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The Kir channel in the nucleus tractus solitarius integrates the chemosensory system with REM sleep executive machinery for homeostatic balance

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The locus coeruleus (LC), nucleus tractus solitarius (NTS), and retrotrapezoid nucleus (RTN) are critical chemosensory regions in the brainstem. In the LC, acid-sensing ion channels and proton pumps serve as H^+ sensors and facilitate the transition from non-rapid eye movement (NREM) to rapid eye movement (REM) sleep. Interestingly, the potassium inward rectifier (KIR) channels in the LC, NTS, and RTN also act as H^+-sensors and are a primary target for improving sleep in obstructive sleep apnea and Rett syndrome patients. However, the role of Kir channels in NREM to REM sleep transition for H^+ homeostasis is not known. Male Wistar rats were surgically prepared for chronic sleep–wake recording and drug delivery into the LC, NTS, and RTN. In different animal cohorts, microinjections of the Kir channel inhibitor, barium chloride ($BaCl_2$), at concentrations of 1 mM (low dose) and 2 mM (high dose) in the LC and RTN significantly increased wakefulness and decreased NREM sleep. However, $BaCl_2$ microinjection into the LC notably reduced REM sleep, whereas it didn't change in the RTN-injected group. Interestingly, $BaCl_2$ microinjections into the NTS significantly decreased wakefulness and increased the percent amount of NREM and REM sleep. Additionally, with the infusion of $BaCl_2$ into the NTS, the mean REM sleep episode numbers significantly increased, but the length of the REM sleep episode didn't change. These findings suggest that the Kir channels in the NTS, but not in the LC and RTN, modulate state transition from NREM to REM sleep.

Keywords Potassium inward rectifier channels, Hypercapnia, REM sleep, Locus coeruleus, Retrotrapezoid nucleus, Solitary tract nucleus, Chemosensory

Rapid eye movement (REM) sleep plays a crucial role in overall health, aiding in ionic homeostasis, brain development, endocrine regulation, and mental well-being[1–5]. It features distinct physiological traits like increased breathing rate, heart rate, and EEG patterns similar to wakefulness[6]. Despite these physiological processes being energy-demanding, their heightened activity during REM sleep remains enigmatic[7–9]. One reason for increased breathing rate during REM sleep could be that REM sleep might function as a sentinel to help maintain normal CO_2 levels during sleep[7]. Extended NREM sleep may cause hypoventilation and increased pCO_2 levels[7]. By regulating CO_2 levels, REM sleep possibly prevents physiological disruptions leading to frequent arousal and fragmented sleep[1,7]. Frequent arousals or fragmented sleep are pathological conditions that can lead to various physiological diseases, including cardiorespiratory disorders[10]. Hence, one of the potential functions of REM sleep could be to facilitate uninterrupted, restorative sleep by regulating bodily CO_2 levels within the normal range, thereby enhancing overall well-being.

The central chemosensory system, comprising neuronal groups in the locus coeruleus (LC), Nucleus tractus solitarius (NTS), and retrotrapezoid nucleus (RTN), plays a crucial role in regulating breathing in response to changes in pH and CO_2 levels[7,11–14]. The LC is important for integration of altered CO_2/pH with breathing modulation, but the NTS and RTN contribute significantly to this chemoregulatory mechanism. Localized acidification of these brainstem areas, using microinjection of acetazolamide or acidic cerebrospinal fluid

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infusion, notably increases breathing frequency and tidal volume[12,14–18]. Furthermore, experiments involving lesions of these central chemosensory brain regions have shown that they are essential for the adaptive respiratory response during hypercapnia[12,14,19–21]. For instance, in rats with kainic acid-induced RTN lesions, while normal breathing patterns remained unaffected, the response to hypercapnia was significantly diminished[22]. Recent studies targeting a substantial portion of Phox2b NTS neurons have revealed an increased threshold to apnea without altering overall CO_2 sensitivity[19,21,23]. In contrast, lesions of the LC or genetic deletion of the dopamine beta-hydroxylase (DBH) enzyme result in profound impairments in hypercapnic responses, regular breathing, and heart rate regulation[18,24]. Moreover, such changes can lead to breathing problems during sleep, which may accompany hypotension[18,24].

The chemosensory machinery also influences sleep–wake architecture. Studies suggest that the LC and RTN are involved in maintaining arousal[12,25–27]. Activation of the LC and RTN neurons through electrical, chemical, and optogenetic stimulation increases wakefulness, whereas their inhibition induces sleep[27–30]. Conversely, the NTS may promote sleep, particularly REM sleep, as most of the NTS neurons are active during REM sleep[31,32]. A low threshold electrical stimulation of the central NTS increases theta and alpha waves and REM sleep[13]. Interestingly, the recurrence of REM sleep increases under a mild hypercapnic (2–4% CO_2) condition[11,33]. The proton pumps and acid-sensing ion channels (ASICs) in the LC are pivotal in mediating the repeated recurrence of REM sleep and initiating the transition from non-rapid eye movement (NREM) to REM sleep states[1,11,34]. These studies suggest that altering the brain's hydrogen ions, and chemosensory machinery influences either REM sleep or the state transition from NREM to REM sleep. Several receptors and channels, including Kir channels, play an essential role in maintaining hydrogen ion homeostatic balance in the brain[11,34,35]. Kir channels play a crucial role in resetting neuronal membrane potential during pH fluctuations[36]. Kir channels are abundantly expressed in the LC, NTS, and RTN neurons[35–39], and overexpression of Kir 4.1/4.5 channels in the LC alters hypercapnia-induced breathing responses, as is noticed in the Rett syndrome[37]. However, the role of Kir channels in the modulation of S-W and sleep state transition from NREM to REM sleep is not known. We hypothesize that the Kir channel in the chemosensory neurons of the LC, NTS, and RTN could have a critical role in promoting the transition from NREM to REM sleep, ensuring smooth and uninterrupted sleep. This mechanism might be essential for maintaining sleep continuity and overall sleep quality.

Materials and methods

Animals

We have used male Wistar rats (n = 20) weighing 250–300 g. The animals were obtained from the Central Laboratory Animal Resources (CLAR) facility and taken to the department's animal room facility. They were kept for one week of habituation. The animals were maintained in a 12-h light–dark (L:D) cycle with lights on at 07:00 AM and off at 07:00 PM. The room temperature was maintained at 23 ± 1 °C. Food and water were given ad libitum. All animals, procedures, and protocols used in this study were approved by the Institutional Animal Ethical Committee (IAEC Protocol # 14/2015) of Jawaharlal Nehru University, New Delhi, India. Furthermore, all the experiments were performed under relevant guidelines and regulations, and data reporting in the manuscript follows the recommendations in the ARRIVE guidelines 2.0. We have made our best efforts to minimize the pain and discomfort of the animals and the number of animals used in the experiments.

