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MAJOR ALTERATION OF MOTOR CONTROL DURING REM SLEEP IN MICE MODELS OF SLEEP DISORDERS

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3 **MAJOR ALTERATION OF MOTOR CONTROL DURING REM SLEEP IN MICE**
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5 **MODELS OF SLEEP DISORDERS**
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ABSTRACT

Alteration of motor control during REM sleep has been extensively described in sleep disorders, in particular in isolated REM sleep behavior disorder (iRBD) and narcolepsy type 1 (NT1). NT1 is caused by the loss of orexin/hypocretin (ORX) neurons. Unlike in iRBD, the RBD comorbid symptoms of NT1 is not associated with alpha-synucleinopathies.

To determine whether the chronic absence of ORX neuropeptides is sufficient to induce RBD symptoms, we analyzed during REM sleep the EMG signal of the prepro-hypocretin knockout mice (ORX^{-/-}), a recognized mouse model of NT1. Then, we evaluated the severity of motor alterations by comparing EMG data of ORX^{-/-} mice to those of mice with a targeted suppression of the sublateralodorsal glutamatergic neurotransmission, a recognized rodent model of iRBD.

We found a significant alteration of tonic and phasic components of EMG during REM sleep in ORX^{-/-} mice, with more phasic events and more REM sleep episodes without atonia compared to the control wild-type mice. However, these phasic events were fewer, shorter and less complex in ORX^{-/-} mice compared to the RBD-like ORX^{-/-} mice.

We thus show that ORX-deficiency, as seen in NT1, is sufficient to impair muscle atonia during REM sleep with a moderate severity of alteration as compared to isolated RBD mice. As described in NT1 patients, we report a major inter-individual variability in the severity and the frequency of RBD symptoms in ORX-deficient mice.

Keywords: Narcolepsy; REM sleep behavior disorder; isolated RBD; Orexin; hypocretin; motor control; sleep; hypothalamus, sublateralodorsal nucleus, muscle atonia

INTRODUCTION

Sleep is a cycling alternation of rapid eye-movements (REM) sleep (also called paradoxical sleep) and non-REM (NREM) sleep episodes in mammals [1]. Importantly, the tonus of skeletal muscles is low during NREM sleep, probably due to the reduction of excitatory inputs on motoneurons, and decreases further to become atonic during REM sleep, due to a hyperpolarizing active process on somatic motoneurons as shown by the abolition of H-reflexes in humans [2] and intracellular recordings in cats [3-5]. Alteration of motor control during REM sleep has been extensively described in sleep disorders, including isolated REM sleep behavior disorder (iRBD) and narcolepsy type 1 (NT1).

Clinical observations of iRBD patients report powerful vocalizations, complex and large body movements, often violent and interpreted as dream-enacting behaviors [6]. iRBD is a middle-age disorder, preponderant in men and strongly associated with neurotoxic alpha-synuclein deposits (namely Lewy Body) within the sublaterodorsal nucleus (SLD) of the dorsal pons where neurons mediating muscle atonia during REM sleep are located, suggesting that iRBD is a prodromal stage of alpha-synucleinopathies [7-9]. It is thought that a targeted neurodegeneration of those neurons may cause the impairment of motor control during REM sleep.

NT1 is a rare neurological disorder with a very different profile, showing excessive daytime sleepiness, disturbed nighttime sleep, hallucinations and cataplexy [10]. It has been reported that 7-63% of NT1 patients show RBD symptoms as comorbidity [11-14]. However, clinical observations of those patients recall RBD as less violent and with movements that are less complex than iRBD patients. It starts at an earlier age, including infancy and is equally seen in men and women [11,12,14]. Moreover, no association was found with alpha-synucleinopathies [15,16]. Taken together, these data suggest a different etiology for RBD in NT1 and iRBD.

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3 NT1 is caused by the loss of orexin/hypocretin (ORX) neuropeptides expressed by neurons
4 restricted to the lateral hypothalamus [17,18], and many symptoms such as cataplexy are clearly
5 due to the absence of ORX [10]. Interestingly, Knudsen et al. have proposed that RBD would
6 be more prevalent in patients with narcolepsy and a low CSF level of ORX suggesting that the
7 lack of ORX may be responsible for the RBD symptoms [19], although ORX neurons are wake-
8 promoting- and REM sleep inhibiting-neurons [20-23].
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17 Based on those observations, we test here whether the chronic impairment of ORX transmission,
18 as seen in NT1, would be sufficient to cause a significant alteration of muscle atonia during
19 REM sleep and may lead to the expression of motor symptoms similar to RBD. Hence, we
20 analyzed EMG recordings in ORX-deficient mice (ORX^{-/-}), a recognized model of NT1 [24]
21 that we compared to those of wild-type (WT) littermates. Next, we evaluated the level of motor
22 control impairment during REM sleep in ORX^{-/-} mice by comparing their EMG signals to those
23 recorded in a mouse model of iRBD, formed of ORX^{-/-} with an impairment of the glutamatergic
24 neurotransmission of the pontine sublaterodorsal nucleus (SLD).
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41 **MATERIEL & METHODS**

44 **Viral Vectors**

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46 Two adeno-associated viral vectors (AAVs; rh10 serotypes; generous gift of M Lazarus and Y.
47 Cherasse, Osaka Bioscience Institute, Japan) were used: one, shGLUT, containing a short-hair
48 pint complementary mRNA sequence of the vesicular glutamate transporter VGluT2 (5'-
49 TGAAACCAGAGATAGCAAATC) that leads to the inactivation of glutamate transmission;
50 the other, shCTRL, containing the same nucleotides as the shGLUT but in a random order as
51 control (5'-GTCAGGCTATCGCGTATCG) [25]. Both sequences were fused with mCherry
52 fluorescent protein and placed under the control of an CMV ubiquitous promoter. The titer of
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3 the viral solution was of 4.5×10^{12} particles/mL for AAV-shCTRL-mCherry and 5.6×10^{12}
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5 particles/mL for AAV-shGLUT-mCherry.
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8 **Animals**

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10 Experiments were performed using 18 males ORX^{-/-} mice of C57Bl/6J genetic background [24]
11
12 and their WT littermates (n=8). Mice were 6-8 weeks of age and weighted 21-28g at surgery.
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15 Mice were divided in 3 experimental groups: (1) a group of 10 mice called “ORX^{-/-}” included 4
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17 ORX^{-/-} mice that received no viral injection, 3 that were transfected with shCTRL in the SLD,
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19 and 3 that were transfected with shCTRL in the ventromedial medulla (VMM); (2) an “shGLUT”
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21 group formed of 8 ORX^{-/-} mice transfected with shGLUT into the SLD; and (3) a control group
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23 with 8 WT mice.
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27 Protocols were approved by the Ethics Research Committee of University-Lyon1 (DR2015-03)
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29 and conducted in accordance to the European Community guidelines for the use of animals for
30
31 research.
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34 **Surgical Procedures**

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37 *Viral injections:* Mice were anesthetized with an intraperitoneal injection of a cocktail of
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39 ketamine/xylazine (100:10mg/kg) then placed on a stereotaxic frame (David Kopf Instruments,
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41 USA). They were bilaterally injected with either 80nL of AAV-shGLUT (n = 8) or AAV-
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43 shCTRL (n = 6) delivered at 60nL/min. Microinjections were performed using a quartz
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45 micropipette (20µm of tip diameter) fixed on a 10µL Hamilton syringe placed into an Ultra
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47 Micro Pump (UMP3 Ultra Micro Pump, WPI) and targeting the SLD or the VMM using
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49 stereotaxic coordinates (SLD: ± 0.88mm lateral to midline (ML), - 6.42mm posterior to Bregma,
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51 -4.45mm from the skull surface with a 20° posterior angle; VMM: ±0.3mm lateral to midline
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53 and -5.9mm from Bregma, -6mm from the skull surface). Then, the micropipette was left in
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55 place for 5 min to allow AAV tissue absorption before being removed.
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3 *Electrodes implantation:* For sleep recordings, all mice were implanted with three
4 electroencephalogram (EEG) electrodes: one above the frontal cortex (1mm ML and 1.5mm
5 anterior to Bregma), one above the parietal cortex (1.7mm ML and -2.5mm posterior to
6 Bregma), and the last one above the cerebellum (-6mm posterior to Bregma) as reference. Two
7 electromyogram (EMG) electrodes were slipped between neck muscles.
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15 **Polysomnographic Recordings**

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18 Seven to ten days after surgery, mice were placed in a Plexiglas recording barrel (30cm diameter)
19 and were connected to a cable plugged to a rotating connector (Bilaney, Plastics One, Germany),
20 to allow free movements during recordings. Mice were acclimated to the recording chamber for
21 at least 3 consecutive days. Note that mice with AAV-shRNA injection were recorded 8 weeks
22 post-injection to maximize shRNAs' efficiency.
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29 Polysomnography and video recordings were collected and synchronized using SleepScore
30 software (Viewpoint, Lyon, France). Each frontal and parietal EEG signal was referenced to
31 the cerebellar EEG electrode, and muscle tone was assessed by a differential EMG signal. EEG
32 and EMG signals were amplified (EEG: 2000x; EMG: 5000x) (16-Channel Model 3500, AM
33 System; US), band filtered with analog filters (EEG: 0.3Hz-100Hz; EMG:10Hz-100Hz) and
34 digitalized at 512Hz (NI Usb 6343 card, National Instrument, Austin, US). Video recordings
35 were continuously collected with a camera fixed above each barrel (Point Grey firefly MV,
36 Black & White, 640x480, 15 fps).
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48 **Analyses**

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51 *Vigilance states:* Vigilance states were scored using a 5-sec window frame of EEG/EMG
52 signals and video recordings according to standard criteria [26]. Note that in rare occasion,
53 ORX^{-/-} mice may have a cataplexy just before the transition to the dark phase. Those cataplexy
54 epochs were excluded from the analysis.
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Analyses were performed using home-made routines on MATLAB version R2021b (MATLAB, Natick, Massachusetts: The MathWorks Inc.) on recordings collected during the 12hrs of the light phase (8 a.m. to 8 p.m.).

EMG analysis during sleep: We considered all REM sleep episodes of at least 10 sec duration that were preceded by a NREM sleep episode of at least 10 sec. The first and last 2 sec of each REM and NREM sleep episode were removed to exclude transition phases. EMG signal was first filtered using a high pass digital filter at 50Hz to remove low frequencies that may interfere, in particular those due to heart bit. Then, EMG signal was rectified and smoothed with a 1 sec resolution sliding window.

First, we split the phasic and the tonic components of the REM sleep EMG signal. For each REM sleep episode, we calculated a threshold based on the 95th percentile of the EMG amplitude of the last 30 sec of the preceding NREM sleep episode (or of its whole duration if ≤ 30 sec). All events above this threshold and lasting for more than 0.5 sec were considered as phasic events. We assessed the number of REM sleep episodes with at least one phasic event, the density in phasic events (ie. the number of phasic events per minute of REM sleep), as well as the density of 3 categories of phasic events: lasting for less than 1 sec, for 1-5 sec and for more than 5 sec. Finally, we calculated for each animal the REM sleep time devoted to phasic events and reported it as percentage of total REM sleep time.

The analysis of the tonic component was obtained by removing all phasic events from the REM sleep EMG. The same procedure was carried out on the last 30 sec of NREM sleep preceding the REM sleep episode. Then, we calculated the ratio between the median value of EMG voltage during REM sleep and NREM sleep (ie. REM sleep^{tone}/NREM sleep^{tone}). This analysis was performed for each of NREM-REM sleep pair, and the median value of this ratio was obtained for each animal.

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3 Note that REM sleep episodes affected by phasic events for more than 75% of their duration
4 were removed from the tonic component analysis. However, because of these episodes are of
5 interest as they may reflect an extreme alteration of motor control, we quantified them for each
6 animal.
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13 **Histology**

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15 *Brain Preparation:* Mice were deeply anesthetized with a lethal dose of pentobarbital (150
16 mg/kg, intraperitoneally, Ceva Santé Animale) and transcardially perfused with Ringer's lactate
17 solution containing 0.1% heparin, followed by 50mL of a 4% paraformaldehyde (PFA) solution.
18 Brains were post-fixed in the same fixative solution at 4°C for 48hrs and sank in a 30% sucrose
19 solution for 48hrs. Then, they were rapidly frozen in a -30°C methylbutane solution and cut in
20 serial 30µm-thick coronal sections with a cryostat (Microm, HM 5500). Free-floating sections
21 were collected in a RNase-free cryoprotectant solution (DEPC 0.05%, Glycerol 20%, Ethylene
22 Glycol 30% in K₂HPO₄ / KH₂PO₄ 50mM buffer pH 7,4) and stored at -20°C until use.
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34 *In situ hybridization:* Antisense and sense digoxigenin-labeled probes against VGluT2 and the
35 vesicular transporter for GABA (VGAT) were synthesized from a recombinant linearized
36 plasmid containing VGluT2 or VGAT cDNA, and using a non-radioactive RNA labelling kit
37 (Roche Diagnostic; [27]). Briefly, brain sections at SLD level were rinsed in a PBST containing
38 a standard saline citrate solution (2xSSC) and 0.2% of RNase inhibitor (ProtectRNA™, Sigma-
39 Aldrich) for 10 min twice. Then, they were incubated overnight at 55°C in the hybridization
40 buffer containing 0.5mg/mL of the digoxigenin-labeled probe. Sections were washed in 1xSSC
41 50% formamide and 0.1% Tween 20, treated at 37°C in a buffer solution containing RNase A
42 (USB Corporation), and finally incubated in a solution of anti-digoxigenin IgG conjugated to
43 alkaline phosphatase (1/2000, Roche Diagnostic). Free-floating sections were rinsed twice in
44 PBST and once in PBS (10mM). Staining was revealed using nitroblue tetrazolium and 5'-
45 bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostic) in a 100mM Tris-HCl buffer at 37°C
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3 for 2hrs. Sections were then mounted on slides and coverslipped using VectaMount TM
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5 (VectorLabs).
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7 Double immunofluorescence: Coronal sections of the VMM that include the gigantocellular
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9 reticular nucleus alpha part and ventral part and the raphe magnus nucleus, were isolated and
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11 incubated for 3 days at 4°C in a PBST-Az containing both rabbit IgG against VGluT2 (1/1000
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13 Synaptic Systems) and rat IgG against mCherry (1/50 000, Synaptic Systems). They were rinsed
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15 in 0.02M PBST for 10 min 3 times and incubated overnight at 4°C in PBST-Az containing a
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17 mixture of secondary antibodies: Alexa Fluor 488 anti-rabbit IgG and Alexa-fluor 594 anti-rat
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19 IgG both made in donkey (1/500, ThermoFisher). After 3 x 10min rinses in PBST, sections
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21 were mounted on slides, and cover-slipped using Fluoromount DAPI TM (Vector Laboratories).
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24 Analyses: The extent of the injection-sites was defined based on the mCherry endogenous
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26 fluorescence using an Axioskop microscope (Zeiss) equipped with a motorized x-y-sensitive
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28 stage and a color video camera connected to a computerized image analysis system (Mercator;
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30 Explora Nova). Photomicrographs were taken using the same system. Double
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32 immunofluorescence of mCherry and VGluT2 in axons was analyzed using confocal
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34 fluorescence microscope SP5 (Leica) at a resolution of 1024x1024 pixels/frame with an
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36 objective 63x (0.5µm image thickness).
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43 **Statistics**

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45 All statistical analyzes were performed using R Statistical Software (v4.2.2; [28]) with R-
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47 Commander (v2.8.0; [29]) and PMCMRplus package (v1.9.6; [30]). To compare our 3 groups,
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49 we used the Kruskal-Wallis non-parametric test [31]. When p-value was lower than the alpha
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51 significance threshold (set at 0.05), we processed to the all-pairs multiple comparison Conover
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53 post-hoc test [32] with Holm's p-value adjustment method [33]. All results of statistical tests
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55 are summarized in Table 1.
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3 We also compare the interindividual variability observed in groups by using the Brown-
4 Forsythe test [34] with Holm's p-value adjustment method.
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10 RESULTS

