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ORIGINAL INVESTIGATION

The retinoid X receptor: a nuclear receptor that modulates the sleep-wake cycle in rats

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Abstract

Rationale The nuclear receptor retinoid X receptor (RXR) belongs to a nuclear receptor superfamily that modulates diverse functions via homodimerization with itself or several other nuclear receptors, including PPARα. While the activation of PPARα by natural or synthetic agonists regulates the sleep-wake cycle, the role of RXR in the sleep modulation is unknown.

Objectives We investigated the effects of bexarotene (Bexa, a RXR agonist) or *UVI 3003* (UVI, a RXR antagonist) on sleep, sleep homeostasis, levels of neurochemical related to sleep modulation, and *c*-Fos and NeuN expression.

Methods The sleep-wake cycle and sleep homeostasis were analyzed after application of Bexa or UVI. Moreover, we also evaluated whether Bexa or UVI could induce effects on dopamine, serotonin, norepinephrine epinephrine, adenosine, and acetylcholine contents, collected from either the nucleus accumbens or basal forebrain. In addition, *c*-Fos and NeuN expression in the hypothalamus was determined after Bexa or UVI treatments.

Results Systemic application of Bexa (1 mM, i.p.) attenuated slow-wave sleep and rapid eye movement sleep. In addition, Bexa increased the levels of dopamine, serotonin, norepinephrine epinephrine, adenosine, and acetylcholine sampled from either the nucleus accumbens or basal forebrain. Moreover, Bexa blocked the sleep rebound period after total sleep deprivation, increased in the hypothalamus the expression of *c*-Fos, and decreased NeuN activity. Remarkably, *UVI 3003* (1 mM, i.p.) induced opposite effects in sleep, sleep homeostasis, neurochemicals levels, and *c*-Fos and NeuN activity.

Conclusions The administration of RXR agonist or antagonist significantly impaired the sleep-wake cycle and exerted effects on the levels of neurochemicals related to sleep modulation. Moreover, Bexa or UVI administration significantly affected *c*-Fos and NeuN expression in the hypothalamus. Our findings highlight the neurobiological role of RXR on sleep modulation.

Keywords Adenosine · Dopamine · Retinoid X receptor · Serotonin · Sleep

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Introduction

The nuclear receptor retinoid X receptor (RXR) belongs to a superfamily of nuclear receptors engaged in a wide variety of biological processes, including protective effects against neurodegenerative diseases (Casali et al., 2018; Loera-Valencia et al., 2019; Martin et al., 2019; Schierle and Merk, 2019; Simandi et al., 2018). The RXR can form both homodimer as well as heterodimer complexes with other nuclear receptors, such as the peroxisome proliferator-activated receptor alpha (PPARα; Kojetin et al., 2015; Lefebvre et al., 2010). The activation of PPARα by natural or synthetic agonists engages gene expression and modulates diverse physiological and clinical conditions, such as cancer, cardiovascular disorders, autism, among others (Barone et al., 2019; Choi, 2019; Laleh et al., 2019; Mirza et al., 2019; Yamashita et al., 2019). Our group has demonstrated that modulation of PPARα activity by the synthetic agonist (Wy14643) or the antagonist (MK-886) exerts effects on the sleep-wake cycle and neurochemicals linked to sleep control (Mijangos-Moreno et al., 2016; Murillo-Rodríguez et al., 2016; Murillo-Rodriguez, 2017).

On the other hand, agonist or antagonists to RXR are used to explore the molecular mechanism of action in medical treatments (Martínez et al., 2019; Krężel et al., 2019; Schierle and Merk, 2019; Wnuk et al., 2018). In this regard, the RXR agonist bexarotene (Bexa) has been tested with positive results for the management of diabetes, cancer, and cardiovascular diseases (Geller et al., 2019; Guleria et al., 2013; Tu et al., 2018). Moreover, multiple pieces of evidence have demonstrated that Bexa shows neuroprotective properties against neurodegenerative diseases, including Alzheimer's disease, multiple sclerosis, and Parkinson's disease as well (Bartzokis, 2014; Fanaee-Danesh et al., 2019; McFarland et al., 2013). Whereas the molecular mechanism of action of Bexa remains to be described in detail, several docking experiments have shown that this drug activates various RXR receptor isoforms (α, β, and γ) as a putative molecular pathway of activation (Nam et al., 2016; Dheer et al., 2018; Chitranshi et al., 2019).

