro & et al., 1999).

2. Material & methods

2.1. Animals

A total of 47 adult male Wistar rats (250–300 g) were individually housed under 12 h light/dark cycles (lights on at 07:00), with water and food provided *ad libitum*. Prior to the beginning of the experiment all animals were manipulated for 7 sessions of 20 min over 4 days, to reduce the stress response to the experimenter. Animal procedures were in accordance with the National Institutes of Health guidelines and received approval from the ethics committee of the Instituto de Ensino e Pesquisa at Hospital Sírio-Libanês (permit # CEUA2006/19). Animals that did not reach sleep-wake criteria for any group or showed poor quality LFP were discarded from the experiment ($n = 17$). For the behavioral task, all 6 groups had 5 animals each. In the molecular experiments, one sample from the Exposed REM group showed aberrant measurements for housekeeping genes, which resulted in inaccurate results for all target genes and therefore the sample was discarded.

2.2. Electrode implantation

Rats were anesthetized with ketamine and xylazine and implanted in the right hemisphere with bipolar electrodes made of Teflon-coated nickel–chromium microwires (180 μ m diameter) in the hippocampus (HP), and varnish-coated nickel–chromium microwires (160 μ m) over the primary somatosensory cortex (SI) (California Fine Wire Co., Grover Beach, CA). Three stainless steel screws and cyanoacrylate glue were used to secure the implant. All screws were soldered to a silver wire, which served as a recording ground. Hippocampal electrodes targeted the dentate gyrus, and the SI electrode targeted the supragranular layers of the whisker barrel field. The following stereotaxic coordinates were used, in millimeters from Bregma with respect to the antero-posterior (AP), medio-lateral (ML), and dorso-ventral (DV) axes Paxinos & Watson, 2005: HP (AP: -3.0 ; ML: $+1.5$; DV: -3.3); SI (AP: -3.0 ; ML: $+5.8$; DV: 0.0). DV measurements were taken with respect to the pial surface. To assess hippocampal electrode positioning, four additional animals were implanted under the same conditions, subsequently recorded to verify signal quality, and then killed with an overdose of ketamine and xylazine, and perfused transcardially with PBS at 37 °C followed by phosphate buffered paraformaldehyde.

hyde at 4 °C. Brains were washed in PBS, followed by 20% buffered saccharose and frozen at –80 °C. Frontal sections (40 µm) were stained with cresyl violet and compared with reference plates (Paxinos & Watson, 2005). HP electrodes correctly targeted the dentate gyrus (Fig. S1). Cortical electrode placement was not assessed because the target was the outer cortical surface.

2.3. Sleep-wake criteria

REM can be reliably identified in rats by a combination of criteria, including the presence of theta rhythm (5–9 Hz) in the hippocampus, rhythmic movement of the vibrissae, and highly irregular breathing during sleep; all of these are absent during SWS, which is characterized by the occurrence of spindles in neocortical LFP, high amplitude hippocampal and neocortical LFP oscillations in the delta frequency (0–4 Hz), quiet breathing and negligible vibrissae movement (Timo-laria et al., 1970) (Fig. S2). Animals grouped as SWS showed at least 5 min of SWS but neither theta rhythm nor any behavior associated with REM. Animals grouped as REM displayed SWS followed by behavioral REM signs, with at least 90s of theta rhythm. Animals that did not fall asleep following sleep deprivation were grouped as WK.

2.4. Electrophysiological and behavioral recordings

After 2–5 days for post-surgery recovery, animals were habituated to the empty recording box for 5 consecutive days, being recorded for 1 h on habituation days 2–4. The recording box consisted of a 50 × 50 × 40 cm wooden box with a steel grid as floor. Experiments consisted of recording animals before, during, and after novel object exploration. Local field potentials (LFP) were filtered (0.1–100 Hz) and digitized at 300 Hz using a Brain Net Bnt-36 (Lynx Tecnologia Eletrônica, São Paulo, Brazil). Behaviors were recorded throughout the entire experiment by a CCD video camera. LFP and video recordings were acquired and synchronized with the software Vídeo Gravações (EMSA Equipamentos Médicos, Rio de Janeiro, Brazil). Electrodes were plugged to the recording cables at 16:30. Animals were then placed inside the recording box and the experiment was initiated. During the first 4 h of recording, animals were kept undisturbed and allowed to sleep freely. At 19:00 visible lights were turned off and behavior recording continued under infrared illumination. There was a 90 min period during which animals were kept undisturbed in the dark, prior to behavioral manipulation. Then, at 20:30, animals in the novelty groups were exposed to 4 novel objects placed in the corners of the recording box, as previously described (Ribeiro et al., 2004, 2007). The objects were novel to the animals and were built to maximize shape, texture, and