11 **Alteration of motor control during REM sleep in ORX^{-/-} versus WT mice**

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14 We first looked at the architecture of the vigilance states during the light phase in ORX^{-/-} mice.
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16 We found no difference in the total amount or the number of episodes of wake, NREM and
17 REM sleep between ORX^{-/-} and WT mice (Fig.1). The only significant difference found was a
18 shorter mean duration of REM sleep episodes in ORX^{-/-} mice compared to WT (46.5 vs. 56 sec
19 respectively; $p = 0.0012$) (Fig.1I).
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28 We found a moderate alteration of muscle tone (Fig.2) during some REM sleep episodes in
29 ORX^{-/-} mice. To objectively assess whether the chronic absence of ORX neuropeptides would
30 alter motor control during REM sleep, we analyzed the phasic and tonic components of the
31 EMG in ORX^{-/-} mice compared to WT mice. We first quantified phasic events for each REM
32 sleep episode (see method for details) (Fig.3) and found a higher percentage of REM sleep
33 episodes affected by phasic movements (18.84 vs 5.91 % respectively; $p = 0.0024$). We also
34 found a higher density of phasic events (ORX^{-/-}: 1.08 vs WT: 0.15; $p = 0.0003$) in ORX^{-/-} mice
35 and when categorizing them by duration (Fig.3C), it appeared that phasic events of all duration
36 were of higher density (<1 sec : $p = 0.0015$; 1-5 sec : $p = 0.0002$; > 5 sec : $p = 0.0043$) in ORX^{-/-}
37 mice compared to WT. Interestingly, 7 of the 10 ORX^{-/-} mice had long duration events (> 5
38 sec) while only 1 out of 8 WT mouse showed 2 events of long duration (Fig.3C). We also
39 quantified the percentage of time spent in phasic activity during REM sleep and found that it
40 was highly and significantly increased in ORX^{-/-} mice compared to WT mice (3.63 vs 0.23 %
41 respectively; $p < 0.0001$) (Fig.3D). However, ORX^{-/-} mice similarly to WT mice had no REM
42 sleep episodes with a very high density of phasic events (>75% of their duration), with the
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3 exception of one ORX^{-/-} mouse showing one single REM sleep episode with such characteristics
4 (Fig.3E). Altogether, these data show that ORX^{-/-} mice have a significantly higher amount of
5 movements of longer duration during REM sleep than their WT littermates.
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10 Next, we assessed the tonic component of the EMG during REM sleep and found that the typical
11 decrease in muscle tone at transition from NREM to REM sleep was altered in ORX^{-/-} mice
12 (Fig.4). Indeed, we calculated the REM sleep^{tone} / NREM sleep^{tone} ratio (see methods for details)
13 and found that it was significantly higher in ORX^{-/-} mice indicating that muscle tone did not
14 decrease or decreased less during REM sleep in ORX^{-/-} mice than in WT (ratio = 0.69 vs 0.5
15 respectively; p = 0.0039) (Fig.4A). Interestingly, the value of the EMG ratio showed a large
16 inter-individual variability in ORX^{-/-} mice and this variability was significantly higher than in
17 WT mice (p = 0.0483).
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21 Additionally, the proportion of REM sleep episodes without atonia (RSWA) (ie. with a ratio>1)
22 was higher in ORX^{-/-} mice compared to WT (5 vs 0.81 % respectively; p = 0.0005) (Fig.4B).
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28 In summary, our results show an alteration of motor control during REM sleep in ORX^{-/-} mice,
29 for both the tonic and phasic components of the EMG. This suggests that the chronic absence
30 of ORX transmission is sufficient to lead to the dysregulation of muscle atonia during REM
31 sleep, along with an increase of phasic motor activities, although the extent of such impairment
32 is highly variable between ORX^{-/-} mice.
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35 36 37 **Validation of a model of iRBD in ORX^{-/-} mice**

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39 A large body of evidence has demonstrated that glutamatergic neurons of the pontine SLD are
40 key players to generate and maintain muscle atonia during REM sleep [25,35-37]. Indeed,
41 blockage of glutamatergic transmission of the SLD in rats dramatically alters muscle atonia
42 with observations of large body movements [25]. To create mice with an iRBD-like phenotype,
43 we applied the same approach as Valencia-Garcia and colleagues [25], altering the
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3 glutamatergic neurotransmission of the SLD using shRNA technology directed against VGluT2.
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5 As illustrated (Fig.5), shRNA expression, visualized by the presence of mCherry reporter
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7 protein, was centered into the SLD in all injected mice. In some animals, we saw mCherry
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9 fluorescent neurons in adjacent pontine regions such as the ventral part of the laterodorsal
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11 tegmental nucleus and the median parabrachial nucleus (Fig.5A,B). Only rare neurons were
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13 transfected in the lateral parabrachial nucleus of 2 shGLUT mice (Fig.5A).

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16 To further determine the efficacy and specificity of the blockage of the glutamatergic
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18 neurotransmission, we performed *in situ* hybridization for VGluT2 and VGAT on SLD brain
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20 sections. As illustrated in Fig.5B, no VGluT2 positive neurons were seen in the SLD after
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22 shGLUT treatment while the surrounding regions were unaffected. In both shCTRL and
23
24 shGLUT mice, VGAT neurons were strongly labeled following VGAT *in situ* hybridization, in
25
26 the SLD and surrounding regions (Fig.5B) demonstrating that GABAergic transmission was
27
28 preserved in all mice. Then, we tested for the expression of VGluT2 proteins within SLD axonal
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30 terminals by confocal microscopy. We found a high density of SLD axonal terminals in the
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32 VMM - as visualized by mCherry expression - of all mice (Fig.5C). Those axons were labeled
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34 for VGluT2 in mice that received shCTRL, but no VGluT2 staining was observed in SLD axons
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36 for mice injected with shGLUT (Fig.5C). Altogether these data indicate, although indirectly,
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38 that SLD glutamatergic neurotransmission was efficiently and specifically blocked in shGLUT
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40 mice and unaltered in shCTRL mice.
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49 **Strong alteration of REM sleep in shGLUT mice**

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51 The total amounts of wakefulness, NREM and REM sleep were unchanged during the light
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53 phase in shGLUT mice compared to WT mice (Fig.1). However, shGLUT mice showed a
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55 strong fragmentation of all vigilance states (ie. a higher number of episodes of shorter mean
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57 duration) compared to WT mice (Fig.1).
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3 Similar to Valencia-Garcia et al. [25], we found a strong alteration of muscle atonia during
4 REM sleep in shGLUT mice (Fig. 2). A large percentage of REM sleep episodes were affected
5 by phasic movements in shGLUT mice compared to WT mice (51.3 vs 5.9% respectively; $p <$
6 0.0001) (Fig.3A) and the density of phasic events was highly increased (4.92 vs 0.15
7 respectively; $p <$ 0.0001) (Fig.3B). Note that all shGLUT mice showed phasic events of long
8 duration (>5 sec) (Fig.3C) and spend a large amount of REM sleep time with phasic activity (28
9 vs 0.23% in WT mice; $p <$ 0.0001) (Fig.3D). Moreover, shGLUT mice had a significant number
10 of REM sleep episodes with a very high density of phasic events (Fig.3E; Fig.2).

11
12 The tonic component of the EMG was also strongly impaired during REM sleep in all shGLUT
13 mice (Fig.4), with a median REM sleep^{tone}/NREM sleep^{tone} ratio close to 1 (shGLUT: 0.84 vs
14 WT: 0.5; $p <$ 0.0001) and with more than 10% of REM sleep episodes qualified as RSWA
15 (shGLUT: 12.58% vs WT: 0.81%; $p <$ 0.0001). Taken together, histology and
16 electrophysiology data show that the glutamatergic transmission of SLD is specifically and
17 efficiency blocked in the shGLUT mice, inducing a major impairment of muscle atonia during
18 REM sleep. We were thus able to reproduce Valencia-Garcia's data obtained in rats [25],
19 creating a suitable iRBD model in the mouse.

40 **Alteration of motor control in ORX^{-/-} group versus shGLUT group**

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42 When comparing mice of the ORX^{-/-} group with those of the shGLUT group, we saw that ORX^{-/-}
43 mice exhibited abnormalities during REM sleep, characterized by excessive head movements
44 despite clear REM-like theta frequency EEG". In the shGLUT group, mice showed in addition
45 body and tail movements.

46
47 We found that both ORX^{-/-} and shGLUT mice show an impairment of muscle atonia during
48 REM sleep compared to WT mice. More interestingly, the level of alteration was different
49 between the two groups of mice.
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3 Indeed, ORX^{-/-} mice had a significantly lower percentage of REM sleep episodes with phasic
4 movements compared to shGLUT mice ($p < 0.0001$) (Fig.3A). They also showed a lower
5 density of phasic movements ($p < 0.0001$) (Fig.3B), independently of their duration (Fig.3C).
6
7 ORX^{-/-} had a lower percentage of REM sleep time with phasic activity ($p < 0.0001$) (Fig.3D)
8 and no expression of REM sleep episodes of very high density of phasic activity (except for
9 one single event in one ORX^{-/-} mouse), while all shGLUT mice expressed such episodes
10 (Fig.3E).

11
12 Furthermore, ORX^{-/-} mice showed a lower REM sleep^{tone}/NREM sleep^{tone} ratio than shGLUT
13 mice ($p = 0.0179$) (Fig.4A) and a lower percentage of RSWA ($p = 0.0069$) (Fig.4B). In other
14 words, ORX^{-/-} mice showed intermediate values to WT and shGLUT mice, for the phasic and
15 tonic components of the EMG, indicating that motor control during REM sleep is less severely
16 altered in ORX^{-/-} than in shGLUT mice. Interestingly, the inter-individual variability for the
17 tonic EMG ratio was significantly higher in ORX^{-/-} than in shGLUT and WT groups ($p = 0.048$
18 and $p = 0.028$ respectively) (Fig.4A)

19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 **DISCUSSION**

40
41 This is the first study comparing REM sleep muscle tone between NT1 and iRBD mouse models.
42
43 Altogether, our data shows that chronic ORX neuropeptide deficiency is sufficient to cause the
44 alteration of muscle atonia during REM sleep, increasing the occurrence of phasic events as
45 well as the amplitude of the tonic component of muscle tone. Of great interest, REM sleep
46 muscle atonia alteration was less pronounced in the ORX^{-/-} group than the shGLUT group,
47 suggesting that ORX deficiency leads to a moderate motor control alteration during REM sleep
48 as compared to the blockade of the glutamatergic transmission within SLD, considered as the
49 generator of muscle atonia specific to REM sleep.
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Role of ORX in motor control during REM sleep

In agreement with our observations, Silvani et al. [38] reported an increased EMG signal during REM sleep in ORX^{-/-} and ORX-ataxin3 mice compared to WT mice although without providing a detailed description of the alteration. Taken together, these results indicate that chronic impairment of ORX neurotransmission (ie. neuropeptide deficiency or neuronal loss), leads to a motor control alteration during REM sleep.

These observations are somehow surprising since ORX neurons fire during active wake and are silent during REM sleep, with the exception of occasional spikes concomitant to the presence of EMG twitches [20,21]. A large amount of data using pharmacology, optogenetics or chemogenetics manipulations shows that ORX neurons promote wakefulness, increase locomotor activity, and inhibit REM sleep possibly via the activation of the ventrolateral periaqueductal gray neurons gating REM sleep [22,39-41]. Conversely, optogenetic or chemogenetic inhibition of ORX neurons decreases the time spent in wakefulness at the benefit of NREM sleep [42,43].

A large body of evidence has demonstrated that descending SLD glutamatergic neurons are critical for maintaining muscle atonia during REM sleep [25,35,36,44]. They send excitatory projections to the glycinergic and GABAergic neurons of the ventromedial medulla that in turn inhibit spinal motoneurons [35,45-47]. ORX neurons project widely throughout the brain [48] and may exert control on muscle tone by modulating its network of regulation at different brain levels.

Interestingly, it was recently reported in mice that a subset of ORX neurons with a significant activity during REM sleep would project to the SLD glutamatergic neurons [49]. The authors further showed that when released in the SLD, ORX reinforces coupling between glutamatergic neurons and strengthen their activity while the chemogenetic inhibition of these SLD projecting ORX neurons significantly impairs muscle atonia during REM sleep [49]. Similarly,

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3 microinjection of ORX in the pons of decerebrated rats, that includes the SLD, provoked the
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5 inhibition of muscle tone similar to that observed during REM sleep [50]. Altogether it suggests
6
7 that the lack of ORX may weaken the ability of SLD glutamatergic neurons to turn on inhibitory
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9 premotor neurons of the medulla during REM sleep, diminishing their ability to inhibit somatic
10
11 motoneurons. As a consequence, it would lower the activation threshold of motoneurons and
12
13 provoke the alteration of muscle atonia during REM sleep. Nevertheless, further experiments
14
15 need to be performed to determine the singularity of this pathway and get a better understanding
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17 of the mechanisms involved. Indeed, it was also shown that ORX-A microinjections in the
18
19 gigantocellular and the dorsal paragigantocellular nuclei would induce bilateral hindlimb
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21 muscle atonia [51]. The pathological and chronic lack of ORX inputs on those nuclei may also
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23 participate to the impairment of muscle atonia during REM sleep in mice and NT1 patients.
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28 However, such effect needs to be confirmed.
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30 31 **Validation of the iRBD mouse model**

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33 We applied in mice the same technology as Valencia-Garcia et al. [25] in rats to create an iRBD
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35 model. As in rats, *in situ* hybridization and immunostainings showed no VGluT2 expression in
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37 the SLD cell bodies and axons, demonstrating that the impairment of the SLD glutamatergic
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39 neurotransmission was extremely efficient. Similar to Valencia-Garcia, we found a major
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41 alteration of muscle atonia during REM sleep. The effect observed is comparable to those seen
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43 when glutamatergic neurotransmission of SLD neurons is inactivated by VGluT2 gene deletion
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45 in SLD neurons or by blocking vesicular transmission of all SLD neurons that projects onto
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47 inhibitory premotor neurons, in WT mice [36,52]. Unlike Valencia-Garcia et al [25] but in
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49 agreement with other studies of SLD glutamatergic impairment in mice [36,44], we found no
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51 reduction of REM sleep amount. Valencia-Garcia et al reported daily amount of REM sleep
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53 while we focused only on the light phase. Interestingly, Krenzer et al [36] found a 40%
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55 reduction of REM sleep quantity over 24hrs but similarly to our data, no reduction during the
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3 light phase. Altogether, these data indicate that we have created a suitable mouse model for
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5 iRBD.
6

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8 We found however a fragmentation of all vigilance states in the shGLUT mice compared to
9
10 WT and not in the ORX^{-/-} mice. Such fragmentation was also reported after the *vglut2* gene
11
12 deletion in the SLD of WT mice, particularly during the light phase [36]. In rats, Valencia-
13
14 Garcia et al. found no fragmentation of vigilance states [25]. As the authors only reported daily
15
16 amounts of vigilance states, it is unknown whether they were fragmented during the light phase
17
18 or if it is specific to mice models.
19

20 21 22 **Muscle atonia impairment during REM sleep in ORX^{-/-} vs shGLUT mice**

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24 This is the first study evaluating and comparing the alteration of muscle atonia during REM
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26 sleep in mouse models of NT1 and iRBD. Interestingly, we found that ORX^{-/-} mice had less
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28 RSWA, a lower density of movements and of REM sleep time spent in activity than shGLUT
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30 mice. REM sleep episodes with high density of phasic activity were found in shGLUT mice
31
32 only. With this approach, we demonstrate that the targeted impairment of the glutamate
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34 neurotransmission generating muscle atonia provokes a severe motor control dysfunction
35
36 whereas chronic ORX deficiency lead to a significant but moderate and variable alteration of
37
38 muscle atonia during REM sleep. Although one has to be cautious when studying animal
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40 models of sleep disorders as they may reproduce only partly the pathophysiology of the diseases,
41
42 it is striking to notice how our observations are in lines with clinical reports. Indeed, the severity
43
44 of RBD symptoms was higher in our mouse model of iRBD than in ORX^{-/-} NT1-like mice,
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46 similarly to previous descriptions of RBD symptoms in NT1 and iRBD patients showing
47
48 RSWA and abnormal motor behaviors in only 7-63% of NT1 patients and with lower severity
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50 than iRBD patients [11,12,14]. Interestingly, it was reported based on an objective evaluation
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52 of RBD symptoms in 63 patients with narcolepsy that RBD symptoms were more prevalent in
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54 patients with low CSF level of ORX-A [19]. By using an animal model with a targeted ORX-
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3 deficiency and an objective method to evaluate motor activity, our results provide new evidence
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5 that chronic ORX-deficiency may be at the origin of RBD symptoms in NT1.
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8 9 **Limitations**

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11 It is important to note that our EMG analysis is limited to neck muscles. It would be of interest
12
13 to look at EMG activity in other muscles, in particular of limb muscles. However, Silvani et al.
14
15 have previously shown that EMG neck muscle activity is a good indicator to evaluate body
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17 motor activity during sleep in mice and to detect alterations during REM sleep in mice models
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19 of NT1 [38].
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22
23 It is to note that we chose to block SLD glutamatergic neurotransmission in ORX^{-/-} mice rather
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25 than WT mice to unravel the additional effect of the targeted SLD glutamatergic blockage
26
27 compared to the ORX-deficiency. Indeed, we found here that ORX^{-/-} deficiency alone does not
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29 entirely reproduce the effect of the SLD blockage. It further demonstrates that RBD symptoms
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31 in ORX^{-/-} are not underlined by a complete alteration of the SLD function.
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35 Finally, the ORX^{-/-} group is a mix of sham mice: a third of them received no injection, a third
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37 had the shCTRL injected in the SLD, the last third received the shCTRL in the VMM. We found
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39 no treatment effect in all performed analyzes as illustrated in supplFig.1, except for the percent
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41 of RSWA that was much higher in mice that had received shCTRL in the VMM than other mice
42
43 of the ORX^{-/-} group. When excluding those 3 mice, the percent of RSWA is still significantly
44
45 different between ORX^{-/-} and WT groups ($p=0.0007$) and ORX^{-/-} and shGLUT groups
46
47 ($p=0.00006$), confirming the difference in severity between ORX^{-/-} and shGLUT mice.
48
49 Importantly, the mix of sham mice in the ORX^{-/-} does not explain the high inter-individual
50
51 variability seen in the ORX^{-/-} group except possibly for the measure of the RSWA percent
52
53 (supplFig.1).
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Proposition of mechanism underlying RBD symptoms in NT1

With this study, we demonstrate that chronic ORX-deficiency is sufficient to induce a RBD phenotype in ORX^{-/-} mice similar to clinical observations made on NT1 patients. Based on Feng et al. [49], it is tempting to propose that the chronic lack of ORX in NT1 may provoke the loss of an excitatory input onto the SLD during REM sleep, resulting in a reduced strength of the SLD output. Since it is a decrease in the strength of the SLD output and not its abolition as it would be with a lesion, the inhibitory input from the medulla to the spinal motoneurons is still present but less efficient. It then results on a moderate and non-systematic expression of RBD symptoms in NT1 patients. Indeed, this mechanism would explain why RBD symptoms have a sporadic occurrence in a given patient and are not experienced by all NT1 patients [12]. This is of great interest because if our hypothesis is correct, the pharmacological replacement of ORX at the right dose, should suppress RBD symptoms in NT1 patients.