Although limited, several antagonists to RXR have been recently studied, including *UVI 3003* (*UVI*; Clemens et al., 2018; Hebert et al., 2017; Mengeling et al., 2018; Watanabe and Kakuta, 2018) which, pharmacologically, behaves as a RXR pan antagonist acting on more than two isoforms (α and γ; Watanabe and Kakuta, 2018; AlSudais et al., 2018). Despite that the role of PPARα on sleep modulation has been explored, the role of RXR in sleep modulation is unknown. Furthermore, the effects of Bexa or UVI on sleep control have not been investigated. Thus, since Bexa activates PPARα (Tunctan et al., 2018; Yu et al., 2015), then we have hypothesized that the sleep-wake cycle may be influenced by the RXR agonist. To achieve this aim, we investigated the involvement of Bexa or UVI in the control of the sleep-wake cycle with focus on their role in sleep ad libitum and sleep homeostasis response. The effects of Bexa or UVI treatment on neurotransmitters, including dopamine, serotonin, norepinephrine epinephrine, adenosine, and acetylcholine, linked to sleep control were collected from either the nucleus accumbens of the basal forebrain for further study. In addition, the activation of neurons has been associated with the activity of immediate expression genes, including *c*-Fos and NeuN (de-la-Cruz et al., 2018; Duan et al., 2016; Farivar et al., 2004; Gallo et al., 2018; Gusel'nikova and Korzhevskiy, 2015; Hight et al., 2010; Huang et al., 2019; Jaworski et al., 2018; Kovács, 2008). Thus, the present study also addressed the potentially effects of Bexa or UVI treatment in sleep-related brain area by addressing the *c*-Fos and NeuN expression in the hypothalamus.

Materials and methods

Experiment 1: Bexarotene or UVI 3003 exert effects on sleep

Ethics approval

The animal experimental procedures were performed in accordance with the domestic and international standards of Animal Welfare including the Mexican Standards Related to Use and Management of Laboratory Animals (DOF. NOM-062-Z00-1999), the National Institutes of Health (NIH Publication No. 80-23, revised 1996), and the ARRIVE (Animal Research: Reporting of in vivo Experiments) guidelines, the commonly accepted "3Rs" Guidelines. Male Wistar rats from Animal Vivarium Center, Universidad Anáhuac Mayab, were used in this study. All the study was approved by the Research and Ethics Committee of Universidad Anáhuac Mayab (Mérida, Yucatán, México). For ethical reasons, the current report used a reduced number of animals.

Animals

Adult Male Wistar rats were used for the experiments ($N = 26$; 250–280 g). Upon arrival to our laboratory, the animals were acclimatized to the experimental conditions and kept under a 12-h light/dark cycle (lights on at 08:00 h; 200 lx), in a temperature (21 ± 1 °C), and humidity-controlled environment (60 ± 10%) with free access to food (Purina Rat Chow, Purina, México) as well as fresh tap water.

Chemicals and materials

Bexarotene (PubChem CID: 82146) and UVI 3003 (PubChem CID: 44566108) were purchased from Sigma-Aldrich (St. Louis, MO. USA). Drug solutions were prepared

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immediately before use in a vehicle (VEH) consisting of dimethyl sulfoxide (DMSO; PubChem CID: 679) followed by phosphate-buffered saline (10% DMSO final concentration; He et al., 2018; Dheer et al., 2019). All other chemicals and reagents were of analytical grade (Sigma-Aldrich, St. Louis, MO. USA). Sleep studies necessitated materials from Plastics One (Roanoke, VA, USA). Microdialysis experiments required materials from Bioanalytical Systems (BAS, West Lafayette, IN, USA) whereas high-performance liquid chromatography (HPLC) depended upon Shimadzu materials (Kyoto, Japan) and Merck Millipore (Darmstadt, Germany). The immunohistochemical analysis demanded reagents from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and Vector Laboratories (Burlingame, CA, USA).

Sleep-recording surgeries

Rats ($n = 8$) were anesthetized (acepromazine [0.75 mg/kg], xylazine [2.5 mg/kg], and ketamine [22 mg/kg]; intraperitoneal [i.p.]) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) for implantation of epidural screw electrodes for recording of the electroencephalogram (EEG), and stainless-steel wires in the neck extensor muscles to record the electromyogram (EMG). The EEG/EMG electrode wires were inserted into a six-pin connector plastic plug (Plastics One, Roanoke, VA. USA) and attached onto the skull by using commercial dental cement. The connector was attached to a 50-cm recording cable (Plastics One, Roanoke, VA. USA) linked to a 6-channel slip-ring commutator (Plastics One, Roanoke, VA, USA) which allowed free moving of animal around in the cage. Upon completion of the EEG/EMG surgeries, animals were placed into individual cages with free access to food and water (Murillo-Rodríguez et al., 2019).

