



Is REM sleep a paradoxical state?: Different neurons are activated in the cingulate cortices and the claustrum during wakefulness and paradoxical sleep hypersomnia

Renato Maciel, Risa Yamazaki, Dianru Wang, Anna de Laet, Sébastien Cabrera, Claudio Agnorelli, Sébastien Arthaud, Paul-Antoine Libourel, Patrice Fort, Hyunsook Lee, et al.

► To cite this version:

Renato Maciel, Risa Yamazaki, Dianru Wang, Anna de Laet, Sébastien Cabrera, et al.. Is REM sleep a paradoxical state?: Different neurons are activated in the cingulate cortices and the claustrum during wakefulness and paradoxical sleep hypersomnia. *Biochemical Pharmacology*, 2021, 191, pp.114514. 10.1016/j.bcp.2021.114514 . hal-03285342

HAL Id: hal-03285342

<https://hal.science/hal-03285342>

Submitted on 13 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Is REM sleep a paradoxical state?: Different neurons are activated in the cingulate cortices and the claustrum during W and paradoxical sleep hypersomnia

Renato Maciel^{1,4*}, Risa Yamazaki^{1,*}, Dianru Wang¹, Anna De Laet¹, Sébastien Cabrera¹, Claudio Agnorelli¹, Sébastien Arthaud¹, Paul-Antoine Libourel¹, Patrice Fort¹, Hyunsook Lee^{1, 2, 3}, Claudio Queiroz⁴[‡], Pierre-Hervé Luppi¹[‡]

¹Team "SLEEP", Centre de Recherche en Neurosciences de Lyon (CRNL), UMR 5292 CNRS/U1028 INSERM and Université de Lyon, Lyon I, Neurocampus, 95 Boulevard Pinel, 69500 Bron, France

²Department of Anatomy and ³Research Institute of Medical Science, School of Medicine, Konkuk University, 05029 Seoul, South Korea

⁴Brain Institute, Federal University of Rio Grande do Norte, Natal, RN, Brazil

*Equally contributed

[‡]Coresponsible

Abbreviations

4-OHT, 4-hydroxytamoxifen; ACA, anterior cingulate cortex; CLA, claustrum; EEG, electroencephalography; EMG, electromyography; PS, paradoxical sleep; PSD, paradoxical sleep deprivation; PSR, paradoxical sleep rebound; RSP, retrosplenial cortex; SWS, slow-wave sleep; TRAP, targeted recombination in active populations; W, wakefulness

a mis en forme : Exposit

a mis en forme : Exposit

a mis en forme : Exposit

Abstract

Michel Jouvet proposed in 1959 that REM sleep is a paradoxical state since it was characterized by the association of a cortical activation similar to wakefulness (W) with muscle atonia. Recently, we showed using cFos as a marker of activity that cortical activation during paradoxical sleep (PS) was limited to a few limbic cortical structures in contrast to W during which all cortices were strongly activated. However, we were not able to demonstrate whether the same neurons are activated during PS and W and to rule out that the activation observed was not linked with stress induced by the flowerpot method of PS deprivation. In the present study, we answered to these two questions by combining tdTomato and cFos immunostaining in the innovative TRAP2 transgenic mice exposed one week apart to two periods of W (W-W mice), PS rebound (PSR-PSR) or a period of W followed by a period of PSR (W-PSR mice). Using such method, we showed that different neurons are activated during W and PSR in the anterior cingulate (ACA) and rostral and caudal retrosplenial (rRSP and cRSP) cortices as well as the claustrum (CLA) previously shown to contain a large number of activated neurons after PSR. Further, the distribution of the neurons during PSR in the rRSP and cRSP was limited to the superficial layers while it was widespread across all layers during W. Our results clearly show at the cellular level that PS and W are two completely different states in term of neocortical activation.

1. Introduction

Paradoxical sleep (PS), also named rapid eye movement (REM) sleep, is paradoxically characterized by the association of rapid eye movements and a cortical EEG activation like during wakefulness (W) with a complete muscle atonia [1]. The concept that cortical activity during PS is close to that seen during W was then further supported by unit recordings showing that the discharge rate of neocortical neurons is higher both during REM sleep and W than during Non-REM sleep [2, 3]. However, neuroimaging studies in human showed that cortical activation was not similar during W and PS [4, 5]. More recently, analyzing the expression of cFos protein as a marker for neuronal activation during PS rebound (PSR) occurring after 72h of PS deprivation using the classical flowerpot method, we showed in rats that only a few limbic cortical structures are activated during PS in contrast to W during which nearly all cortices

are strongly activated [6]. In the neocortex, the anterior cingulate (ACA) and moreover the retrosplenial (RSP) cortices were strongly activated during PSR [6]. Further, we showed that the claustrum (CLA) was the only subcortical structure containing activated neurons during PSR projecting to the ACA and RSP and we therefore proposed that these neurons are responsible for their activation during PS [6].

Our results indicated that W and PS are very different state in term of neocortical activation, *although* we were not able to determine whether the same or different neurons were activated in ACA, RSP and CLA during W and PSR. Further, we could not rule out that the activation occurring during PSR was not due to the semi-chronic stress induced by the exposure of the rats to the flowerpot method [7].

To answer to these two questions, we induced wakefulness and a PS hypersomnia in a new 'targeted recombination in active populations' (TRAP) transgenic mice that allow to permanently tag neurons activated by defined stimuli [8-10]. We validated such approach in our recent study on the activation of lateral hypothalamic neurons during PSR and W [11]. The transgenic TRAP2 mice express tamoxifen-dependent CreER^{T2} recombinase under cFos promoter. CreER^{T2} can only undergo recombination when 4-hydroxytamoxifen (4-OHT, an active metabolite of tamoxifen) is present with a time window of approximately 4h centered on the injection time, resulting in a permanent CreER^{T2}-dependent reporter gene (tdTomato) expression in activated neurons. To induce a strong PS hypersomnia or W during the time-window of the CreER^{T2}-driven recombination, we deprived selectively of PS using our innovative automatic PS deprivation system [11, 12] or put the mice in an open field, respectively. With such approach, we were able to demonstrate for the first time that different neurons are activated in the ACA, RSP and CLA during PSR and W.

