Neural circuitry of the suprachiasmatic nucleus and retrochiasmatic area regulating arousal

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Abstract

The suprachiasmatic nucleus (SCN) and retrochiasmatic area (RCA) innervate the paraventricular nucleus of hypothalamus (PVH) that projects to the brainstem arousal parabrachial nucleus (PB), which may form a neural circuit regulating arousal, independent from the circadian control circuit of arousal. To test the hypothesis, we used DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) to activate these regions and examine wakefulness. We found that chemo-stimulation of the PVH, the SCN, the RCA, and the PVH-PB pathway all significantly promoted wakefulness, suggesting that a neural circuit consisting of these nodes regulating arousal.

Introduction

The master circadian pacemaker, the suprachiasmatic nucleus (SCN), controls circadian timing of sleep and wakefulness. SCN ablations in rodents, despite completely abolishing circadian rhythms of sleep-wake behavior, do not alter total sleep or wake amounts (Ibuka & Kawamura, 1975) . However, lesions of the SCN in squirrel monkeys eliminate circadian rhythms of sleep-wake behavior but also reduce total wake time by 15% (Edgar *et al.* , 1993) , suggesting that the SCN may promote wake in rodents. Delineating the circadian independent pathways by which the SCN promotes arousal will enrich the mechanisms of SCN control of arousal. The paraventricular nucleus of hypothalamic (PVH) regulates autonomic function (Leibowitz *et al.* , 1981) and food intake (Atasoy *et al.* , 2012; Garfield *et al.* , 2015) , it receives direct inputs from the SCN (Ono *et al.* , 2020) , and mediates circadian control of the SCN on pineal melatonin and corticosterone release (Klein *et al.,* 1983; Jones *et al.* , 2021). It has been reported that corticotropin-releasing factor (CRF) neurons in the PVH mediate wakefulness via the SCN projection (Ono*et al.* , 2020; Chen *et al.* , 2021). As the PVH projects to the parabrachial nucleus (PB), a key brainstem arousal center (Qiu*et al.* , 2016; Lv *et al.* , 2023), the PVH may mediates arousal via the PB.

The retrochiasmatic area (RCA), an area situated immediately ventral to the third ventricle, behind the SCN and in front of the arcuate nucleus receives massive SCN inputs and is involved in control of the circadian amplitude of N-acetylserotonin and melatonin by the pineal gland (Campos*et al.* , 2014) . Both SCN and RCA are critical for pulsatile luteinizing hormone (LH) secretion (Arendash & Gallo, 1979). We hypothesize that the SCN, RCA, PVH and PB constitute an arousal network. We used designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to examine the effects of activation of the RCA, SCN, PVH and PVH-PB pathway on wake behavior.

Materials and Methods

Animals

Animal protocols were approved by Beth Israel Deaconess Medical Center Animal Care and Use committees. Male Sprague Dawley rats (300-325 g; Harlan Sprague Dawley, Indianapolis, IN) were housed under controlled conditions (LD=12:12, light on = 07:00 AM, 100 lux) in an isolated ventilated chamber maintained at 20–22°C, with ad libitum food and water.

Surgery

AAV injection and implantation for polysomnographic recording in rats were performed as described previously (Anaclet *et al.* , 2012; Anaclet *et al.* , 2014). Under deep ketamine/xylazine anesthesia, 18–60 nl of AAV10-hSyn-DIO-hM3Dq-mCherry (AAV-M3-mCherry) virus were injected into the PVH, RCA or SCN using glass micropipettes and a compressed air delivery system described previously (Anaclet *et al.* , 2014; Anaclet *et al.* , 2015; Gompf *et al.* , 2015; Chen*et al.* , 2016). Glass pipettes had small tip diameters (<20 mm) and did not cause visible tissue damage. Sham control rats were injected with 15 nl of saline. The coordinates for the injections were as follows: PVH [AP= -1.8 mm, ML= ± 0.4 mm, DV=-6.6 mm], RCA [AP=-0.8 mm, ML= +- 0.2 mm, DV= -8.3 mm] and SCN [AP= -1.0 mm, ML= +- 0.2 mm, DV= -8.3 mm]. The pathway experiment animals received AAV6-cre (Children Hospital, Boston) injection into the PB [AP= -9.0 mm, ML= +-2.0 mm, DV= -5.1 mm]. AAV6-cre has a unique property of retrograde transporting. In our case, cre is expected to be in the sites projecting to the PB. In the same surgery, cre dependent AAV-hM3Dq-mCherry virus was also injected into the PVH using the coordinates above. This approach selectively inserts M3 receptors in the PVH neurons projecting to the PB. All rats were implanted with EEG/EMG electrodes as described previously (Anaclet *et al.* , 2012; Anaclet*et al.* , 2014).

