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**Targeted recombination in active populations (TRAP) as a new mice genetic model to study sleep-active neuronal populations: demonstration that Lhx6+ neurons in the ventral zona incerta are activated during paradoxical sleep hypersomnia**

**Running title:** Lhx6+ neurons activated during PS hypersomnia

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## SUMMARY

The cFos immunostaining allowed the identification of multiple populations of neurons involved in the generation of paradoxical sleep (PS). We adopted in this study new transgenic (so-called targeted recombination in active populations, TRAP) mice which allow expression of a reporter gene in a Cre-dependent manner by means of injecting tamoxifen, thus inducing mCherry in the neurons which express cFos during waking (W) or PS hypersomnia following automatic PS deprivation. Three groups of mice were subjected (one week apart) to two periods of W (i.e., W-W), one period of W and one of PS hypersomnia (W-PS), or two periods of PS hypersomnia (PS-PS). A high percentage of double-labeled neurons was observed in W-W and PS-PS mice, but not in W-PS animals. MCH neurons in the lateral hypothalamus (LH) and LIM homeobox 6 (Lhx6)-immunoreactive cells in the zona incerta (ZI) constituted  $5.7\pm 1.5\%$  and  $8.8\pm 2.3\%$  of all mCherry and  $20.6\pm 4.8\%$  and  $24.6\pm 5.9\%$  of all cFos in PS-PS animals. In addition, MCH and Lhx6+ neurons rarely expressed mCherry (or Fos) in the W condition in contrast to orexin neurons which constituted approximately 30% of mCherry and cFos neurons. Our results validate the TRAP methodology and open the way to use it for identifying the neurons activated during W and PS hypersomnia. Furthermore, they indicate for the first time that Lhx6+ neurons in the ZI, like MCH cells in the LH, are activated during PS hypersomnia but not during W. These results indicate that Lhx6+ neurons might play a role in the control of PS like the MCH cells.

**Keywords:** sleep-wake cycle, hypothalamic neuropeptides, paradoxical sleep hypersomnia

## INTRODUCTION

The cFos immunostaining has long been utilized to identify the neurons activated during waking (W), slow wave sleep (SWS) and paradoxical (i.e., rapid eye movement, REM) sleep (PS). By this means, we and others (Verret et al., 2003; Gao, 2009; Jengo et al., 2013) identified multiple populations of neurons playing a role in the generation of PS like the melanin-concentrating hormone (MCH) cells located in the lateral hypothalamus (LH) and zona incerta (ZI). It has been later confirmed using unit recordings that these neurons are specifically active during PS and that their activation induces an increase in PS quantities (Hassani et al., 2009; Peever and Fuller, 2016; Blanco-Centurion et al., 2019). These results validated the strategy of using the cFos staining to identify populations of neurons controlling PS. However, the vast majority of cFos-labeled neurons localized in the LH and ZI after PS hypersomnia have not been functionally characterized although it has been shown that a large number expresses GAD67 and are thus GABAergic (Sapin et al., 2009; 2010; Brown et al., 2017). However, optogenetic stimulation of all GABAergic neurons in the region induced waking rather than PS likely because part of the GABA neurons in the LH and ZI are wake-inducing (Herrera et al., 2016). Interestingly, it has been recently reported that GABAergic neurons co-containing LIM homeobox 6 (Lhx6) in the ventral ZI express cFos at the end of the dark period or during total sleep deprivation and that their activation (or inhibition) increases (or decreases) SWS and PS quantities (Liu et al., 2017).