2. Materials and Methods

2.1. Animals

All experiments were conducted in accordance with the French and European Community guidelines for using animals in research and were approved by the institutional animal care and committee of the

University of Lyon 1 (Project APAFIS#21351). Both male and female of double heterozygous Fos^{2A-iCreER};R26^{AI14} (TRAP2) mice, kindly gifted by Dr. Liqun Luo from Stanford University were used. TRAP2-RED mice were generated by crossing Fos^{2A-iCreER/+} (TRAP2) mice to R26^{AI14/+} (AI14) mice [10]. In all experiments, age 8-12 weeks old mice underwent surgery. Transgenic mice were housed individually and placed under a constant light/dark cycle (light on from 8:00 a.m. to 8:00 p.m.) and in a room temperature at 23°C ± 1°C

2.2. Experimental design

In order to evaluate the pattern of neuronal activation during PS compared to wakefulness, 2h of PS hypersomnia (PSR-PSR group) or 4h of permanent wakefulness (W-W and W-PSR groups) were induced during the first experimental block (1st condition, Fig. 1B). Then, all mice immediately received one 4-OHT injection for inducing the transport inside the nuclei of activated neurons of CreERT2 and the removal of the stop codon of the tdTomato gene and the expression of the tdTomato mRNA and protein (further details about the methodology can be found in [11]). One week later, the mice were submitted again to 4h of W or 2h of PSR. Euthanasia was performed in W-W group immediately at the end of the 4th hour of W, and in the W-PSR and PSR-PSR groups, 120 minutes after the end of the 2nd hour of PS hypersomnia.

2.3. Surgery

Mice were kept anesthetized under a cocktail of ketamine and xylazine (100/10 mg/kg. .p.; [Imalgene 1000, Merial and Rompun 2%, Bayer](#)). Then, the top of the head was shaved and mice were placed in a stereotactic frame with a heating pad underneath. Two EEG electrodes were fixed in the parietal (AP: -2.0 mm, ML: 1.5 mm from bregma) and frontal (AP: +2.0 mm, ML: 1.0 mm from bregma) parts of the skull, whereas the reference electrode was fixed above the cerebellum (AP: -5.0 mm, ML: 0.0 mm from bregma). Two wire electrodes were inserted into the neck muscles for differential EMG recordings. All leads were connected to a miniature plug (Plastics One, Roanoke, VA) that was cemented on the skull.

2.4. Polysomnography and analysis of vigilance states

Animals were allowed to recover from surgery for 7 to 10 days in their home cage before being habituated to the recording conditions for at least 3 days. Unipolar EEG and bipolar EMG signals were amplified 1:5000 V/V and 1:2000 V/V, respectively (MCP-PLUS, Alpha-Omega Engineering, Israel), digitized at 1024 Hz, and transferred to Slip Analysis v 2.9.8 software (View Point, Civrieux, France). The analysis was done as previously described in Renouard et al. [6] with slight modifications. Briefly, the vigilance states of 5 s episodes were identified by visual inspection of polysomnographic signals according to the criteria described in detail elsewhere ([12, 13], example of representative EEG/EMG signals from each state are shown in Fig. 1C). Wakefulness, slow-wave sleep and paradoxical sleep (PS) were quantified during the last 2 hours (10:00 a.m. to 12:00) before euthanasia and in all PSR animals. The amount of the three vigilance states of baseline recordings from the same animals were quantified as a control condition (Fig. 1F).

2.5. Drug preparation for TRAPing

4-hydroxytamoxifen (4-OHT) was prepared as described previously [9]. Briefly, 4-OHT (Cat# H6278 Sigma Aldrich, St. Luis, MO) was dissolved at 20mg/mL in absolute ethanol by ultrasonic water bath at 37°C for 10 min, and was then aliquoted and stored at -20°C as a stock solution. Before using, corn oil (Sigma Aldrich) was added to the thawed stock solution to replace the ethanol and to obtain 10 mg/mL 4-OHT, and then the ethanol was evaporated at 37°C. The aliquots containing 4-OHT at 10mg/mL were stored under +4 °C and used up to 24 h after preparation.

2.6. Wakefulness

The waking protocol was used to induce a continuous period of wakefulness under lights ON during 4 hours from 10:00 a.m. (ZT2) to 2:00 p.m (ZT6), 4-OHT was injected at 12:00 (ZT4, at noon). To maintain animals awake, two mice were placed into a square 45*45cm white open field box together

with wood tips and small objects. Food and water were freely available in the box. During 4h, the animals were permanently monitored by the web-camera from a different room to check whether they were awake. The animals were gently touched by a soft tissue when they became inactive/drowsy.

2.7. Paradoxical sleep deprivation and rebound

The PS deprivation was performed as described in our previous report [11, 12]. Briefly, the mouse was placed in a transparent individual barrel with a movable floor connected to a small piston controlled by an electromagnet. The automatic detection of PS episodes was achieved by means of a learning file based on the parameters of the sleep-wake cycle states previously defined during baseline recordings. All deprivations started at 10:00 a.m. (ZT 2). During the recordings, as soon as PS appeared and was detected computationally and in real time by an algorithm, a digital transistor-transistor logical (TTL) pulse was sent to a current generator controlling the instantaneous onset of the electromagnet. It caused a few slight movements (up/down) of the floor that woke up the animal after only 5 seconds from the detection of a PS [12, 13]. After 48h of PS deprivation, the system automatically stopped at 10:00 a.m. Then animals were allowed to recover and to display PS hypersomnia in the same barrel.

2.8. Perfusion

All animals were deeply anesthetized with sodium pentobarbital (400 mg/kg, i.p.; Euthasol) and perfused with heparin-added Ringer's lactate solution followed by 4% paraformaldehyde/PBS (pH 7.4) for pre-fixation. The brains were post-fixed with 4% paraformaldehyde for one night at 4°C and then stored in 30% sucrose/PB for at least two days at 4°C.