Surgical procedures for polysomnographic recording and cannula implantation for microinjection of drugs in the LC, NTS, and RTN

Rats were randomly divided into three groups: (i) the LC group, (ii) the NTS group, and (iii) the RTN group. All animals were surgically prepared for chronic sleep-wakefulness (S-W) recording (for details, see Mir and Jha 2021)[11]. Under sterile conditions, the animal was anesthetized by inhalation of isoflurane anesthesia (Baxter Healthcare, India) with 4% initiation and 0.5–2% maintenance using a facemask. The head was shaved and cleaned with iodine, followed by ethanol. The animal's head was fixed in stereotaxic apparatus (RWD Life Science, China), and a midline incision was made with a sterile surgical blade. The skin was moved aside to expose the skull for electrode implantation. Two pairs of small, stainless-steel screw electrodes were affixed on the skull above the frontal and parietal cortices to record electroencephalogram (EEG). Three electrodes (flexible insulated wires except at the tip) were implanted in the dorsal neck muscles to record bipolar electromyogram (EMG) (a third EMG was implanted as a safeguard). One stainless steel screw electrode was fixed laterally to the midline in the nasal bone as a reference electrode. A pair of bilateral stainless-steel cannulas for drug/vehicle infusions was implanted into the LC (at brain co-ordinate from bregma: – 9.8 mm, 1.3 ML, DV 6 from skull surface) in the LC group of animals; in the NTS (at brain co-ordinate from bregma: – 13.5 mm from bregma, 0.8 mm ML, DV 7.5 mm) in the NTS group of animals and in the RTN (at brain co-ordinate from bregma: – 11.8 mm from bregma, 1.6 ML, DV 10.5 mm) in the RTN group of animals respectively. An obturator (blocker wire) was inserted into the guide cannula to prevent occlusion and blocking of the guide cannula. Free ends of EEG, EMG, and reference electrodes were connected to a 9-pin miniature connector, which was cemented onto the skull with dental acrylic cement. Finally, the neck skin was sutured. The animal was removed from the stereotaxic apparatus. The animal was kept on a heating pad and observed closely until it emerged from the anesthesia. An oral analgesic (Ibugesic Plus) was given to the animal for pain management. The animal received post-operative treatment for 3–4 consecutive days with intraperitoneal injections of dexamethasone (1.5 mg/kg) and gentamicin (2 mg/kg), along with the application of nebaself powder (an antibiotic) on the suture site to minimize brain inflammation and prevent infection. After the recovery period from surgery, the animal underwent acclimatization to the recording setup, experimental handling, and mock microinjections to minimize stress during subsequent experiments.

Experimental procedures (drug infusions)

We studied the effects of the Kir channel blocker Barium Chloride ($BaCl_2$), for which two different doses of the drug, low dose (1 mM) and high dose (2 mM), were used (based on reported IC50 values of the drug). Freshly prepared drug solutions and sterile saline (0.9%) were made, and a 200 nl volume was microinjected into each side of the LC, NTS, or RTN brain areas in their respective groups of animals. All drug doses and the respective vehicle solution were microinjected into the respective brain areas randomly on alternative days before sleep–wake recording. We kept a two-day interval between the low and high-dose microinjections to ensure complete clearance of the drug from the previous injection. $BaCl_2$ (Sigma Aldrich) at a low dose of 1 mM and a high dose of 2 mM was dissolved in 0.9% saline, and the pH was adjusted to 7.4. Then 200 nl of this solution was microinjected on each side (200 nl /side) into the respective nuclei over two min. The microinjection of nano volume over two minutes was chosen to restrict the diffusion within the nuclei. For the vehicle injection, 0.9% saline was microinjected in the same manner as the drug.

Polysomnographic recordings

After recovery from the surgery, rats were habituated for 2–3 days in a well-ventilated, and sound-dampened sleep recording chamber (black-colored plexiglass, size: 48″ × 24″ × 24″; illuminated with 40 Lux light). The animal was provided with food and water ad libitum. During the habituation days (Days 1 and 2), the animal was tethered to the recording cable from 11:00 AM to 5:00 PM daily to acclimatize the animals to the setup. The EEG and EMG signals were examined in a computer using "Somnologica Science" software and Embla A10 Medcare Flaga, Iceland. On the subsequent two days, Day 3 and 4, the animal was tethered to the recording cable, and two baselines of S-W were recorded for 6 h (11:00 AM to 5:00 PM). On days 5, 6, and 7, sleep–wake cycles were recorded following microinjections of either a vehicle, a low dose, or a high dose of $BaCl_2$ in a random order for 6 h (11:00 AM to 5:00 PM). Microinjections were always performed after a gap of two days between two drug injections in a randomized fashion to allow the clearance of the drug traces from the brain. EEG signals were recorded using high-pass (0.1 Hz) and low-pass (40 Hz) filters, while EMG signals were recorded with high-pass (10 Hz) and low-pass (90 Hz) filters, all digitized at a 100 Hz sampling rate. All recordings were saved and analyzed offline.

Microinjection procedure for drug infusion

The $BaCl_2$ solution was freshly prepared before injection at the desired concentration and adjusted to 7.4 pH. Before microinjection, animals were manually restrained, and the blocker wires of the cannula were removed and replaced with the injector cannula (30-gauge; 18 mm in length), which was connected to a short silicone tubing, further linked to the microinjector Hamilton syringe (10 µl; Kent Scientific, USA). The injector cannula was inserted 1 mm beyond the tip of the guide cannula to ensure contact with the dorsal surface of the targeted site for drug/vehicle delivery. Subsequently, 200 nl of vehicle (0.9% saline) or $BaCl_2$ solution (low dose: 1 mM or high dose: 2 mM) was injected over 2 min at a 100 nl/min flow rate. The injector needle remained in the guide cannula for an additional 2 min before being slowly withdrawn, and blocker wires were reinserted into the guide cannula. Following microinjections, animals were promptly subjected to polysomnographic recording.

Histological identification of cannula and injector sites

After the completion of all experiments, the rats were sacrificed with an overdose of thiopentone anesthesia (80 mg/kg) and perfused transcardially with 0.9% saline and 10% formalin solution for 15 min each. Later, the brain was extracted and stored in 10% formalin for further histological analysis. Before histology, the brain was dipped in 30% sucrose solution at room temperature for about a day or two. Sectioning was done by taking 40 µM coronal brain sections using a cryostat (Thermo Fisher Scientific, USA) and mounted on the subbed glass slides. The sections were then stained with 0.1% cresyl violet, and the injection site was identified under a microscope and demonstrated in a reconstruction diagram (Figs. 1, 4, and 7).

Data analysis

The polysomnographic records were scored offline using "Somnologica Science Software" (Medcare Flaga, Iceland). Sleep recordings were manually scored using a four-second epoch length, employing the standard criteria for the rats[40]. Low voltage and high-frequency EEG waves associated with increased motor activity were marked as wake; high voltage, low-frequency EEG waves (0.5–4 Hz) and decreased motor activity were marked as NREM sleep and low voltage, high-frequency EEG waves with a prominent theta peak (5–9 Hz) and nuchal muscle atonia were marked as REM sleep. The total time spent in the wake, NREM, and REM sleep was calculated. These values were expressed as percent amounts from hourly and mean values for the total recording time. The differences in the vigilant states after drug and vehicle microinjections were compared statistically with the baseline and vehicle values by one-way RM-ANOVA followed by the Tukey post hoc test.

Results

Effects of $BaCl_2$ microinjections in the LC on sleep–wake architecture

Microinjections of potassium inward rectifier channel inhibitor, $BaCl_2$ in the LC (Fig. 1), n = 7) significantly increased wakefulness ($p < 0.001$, $F_{(3,27)} = 31.02$) and decreased NREM sleep ($p < 0.001$, $F_{(3,27)} = 22.25$), as well as REM sleep ($p < 0.001$, $F_{(3,27)} = 152.84$) (one-way RM-ANOVA followed by Tukey post hoc) (Fig. 2A–C). The sites of microinjections of $BaCl_2$ in 7 animals were within LC, and 2 animals were located outside the LC (Fig. 1) (see Supplementary results). Microinjection of $BaCl_2$ outside the LC did not change wake, NREM sleep, and REM sleep amount (see Supplementary results and Fig. S1).