Sleep–wake recording and analysis

In three weeks after surgery, the rats were connected via flexible recording cables and a commutator (Plastics One) to an analog amplifier (A-M Systems) and computer, with an analog-to-digital converter card and running Vital Recorder (Kissei Pharmaceutical). Rats were habituated to the recording cable for 2–3 days before starting EEG/EMG and video recording.

We used clozapine-N-oxide (CNO) to activate G protein-coupled receptors (cholinergic M3 receptors). Rat EEG/EMG were recorded for 48 hours. On day 1, the rats were treated with saline (i.p.), and on the other day rats were treated with CNO (Sigma-Aldrich; 0.2 mg/kg in saline, i.p.) injections at 8:00 (ZT 01, light-on at 7:00) or 20:00 (ZT13). A dim red flashlight (8 lux at 25 cm) was used during injections performed in the dark. The vigilance states were automatically identified and analyzed off-line in 10 s epochs into REM sleep, NREM sleep, and wakefulness using SLEEPSIGN for Animal (Kissei Pharmaceutical, Nagano, Japan) (Chen et al., 2015). EEG/EMG signals were amplified and filtered (0.5–30 Hz for EEG, 40–200 Hz for EMG), and were then digitized at 128 Hz and recorded. Wakefulness was considered to have desynchronized EEG and high levels of EMG activity, NREM sleep was considered to have synchronized, high-amplitude, low-frequency (0.5–4 Hz) EEG signals in the absence of motor activity; and REM sleep was considered to have pronounced theta-like (4–9 Hz) EEG activity and muscle atonia. As a final step, defined sleep–wake stages were examined visually and corrected if necessary.

The percentage of time spent in W, NREM and REM sleep, as well as the number and the average durations of the episode was summarized for each group and each condition. The sleep latency is defined as the time between CNO or saline injection and the onset of NREM episode lasting >20 s.

Histology

At least one week after recordings, rats were perfused for histology and characterization of injection areas. Three hours after CNO injection rats were deeply anesthetized by chloral hydrate (500 mg/kg), perfused with 0.9% saline followed by 10% neutral buffered formalin (Sigma) through the heart. The brains were removed, and then equilibrated in PBS with 20% sucrose overnight. The brains were sectioned on a freezing microtome at 40μm into 4 series. For all c-Fos and mCherry immunohistochemical staining, visualization was based on diaminobenzidine reaction. On the first day, the sections were incubated overnight with rabbit anti-c-Fos, 1:50,000 (Oncogene) and then incubated in the secondary antibodies for 1h, followed by incubation in

ABC reagents (1:1,000; Vector Laboratories) for 1h, then washed again and incubated in a 0.06% solution of 3,3-diaminobenzidinetetrahydrochloride (DAB, Sigma-Aldrich) with 0.05% CoCl2 and 0.01% NiSO4 (NH4) in PBS plus 0.02% H_2O_2 for 5-10 min. On the second day, the same sections were incubated overnight with anti-mCherry (1:10 000; Clontech), and follow the same protocol above mentioned. Then, the sections incubated in a 0.06% solution of DAB in PBS plus 0.02% H_2O_2 for 5-10 min. To delineate nuclear boundaries, sections mounted on glass slides were Nissl counterstained in 0.25% thionin in 0.2 M acetate buffer, pH 4.5, for 1 min, placed in 1% acetic acid for 1–5 min to differentiate stained structures, dehydrated in graded alcohols, cleared in xylene, and coverslipped (Chen et al., 2021). The pattern of Fos immunoreactivity was examined for SCN, PVH and RCA, bilateral counts were taken on three consecutive sections, 120 μm apart, that contained the largest nuclear areas, and these six counts were averaged.