2.9. Immunohistochemistry

Brains were frozen with 2-methylbutane placed on a dry ice at around -33°C. Then the brain was sliced in 30 µm thick coronal sections, and stored at -20°C in cryoprotective solution containing 20% glycerol

a mis en forme : Police : (Par défaut) Times New Roman

a mis en forme : Police : (Par défaut) Times New Roman

a mis en forme : Police :

a mis en forme : Police : (Par défaut) Times New Roman

a mis en forme : Police :

and 30% ethylene glycol in 0.05 M PB (pH 7.4). They were successively incubated in (i) rabbit anti-cFos primary antibody ([1:15000](#), #ABE457, Millipore, Burlington, MA) in PBST for 2 days at 4°C; (ii) a biotinylated anti-rabbit IgG ([1:1000](#), #BA-9500, Vector Laboratories, Burlingame, CA); and (iii) an ABC-horseradish peroxidase solution ([1:1000](#), Elite kit #PK-6100, Vector Laboratories) for 90 min at room temperature. Finally, the sections were immersed in DAB solution (0.025% 3,3'-diaminobenzidine-4 HCl (DAB; Sigma Aldrich) and 0.003% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6) with 0.6% nickel ammonium sulfate to obtain a black reaction product. After washing three times during 30 min each one with PBST, the cFos-stained sections were incubated in the rat anti-mCherry antibody ([1:100000](#), #M11217, Invitrogen, Carlsbad, CA), biotinylated anti-rat IgG ([1:1000](#), Vector Laboratories, Burlingame, BA-4000), and an ABC-horseradish peroxidase solution. After washing the sections three times again for 30 min, they were immersed in the DAB solution to obtain a brown reaction product. In order to stop completely the reaction before mounting all sections were left overnight in an PBST solution. After mounting the sections on slide glasses, the slides were sequentially submerged in 70%, 90% and 100 % ethanol for 2 min each. Lastly, the slide was submerged in toluene for 2 min preceded by an immersion in a xylene and alcohol substitute (Ottix®, DiaPath, Martinengo, Italy) for the same amount of time. Finally, the slides were cover-slipped with a slide mounting medium (DPX Mountant, Sigma Aldrich). The data acquisition was done by using Morphotrider software (Explora Nova®, La Rochelle, France) with Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

2.10. Immunofluorescence

Sections were incubated with the same rabbit anti-cFos primary antibody ([1:250](#), #ABE457, Millipore, Burlington, MA) that for DAB staining in PBST for 24-48h at 4 °C. Following washes, they were incubated in Alexa Fluor 488-conjugated secondary IgG ([1:1000](#), Jackson ImmunoResearch Lab., West Grove, PA) in PBST for 2h at room temperature. Following washes with phosphate buffer (PB), sections were mounted, dried, and cover-slipped with prolong Gold anti-fading reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) and stored at 4 °C.

For image processing, we utilized ZEN 2010 software with confocal laser scanning microscope (LSM 800, Carl Zeiss) equipped with blue argon (488 nm), green helium neon (543 nm) and red helium neon (633 nm) lasers. For image acquisition, 'best signal' was selected in SMART-setup tool. Z-stack procedure with an interval of 1.0 μm was employed and processed with ZEN software to stack the images.

2.11. *Cell counting*

The atlas of George Paxinos and the Allen Brain Reference Atlases (Adult Mouse) were used as references for all structures. For the DAB-stained sections, drawings of structures with plotting of cFos⁺ and/or tdTomato⁺ neurons from one W-PSR representative mouse were made with an Axioskop microscope (Carl Zeiss) equipped with a motorized XY-sensitive stage and a video camera connected to a computerized image analysis system. Mercator and Morphotrider softwares (ExploraNova®, France) were used to obtain low and high power photomicrographs. cFos⁺, tdTomato⁺ and double labeled neurons (cFos⁺/tdTomato⁺) were plotted and counted manually with the Mercator (ExploraNova®, France) software in animals on hemi-sections taken at the same level. For the immunofluorescent sections, double-labeled neurons were confirmed for genuine labeling using a single channel illumination. GIMP (GNU Image Editor, version 2.10.8) and ImageJ were used for the analyses of the photomicrographs; cells were considered labeled when they exhibited clear cytoplasmic (i.e., tdTomato) or nuclear (i.e., cFos) staining.

2.12. *Data analysis*

Normality was assessed using Shapiro test. Unpaired Mann-Whitney T-test was performed on the difference in the amount of each state in control condition and in PSR condition 2h before perfusion (Fig. 1F, n = 8, Ctrl group and n = 11, PSR group). The time spent in PS during the 1st (n = 5) and 2nd PSR (n = 11) was compared with the amount of PS during the same baseline control recordings (n = 8). The data was obtained from the same individuals, at the same time of the day, on different days of

experiment. In order to compare the number of tdTomato and cFos+ cells, and the percentage of double-labeled cells, [the data are presented as mean ± SEM using](#) unpaired two-tailed Mann-Whitney test set at significance level of 5 %.

3. Results

3.1. Sleep analysis and controls

Mice were divided into 3 groups as illustrated in Fig. 1B: the W-W, the PSR-PSR, and the W-PSR groups.[12, 13]

Two hours before perfusion all mice showed a significant increase of PS, lower amount of W and a slight increase in SWS compared to the control group (n = 8 (Ctrl) and n = 11 (PSR); $p < 0.0001$, $p < 0.0001$, and $p = 0.0214$ for PS, W, and SWS, respectively) (Fig. 1F). During PSR, the quantity of PS peaked during the first 30 min of PSR ~ 30 % of total time (PSR before 4-OHT injection versus during basal control condition, $p = 0.0016$; PSR before perfusion versus control, $p < 0.0001$). Then, it progressively decreased but stayed higher than control at least throughout the first 2h of PSR . (Fig. 1D and E). We then verified that in all brain structures examined, the number of cFos+ neurons was statistically identical in the PSR-PSR and the W-PSR groups as well as that of tdTomato+ neurons in W-W and W-PSR groups (Table 1), indicating the high reproducibility of the cFos and tdTomato staining when obtained for PSR or W in two different groups of mice. Therefore, for all our analyses (Fig. 2-5), the mean number of cFos+ neurons during PSR was calculated by merging the PSR-PSR and W-PSR groups and that of tdTomato+ neurons during W by merging the W-W and the W-PSR groups.