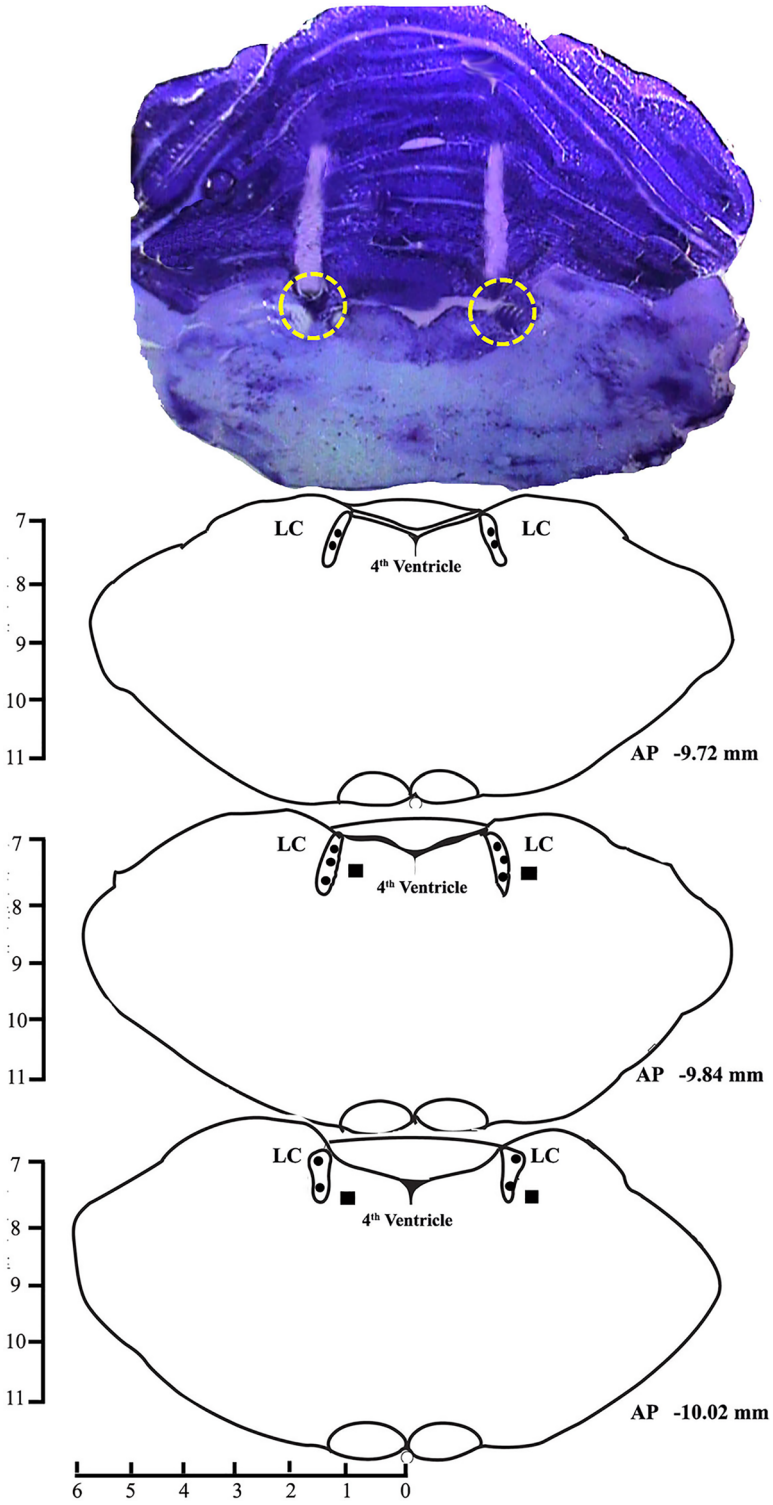

Fig. 1. A photomicrograph of 40 µm cresyl violet stained section and reconstruction diagram showing cannula tract and injection sites in the LC. Filled circles (•) in the reconstruction diagram denote the site of injections within the LC and filled boxes (■) denote the site of injections outside the LC.

The low dose of $BaCl_2$ (1 mM) increased wakefulness by 38% compared to baseline (Tukey, $p < 0.001$) and 40% compared to vehicle (Tukey, $p < 0.001$), respectively. Microinjection of a high dose of $BaCl_2$ (2 mM) significantly increased the amount of wakefulness by 54.6% compared to baseline (Tukey, $p < 0.001$) and 56.5% compared to vehicle (Tukey, $p < 0.001$), respectively (Fig. 2A).

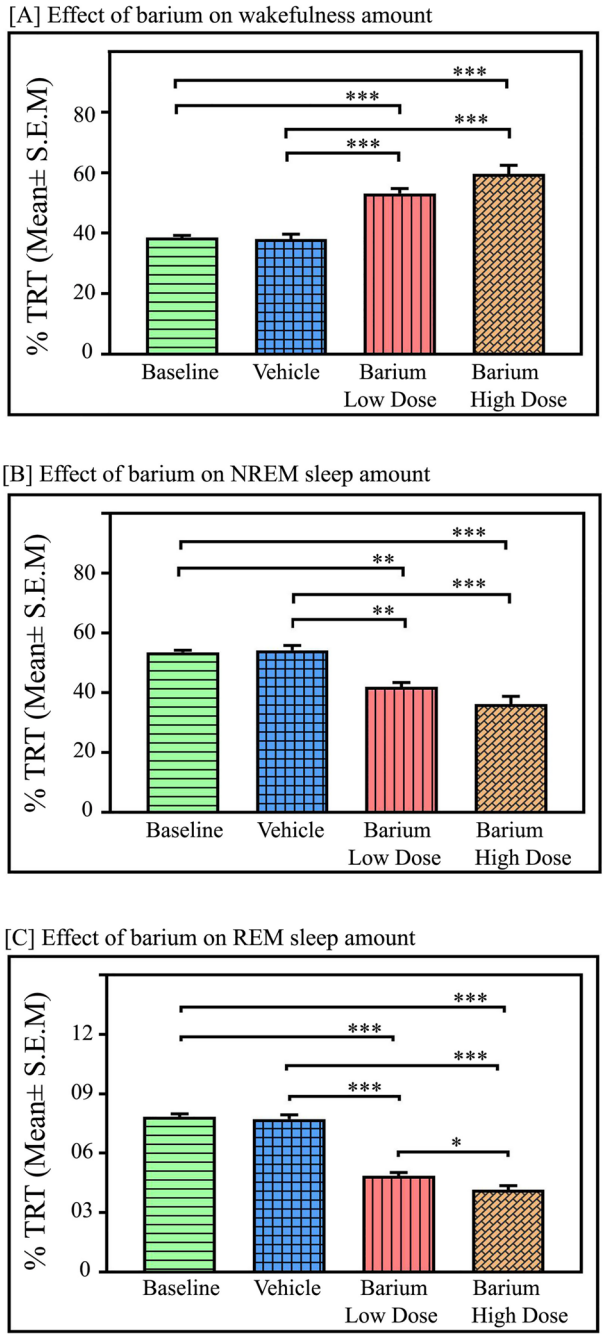

Fig. 2. Effects of Kir channel inhibition in the LC on sleep–wake architecture. Microinjections of Kir channel blocker $BaCl_2$ in the LC (n = 7) significantly augmented wake and reduced NREM and REM sleep amounts. Microinjections at a low dose (1 mM) and high dose (2 mM) of $BaCl_2$ in the LC significantly increased (**A**) wakefulness ($p < 0.001$; $F_{(3,27)} = 31.02$), One-way RM ANOVA followed by Tukey post-hoc test. (**B**) Kir inhibition in LC significantly decreased NREM sleep ($p < 0.001$, $F_{(3,27)} = 22.25$). (**C**) Kir inhibition in LC significantly decreased REM sleep ($p < 0.001$, $F_{(3,27)} = 152.84$) (One Way RM ANOVA followed by Tukey post hoc test). * denotes < 0.05, ** denotes < 0.01, *** denotes $p < 0.001$.

In contrast, microinjections of $BaCl_2$ in the LC significantly decreased the NREM sleep amount ($p < 0.001$, $F_{(3,27)} = 22.25$) (one-way RM-ANOVA followed by Tukey post-hoc (Fig. 2B). The low dose of $BaCl_2$ decreased NREM sleep by 22% compared to baseline (Tukey, $p < 0.01$) and 23% compared to vehicle (Tukey, $p < 0.01$). Whereas a high dose of $BaCl_2$ (2 mM) significantly decreased NREM sleep by 32.4% compared to baseline (Tukey, $p < 0.001$) and 33.2% compared to vehicle (Tukey, $p < 0.001$) (Fig. 2B).

