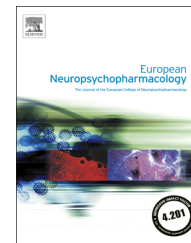




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D2 dopamine receptor regulation of learning, sleep and plasticity

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Abstract

Dopamine and sleep have been independently linked with hippocampus-dependent learning. Since D2 dopaminergic transmission is required for the occurrence of rapid-eye-movement (REM) sleep, it is possible that dopamine affects learning by way of changes in post-acquisition REM sleep. To investigate this hypothesis, we first assessed whether D2 dopaminergic modulation in mice affects novel object preference, a hippocampus-dependent task. Animals trained in the dark period, when sleep is reduced, did not improve significantly in performance when tested 24 h after training. In contrast, animals trained in the sleep-rich light period showed significant learning after 24 h. When injected with the D2 inverse agonist haloperidol immediately after the exploration of novel objects, animals trained in the light period showed reduced novelty preference upon retesting 24 h later. Next we investigated whether haloperidol affected the protein levels of plasticity factors shown to be up-regulated in an experience-dependent manner during REM sleep. Haloperidol decreased post-exploration hippocampal protein levels at 3 h, 6 h and 12 h for phosphorylated Ca²⁺/calmodulin-dependent protein kinase II, at 6 h for Zif-268; and at 12 h for the brain-derived neurotrophic factor. Electrophysiological and kinematic recordings showed a significant decrease in the amount of REM sleep following haloperidol injection, while slow-wave sleep remained unaltered. Importantly, REM sleep decrease across animals was strongly correlated with deficits in novelty preference ($Rho=0.56$, $p=0.012$). Altogether, the

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results suggest that the dopaminergic regulation of REM sleep affects learning by modulating post-training levels of calcium-dependent plasticity factors.

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1. Introduction

The neurotransmitter dopamine is involved with the acquisition of hippocampal-dependent memories (Rossato et al., 2009). While most studies have focused on D1/D5 receptors (Lisman and Grace, 2005; Lemon and Manahan-Vaughan, 2006; Rossato et al., 2009), a role for D2 receptors in hippocampus-dependent memory acquisition and/or consolidation has been proposed (Manahan-Vaughan and Kulla, 2003; De Lima et al., 2011). D2 receptors are also known to control the sleep-wake cycle (Dzirasa et al., 2006; Monti and Monti, 2007; Lima et al., 2007; Morice et al., 2007; Lima et al., 2008). Hyperdopaminergic mice knockout for the dopamine transporter display robust REM sleep (Dzirasa et al., 2006), but REM sleep disappears when animals are treated with alpha-methyl-tyrosine, a dopamine synthesis inhibitor (Dzirasa et al., 2006). Importantly, a D2 (but not D1) receptor agonist is able to rescue REM sleep (Dzirasa et al., 2006). These findings are consistent with the evidence that blockade of D2 receptors specifically decreases REM sleep (Lima et al., 2008). The acute blockade of D2 receptors by haloperidol is known to impair learning in the novel object preference test in rats (Proença et al., 2014) and mice (França et al., 2014). Could the effects of dopamine on learning be mediated by REM sleep?

Hippocampus-dependent learning increases the duration and number of REM sleep episodes (Binder et al., 2012). Several studies have reported the up-regulation of calcium-dependent plasticity factors during post-acquisition REM sleep (Ribeiro et al., 1999, 2002, 2007; Ulloor and Datta, 2005), in line with evidence that sleep deprivation impairs cAMP signaling in the hippocampus (Vecsey et al., 2009). Neural plasticity related to learning is associated with both molecular and electrophysiological events that lead to gradual changes in synaptic strength and morphology (Kandel et al., 2014; Whitlock et al., 2006). Key molecular events include the phosphorylation of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) (Lisman et al., 2002), and up-regulation of the protein levels of immediate-early gene Zif-268 (Guzowski, 2002) and brain-derived neurotrophic factor (BDNF) (Panja and Bramham, 2014). The early increase in pCaMKII levels immediately after memory acquisition is followed by a rise of Zif-268 protein levels after 1-2 h, and then increased BDNF levels after 12 h (Bekinschtein et al., 2007; Medina et al., 2008). These events are necessary for the consolidation of long-lasting memories (Bozon et al., 2003; Xia and Storm, 2005), and for the long-term potentiation (LTP) of electrophysiological responses implicated as the cellular basis of learning and memory (Jones et al., 2001; Whitlock et al., 2006).

To gain insight into the relation of dopamine, sleep and learning, we set out to investigate whether the D2 regulation of REM sleep correlates with changes in the consolidation of the object recognition task, a hippocampus-dependent task (Piterkin et al., 2008). To this end, we assessed electrophysiological and behavioral alterations induced by haloperidol in mice subjected to the object recognition task.

We also investigated how the post-training administration of haloperidol modulates the hippocampal levels of pCaMKII, Zif-268 and BDNF. Finally, we combined behavioral, electrophysiological and kinematic recordings to determine the relationship between learning deficits and haloperidol-induced changes in sleep. The results suggest that D2 dopaminergic transmission affects learning by way of changes in REM sleep and calcium-dependent plasticity factors.

2. Experimental procedures

2.1. Animals

A total of 116 adult male mice were used (C57Bl-6 strain, 2-5 months). After surgery, animals were housed in cages under a 12 h/12 h light/dark schedule, with lights on at 07:00, and food and water *ad libitum*. Animals were daily handled 10 times for 5 min before the experiments, in order to decrease stress responses. Housing, surgical and behavioral procedures were in accordance with the guidelines of the National Institutes of Health, and were approved by the ELS-IINN Ethics Committee (Protocol number 08/2010).

2.2. Novelty preference task

The task was based on the spontaneous tendency of rodents to explore novelty (Hughes, 2007). Our task employed 6 different objects presented over 2 consecutive days; 4 objects were presented during the initial exploration session (training session, 10 min); and 2 unfamiliar objects replaced 2 familiar objects during the second exploration session, 24 h later (testing session, 10 min). To evaluate memory consolidation, an object preference ratio was calculated (time spent with unfamiliar/familiar objects). Behavioral recordings began at 10:00 or 22:00 in an open field apparatus (50 cm diameter and 30 cm high). Following object exploration, animals were injected with haloperidol or vehicle, and were then allowed to behave freely in their home cages until the second exploration session (Figure 1A). For animals subjected to electrophysiological recordings, two training-testing pairs of session were performed within seven days of each other, the first with vehicle and the second with haloperidol. Locomotion was estimated as the total distance traveled per session. In behavioral sessions, experiments during the dark period were conducted under white light.