Although cFos immunostaining allowed the identification of neurons activated during PS in the LH, ZI, and many other structures, the identification of their role was strongly limited due to the lack of specific markers. A new genetic strategy, so-called targeted recombination in active populations (TRAP), might be an ideal choice to study these neurons (Guenthner et al., 2013; DeNardo and Luo, 2017). The TRAP mice have been indeed already used to characterize neurons involved in sensory coding, motor behavior, memory, valence-encoding, and SWS genesis (Allen et al., 2017; Girasole et al., 2018; Tasaka et al., 2018; DeNardo et al., 2019; Zhang et al., 2019). The striking advantage of such transgenic mice is that they allow Cre-dependent reporter constructs in the neurons expressing cFos at a given time by means of injecting 4-OH-tamoxifen (4-OHT). In particular, permanent Golgi-like labeling (using mCherry as a reporter gene) of previously activated neurons can be obtained, which can be combined with cFos labeling if the animals were perfused right after the second stimulus which occurred days (or weeks) after the first stimulus.

The aim of the present study was to validate the TRAP methodology in studying the role of LH and ZI neurons activated during PS hypersomnia and waking. To this aim, we allowed the expression of mCherry and cFos in three groups of mice. The first group was put twice (one-week apart) in an open field. It received 4-OHT after the first W period and was perfused at the end of the second W period. By this means, we were able to determine whether the same cells were labeled with mCherry and cFos. We also combined staining of both markers with that of orexin (ORX) to confirm that the

labeled neurons were W-active neurons. In the second group, we performed two (one-week apart) PS deprivation-rebound (PSR) procedures, which allowed us to show whether mCherry-labeled neurons were expressing cFos during the second PS hypersomnia. We verified that these neurons might correspond to neurons activated during PS hypersomnia by combining mCherry/cFos-labeling with MCH staining. The third group of mice was first exposed to the open field and then one week later before perfusion exposed to PSR. Double-labeling in these mice allowed us to confirm that neurons activated during W were not activated during PS hypersomnia. We finally determined whether Lhx6+ neurons are active during W or PS hypersomnia.

## METHODS

### Animals

We followed EU as well as French guidelines for animal care in scientific research. The experimental protocol was approved by the research ethical committee of University-Lyon 1. Mice were housed in groups of two before surgery and in individual barrels afterwards. Animals were maintained at  $22\pm 1^\circ\text{C}$  under 12 hr light-dark cycle (lights on from 8 am to 8 pm) with free access to food and water. A new knock-in mice (*TRAP2-RED*) for activity-dependent genetic labeling were kindly donated by Dr. Liqun Luo from Stanford University. Briefly, *Fos*<sup>2A-iCreER/+</sup> (*TRAP2*) mice were crossed to *R26*<sup>AI14/+</sup> (*AI14*) mice to obtain double heterozygous (*TRAP2;Ai14*, *TRAP-RED*) mice (Allen et al., 2017; DeNardo et al., 2019). Activity-dependent expression of double transgenes is described in Fig. 1A. Animals at 8-12 weeks of age (n=21; 25-30 g) were utilized, where every effort was made to minimize the number of mice sacrificed (W-W, n=3; W-PSR, n=8; PSR-PSR, n=10). Among them, four W-PSR (M1270, 1283, M1301 and 1361) and three PSR-PSR (M1268, 1269, and 1312) mice were selected. The case (M1301) was added to the analysis since we ran out of tissue sections from the three cases of W-PSR (M1270, 1283, and 1361) due to our previous TRAP-related studies. Criteria of inclusion for analysis were mice which exhibit at least 24% of PS amount during the first 30 min period among 2-hr PS hypersomnia; after perfusion, these animals did exhibit optimal cFos and mCherry staining (Figs. 1-5; Tables 1 and 2).

### Surgical procedures

A mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) was injected intraperitoneally to induce anesthesia. Using a stereotaxic device, three electroencephalogram (EEG) electrodes were implanted; two above the frontal (1 mm lateral to midline and 3 mm anterior to bregma) and parietal (3 mm lateral to midline and -4 mm from bregma) cortices and the third above the cerebellum (3 mm lateral to midline and -12 mm from bregma) as reference. Electrodes for electromyogram (EMG) were inserted into neck muscles (Clement et al., 2011).