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REFERENCE LIST

1. Peyron C, Arthaud S, Villalba M, P. F: Defining and measuring paradoxical (REM) sleep in animal models of sleep disorders. *Current opinion in Physiology* 2020, 15:203-209.
2. Hodes R, Dement WC: Depression of Electrically Induced Reflexes ("H-Reflexes") in Man during Low Voltage Eeg "Sleep". *Electroencephalogr Clin Neurophysiol* 1964, 17:617-629.
3. Soja PJ, Lopez-Rodriguez F, Morales FR, Chase MH: The postsynaptic inhibitory control of lumbar motoneurons during the atonia of active sleep: effect of strychnine on motoneuron properties. *J Neurosci* 1991, 11:2804-2811.
4. Morales FR, Chase MH: Intracellular recording of lumbar motoneuron membrane potential during sleep and wakefulness. *Exp Neurol* 1978, 62:821-827.
5. Nakamura Y, Goldberg LJ, Chandler SH, Chase MH: Intracellular analysis of trigeminal motoneuron activity during sleep in the cat. *Science* 1978, 199:204-207.
6. Schenck CH, Bundlie SR, Ettinger MG, Mahowald MW: Chronic behavioral disorders of human REM sleep: a new category of parasomnia. *Sleep* 1986, 9:293-308.
7. Boucetta S, Salimi A, Dadar M, Jones BE, Collins DL, Dang-Vu TT: Structural Brain Alterations Associated with Rapid Eye Movement Sleep Behavior Disorder in Parkinson's Disease. *Sci Rep* 2016, 6:26782.
8. Iranzo A, Santamaria J, Tolosa E: The clinical and pathophysiological relevance of REM sleep behavior disorder in neurodegenerative diseases. *Sleep Med Rev* 2009, 13:385-401.
9. Hogl B, Stefani A, Videnovic A: Idiopathic REM sleep behaviour disorder and neurodegeneration - an update. *Nat Rev Neurol* 2018, 14:40-55.

10. Bassetti CLA, Adamantidis A, Burdakov D, Han F, Gay S, Kallweit U, Khatami R, Koning F, Kornum BR, Lammers GJ, et al.: Narcolepsy - clinical spectrum, aetiopathophysiology, diagnosis and treatment. *Nat Rev Neurol* 2019, 15:519-539.
11. Dauvilliers Y, Jennum P, Plazzi G: Rapid eye movement sleep behavior disorder and rapid eye movement sleep without atonia in narcolepsy. *Sleep Med* 2013, 14:775-781.
12. Antelmi E, Pizza F, Franceschini C, Ferri R, Plazzi G: REM sleep behavior disorder in narcolepsy: A secondary form or an intrinsic feature? *Sleep Med Rev* 2020, 50:101254.
13. Schenck CH, Mahowald MW: Motor dyscontrol in narcolepsy: rapid-eye-movement (REM) sleep without atonia and REM sleep behavior disorder. *Ann Neurol* 1992, 32:3-10.
14. Mattarozzi K, Bellucci C, Campi C, Cipolli C, Ferri R, Franceschini C, Mazzetti M, Russo PM, Vandi S, Vignatelli L, et al.: Clinical, behavioural and polysomnographic correlates of cataplexy in patients with narcolepsy/cataplexy. *Sleep Med* 2008, 9:425-433.
15. Antelmi E, Pizza F, Donadio V, Filardi M, Sosero YL, Incensi A, Vandi S, Moresco M, Ferri R, Marelli S, et al.: Biomarkers for REM sleep behavior disorder in idiopathic and narcoleptic patients. *Ann Clin Transl Neurol* 2019, 6:1872-1876.
16. Jennum PJ, Ostergaard Pedersen L, Czarna Bahl JM, Modvig S, Fog K, Holm A, Rahbek Kornum B, Gammeltoft S: Cerebrospinal Fluid Biomarkers of Neurodegeneration Are Decreased or Normal in Narcolepsy. *Sleep* 2017, 40. doi: 10.1093/sleep/zsw006.
17. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, et al.: A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 2000, 6:991-997.
18. Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM: Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 2000, 27:469-474.

- 1
2
3 19. Knudsen S, Gammeltoft S, Jennum PJ: Rapid eye movement sleep behaviour disorder in
4 patients with narcolepsy is associated with hypocretin-1 deficiency. *Brain* 2010,
5 133:568-579.
6
7
8
9
- 10 20. Mileykovskiy BY, Kiyashchenko LI, Siegel JM: Behavioral correlates of activity in
11 identified hypocretin/orexin neurons. *Neuron* 2005, 46:787-798.
12
13
- 14 21. Lee MG, Hassani OK, Jones BE: Discharge of identified orexin/hypocretin neurons across
15 the sleep-waking cycle. *J Neurosci* 2005, 25:6716-6720.
16
17
18
- 19 22. Roman A, Meftah S, Arthaud S, Luppi PH, Peyron C: The inappropriate occurrence of rapid
20 eye movement sleep in narcolepsy is not due to a defect in homeostatic regulation of
21 rapid eye movement sleep. *Sleep* 2018, 41. doi: 10.1093/sleep/zsy046.
22
23
24
25
- 26 23. Takahashi K, Lin JS, Sakai K: Neuronal activity of orexin and non-orexin waking-active
27 neurons during wake-sleep states in the mouse. *Neuroscience* 2008, 153:860-870.
28
29
- 30 24. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA,
31 Williams SC, Xiong Y, Kisanuki Y, et al.: Narcolepsy in orexin knockout mice:
32 molecular genetics of sleep regulation. *Cell* 1999, 98:437-451.
33
34
35
36
- 37 25. Valencia Garcia S, Libourel PA, Lazarus M, Grassi D, Luppi PH, Fort P: Genetic
38 inactivation of glutamate neurons in the rat sublaterodorsal tegmental nucleus
39 recapitulates REM sleep behaviour disorder. *Brain* 2017, 140:414-428.
40
41
42
43
- 44 26. Franken P, Malafosse A, Tafti M: Genetic determinants of sleep regulation in inbred mice.
45 *Sleep* 1999, 22:155-169.
46
47
48
- 49 27. Clement O, Sapin E, Berod A, Fort P, Luppi PH: Evidence that neurons of the
50 sublaterodorsal tegmental nucleus triggering paradoxical (REM) sleep are
51 glutamatergic. *Sleep* 2011, 34:419-423.
52
53
54
- 55 28. team R: R: A language and environment for statistical computing. . Edited by: R
56 Foundation for statistical computing, Vienna Austria; 2022.
57
58
59
60

- 1
2
3 29. Fox J, Bouchet-Valat M: Rcmdr: R commander., edn 2.8-0. Edited by; 2022:R package.
4
5 30. Pohlert T: PMCMRplus: Calculate pairwise multiple comparisons of mean rank sums
6
7 extended. edn 1.9.6. Edited by; 2022:R package.
8
9
10 31. Kruskal WH, Wallis WA: Use of ranks in one-criterion variance analysis. Journal of the
11
12 American Statistical association 1952, 47:583-621.
13
14 32. Conover WJ, Iman RL: On multiple comparisons procedures. Edited by. Tech. Rep. LA-
15
16 7677-MS, Los Alamos scientific laboratory; 1979.
17
18 33. Holm S: A simple sequentially rejective multiple test procedure. Scandinavian journal of
19
20 statistics 1979, 6:65-70.
21
22
23 34. Brown MB, Forsythe AB: Robust tests for the equality of variances. Journal of the American
24
25 Statistical association 1974, 69:364-367.
26
27
28 35. Boissard R, Gervasoni D, Schmidt MH, Barbagli B, Fort P, Luppi PH: The rat ponto-
29
30 medullary network responsible for paradoxical sleep onset and maintenance: a
31
32 combined microinjection and functional neuroanatomical study. Eur J Neurosci 2002,
33
34 16:1959-1973.
35
36
37 36. Krenzer M, Anacleto C, Vetrivelan R, Wang N, Vong L, Lowell BB, Fuller PM, Lu J:
38
39 Brainstem and spinal cord circuitry regulating REM sleep and muscle atonia. PLoS One
40
41 2011, 6:e24998.
42
43
44 37. Arrigoni E, Chen MC, Fuller PM: The anatomical, cellular and synaptic basis of motor
45
46 atonia during rapid eye movement sleep. J Physiol 2016, 594:5391-5414.
47
48
49 38. Silvani A, Ferri R, Lo Martire V, Bastianini S, Berteotti C, Salvade A, Plazzi G, Zucconi
50
51 M, Ferini-Strambi L, Bassetti CL, et al.: Muscle Activity During Sleep in Human
52
53 Subjects, Rats, and Mice: Towards Translational Models of REM Sleep Without Atonia.
54
55 Sleep 2017, 40. doi: 10.1093/sleep/zsx029.
56
57
58
59
60

- 1
2
3 39. Kaur S, Thankachan S, Begum S, Liu M, Blanco-Centurion C, Shiromani PJ: Hypocretin-
4 2 saporin lesions of the ventrolateral periaqueductal gray (vlPAG) increase REM sleep
5 in hypocretin knockout mice. *PLoS One* 2009, 4:e6346.
6
7
8
9
10 40. Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L: Neural substrates of
11 awakening probed with optogenetic control of hypocretin neurons. *Nature* 2007,
12 450:420-424.
13
14
15
16 41. Bourgin P, Huitron-Resendiz S, Spier AD, Fabre V, Morte B, Criado JR, Sutcliffe JG,
17 Henriksen SJ, de Lecea L: Hypocretin-1 modulates rapid eye movement sleep through
18 activation of locus coeruleus neurons. *J Neurosci* 2000, 20:7760-7765.
19
20
21
22 42. Tsunematsu T, Kilduff TS, Boyden ES, Takahashi S, Tominaga M, Yamanaka A: Acute
23 optogenetic silencing of orexin/hypocretin neurons induces slow-wave sleep in mice. *J*
24 *Neurosci* 2011, 31:10529-10539.
25
26
27
28 43. Sasaki K, Suzuki M, Mieda M, Tsujino N, Roth B, Sakurai T: Pharmacogenetic modulation
29 of orexin neurons alters sleep/wakefulness states in mice. *PLoS One* 2011, 6:e20360.
30
31
32
33 44. Torontali ZA, Fraigne JJ, Sanghera P, Horner R, Peever J: The Sublaterodorsal Tegmental
34 Nucleus Functions to Couple Brain State and Motor Activity during REM Sleep and
35 Wakefulness. *Curr Biol* 2019, 29:3803-3813 e3805.
36
37
38
39 45. Sulaman BA, Wang S, Tyan J, Eban-Rothschild A: Neuro-orchestration of sleep and
40 wakefulness. *Nat Neurosci* 2023, 26:196-212.
41
42
43
44 46. Valencia Garcia S, Brischoux F, Clement O, Libourel PA, Arthaud S, Lazarus M, Luppi
45 PH, Fort P: Ventromedial medulla inhibitory neuron inactivation induces REM sleep
46 without atonia and REM sleep behavior disorder. *Nat Commun* 2018, 9:504.
47
48
49
50 47. Valencia Garcia S, Luppi PH, Fort P: A Particular Medullary-Spinal Inhibitory Pathway is
51 Recruited for the Expression of Muscle Atonia During REM Sleep. *J Exp Neurosci* 2018,
52 12:1179069518808744.
53
54
55
56
57
58
59
60

- 1
2
3 48. Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS:
4
5 Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci*
6
7 1998, 18:9996-10015.
8
9
- 10 49. Feng H, Wen SY, Qiao QC, Pang YJ, Wang SY, Li HY, Cai J, Zhang KX, Chen J, Hu ZA,
11
12 et al.: Orexin signaling modulates synchronized excitation in the sublaterodorsal
13
14 tegmental nucleus to stabilize REM sleep. *Nat Commun* 2020, 11:3661.
15
16
- 17 50. Kiyashchenko LI, Mileykovskiy BY, Lai YY, Siegel JM: Increased and decreased muscle
18
19 tone with orexin (hypocretin) microinjections in the locus coeruleus and pontine
20
21 inhibitory area. *J Neurophysiol* 2001, 85:2008-2016.
22
23
- 24 51. Mileykovskiy BY, Kiyashchenko LI, Siegel JM: Muscle tone facilitation and inhibition
25
26 after orexin-a (hypocretin-1) microinjections into the medial medulla. *J Neurophysiol*
27
28 2002, 87:2480-2489.
29
30
- 31 52. Uchida S, Soya S, Saito YC, Hirano A, Koga K, Tsuda M, Abe M, Sakimura K, Sakurai T:
32
33 A Discrete Glycinergic Neuronal Population in the Ventromedial Medulla That Induces
34
35 Muscle Atonia during REM Sleep and Cataplexy in Mice. *J Neurosci* 2021, 41:1582-
36
37 1596.
38
39
40
41
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43
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LEGENDS

Figure 1. Quantification of vigilance states during the light phase.

A-C, Total amount of time (min) spent in Wake, NREM sleep and REM sleep. **D-F**, Total number of Wake, NREM sleep and REM sleep episodes. **G-I**, Mean duration (sec) of Wake, NREM sleep and REM sleep bouts. Graphs represent data obtained on wild-type mice (WT), and narcoleptic mice without (ORX^{-/-}) and with (shGLUT) impairment of glutamatergic transmission of the SLD. Each symbol represents the value of an individual mouse. Box plots show the median value and the interquartile range for each experimental group. Statistics was performed using Kruskal-Wallis test followed by Conover post-hoc comparison and Holm correction. Significance was set at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2. Representative EEG and EMG signals for mice of the WT, ORX^{-/-} and shGLUT groups. In the representative WT mouse, the EMG signal is unaltered with a decrease in amplitude in REM sleep compared to prior NREM sleep. The REM sleep EMG signal of the representative ORX^{-/-} mouse shows a mild alteration with an increase amplitude for the tonic component and the presence of phasic events. The representative shGLUT mouse shows a major alteration of the EMG signal during REM sleep.

Figure 3. Quantification of EMG phasic events during REM sleep.

A, Percentage of REM sleep episodes with at least one phasic event detected. **B**, Density in phasic events (number of phasic events per minute of REM sleep). **C**, Density of phasic events by subcategories defined on the basis of the phasic event duration (short: < 1 sec ; medium : 1–5 sec ; long: >5 sec). **D**, Total REM sleep time represented by phasic activity, expressed as percentage of the total amount of REM sleep. **E**, Percentage of REM sleep episode with presence of phasic events during more than 75% of the REM sleep episode duration. Graphs

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3 represent data obtained on wild-type mice (WT) and narcoleptic mice without (ORX^{-/-}) and
4 with (shGLUT) impairment of glutamatergic transmission of the SLD. Each symbol represents
5 the value of an individual mouse. Histograms show the median value for each group with the
6 interquartile range. Statistics comparison was performed using the Conover post-hoc
7 comparison (with Holm correction) after a Kruskal-Wallis test. Significance is set at * p < 0.05 ;
8 ** p < 0.01 ; *** p < 0.001.

19 **Figure 4. Quantification of EMG tonic component during REM sleep**

21 **A**, EMG tonic ratio illustrating the difference in muscle tone during REM sleep compared to
22 NREM sleep ($REM\ sleep\ tone / NREM\ sleep\ tone$). This analysis was performed for each of
23 NREM-REM sleep pair, and the median value of this ratio was obtained for each animal. Values
24 displayed are the median and quartiles of the EMG tonic ratio obtained for each experimental
25 group. **B**, Percentage of REM sleep episode without atonia (RSWA). RSWA is defined by an
26 EMG tonic ratio above 1. Graphs represent data obtained on wild-type mice (WT) and
27 narcoleptic mice without (ORX^{-/-}) and with (shGLUT) impairment of glutamatergic
28 transmission of the SLD. Each symbol represents the value of an individual mouse and box
29 plots report the median value and the interquartile range for each group. Statistic comparison
30 was performed using a Kruskal-Wallis test followed by the Conover post-hoc comparison with
31 Holm correction. Significance is set at * p < 0.05 ; ** p < 0.01 ; *** p < 0.001.