2.3. Single object exposure and perfusion times for histochemical analyses

For histochemical analyses, we used mice previously exposed to 10 sessions of handling and a single exposure of 10 min to 4 novel objects, identical to the training session described above. All experiments in this case were conducted from 8:00 to 10:00; animals ($N=7-10$ per group) received 0.1 ml/10 g injections of haloperidol (0.3 mg/kg) or vehicle (saline) immediately after object exploration. An additional group (naïve; $N=7$) was studied in which the mice were immediately perfused after being removed from their cages, without object exploration. With the exception of the naïve group, animals were returned to their home cages after injection and allowed to cycle freely through waking (WK) and sleep states for 3 h ($n=10$).

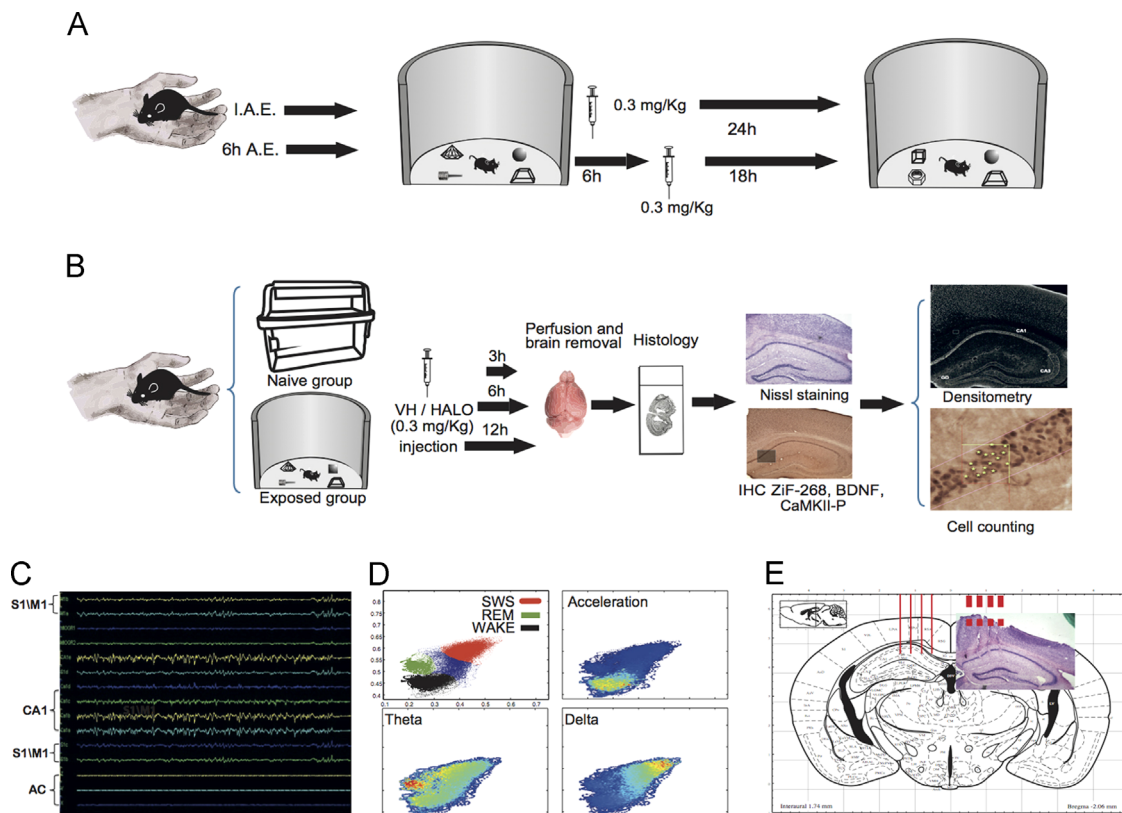


Figure 1 Behavioral task, pharmacology, immunohistochemistry and LFP recordings. (A) Behavioral and pharmacological procedures. C57BL-6 mice were presented to 4 novel objects at light or dark periods (training session). Haloperidol or vehicle was injected immediately after exploration (I.A.E.) or 6 h after exploration (6 h A.E.) of novel objects. One day after training, animals were exposed to 2 novel objects and 2 familiar objects. (B) Immunohistochemistry and histology. At 3, 6 or 12 h after injection, animals were euthanized, and the brains were processed. Frontal sections were cresyl-stained or subjected to immunohistochemistry for the transcription factor Zif-268, phosphorylated calcium-calmodulin kinase II (pCaMKII), and brain-derived neurotrophic factor (BDNF). Labeling quantification in hippocampal regions DG, CA3, and CA1 comprised densitometry for Zif-268, pCaMKII, and BDNF, as well as cell countings for Zif-268, which shows nuclear staining. (C) Local field potential recordings (LFP) were performed in the primary somatosensory (S1) and motor cortices (M1), and in CA1. Inertial recordings from an accelerometer were also obtained. (D) Spectral maps used to quantitatively sort the major behavioral states of the sleep-wake cycle (Gervasoni et al., 2004). Top left panel indicates waking (WK, in black), slow wave sleep (SWS, in red), and rapid-eye-movement sleep (REM, in green); blue denotes state transitions. Top right panel show accelerometer data. High acceleration (hot colors) was only observed during WK. Bottom left panel shows LFP power in the theta range (6-12 Hz); peak theta power occurs during REM. Bottom right panel show LFP power in the delta range (1-4.5 Hz); peak delta power occurs during SWS. (E) Placement of the microelectrode arrays on neuroanatomical diagram overlaid with cresyl-violet stained section. Note electrode tracks in CA1.

VH/haloperidol), 6 h ($n=7$ VH/haloperidol) or 12 h ($n=7$ VH/haloperidol); at the criterion time, animals were deeply anesthetized and perfused (Figure 1B).

2.4. Immunohistochemistry

Animals were anesthetized with isoflurane and perfused with paraformaldehyde 4% in phosphate buffer (PB). The brains were removed and placed in a solution of 30% sucrose at 4 °C for 24 h. Subsequently the brains were frozen, frontally sectioned at 30 μ m in a criostat (Zeiss), and thaw-mounted over glass slides. Sections were incubated as a single batch per plasticity factor in blocking buffer solution (0.5% fresh skim milk and 0.3% Triton X-100 in 0.1 M PB) for 30 min, and then incubated overnight at 18 °C in primary antibody (pCaMKII - 1:200, Millipore; Zif-268-1:100, Santa Cruz Biotechnology, USA) diluted in blocking buffer. Next, the sections were washed in PB for 15 min, incubated with a biotinylated

secondary antibody for 2 h, washed again in PB for 15 min, and then incubated in avidin-biotin-peroxidase solution (Vector Labs, USA) for another 2 h. Slides were then placed in a solution containing 0.03% DAB and 0.001% hydrogen peroxide in 0.1 M PB, dehydrated and cover-slipped with Entellan (Merck, USA). In order to confirm labeling specificity, the primary antibodies were replaced by blocking buffer in test sections. For BDNF staining, some changes were made in the protocol. First, we used an antigenic recovery protocol that consisted in immersing sections in borate buffer (0.1 M, pH 9.0) and then heating them in a microwave oven, for two periods of 30 s. Sections were washed in PB for 15 min. To block endogenous peroxidase, sections were then incubated for 30 min in 3% H_2O_2 diluted in 20% methanol. The sections were then washed in PB for 15 min, incubated in blocking buffer for 2 h and incubated for 72 h in primary antibody (BDNF - 1:100, Santa Cruz Biotechnology, USA). After this procedure the steps were the same as described above (Figure 1B).