behavioral value differences (Ribeiro et al., 2004, 2007). Objects were presented when visible lights were off, so as to maximize the drive for the whisker-based tactile exploration of the environment (Fig. S2). The experiment consisted of a naturalistic behavioral paradigm involving novel sensory and spatial cues, designed to maximize changes induced by exposure to novel objects, as opposed to changes related to repeated behavioral training. Exploratory behavior was measured and defined as the animal directing its nose toward the object at a distance of less than 4 cm. Rearing with the head oriented upward was also included if at least one forepaw was on the object. Any other behavior, such as gazing around while resting or leaning against an object, was not considered to be exploration. After novel object exposure the animals were sleep deprived for 60 min in order to separate the effects of novel sensory stimulation from sleep-dependent changes in gene expression. Sleep deprivation was achieved by gently tapping the recording box before animals transited into target states. Animals in the WK group were not allowed to have undisturbed sleep, animals in the SWS group were repeatedly woken up before transiting into REM, and animals in the REM group were allowed to sleep freely (Figs. 1 and S2). Animals assigned to the SWS and REM groups were killed 30 min after reaching the criteria for the respective state. WK animals were killed immediately after sleep deprivation (Fig. 1). With this procedure, 6 groups ($n = 5$ rats/group) were created: Exposed Waking, Control Waking, Exposed SWS, Control SWS, Exposed REM and Control REM. Similar behavioral protocols have been successfully used by our group in the past (Ribeiro et al., 1999, 2002, 2007). Animals assigned to the REM and SWS groups were killed 30 min after reaching criteria for the respective state. WK animals were killed immediately after sleep deprivation (Fig. 1). For killing, animals were anesthetized with halothane and instantly decapitated. The brains were rapidly removed, the hippocampus and the SI cortex were dissected bilaterally, sampled with a sterile scalpel, quickly frozen on dry ice, and stored at –80 °C. The entire hippocampus (including dorsal and ventral portions) was included, but the subiculum was excluded.

2.5. Gene expression analysis

Frozen tissue samples (20 mg) were homogenized with a Polytron (Fisher Scientific, Houston, TX) for 30 s. Total RNA was isolated using TRIzol (Life Technologies, Carlsbad, CA). RNA quality and quantity were determined by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) followed by agarose gel electrophoresis to assess RNA integrity. Only samples with $A_{260nm}/280nm$ and $A_{260nm}/230nm$ larger than 1.75 were used. One microgram of total RNA from each sample was reverse-transcribed

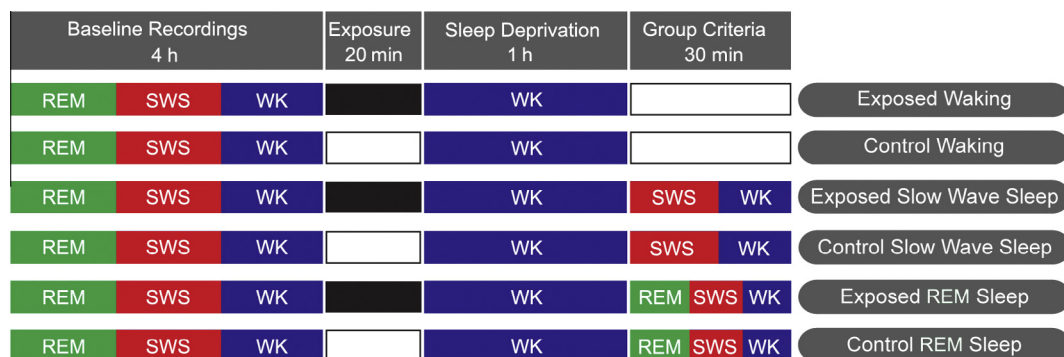


Fig. 1. Experimental design to probe experience-dependent gene expression across the sleep-wake cycle. After baseline recordings (4 h), novel objects were introduced in the box for 20 min in the case of animals in the exposed groups (black boxes); controls remained in the box without any novel stimuli (white boxes). Immediately following the removal of the objects, animals were sleep deprived for one hour. WK animals were killed immediately after sleep deprivation while those belonging to the SWS and REM groups were killed 30 min after achieving sleep criteria for their respective group. Note that REM animals were the only ones allowed uninterrupted sleep in the last min of the experiment, while in the other groups sleep was interrupted after reaching sleep criteria.