49 **Figure 5. Histological validation of the shGLUT mouse model**

51 **A – Schematic drawings illustrating the extent of shGLUT injections sites. B (top panels)-**
52 Low power photomicrographs of mCherry fluorescent signal after shRNA transfection in
53 representative control (shCTRL) and altered (shGLUT) mice. Scale bar: 150µm. B (bottom
54 panel) – **B (mid-panels)**, Low power and enlargement microphotographs illustrating coronal
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3 brain sections treated with VGluT2 *in situ hybridization* of a representative shCTRL (left) and
4 shGLUT (right) mice. Scale bars: 150 μ m (low power) and 80 μ m (enlargement). **B (lower**
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6 **panels)**, Low power and enlargement microphotographs illustrating coronal brain sections
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8 treated with VGAT *in situ hybridization* of a representative shCTRL (left) and shGLUT (right)
9
10 mice. Scale bars: 150 μ m (low power) and 80 μ m (enlargement). **C-** Confocal images of the
11
12 ventromedial medulla region (VMM) representing the expression of VGluT2 proteins (green),
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14 mCherry (red) and DAPI (blue). On the merge image, co-expression of vGluT2 and mCherry
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16 (white arrowheads) indicate axons coming from the SLD neurons and of glutamatergic nature.
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18 Note that co-expression is seen only in control condition (shCTRL). None are observed in
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20 animals treated with shGLUT demonstrating that VGluT2 proteins are absent and glutamatergic
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22 transmission impaired. Scale bar: 10 μ m.
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28 *Abbreviations:* Aq, aqueduct ; DTg, dorsal tegmental nucleus ; LDT, laterodorsal tegmental
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30 nucleus ; LPB, lateral parabrachial nucleus ; Mo5, motor trigeminal nucleus ; MPB, medial
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32 parabrachial nucleus ; PnC, Pontine caudalis area ; SLD, sublateralodorsal nucleus ; VLPAG,
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34 ventrolateral periaqueductal gray.
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40 **Table 1. Summary of statistical results**

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42 Statistical results with p-value obtained for data on Figure 1, Figure 3 and Figure 4. Each p-
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44 value of Conover test column are associated with a label for post-hoc groups comparison: 1 for
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46 WT vs ORX^{-/-} ; 2 for WT vs shGLUT ; 3 for ORX^{-/-} vs shGLUT. Significance was set at * p <
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48 0.05 ; ** p < 0.01 ; *** p < 0.001.
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54 **Supplementary figure 1: Illustration of the distribution of individual mice of the ORX^{-/-}**
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56 **group using a color-code to identify each subgroup of mice.**
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3 **A-C**, Total amount of minutes spent in Wake, in NREM sleep and in REM sleep over the 12hrs
4 of the light phase. **D-F**, Total number of episodes of Wake, NREM sleep, and REM sleep. **G-I**,
5 Mean duration in seconds of Wake, NREM sleep, and REM sleep episodes. **K**, EMG tonic ratio
6 representing the evolution of muscle tone during REM sleep compared to NREM sleep. Values
7 displayed are the median of EMG tonic ratio obtained for each REM sleep episodes. **L**,
8 Percentage of REM sleep episode without atonia (RSWA). RSWA are defined by an EMG tonic
9 ratio above 1. **M**, Percentage of REM sleep episode with at least one phasic event detected (100%
10 is the total number of REM sleep episodes for which the EMG analysis was carried out). **N**,
11 Phasic events density, defined as the number of phasic events per minutes of REM sleep. **O**,
12 Phasic events density for each category of phasic events (short duration : < 1 sec ; mid duration :
13 1 sec – 5 sec ; long duration : 5 sec). Graphs represent data obtained from mice of the ORX^{-/-}
14 group. Dark blue squares represent ORX^{-/-} mice with no viral injection (n=4), red squares the
15 ORX^{-/-} mice with injection of shCTRL in the SLD (n=3) and the light pink squares the ORX^{-/-}
16 mice with injection of shCTRL in VMM.
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3 **MAJOR ALTERATION OF MOTOR CONTROL DURING REM SLEEP IN MICE**
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5 **MODELS OF SLEEP DISORDERS**
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ABSTRACT

Alteration of motor control during REM sleep has been extensively described in sleep disorders, in particular in isolated REM sleep behavior disorder (iRBD) and narcolepsy type 1 (NT1). NT1 is caused by the loss of orexin/hypocretin (ORX) neurons. Unlike in iRBD, the RBD comorbid symptoms of NT1 is not associated with alpha-synucleinopathies.

To determine whether the chronic absence of ORX neuropeptides is sufficient to induce RBD symptoms, we analyzed during REM sleep the EMG signal of the prepro-hypocretin knockout mice (ORX^{-/-}), a recognized mouse model of NT1. Then, we evaluated the severity of motor alterations by comparing EMG data of ORX^{-/-} mice to those of mice with a targeted suppression of the sublateralodorsal glutamatergic neurotransmission, a recognized rodent model of iRBD.

We found a significant alteration of tonic and phasic components of EMG during REM sleep in ORX^{-/-} mice, with more phasic events and more REM sleep episodes without atonia compared to the control wild-type mice. However, these phasic events were fewer, shorter and less complex in ORX^{-/-} mice compared to the RBD-like ORX^{-/-} mice.

We thus show that ORX-deficiency, as seen in NT1, is sufficient to impair muscle atonia during REM sleep with a moderate severity of alteration as compared to isolated RBD mice. As described in NT1 patients, we report a major inter-individual variability in the severity and the frequency of RBD symptoms in ORX-deficient mice.

Keywords: Narcolepsy; REM sleep behavior disorder; isolated RBD; Orexin; hypocretin; motor control; sleep; hypothalamus, sublateralodorsal nucleus, muscle atonia

INTRODUCTION

Sleep is a cycling alternation of rapid eye-movements (REM) sleep (also called paradoxical sleep) and non-REM (NREM) sleep episodes in mammals [1]. Importantly, the tonus of skeletal muscles is low during NREM sleep, probably due to the reduction of excitatory inputs on motoneurons, and decreases further to become atonic during REM sleep, due to a hyperpolarizing active process on somatic motoneurons as shown by the abolition of H-reflexes in humans [2] and intracellular recordings in cats [3-5]. Alteration of motor control during REM sleep has been extensively described in sleep disorders, including isolated REM sleep behavior disorder (iRBD) and narcolepsy type 1 (NT1).

Clinical observations of iRBD patients report powerful vocalizations, complex and large body movements, often violent and interpreted as dream-enacting behaviors [6]. iRBD is a middle-age disorder, preponderant in men and strongly associated with neurotoxic alpha-synuclein deposits (namely Lewy Body) within the sublaterodorsal nucleus (SLD) of the dorsal pons where neurons mediating muscle atonia during REM sleep are located, suggesting that iRBD is a prodromal stage of alpha-synucleinopathies [7-9]. It is thought that a targeted neurodegeneration of those neurons may cause the impairment of motor control during REM sleep.

NT1 is a rare neurological disorder with a very different profile, showing excessive daytime sleepiness, disturbed nighttime sleep, hallucinations and cataplexy [10]. It has been reported that 7-63% of NT1 patients show RBD symptoms as comorbidity [11-14]. However, clinical observations of those patients recall RBD as less violent and with movements that are less complex than iRBD patients. It starts at an earlier age, including infancy and is equally seen in men and women [11,12,14]. Moreover, no association was found with alpha-synucleinopathies [15,16]. Taken together, these data suggest a different etiology for RBD in NT1 and iRBD.

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3 NT1 is caused by the loss of orexin/hypocretin (ORX) neuropeptides expressed by neurons
4 restricted to the lateral hypothalamus [17,18], and many symptoms such as cataplexy are clearly
5 due to the absence of ORX [10]. Interestingly, Knudsen et al. have proposed that RBD would
6 be more prevalent in patients with narcolepsy and a low CSF level of ORX suggesting that the
7 lack of ORX may be responsible for the RBD symptoms [19], although ORX neurons are wake-
8 promoting- and REM sleep inhibiting-neurons [20-23].
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17 Based on those observations, we test here whether the chronic impairment of ORX transmission,
18 as seen in NT1, would be sufficient to cause a significant alteration of muscle atonia during
19 REM sleep and may lead to the expression of motor symptoms similar to RBD. Hence, we
20 analyzed EMG recordings in ORX-deficient mice (ORX^{-/-}), a recognized model of NT1 [24]
21 that we compared to those of wild-type (WT) littermates. Next, we evaluated the level of motor
22 control impairment during REM sleep in ORX^{-/-} mice by comparing their EMG signals to those
23 recorded in a mouse model of iRBD, **formed of ORX^{-/-} with an impairment of the glutamatergic**
24 **neurotransmission of the pontine sublaterodorsal nucleus (SLD).**
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41 **MATERIEL & METHODS**

44 **Viral Vectors**

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46 Two adeno-associated viral vectors (AAVs; rh10 serotypes; generous gift of M Lazarus and Y.
47 Cherasse, Osaka Bioscience Institute, Japan) were used: one, shGLUT, containing a short-hair
48 pint complementary mRNA sequence of the vesicular glutamate transporter VGluT2 (5'-
49 TGAAACCAGAGATAGCAAATC) that leads to the inactivation of glutamate transmission;
50 the other, shCTRL, containing the same nucleotides as the shGLUT but in a random order as
51 control (5'-GTCAGGCTATCGCGTATCG) [25]. Both sequences were fused with mCherry
52 fluorescent protein and placed under the control of an CMV ubiquitous promoter. The titer of
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3 the viral solution was of 4.5×10^{12} particles/mL for AAV-shCTRL-mCherry and 5.6×10^{12}
4 particles/mL for AAV-shGLUT-mCherry.
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8 **Animals**

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10 Experiments were performed using 18 males ORX^{-/-} mice of C57Bl/6J genetic background [24]
11 and their WT littermates (n=8). Mice were 6-8 weeks of age and weighted 21-28g at surgery.
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13 Mice were divided in 3 experimental groups: (1) a group of 10 mice called “ORX^{-/-}” included 4
14 ORX^{-/-} mice that received no viral injection, 3 that were transfected with shCTRL in the SLD,
15 and 3 that were transfected with shCTRL in the ventromedial medulla (VMM); (2) an “shGLUT”
16 group formed of 8 ORX^{-/-} mice transfected with shGLUT into the SLD; and (3) a control group
17 with 8 WT mice.
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Protocols were approved by the Ethics Research Committee of University-Lyon1 (DR2015-03)
and conducted in accordance to the European Community guidelines for the use of animals for
research.

35 **Surgical Procedures**

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Viral injections: Mice were anesthetized with an intraperitoneal injection of a cocktail of
ketamine/xylazine (100:10mg/kg) then placed on a stereotaxic frame (David Kopf Instruments,
USA). They were bilaterally injected with either 80nL of AAV-shGLUT (n = 8) or AAV-
shCTRL (n = 6) delivered at 60nL/min. Microinjections were performed using a quartz
micropipette (20µm of tip diameter) fixed on a 10µL Hamilton syringe placed into an Ultra
Micro Pump (UMP3 Ultra Micro Pump, WPI) and targeting the SLD or the VMM using
stereotaxic coordinates (SLD: ± 0.88mm lateral to midline (ML), - 6.42mm posterior to Bregma,
-4.45mm from the skull surface with a 20° posterior angle; VMM: ±0.3mm lateral to midline
and -5.9mm from Bregma, -6mm from the skull surface). Then, the micropipette was left in
place for 5 min to allow AAV tissue absorption before being removed.

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3 *Electrodes implantation:* For sleep recordings, all mice were implanted with three
4 electroencephalogram (EEG) electrodes: one above the frontal cortex (1mm ML and 1.5mm
5 anterior to Bregma), one above the parietal cortex (1.7mm ML and -2.5mm posterior to
6 Bregma), and the last one above the cerebellum (-6mm posterior to Bregma) as reference. Two
7 electromyogram (EMG) electrodes were slipped between neck muscles.
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15 **Polysomnographic Recordings**

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18 Seven to ten days after surgery, mice were placed in a Plexiglas recording barrel (30cm diameter)
19 and were connected to a cable plugged to a rotating connector (Bilaney, Plastics One, Germany),
20 to allow free movements during recordings. Mice were acclimated to the recording chamber for
21 at least 3 consecutive days. Note that mice with AAV-shRNA injection were recorded 8 weeks
22 post-injection to maximize shRNAs' efficiency.
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29 Polysomnography and video recordings were collected and synchronized using SleepScore
30 software (Viewpoint, Lyon, France). Each frontal and parietal EEG signal was referenced to
31 the cerebellar EEG electrode, and muscle tone was assessed by a differential EMG signal. EEG
32 and EMG signals were amplified (EEG: 2000x; EMG: 5000x) (16-Channel Model 3500, AM
33 System; US), band filtered with analog filters (EEG: 0.3Hz-100Hz; EMG:10Hz-100Hz) and
34 digitalized at 512Hz (NI Usb 6343 card, National Instrument, Austin, US). Video recordings
35 were continuously collected with a camera fixed above each barrel (Point Grey firefly MV,
36 Black & White, 640x480, 15 fps).
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48 **Analyses**

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51 *Vigilance states:* Vigilance states were scored using a 5-sec window frame of EEG/EMG
52 signals and video recordings according to standard criteria [26]. Note that in rare occasion,
53 ORX^{-/-} mice may have a cataplexy just before the transition to the dark phase. **Those cataplexy**
54 **epochs were excluded from the analysis.**
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Analyses were performed using home-made routines on MATLAB version R2021b (MATLAB, Natick, Massachusetts: The MathWorks Inc.) on recordings collected during the 12hrs of the light phase (8 a.m. to 8 p.m.).

EMG analysis during sleep: We considered all REM sleep episodes of at least 10 sec duration that were preceded by a NREM sleep episode of at least 10 sec. The first and last 2 sec of each REM and NREM sleep episode were removed to exclude transition phases. EMG signal was first filtered using a high pass digital filter at 50Hz to remove low frequencies that may interfere, in particular those due to heart bit. Then, EMG signal was rectified and smoothed with a 1 sec resolution sliding window.

First, we split the phasic and the tonic components of the REM sleep EMG signal. For each REM sleep episode, we calculated a threshold based on the 95th percentile of the EMG amplitude of the last 30 sec of the preceding NREM sleep episode (or of its whole duration if ≤ 30 sec). All events above this threshold and lasting for more than 0.5 sec were considered as phasic events. We assessed the number of REM sleep episodes with at least one phasic event, the density in phasic events (ie. the number of phasic events per minute of REM sleep), as well as the density of 3 categories of phasic events: lasting for less than 1 sec, for 1-5 sec and for more than 5 sec. Finally, we calculated for each animal the REM sleep time devoted to phasic events and reported it as percentage of total REM sleep time.

The analysis of the tonic component was obtained by removing all phasic events from the REM sleep EMG. The same procedure was carried out on the last 30 sec of NREM sleep preceding the REM sleep episode. Then, we calculated the ratio between the median value of EMG voltage during REM sleep and NREM sleep (ie. REM sleep^{tone}/NREM sleep^{tone}). This analysis was performed for each of NREM-REM sleep pair, and the median value of this ratio was obtained for each animal.

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3 Note that REM sleep episodes affected by phasic events for more than 75% of their duration
4 were removed from the tonic component analysis. However, because of these episodes are of
5 interest as they may reflect an extreme alteration of motor control, we quantified them for each
6 animal.
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13 **Histology**

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15 Brain Preparation: Mice were deeply anesthetized with a lethal dose of pentobarbital (150
16 mg/kg, intraperitoneally, Ceva Santé Animale) and transcardially perfused with Ringer's lactate
17 solution containing 0.1% heparin, followed by 50mL of a 4% paraformaldehyde (PFA) solution.
18 Brains were post-fixed in the same fixative solution at 4°C for 48hrs and sank in a 30% sucrose
19 solution for 48hrs. Then, they were rapidly frozen in a -30°C methylbutane solution and cut in
20 serial 30µm-thick coronal sections with a cryostat (Microm, HM 5500). Free-floating sections
21 were collected in a RNase-free cryoprotectant solution (DEPC 0.05%, Glycerol 20%, Ethylene
22 Glycol 30% in K₂HPO₄ / KH₂PO₄ 50mM buffer pH 7,4) and stored at -20°C until use.
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34 In situ hybridization: Antisense and sense digoxigenin-labeled probes against VGluT2 and the
35 vesicular transporter for GABA (VGAT) were synthesized from a recombinant linearized
36 plasmid containing VGluT2 or VGAT cDNA, and using a non-radioactive RNA labelling kit
37 (Roche Diagnostic; [27]). Briefly, brain sections at SLD level were rinsed in a PBST containing
38 a standard saline citrate solution (2xSSC) and 0.2% of RNase inhibitor (ProtectRNA™, Sigma-
39 Aldrich) for 10 min twice. Then, they were incubated overnight at 55°C in the hybridization
40 buffer containing 0.5mg/mL of the digoxigenin-labeled probe. Sections were washed in 1xSSC
41 50% formamide and 0.1% Tween 20, treated at 37°C in a buffer solution containing RNase A
42 (USB Corporation), and finally incubated in a solution of anti-digoxigenin IgG conjugated to
43 alkaline phosphatase (1/2000, Roche Diagnostic). Free-floating sections were rinsed twice in
44 PBST and once in PBS (10mM). Staining was revealed using nitroblue tetrazolium and 5'-
45 bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostic) in a 100mM Tris-HCl buffer at 37°C
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3 for 2hrs. Sections were then mounted on slides and coverslipped using VectaMount TM
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5 (VectorLabs).
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7 Double immunofluorescence: Coronal sections of the VMM that include the gigantocellular
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9 reticular nucleus alpha part and ventral part and the raphe magnus nucleus, were isolated and
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11 incubated for 3 days at 4°C in a PBST-Az containing both rabbit IgG against VGluT2 (1/1000
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13 Synaptic Systems) and rat IgG against mCherry (1/50 000, Synaptic Systems). They were rinsed
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15 in 0.02M PBST for 10 min 3 times and incubated overnight at 4°C in PBST-Az containing a
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17 mixture of secondary antibodies: Alexa Fluor 488 anti-rabbit IgG and Alexa-fluor 594 anti-rat
18
19 IgG both made in donkey (1/500, ThermoFisher). After 3 x 10min rinses in PBST, sections
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21 were mounted on slides, and cover-slipped using Fluoromount DAPI TM (Vector Laboratories).
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24 Analyses: The extent of the injection-sites was defined based on the mCherry endogenous
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26 fluorescence using an Axioskop microscope (Zeiss) equipped with a motorized x-y-sensitive
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28 stage and a color video camera connected to a computerized image analysis system (Mercator;
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30 Explora Nova). Photomicrographs were taken using the same system. Double
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32 immunofluorescence of mCherry and VGluT2 in axons was analyzed using confocal
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34 fluorescence microscope SP5 (Leica) at a resolution of 1024x1024 pixels/frame with an
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36 objective 63x (0.5µm image thickness).
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43 **Statistics**

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45 All statistical analyzes were performed using R Statistical Software (v4.2.2; [28]) with R-
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47 Commander (v2.8.0; [29]) and PMCMRplus package (v1.9.6; [30]). To compare our 3 groups,
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49 we used the Kruskal-Wallis non-parametric test [31]. When p-value was lower than the alpha
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51 significance threshold (set at 0.05), we processed to the all-pairs multiple comparison Conover
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53 post-hoc test [32] with Holm's p-value adjustment method [33]. All results of statistical tests
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55 are summarized in Table 1.
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3 We also compare the interindividual variability observed in groups by using the Brown-
4 Forsythe test [34] with Holm's p-value adjustment method.
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10 RESULTS

11 **Alteration of motor control during REM sleep in ORX^{-/-} versus WT mice**

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14 We first looked at the architecture of the vigilance states during the light phase in ORX^{-/-} mice.
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16 We found no difference in the total amount or the number of episodes of wake, NREM and
17 REM sleep between ORX^{-/-} and WT mice (Fig.1). The only significant difference found was a
18 shorter mean duration of REM sleep episodes in ORX^{-/-} mice compared to WT (46.5 vs. 56 sec
19 respectively; $p = 0.0012$) (Fig.1I).