2.5. Staining quantification

Densitometric measurements of pCaMKII, Zif-268 and BDNF staining were performed with ImageJ software (3 sections/animal, 1.46–2.06 mm posterior to bregma). Measurements of the corpus callosum were used to subtract staining background values in each section. Densitometric measurements of the different hippocampal regions of interest (DG, CA3, CA1) were then normalized by dividing the mean grey value (in black and white photographs) measured for each animal by the average of these values from all compared groups. Unlike pCaMKII and BDNF, which present diffuse cytoplasmic staining, Zif-268 exhibits nuclear staining. For the cellular quantification of Zif-268 staining, labeled nuclei were counted using Stereo Investigator software (MBF bioscience, USA). In each region of interest (3 sections/animal, 1.46 mm–2.06 mm posterior to Bregma), labeled cells were counted within $50 \times 50 \mu\text{m}^2$ grid squares. The number of labeled cells per grid (R1) was obtained for each section. Individual values were then normalized by the average value of all groups: naïve, VH and haloperidol for each time point, as well as VH and haloperidol inter-time comparisons (Figure 1B). Results are presented as the average of the normalized staining ratio across the three regions of interest (Figure 4), as well as separated by anatomical region (Supplementary Figure 1).

2.6. Behavioral and kinematic recordings

Behaviors were recorded using a Panasonic camera and AMcap 9.21 free software in behavioral groups. Video recordings were synchronized to local field potentials (LFPs) and kinematic data obtained with a three axis accelerometer sensor (ADXL330, Analog Devices) tightly installed over the multi-electrode implant.

2.7. Multi-electrode implantation and recordings

For intracranial LFP recordings (Figure 1C), 9 animals were chronically implanted with 5 electrodes in the hippocampus, 4 electrodes in the primary motor cortex (M1), and 4 electrodes in the primary somatosensory cortex (S1). Each multi-electrode array was $0.9 \times 2.10 \text{ mm}^2$ with length 1.5 mm, composed of $50 \mu\text{m}$ diameter tungsten wires coated with polyamide, attached to an 18-pin connector (Omnetics A79040-001). Arrays were implanted under isoflurane through an opening in the skull (Bregma coordinates: 0.55 and 1.65 ML, 0.0 and -2.2 mm AP). An acrylic cap built over the head was secured by 3 screws attached to the skull. One of the screws touched the duramater and was used as recording ground soldered to a silver wire. A 10-fold pre-amplification circuitry was placed 4 cm distant from the animal's head, in order to reduce noise. LFP signals sampled at a 1000 Hz were pre-amplified $500 \times$ and recorded in a 32-channel system for neural recording analysis (MAP, Plexon Inc).

2.8. Identification of wake-sleep states

LFP, video and kinematic recordings were combined to sort the different sleep-wake states. Online LFP spectral analysis of the sleep-wake cycle (Gervasoni et al., 2004; Ribeiro et al., 2007) was used to identify and quantify occurrence of WK, slow wave-sleep (SWS) and REM sleep (Figure 1D). Animal behavior and LFPs were continuously observed and recorded in real time for 12 h. The first 4 h of recording were used for comparisons among treatments.

2.9. Haloperidol treatment in behavioral groups

Animals received i.p. injections of haloperidol or vehicle (saline 0.9%). Haloperidol doses of 0.3 mg/kg i.p. were used, based on previous studies (Dzirasa et al., 2006; Morice et al., 2007). Depending on the

animal group (Figure 1A), drug or vehicle was applied immediately after exploration (I.A.E.) or 6 h after exploration (6 h A.E.).

2.10. Haloperidol treatment in electrophysiological groups

Animals subjected to object exploration received haloperidol (0.3 mg/kg i.p.) or vehicle I.A.E., defining the Exploration/Haloperidol and Exploration/Vehicle groups, respectively. Animals not subjected to object exploration were injected with haloperidol (0.3 mg/kg, group Control/Haloperidol) or vehicle (group Control/Vehicle).

2.11. Histological confirmation of electrode placement

In order to confirm electrode positioning, animals subjected to multi-electrode implantation were subjected to post-mortem analysis by Nissl staining. In all cases the electrode tips were located at the depth of 1.5 mm for cortical electrodes, or in the CA1 layer in the case of hippocampal electrodes. A representative example is shown in Figure 1E.

2.12. Statistical analysis

The data were first subjected to the Shapiro-Wilk and Kolmogorov-Smirnov normality tests. The data showed parametric distributions, and were expressed as mean \pm the standard error of mean (SEM), with statistical significance set at $\alpha=0.05$. Two-way ANOVA comparisons followed by Bonferroni post-hoc tests were used for behavioral analyses (period and treatment as independent variables, in Figure 2A), for immunohistochemical analyses (using time of injection and treatment as independent variables in Figure 4) and in electrophysiology groups (with object exposure and treatment as independent variables in Figure 6A). Bonferroni-corrected one-way ANOVA followed by Bonferroni post-hoc tests were used for multiple comparisons among the cell counting groups (Figure 5) and the staining ratio of separate hippocampal regions (Supplementary Figure 1). Spearman's correlations were used for verifying the relation between REM sleep duration and novelty preference ratio (Figure 6C). The descriptive statistics comprise F , p values and degrees of freedom (DF) of corresponding one or two-way ANOVAs, followed by mean \pm SEM and summary of p values for each post-hoc test (Table 1, Supplementary Tables 1–4).

3. Results

3.1. Time-dependent effect of haloperidol injection in the object recognition task

First, we measured the effect of haloperidol on performance of the object recognition task. A two-way ANOVA (Time of injection: $F(1, 28)=22.24$, $p<0.0001$; Treatment: $F(1, 28)=11.44$, $p=0.0021$; Interaction: $F(1, 28)=5.4$, $p=0.027$) revealed that animals injected with haloperidol and subjected to the task during the dark period showed significantly less object recognition in comparison to animals treated during the light period (Figure 2A).