into double-stranded cDNA using an oligo-dT-15 primer and Improm-II Reverse Transcriptase (Promega Corporation, Madison, WI). Real-time PCR experiments were performed using TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA) for *Arc*, *Bdnf*, *Camk4*, *Creb1*, *Egr1*, *Fos*, *Nr4a1*, *Ppp2ca* and *Ppp2r2d* as target genes. In accordance with geNorm software (Vandesompele & et al., 2002), *Gusb* and *Rpl19* were chosen as housekeeping genes for our samples. Assay efficiency and data normalization were performed in qBase v1.3.5 software (Hellemans & et al., 2007). All samples were analyzed in triplicates and the mean value was used.

2.6. Statistical analysis

The presence of outliers was tested (Motulsky, 1997). One sample from group Exposed REM was considered an outlier for the housekeeping genes and was excluded from the analysis. A bootstrap-based non-parametric two-way ANOVA (NANOVA) (Zhou & Wong, 2011; Zhou et al., 2010) was implemented using the TANOVA package v1.0.2 for R (available at <http://gluegrant1.stanford.edu/TANOVA>). Sleep stage and exposure to enriched environment were used as fixed-factors, and the number of bootstraps was set to 1,000. A Bonferroni correction for the multiple genes was performed. The NANOVA was followed by Tukey's test for group comparisons when appropriate. *P*-values lower than 0.05 were considered to be statistically significant. Pearson correlations were used to assess the relationship between mRNA levels for gene pairs, and between REM amounts and mRNA levels across animals. Bonferroni correction for multiple comparisons was used to determine statistical significance.

3. Results

3.1. Behavior

The experiment was designed to keep animals within their regular sleep cycle during the baseline recording period, and then to enrich a target sleep-wake state prior to sacrifice, so as to maximize differences in gene expression across states. Indeed, the distribution of behavioral states was similar across groups during the baseline recording (Fig. S3A). As predicted by the experimental design, we observed an enhancement in the amount of the target sleep state assigned to each group in the 30 min period that preceded killing (Figs. 2 and S3B).

An analysis of the time elapsed between the beginning of sleep deprivation and killing showed a significant difference between WK and sleep groups, with more time elapsed in the SWS and REM groups than in the WK group (Fig. S4; $F(5,29) = 16.122$; $p < 0.001$). This was expected since WK animals were killed immediately after the sleep deprivation period, while SWS and REM groups had to spontaneously enter sleep and then fulfill criteria for state grouping. No significant differences in the interval between the beginning of sleep deprivation and killing were found between the SWS and REM groups (Fig. S4). No difference was found between the control and exposed groups within each state (Fig. S4; $F(2,29) = 1.237$; $p = 0.277$), nor was there an interaction between sleep and object exposure ($F(2,29) = 0.522$; $p = 0.60$). To determine whether the groups differed in exploratory behavior during object presentation, we compared the total time spent in object exploration across groups. No significant differences were detected (Fig. 3; $F(2,13) = 1466$; $p = 0.273$).

3.2. Gene expression

In the hippocampus, 6 genes presented significant effects for novel object exposure and/or wake-sleep state (Fig. 4): *Arc*

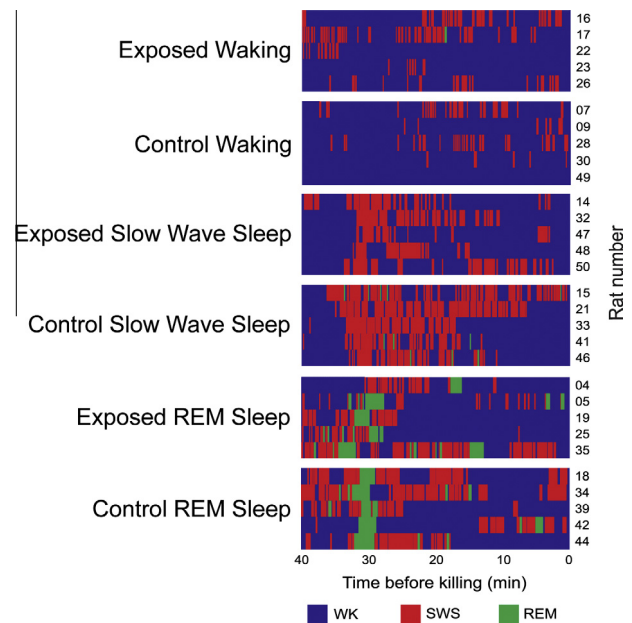


Fig. 2. Hypnograms. Sleep–wake architecture of the last 40 min of the experiment in the 30 animals in which IEG expression was assessed. The different sleep states were prevented or allowed in each group, leading to an enrichment of specific states in different groups.