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21 We found a moderate alteration of muscle tone (Fig.2) during some REM sleep episodes in
22 ORX^{-/-} mice. To objectively assess whether the chronic absence of ORX neuropeptides would
23 alter motor control during REM sleep, we analyzed the phasic and tonic components of the
24 EMG in ORX^{-/-} mice compared to WT mice. We first quantified phasic events for each REM
25 sleep episode (see method for details) (Fig.3) and found a higher percentage of REM sleep
26 episodes affected by phasic movements (18.84 vs 5.91 % respectively; $p = 0.0024$). We also
27 found a higher density of phasic events (ORX^{-/-}: 1.08 vs WT: 0.15; $p = 0.0003$) in ORX^{-/-} mice
28 and when categorizing them by duration (Fig.3C), it appeared that phasic events of all duration
29 were of higher density (<1 sec : $p = 0.0015$; 1-5 sec : $p = 0.0002$; > 5 sec : $p = 0.0043$) in ORX^{-/-}
30 mice compared to WT. Interestingly, 7 of the 10 ORX^{-/-} mice had long duration events (> 5
31 sec) while only 1 out of 8 WT mouse showed 2 events of long duration (Fig.3C). We also
32 quantified the percentage of time spent in phasic activity during REM sleep and found that it
33 was highly and significantly increased in ORX^{-/-} mice compared to WT mice (3.63 vs 0.23 %
34 respectively; $p < 0.0001$) (Fig.3D). However, ORX^{-/-} mice similarly to WT mice had no REM
35 sleep episodes with a very high density of phasic events (>75% of their duration), with the
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3 exception of one ORX^{-/-} mouse showing one single REM sleep episode with such
4 characteristics (Fig.3E). Altogether, these data show that ORX^{-/-} mice have a significantly
5 higher amount of movements of longer duration during REM sleep than their WT littermates.
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10 Next, we assessed the tonic component of the EMG during REM sleep and found that the typical
11 decrease in muscle tone at transition from NREM to REM sleep was altered in ORX^{-/-} mice
12 (Fig.4). Indeed, we calculated the REM sleep^{tone} / NREM sleep^{tone} ratio (see methods for details)
13 and found that it was significantly higher in ORX^{-/-} mice indicating that muscle tone did not
14 decrease or decreased less during REM sleep in ORX^{-/-} mice than in WT (ratio = 0.69 vs 0.5
15 respectively; p = 0.0039) (Fig.4A). Interestingly, the value of the EMG ratio showed a large
16 inter-individual variability in ORX^{-/-} mice and this variability was significantly higher than in
17 WT mice (p = 0.0483).

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22 Additionally, the proportion of REM sleep episodes without atonia (RSWA) (ie. with a ratio>1)
23 was higher in ORX^{-/-} mice compared to WT (5 vs 0.81 % respectively; p = 0.0005) (Fig.4B).

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In summary, our results show an alteration of motor control during REM sleep in ORX^{-/-} mice,
for both the tonic and phasic components of the EMG. This suggests that the chronic absence
of ORX transmission is sufficient to lead to the dysregulation of muscle atonia during REM
sleep, along with an increase of phasic motor activities, although the extent of such impairment
is highly variable between ORX^{-/-} mice.

Validation of a model of iRBD in ORX^{-/-} mice

A large body of evidence has demonstrated that glutamatergic neurons of the pontine SLD are
key players to generate and maintain muscle atonia during REM sleep [25,35-37]. Indeed,
blockage of glutamatergic transmission of the SLD in rats dramatically alters muscle atonia
with observations of large body movements [25]. To create mice with an iRBD-like phenotype,
we applied the same approach as Valencia-Garcia and colleagues [25], altering the

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3 glutamatergic neurotransmission of the SLD using shRNA technology directed against VGluT2.
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5 As illustrated (Fig.5), shRNA expression, visualized by the presence of mCherry reporter
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7 protein, was centered into the SLD in all injected mice. In some animals, we saw mCherry
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9 fluorescent neurons in adjacent pontine regions such as the ventral part of the laterodorsal
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11 tegmental nucleus and the median parabrachial nucleus (Fig.5A,B). Only rare neurons were
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13 transfected in the lateral parabrachial nucleus of 2 shGLUT mice (Fig.5A).

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15 To further determine the efficacy and specificity of the blockage of the glutamatergic
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17 neurotransmission, we performed *in situ* hybridization for VGluT2 and VGAT on SLD brain
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19 sections. As illustrated in Fig.5B, no VGluT2 positive neurons were seen in the SLD after
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21 shGLUT treatment while the surrounding regions were unaffected. In both shCTRL and
22
23 shGLUT mice, VGAT neurons were strongly labeled following VGAT *in situ* hybridization, in
24
25 the SLD and surrounding regions (Fig.5B) demonstrating that GABAergic transmission was
26
27 preserved in all mice. Then, we tested for the expression of VGluT2 proteins within SLD axonal
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29 terminals by confocal microscopy. We found a high density of SLD axonal terminals in the
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31 VMM - as visualized by mCherry expression - of all mice (Fig.5C). Those axons were labeled
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33 for VGluT2 in mice that received shCTRL, but no VGluT2 staining was observed in SLD axons
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35 for mice injected with shGLUT (Fig.5C). Altogether these data indicate, although indirectly,
36
37 that SLD glutamatergic neurotransmission was efficiently and specifically blocked in shGLUT
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39 mice and unaltered in shCTRL mice.

49 **Strong alteration of REM sleep in shGLUT mice**

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51 The total amounts of wakefulness, NREM and REM sleep were unchanged during the light
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53 phase in shGLUT mice compared to WT mice (Fig.1). However, shGLUT mice showed a
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55 strong fragmentation of all vigilance states (ie. a higher number of episodes of shorter mean
56
57 duration) compared to WT mice (Fig.1).

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3 Similar to Valencia-Garcia et al. [25], we found a strong alteration of muscle atonia during
4 REM sleep in shGLUT mice (Fig. 2). A large percentage of REM sleep episodes were affected
5 by phasic movements in shGLUT mice compared to WT mice (51.3 vs 5.9% respectively; $p <$
6 0.0001) (Fig.3A) and the density of phasic events was highly increased (4.92 vs 0.15
7 respectively; $p <$ 0.0001) (Fig.3B). Note that all shGLUT mice showed phasic events of long
8 duration (>5 sec) (Fig.3C) and spend a large amount of REM sleep time with phasic activity (28
9 vs 0.23% in WT mice; $p <$ 0.0001) (Fig.3D). Moreover, shGLUT mice had a significant number
10 of REM sleep episodes with a very high density of phasic events (Fig.3E; Fig.2).

11
12 The tonic component of the EMG was also strongly impaired during REM sleep in all shGLUT
13 mice (Fig.4), with a median REM sleep^{tone}/NREM sleep^{tone} ratio close to 1 (shGLUT: 0.84 vs
14 WT: 0.5; $p <$ 0.0001) and with more than 10% of REM sleep episodes qualified as RSWA
15 (shGLUT: 12.58% vs WT: 0.81%; $p <$ 0.0001). Taken together, histology and
16 electrophysiology data show that the glutamatergic transmission of SLD is specifically and
17 efficiency blocked in the shGLUT mice, inducing a major impairment of muscle atonia during
18 REM sleep. We were thus able to reproduce Valencia-Garcia's data obtained in rats [25],
19 creating a suitable iRBD model in the mouse.

40 41 **Alteration of motor control in ORX^{-/-} group versus shGLUT group**

42
43 **When comparing mice of the ORX^{-/-} group with those of the shGLUT group, we saw that ORX^{-/-}**
44 **mice exhibited abnormalities during REM sleep, characterized by excessive head movements**
45 **despite clear REM-like theta frequency EEG". In the shGLUT group, mice showed in addition**
46 **body and tail movements.**

47
48 We found that both ORX^{-/-} and shGLUT mice show an impairment of muscle atonia during
49 REM sleep compared to WT mice. More interestingly, the level of alteration was different
50 between the two groups of mice.
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3 Indeed, ORX^{-/-} mice had a significantly lower percentage of REM sleep episodes with phasic
4 movements compared to shGLUT mice ($p < 0.0001$) (Fig.3A). They also showed a lower
5 density of phasic movements ($p < 0.0001$) (Fig.3B), independently of their duration (Fig.3C).
6
7 ORX^{-/-} had a lower percentage of REM sleep time with phasic activity ($p < 0.0001$) (Fig.3D)
8 and no expression of REM sleep episodes of very high density of phasic activity (except for
9 one single event in one ORX^{-/-} mouse), while all shGLUT mice expressed such episodes
10 (Fig.3E).

11
12 Furthermore, ORX^{-/-} mice showed a lower REM sleep^{tone}/NREM sleep^{tone} ratio than shGLUT
13 mice ($p = 0.0179$) (Fig.4A) and a lower percentage of RSWA ($p = 0.0069$) (Fig.4B). In other
14 words, ORX^{-/-} mice showed intermediate values to WT and shGLUT mice, for the phasic and
15 tonic components of the EMG, indicating that motor control during REM sleep is less severely
16 altered in ORX^{-/-} than in shGLUT mice. Interestingly, the inter-individual variability for the
17 tonic EMG ratio was significantly higher in ORX^{-/-} than in shGLUT and WT groups ($p = 0.048$
18 and $p = 0.028$ respectively) (Fig.4A)

38 DISCUSSION

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40 This is the first study comparing REM sleep muscle tone between NT1 and iRBD mouse models.
41
42 Altogether, our data shows that chronic ORX neuropeptide deficiency is sufficient to cause the
43 alteration of muscle atonia during REM sleep, increasing the occurrence of phasic events as
44 well as the amplitude of the tonic component of muscle tone. Of great interest, REM sleep
45 muscle atonia alteration was less pronounced in the ORX^{-/-} group than the shGLUT group,
46 suggesting that ORX deficiency leads to a moderate motor control alteration during REM sleep
47 as compared to the blockade of the glutamatergic transmission within SLD, considered as the
48 generator of muscle atonia specific to REM sleep.
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Role of ORX in motor control during REM sleep

In agreement with our observations, Silvani et al. [38] reported an increased EMG signal during REM sleep in ORX^{-/-} and ORX-ataxin3 mice compared to WT mice although without providing a detailed description of the alteration. Taken together, these results indicate that chronic impairment of ORX neurotransmission (ie. neuropeptide deficiency or neuronal loss), leads to a motor control alteration during REM sleep.

These observations are somehow surprising since ORX neurons fire during active wake and are silent during REM sleep, with the exception of occasional spikes concomitant to the presence of EMG twitches [20,21]. A large amount of data using pharmacology, optogenetics or chemogenetics manipulations shows that ORX neurons promote wakefulness, increase locomotor activity, and inhibit REM sleep possibly via the activation of the ventrolateral periaqueductal gray neurons gating REM sleep [22,39-41]. Conversely, optogenetic or chemogenetic inhibition of ORX neurons decreases the time spent in wakefulness at the benefit of NREM sleep [42,43].

A large body of evidence has demonstrated that **descending** SLD glutamatergic neurons are critical for maintaining muscle atonia during REM sleep [25,35,36,44]. They send excitatory projections to the glycinergic and GABAergic neurons of the ventromedial medulla that in turn inhibit spinal motoneurons [35,45-47]. ORX neurons project widely throughout the brain [48] and may exert control on muscle tone by modulating its network of regulation at different brain levels.

Interestingly, it was recently reported in mice that a subset of ORX neurons with a significant activity during REM sleep would project to the SLD glutamatergic neurons [49]. The authors further showed that when released in the SLD, ORX reinforces coupling between glutamatergic neurons and strengthen their activity while the chemogenetic inhibition of these SLD projecting ORX neurons significantly impairs muscle atonia during REM sleep [49]. Similarly,

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3 microinjection of ORX in the pons of decerebrated rats, that includes the SLD, provoked the
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5 inhibition of muscle tone similar to that observed during REM sleep [50]. Altogether it suggests
6
7 that the lack of ORX may weaken the ability of SLD glutamatergic neurons to turn on inhibitory
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9 premotor neurons of the medulla during REM sleep, diminishing their ability to inhibit somatic
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11 motoneurons. As a consequence, it would lower the activation threshold of motoneurons and
12
13 provoke the alteration of muscle atonia during REM sleep. Nevertheless, further experiments
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15 need to be performed to determine the singularity of this pathway and get a better understanding
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17 of the mechanisms involved. Indeed, it was also shown that ORX-A microinjections in the
18
19 gigantocellular and the dorsal paragigantocellular nuclei would induce bilateral hindlimb
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21 muscle atonia [51]. The pathological and chronic lack of ORX inputs on those nuclei may also
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23 participate to the impairment of muscle atonia during REM sleep in mice and NT1 patients.
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25 However, such effect needs to be confirmed.
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31 **Validation of the iRBD mouse model**

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33 We applied in mice the same technology as Valencia-Garcia et al. [25] in rats to create an iRBD
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35 model. As in rats, *in situ* hybridization and immunostainings showed no VGluT2 expression in
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37 the SLD cell bodies and axons, demonstrating that the impairment of the SLD glutamatergic
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39 neurotransmission was extremely efficient. Similar to Valencia-Garcia, we found a major
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41 alteration of muscle atonia during REM sleep. The effect observed is comparable to those seen
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43 when glutamatergic neurotransmission of SLD neurons is inactivated by VGluT2 gene deletion
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45 in SLD neurons or by blocking vesicular transmission of all SLD neurons that projects onto
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47 inhibitory premotor neurons, in WT mice [36,52]. Unlike Valencia-Garcia et al [25] but in
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49 agreement with other studies of SLD glutamatergic impairment in mice [36,44], we found no
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51 reduction of REM sleep amount. Valencia-Garcia et al reported daily amount of REM sleep
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53 while we focused only on the light phase. Interestingly, Krenzer et al [36] found a 40%
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55 reduction of REM sleep quantity over 24hrs but similarly to our data, no reduction during the
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3 **light phase**. Altogether, these data indicate that we have created a suitable mouse model for
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5 iRBD.
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8 We found **however** a fragmentation of all vigilance states in the shGLUT mice compared to
9
10 WT and not in the ORX^{-/-} mice. Such fragmentation was also reported after the *vglut2* gene
11
12 deletion in the SLD of WT mice, particularly during the light phase [36]. In rats, Valencia-
13
14 Garcia et al. found no fragmentation of vigilance states [25]. As the authors only reported daily
15
16 amounts of vigilance states, it is unknown whether they were fragmented during the light phase
17
18 or if it is specific to mice models.
19

20 21 22 **Muscle atonia impairment during REM sleep in ORX^{-/-} vs shGLUT mice**

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24 This is the first study evaluating and comparing the alteration of muscle atonia during REM
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26 sleep in mouse models of NT1 and iRBD. Interestingly, we found that ORX^{-/-} mice had less
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28 RSWA, a lower density of movements and of REM sleep time spent in activity than shGLUT
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30 mice. REM sleep episodes with high density of phasic activity were found in shGLUT mice
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32 only. With this approach, we demonstrate that the targeted impairment of the glutamate
33
34 neurotransmission generating muscle atonia provokes a severe motor control dysfunction
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36 whereas chronic ORX deficiency lead to a significant but moderate and variable alteration of
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38 muscle atonia during REM sleep. Although one has to be cautious when studying animal
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40 models of sleep disorders as they may reproduce only partly the pathophysiology of the diseases,
41
42 it is striking to notice how our observations are in lines with clinical reports. Indeed, the severity
43
44 of RBD symptoms was higher in our mouse model of iRBD than in ORX^{-/-} NT1-like mice,
45
46 similarly to previous descriptions of RBD symptoms in NT1 and iRBD patients showing
47
48 RSWA and abnormal motor behaviors in only 7-63% of NT1 patients and with lower severity
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50 than iRBD patients [11,12,14]. Interestingly, it was reported based on an objective evaluation
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52 of RBD symptoms in 63 patients with narcolepsy that RBD symptoms were more prevalent in
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54 patients with low CSF level of ORX-A [19]. By using an animal model with a targeted ORX-
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3 deficiency and an objective method to evaluate motor activity, our results provide new evidence
4 that chronic ORX-deficiency may be at the origin of RBD symptoms in NT1.
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8 **Limitations**

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10 It is important to note that our EMG analysis is limited to neck muscles. It would be of interest
11 to look at EMG activity in other muscles, in particular of limb muscles. However, Silvani et al.
12 have previously shown that EMG neck muscle activity is a good indicator to evaluate body
13 motor activity during sleep in mice and to detect alterations during REM sleep in mice models
14 of NT1 [38].
15

16 It is to note that we chose to block SLD glutamatergic neurotransmission in ORX^{-/-} mice rather
17 than WT mice to unravel the additional effect of the targeted SLD glutamatergic blockage
18 compared to the ORX-deficiency. Indeed, we found here that ORX^{-/-} deficiency alone does not
19 entirely reproduce the effect of the SLD blockage. It further demonstrates that RBD symptoms
20 in ORX^{-/-} are not underlined by a complete alteration of the SLD function.
21

22 Finally, the ORX^{-/-} group is a mix of sham mice: a third of them received no injection, a third
23 had the shCTRL injected in the SLD, the last third received the shCTRL in the VMM. We
24 found no treatment effect in all performed analyzes as illustrated in supplFig.1, except for the
25 percent of RSWA that was much higher in mice that had received shCTRL in the VMM than
26 other mice of the ORX^{-/-} group. When excluding those 3 mice, the percent of RSWA is still
27 significantly different between ORX^{-/-} and WT groups (p= 0.0007) and ORX^{-/-} and shGLUT
28 groups (p=0.00006), confirming the difference in severity between ORX^{-/-} and shGLUT mice.
29 Importantly, the mix of sham mice in the ORX^{-/-} does not explain the high inter-individual
30 variability seen in the ORX^{-/-} group except possibly for the measure of the RSWA percent
31 (supplFig.1).
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Proposition of mechanism underlying RBD symptoms in NT1

With this study, we demonstrate that chronic ORX-deficiency is sufficient to induce a RBD phenotype in ORX^{-/-} mice similar to clinical observations made on NT1 patients. Based on Feng et al. [49], it is tempting to propose that the chronic lack of ORX in NT1 may provoke the loss of an excitatory input onto the SLD during REM sleep, resulting in a reduced strength of the SLD output. Since it is a decrease in the strength of the SLD output and not its abolition as it would be with a lesion, the inhibitory input from the medulla to the spinal motoneurons is still present but less efficient. It then results on a moderate and non-systematic expression of RBD symptoms in NT1 patients. Indeed, this mechanism would explain why RBD symptoms have a sporadic occurrence in a given patient and are not experienced by all NT1 patients [12]. This is of great interest because if our hypothesis is correct, the pharmacological replacement of ORX at the right dose, should suppress RBD symptoms in NT1 patients.