### **Polysomnography**

Animals were allowed to recover from surgery for 1 week in their home cage before being habituated to the recording conditions for 4 days. They were connected to a cable attached to a slip-ring commutator to allow free movement within the barrel. Unipolar EEG and bipolar EMG signals were amplified (MCP-PLUS, Alpha-Omega Engineering, Israel), digitized at 250 Hz, and transferred to Slip Analysis v 2.9.8 software. Vigilance states were scored using a 5-s window frame according to standard criteria (Libourel et al., 2015; Renouard et al., 2015).

### **Tamoxifen injection**

A stock solution (20mg/mL) of 4-OHT (Sigma, H6278) was prepared by adding 200-proof ethanol, vortexed, and placed on a shaker at 37°C for 15 mins until it dissolved. It was covered with aluminum foil to minimize light exposure. Working solution (10 mg/mL) was prepared in oil; the 4-OHT/ethanol mixture combined with corn oil (Sigma, C8267) was put in a speed-vac for 2-3 hrs until ethanol evaporated. The drug was delivered intraperitoneally based on the time schedule (Fig. 1B).

### **Protocols for PS deprivation and rebound and W induction**

PS was deprived by an automated method (Libourel et al., 2015), employing an online signal analysis with Spike2 (CED, UK) or Sleepscore (Viewpoint, France) software. Briefly, several discriminant parameters of EEG/EMG recordings were calculated based on manual scoring of the recordings in order to automatically assign a vigilance state (W, SWS or PS) for every 1-s epoch. When a PS episode was detected, a transistor–transistor logic (TTL) signal was sent to a stimulation box through a software developed under Matlab, waking up the animal by vertical shaking of the barrel floor via electromagnet. Automatic PS-deprivation for 48 hrs was highly efficient in mice, leaving a residual PS amount of only 2.2% (Arthaud et al., 2015). PS deprivation started at 10:00 am, when the PS amount is high. After 48 hrs of PS deprivation, mice were returned to their home barrel, where they could sleep *ad libitum* during 2 hr recovery period (Fig. 1B). In PSR-PSR animals, 4-OHT was injected after 2 hrs of PSR. One week later, the animals were subjected to PS deprivation and perfused after 2 hrs of PSR. In W-PSR or W-W animals, mice were placed during 2 hrs in a white open-field box (base, 45x45cm) containing a variety of toys. The animals were monitored via video camera and polysomnography (Libourel et al., 2015). Whenever asleep, they were awakened by slow re-positioning of toys or by gentle touch using a soft tissue. One week later, W-W animals were submitted to the same procedure while the W-PS ones were PS-deprived for 48 hrs and allowed to recover for 2 hrs before perfusion.

### **Perfusion and cryostat sectioning**

Mice were trans-cardially perfused with heparin-added saline and 4% of paraformaldehyde (0.01 M phosphate-buffered saline, PBS; pH 7.4). Brains were removed and stored in 30% sucrose until they settled to the bottom. Using a cryostat, eight consecutive series of hypothalamic sections (extending from the caudal pole of paraventricular nucleus to the rostral pole of tuberomammillary nucleus) were obtained at the thickness of 30  $\mu\text{m}$ , thus each well contained a series of 240  $\mu\text{m}$ -apart sections.

### **Immunofluorescence**

Sections were washed with PBS containing 0.3% Triton X-100 (PBST) and then incubated in 10% normal serum for 1 hr. They were then reacted with primary antibodies including rabbit anti-cFos (1:2,500; Millipore, ABE457), goat anti-MCH (1:250; Santa Cruz, 14509), goat anti-orexin (1:250; Santa Cruz, 8070), or mouse anti-Lhx6 (1:250; Santa Cruz, 271433) for 48 hrs (4°C). Following washes, sections were incubated in 1:500 dilution of AlexaFluor 488- or 647-conjugated secondary antibodies (Jackson ImmunoResearch) for 2 hrs. Sections were mounted, cover-slipped with Prolong Gold (Molecular Probes, P36930), and cured overnight in the dark (4°C). Positive control experiment for the immunostaining was performed by identifying positive immunoreaction in other brain regions which has been known to contain the antigen. Negative control was done by incubating sections in the increasing dilutions of primary antibodies until immunoreaction disappeared in the absence of the antibody.