Habituation processes

After EEG/EMG surgeries, animals were habituated to experimenters by daily 10 min of handling and removal from the sleep-recording system across 7 days. When signs of no stress (aggression, droppings, and/or urine) were observed when researcher handled the animals, the habituation period was considered complete.

Drugs preparation and administrations

Seven days after EEG/EMG surgeries (habituation/post-surgery recovery period), rats were randomly assigned to the following groups: control (vehicle [VEH]: DMSO and phosphate-buffered saline [10% DMSO final concentration]), bexarotene (Bexa), or UVI 3003 (UVI). The final concentration of Bexa and UVI was the same (1 mM). The solutions of Bexa (5 mg/kg, i.p.) and UVI (3 mg/kg, i.p.) were injected as reported by others (Dheer et al., 2019; He et al., 2018; Zhang et al., 2019). To avoid experimental biases, such as circadian influences in sleep results, the pharmacological challenges were given 1 h after the start of the beginning of either the lights-on or the lights-off period. In addition, one blind observer to the nature of treatments applied the administrations. Once experimental challenges were administered, rats were reattached to the sleep-recording system and sleep data were collected across the next 4 h. For ethical reasons, a reduced number of animals were used in the whole project. Thus, to achieve our experimental hypothesis, a Latin square crossover design was used then (Table 1). The Latin square is an experimental design in which each treatment is assigned to a specific period, with a defined number of interventions given to each subject. The carryover effects are controlled by using the Latin square experimental design that are "counterbalanced." In addition, treatments were applied to rats with an interval between administrations of 48 h.

Analysis of sleep recordings

The EEG/EMG signals were scored in 12 s epochs and amplified, filtered (70 Hz [low-pass filter] and 0.3 Hz [high-pass filter]; Model M15LT 15A54, Grass Instruments, Quincy, MA, USA), and digitized at a sampling rate of 128 Hz by using a 100-bit analog-to-digital converter board (NI PCI-6033E Multifunction I/O Board and NI-DAQ Software, SCB-100 Shielded Connector Block, National Instruments, Austin, TX, USA) recorded and visually scored with the ICELUS software. The EEG/EMG data were characterized in wakefulness (W) when the presence of desynchronized EEG with high EMG activity was observed in sleep recordings whereas the slow-wave sleep (SWS) defined as high-amplitude slow waves with a low EMG tone relative to W was found in the EEG/EMG charts. Lastly, rapid eye movement sleep (REMS) was identified by regular theta activity

Table 1 The Latin square design used in the current study. The Latin square design was a method used for placing treatments in a balanced fashion within a square block or field. The treatments are assigned randomly once in each row and column. The Latin square design included equal numbers of rows, columns, and treatments

Subjects	Treatment period 1	Treatment period 2	Treatment period 3
Rat 1	VEH	UVI	Bexa
Rat 2	Bexa	VEH	UVI
Rat 3	UVI	Bexa	VEH
Rat 4	VEH	UVI	Bexa
Rat 5	Bexa	VEH	UVI
Rat 6	UVI	Bexa	VEH
Rat 7	VEH	UVI	Bexa
Rat 8	Bexa	VEH	UVI

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across the EEG associated with low EMG signal. One observer blinded to the experimental codes of the animals scored the sleep data (Murillo-Rodríguez et al., 2019).

Statistical analysis

All values are expressed as mean ± standard error of the mean. Statistical analyses were performed using StatView (version 5.0.0, SAS Institute. USA). The significance of differences for the experimental groups was determined using one-way analysis of variance (ANOVA) followed by Bonferroni test. $P < 0.05$ was considered statistically significant.

Experiment 2: Sleep homeostasis under influence of Bexarotene or UVI 3003

Ethics approval, animals, chemicals and materials, sleep-recording surgeries, and habituation processes

As described in Experiment 1.

Sleep deprivation procedure

In addition to the characterization of the pharmacological effects of Bexa or UVI on the sleep-wake cycle, we tested whether the RXR modulators would modify the sleep homeostasis. To achieve this goal, animals ($n = 8$) were subjected to total sleep deprivation (TSD) during 6 h by maintaining them on continuous waking from 08:00 to 14:00 h. The sleep deprivation procedure was developed by generating sounds (tapping the cages) or by placing novel objects into the sleep chambers (Murillo-Rodríguez et al., 2017). Upon the TSD was completed and under a blinded procedure, pharmacological challenges were administered to rats allowing them to sleep ad libitum for the next 4 h (sleep rebound period). The characterization and scoring the states of vigilance during the sleep rebound period were carried out as previous criteria (Murillo-Rodríguez et al., 2017). The sleep stages during the sleep rebound period were analyzed by one observer blinded to the code of the rats.