3.2. Ratio of tdTomato+ and cFos+ neurons

As shown in Table 1, during W, the number of tdTomato+ neurons was around eight to twelve times smaller than the number of cFos+ neurons in the cingulate cortices, as shown by the numbers of tdTomato+ / cFos+ neurons (ACA, $79.33 \pm 23.95 / 646 \pm 31.8$; cRSP, $91.33 \pm 39.82 / 1132.0 \pm 120.2$; rRSP,

74.67±25.76 / 583.0±164.98). In contrast, the ratio was only of four for the CLA (33.17±10.23 / 145.67±27.12). During PSR, the number of tdTomato+ neurons was only around three times smaller than that of cFos+ neurons for the CLA, rRSP and ACA (CLA, 18.20±4.40 / 48.20±13.69; rRSP, 103.0±23.79 / 319.80±49.88; ACA, 61.60±15.87 / 188.80±77.62). In contrast, there was nine time more cFos than tdTomato in the cRSP (56.80±9.69 / 532.8±188.05).

3.3. Anterior cingulate cortex (ACA)

The distribution of the tdTomato+ and cFos+ cells was quite homogenous in the ACA for W and PSR (Fig. 2A,C). There was around four times more cFos+ neurons during W than PSR (W: 646±31.8 / PSR: 126.0±42.27, $p = 0.007$) (Fig. 2B). The number of tdTomato+ neurons in the ACA was higher during W than PSR, although it did not reach statistical significance (W: 117.7±22.22; PSR, 61.6±15.87, $p = 0.0932$) (Fig. 2B). A significant higher number of double-labelled neurons was observed in the W-W group (41.33±13.86) than in the W-PSR group (3.6±0.86, $p = 0.0357$) and the PSR-PSR group (3.4±0.75, $p = 0.0357$) (Fig. 2E). There was no statistical difference between the W-PSR and the PSR-PSR groups ($p > 0.9999$). The percentage of double-labeled cells over the total number of tdTomato + cells was also significantly superior in the W-W condition (53.6±9.56) compared to the W-PSR (2.64±0.56, $p = 0.0357$) and PSR-PSR (7.50±3.05, $p = 0.0179$) conditions (Fig. 2E). Importantly, the percentage of double-labeled neurons over the total number of tdTomato + cells was significantly higher in the PSR-PSR than in the W-PSR group ($p = 0.0476$).

3.4. Rostral retrosplenial cortex (rRSP)

In the rostral RSP, the distribution of the cFos+ and tdTomato neurons was strikingly different between W and PSR. Indeed, although during W, there were more neurons in the superficial layers, there was also a large number of neurons in the deep layers of the cortex in contrast to PSR during which labelled neurons were mainly localized in the superficial layers (Fig. 3A, B). The number of cFos+ cells was around three times superior during W than PSR (W: 583±164.98, PSR: 226.8±43.3, $p = 0.0490$, Fig. 3C,

top). In contrast, the number of tdTomato+ neurons was similar during W and PSR (W: 112 ± 33.99 , PSR: 103 ± 23.79 , $p = 0.8329$, Fig. 3C, bottom). Although the number of double-labelled neurons was higher in the W-W group (23 ± 10.26) compared to the W-PSR (4.3 ± 1.45) and PSR-PSR (11.6 ± 2.98) groups, it did not reach statistical significance (W-W vs W-PSR: $p = 0.0714$; W-W vs PSR-PSR: $p = 0.4286$, Fig. 3E, left). However, the percentage of double-labeled cells over the total number of tdTomato+ cells was superior in W-W mice (29.2 ± 3.39) compared to W-PSR (3.49 ± 0.96 , $p = 0.0357$) and PSR-PSR (11.71 ± 2.1 , $p = 0.0357$) mice (Fig. 3E, right). Importantly, the percentage of double-labeled cells was higher in the PSR-PSR than in the W-PSR mice ($p = 0.0079$, Fig. 3E).

3.5. Caudal retrosplenial cortex (cRSP)

In the cRSP, like for the rRSP, the distribution of the cFos+ and tdTomato+ neurons was completely different during W and PSR (Fig. 4A,B). During W, there was a high number of cFos+ neurons in the most superficial layer of the cortex but there was also a high number of cells in all layers. In contrast during PSR, most of the cFos+ and tdTomato+ cells were localized in the superficial layers of the cortex. The number of cFos+ neurons was also four times superior in the W (1132 ± 120.2) than in the PSR condition (362.2 ± 108.5 , $p = 0.0280$) (Fig. 4C, top). Twice more tdTomato was observed in the W (154 ± 50.11) than in the PSR condition (56.8 ± 9.69 , $p = 0.0295$, Fig. 4C, bottom). The number of double-labelled neurons in the W-W group (29.67 ± 12.72) was significantly higher than in the PSR-PSR group (7.4 ± 1.29 , $p = 0.0357$). It was also higher compared to the W-PSR group but it did not reach statistical significance (7.4 ± 2.01 , $p = 0.0714$) (Fig. 4F, left). Finally, the percentage of double-labeled neurons over the total number of tdTomato+ neurons was higher in the W-W (35.05 ± 6.31) than in the W-PSR (4.53 ± 0.88 , $p = 0.0357$) and PSR-PSR (14.09 ± 2.49 , $p = 0.0357$) groups (Fig. 4F, right). Importantly, the percentage of double-labeled neurons was also significantly higher in the PSR-PSR than in the W-PSR mice ($p = 0.0079$).