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REFERENCE LIST

1. Peyron C, Arthaud S, Villalba M, P. F: Defining and measuring paradoxical (REM) sleep in animal models of sleep disorders. *Current opinion in Physiology* 2020, 15:203-209.
2. Hodes R, Dement WC: Depression of Electrically Induced Reflexes ("H-Reflexes") in Man during Low Voltage Eeg "Sleep". *Electroencephalogr Clin Neurophysiol* 1964, 17:617-629.
3. Soja PJ, Lopez-Rodriguez F, Morales FR, Chase MH: The postsynaptic inhibitory control of lumbar motoneurons during the atonia of active sleep: effect of strychnine on motoneuron properties. *J Neurosci* 1991, 11:2804-2811.
4. Morales FR, Chase MH: Intracellular recording of lumbar motoneuron membrane potential during sleep and wakefulness. *Exp Neurol* 1978, 62:821-827.
5. Nakamura Y, Goldberg LJ, Chandler SH, Chase MH: Intracellular analysis of trigeminal motoneuron activity during sleep in the cat. *Science* 1978, 199:204-207.
6. Schenck CH, Bundlie SR, Ettinger MG, Mahowald MW: Chronic behavioral disorders of human REM sleep: a new category of parasomnia. *Sleep* 1986, 9:293-308.
7. Boucetta S, Salimi A, Dadar M, Jones BE, Collins DL, Dang-Vu TT: Structural Brain Alterations Associated with Rapid Eye Movement Sleep Behavior Disorder in Parkinson's Disease. *Sci Rep* 2016, 6:26782.
8. Iranzo A, Santamaria J, Tolosa E: The clinical and pathophysiological relevance of REM sleep behavior disorder in neurodegenerative diseases. *Sleep Med Rev* 2009, 13:385-401.
9. Hogl B, Stefani A, Videnovic A: Idiopathic REM sleep behaviour disorder and neurodegeneration - an update. *Nat Rev Neurol* 2018, 14:40-55.

10. Bassetti CLA, Adamantidis A, Burdakov D, Han F, Gay S, Kallweit U, Khatami R, Koning F, Kornum BR, Lammers GJ, et al.: Narcolepsy - clinical spectrum, aetiopathophysiology, diagnosis and treatment. *Nat Rev Neurol* 2019, 15:519-539.
11. Dauvilliers Y, Jennum P, Plazzi G: Rapid eye movement sleep behavior disorder and rapid eye movement sleep without atonia in narcolepsy. *Sleep Med* 2013, 14:775-781.
12. Antelmi E, Pizza F, Franceschini C, Ferri R, Plazzi G: REM sleep behavior disorder in narcolepsy: A secondary form or an intrinsic feature? *Sleep Med Rev* 2020, 50:101254.
13. Schenck CH, Mahowald MW: Motor dyscontrol in narcolepsy: rapid-eye-movement (REM) sleep without atonia and REM sleep behavior disorder. *Ann Neurol* 1992, 32:3-10.
14. Mattarozzi K, Bellucci C, Campi C, Cipolli C, Ferri R, Franceschini C, Mazzetti M, Russo PM, Vandi S, Vignatelli L, et al.: Clinical, behavioural and polysomnographic correlates of cataplexy in patients with narcolepsy/cataplexy. *Sleep Med* 2008, 9:425-433.
15. Antelmi E, Pizza F, Donadio V, Filardi M, Sosero YL, Incensi A, Vandi S, Moresco M, Ferri R, Marelli S, et al.: Biomarkers for REM sleep behavior disorder in idiopathic and narcoleptic patients. *Ann Clin Transl Neurol* 2019, 6:1872-1876.
16. Jennum PJ, Ostergaard Pedersen L, Czarna Bahl JM, Modvig S, Fog K, Holm A, Rahbek Kornum B, Gammeltoft S: Cerebrospinal Fluid Biomarkers of Neurodegeneration Are Decreased or Normal in Narcolepsy. *Sleep* 2017, 40. doi: 10.1093/sleep/zsw006.
17. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, et al.: A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 2000, 6:991-997.
18. Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM: Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 2000, 27:469-474.

- 1
2
3 19. Knudsen S, Gammeltoft S, Jennum PJ: Rapid eye movement sleep behaviour disorder in
4 patients with narcolepsy is associated with hypocretin-1 deficiency. *Brain* 2010,
5 133:568-579.
6
7
8
9
- 10 20. Mileykovskiy BY, Kiyashchenko LI, Siegel JM: Behavioral correlates of activity in
11 identified hypocretin/orexin neurons. *Neuron* 2005, 46:787-798.
12
13
- 14 21. Lee MG, Hassani OK, Jones BE: Discharge of identified orexin/hypocretin neurons across
15 the sleep-waking cycle. *J Neurosci* 2005, 25:6716-6720.
16
17
18
- 19 22. Roman A, Meftah S, Arthaud S, Luppi PH, Peyron C: The inappropriate occurrence of rapid
20 eye movement sleep in narcolepsy is not due to a defect in homeostatic regulation of
21 rapid eye movement sleep. *Sleep* 2018, 41. doi: 10.1093/sleep/zsy046.
22
23
24
- 25 23. Takahashi K, Lin JS, Sakai K: Neuronal activity of orexin and non-orexin waking-active
26 neurons during wake-sleep states in the mouse. *Neuroscience* 2008, 153:860-870.
27
28
29
- 30 24. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA,
31 Williams SC, Xiong Y, Kisanuki Y, et al.: Narcolepsy in orexin knockout mice:
32 molecular genetics of sleep regulation. *Cell* 1999, 98:437-451.
33
34
35
36
- 37 25. Valencia Garcia S, Libourel PA, Lazarus M, Grassi D, Luppi PH, Fort P: Genetic
38 inactivation of glutamate neurons in the rat sublaterodorsal tegmental nucleus
39 recapitulates REM sleep behaviour disorder. *Brain* 2017, 140:414-428.
40
41
42
43
- 44 26. Franken P, Malafosse A, Tafti M: Genetic determinants of sleep regulation in inbred mice.
45 *Sleep* 1999, 22:155-169.
46
47
48
- 49 27. Clement O, Sapin E, Berod A, Fort P, Luppi PH: Evidence that neurons of the
50 sublaterodorsal tegmental nucleus triggering paradoxical (REM) sleep are
51 glutamatergic. *Sleep* 2011, 34:419-423.
52
53
54
- 55 28. team Rc: R: A language and environment for statistical computing. . Edited by: R
56 Foundation for statistical computing, Vienna Austria; 2022.
57
58
59
60

- 1
2
3 29. Fox J, Bouchet-Valat M: Rcmdr: R commander., edn 2.8-0. Edited by; 2022:R package.
4
5 30. Pohlert T: PMCMRplus: Calculate pairwise multiple comparisons of mean rank sums
6
7 extended. edn 1.9.6. Edited by; 2022:R package.
8
9
10 31. Kruskal WH, Wallis WA: Use of ranks in one-criterion variance analysis. Journal of the
11
12 American Statistical association 1952, 47:583-621.
13
14 32. Conover WJ, Iman RL: On multiple comparisons procedures. Edited by. Tech. Rep. LA-
15
16 7677-MS, Los Alamos scientific laboratory; 1979.
17
18 33. Holm S: A simple sequentially rejective multiple test procedure. Scandinavian journal of
19
20 statistics 1979, 6:65-70.
21
22 34. Brown MB, Forsythe AB: Robust tests for the equality of variances. Journal of the
23
24 American Statistical association 1974, 69:364-367.
25
26 35. Boissard R, Gervasoni D, Schmidt MH, Barbagli B, Fort P, Luppi PH: The rat ponto-
27
28 medullary network responsible for paradoxical sleep onset and maintenance: a
29
30 combined microinjection and functional neuroanatomical study. Eur J Neurosci 2002,
31
32 16:1959-1973.
33
34 36. Krenzer M, Anacleto C, Vetrivelan R, Wang N, Vong L, Lowell BB, Fuller PM, Lu J:
35
36 Brainstem and spinal cord circuitry regulating REM sleep and muscle atonia. PLoS One
37
38 2011, 6:e24998.
39
40 37. Arrigoni E, Chen MC, Fuller PM: The anatomical, cellular and synaptic basis of motor
41
42 atonia during rapid eye movement sleep. J Physiol 2016, 594:5391-5414.
43
44 38. Silvani A, Ferri R, Lo Martire V, Bastianini S, Berteotti C, Salvade A, Plazzi G, Zucconi
45
46 M, Ferini-Strambi L, Bassetti CL, et al.: Muscle Activity During Sleep in Human
47
48 Subjects, Rats, and Mice: Towards Translational Models of REM Sleep Without Atonia.
49
50 Sleep 2017, 40. doi: 10.1093/sleep/zsx029.
51
52
53
54
55
56
57
58
59
60

- 1
2
3 39. Kaur S, Thankachan S, Begum S, Liu M, Blanco-Centurion C, Shiromani PJ: Hypocretin-
4 2 saporin lesions of the ventrolateral periaqueductal gray (vlPAG) increase REM sleep
5 in hypocretin knockout mice. *PLoS One* 2009, 4:e6346.
6
7
8
9
10 40. Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L: Neural substrates of
11 awakening probed with optogenetic control of hypocretin neurons. *Nature* 2007,
12 450:420-424.
13
14
15
16 41. Bourgin P, Huitron-Resendiz S, Spier AD, Fabre V, Morte B, Criado JR, Sutcliffe JG,
17 Henriksen SJ, de Lecea L: Hypocretin-1 modulates rapid eye movement sleep through
18 activation of locus coeruleus neurons. *J Neurosci* 2000, 20:7760-7765.
19
20
21
22
23 42. Tsunematsu T, Kilduff TS, Boyden ES, Takahashi S, Tominaga M, Yamanaka A: Acute
24 optogenetic silencing of orexin/hypocretin neurons induces slow-wave sleep in mice. *J*
25 *Neurosci* 2011, 31:10529-10539.
26
27
28
29
30 43. Sasaki K, Suzuki M, Mieda M, Tsujino N, Roth B, Sakurai T: Pharmacogenetic modulation
31 of orexin neurons alters sleep/wakefulness states in mice. *PLoS One* 2011, 6:e20360.
32
33
34
35 44. Torontali ZA, Fraigne JJ, Sanghera P, Horner R, Peever J: The Sublaterodorsal Tegmental
36 Nucleus Functions to Couple Brain State and Motor Activity during REM Sleep and
37 Wakefulness. *Curr Biol* 2019, 29:3803-3813 e3805.
38
39
40
41 45. Sulaman BA, Wang S, Tyan J, Eban-Rothschild A: Neuro-orchestration of sleep and
42 wakefulness. *Nat Neurosci* 2023, 26:196-212.
43
44
45
46 46. Valencia Garcia S, Brischoux F, Clement O, Libourel PA, Arthaud S, Lazarus M, Luppi
47 PH, Fort P: Ventromedial medulla inhibitory neuron inactivation induces REM sleep
48 without atonia and REM sleep behavior disorder. *Nat Commun* 2018, 9:504.
49
50
51
52 47. Valencia Garcia S, Luppi PH, Fort P: A Particular Medullary-Spinal Inhibitory Pathway is
53 Recruited for the Expression of Muscle Atonia During REM Sleep. *J Exp Neurosci* 2018,
54 12:1179069518808744.
55
56
57
58
59
60

- 1
2
3 48. Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS:
4
5 Neurons containing hypocretin (orexin) project to multiple neuronal systems. J
6
7 Neurosci 1998, 18:9996-10015.
8
9
- 10 49. Feng H, Wen SY, Qiao QC, Pang YJ, Wang SY, Li HY, Cai J, Zhang KX, Chen J, Hu ZA,
11
12 et al.: Orexin signaling modulates synchronized excitation in the sublaterodorsal
13
14 tegmental nucleus to stabilize REM sleep. Nat Commun 2020, 11:3661.
15
16
- 17 50. Kiyashchenko LI, Mileykovskiy BY, Lai YY, Siegel JM: Increased and decreased muscle
18
19 tone with orexin (hypocretin) microinjections in the locus coeruleus and pontine
20
21 inhibitory area. J Neurophysiol 2001, 85:2008-2016.
22
23
- 24 51. Mileykovskiy BY, Kiyashchenko LI, Siegel JM: Muscle tone facilitation and inhibition
25
26 after orexin-a (hypocretin-1) microinjections into the medial medulla. J Neurophysiol
27
28 2002, 87:2480-2489.
29
30
- 31 52. Uchida S, Soya S, Saito YC, Hirano A, Koga K, Tsuda M, Abe M, Sakimura K, Sakurai T:
32
33 A Discrete Glycinergic Neuronal Population in the Ventromedial Medulla That Induces
34
35 Muscle Atonia during REM Sleep and Cataplexy in Mice. J Neurosci 2021, 41:1582-
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37 1596.
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LEGENDS

Figure 1. Quantification of vigilance states during the light phase.

A-C, Total amount of time (min) spent in Wake, NREM sleep and REM sleep. **D-F**, Total number of Wake, NREM sleep and REM sleep episodes. **G-I**, Mean duration (sec) of Wake, NREM sleep and REM sleep bouts. Graphs represent data obtained on wild-type mice (WT), and narcoleptic mice without (ORX^{-/-}) and with (shGLUT) impairment of glutamatergic transmission of the SLD. Each symbol represents the value of an individual mouse. Box plots show the median value and the interquartile range for each experimental group. Statistics was performed using Kruskal-Wallis test followed by Conover post-hoc comparison and Holm correction. Significance was set at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2. Representative EEG and EMG signals for mice of the WT, ORX^{-/-} and shGLUT groups. In the representative WT mouse, the EMG signal is unaltered with a decrease in amplitude in REM sleep compared to prior NREM sleep. The REM sleep EMG signal of the representative ORX^{-/-} mouse shows a mild alteration with an increase amplitude for the tonic component and the presence of phasic events. The representative shGLUT mouse shows a major alteration of the EMG signal during REM sleep.

Figure 3. Quantification of EMG phasic events during REM sleep.

A, Percentage of REM sleep episodes with at least one phasic event detected. **B**, Density in phasic events (number of phasic events per minute of REM sleep). **C**, Density of phasic events by subcategories defined on the basis of the phasic event duration (short: < 1 sec ; medium : 1–5 sec ; long: >5 sec). **D**, Total REM sleep time represented by phasic activity, expressed as percentage of the total amount of REM sleep. **E**, Percentage of REM sleep episode with presence of phasic events during more than 75% of the REM sleep episode duration. Graphs

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3 represent data obtained on wild-type mice (WT) and narcoleptic mice without (ORX^{-/-}) and
4 with (shGLUT) impairment of glutamatergic transmission of the SLD. Each symbol represents
5 the value of an individual mouse. Histograms show the median value for each group with the
6 interquartile range. Statistics comparison was performed using the Conover post-hoc
7 comparison (with Holm correction) after a Kruskal-Wallis test. Significance is set at * p < 0.05 ;
8 ** p < 0.01 ; *** p < 0.001.

19 **Figure 4. Quantification of EMG tonic component during REM sleep**

21 **A**, EMG tonic ratio illustrating the difference in muscle tone during REM sleep compared to
22 NREM sleep ($REM\ sleep\ tone / NREM\ sleep\ tone$). This analysis was performed for each of
23 NREM-REM sleep pair, and the median value of this ratio was obtained for each animal. Values
24 displayed are the median and quartiles of the EMG tonic ratio obtained for each experimental
25 group. **B**, Percentage of REM sleep episode without atonia (RSWA). RSWA is defined by an
26 EMG tonic ratio above 1. Graphs represent data obtained on wild-type mice (WT) and
27 narcoleptic mice without (ORX^{-/-}) and with (shGLUT) impairment of glutamatergic
28 transmission of the SLD. Each symbol represents the value of an individual mouse and box
29 plots report the median value and the interquartile range for each group. Statistic comparison
30 was performed using a Kruskal-Wallis test followed by the Conover post-hoc comparison with
31 Holm correction. Significance is set at * p < 0.05 ; ** p < 0.01 ; *** p < 0.001.