Statistical analysis

As described in Experiment 1.

Experiment 3: Neurochemical response to Bexarotene or UVI 3003

Ethics approval, animals, chemicals and materials, habituation processes, and drugs preparation and administrations

As described in Experiment 1.

Microdialysis surgeries

Studies from our group have demonstrated reliable measurements of monoamines (dopamine [DA], serotonin [5-HT], norepinephrine [NE], and epinephrine [EP]) sampled from nucleus accumbens (AcbC; Murillo-Rodríguez et al., 2017, 2019). Therefore, we determined whether Bexa or UVI would induce changes in the extracellular contents of monoamines collected by microdialysis means from AcbC and determined by analytical approaches. To achieve this goal, a new group of rats ($n = 6$) were anesthetized and mounted into the stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) for implantation of a microdialysis guide cannula (IC guide; Bioanalytical Systems [BAS], West Lafayette, IN, USA) aimed unilaterally into the AcbC (coordinates $A = +1.2$, $L = +2.0$, and $H = -7.0$ mm, with reference to bregma [Paxinos and Watson, 2005]). The microdialysis probe was attached onto the skull by dental cement. Once the surgery was completed, rats were placed into the microdialysis bowl (Raturn Microdialysis Stand-Alone System, MD-1404, BAS, West Lafayette, IN, USA) during 7 days for post-surgery recovery as well as habituation for the experimental conditions. All surgical procedures of microdialysis surgery were accomplished as previously reported (Murillo-Rodríguez et al., 2017, 2019).

Microdialysis sampling procedures

Once the recovery and habituation period of time was attained, rats were removed from the microdialysis bowls for withdrawing the stylet from guide cannula. Next, the microdialysis probe (1 mm of length; polyacrylonitrile, MWCO = 30,000 Da; 340 μm OD; BAS, West Lafayette, IN, USA) was inserted at 08:00 h. Then, artificial cerebrospinal fluid (aCSF [composition: NaCl [147 mM], KCl [3 mM], $CaCl_2$ [1.2 mM], $MgCl_2$ [1.0 mM], pH 7.2]) was perfused through a miniature tube (0.65 mm OD × 0.12 mm ID; BAS, West Lafayette, IN, USA) attached to a 2.5-mL syringe (BAS, West Lafayette, IN, USA) using a pump (flow rate 0.25 μL/min; BAS Bee, West Lafayette, IN, USA). The probe was placed in the AcbC at least 24 h before the experiments in order to minimize the damage made by the insertion and allow the probe's stabilization. The samples from stabilization period were excluded for the final analyses. In addition, the probes in all microdialysis studies across our study were used < 5 days since previous studies have showed tissue damage, resulting in findings of gliosis at the implantation site between 3 and 7 days after implantation of microdialysis probe (Anderzhanova and Wotjak, 2013; Hammarlund-Udenaes, 2017; Murillo-Rodríguez et al., 2004). Twenty-four hours after the insertion of the probe, animals received the experimental trials, and samples were collected every 20 min at the beginning of each hour during the following 4 h. Later, all samples were stored

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(− 80 °C; Revco Ultima PLUS, Thermo Scientific, Waltham, MA, USA) for further analysis. The microdialysis sampling procedure was developed as previously reported (Murillo-Rodríguez et al., 2017, 2019).

Measurements of monoamines using HPLC

The collected microdialysis samples from treatments applied at either the lights-on or lights-off period were filtered (Millipore 0.22 μm; Merck Millipore, Darmstadt, Germany) and injected into high-performance liquid chromatography (HPLC; Modular Prominence, Shimadzu, Kyoto, Japan). The detection of DA, 5-HT, NE, and EP required a mobile phase of NaH_2PO_4 (7 mM, pH 3.0), plus MEOH (3.5%), which was perfused into the HPLC at a flow rate of 80 μL/min (pump LC-20AT, Shimadzu, Kyoto, Japan). The separation of molecules was achieved by using a microbore column (octadecyl silica [3 μm, 100 × 1 mm], BAS, West Lafayette, IN, USA), under controlled temperature (22 °C; oven CTO-20A, Shimadzu, Kyoto, Japan). The detection of monoamines was obtained by an electrochemical detector (LC-4C; BAS, West Lafayette, IN, USA). The chromatographic data were stored on a personal computer (via computer controller CBM-20A, Shimadzu, Kyoto, Japan), and the concentrations of monoamines in analyzed samples were obtained by comparing the external known concentration of standards for DA, 5-HT, EP, and NE. The procedure for the measurements of monoamines was developed as described previously (Murillo-Rodríguez et al., 2017, 2019). The determination of contents of monoamines was achieved under a blinded procedure.