3.6. Claustrum (CLA)

The results obtained for the CLA are shown in Figure 5. The neurons were homogenously distributed in the CLA (Fig. 5A). The number of cFos+ neurons was around three times superior during W (145.67 ± 27.12) than during PSR (39.3 ± 8.458 , $p = 0.0035$). In contrast, the number of tdTomato+ neurons was only twice superior during W (33.75 ± 4.109 , $p = 0.0396$) than PSR (18.2 ± 4.40). There was also a much larger number of double-labeled cells in the W-W mice (22 ± 9.61) compared to the W-PSR (1.3 ± 0.58) and PSR-PSR (1.8 ± 0.58) mice (W-W versus W-PSR: $p = 0.0179$ and W-W versus PSR-PSR: $p = 0.0357$) (Fig. 5E, left). The percentage of double-labeled neurons over the total number of tdTomato+ neurons was also much higher in the W-W group (62.69 ± 11.44) compared to W-PSR (4.0 ± 1.84 , $p = 0.0357$) and PSR-PSR (10.16 ± 4.62 , $p = 0.0357$) groups. Although the percentage of double labeled neurons was higher in the PSR-PSR than in the W-PSR group, it did not reach statistical significance ($p = 0.2381$).

4. Discussion

Here we showed using the innovative TRAP mice method that different neurons are activated during PSR and W in the RSP, ACA and the CLA. Further, we showed that in the rostral and caudal RSP but not in ACA and CLA, the distribution of the neurons activated during PSR and W was very different. In addition, we showed that in these structures, there is no reactivation during PSR of neurons activated one week before during W. Altogether, our results demonstrate for the first time that W and PS are completely different states at the neocortical level.

4.1. *Is cFos expression during PS rebound specifically induced in neurons activated during PS?*

The distribution and number of cFos+ neurons reported in the ACA, RSP and CLA after PSR obtained with automatic PS deprivation in the present study in mice is similar to that obtained in rats using the flowerpot PS deprivation method [6]. It indicates that the activation observed in the ACA, RSP and CLA during PSR is not species dependent and is not due to the use of the flowerpot deprivation method. It is

a mis en forme : Police : (Par défaut) + Corps (Calibri), Non Gras

important since it has been previously shown that corticosterone levels increase after PS deprivation by the flowerpot method and that animals start to sleep only after 30 min to 1h of waking [7]. In contrast, with the automatic PS deprivation system based on EEG/EMG signals [12] used in the present experiment, corticosterone levels were not increased and the mice immediately started to sleep at the end of PS deprivation [12]. Altogether, these results indicate that cFos+ neurons during PSR likely correspond to neurons specifically activated during PS and that such activation does not depend on species or the exposure of the animals to a specific stimulus or condition.

4.2. TRAP method validation

TdTomato expression is induced in the TRAP mice by the removal by CreERT2 of the stop codon in the tdTomato transgene. The entrance of CreERT2 in the nuclei of neurons occurs when it is binding to 4OHT. Without 4-OHT, CreERT2 expression which depends on the cFos promoter remains localized in the cytoplasm [14]. Theoretically, all neurons expressing cFos should also express tdTomato. However, this seems unlikely since [there](#) are many steps [which](#) might be limiting to induce the expression of tdTomato. In our first study using the TRAP mice, the percentage of neurons in the lateral hypothalamic area labelled with tdTomato and expressing cFos when the animals were re-exposed to the same conditions (i.e., W-W or PSR-PSR) just before perfusion was of 37.8% during W and 14.4% during PSR [11]. In contrast, the proportions were almost negligible when the animals were exposed to two different conditions (i.e., W-PSR). In the present study, we found that in the W-W mice, the ratio between the number of tdTomato+ and cFos+ neurons was of 1/8 for the ACA and [rRSP](#) and 1/4 for the claustrum. In contrast in PSR-PSR mice, the ratio was of 1/3 for all structures excepting for the cRSP for which it was of 1/9. These results indicate that the efficiency of the tdTomato transcription is highly variable depending on the state and on the structure under study. Such variability in the TRAPing is in line with previous studies. Indeed, low TRAPing was reported previously in CA1 by Deng et al [15] while high level of TRAPing was reported in the thirst circuit after water-deprivation with a percentage of double labeled neurons/total tdTomato+ of almost one [10]. In DeNardo et al. [9], after contextual fear conditioning training and test, the ratio of tdTomato+/cFos+ neurons was around of 0.4 in the prelimbic cortex. These and our results indicate that the level of tdTomato expression is close to that for

cFos in most subcortical structures while it is lower but still high in most cortical structures (around 25%) during PSR but not during W during which it is very low in ACA and RSP. The mechanisms underlining the variability in the efficiency of TRAPing across structures is unclear and could be due to the type of neurons, the level of transgene expression or the local availability of 4-OHT. One way to test such hypothesis would be to inject it ICV. In conclusion, our results indicate that the TRAP method is efficient and can be used to map neurons expressing cFos.

4.3. Comparison with previous studies

In agreement with Renouard et al. [6], in the RSP, ACA and the CLA, a very large number of cFos+ neurons were observed during W and a large number during PSR. There was from 2.5 to 5 times less cFos+ neurons during PSR than during W. For tdTomato, there was also more neurons during W than PSR in the RSPc, ACA and CLA with a ratio around two while it was surprisingly only of 1 for the RSPr. In addition to Renouard et al. [6], our data suggest that different neurons are activated during W and PSR in the ACA, RSP and CLA since double-stained neurons were rarely observed in these structures in the W-PSR mice in contrast to W-W and PSR-PSR mice. Further, we showed that in the rRSP and cRSP, the distribution of the neurons was different between PSR and W with a localization limited to superficial layers during PSR compared to a more dispersed distribution across layers in W. These results indicate that the activation of the RSC is different during W and PSR. In addition to Renouard et al. [6] in which only the caudal RSC was examined, we analyzed for the first time in the present study the rostral part of the RSC. Our results indicate that the rRSP shows similar trends that the cRSP in term of number of cFos+ and tdTomato+ neurons and double-labeled neurons both during W and PSR. It strongly suggests that both the rostral and caudal parts of the RSP are strongly activated during PS. Finally, although the percentage of double-labeled neurons was higher in all structures excepting the CLA in the PSR-PSR mice compared to the W-PSR ones, the percentage did not exceed 14%. It suggests that mostly different sets of cortical neurons are activated during two periods of PS hypersomnia separated by one week in contrast to two periods of W.