49 **Figure 5. Histological validation of the shGLUT mouse model**

51 **A – Schematic drawings illustrating the extent of shGLUT injections sites. B (top panels)-**
52 Low power photomicrographs of mCherry fluorescent signal after shRNA transfection in
53 representative control (shCTRL) and altered (shGLUT) mice. Scale bar: 150µm. B (bottom
54 panel) – **B (Mid- panels)**, Low power and enlargement microphotographs illustrating coronal
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3 brain sections treated with VGluT2 *in situ hybridization* of a representative shCTRL (left) and
4 shGLUT (right) mice. Scale bars: 150 μ m (low power) and 80 μ m (enlargement). **B (lower**
5 **panels), Low power and enlargement microphotographs illustrating coronal brain sections**
6 **treated with VGAT *in situ hybridization* of a representative shCTRL (left) and shGLUT (right)**
7 **mice. Scale bars: 150 μ m (low power) and 80 μ m (enlargement). C-** Confocal images of the
8 ventromedial medulla region (VMM) representing the expression of VGluT2 proteins (green),
9 mCherry (red) and DAPI (blue). On the merge image, co-expression of vGluT2 and mCherry
10 (white arrowheads) indicate axons coming from the SLD neurons and of glutamatergic nature.
11 Note that co-expression is seen only in control condition (shCTRL). None are observed in
12 animals treated with shGLUT demonstrating that VGluT2 proteins are absent and glutamatergic
13 transmission impaired. Scale bar: 10 μ m.

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28 *Abbreviations:* Aq, aqueduct ; DTg, dorsal tegmental nucleus ; LDT, laterodorsal tegmental
29 nucleus ; LPB, lateral parabrachial nucleus ; Mo5, motor trigeminal nucleus ; MPB, medial
30 parabrachial nucleus ; PnC, Pontine caudalis area ; SLD, sublaterodorsal nucleus ; VLPAG,
31 ventrolateral periaqueductal gray.

32 33 34 35 36 37 38 39 **Table 1. Summary of statistical results**

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42 Statistical results with p-value obtained for data on Figure 1, Figure 3 and Figure 4. Each p-
43 value of Conover test column are associated with a label for post-hoc groups comparison: 1 for
44 WT vs ORX^{-/-} ; 2 for WT vs shGLUT ; 3 for ORX^{-/-} vs shGLUT. Significance was set at * p <
45 0.05 ; ** p < 0.01 ; *** p < 0.001.

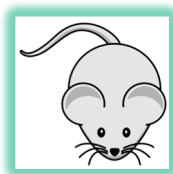
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54 **Supplementary figure 1: Illustration of the distribution of individual mice of the ORX^{-/-}**
55 **group using a color-code to identify each subgroup of mice.**

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3 **A-C**, Total amount of minutes spent in Wake, in NREM sleep and in REM sleep over the 12hrs
4 of the light phase. **D-F**, Total number of episodes of Wake, NREM sleep, and REM sleep. **G-**
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6 **I**, Mean duration in seconds of Wake, NREM sleep, and REM sleep episodes. **K**, EMG tonic
7 ratio representing the evolution of muscle tone during REM sleep compared to NREM sleep.
8 Values displayed are the median of EMG tonic ratio obtained for each REM sleep episodes. **L**,
9 Percentage of REM sleep episode without atonia (RSWA). RSWA are defined by an EMG
10 tonic ratio above 1. **M**, Percentage of REM sleep episode with at least one phasic event detected
11 (100% is the total number of REM sleep episodes for which the EMG analysis was carried out).
12 **N**, Phasic events density, defined as the number of phasic events per minutes of REM sleep. **O**,
13 Phasic events density for each category of phasic events (short duration : < 1 sec ; mid duration :
14 1 sec – 5 sec ; long duration : 5 sec). Graphs represent data obtained from mice of the ORX^{-/-}
15 group. Dark blue squares represent ORX^{-/-} mice with no viral injection (n=4), red squares the
16 ORX^{-/-} mice with injection of shCTRL in the SLD (n=3) and the light pink squares the ORX^{-/-}
17 mice with injection of shCTRL in VMM.
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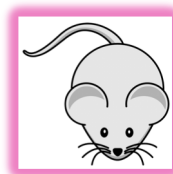
Major alteration of motor control during REM sleep in mouse models of sleep disorders



Wild-type (WT)



ORX-/-
(Model for narcolepsy type 1, NT1)



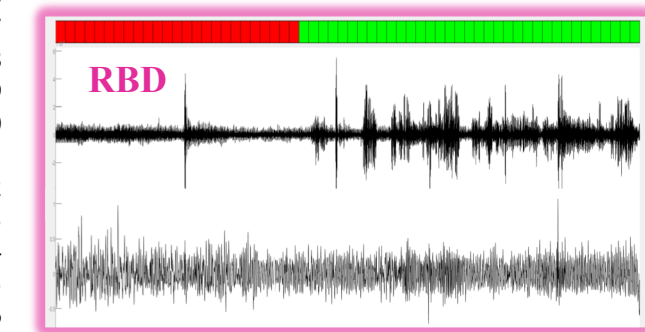
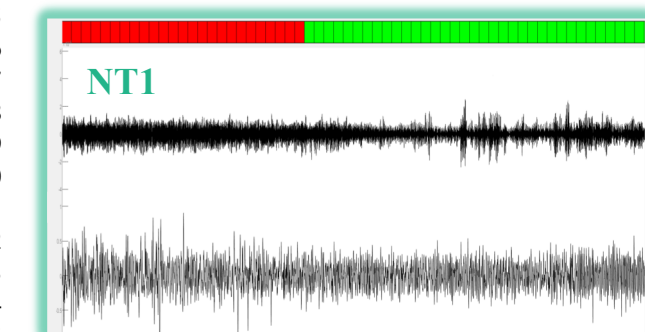
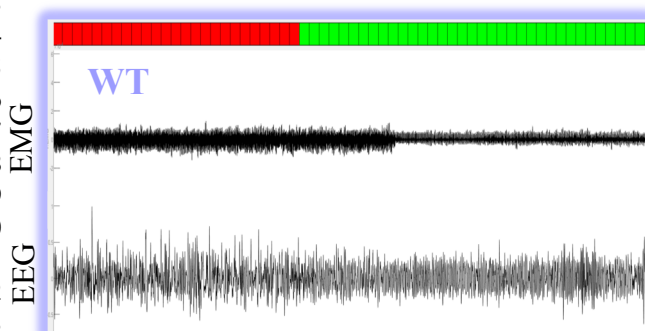
shGLUT
(Model for REM sleep behavior disorder, RBD)