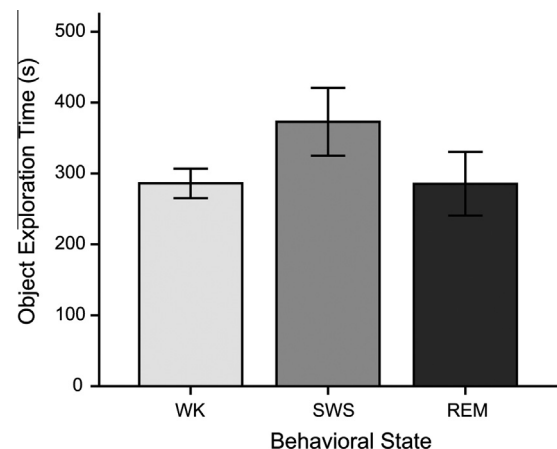


Fig. 3. Total time spent in object exploration in the WK, SWS and REM groups exposed to novel objects. No significant differences were detected (mean $\pm$ standard error of the mean).

($p = 0.009$), *Egr1* ($p < 0.001$), *Fos* ($p < 0.001$), *Nr4a1* ($p = 0.027$), *Ppp2ca* ($p = 0.018$) and *Ppp2r2d* ($p < 0.001$). Data from genes found to have significant effects were tested for interactions between behavioral states and novel object exposure. All the tested genes showed significant interactions: *Arc* ($p = 0.012$), *Egr1* ($p = 0.012$), *Fos* ($p = 0.018$), *Nr4a1* ($p = 0.035$), *Ppp2ca* ($p < 0.001$) and *Ppp2r2d* ($p = 0.035$). Post-hoc analysis revealed a significant increase in the expression of *Arc*, *Egr1* and *Fos* in the Exposed Waking group, in comparison to the Control Waking group (*Arc* $p = 0.001$; *Egr1* $p = 0.005$; *Fos* $p < 0.001$; *Nr4a1* $p = 0.006$). IEG expression was re-induced during REM in an experience-dependent manner, as shown by the comparison of the Exposed REM group with the Control REM group (*Arc* $p = 0.030$; *Egr1* $p = 0.001$; *Fos* $p = 0.011$). Similar results were observed for genes coding for PP2A subunits, with a significant increase of *Ppp2ca* mRNA levels in exposed groups during WK ($p = 0.018$) and REM ($p = 0.037$). *Ppp2r2d* expression was significantly increased in the Exposed REM group versus the