Statistical analysis

All data were expressed as the means ± SEM. Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL). Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, Inc., CA, USA). Time-course of the hourly amounts of each stage, histograms of sleep/wake amounts, sleep/wake stage transition number, number and duration of sleep/wake bouts were analyzed by the paired t -test, with each animal serving as its own control. For the sleep latency, total numbers of each vigilance stage were analyzed by two-way repeated measures analysis of variance (ANOVA) followed by the Fisher probable least-squares difference (PLSD) test to determine whether the difference among groups was statistically significant. The significance level was set at $P<0.05$ for all statistical tests.

Results

Chemogenetic stimulation of the PVH increases wakefulness

Rats (N=8) with AAV10-hSyn-DIO-hM3Dq-mCherry (AAV-M3-mCherry) injections in bilateral PVH showed a normal pattern of sleep-wake behavior following saline injection at 8:00 (Fig. 1B). However, following CNO (0.2 mg/kg) administration at 8:00 that stimulated the PVH neurons, the same rats showed long bouts of wake, with NREM sleep latency ($F_{[2,23]}$=19.74, P<0.001) significantly lengthened (CNO= 95.2±5.0min; saline= 23.4±2.8min) (Fig.1B, D, E). Sleep-wake transition analysis (Fig. 1D) showed that CNO decreased the number of state transitions from NREM to REM sleep, REM sleep to wake and from wake to NREM. Wake, NREM, REM bouts number were significantly decreased and wake duration increased over 6 hours (Fig.1E). The mean number of REM bouts was significantly decreased from 20.3±1.8 to 6.4±2.0 by CNO (p< 0.001), and wake duration increased from 60.3±4.9 to 163.1±28.8 s (p< 0.001) (Fig.1E). REM and NREM sleep duration were not significantly influenced (Fig.1E).

Sleep and wake amount analysis revealed a significant increase in hourly waking amounts by CNO (Fig. 2), lasting about 6 hours by CNO at 8:00 (Fig. 2A) and 4 hours by CNO at 20:00 (Fig. 2C). The amount of wakefulness during 6, 12 hours by CNO at 8:00 or 20:00 also significantly increased (Fig. 2B, D).

PVH-PB pathway activation increase wakefulness

To examine if the PVH promotes wakefulness via its projections to the PB, we injected cre dependent AAV-M3-mCherry into the PVH and AAV6-Cre bilaterally in the PB in 4 rats (Fig. 1C). In this model system, AAV6-cre injections resulted in cre expression in neurons that project to the PB, and cre was in PVH neurons that were exposed to the cre-dependent AAV-M3-mCherry, M3 was inserted in the PVH neurons that project to the PB. Only neurons with both AAV-M3-mCherry and cre expressed the M3 DREADD; CNO then activated the subset of PVH neurons that project to the PB, i.e., selectively activating the PVH-PB pathway. Activation of the PVH-PB pathway was confirmed by Fos expression in the PVH and cre in the PB (Fig. 1C, F1-3). PVH-PB pathway activation by CNO induced prolonged wakefulness (Fig. 1G) compared to saline injection at 8:00. CNO injection (0.2 mg/kg) at 8:00 significantly increased wake duration, but decreased NREM and REM duration and bouts (Fig. 1H). Consistent with this, wake and NREM amounts were also significantly different between the CNO and saline groups (Fig. 3) when CNO was injected at 8:00 or 20:00. These results indicate that the PVH-PB pathway promotes arousal during active

and inactive period.

RCA activation increases wakefulness

Like the PVH, the RCA also receives input from the SCN and projects to the PB; the PVH together with the RCA may relay SCN signals to the PB for control of arousal. We next examined the role of the RCA in arousal by injecting AAV-M3 into the RCA bilaterally. We had 8 rats with AAV-M3-mCherry in bilateral RCA without the SCN expression of mCherry (Fig. 4A). Compared to saline injection, CNO injections in these animals at 8:00 significantly increased wakefulness and reduced NREM sleep and REM sleep in first three hours post CNO injections (Fig. 5A). Wake increase by CNO at 20:00 was only seen in the first hour post CNO injection (Fig. 5C). No sleep rebound was seen after the CNO arousal effects at either time. The amount of wakefulness during the first 3 and 12 hours by CNO at 8:00 significantly increased (Fig. 5B), but the amount of wakefulness only increase 3 hours at 20:00 (Fig. 5D). Consistent with these results, CNO injection increased wake duration or NREM sleep latency ($F_{[2,23]}$=14.46, P<0.001, saline=15.5±2.0min , CNO= 66.2±6.0min.), and decreased number of bouts and stage transition of wake, NREM and REM sleep (Fig.4C-D).