Additionally, microinjections of $BaCl_2$ in the LC significantly decreased the amount of REM sleep in a dose-dependent manner [($p < 0.001$, $F_{(3,27)} = 152.84$) (one-way RM-ANOVA followed by Tukey post-hoc Fig. 2C)].

The low dose of $BaCl_2$ decreased REM sleep by 38% compared to baseline, (Tukey $p < 0.001$) and 37% compared to vehicle, (Tukey, $p < 0.001$). Whereas a high dose of $BaCl_2$ (2 mM) significantly decreased REM sleep by 47% compared to baseline (Tukey, $p < 0.001$) and 46% compared to vehicle (Tukey, $p < 0.001$) (Fig. 2C). Furthermore, a low dose of $BaCl_2$ (1 mM) decreased REM sleep amount by 15% compared to the high dose of $BaCl_2$ (2 mM) microinjections ($p < 0.05$), (one-way RM-ANOVA followed by Tukey post-hoc (Fig. 2C). Moreover, microinjection of both low and high doses of $BaCl_2$ in the LC significantly reduced the number of REM sleep episodes compared to the baseline and vehicle ($p < 0.001$, $F_{(3,27)} = 28.83$) (one-way RM-ANOVA followed by Tukey post-hoc) [Fig. 3A]. The low dose of $BaCl_2$ decreased the number of REM sleep episodes by 52.4% compared to baseline (Tukey, $p < 0.001$) and 54% compared to vehicle (Tukey, $p < 0.001$). Whereas a high dose of $BaCl_2$ (2 mM) significantly decreased the number of REM sleep episodes by 61% compared to baseline (Tukey, $p < 0.001$) and 63% compared to vehicle (Tukey, $p < 0.001$) (Fig. 3A). However, the length of REM sleep episodes did not change with low or high doses of $BaCl_2$ microinjections into the LC (Fig. 3B). Additionally, both low and high doses of $BaCl_2$ microinjections into the LC significantly increased the mean latency of REM sleep ($p < 0.001$, $F_{(3,27)} = 51.08$) (one-way RM-ANOVA followed by Tukey post-hoc (Fig. 3C). The low dose of $BaCl_2$ increased the mean latency of REM sleep by 28% compared to baseline (Tukey, $p < 0.01$) and 34% compared to vehicle (Tukey, $p < 0.01$). Whereas a high dose of $BaCl_2$ (2 mM) significantly increased the mean latency of REM sleep by 74% compared to baseline (Tukey, $p < 0.001$) and 82% compared to vehicle (Tukey, $p < 0.001$) (Fig. 3C).

Effects of $BaCl_2$ microinjections in the NTS on sleep–wake

Microinjections of potassium inward rectifier channel inhibitor $BaCl_2$ into the NTS (Fig. 4, n = 7) significantly decreased wakefulness ($p < 0.001$, $F_{(3,27)} = 91.26$). However, NREM sleep ($p < 0.001$, $F_{(3,27)} = 58.92$), as well as REM sleep ($p < 0.001$, $F_{(3,27)} = 183.70$) significantly increased (one-way RM-ANOVA followed by Tukey post hoc, Fig. 5A–C). The low dose of $BaCl_2$ (1 mM) decreased wakefulness by 29% compared to baseline (Tukey, $p < 0.001$) and 28% compared to vehicle (Tukey, $p < 0.001$), respectively. Whereas microinjection of a high dose of $BaCl_2$ (2 mM) significantly decreased the amount of wakefulness by 42% compared to baseline (Tukey, $p < 0.001$) and 41% compared to vehicle (Tukey, $p < 0.001$), respectively (Fig. 5A). Additionally, a high dose of $BaCl_2$ (2 mM) decreased wakefulness by 18% compared to a low dose of $BaCl_2$ in a dose-dependent manner (Tukey, $p < 0.01$) (Fig. 5A).

However, in contrast to the LC, microinjections of $BaCl_2$ in the NTS significantly increased NREM sleep ($p < 0.001$, $F_{(3,27)} = 58.92$), (one-way RM-ANOVA followed by Tukey post-hoc (Fig. 5B). The low dose of $BaCl_2$ increased NREM sleep by 19% compared to baseline (Tukey, $p < 0.001$) and 17% compared to vehicle (Tukey, $p < 0.001$). Whereas a high dose of $BaCl_2$ (2 mM) significantly increased NREM sleep by 26% compared to baseline (Tukey, $p < 0.001$) and 24.2% compared to vehicle (Tukey, $p < 0.001$) (Fig. 5B).

Interestingly, microinjections of $BaCl_2$ in the NTS significantly augmented REM sleep amount in a dose-dependent manner ($p < 0.001$, $F_{(3,27)} = 183.70$) (one-way RM-ANOVA followed by Tukey post-hoc (Fig. 5C). The low dose of $BaCl_2$ increased REM sleep by 29.4% compared to baseline (Tukey, $p < 0.001$) and 30.5% compared to vehicle (Tukey, $p < 0.001$). Whereas a high dose of $BaCl_2$ (2 mM) significantly increased REM sleep by 44% compared to baseline (Tukey, $p < 0.001$) and 45% compared to vehicle (Tukey, $p < 0.001$) (Fig. 5C). Moreover, a high dose of $BaCl_2$ (2 mM) significantly increased REM sleep amount by 12% compared to a low dose (Tukey, $p < 0.01$) in a dose-dependent manner. Additionally, microinjection of both low and high doses of $BaCl_2$ into the NTS significantly increased the mean number of REM sleep episodes compared to the baseline and vehicle ($p < 0.001$, $F_{(3,27)} = 9.54$) (one-way RM-ANOVA followed by Tukey post-hoc (Fig. 6A). The low dose of $BaCl_2$ increased the number of REM sleep episodes by 44% compared to baseline (Tukey, $p < 0.05$) and 42.5% compared to vehicle (Tukey, $p < 0.05$). Whereas a high dose of $BaCl_2$ (2 mM) significantly increased the number of REM sleep episodes by 57% compared to baseline (Tukey, $p < 0.01$) and 55.7% compared to vehicle (Tukey, $p < 0.01$) (Fig. 6A). However, both doses of the $BaCl_2$ microinjections into the NTS did not change REM sleep episode length (Fig. 6B), suggesting that kir channel inhibition in the NTS enhances REM sleep primarily by transiting REM sleep episode numbers from NREM sleep.

Additionally, both low and high doses of $BaCl_2$ microinjections into the NTS significantly decreased the mean latency of REM sleep ($p < 0.001$, $F_{(3,27)} = 39.80$) (one-way RM-ANOVA followed by Tukey post-hoc (Fig. 6C). The low dose of $BaCl_2$ decreased the mean latency of REM sleep by 35.5% compared to baseline (Tukey, $p < 0.001$) and 31.3% compared to vehicle (Tukey, $p < 0.001$). Whereas a high dose of $BaCl_2$ (2 mM) significantly decreased the mean latency of REM sleep by 48% compared to baseline (Tukey, $p < 0.001$) and 45% compared to vehicle (Tukey, $p < 0.001$) (Fig. 6C).