### **Confocal microscopy and cell counting**

ZEN 2010 software was utilized with confocal laser scanning microscope (LSM 800), which was equipped with blue argon (488 nm), green helium neon (543 nm), and red helium neon (633 nm) lasers. In the SMART setup, 'best signal' was chosen. To obtain high-resolution images, specific frame size (i.e.,  $x*y = 1024x1024$ ) at speed 4 was selected with averaging process ( $n=4$ ). Double- or triple-labeled neurons were re-checked for genuine labeling using a single channel illumination. Optical section thickness was between 0.6-1.8  $\mu\text{m}$ . Tile scan was selected to obtain a wider view; three in the LH were taken for ORX or MCH neurons, while three in the ZI were taken for Lhx6+ cells. The counting tool in the Adobe Photoshop CS6 (64 Bit) was used; cells were considered labeled when they exhibited clear cytoplasmic (i.e., mCherry, MCH and ORX) or nuclear (i.e., cFos and Lhx6) morphology.

### **Data analysis**

The paired t-test was employed to analyze the values in Table 1, since the data were obtained from the same individuals, at the same time of the day, and on different days. The Mann-Whitney U test was employed to analyze the data shown in Table 2.

## **RESULTS**

The TRAP mice were used in this study to identify the neurons in the LH and ZI activated during W and PS hypersomnia. The methodology allowed us to label neurons expressing cFos at two different time points. The mCherry labeling (so-called TRAPing) was induced in activated neurons when 4-OHT was injected (Fig. 1A). Neurons were labeled with cFos when they were activated just before perfusion. Three protocols were used: PSR-PSR, W-PSR, and W-W (Fig. 1B). Based on polysomnographic data (Table 1), the efficiency of 48 hr PS deprivation was assessed as a mean of inducing PS hypersomnia (Fig. 1C). Seven representative PSR animals were selected for analysis; the proportions of PS amount during the first 30 min period varied from 0 to 17.78% in control condition, while they were between 24.17 and 53.06% during PSR condition (Table 1). PS amount was significantly higher in PSR (Fig. 1C, blue line) than in control condition (Fig. 1C, black line) during all four 30 min periods. Thus, 48-hr PS deprivation was efficient to induce a significant PS hypersomnia during the subsequent 2 hr rebound period.

We examined the distribution of mCherry+ (or cFos+) ORX neurons in W-W vs. W-PSR animals (Fig. 2). In W-W animal (Fig. 2A-B<sub>4</sub>), many ORX neurons were either cFos- or mCherry-labeled. A large number of cells were triple-labeled, exhibiting a white nucleus with violet cytoplasm. In W-PSR animal (Fig. 2C-D<sub>4</sub>), mCherry-labeled ORX neurons representing cells activated during W were numerous, while cFos-labeled ORX cells were rare. Triple-labeled neurons were not observed in this case.