SCN activation increases wakefulness

We injected 18 nl AAV-M3-mCherry bilaterally in more than 20 rats, but only 5 rats were successfully expressed in the SCN (Fig. 4E). The location and size of the SCN made it difficult to achieve relatively confined and complete bilateral injections of AAV-M3. As a result, we only obtained 5 rats with unilateral transfection in the SCN. Despite the unilateral nature of the expression in these animals, CNO injection at 8:00 but not at 20:00 significantly increased wake amounts during the first three hours compared to saline injection (Fig. 6A,C) . CNO significantly increased the NREM sleep latency (p< 0.01, saline=16.2±2.7 min, CNO=60.1±17.7 min, Fig. 4F,G). As compared with saline-injected controls, CNO markedly increased the amount of wake, decreased the amount of NREM sleep and REM sleep during light condition (Fig. 6B). There was no disruption of the sleep architecture during the subsequent period (Fig. 6A). Of the rats where injections missed the SCN completely as the sham control, AAV-M3-mCherry was often expressed in the third ventricle; CNO also did not affect sleep in this group (n=5), similar to saline-injected controls.

Dual immunostaining for Fos, a marker of neuronal activation, and mCherry showed transfected neurons and Fos in the SCN and Fos in the PVH and RCA ipsilateral to SCN activation (compared to the missed injection side) (Fig. 7). SCN activation significantly increased expression of Fos in the SCN, RCA and PVH (Fig. 7C) compared to the sham group (Fig. 7A,B). We obtained a few unilateral injection cases, which showed that the one side SCN activates other side SCN (Fig.7.D-F); the PVH stimulation did not affect the SCN or RCA Fos expression (Fig.7. G-I); ipsilateral RCA activation potently stimulates both side SCN (Fig. 7 J-L).

Discussion

Using DREADDs, we showed that chemo-activation of the SCN, the RCA, the PVH and PVH projecting neurons to the PB, all promoted arousal. We hypothesize that SCN-RCA-PVH-PB forming an arousal network promotes arousal.

Lesions of the medial PB and lateral PB result in 40% and 10% increase in sleep, respectively, and lesions of the entire PB produce a coma-like state (Fuller *et al.* , 2011). PVH projections to the PB are mostly glutamatergic (Chen *et al.* , 2021) and RCA projections to the PB may also be glutamatergic. Given that both PVH and RCA receive SCN inputs, chemo-stimulation of the SCN induces Fos in RCA and PVH, and chemo-stimulations of these sites promoted arousal, it is speculative that the SCN activating RCA-PVH-PB network promotes arousal.

Single-unit recordings of the SCN *in vivo* in freely moving rats reveals not only circadian firing pattern (Kawamura & Hashimoto, 1979) ,but also mixed populations of sleep and wake dependent firing in mouse, with predominantly wake-active neurons (Sakai, 2014). A small sample of single-unit recording in the SCN in rats demonstrates wake-REM sleep active firing (Deboer *et al.* , 2003). These wake-active neurons may

mediate retinal inputs on arousal but may not be critical for circadian regulation. It is not clear whether these neurons receive direct retinal inputs. SCN wake-active neurons may particularly be important in diurnal species such as primates (Mistlberger, 2005). Despite of existence of small amounts of sleep-active neurons in the SCN, we never observed sleep promotion effects by stimulation of the SCN, PVH, or RCA.

Although DREADD clozapine-N-oxide (CNO) on sleep have been systematically tested, it intraperitoneal injections of commonly used mouse CNO doses (1, 5, and 10 mg/kg) dose-dependent suppression of rapid eye movement (REM) sleep, alter sleep in male mice (Traut et al., 2023), CNO at much lower dose 0.2 mg/kg did not alter sleep architecture in rats (Wen et al., 2020).