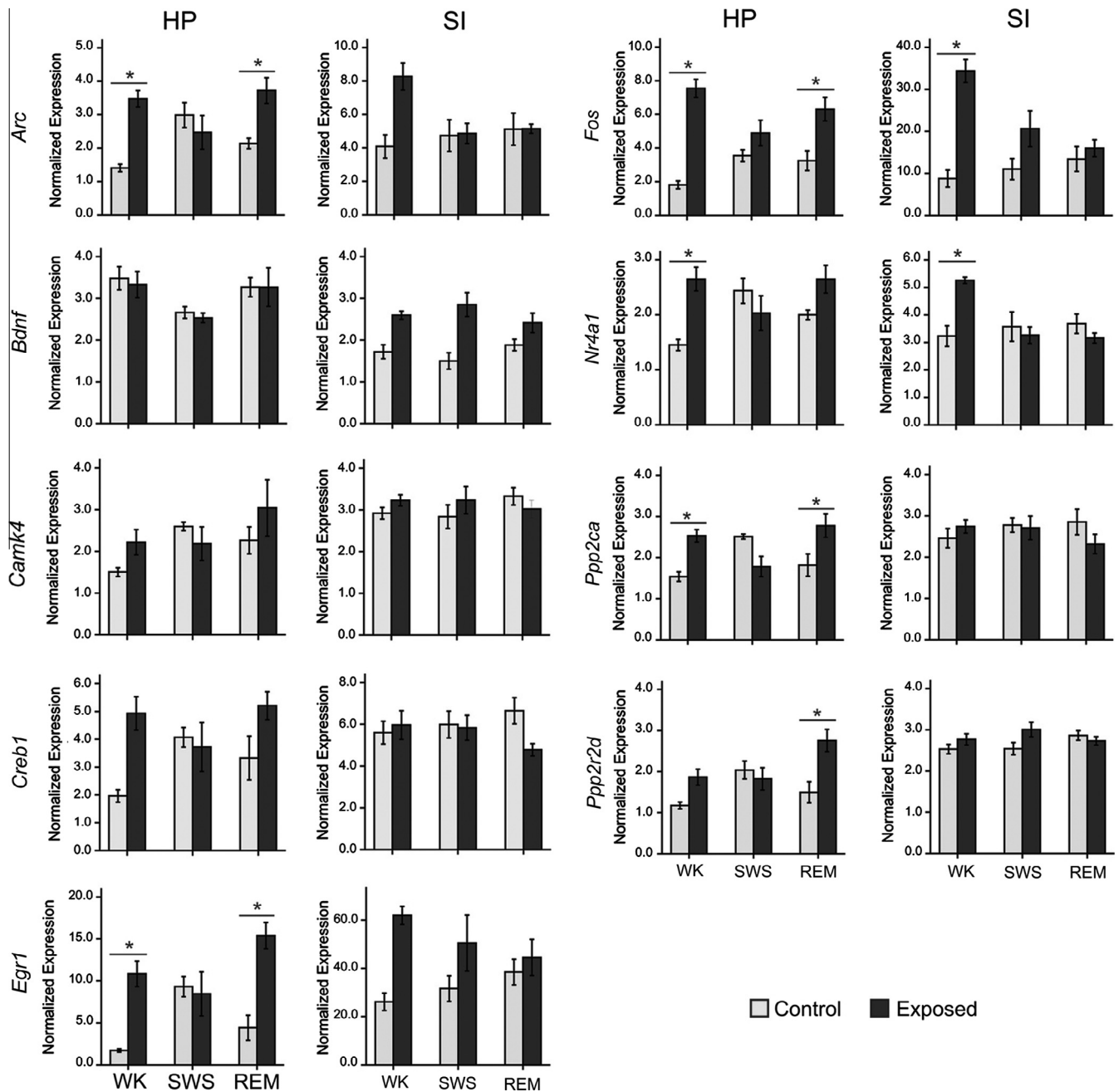


Fig. 4. REM that follows novelty exposure induces transcriptional upregulation of multiple plasticity factors in the hippocampus. Bar charts show the real-time PCR expression levels of plasticity factors, normalized by the expression levels of housekeeping genes (mean $\pm$ standard error of the mean). The mRNA levels of *Arc*, *Fos*, *Egr1*, *Ppp2ca* and *Ppp2r2d* were significantly increased in the hippocampus during REM that followed object exploration. Asterisk indicates $p < 0.05$ corrected for the number of comparisons. Hippocampus (HP), primary somatosensory cortex (SI).

Control REM group ($p = 0.010$); a non-significant trend was observed during WK ($p = 0.265$). *Nr4a1* expression was significantly increased by novel object exposure in WK ($p = 0.006$). In addition, *Nr4a1* mRNA levels were significantly higher in Control SWS than in Control WK ($p = 0.031$).

Other hippocampal comparisons also showed significant differences. For instance, *Arc* and *Egr1* mRNA levels were higher in Control SWS than in Control Waking (*Arc* $p = 0.019$; *Egr1* $p = 0.026$). Regarding *Fos* expression, levels were significantly higher in Exposed Waking than in Exposed SWS ($p = 0.023$). For *Ppp2ca* there was also a significant increase in Exposed REM versus Exposed SWS ($p = 0.028$), and in Control SWS versus Control Waking ($p = 0.021$).

In the SI cortex we observed a significant effect of novelty exposure and/or wake-sleep state for *Bdnf* ($p < 0.001$), *Fos* ($p < 0.001$)

and *Nr4a1* ($p = 0.009$) but not for *Arc* ($p = 0.166$), *Camk4* ($p = 1.000$), *Creb1* ($p = 0.987$), *Egr1* ($p = 0.119$), *Ppp2ca* ($p = 1.000$) and *Ppp2r2d* ($p = 0.720$). *Fos* ($p = 0.006$) and *Nr4a1* ($p = 0.003$) presented a significant interaction, while *Bdnf* did not ($p = 0.348$). Post-hoc tests revealed a significant increase in *Fos* and *Nr4a1* expression in the Exposed Waking group, in comparison with the other groups. Since we could find no interaction effects for *Bdnf*, we compared the main effects. *Bdnf* expression was increased in animals exposed to novelty versus controls ($p < 0.001$), but there was no sleep-wake effect ($p = 0.994$).