Quantitative analysis was performed to characterize the neurons activated in W-W and W-PSR conditions (Fig. 3, the top row; Table 2, rows 3-10). In W-W mice, the average numbers of mCherry+ and cFos+ neurons in the LH were  $325 \pm 93$  (n=3) and  $405 \pm 41$  (n=3), respectively. The average number of double-labeled (mCherry+ cFos+) cells was  $123 \pm 22$ , which corresponds to approximately 37.8% of the mCherry+ and 30.4% of the cFos+ cells. In W-PSR mice, the average numbers of mCherry+ and cFos+ neurons were  $281 \pm 40$  and  $71 \pm 22$ , respectively. On average, only 3 neurons were double-labeled (mCherry+ cFos+), indicating that different groups of cells are activated during W and PSR. Based on the number of labeled cells (Fig. 3, the top row), the proportions of labeled neuronal groups were analyzed (Table 2, rows 3-10). The average percentages of double-labeled (mCherry+ ORX+) cells over total mCherry and over total ORX neurons in W-W animals were  $31.1 \pm 4.2\%$  and  $32.3 \pm 5.0\%$ , respectively. The values were in the same range in W-PSR animals. The percentages of cFos-labeled ORX neurons over total cFos and over total ORX in W-W animals were  $30.7 \pm 0.5\%$  and  $42.9 \pm 4.3\%$ , respectively. In contrast, in W-PSR mice, the percentages were  $6.2 \pm 0.9\%$  and  $1.6 \pm 0.2\%$ , respectively, indicating that ORX are not activated during PSR. In line with this assumption, the average number of triple-labeled (mCherry+ cFos+ ORX+) neurons was  $57 \pm 12$  in W-W animals (Fig. 3, the top row), but it was zero in the W-PSR mice. Impressively, the percentage of triple-labeled neurons in W-W mice constituted  $19.6 \pm 3.6\%$  of all ORX neurons (Table 2).



We then determined whether the neurons activated during PSR correspond to MCH neurons. The micrograph (Fig. 4A-B<sub>4</sub>) shows in a PSR-PSR animal a large number of cFos<sup>+</sup> (or mCherry<sup>+</sup>) MCH neurons but only a few triple-labeled MCH cells. In a W-PSR animal, however, a large proportion of MCH cells are cFos-labeled, but not mCherry-labeled (Fig. 4C-D<sub>4</sub>), indicating that MCH neurons are activated during PSR but not during W.

Quantitative analysis was performed to compare the number of MCH neurons activated in PSR-PSR vs. W-PSR mice (Fig. 3, the second row; Table 2, rows 11-18). In PSR-PSR animals, the average numbers of mCherry<sup>+</sup> and cFos<sup>+</sup> neurons in the LH were  $369 \pm 27$  (n=3) and  $192 \pm 64$  (n=3), respectively (Fig. 3, the second row). The number of double-labeled (mCherry<sup>+</sup> cFos<sup>+</sup>) cells was  $53 \pm 11$ , which corresponds to 14.4% of the mCherry and 27.6% of the cFos cells. In W-PSR animals, the numbers of mCherry<sup>+</sup> and cFos<sup>+</sup> neurons were  $273 \pm 29$  and  $197 \pm 27$ , respectively. On average, only 10 neurons were double-labeled in W-PSR animals, indicating that different populations of neurons are activated during W and PSR. Based on the number of labeled cells (Fig. 3, the second row), the proportions of labeled neuronal groups were analyzed (Table 2, rows 11-18). The average percentages of mCherry<sup>+</sup> MCH cells over total mCherry and over total MCH in PSR-PSR animals were  $5.7 \pm 1.5\%$  and  $9.1 \pm 2.7\%$ , respectively. In contrast, these values were only  $1.6 \pm 0.3\%$  and  $2.0 \pm 0.1\%$  in W-PSR animals. Finally, the percentages of cFos<sup>+</sup> MCH neurons over total cFos and over total MCH in PSR-PSR animals were  $20.6 \pm 4.8\%$  and  $16.6 \pm 7.5\%$ , respectively. These values were similar in W-PSR animals (i.e.,  $23.7 \pm 1.2\%$  and  $21.9 \pm 1.6\%$ ). Extremely limited number of triple-labeled cells were observed only in one PSR-PSR animal (Table 2, M1312).

We finally examined whether Lhx6<sup>+</sup> neurons mainly localized in the ZI are activated during W or PS hypersomnia. The micrograph (Fig. 5A-C<sub>4</sub>) shows a substantial number of triple-labeled Lhx6<sup>+</sup> neurons in a PSR-PSR animal. Since Lhx6 is a nuclear protein (like cFos), triple-labeled cells in the ventral ZI exhibited red (i.e., mCherry-labeled) cytoplasm with a white nucleus; many mCherry<sup>+</sup> (or cFos<sup>+</sup>) Lhx6 neurons are also visible in the region. In a W-PSR animal, however, many cFos-labeled Lhx6 neurons are visible but only a few mCherry-labeled Lhx6 cells (Fig. 5D-F<sub>4</sub>).