Next we compared the effect of haloperidol at two different time points during the light phase: I.A.E. and 6 h A.E. Figure 2B shows that the only treatment to impair object recognition was the administration of haloperidol 0.3 mg/kg I.A.E. (two-way ANOVA: Time of injection: $F(1, 28)=2.30$, $p=0.14$, Treatment: $F(1, 28)=5.04$, $p=0.037$; Interaction: $F(1, 28)=9.5$,

$p=0.0044$). This result indicates that haloperidol impaired object discrimination within the initial hours after injection.

3.2. Haloperidol has a time-dependent effect on pCaMKII, Zif-268 and BDNF levels

The protein levels of the plasticity factors assessed varied substantially across naïve, vehicle-treated and haloperidol-treated animals (Figure 3). 3D illustrates regions of interest differentially stained for pCaMKII, Zif-268 and BDNF, respectively. Quantitative results are shown in Figure 4, Supplementary Figure 1 (densitometry) and Figure 5 (cell counting, only for zif-268).

Figure 4 shows that pCaMKII levels in pooled hippocampal data 3 h after injection were lower in the haloperidol and vehicle groups, in comparison with naïve animals. Haloperidol-treated animals showed lower levels than naïve and vehicle animals 6 h after injection. Animals killed 12 h after injection showed lower levels of pCaMKII in the haloperidol group, in comparison with the vehicle group (statistics in Table 1). In the analysis of separate hippocampal regions 3 h after injection, a significant difference was observed only in the CA3 region (Supplementary Figure 1A, $F=5.58$, $p=0.049$, statistics in Supplementary Table 2). At 6 h post-injection, a decrease of pCaMKII levels was detected in the haloperidol group in CA1 ($F=33.22$; $p=0.0003$), CA3 ($F=28.28$; $p=0.0003$) and DG ($F=11.56$; $p=0.0027$) (Supplementary Figure 1A; statistics in Supplementary Table 3). At 12 h post-injection, pCaMKII levels were lower in the haloperidol group in comparison with the vehicle group in CA1 ($F=9.23$; $p=0.0051$), CA3 ($F=14.24$; $p=0.0003$) and DG ($F=11.12$; $p=0.0024$; Supplementary Figure 1A statistics in Supplementary Table 4).

Significant differences in Zif-268 levels were observed only at 6 h post-injection. The haloperidol group showed lower Zif-268 levels than the vehicle and naïve groups (two-way ANOVA: Time: $F(2, 45)=0.14$, $p=0.86$, Treatment: $F(2, 45)=7.82$, $p<0.0012$; Interaction: $F(4, 45)=5.58$, $p<0.0007$; see Table 1). Similar results were observed when we analyzed the different hippocampal regions: at 6 h post injection,

Zif-268 staining was significantly decreased in the haloperidol group in CA1 ($F=14.27$; $p=0.0009$) and CA3 ($F=9.92$; $p=0.0054$), in comparison with naïve and vehicle groups (Supplementary Figure 1B; statistics in Supplementary Table 3).

The effect of haloperidol in BDNF levels was observed only at 12 h post injection. Staining in the haloperidol group was significant lower than in the vehicle group (two-way ANOVA: Time: $F(2, 43)=0.22$, $p=0.80$, Treatment: $F(2, 43)=5.18$, $p<0.009$; Interaction: $F(4, 43)=0.91$, $p=0.91$, statistics in Table 1). A non-significant statistical trend was observed in the DG at 12 h post-injection, with lower levels in the haloperidol group than in animals injected with vehicle ($F=2.06$, $p=0.056$) (Supplementary Figure 1C, statistics in Supplementary Table 4).

3.3. VH and haloperidol inter-time comparisons

We then analyzed the data through separate comparisons over time for the VH and haloperidol groups. For pCaMKII levels, ANOVA revealed lower immunostaining in the VH 3 h group, in comparison with the other groups (naïve, 6 h and 12 h), in DG ($F=8.346$; $p=0.0009$), CA1 (compared only to 6 h and 12 groups; $F=10.28$; $p=0.0002$) and CA3 (compared only to 6 h and 12 h groups; $F=9.218$; $p=0.0004$). No differences were found for the VH inter-time comparisons of Zif-268 and BDNF staining among naïve, 3 h, 6 h and 12 h groups (Figure 4). For inter-time comparisons among groups injected with haloperidol, there was decreased immunoreactivity for pCaMKII levels at 6 h compared in DG ($F=6.708$; $p=0.0026$, comparison to naïve), and CA1 ($F=16.34$; $p=0.0001$, comparison to naïve and haloperidol 12 h). In CA3 there was decreased pCaMKII labeling in haloperidol 3 h and haloperidol 6 h in comparison to the naïve group. ($F=9.400$; $p=0.0004$). For Zif-268 staining, ANOVA revealed a decrease at 6 h when compared to all other time points, in both CA1 ($F=12.42$; $P<0.0001$) and CA3 ($F=9.672$; $p=0.0004$; Figure 4). For BDNF, ANOVA revealed no differences in haloperidol inter-time comparisons (Figure 4).

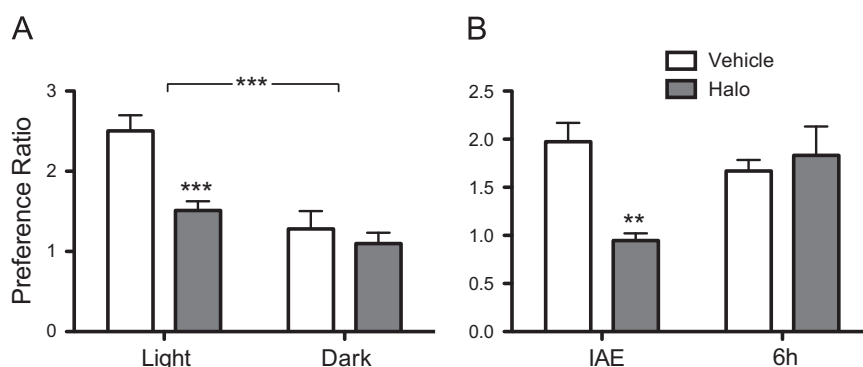


Figure 2 Haloperidol modulates memory for novel objects in mice ($N=7-8$ animals per group) Preference ratio was calculated as the exploration time of unfamiliar/familiar objects. (A) Animals trained during the light phase (morning) showed clear preference for novel objects when injected with vehicle immediately after training, but not when injected with haloperidol. Animals trained during the dark phase (night) failed to show novel object preference for either treatment. Two-way ANOVA followed by Bonferroni post-hoc test was applied for the comparisons. (B) Effect on preference ratio of haloperidol injected in the light phase. Two-way ANOVA followed by Bonferroni post-hoc test were applied. Haloperidol impairs learning when injected immediately after training, but this effect disappeared when animals were injected 6 h after training. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 1 Statistical summary of the significant results in the densitometry analysis for pCaMKII, Zif-268 and BDNF staining. MANOVA comparisons of labeling measurements in the hippocampus were followed by Bonferroni post-hoc tests. Comparisons were performed considering two independent variables: treatment and time of injection.