To gain insight into the joint expression of multiple plasticity factors in the hippocampus, we calculated the Pearson correlation of mRNA levels for all gene pairs considered, pooling data from all wake-sleep states (Table 1). For Control groups we found a very

Table 1
Pearson correlations (*R*) of hippocampal mRNA levels for all gene pairs considered. Data pooled from all wake-sleep states. Red indicates significant correlations with Bonferroni correction.

Exposed Control	Arc	Bdnf	Camk4	Creb1	Egr1	Fos	Nr4a1	Ppp2ca	Ppp2r2d
Arc	1.000	−0.126	0.406	0.415	0.891	0.818	0.887	0.405	0.559
Bdnf	0.516	1.000	0.453	0.383	−0.087	−0.071	−0.064	0.528	0.184
Camk4	0.619	−0.505	1.000	0.725	0.584	0.490	0.442	0.694	0.779
Creb1	0.573	−0.663	0.916	1.000	0.481	0.385	0.470	0.741	0.686
Egr1	0.816	−0.650	0.766	0.826	1.000	0.877	0.763	0.524	0.693
Fos	0.910	−0.510	0.623	0.623	0.902	1.000	0.852	0.406	0.510
Nr4a1	0.931	−0.559	0.683	0.646	0.891	0.923	1.000	0.489	0.526
Ppp2ca	0.514	−0.523	0.848	0.898	0.760	0.551	0.634	1.000	0.632
Ppp2r2d	0.730	−0.679	0.878	0.896	0.858	0.705	0.777	0.808	1.000

Table 2
Pearson correlations (*R*) between REM amounts and mRNA levels across animals. No significant correlations with Bonferroni correction were detected for any of the genes tested.

Exposed Control	Arc	Bdnf	Camk4	Creb1	Egr1	Fos	Nr4a1	Ppp2ca	Ppp2r2d
<i>Hippocampus</i>									
40–30 min	0.223	0.269	0.518	0.307	0.492	0.106	0.135	0.496	0.619
30–20 min	0.328	−0.087	0.074	0.061	0.409	−0.205	0.201	0.178	0.491
<i>Hippocampus</i>									
40–30 min	0.053	0.345	0.450	0.366	0.159	0.258	0.077	0.089	0.234
30–20 min	0.004	0.071	−0.180	−0.064	−0.160	0.020	0.052	−0.180	−0.167
<i>SI Cortex</i>									
40–30 min	−0.278	−0.239	−0.131	−0.297	−0.189	−0.461	−0.435	−0.250	−0.184
30–20 min	−0.367	−0.335	−0.250	−0.453	−0.375	−0.588	−0.488	−0.357	−0.210
<i>SI Cortex</i>									
40–30 min	−0.113	0.270	0.065	−0.030	0.119	−0.037	−0.159	−0.057	0.338
30–20 min	−0.161	0.037	0.230	0.334	0.174	−0.079	−0.007	0.426	0.324

high degree of significant positive or negative correlations across all gene pairs. In contrast, Exposed groups display clusters of positive significant correlations that differentiate the combined expression of *Arc*, *Egr1*, *Fos* and *Nr4a1* from that of *Bdnf*, *Creb1*, *Camk4*, *Ppp2ca*, and *Ppp2r2d*.

Finally we searched for correlations across animals between REM amounts and mRNA levels. We calculated Pearson correlations between gene expression levels and REM amounts for 10 min intervals during the period of peak mRNA expression (40–20 min before killing; Bozon et al., 2003; Bramham et al., 2010; Guzowski et al., 1999, 2001; Mello & Clayton, 1994; Tzingounis & Nicoll, 2006). No significant correlations were detected for any of the genes tested (Table 2).