5. Functional significance

Overall, our results are very surprising since they indicate that activation of the same neuron in ACA, RSP and CLA does not occur during W and PSR.

Our results first indicate that the CLA contains two types of neurons activated specifically during W or during PSR. They also indicate that approximately 5 times more neurons of the CLA are activated during W than during PS. We previously showed in rats that after PS rebound, the CLA was the only subcortical structure containing a significant number of retrogradely labeled neurons expressing cFos when injecting a retrograde tracer in the ACA and cRSP [6]. In addition, it has been shown that CLA neurons projecting to the cortex are glutamatergic [16]. From these results, we proposed that activation of neurons of the ACA and the cRSP is due to a projection from glutamatergic PS-active neurons in the CLA [6]. Besides, it has been recently shown that a large subpopulation of CLA neurons is activated during slow wave sleep and not during W. Stimulation of these neurons was shown to increase slow waves while their inhibition was shown to decrease them [17]. It was further shown that these CLA neurons are glutamatergic and excite local interneurons in the neocortex. It remains to be determined whether these neurons are corresponding to the neurons activated during W or PSR reported in the present study or correspond to a third population of neurons. Additional unit recordings studies are required to determine the activity of claustral neurons during the different sleep-waking states. Our hypothesis is that a subpopulation of PS-active neurons in the CLA activate glutamatergic PS-active neurons localized in the ACA and the RSP while other CLA neurons are activated during W and regulate cognition, attention, and impulsivity [18].

Our results also suggests that different set of neurons are activated in the neocortex during W and PS. It is in line with our unit recording study of the activity of ACA and RSC neurons during the three vigilance states. Indeed, neurons recorded in the two structures were belonging to two groups: neurons active more during PS than the two other states or conversely neurons more active during W than the two other states [19]. In our W-PSR mice, W was induced by putting the mice in an open field for four hours and one week later a PS rebound was induced. In these mice, there is no reactivation in ACA and RSP during PSR of the neurons activated during exposure to the open field one week before. It suggests that reactivation of a memory engram of the open field does not occur in these structures during PS one week

later. In contrast, reactivation of a memory engram in the RSP during sleep one day after contextual fear conditioning using a c-Fos-based genetic tagging system to selectively express channelrhodopsin and optogenetically induced memory consolidation

[20]. From these and our results, it can be proposed that replay during PS might occur preferentially during the first 24h

In summary, based on the present results and previous data, it can be hypothesized that the strong activation of RSP and ACA neurons during PS could correspond to the activation of multiple memory engrams. We hypothesize that such reactivation is induced by the projection from the CLA and plays a role in memory consolidation and the generation of dreams. Additional studies are necessary to confirm such hypothesis.

6. References

- [1] M. Jouvet, F. Michel, J. Courjon, Sur un stade d'activité électrique cérébrale rapide au cours du sommeil physiologique, *CR Seances Soc Biol* 153 (1959) 1024-1028.
- [2] V.V. Vyazovskiy, U. Olcese, Y.M. Lazimy, U. Faraguna, S.K. Esser, J.C. Williams, C. Cirelli, G. Tononi, Cortical firing and sleep homeostasis, *Neuron* 63(6) (2009) 865-78.
- [3] M. Steriade, I. Timofeev, F. Grenier, Natural waking and sleep states: a view from inside neocortical neurons, *J Neurophysiol* 85(5) (2001) 1969-85.
- [4] P. Maquet, J. Peters, J. Aerts, G. Delfiore, C. Degueldre, A. Luxen, G. Franck, Functional neuroanatomy of human rapid-eye-movement sleep and dreaming, *Nature* 383(6596) (1996) 163-6.
- [5] E.A. Nofzinger, M.A. Mintun, M. Wiseman, D.J. Kupfer, R.Y. Moore, Forebrain activation in REM sleep: an FDG PET study, *Brain Res* 770(1-2) (1997) 192-201.
- [6] L. Renouard, F. Billwiller, K. Ogawa, O. Clément, N. Camargo, M. Abdelkarim, N. Gay, C. Scoté-Blachon, R. Touré, P.-A. Libourel, P. Ravassard, D. Salvert, C. Peyron, B. Claustrat, L. Léger, P. Salin, G. Malleret, P. Fort, P.-H. Luppi, The supramammillary nucleus and the claustrum activate the cortex during REM sleep, *Science Advances* 1(3) (2015).
- [7] S. Arthaud, C. Varin, N. Gay, P.A. Libourel, F. Chauveau, P. Fort, P.H. Luppi, C. Peyron, Paradoxical (REM) sleep deprivation in mice using the small-platforms-over-water method: polysomnographic analyses and melanin-concentrating hormone and hypocretin/orexin neuronal activation before, during and after deprivation, *J Sleep Res* 24(3) (2015) 309-19.
- [8] C.J. Guenther, K. Miyamichi, H.H. Yang, H.C. Heller, L. Luo, Permanent genetic access to transiently active neurons via TRAP: targeted recombination in active populations, *Neuron* 78(5) (2013) 773-84.
- [9] L.A. DeNardo, C.D. Liu, W.E. Allen, E.L. Adams, D. Friedmann, L. Fu, C.J. Guenther, M. Tessier-Lavigne, L. Luo, Temporal evolution of cortical ensembles promoting remote memory retrieval, *Nat Neurosci* 22(3) (2019) 460-469.