pCaMKII			
Source of variation	MANOVA		
	<i>F</i>	<i>P</i> value	DF
Interaction	7.87	0.0001	(4,54)
Treatment	42.88	0.0001	(2,54)
Time	1.67	0.19	(2,54)
Time	Bonferroni		
	Groups	Mean ± SEM; N	<i>P</i> value
3 h	NV × Halo	1.08 ± 0.05; N=7	<i>P</i> < 0.001
		0.83 ± 0.05; N=6	
	NV × VH	1.08 ± 0.05; N=6	<i>P</i> < 0.05
6 h		0.92 ± 0.04; N=5	
	NV × Halo	1.05 ± 0.05; N=7	<i>P</i> < 0.001
	VH × Halo	1.17 ± 0.01; N=6	<i>P</i> < 0.001
12 h		0.70 ± 0.02; N=6	
		0.70 ± 0.02; N=6	
	NV × halo	1.14 ± 0.01; N=7	<i>P</i> < 0.01
		0.87 ± 0.03; N=7	
Zif-268			
Source of variation	MANOVA		
	<i>F</i>	<i>P</i> value	DF
Interaction	5.58	0.0007	(4,45)
Treatment	7.82	0.0012	(2,45)
Time	0.14	0.86	(2,45)
Time	Bonferroni		
	Groups	Mean ± SEM; N	<i>P</i> value
6 h	NV × Halo	1.17 ± 0.05; N=6	<i>P</i> < 0.001
		0.65 ± 0.08; N=6	
	VH × Halo	1.09 ± 0.07; N=6	<i>P</i> < 0.001
		0.65 ± 0.08; N=6	
BDNF			
Source of variation	MANOVA		
	<i>F</i>	<i>P</i> value	DF
Interaction	0.24	0.91	(4,43)
Treatment	5.18	0.009	(2,43)
Time	0.22	0.80	(2,43)
Time	Bonferroni		
	Groups	Mean ± SEM; N	<i>P</i> value
12 h	VH × Halo	1.14 ± 0.06; N=7	<i>P</i> < 0.05
		0.89 ± 0.04; N=7	

3.4. IHC quantification of Zif-268 by cell counting

Most of the results obtained by cell counting (Figure 5) were in accordance with the densitometry measurements. For the 3 h post-injection time, we observed increased Zif-268 reactivity

in the VH group in CA1 comparison to the naïve group ($F=5.323$; $p=0.066$), and in CA3 when compared to naïve and haloperidol groups ($F=12.59$; $p=0.003$). At 6 h post-injection, we found a decrease in CA1 in the haloperidol group, when compared to naïve and vehicle groups ($F=24.97$;

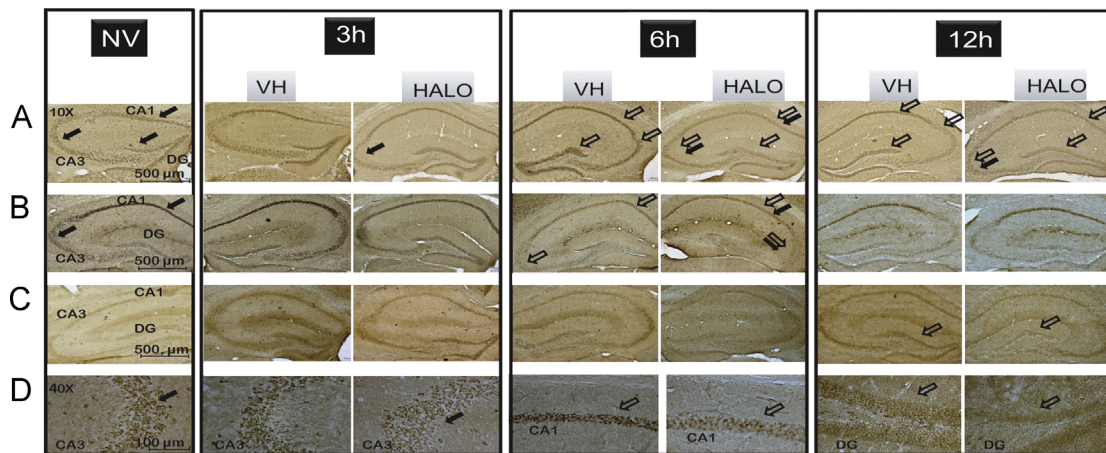


Figure 3 Haloperidol injection decreases the hippocampal levels of pCaMKII, Zif-268 and BDNF according to a temporal gradient. The columns exemplify representative data of different groups. Arrows indicate hippocampal regions (DG, CA3, CA1) with significant labeling differences in the haloperidol group, in comparison with naïve and vehicle groups (empty and filled arrows, respectively). Non-exposed, not injected naïve animals (NV), and animals injected with haloperidol (HALO) or vehicle (VH) immediately after training were euthanized at 3 h, 6 h and 12 h post-exploration. The first three rows represent frontal hippocampal sections at 40 × labeled for pCaMKII (A), Zif-268 (B) or BDNF (C). The last row at 200 × focuses on regions where significant differences were detected (D). Panels show differential labeling for pCaMKII (naïve and 3 h), Zif-268 labeling at 6 h (vehicle), and BDNF at 12 h (vehicle). Please note that haloperidol decreased the hippocampal levels of pCaMKII at all time points. Decreased Zif-268 levels in the haloperidol group were detected only after 6 h, while decreased BDNF levels occurred only after 12 h.

$P < 0.0001$). At 12 h post-injection, no significant differences were found among groups in CA1 and CA3. In VH inter-time comparisons, we observed no differences among naïve, 3 h, 6 h and 12 h groups. Nevertheless, haloperidol-injected animals presented differential staining at these time points (ANOVA $F = 7.689$; $p = 0.004$). In CA1, we observed a decrease in Zif-268 at 6 h when compared to naïve ($P > 0.01$) haloperidol 3 h ($P < 0.01$) and 12 h groups ($P < 0.05$). At CA3 we observed a similar reduction in animals injected with haloperidol at a 6 h time point (ANOVA $F = 9.181$ and $p = 0.002$), when compared to 3 h ($P < 0.05$) and 12 h groups ($P < 0.05$), and a reduction in naïve animals compared to the haloperidol 3 h group ($P < 0.05$).