Statistical analysis

The total value obtained for each molecule consisted in the sum of hourly collected samples from each neurotransmitter across 4 h post-treatments. Data from microdialysis experiments were represented as mean ± SEM, and the statistical differences among the experimental groups were determined by one-way ANOVA followed by Bonferroni's post hoc test. All statistical analyses were performed using the StatView software (version 5.0.0, SAS Institute, USA), and statistical differences among groups were determined if $P < 0.05$.

Experiment 4: Bexarotene or UVI 3003 modulate adenosine contents

Ethics approval, animals, chemicals and materials, habituation processes, drugs preparation and administrations

As described in Experiments 1 and 3.

Microdialysis surgeries

Several pieces of evidence have suggested that adenosine (AD) is measurable from the basal forebrain (Kalinchuk et al., 2011; Sharma et al., 2017; Vazquez-DeRose et al., 2016). Then, in this part of our study, a new group of rats ($n = 6$) were anesthetized and mounted into the stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) for unilateral implantation of a microdialysis guide cannula (IC guide; Bioanalytical Systems [BAS], West Lafayette, IN, USA) aimed into the basal forebrain ($A = -0.35$; $L = 2.0$; and $H = -7.5$ mm, with reference to bregma [Paxinos and Watson, 2005]). All microdialysis surgery procedure was developed as described in Experiment 3.

Microdialysis sampling procedures

As described in Experiment 3.

Analytical determination of adenosine

As described in Experiment 3, with the following differences, mobile phase was prepared with NaH_2PO_4 (10 mM; pH 4.5) and MEOH (9%) perfused at a flow rate of 80 mL/min using a pump (LC-20AT, Prominence HPLC, Shimadzu, Japan). The detection of AD was achieved by using an UV detector (SPD-20A Prominence, Shimadzu, Kyoto, Japan) set to a wavelength of 254 nm (deuterium lamp). The HPLC procedure for detection of AD was developed as previously reported (Murillo-Rodríguez et al., 2017, 2019). To avoid interpretive bias, the dialysates were analyzed under a blinded code. For statistical analysis, the randomization code was then broken.

Statistical analysis

As described in Experiment 3.

Experiment 5: Acetylcholine contents after administration Bexarotene or UVI 3003

Ethics approval, animals, chemicals and materials, habituation processes, drugs preparation and administrations, microdialysis surgeries

As described in Experiments 1 and 4.

Microdialysis sampling procedure

As described in Experiment 4 with exception of aCSF which was composed by KCl (2.4 nM), Na_2SO_4 (0.5 nM), NaCl (126.5 nM), $CaCl_2$ (1.2 nM), $NaHCO_3$ (27.5 nM), KH_2PO_4 (0.5 nM), $MgCl_2$ (0.8 nM), and dextrose (5.0 nM) at pH 6.8, the aCSF was perfused through a miniature tubing (0.65 mm

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OD; 0.12 mm ID; BAS, West Lafayette, IN, USA) attached to a 2.5-mL syringe (BAS, West Lafayette, IN, USA) with a pump (flow rate 2 mL/min; BAS Bee, West Lafayette, IN, USA). All microdialysis procedures were developed as previously reported (Murillo-Rodríguez et al., 2018).

Acetylcholine measurement

The HPLC settings for ACh detection was used as follows: $C_{12}H_{19}BrN_2O_2$ (100 nM) was added to the aCSF (Murillo-Rodríguez et al., 2018). In addition, an acetylcholine-choline assay kit (MF-8910; BAS, West Lafayette, IN, USA) was included for ACh detection. The separation of molecule was achieved by injecting samples into the HPLC at a flow rate of 1 mL/min, under temperature of 28 °C (oven CTO-20A, Shimadzu, Kyoto, Japan), using a 10-cm analytical column (MF-6150, BAS, West Lafayette, IN, USA) and a mobile phase (Na_2HPO_4 [35 nM], EDTA [0.1 nM], and ProClin 150 preservative (0.005%); BAS, West Lafayette, IN, USA) with pH 8.5. An electrochemical detector allowed the ACh determination (LC-4C; BAS, West Lafayette, IN, USA) maintaining the potential at + 0.5 V. The procedure to determine ACh was developed as previously reported (Grupe et al., 2013; Murillo-Rodríguez et al., 2018). A blinded analysis strategy was used to minimize the possibility of bias in experimental results.