Motor control alteration in REM sleep

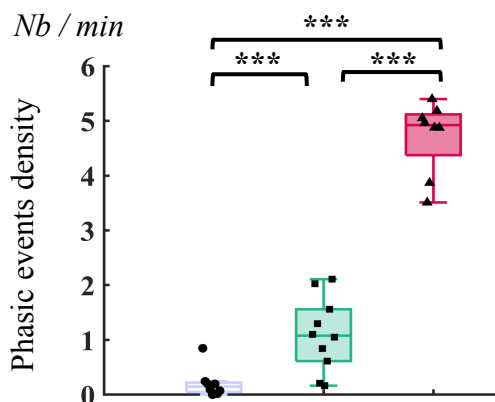
EMG / EEG signals

NREM sleep

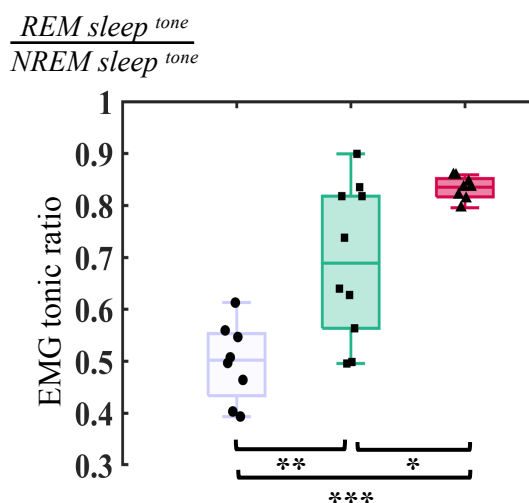
REM sleep



Phasic activity during REM sleep



Tonic activity during REM sleep



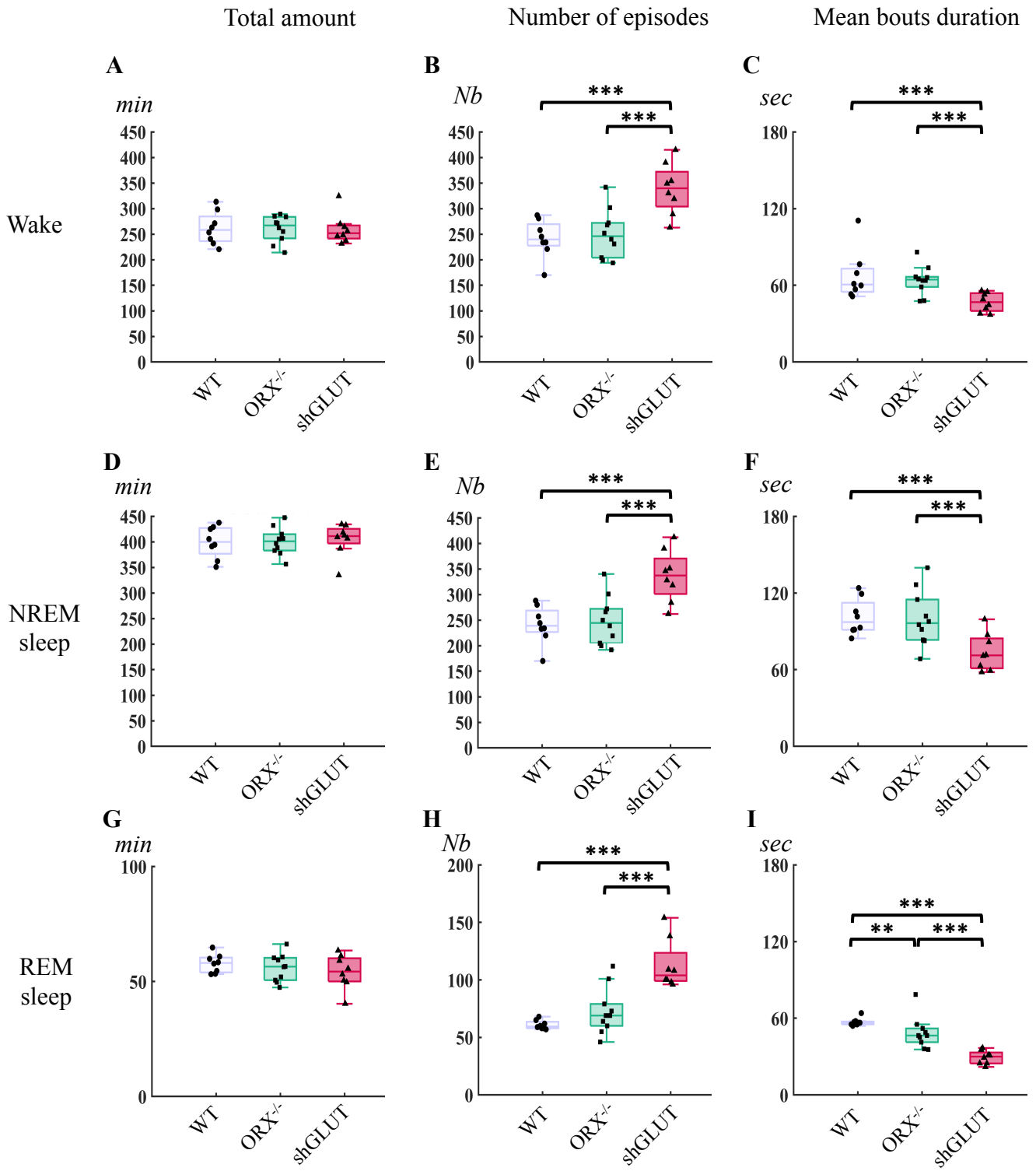


Figure 1

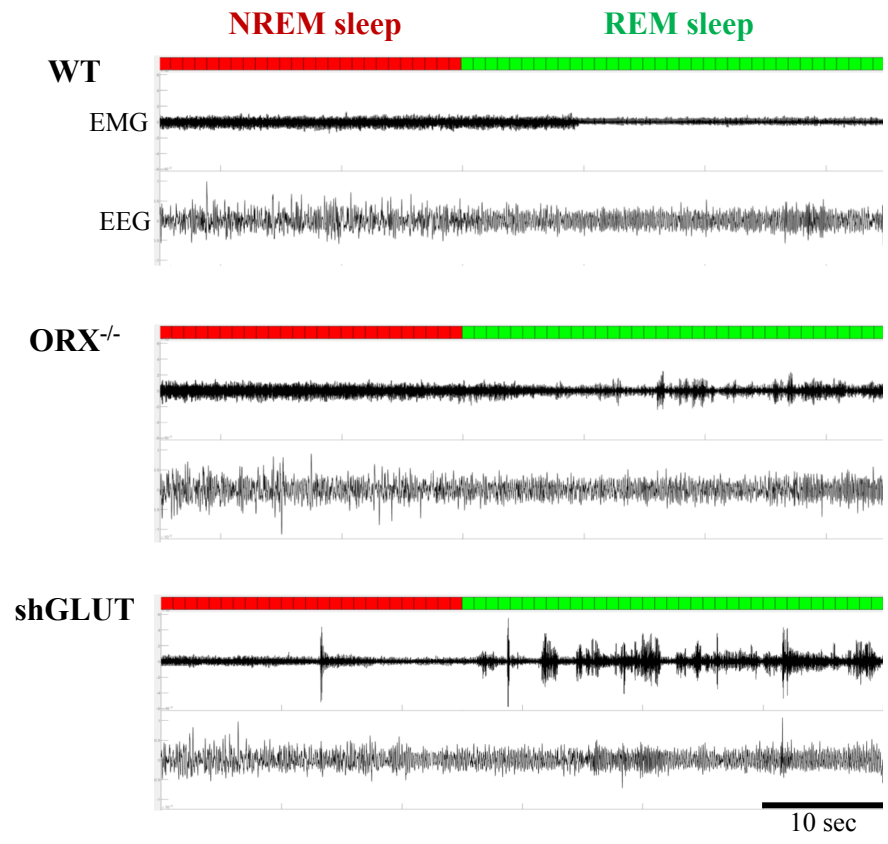


Figure 2

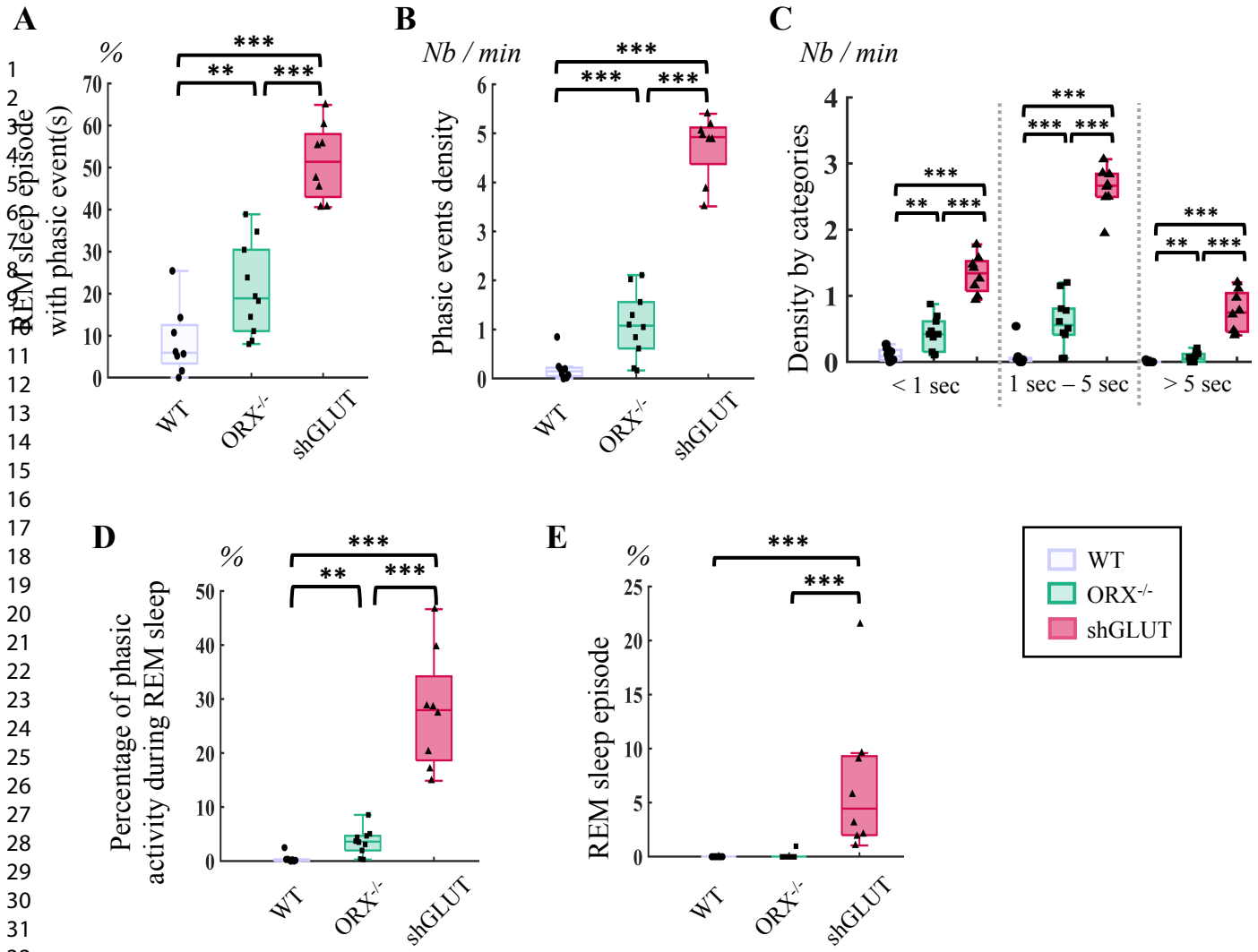


Figure 3

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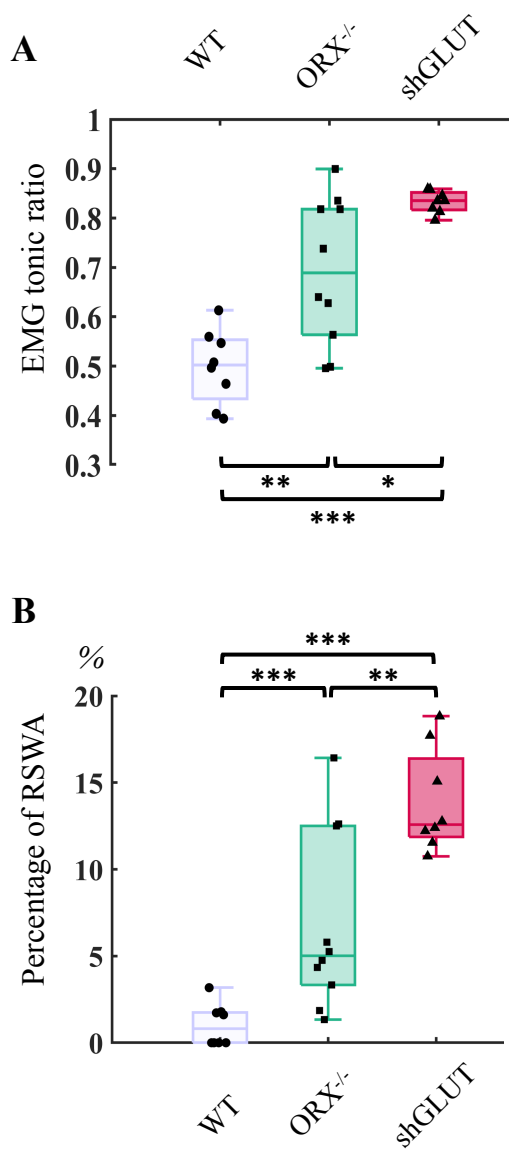
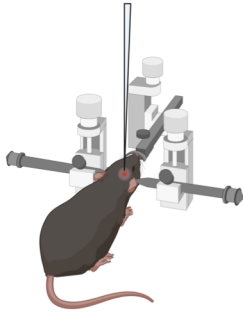
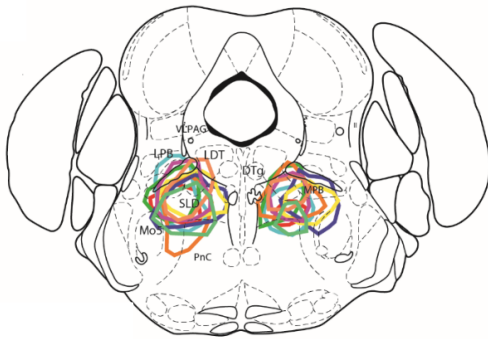


Figure 4

AAV10-shGLUT-mCherry or
AAV10-shCTRL-mCherry

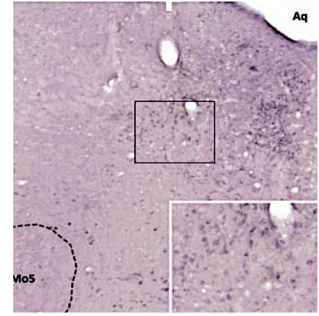
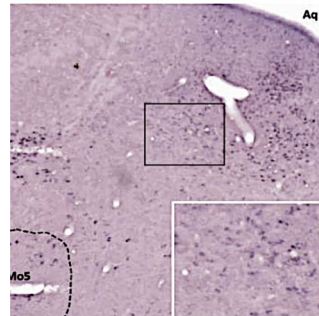
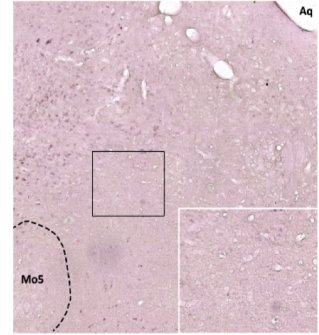
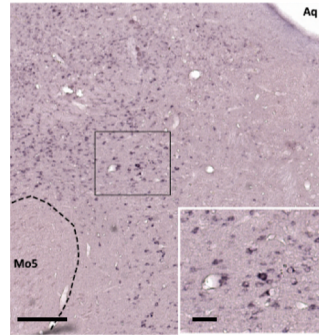
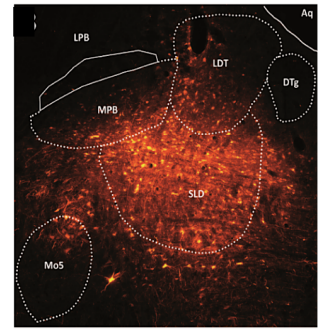
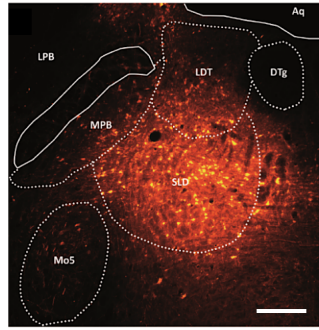


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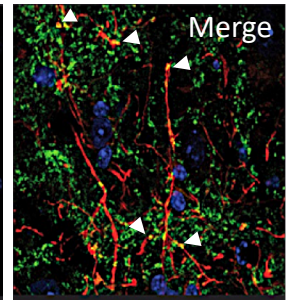
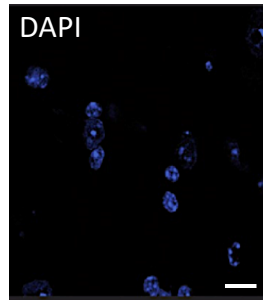
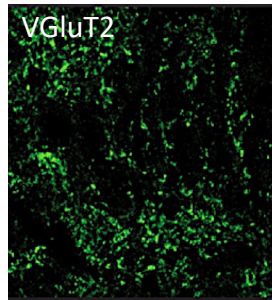
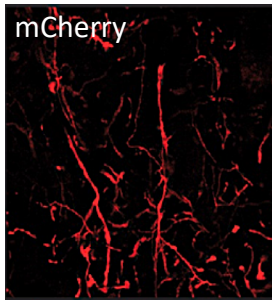
VGLuT2

VGAT



C

shCTRL



shGLUT

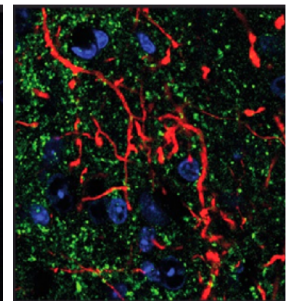
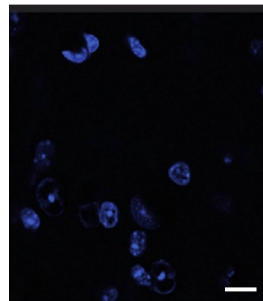
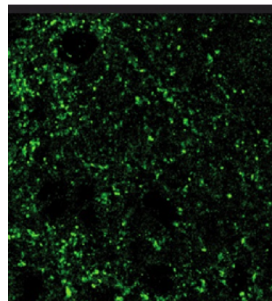
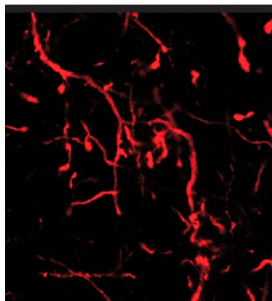
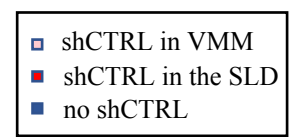
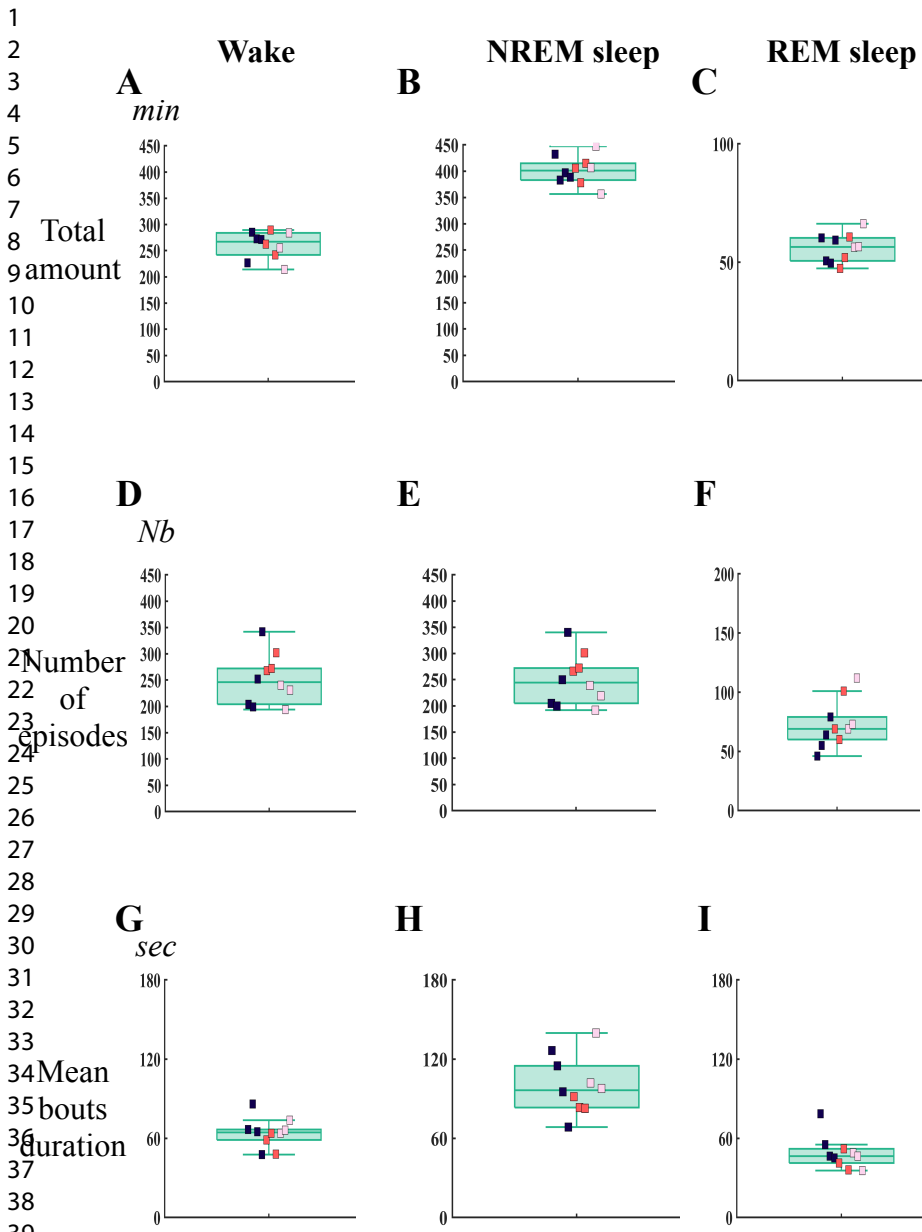


Figure 5

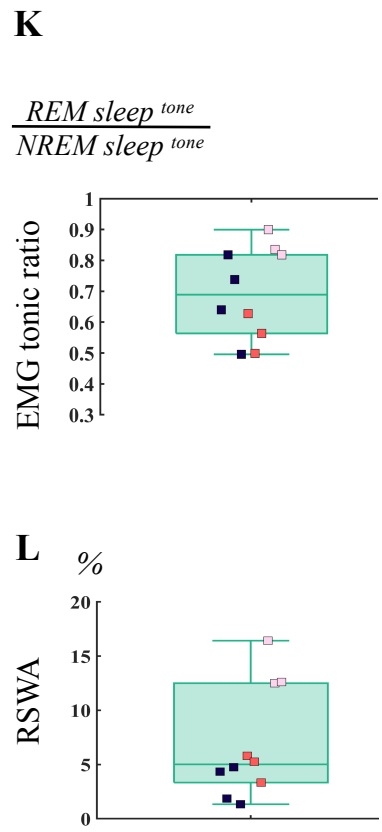
Variable	Figure	Kruskal-Wallis test	Post-hoc comparison	Post-hoc test of Conover (Holm correction)
Total amount of Wake	1A	0.9042	/	/
Number of Wake episode	1B	0.0025	1	0.7178
			2	0.0014 **
			3	0.0014 **
Mean duration of Wake episodes	1C	0.0046	1	0.9862
			2	0.0036 **
			3	0.0032 **
Total amount of NREM sleep	1D	0.7347	/	/
Number of NREM sleep episodes	1E	0.0031	1	0.8170
			2	0.0022 **
			3	0.0022 **
Mean duration of NREM sleep episodes	1F	0.0090	1	0.7345
			2	0.0086 **
			3	0.0086 **
Total amount of REM sleep	1G	0.5399	/	/
Number of REM sleep episodes	1H	0.0009	1	0.0519
			2	< 0.0001 ***
			3	0.0035 **
Mean duration of REM sleep bouts	1I	< 0.0001	1	0.0012 **
			2	< 0.0001 ***
			3	< 0.0001 ***
Percentage of REM sleep episodes with phasic event(s)	2A	< 0.0001	1	0.0024 **
			2	< 0.0001 ***
			3	< 0.0001 ***
Phasic events density	2B	< 0.0001	1	0.0003 ***
			2	< 0.0001 ***
			3	< 0.0001 ***
Density of phasic events, <1sec	2C_left	< 0.0001	1	0.0015 **
			2	< 0.0001 ***
			3	< 0.0001 ***
Density of phasic events, 1-5sec	2C_middle	< 0.0001	1	0.0002 ***
			2	< 0.0001 ***
			3	< 0.0001 ***
Density of phasic events, >5sec	2C_right	< 0.0001	1	0.0043 **
			2	< 0.0001 ***
			3	< 0.0001 ***
Percentage of phasic activity during REM sleep	2D	< 0.0001	1	< 0.0001 ***
			2	< 0.0001 ***
			3	< 0.0001 ***
Percentage of REM sleep episode	2E	< 0.0001	1	0.4047
			2	< 0.0001 ***
			3	< 0.0001 ***
EMG tonic ratio	3A	0.0006	1	0.0039 **
			2	< 0.0001 ***
			3	0.0179 *
Percentage of RSWA	3B	0.0002	1	0.0005 ***
			2	< 0.0001 ***
			3	0.0069 **

Table 1. Summary of statistical results <https://doi.org/10.1177/0898010121101111>

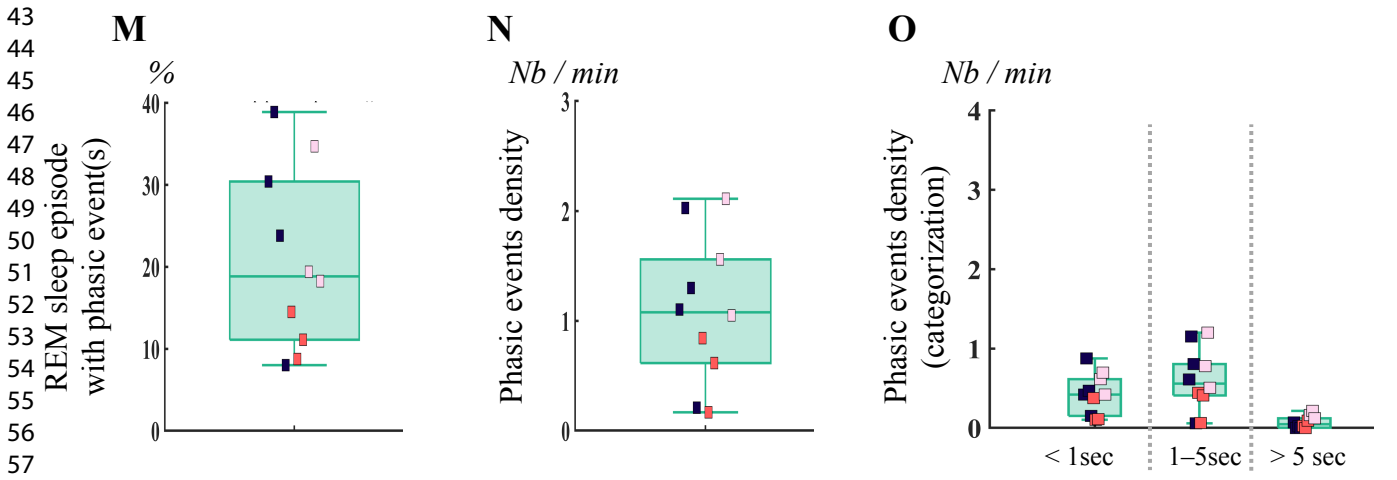
I. Vigilance states



II. Tonic component of the EMG signal



III. Phasic components of EMG



Supplementary Figure 1.