3.5. Haloperidol impairs memory recognition and decreases REM sleep duration

To test the influence of haloperidol on specific sleep states, we subjected animals to the object recognition task, injected either haloperidol or vehicle, and performed subsequent electrophysiological recordings across the sleep-wake cycle. Two-Way ANOVA was used to compare exposed animals (vehicle and haloperidol 0.3 mg/kg I.A.E.) to controls (vehicle and haloperidol 0.3 mg/kg I.A.E.). Comparisons were independently made for the duration of each state (WK, SWS, REM) versus the independent variables treatment (haloperidol/vehicle) and task (exposure and control). For WK and SWS, the two-way ANOVA detected no difference regarding treatment ($F(1, 14) = 1.76$ $p = 0.21$ for WK; $F(1, 14) = 0.07$ $p = 0.80$ for SWS) or task ($F(1, 14) = 3.63$ $p = 0.07$ for WK; $F(1, 14) = 0.50$ $p = 0.49$ for SWS). For REM sleep, however, significant differences were detected for both treatment ($F(1, 14) = 7.69$ $p = 0.014$) and task: ($F(1, 14) = 32.85$ $P < 0.0001$). Bonferroni *post hoc* tests pointed to decreased REM sleep duration in both haloperidol and

vehicle control groups, in comparison with corresponding object-exposed groups (Figure 6A). We also detected a significant reduction of the preference ratio in haloperidol-injected I.A.E. mice (Figure 6B).

Finally, to investigate the relationship between object recognition and REM sleep duration, we calculated the Spearman correlation between normalized preference ratio and normalized REM sleep duration (Haloperidol or Vehicle divided by [Haloperidol+Vehicle]). We found that lower preference indexes in haloperidol-injected animals were significantly correlated with lower REM sleep durations, just like the higher preference and higher REM sleep durations verified in control animals were also correlated (Figure 6C; $Rho = 0.56$ $p = 0.0125$).

4. Discussion

In the present work we aimed at a better understanding of the biological mechanisms that link sleep, memory formation and dopamine receptor regulation. The behavioral and electrophysiological data showed that haloperidol impairs novel object recognition when injected immediately after training, but has no effect when injected 6 h later. Haloperidol also decreases total REM sleep duration for up to 4 h after training. Importantly, for injections immediately after training, learning and REM sleep duration showed strong positive correlation, suggesting a possible common cause linking REM sleep reduction and learning deficits. Mechanistic insight was sought through an assessment of calcium-dependent plasticity factors. We found that haloperidol injection immediately after training decreased the hippocampal levels of these factors for several hours after treatment, starting with pCaMKII at 3 h, Zif-268 at 6 h and then BDNF at 12 h. Our data point to the calcium-dependent plasticity pathway as a candidate to mediate the effects of haloperidol on both sleep and learning.

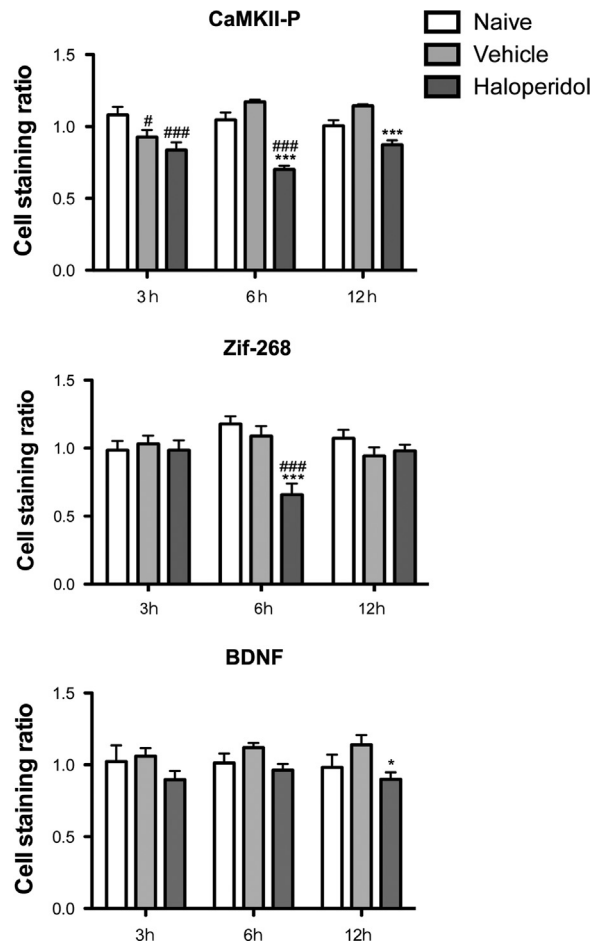


Figure 4 Densitometry quantification of pCaMKII, Zif-268 and BDNF staining at three distinct time points. The three graphs represent pCaMKII, Zif-268 and BDNF staining measures at different time points (3 h, 6 h and 12 h as indicated). Vehicle and haloperidol were injected immediately after exploration. Naïve animals did not receive any injection, nor were presented to objects. Two-way ANOVAs and Bonferroni post-hoc tests were performed for comparisons among these groups for each molecule, considering time and treatment as independent variables. * $p < 0.05$, *** $p < 0.001$ related to vehicle groups; $p < 0.05$, $p < 0.001$ related to control groups.

The possible involvement of the dopaminergic system in the consolidation of memories has been previously reported in the literature. While haloperidol impairs water maze learning (Morice et al., 2007), dopamine D2 agonists decrease memory consolidation in fear conditioning (Nader and LeDoux, 1999), and impair the extinction of conditioned fear memory (Ponnusamy et al., 2005). Both antagonists and agonists of D1 receptors reduce memory consolidation in a fear-conditioning task (Rossato et al., 2009). Haloperidol works as an inverse agonist of D2 receptors, disinhibiting adenylyl cyclase activity and therefore leading to elevated cyclic AMP levels (Konradi and Heckers, 1995). Zif-268 and BDNF are thought to be directly influenced by prior activation of pCaMKII-mediated signaling pathways (Hasbi et al., 2009). It is therefore expected that the levels of cAMP-influenced targets such as pCaMKII (Blitzer et al., 1998), Zif-268 (Kang et al., 2007) and BDNF (Ji et al., 2005; Bekinschtein et al.,

2007) are decreased after haloperidol treatment. The putative mechanism to explain the dopaminergic influence on memory-related plasticity factors is the regulation of non-NMDA glutamatergic receptor activation by D2 dopamine receptor (Hakansson et al., 2006). Since these factors show experience-dependent up-regulation during REM sleep (Ribeiro et al., 1999, 2007; Ulloor and Datta, 2005), the decrease in REM sleep promoted by haloperidol is likely to contribute further to the decrease in the levels of plasticity factors.