Effects of $BaCl_2$ microinjections in the RTN on sleep–wake architecture

We next investigated if Kir channel inhibition in RTN neurons also modulates sleep–wake since it has been reported that both neurons and astrocytes of RTN express Kir channels abundantly. Microinjections of potassium inward rectifier channel inhibitor, $BaCl_2$, into the RTN (Fig. 7, n = 6) significantly increased wakefulness ($p < 0.001$, $F_{(3,23)} = 78.85$). However, NREM sleep significantly decreased ($p < 0.001$, $F_{(3,23)} = 88.61$) (one-way RM-ANOVA followed by Tukey post-hoc) (Fig. 8). We did not find any significant change in REM sleep amount after microinjections of either low or high dose of $BaCl_2$ in the RTN (Fig. 8C). The low dose of $BaCl_2$ microinjection (1 mM) increased wakefulness by 29% compared to baseline (Tukey, $p < 0.001$) and 27% compared to vehicle (Tukey, $p < 0.001$), respectively (Fig. 8A). Microinjection of high dose of $BaCl_2$ (2 mM) significantly increased the amount of wakefulness by 41% compared to baseline, (Tukey, $p < 0.01$) and 38% compared to vehicle, (Tukey, $p < 0.01$), respectively (Fig. 8A). Further, a high dose of $BaCl_2$ microinjections into the RTN increased the amount of wakefulness by 10% compared to the low dose (Tukey, $p < 0.05$) (Fig. 8A). The sites of microinjections of $BaCl_2$

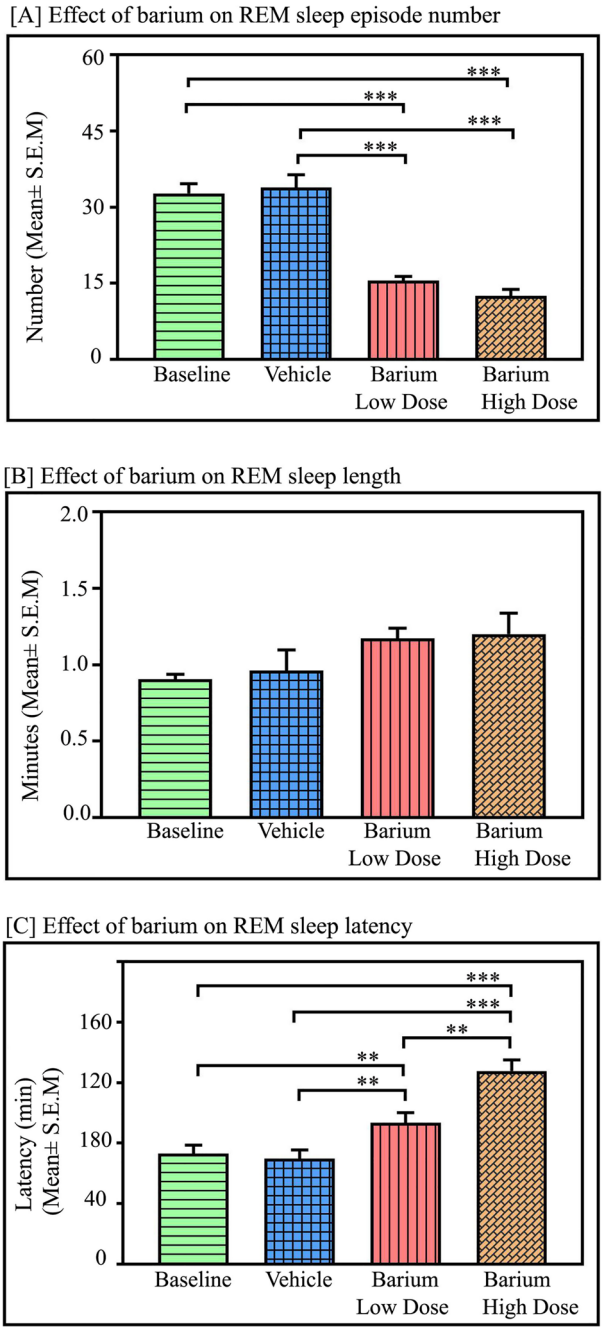

Fig. 3. Effects of Kir channel inhibition in the LC on REM sleep parameters: Effects of Kir channel inhibition in the LC on REM sleep parameters. (**A**) Microinjections of low and high doses of $BaCl_2$ (n = 7) in the LC significantly decreased the mean number of REM sleep episodes in contrast to the baseline and vehicle ($p < 0.001$, $F_{(3,27)} = 28.83$), (one-way RM-ANOVA followed by Tukey post-hoc test. (**B**) However, REM sleep episode length did not change with low dose or high dose of $BaCl_2$ microinjections into the LC when compared to the vehicle ($p > 0.05$, $F_{(3,27)} = 68.38$). (**C**) REM sleep latency was significantly increased after microinjections of low and high doses of $BaCl_2$ into the LC ($p < 0.001$, $F_{(3,27)} = 51.08$) (one-way RM-ANOVA followed by Tukey post-hoc test. ** denotes $p < 0.01$, *** denotes $p < 0.001$.

in 6 animals were within RTN, and 2 animals were outside the RTN (Fig. 7). Microinjection of $BaCl_2$ outside the RTN did not change wake, NREM sleep, and REM sleep amount (see Supplementary results and Fig. S2).

Microinjection of $BaCl_2$ in the RTN significantly decreased the amount of NREM sleep ($p < 0.001$, $F_{(3,23)} = 88.61$), (one-way RM-ANOVA followed by Tukey post hoc, Fig. 8B). The low dose of $BaCl_2$ decreased NREM sleep by 20% compared to baseline, (Tukey, $p < 0.001$) and 19% compared to vehicle, (Tukey, $p < 0.001$). Whereas a high dose of $BaCl_2$ (2 mM) significantly decreased NREM sleep by 28% compared to baseline (Tukey, $p < 0.001$) and 26.5% compared to vehicle (Tukey, $p < 0.001$) (Fig. 8B). Additionally, a high dose of $BaCl_2$

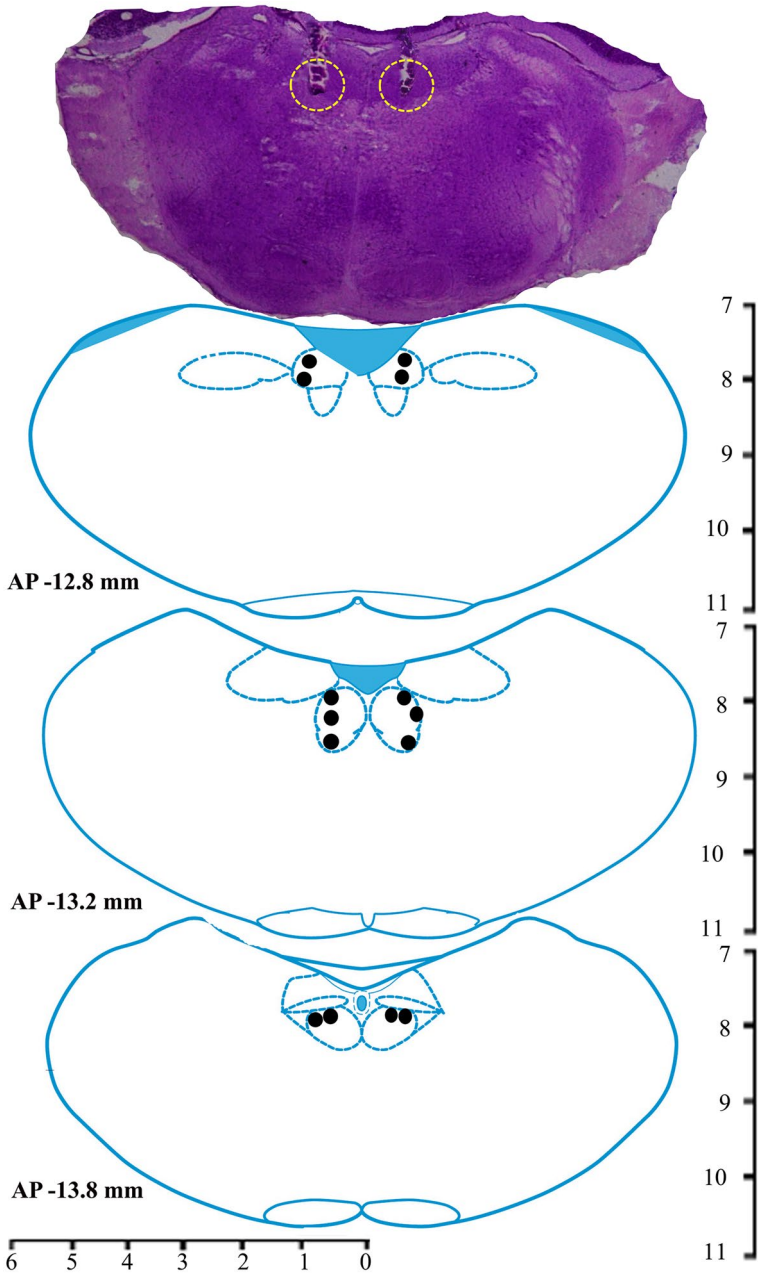

Fig. 4. A photomicrograph of 40 µm cresyl violet stained section and reconstruction diagram showing cannula tract and injection sites in the NTS. Filled circles (•) in the reconstruction diagram denote the site of injections within the NTS.

microinjections into the RTN decreased the amount of NREM sleep by 10% compared to the low dose (Tukey, $p < 0.01$) (Fig. 8B). However, microinjections of $BaCl_2$ in the RTN did not significantly change the amount of REM sleep compared to the baseline and vehicle groups (Fig. 8C).