- [10] W.E. Allen, L.A. DeNardo, M.Z. Chen, C.D. Liu, K.M. Loh, L.E. Fenno, C. Ramakrishnan, K. Deisseroth, L. Luo, Thirst-associated preoptic neurons encode an aversive motivational drive, *Science* 357(6356) (2017) 1149-1155.
- [11] H.S. Lee, R. Yamazaki, D. Wang, S. Arthaud, P. Fort, L.A. DeNardo, P.H. Luppi, Targeted recombination in active populations as a new mouse genetic model to study sleep-active neuronal populations: Demonstration that *Lhx6*⁺ neurons in the ventral zona incerta are activated during paradoxical sleep hypersomnia, *J Sleep Res* (2020) e12976.
- [12] S. Arthaud, P.A. Libourel, P.H. Luppi, C. Peyron, Insights into paradoxical (REM) sleep homeostatic regulation in mice using an innovative automated sleep deprivation method, *Sleep* (2020).
- [13] P.A. Libourel, A. Corneillie, P.H. Luppi, G. Chouvet, D. Gervasoni, Unsupervised online classifier in sleep scoring for sleep deprivation studies, *Sleep* 38(5) (2015) 815-28.
- [14] L. DeNardo, L. Luo, Genetic strategies to access activated neurons, *Curr Opin Neurobiol* 45 (2017) 121-129.
- [15] W. Deng, M. Mayford, F.H. Gage, Selection of distinct populations of dentate granule cells in response to inputs as a mechanism for pattern separation in mice, *Elife* 2 (2013) e00312.
- [16] J. Jackson, M.M. Karnani, B.V. Zemelman, D. Burdakov, A.K. Lee, Inhibitory Control of Prefrontal Cortex by the Claustrum, *Neuron* 99(5) (2018) 1029-1039 e4.
- [17] K. Narikiyo, R. Mizuguchi, A. Ajima, M. Shiozaki, H. Hamanaka, J.P. Johansen, K. Mori, Y. Yoshihara, The claustrum coordinates cortical slow-wave activity, *Nat Neurosci* 23(6) (2020) 741-753.
- [18] J. Liu, R. Wu, B. Johnson, J. Vu, C. Bass, J.X. Li, The Claustrum-Prefrontal Cortex Pathway Regulates Impulsive-Like Behavior, *J Neurosci* 39(50) (2019) 10071-10080.
- [19] B.D.V. Koike, K.S. Farias, F. Billwiller, D. Almeida-Filho, P.A. Libourel, A. Tiran-Cappello, R. Parmentier, W. Blanco, S. Ribeiro, P.H. Luppi, C.M. Queiroz, Electrophysiological Evidence That the Retrosplenial Cortex Displays a Strong and Specific Activation Phased with Hippocampal Theta during Paradoxical (REM) Sleep, *J Neurosci* 37(33) (2017) 8003-8013.
- [20] A.F. de Sousa, K.K. Cowansage, I. Zutshi, L.M. Cardozo, E.J. Yoo, S. Leutgeb, M. Mayford, Optogenetic reactivation of memory ensembles in the retrosplenial cortex induces systems consolidation, *Proc Natl Acad Sci U S A* 116(17) (2019) 8576-8581.

Acknowledgements

We thank Dr. Liqun Luo (Stanford University, CA) for providing us the TRAP2-RED mice. This work is supported by CNRS (UMR5292), INSERM (U1028), SFRMS, University Lyon 1, [Labex Cortex](#) and Coordination for the Improvement of Higher Education Personnel (CAPES), Brazil.

Figure Legends

Fig. 1: TRAP method, experimental design and paradoxical sleep rebound. (A, B) Scheme of TRAP method and experimental design. 4-OHT was injected after W or PSR (50 mg/Kg, i.p.) for TRAPing (tdTomato labeling). To obtain cFos expression, mice were anaesthetized and perfused 120 minutes after W or PSR. Brains were cut in a cryostat to obtain 30 μ m thick coronal sections. (C) Representative EEG

(40 Hz lowpass filter) and EMG (30 – 300 bandpass filter) traces depicting the three sleep-wake states: wakefulness, slow-wave sleep (SWS) and paradoxical sleep (PS). (D) Percentage of time spent in PS two hours before 4-OHT injection, and (E) Percentage of time spent in PS 2 hours before perfusion. (F) The amounts of each state (wake, SWS and PS) in basal condition (n = 8) and during PSR (n = 11). * p < 0.05; ** p < 0.0001. Statistical data is depicted as mean±SEM. Black open circles represent the value for each individual animal.

Fig. 2: Distinct ACA neurons are activated during W and PSR (A) Representative drawing showing the distribution of cFos+ (black squared dots), tdTomato+ (blue open circles) and double-labeled neurons (red close circles) in ACA localized on the section shown on the left. (B) Histograms showing the mean±SEM and the individual (open circles) number of cFos+ and tdTomato+ cells during wakefulness (W-W group) compared to PS rebound (W-PSR and PSR-PSR groups) (C) Representative low-power photomicrographs (10x) showing the distribution of cFos+ and tdTomato+ neurons in ACA in W-W, W-PSR and PSR-PSR representative mice. (D) High power (40x) representative photomicrographs of sections from W-PSR and PSR-PSR mice. Red arrows point out double labeled neurons (E) Histograms showing the high number of double-labeled neurons and percentage of double-labeled neurons over the total number of tdTomato+ neurons in the W-W mice compared to both W-PSR and PSR-PSR mice. PSR-PSR mice exhibit higher percentage of double-labeled cells over the total number of tdTomato+ cells compared to W-PSR mice. Statistical data are depicted as mean±SEM. * p<0.05, Mann-Whitney U-test. Black open circles represent the value of each individual animal.