Relative recovery of the microdialysis probes

The in vitro relative recovery experiments in all microdialysis studies (monoamines, AD, and ACh sampling) were achieved by inserting the microdialysis probes into a solution containing external known standards of tested molecules (DA, 5-HT, NE, EP, AD, and ACh) under similar conditions as Experiments 3–5 were developed. The dialysates were collected in a triplicate and each sample was analyzed. Then, the peak area ratio for monoamines, AD, or ACh was calculated against the external known standards. The relative recovery data were calculated as follows: Recovery rate (%) D (the peak area ratio of the dialysate) / (the peak area ratio of the dialysate in the test solution). The in vitro relative recovery procedure was developed as previously reported (Huang et al., 2013; Kho et al., 2017; Kou et al., 2019; Murillo-Rodríguez et al., 2004; Porkka-Heiskanen et al., 2000).

Histological verification of microdialysis probes implantation

The placement of microdialysis probes into target areas was verified as described previously (Blanco-Centurion et al. 2006). Briefly, after the microdialysis study, rats were sacrificed by a lethal dose of pentobarbital for the standard procedure of vascular perfusion (saline solution [0.9%] followed by formaldehyde [4%]). The brain was collected and post-fixed overnight in formaldehyde (4%) followed by 10, 20, and 30% sucrose/0.1 M PBS during 24 h/each concentration. Later, brains were cut in coronal sections (20 μm) using a Portable Bench-top Cryostat (Leica CM1100, Wetzlar, Germany) and collected in 1:5 serial order. For verification of probe location, one series was used for plotting the targeted brain area (Paxinos and Watson, 2005). Sections that showed the microdialysis probe localization outside of target area were excluded for the final neurochemical analysis. The histological procedures were developed as previously reported (Blanco-Centurion et al. 2006; Murillo-Rodríguez et al., 2004).

Statistical analysis

As described in Experiment 3.

Experiment 6: Bexarotene or UVI 3003 affects *c*-Fos expression in hypothalamus

Ethics approval, animals, chemicals and materials, habituation processes, and drugs preparation and administrations

As described in Experiment 1.

Immunohistochemical studies

The expression of *c*-Fos by individual neurons normally is used as a marker of cell activation allowing to elucidate a functional neuroanatomical mapping in response to specific stimuli (Perrin-Terrin et al., 2016). Thus, to characterize the effects of Bexa or UVI on molecular activity in a brain area related to the sleep-wake cycle control, such as the hypothalamus, we analyzed the *c*-Fos expression in such region. This brain area was selected since it has been suggested as a key element in the modulation of the sleep-wake cycle (Arrigoni et al., 2019; Jones, 2019). Then, a new set of animals ($n = 24$) were used for the *c*-Fos studies. One group of rats received at 09:00 h a systemic (i.p.) injection of either control ($n = 4$), Bexa ($n = 4$), or UVI ($n = 4$), and 1 h later, they were sacrificed by a lethal dose of pentobarbital for the standard procedure of vascular perfusion. Same experimental condition was carried out at lights-off period: At 21:00 h, animals were sacrificed 1 h later after receiving an application of control ($n = 4$), Bexa ($n = 4$) or UVI ($n = 4$). Brains were collected and processed for *c*-Fos immunostaining. The identification of the hypothalamus included slides collected from coordinates − 0.12 to − 3.48 mm (from bregma according to the Rat Brain Atlas [Paxinos and Watson, 2005]). The Fos staining was developed as follows: we used standardized procedures for immunohistochemically detection of *c*-Fos by conventional avidin-biotin-immunoperoxidase chemicals to localize an