Discussion

Our results show differential effects of Kir channel inhibition in the LC, NTS, and RTN on sleep–wake architecture. In the LC and RTN, inhibition of Kir channels led to an increased wakefulness and decreased NREM sleep. Conversely, inhibition of Kir channels in the NTS resulted in decreased wakefulness and increased NREM and REM sleep. Inhibiting Kir channels in the LC led to a significant reduction in REM sleep, while similar inhibition in the RTN did not produce notable changes in REM sleep. Interestingly, inhibiting Kir channels in the NTS increased REM sleep, primarily due to higher frequency of REM sleep episodes and a shorter latency to REM sleep. It can be surmised that the Kir channels in the NTS may play a crucial role in initiating REM sleep rather than its maintenance. Similar results were not observed when $BaCl_2$ was microinjected outside the LC and RTN

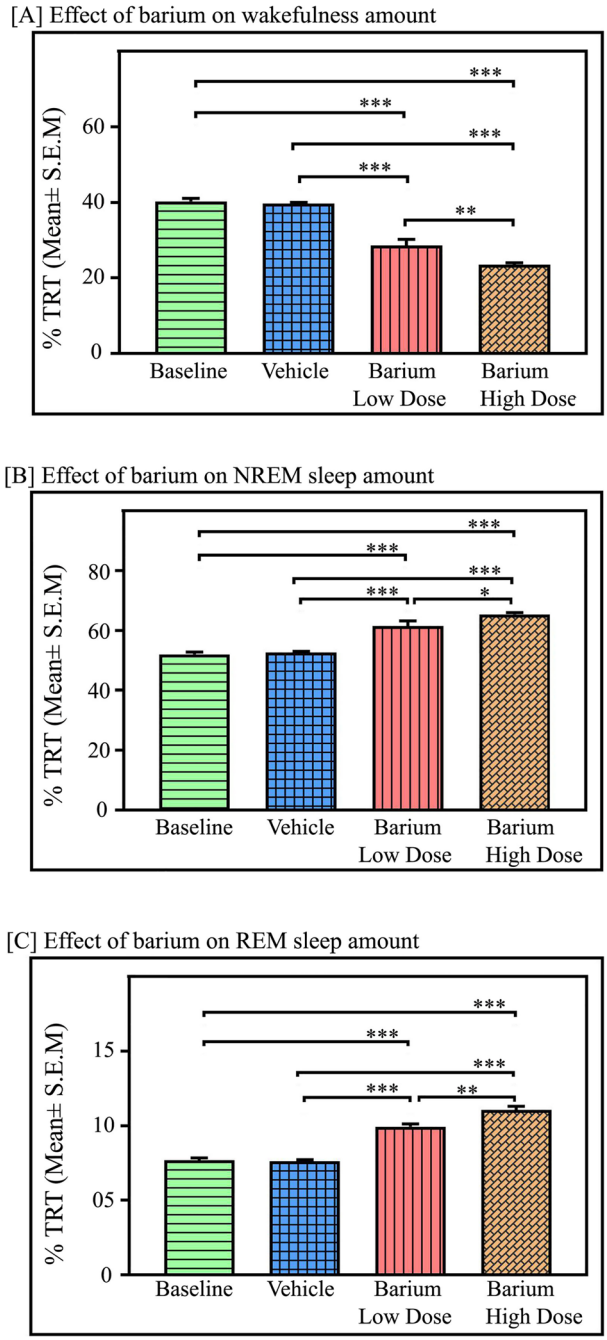

Fig. 5. Effects of Kir channel inhibition in the NTS on sleep–wake architecture. Microinjections of Kir channel blocker $BaCl_2$ in the NTS (n = 7) significantly decreased the percent amount of wake but increased NREM and REM sleep. (**A**) Microinjections of low dose (1 mM) and high dose (2 mM) of $BaCl_2$ in the NTS significantly decreased wakefulness ($p < 0.001$, $F_{(3,27)} = 91.26$), compared to the baseline and vehicle microinjections. (**B**) However, compared to baseline and vehicle, both the dose of $BaCl_2$ microinjections into the NTS significantly augmented NREM sleep amount ($p < 0.001$, $F_{(3,27)} = 58.92$), (**C**) Further, microinjections of low as well as high doses of $BaCl_2$ into the NTS significantly increased REM sleep amount in a dose-dependent manner ($p < 0.001$, $F_{(3,27)} = 183.70$, one-way RM-ANOVA followed by Tukey post hoc test). ** denotes $p < 0.01$, *** denotes $p < 0.001$.

(Supplementary results). These findings indicate that Kir channels in the LC, RTN, and NTS chemosensory regions affect sleep–wake patterns. Additionally, they suggest that Kir channels in the NTS, but not in the LC or RTN, regulate the transition from NREM to REM sleep.

The Kir channels are the potential central CO_2/H^+ sensor[41]. When CO_2/H^+ is detected, the inhibition of K^+ current plays a critical role in chemosensory neuronal membrane depolarization and subsequent processes like Ca^{2+} influx and neurotransmitter release[41,42]. Kir channels are inhibited when the surrounding pH is decreased[43].