4. Discussion

The present real-time PCR study corroborates previous *in situ* hybridization findings regarding the reinduction of *Egr1*, *Fos* and *Arc* transcription in the hippocampus during post-learning REM (Ravassard et al., 2014; Ribeiro et al., 1999, 2002, 2007; Ulloor & Datta, 2005). The observation of experience-dependent IEG reinduction during REM was also extended to *Ppp2ca* and *Ppp2r2d*, providing a broader view of the experience-dependent changes in gene expression that take place during sleep. The reinduction of phosphatases altogether with *Egr1*, *Fos* and *Arc* leads to a more complex view of the phenomenon. In most cases, gene expression is increased both in WK and in REM groups. Such U-shaped pattern of gene expression across WK, SWS and REM was actually expected from our previous studies of *Egr1* and *Arc* (Ribeiro et al., 1999, 2002, 2007), which show experience-dependent IEG reinduction during REM but not SWS.

The mRNA levels of *Arc*, *Egr1*, *Fos* and *Nr4a1* were tightly correlated across animals (Table 1), reflecting the transcriptional upregulation of multiple genes by similar underlying mechanisms. For all but one of the genes that showed experience-dependent reinduction during REM, expression was also increased during WK that followed novel object exposure. *Ppp2r2d* was the only gene with increased expression in the Exposed group during REM but not during WK. This suggests that post-novelty *Ppp2r2d* expression is specifically triggered by REM. Indeed, the largest correlation detected between gene expression and REM amount corresponded to *Ppp2r2d* ($R = 0.619$). Note however that there was a trend toward increased WK expression in the Exposed group.

In the SI cortex, no significant differences in gene expression were detected between Exposed and Control REM groups. In some cases this occurred despite a large mean difference between conditions, because ANOVA did not reach significance. For instance, *Egr1* and *Arc* showed non-significant trends ($p = 0.119$ and 0.166 , respectively). This lack of statistical differences across states in the SI cortex contrasts with previous *in situ* hybridization results (Ribeiro et al., 1999, 2002, 2007). The discrepancy may be related to the substantially different post-stimulation intervals before killing in these different studies (60 min in the present study, >180 min in the other studies). Altogether, the available evidence suggests that short post-stimulation intervals lead to transient experience-dependent changes in the hippocampus, while longer stimulation intervals lead to progressively more corticalized experience-dependent changes (Ribeiro, 2012; Ribeiro & Nicolelis, 2004; Ribeiro et al., 1999, 2002, 2007).

At variance with previous studies of protein levels (Datta, Li, & Auerbach, 2008; Ulloor & Datta, 2005), we did not detect significant sleep-dependent differences in the mRNA levels of *Creb* and *Bdnf* following exposure to novel stimuli. This is in agreement with

the fact that CREB is expressed constitutively and activated by post-translational phosphorylation in response to specific signaling (Alberini, 2009). The differences observed for *Bdnf* between previous studies (protein) and the present study (mRNA) may also be related to differences in the temporal dynamics of the experiments, since we assessed changes in *Bdnf* expression 30 min after sleep, while the previous studies used a 1–3 h interval. These differences may also be related to the distinct stimulation paradigms employed, milder in our paradigm (novel objects exploration) in comparison with the two way avoidance task employed earlier.

The overall non-significant degree of correlation between gene expression levels and REM amounts (Table 2) is not likely explained by insufficient sample size ($N = 15$ for controls, $N = 14$ for exposed animals). We have previously observed a similar lack of correlation between time spent in REM and *Egr-1*/*Arc* expression (Ribeiro et al., 2007). The present results add to the notion that REM plays a brief and mostly permissive role on gene expression upregulation, in agreement with the fact that birds and crocodilians exhibit very short REM episodes (Siegel, 1995; Vorster & Born, 2014).

To our knowledge, the present study reports for the first time a sleep-dependent upregulation of *Ppp2r2d* and *Ppp2ca*, which code for PP2A subunits. In the hippocampus, PP2A is involved with LTD (Norman et al., 2000; Thiels et al., 2000). In the cerebellum, PP2A activation is necessary for LTD induction, and its persistent inhibition is necessary for LTP induction and maintenance (Belmeguenai & Hansel, 2005). We speculate that the upregulation of *Ppp2r2d* and *Ppp2ca* following novelty exposure may be mediated by CREB, since the expression of *Ppp2ca* in T cells is regulated by phosphorylated CREB (Sunahori, Juang, & Tsokos, 2009). In line with this hypothesis, mutations in the genes coding for PP2A and CREB have been shown to disrupt the sleep induction triggered by novelty in fruit-flies (Ganguly-Fitzgerald et al., 2006). Further experimentation must be carried on to test this hypothesis.