One important aspect to consider is the possible motor effect of haloperidol. At high doses, haloperidol impairs motility for several hours after injection. However, no motility differences were detected when total travelled distance was assessed one day after drug injection (data not shown). Furthermore, haloperidol-injected animals injected immediately after training exhibited no object preference 24 h later, indicating that these learning deficits are not related to a possible decrease in motor activity during exploration of the novel objects, but rather reflect an impairment in post-exploration memory consolidation.

A time course analysis of the molecular data indicates that haloperidol impairs calcium signaling through a broad cascade of events distributed over time (Figure 4). At 3 h post-exploration, haloperidol decreased pCaMKII levels in the CA3 field; at 6 h and 12 h post-exploration, the decrease in pCaMKII levels occurred in all hippocampal regions (Supplementary Figure 1, left panels). Zif-268 levels were significantly reduced by haloperidol 6 h after training (Supplementary Figure 1, middle panels), and BDNF levels were decreased by haloperidol 12 h after training in the DG field (Supplementary Figure 1, right panels).

Although several studies have reported increased pCaMKII activity after task training or LTP induction, the exact time window for the activation of this kinase after stimulus remains controversial. A study using *in vitro* LTP recordings found a significant increase in pCaMKII in the CA1 field 30 min after tetanic stimulation (Ouyang et al., 1997), whereas another study revealed that CaMKII remained in a specific, but enhanced phosphorylated state during the induction, and throughout early- and late-LTP, maintaining high levels for 8 h (Ahmed and Frey, 2005). The use of different tetanization paradigms in those studies possibly resulted in large calcium influx changes, affecting other signaling mechanisms in a dose-dependent manner. Additionally, *in vitro* CaMKII phosphorylation in the hippocampus was increased 30 min after inhibitory avoidance, but not after 120 min (Cammarota et al., 1998). CaMKII plays a role in plasticity-related synaptic tagging, a mechanism whereby suitable synapses are sorted for subsequent strengthening (Hernandez and Abel, 2011).

Zif-268 levels were lower in animals treated with haloperidol at 6 h (Figures 4, 5 and Supplementary 1). Since Zif-268 levels in the hippocampus are increased 1-2 h after a novel stimulus (Barbosa et al., 2013), we believe that a 6 h increase in vehicle-injected animals could be related to a second wave of Zif-268 transcription and translation. This re-induction is probably related to the occurrence of REM sleep in vehicle-injected animals, but not in haloperidol-injected animals, during the 4 h interval following object exposure.

Our results corroborate the notion of a late phase of BDNF synthesis in the rat hippocampus 12 h after training, related to the long-term persistence of memories (Bekinschtein et al., 2007, 2014). A significant decrease of BDNF levels

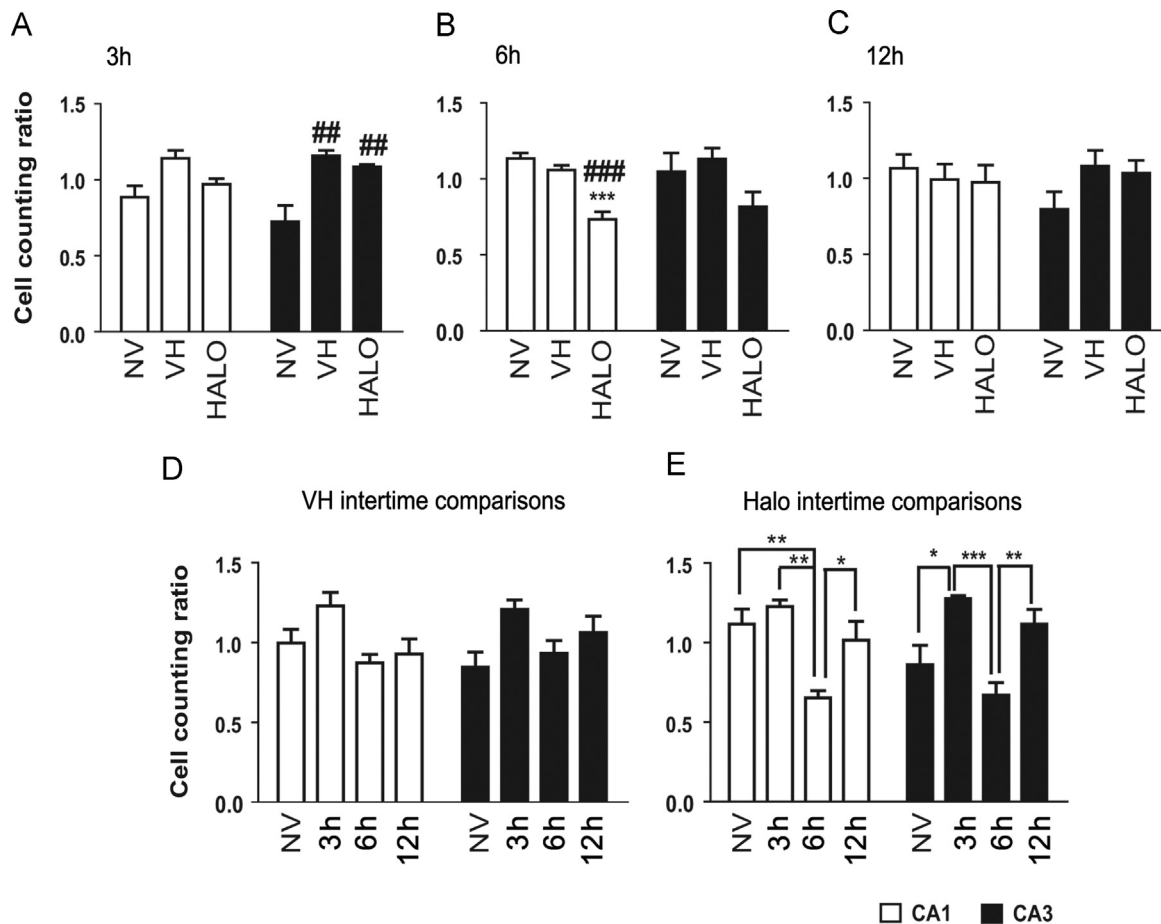


Figure 5 Cell counting quantification of Zif-268 staining at three distinct time points. The first three plots from left to right illustrate Zif-268 individual cell staining of naïve (NV), vehicle (VH) and haloperidol (Halo) groups at different time points, set at 3 h (A), 6 h (B) and 12 h (C) after injection, using Bonferroni-corrected ANOVAs followed by Bonferroni post-hoc tests. The two last plots on the right illustrate inter-time ANOVA followed by Bonferroni post-hoc comparisons in vehicle groups (D) and Halo groups (E) performed to evaluate the time course of Zif-268 protein levels in CA1 and CA3. Bars in black and white represent CA3 and CA1, respectively. In A, B, C, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ related to vehicle groups; $p < 0.05$, $p < 0.01$, $p < 0.001$ related to control groups. In D and E, the differences are indicated by linked lines.

in the hippocampus of animals treated with haloperidol was detected in the pooled data (Figure 4). Injections of anti-BDNF antibodies into the DG demonstrated BDNF's essential role in the spontaneous location recognition task, impairing task execution when animals had to disambiguate two similar locations within an open field, but not when these locations were made less similar (Bekinschtein et al., 2014). Injection of recombinant BDNF into the DG enhanced pattern separation. Furthermore, exposure of the animals to either similar or dissimilar objects led to a 4-fold increase in BDNF levels in the DG when rats explored two similar locations, but not when dissimilar locations were explored (Katche et al., 2010; Bekinschtein et al., 2014).