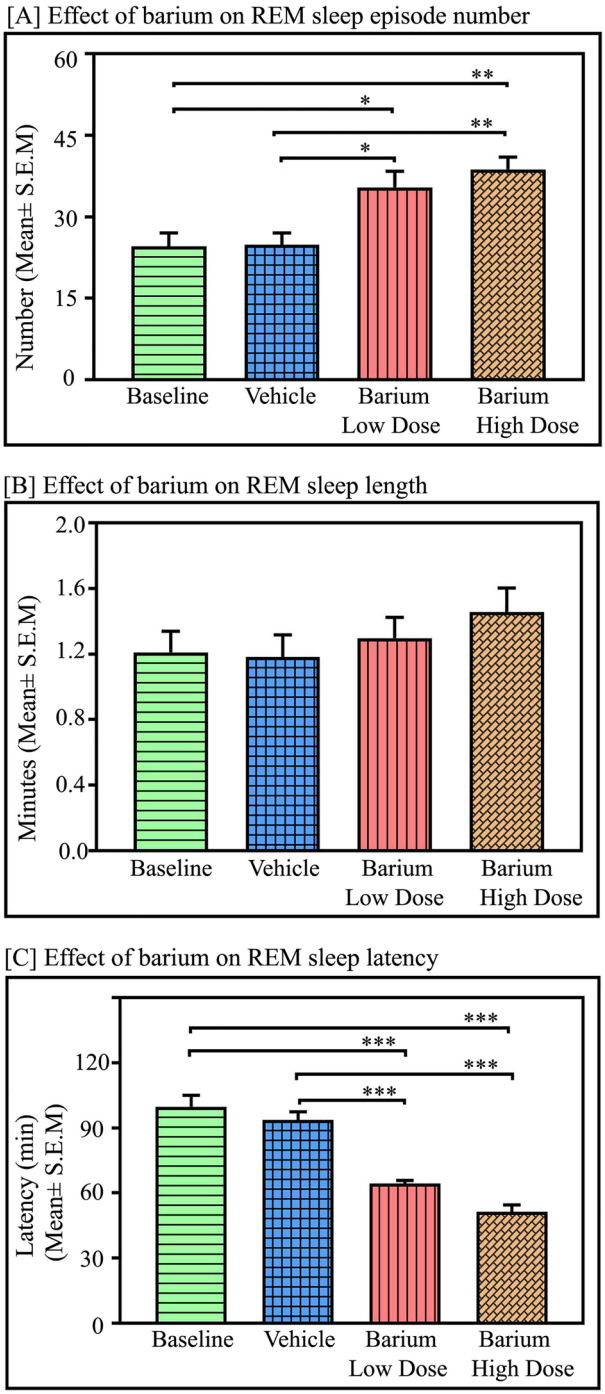

Fig. 6. Effects of Kir channel inhibition in the NTS on REM sleep parameters. Microinjections of kir channel blocker $BaCl_2$ in the NTS (n = 7) significantly increased (**A**) the average number of REM sleep episodes ($p < 0.001$, $F_{(3,27)} = 9.54$) but we did not observe any significant change in REM sleep episode length (**B**). However, kir channel inhibition in the NTS by low as well as high doses of $BaCl_2$ significantly decreased the average latency of REM sleep episodes ($p < 0.001$, $F_{(3,27)} = 39.80$, One-way RM-ANOVA followed by Tukey post-hoc test). *Denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

The conductance through Kir channels in the LC neurons is reduced in response to hypercapnic acidosis[43]. Furthermore, hypoxia and normoxic-hypercapnia-mediated changes in ventilatory responses are totally abolished in Kir5.1 knockout mice[44]. Conversely, Ca^{2+}-activated K^+ channels are inhibited during hypercapnic hypoxia[45]. These findings suggest that Kir channels in the chemosensitive neurons are crucial in detecting hypercapnic acidosis[43,46]. Additionally, Kir channel conductance increases with hyperpolarization, and the closure of these channels at depolarized potentials permits more prolonged action potentials[47]. Therefore, adjusting Kir channel

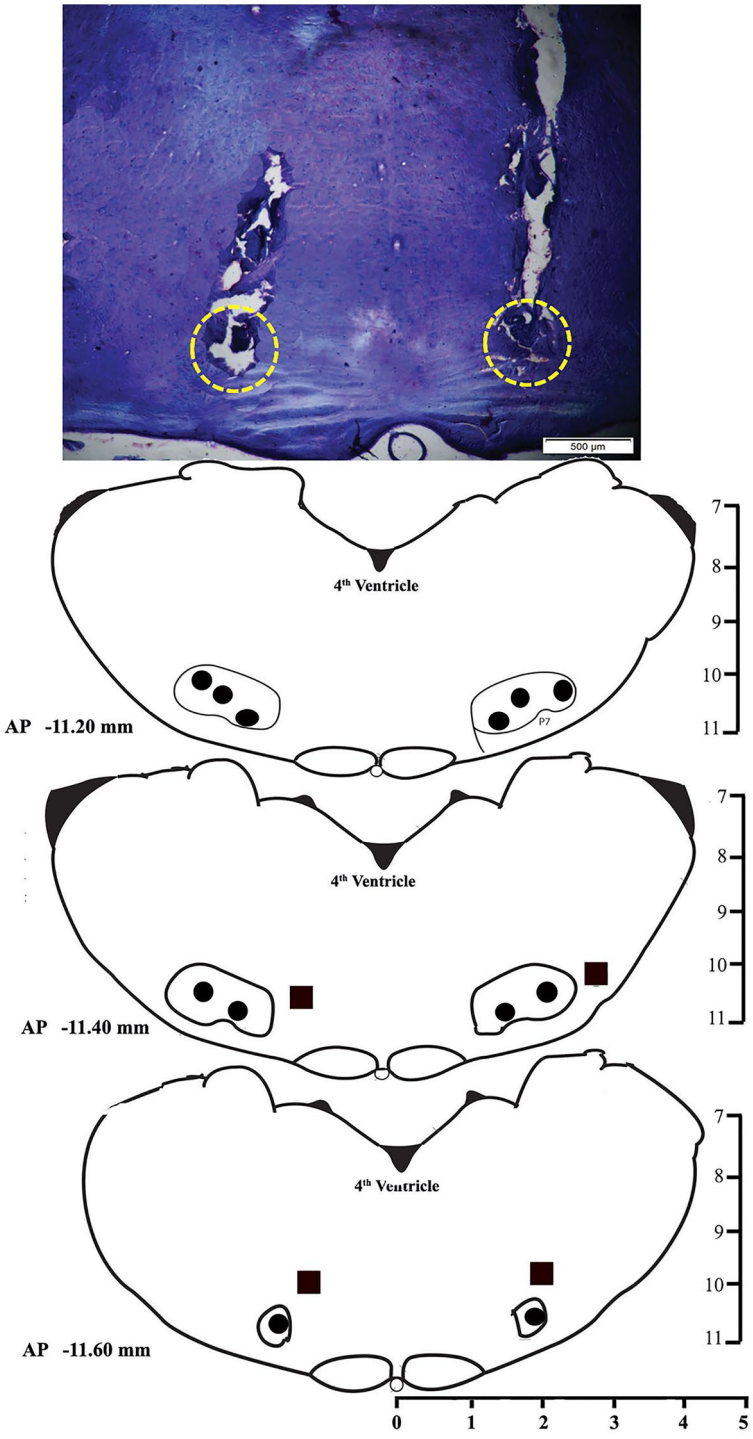

Fig. 7. A photomicrograph of 40 µm cresyl violet stained section and reconstruction diagram showing cannula tract and injection sites in the RTN. Filled circles (•) in the reconstruction diagram denote the site of injections within the RTN, and filled boxes (■) denote the site of injections outside of the RTN.

conductance can effectively regulate a neuron's membrane potential and neuronal excitability[47]. These studies suggest that Kir channels regulate ionic homeostasis at the membrane of various neurons and are also involved in H^+ sensing processes and central chemoregulation.

Fluctuations in bodily pH/CO_2 levels, resulting from different physiological and environmental factors, can alter the overall sleep–wake architecture. Alteration in bodily pH after meal, respiratory disorders like sleep apnea, central hypoventilation syndrome, and moderately increased CO_2 levels inside the room alter sleep–wake architecture profoundly[7,48–50]. Similarly, moderate hypercapnia induced by infusion of 2–4% CO_2 in breathing air