Quantitative analysis was performed to examine the activation of Lhx6 neurons in PSR-PSR vs. W-PSR animals (Fig. 3, the bottom row; Table 2, rows 19-26). In PSR-PSR animals, the average numbers of mCherry<sup>+</sup> and cFos<sup>+</sup> neurons in the ZI were  $193 \pm 21$  (n=3) and  $100 \pm 27$  (n=3), respectively (Fig. 3, the bottom row). The number of double-labeled (mCherry<sup>+</sup> cFos<sup>+</sup>) cells was  $17 \pm 2$ , which corresponds to 8.8% of the mCherry and 17.0% of the cFos cells. In W-PSR animals, the average numbers of mCherry<sup>+</sup> and cFos<sup>+</sup> neurons were  $218 \pm 15$  and  $147 \pm 51$ , respectively. On average, only 9 neurons were double-labeled (mCherry<sup>+</sup> cFos<sup>+</sup>), indicating that different groups of neurons are activated during W and PSR. Based on the number of labeled cells (Fig. 3, the bottom row), the proportions of labeled neuronal groups were analyzed (Table 2, rows 19-26). The average percentages of mCherry<sup>+</sup> Lhx6 cells over total mCherry and over total Lhx6 in PSR-PSR animals were  $8.8 \pm 2.3\%$

and  $4.6 \pm 1.0\%$ , respectively. These values were significantly ( $P < 0.05$ ) higher than those seen in W-PSR animals (i.e.,  $2.1 \pm 0.4\%$  and  $1.7 \pm 0.4\%$ ) indicating that Lhx6 neurons are activated during PSR rather than during W. In addition, the percentages of double-labeled (cFos+ Lhx6+) neurons over total cFos and over total Lhx6 were  $24.6 \pm 5.9\%$  and  $5.9 \pm 0.7\%$ , respectively. These values were in the same range as W-PSR animals (i.e.,  $23.0 \pm 5.4\%$  and  $10.2 \pm 1.0\%$ ). Altogether, these results indicate that a substantial proportion of the ZI neurons activated during PSR (but not during W) correspond to Lhx6 neurons. In line with these results, the average percentage of triple-labeled neurons over total Lhx6 cells was significantly ( $P < 0.05$ ) larger in PSR-PSR than in W-PSR animals (Table 2, the last column).

## DISCUSSION

In the present study, we first report that a large proportion of neurons labeled with mCherry during W (37.8%) or PSR (14.4%) are expressing cFos when the animals are re-exposed to the same conditions (i.e., W-W or PSR-PSR) just before perfusion while the proportions are much smaller (i.e., 1.1% and 3.7%) when the animals are exposed to two different conditions (i.e., W-PSR). In addition, we found that ORX neurons known to be specifically active during W (Lee et al., 2005) expressed cFos during W but not during PSR while those expressing MCH known to be specifically active during PSR (Verret et al., 2003) are mCherry-positive during PSR and not during W. These results first indicate that a large proportion of the neurons expressing mCherry express cFos when the animals are reexposed to the same condition validating the TRAP methodology for studying neurons activated during W or PSR. In addition, we observed that 8.8% of ZI neurons labeled with mCherry during PSR expressed Lhx6 in contrast to the W condition in which only 2.1% of the mCherry-labeled cells expressed Lhx6. These results indicate that Lhx6+ neurons are specifically activated during PSR. These values are even higher than those seen with MCH, suggesting that Lhx6+ neurons might be specifically active during PS like the MCH cells.