Fig. 3: Rostral RSP neurons activated during PSR are different than those activated during W and show also a distinct distribution. (A) Drawings of representative sections showing the distributions of cFos+ (black squared dots), tdTomato+ (blue open circles) and double-labeled neurons (red close circles) in the rostral RSP in the three groups of mice. The level of the sections drawn is

displayed on the atlas section shown on the left. Note the different distribution of neurons during PSR and W (B) Low power photomicrographs of sections immunostained for cFos (black) and tdTomato (brown) from three representative mice of each group (W-W, W-PSR and PSR-PSR). (C) Histograms showing the mean \pm SEM and the individual (open circles) numbers of cFos+ and tdTomato+ neurons during W and PSR in all mice (D) High power photomicrographs (40x) from representative sections of W-PSR and PSR-PSR mice. High number of cFos+ (black nuclei) and tdTomato+ (brown cytoplasm) neurons are visible in W-PSR and PSR-PSR mice. A double-labeled cells (red arrow) is visible only on the section of the PSR-PSR mice. (E) Histograms showing the mean \pm SEM and the individual number of double-labeled cells and the percentage of double-labeled cells over the total number of tdTomato+ neurons in the three groups. Note that the percentage of double-labeled cells over the total number of tdTomato+ cells is higher in PSR-PSR than in W-PSR mice. * $p<0.05$, Mann-Whitney U-test.

Fig. 4: TdTomato and cFos+ neurons activated during PSR and W in the caudal RSP are different and show a distinct distribution. (A) Drawings of representative sections showing the distributions of cFos+ (black squared dots), tdTomato+ (blue open circles) and double-labeled (red close circles) neurons in the caudal RSP in the three groups of mice. The level of the sections drawn is displayed on the atlas section shown on the left. (B) Low power photomicrographs showing double-labeled sections from three representative mice from each group (W-W, W-PS and PS-PS).(C) Histogram showing the mean \pm SEM and the individual (open circles) number of cFos+ and tdTomato+ neurons during W and PSR in all mice. Note the higher number of labeled neurons during W compared to PSR. (D) High power photomicrographs (40x) from representative double-labeled sections of W-PSR and PSR-PSR mice. High number of cFos+ (black nuclei) and tdTomato+ (brown cytoplasm) neurons are visible in W-PSR and PSR-PSR mice. Double-labeled cells (red arrows) are visible only on the section of the PSR-PSR mice. (E) Photomicrographs of sections with fluorescence staining of tdTomato+(red) and cFos+ cells (green) from representative sections in caudal RSP of W-W and W-PSR mice. White arrows point out the numerous double-labeled neurons (yellow) visible specifically in the W-W mice. (F) Histograms showing the mean \pm SEM and the individual number of double-labeled cells and the percentage of

double-labeled cells over the total number of tdTomato+ neurons in the three groups. Note that the percentage of double-labeled cells over the total number of tdTomato+ cells is higher in PSR-PSR than in W-PSR mice. * $p < 0.05$, Mann-Whitney U-test.

Fig 5: Different CLA neurons are activated during W and PSR. (A) Drawings of representative sections showing the distributions of cFos+ (black squared dots), tdTomato+ (blue open circles) and double labeled (red close circles) neurons in the CLA in the three groups of mice. The level of the sections drawn is displayed on the atlas section shown on the left. (B) Photomicrographs at low (10x) and high (40x) power magnification (enlargement of the region inside the square drawn on the low power photomicrograph) of representative sections at the level of the CLA from two mice from the W-W and PSR-PSR groups. Note the large number of double-labeled (red arrows) and singly labeled cFos+ neurons and the unique tdTomato+ cell (blue arrow) in the W-W mice. In contrast, only one double-labeled neuron is visible in the PSR-PSR mice (C) Histogram showing the mean \pm SEM and the individual (open circles) number of cFos+ and tdTomato+ neurons during W and PSR in all mice. Note the higher number of labeled neurons during W compared to PSR. (D) Photomicrographs of sections with fluorescence staining of tdTomato+(red) and cFos+ cells (green) from representative sections of CLA from W-W, W-PSR and PSR-PSR mice. White arrows point out the double-labeled neurons (yellow). (E) Histograms showing the mean \pm SEM and the individual (open circles) number of double-labeled cells and the percentage of double-labeled cells over the total number of tdTomato+ neurons in the three groups. * $p < 0.05$, Mann-Whitney U-test.

Table 1: Total number of cFos+, tdTomato+ and double-labelled neurons (cFos+ and tdTomato+) in W-W, W-PS and PS-PS according to the anatomical structure. The values are presented as mean \pm SEM. The ratio (last column) is calculated between the number of cFos+ and tdTomato+ cells.

Region/ExpGroup		WW							
	Total <i>nlTomato</i> +	SEM (+ -)	Total <i>c Fos</i>	SEM (+ -)	double	SEM (+ -)	%double/Trap	SEM (+ -)	ratio <i>nlTomato/c Fos</i>
RSPr	74.67	25.76	583.00	164.98	23.00	10.26	29.20	3.39	1:8
RSPc	91.33	39.82	1132.00	120.20	29.67	12.72	35.05	6.31	1:12
CLA	33.17	10.23	145.67	27.12	22.00	9.61	62.60	11.44	1:4
ACA	79.33	23.95	640.00	31.80	41.33	13.06	53.60	9.56	1:8

Region/ExpGroup		WPS							
	Total <i>nlTomato</i> +	SEM (+ -)	Total <i>c Fos</i>	SEM (+ -)	double	SEM (+ -)	%double/Trap	SEM (+ -)	ratio <i>nlTomato/c Fos</i>
RSPr	134.45	52.02	133.75	40.25	4.30	1.45	3.49	0.96	--
RSPc	191.60	74.92	191.60	54.96	7.40	2.01	4.53	0.88	--
CLA	33.90	3.89	30.20	9.81	1.30	0.58	4.00	1.84	--
ACA	140.60	29.30	63.25	6.88	3.60	0.86	2.64	0.56	--

Region/ExpGroup		PSPS							
	Total <i>nlTomato</i> +	SEM (+ -)	Total <i>c Fos</i>	SEM (+ -)	double	SEM (+ -)	%double/Trap	SEM (+ -)	ratio <i>nlTomato/c Fos</i>
RSPr	103.00	23.79	319.80	49.88	11.60	2.98	11.71	2.10	1:3
RSPc	56.80	9.69	532.80	188.05	7.40	1.29	14.09	2.49	1:9
CLA	18.20	4.40	48.20	13.69	1.80	0.58	10.16	4.62	1:3
ACA	61.60	15.87	188.80	77.62	3.40	0.75	7.50	3.05	1:3