Various studies have described the relation of BDNF with other molecules involved in synaptic and cellular plasticity. Activity-dependent BDNF upregulation may depend on interaction with the NMDA receptor, which in turn interacts with CaMKII (Sanhueza et al., 2011). The blockade of NMDA and pCaMKII fully abolishes exercise-induced increases in synapsin I and TrkB mRNA levels, promoting a decrease of CREB and BDNF mRNA levels (Vaynman et al., 2003). Complementarily, BDNF blockade 12 h after a behavioral

task prevents a late increase in c-Fos and Zif-268 proteins, 24 h after the initial stimulus (Bekinschtein et al., 2007).

The cyclic reactivation of plasticity-related proteins during the post-training time may be essential to consolidate certain kinds of memory (Ribeiro and Nicoletis 2004; Hernandez and Abel, 2011). For instance, the long-term persistence of some memories depends on the circadian reactivation of the cAMP/MAPK/CREB transcriptional pathway in the hippocampus (Eckel-Mahan et al., 2008). This pathway can be modulated by sleep deprivation, circadian rhythms, and neurotransmitter systems involved in sleep-wake regulation (Hernandez and Abel, 2011). The dopaminergic regulation of calcium-dependent signaling pathways is a likely candidate mechanism to causally connect sleep deficits and learning impairment.

Conflicts of interest

We declare no conflicts of interest with regard to the study “D2 dopamine receptor regulation of learning, sleep and plasticity”.

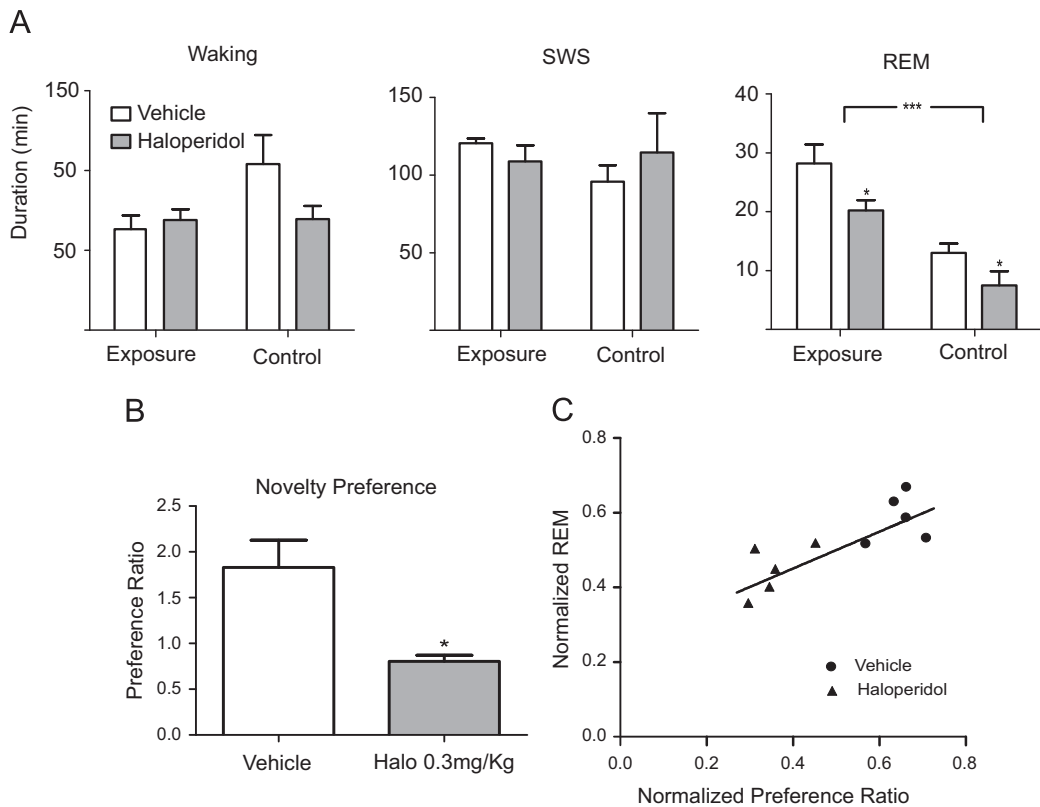


Figure 6 Haloperidol reduces REM sleep and proportionally impairs novelty preference. (A) Duration (minutes over a 4 h interval) of WK, SWS and REM sleep in control versus exposed animals, injected with either vehicle or haloperidol immediately after exploration. Haloperidol treatment or novelty exposure had no effect on the duration of WK or SWS (two-way ANOVA). In contrast, REM sleep was significantly affected by both factors, with increased REM sleep duration after novelty exposure, and decreased REM duration after haloperidol. (B) Animals injected with haloperidol showed decreased novelty preference ratio in comparison with vehicle-injected controls group. (C) Normalized REM sleep duration and normalized preference ratio (Halo or Vehicle/Halo+Vehicle) are positively correlated (Spearman's regression $R=0.56$, $p=0.0127$). * $p<0.05$ and *** $p<0.001$.

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Contributors

Arthur S. C. França helped design the experimental procedures, participated in the acquisition of the electrophysiological and behavioral data, performed data analysis, prepared figures and helped to edit the manuscript. Bruno Lobão-Soares supervised the experimental design and data acquisition, helped in the analysis and helped write the last version of the manuscript. Larissa Muratori collected and analyzed histochemical and immunohistochemical data, prepared figures and helped to edit the manuscript. George Nascimento participated in surgical procedures and manufactured the head-stage used in the electrophysiological recordings. Jessica Winne helped to collect immunohistochemical data, Catia Pereira conducted behavioral and immunohistochemical experiments, Selma Jeronimo supervised histochemical and immunohistochemical procedures and contributed to the experimental design. Sidarta Ribeiro coordinated the experimental design, data analysis, figure preparation, and manuscript writing.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.euroneuro.2015.01.011>.

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