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antiserum raised against a synthetic N-terminal fragment of human Fos protein (de-la-Cruz et al., 2018). The selected sections were washed 3 times in PBS (0.1 M, pH 7.3) and incubated with the endogenous peroxidase (0.28% for 1 min) at room temperature and with H_2O_2 (3%) and MEOH (10%) in PBS (0.1 M) for 20 min at room temperature. Later, slides were washed 3 times in PBS (0.1 M, pH 7.3) and blocked with 10% donkey or goat serum diluted in PBS (containing 0.2% Triton X-100; Sigma-Aldrich, St Louis, MO, USA). Subsequently, the sections were incubated overnight with the corresponding primary antibody at 4 °C (goat anti-c-fos 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). On the next day, slides were again washed 3 times in PBS (0.1 M, pH 7.3) and incubated for 2 h at room temperature with the respective biotinylated secondary antibody (1:250, goat anti-mouse IgG, 125 K6063; Sigma-Aldrich, St Louis, MO, USA; donkey anti-rabbit IgG; Vector Laboratories, Burlingame, CA, USA). Next, the sections were washed other 3 times in PBS (0.1 M, pH 7.3) and incubated with the peroxidase complex (1:2000; Sigma-Aldrich, St. Louis, MO, USA) for 1 h in the dark. Lastly, following 3 washes in PBS, immunolabeling was revealed by exposing the slides to 0.05% diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA) and H_2O_2 (0.03%) in PBS. The reaction was stopped using PBS and sections were then washed several times again in PBS. The sections were mounted onto chrome alum gelatin-coated slides, dehydrated through graded alcohols, cleared in xylene, and cover slipped using DPX Mountant (Sigma-Aldrich, St Louis, MO, USA). The reproducibility of the *c*-Fos protocol was confirmed by repeating the whole procedure with additional series that contained approximately the same number of sections from all experimental groups using the same primary antibody. The whole *c*-Fos procedure was developed as previously reported (de-la-Cruz et al., 2018). To minimize bias, experimenters were not aware of the identity of the treatments in the immunohistochemical analysis.

The expression pattern of *c*-Fos in response to Bexarotene or UVI 3003

A similar number of sections of the hypothalamus (coordinates from − 0.12 to − 3.48 mm, according to the Rat Brain Atlas [Paxinos and Watson, 2005]) of both hemispheres of each rat, from all experimental groups, were included in the counting for *c*-Fos staining. Bright field images were photographed with a digital camera (AxioCam ICc1; Carl Zeiss Microscopy, Oberkochen, Germany) on a microscope (× 100 objective lens; Zeiss Imager A.2. Carl Zeiss Microscopy, Oberkochen, Germany) with the image analysis computer software (ZEN 2012, Blue Edition, Carl Zeiss Microscopy, Oberkochen, Germany). The analysis of images included *c*-Fos immunohistochemical patterns from slides containing the hypothalamus. We counted in 3 consecutive sections (coordinates − 0.12 to − 3.48 mm; from bregma according to the Rat Brain Atlas [Paxinos and Watson, 2005]) to perform these analyses in 4 animals per group. To minimize experimental bias, the tally of *c*-Fos expression was developed under a blind code, meaning that experimenters were unaware of the identity of treatments. The whole *c*-Fos study was carried out as described previously (de-la-Cruz et al., 2018).

Statistical analysis

As described in Experiment 1.

Experiment 7: Characterization of neurons expressing neuronal nuclear protein (NeuN) in hypothalamus after the application of Bexarotene or UVI 3003

Ethics approval, animals, chemicals and materials, habituation processes, and drugs preparation and administrations

As described in Experiment 1.

Immunohistochemical studies

Several studies have reported the activity of NeuN as a reliable marker of postmitotic neurons as well as neuronal differentiation (Duan et al., 2016; Gusel'nikova and Korzhevskiy, 2015). Having this in mind, we characterized whether administrations of Bexa or UVI might exert effects on NeuN expression in hypothalamus. To achieve this aim, standardized protocols for immunohistochemically detection of NeuN were used (de-la-Cruz et al., 2018; Hight et al., 2010). In detail, sections containing the hypothalamus were washed 3 times in PBS (0.1 M, pH 7.3) and blocked with 10% donkey or goat serum diluted in PBS (containing 0.2% Triton X-100). Subsequently, sections were incubated overnight with the corresponding primary antibody at 4 °C (mouse anti-NeuN; 1:500; Millipore, Billerica, MA, USA). On the next day, the sections were washed 3 times in PBS (0.1 M, pH 7.3) and incubated for 2 h at room temperature with the respective biotinylated secondary antibody (1:250; goat anti-mouse IgG, 125 K6063; Sigma, donkey anti-rabbit IgG; Vector Laboratories, Burlingame, CA, USA). Next, the sections were washed 3 times in PBS (0.1 M, pH 7.3) and incubated with the peroxidase complex (1:2000, Sigma-Aldrich, St Louis, MO, USA) for 1 h in the dark. Lastly, following 3 washes in PBS, sections were exposed for revelation to 0.05% diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA) and 0.03% H_2O_2 in PBS. The NeuN immunohistochemical studies were developed as previously reported (de-la-Cruz et al., 2018).