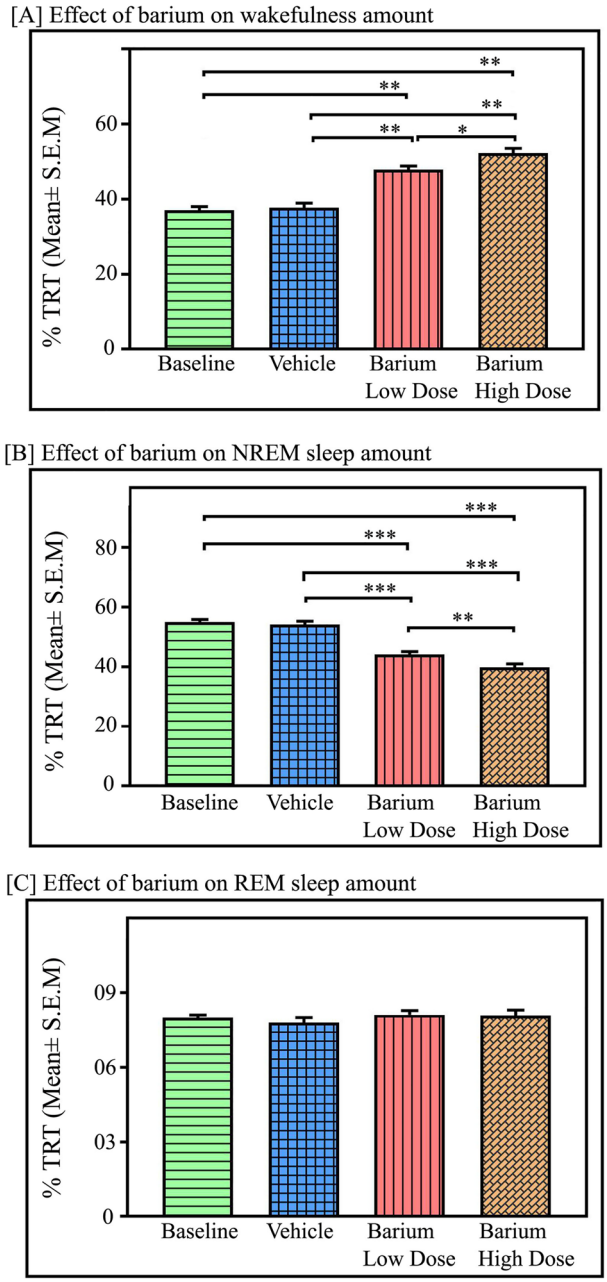

Fig. 8. Effects of Kir channel inhibition in the RTN on sleep–wake architecture. Microinjections of kir channel blocker $BaCl_2$ in the RTN (n = 6) significantly increased (**A**) wakefulness amount compared to the baseline and vehicle $p < 0.01$, $F_{(3,23)} = 78.85$), One way RM ANOVA, followed by post-hoc Tukey test. (**B**) However, $BaCl_2$ microinjections with low dose (1 mM) and high dose (2 mM) in the RTN significantly decreased NREM sleep amount compared to baseline and vehicle ($p < 0.001$, $F_{(3,23)} = 88.61$). (**C**) However, REM sleep amount did not change either with a low dose or a high dose of $BaCl_2$ microinjections into the RTN compared to baseline or vehicle ($p > 0.05$; $F_{(3,23)} = 40.39$). *Denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

increases NREM sleep amount and REM sleep episode numbers[11,33,51]. Studies suggest that during NREM sleep, reduced breathing rates lead to a rise in bodily CO_2 levels, which are then regulated back to normal through increased breathing[7,9]. Consequently, it is plausible that the accumulation of CO_2 within a narrow range during NREM sleep could facilitate the transition into REM sleep to help expel excess CO_2 without disrupting sleep[1,7]. However, if the CO_2 is accumulated to a greater extent, it may cause either micro or macro arousal[1,11,34]. The execution of such a shift from NREM to REM sleep under increased CO_2 levels unquestionably depends on the function of chemosensory machinery in the brainstem. Our recent findings are consistent with this supposition that chemosensory neurons in the LC, with the help of proton pumps and acid-sensing ion channels, help initiate the transition from NREM to REM sleep under normocapnic as well as hypercapnic conditions[11].

The brainstem's chemosensory machinery possibly play dualistic and complementary functions[11,23,52]. For example, the LC is one of the essential chemosensory areas with a well-established role as one of the wake-centers in the brain. Its neuronal activation causes arousal and inhibition or lesion promotes sleep[28,29,53,54]. Similarly, the NTS has dual functional chemoregulatory and sleep-promoting circuitries[14,20,31,32]. Studies suggest that high-frequency electrical stimulation of NTS induces REM sleep[13]. A group of neurons in the NTS exhibits an increased firing rate during NREM sleep, and possibly endogenous opioid receptors contribute to NREM sleep induction[32,55]. For the RTN, studies suggest that optogenetic stimulation of these neurons triggers sleep-dependent cardio-respiratory changes that ultimately lead to arousal from sleep[12,27]. Furthermore, the RTN acidification or optogenetic activation causes arousal and increased breathing during wake and NREM sleep but not during REM sleep, suggesting that the RTN also plays an essential role in NREM sleep-dependent breathing drive[56]. It is, however, not clear why different chemosensory brain areas influence S–W differently, but it can be attributed to their optimal capacity to sense the changes in pH. The sensitivity of neurons in these three areas to pH changes varies significantly. A narrow range of pH changes activates some neurons, while others only respond to substantial pH reductions, indicating they are sensitive to a broader pH range[57]. Therefore, it is likely that shifts in vigilance states from NREM sleep to wakefulness or REM sleep might be driven by excessive or minute alterations in pH levels respectively. For example, a major alteration in the pH level would induce wake, while mild hypercapnia would induce REM sleep. The neural system cannot sustain a prolonged hypercapnic state during NREM sleep. It must rapidly be corrected by increasing the breathing rate, which occurs during either wakefulness or REM sleep. Frequent awakenings from NREM sleep are not typical of normal physiology, as they can result in medical conditions like sleep apnea. Hence, it has been proposed that one of the functions of REM sleep is to maintain a normal brain CO_2 level for sustained and unperturbed sleep[7].

This study further enhances our understanding of the role of Kir channels in the brainstem's chemosensory system and its integration with the regulation of sleep–wake cycles. Previously, studies have reported that kir channels on cranial motor neurons regulate genioglossal muscle activity, thereby regulating breathing during REM sleep[57,58]. Further studies have also reinforced the role of Kir channels in the pCO_2/pH-dependent modulation of cardiovascular and respiratory physiology[59]. Kir channels also play a role in the pathophysiology of obstructive sleep apnea. Kir gene expression has shown a significant correlation with the severity of sleep apnea, suggesting their involvement in apneic events[60]. Additionally, the increase in circulating Kir proteins following positive airway pressure therapy further implies their role in obstructive sleep apnea[60]. Interestingly, sleep apnea is associated with sleep fragmentation and repeated arousals from sleep, which pose a greater risk for the development of cardio-respiratory diseases as well as cognitive impairments[9,61]. On the other hand, studies suggest that sleep and anesthesia drastically reduced cortical extracellular levels of K^+ ions, and extracellular application of K^+ by micro-iontophoresis causes arousal from sleep, implying that K^+-dependent sleep-state transitions occur quickly[62]. It is interesting to note that during sleep, extracellular K^+ levels decrease; however, the underlying mechanism is not known. Kir channels are inward-rectifiers that move K^+ inside more swiftly than outside; hence, they could be involved in vigilance state-dependent K^+ homeostatic balance. All these studies suggest that kir channels in the brainstem may be involved in the sleep-to-wake transition. This study expands our understanding by showing that Kir channels in the NTS, but not in the LC and RTN, influence the transition from NREM to REM sleep.

In conclusion, our study provides insights into the role of chemosensory machinery in regulating state transitions from NREM sleep to REM sleep/wakefulness during the sleep time of the circadian phase. This mechanism is crucial to a 'brainstem alarm' executive circuit that may govern state transition responses mediated by hypercapnia, such as (i) abrupt changes in pH can trigger wakefulness from NREM sleep, and (ii) mild changes in pH can induce REM sleep from NREM sleep. The dynamic transition from NREM to REM sleep may restore H^+ homeostasis by increasing breathing rates to expel excessive CO_2. This transition helps prevent repeated arousals from sleep, which pose significant risks to cardiovascular and respiratory health and cognitive function[63]. Further, chemosensory dysfunctions may contribute to various sleep-related breathing disorders, such as sleep apnea, congenital central hypoventilation syndrome, sudden infant death syndrome, etc. Future studies should identify specific subsets of neurons within central chemosensory areas that play dualistic roles in regulating the initiation or termination of sleep states based on bodily CO_2 levels and other ionic disturbances. Such findings hold promise for developing novel treatment strategies and management approaches for various sleep-related breathing disorders.

Data availability

All the data used in the current study will be available from the corresponding and co-author upon reasonable request.

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Author contributions

F.A.M. performed and designed the experiments, analyzed the data, and prepared the manuscript. S.K.J. conceived and conceptualized the ideas, designed the experiments, analyzed the data, and finalized the manuscript. All authors reviewed the manuscript before submission.

Competing interests

The authors declare no competing interests.

Additional information

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