Compared to wake effects of PVH or PVH-PB pathway stimulation as well as other arousal neurons such as orexinergic neurons, unilateral SCN stimulation showed weaker effects and only effective during the daytime, the highest sleep period. On the other hand, we expect that the bilateral SCN stimulation would show stronger arousal effects and may promote arousal during the night time. In the PVH, melanocortin-4 receptor containing glutamatergic neurons that project to the PB regulate appetite (Garfield *et al.* , 2015). These neurons may regulate arousal and serve as a neural circuit by which hunger and appetite influence on sleep-wake behavior. The PVH-PB pathway may mediate arousal by other neural factors such as stress (Radley *et al.* , 2009), dehydration (Watts *et al.* , 1999) and cold exposure (Cano *et al.* , 2003).

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CONFLICT OF INTEREST

The authors of this paper declare that they do not have any conflict of interest.

AUTHORS CONTRIBUTION

JL conceptualized and designed the study and approved the final manuscript. CR Chen performed the experiments and the acquisition of the data, drafted the initial manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure 1. Chemogenetic stimulation of the PVH and PVH-PB pathway promotes arousal.

Outlines of AAV-M3-mCherry transfection in the PVH in 8 cases (A), EEG/EMG recording and corresponding hypnograms (8:00-11:00) in a rat with saline and CNO injection (B) and Fos induced by CNO and mCherry labeled neurons in the PVH (C), and effect of CNO on NREM sleep latency, sleep-wake episodes transition, total number and mean duration of waking, NREM, and REM bouts in a 6 h period (D-E). AAV6-cre injected in the PB was labeled by cre (F1) while CNO induced Fos in mCherry neurons were seen in the PVH where cre dependent AAV-M3-mCherry was injected (F2-F3). EEG/EMG recordings and corresponding hypnograms in a rat with activation of PVH-PB circuit by CNO (G). Effect of CNO on NREM sleep latency, sleep-wake episodes transition, total number and mean duration of waking, NREM, and REM bouts in a 2 h period (H).

Figure 2. Characterizations of sleep-wake effects by PVH chemo-activation

CNO injection at 8:00 and 20:00 increased wake amounts and reduces sleep in hourly time-course (A and C), and averaged amounts of 6,12, 12 hours (B and D). Each circle represents the hourly mean ± SEM. *P<0.05, **P<0.01. No sleep rebound is seen after CNO injection of 8:00 and 20:00.

Figure 3. Characterizations of sleep-wake effects by PVH-PB pathway activation

CNO injection at 8:00 and 20:00 increases wake amounts and reduces sleep in hourly time-course (A and C), and averaged amounts of 6,12, 12 hours (B and D). Each circle represents the hourly mean ± SEM. *P<0.05, **P<0.01. No sleep rebound is seen after CNO injection of 8:00 and 20:00.

Figure 4. Chemogenetic stimulation of the RCA and SCN promote arousal

Selective cases that outline AAV-M3-mCherry injection in the RCA (A). EEG/EMG and sleep-wake stage change after saline or CNO injection at 8:00 (B). Effects of RCA activation on NREM sleep latency, sleep-wake episodes transition, total number and mean duration of waking, NREM, and REM bouts during 8:00-11:00 (C and D). Selective cases that outline AAV-M3-mCherry injection in the SCN (E). EEG/EMG and stage changes saline or CNO injection at 8:00 (F). Effects of SCN activation on NREM sleep latency (G).

Figure 5. Characterizations of sleep-wake effects by RCA activation

CNO injection at 8:00 and 20:00 increases wake amounts and reduces sleep in hourly time-course (A and C), and averaged amounts of 6,12, 12 hours (B and D).

Figure 6. Characterizations of sleep-wake effects by SCN activation

CNO at 8:00 but not 20:00 increases wake amounts and reduces sleep amounts shown in hourly time-course (A and C), and averaged amounts of 6,12, 12 hours (B and D). No sleep rebound is seen after CNO injection of 8:00.

Figure 7. Fos expression in the PVH, RCA and SCN after unilateral chemo-stimulation in the SCN, or RCA or PVH.

Chemogenetic activation of the unilateral SCN increases Fos expression in ipsilateral PVH and RCA (D-F), compared to sham group (A-B). Fos-ir positive cell in the ipsilateral PVH, RCA and l SCN were induced after unilateral SCN, PVH or RCA activation (C). Chemogenetic activation of the unilateral PVH did not alter Fos expression in contralateral PVH or SCN or RCA (G-I). Chemogenetic activation of unilateral RCA did not influence Fos expression in the PVH, but appeared to induce Fos expression in SCN (J-L).

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