The TRAP method has never been utilized to study neurons activated during PS hypersomnia and waking. We showed here that a large percentage of the neurons labeled with mCherry are cFos positive in W-W and PSR-PSR but not in W-PSR conditions. Further, we showed that a large percentage of the mCherry- and cFos-positive cells labeled after W condition express ORX and not MCH indicating that mCherry is expressed in W-active neurons thus validating the specificity of the staining obtained. Further, we confirmed the specificity for activated neurons of the mCherry labeling obtained after PSR since a large number of the mCherry-positive neurons were expressing MCH. In addition, the fact that nearly no ORX neurons were labeled in the PSR condition indicates that although there are some W episodes during PSR, it is not sufficient to induce mCherry or cFos staining. In summary, our results clearly indicate that the TRAP method can be used to study neurons activated during PSR and waking,

introducing a powerful new method to study such neurons. It is in line with a recent study using TRAP mice to identify a previously unknown population of glutamatergic neurons involved in the generation of SWS (Zhang et al., 2019). Our study opens the possibility to fully characterize PS-activated neurons across the brain combining the TRAP methodology with mRNAs sequencing, “in vitro” recordings, optogenetic and chemogenetic. Furthermore, we demonstrated for the first time that Lhx6<sup>+</sup> neurons of the ZI were activated during PSR like the MCH neurons and not during W. We previously showed that most of the neurons expressing cFos in the ZI during PSR express GAD67 (Sapin et al., 2010). It has been recently shown that Lhx6<sup>+</sup> neurons of the ZI express vGAT and GAD67 and are distinct from MCH and ORX neurons (Liu et al., 2017; Mickelsen et al., 2019). It is therefore likely that the Lhx6<sup>+</sup> neurons constitute a sub-population of the PS-activated GABAergic neurons localized in the ZI.

The previous report (Liu et al., 2017) demonstrated that cFos expression is increased in Lhx6<sup>+</sup> neurons at light onset and after 6 hrs of total sleep deprivation with or without including 1 h of sleep recovery. From these results, they concluded that Lhx6<sup>+</sup> neurons are activated by sleep pressure. Although it is not directly comparable to our results, we did find that cFos expression was strongly increased after 2 hrs of PSR and not after 2 hrs of W. Our results therefore indicate that Lhx6<sup>+</sup> neurons are more active during PSR than during W. Unit recordings of these neurons are eventually needed to confirm this hypothesis. Liu et al (2017) further showed that the loss of Lhx6<sup>+</sup> neurons (which was directly induced by gene deletion from developing diencephalon) caused an increase in W and a reduction of SWS and PS with a relative decrease of PS greater than that of SWS (Liu et al., 2017). Likewise, clozapine N oxide (CNO) injection after transfecting Lhx6<sup>+</sup> neurons with excitatory designer receptors exclusively activated by designer drugs (DREADDS) induced an increase in PS and to a minor extent in SWS, while inhibition of Lhx6<sup>+</sup> cells transfected with inhibitory DREADDS reduced sleep (in particular PS). They further showed that Lhx6<sup>+</sup> cells project to the ORX and monoaminergic W-active neurons and likely induce sleep by inhibiting them. These observations are similar to what has been reported for the activation or inhibition of MCH neurons (Verret et al., 2003; Jégo et al., 2013; Varin et al., 2018). Altogether, these and our results suggest that like MCH neurons, Lhx6<sup>+</sup> neurons of the ZI might contribute to the generation of PS.

In addition to these two populations of neurons, other neurons in the LH and ZI express cFos following PSR; thus, further experiments are needed to identify these neurons and characterize their specific functions. In conclusion, the present findings demonstrated that TRAP methodology combined with our automatic PS deprivation system constitutes a promising new strategy to identify various types of neurons involved in the initiation and/or maintenance of PS.

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#### **CONFLICT OF INTEREST**

The authors have no conflict of interest.

#### **AUTHOR CONTRIBUTIONS**

PHL, PF, and LAD designed the experiment, while RY, DW, and SA performed physiological recordings. HSL performed anatomical works with data analysis. HSL and PHL wrote the manuscript.

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