Gene expression follows precise temporal dynamics, therefore it could be postulated that differences in gene expression observed during sleep could be reminiscent of activity generated by the exposure itself. In this case we would expect different time intervals between object exploration and killing for the REM and SWS groups, which did not occur (Fig. S4). Denial of this assumption is further supported by the expression pattern of *Arc*, *Egr1*, *Fos* and *Ppp2ca*, which showed increased expression after both waking and REM, but not after SWS. This interpretation is also supported by the evidence that *Arc* and *Egr1* mRNA levels peak ~30 min after a reference state or stimulus (Bozon et al., 2003; Bramham et al., 2010; Guzowski et al., 1999, 2001; Mello & Clayton, 1994; Tzingounis & Nicoll, 2006). Another possible caveat would be that differences in gene expression are related to differences in learning and/or object exploration. This may also not be the case because exploration time was similar for all groups, and also similar to exploration levels observed by us in the past (Ribeiro et al., 2007). In addition the waking group can serve as an indication that the type of exploration used here is associated with increases in gene expression in the hippocampus, a result that corroborate many previously published results for *Arc*, *Egr1* and *Fos* (Ribeiro et al., 1999, 2002, 2007; Ulloor & Datta, 2005). Additional experiments are also welcome to manipulate the expression of these genes and therefore examine more directly their function during sleep-related memory consolidation. Circadian rhythms are important to take into account when dealing with sleep-related procedures. Although the habituation phase started during daylight, all experimental manipulations occurred at least 100 min after lights were dimmed.

The processing and storage of information in the nervous system have been shown to depend on both synaptic upscaling and

downscaling (Jones et al., 2001; Nicholls et al., 2008; Shepherd & Bear, 2011). The transcriptional upregulation during REM of the gene coding for PP2A indicates an active reduction in synaptic strength during REM that follows novel experience. This phenomenon seems to occur in parallel with enhanced synaptic strength, as suggested by the increase in the expression of LTP-related genes *Fos* and *Egr1* (Alberini, 2009; Bliss & Collingridge, 2013; Racaniello et al., 2010). In this way it is also interesting to observe that *Arc*, which has been implicated in different forms of synaptic plasticity, is also reinduced during REM. These findings support a role for sleep in memory consolidation and provide original evidence that synaptic upscaling and downscaling may occur concomitantly during sleep. Additional evidence supporting this hypothesis comes from monocular deprivation experiments in newly-born kitten. Monocular deprivation promotes LTP in the neocortex contralateral to the deprived eye (Heynen & et al., 2003), and LTD in the ipsilateral cortex (Sawtell & et al., 2003). Furthermore, 6 h of sleep deprivation promote an increase in ocular dominance, which is equivalent to that obtained after the same time of monocular deprivation (Frank, Issa, & Stryker, 2001). This effect is dependent on NMDA receptors (NMDARs), cAMP-dependent protein kinase (PKA), CREB-mediated gene expression and protein synthesis, which are all required for the calcium-dependent potentiation of glutamatergic synapses (Aton & et al., 2009).

5. Conclusions

In summary, our study demonstrates the experience-dependent reinduction in the hippocampus of genes related to synaptic plasticity (*Arc*, *Egr1*, *Fos*, *Ppp2r2d*, *Ppp2ca*) during REM. These findings support a role for sleep in memory consolidation, strengthen the link between REM and learning, and provide original evidence that synaptic upscaling and downscaling may occur concomitantly during sleep. At present, two conflicting theories attempt to explain the mechanisms underlying the role of sleep for hippocampus-dependent memory processing. One theory proposes that sleep-dependent memory consolidation is caused by a global reduction in synaptic connectivity induced by slow oscillatory activity during SWS (Tononi & Cirelli, 2003, 2012). According to this view, sleep would be necessary for “an overall balance of synaptic strength”, leading to “global synaptic depression (Vyazovskiy et al., 2008). An alternative theory (Active Systems Consolidation Hypothesis) argues that specific patterns of neuromodulatory activity and LFP oscillations during SWS and REM respectively support neuronal reverberation and synaptic plasticity, leading to a synaptic embossing of memories (Diekelmann & Born, 2010; Poe et al., 2010; Ribeiro & Nicolelis, 2004). Our results argue against the global synaptic downscaling theory. Instead, our data constitute original evidence that sleep harbors both synaptic potentiation and depression following novel experience, providing a mechanism for the synaptic embossing of memories. Future experiments shall determine whether LTP and LTD factors are upregulated in the same neurons, or in separate but adjacent circuits.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.nlm.2015.01.002>.

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