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The counting number of neurons expressing NeuN

As described in Experiment 6.

Statistical analysis

As described in Experiment 1.

Results

Bexarotene and UVI-3003 control the sleep-wake cycle

To determine the effect of RXR on sleep, Bexa or UVI was systemically (i.p.) administered and sleep-wake cycle was recorded and analyzed. As shown in Fig. 1 (panel a), application of Bexa at the beginning of the lights-on period enhanced W ($F_{(2, 21)} = 72.113$, $P < 0.0001$) and decreased SWS ($F_{(2, 21)} = 68.356$, $P < 0.0001$) while REMS was found with no statistical changes ($F_{(2, 21)} = 1.009$, $P = 0.3$). When UVI was injected at the beginning of the lights-on period, no significant differences were seen in W, SWS, or REMS (Fig. 1, panel a). Bonferroni test showed significant differences in alertness and SWS between VEH vs. Bexa ($P < 0.0001$) and Bexa vs. UVI ($P < 0.0001$).

Subjects	Treatment Period 1	Treatment Period 2	Treatment Period 3
Rat 1	VEH	UVI	Bexa
Rat 2	Bexa	VEH	UVI
Rat 3	UVI	Bexa	VEH
Rat 4	VEH	UVI	Bexa
Rat 5	Bexa	VEH	UVI
Rat 6	UVI	Bexa	VEH
Rat 7	VEH	UVI	Bexa
Rat 8	Bexa	VEH	UVI

Fig. 1 Effects of total time (4 h) of wakefulness (W), slow-wave sleep (SWS), and rapid eye movement sleep (REMS) after the administration (i.p.) at the beginning of either the lights-on (panel **a**) or lights-off period (panel **b**) of vehicle, bexarotene (Bexa), or UVI 3003 (UVI; 1 mM each compound). Application of Bexa at the beginning of the lights-on period increased W ($P < 0.0001$), decreased SWS ($P < 0.0001$), and caused no changes in REMS ($P = 0.3$; panel **a**) whereas UVI caused opposite effects in W and SWS (panel **a**). Bonferroni test showed significant differences in W and SWS between VEH vs. Bexa ($P < 0.0001$) and Bexa vs. UVI ($P < 0.0001$). On the contrary, Bexa injected at the beginning of the lights-off period (panel **b**) decreased W ($P < 0.0002$) and enhanced SWS ($P < 0.0006$) whereas REMS remained with no changes ($P = 0.5$). In addition, UVI exerted no significant changes if injected at the dark period in W, SWS, or REMS. Bonferroni test showed differences in W and SWS among VEH vs. Bexa ($P < 0.0001$) and Bexa vs. UVI ($P < 0.0001$; *vs. control; #vs. Bexa)

Next, we assessed the effect of Bexa or UVI on the sleep-wake cycle if injected at the beginning of the dark period (Fig. 1, panel b). The results showed that application of Bexa decreased W ($F_{(2, 21)} = 12.863$, $P < 0.0002$) and enhanced SWS ($F_{(2, 21)} = 10.779$, $P < 0.0006$) whereas REMS remained with no changes ($F_{(2, 21)} = 0.535$, $P = 0.5$). In addition, UVI-treated animals showed no statistical differences in W, SWS, or REMS. Further disparities addressed by Bonferroni test showed significant differences in W and SWS among VEH vs. Bexa ($P < 0.0001$) and Bexa vs. UVI ($P < 0.0001$). We conclude that RXR modulates sleep in a time-dependent fashion.

Sleep homeostasis is affected by application of bexarotene or UVI-3003

In addition to the effects on sleep during the lights-on or lights-off period, RXR caused marked changes in sleep homeostasis (Fig. 2). Six hours after TSD, rats received an intraperitoneal injection of either Bexa or UVI and sleep rebound was analyzed. The application of the RXR agonist increased the W amount during the sleep rebound ($F_{(2, 21)} = 52.59$, $P < 0.0001$) and no changes were observed in SWS ($F_{(2, 21)} = 15.345$, $P < 0.001$) while decreased REMS ($F_{(2, 21)} = 14.028$, $P < 0.0001$). Moreover, UVI did not modify the W amount but enhanced the SWS amount and decreased REMS. Bonferroni post hoc test showed significant differences in W (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), SWS (VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0003$), and REMS (VEH vs. Bexa, $P < 0.04$; VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.005$). These findings suggest that RXR modulates the sleep homeostasis elicited by the sleep rebound after a TSD.

Neurochemical analysis of DA, 5-HT, NE, EP, AD, and ACh contents in response to bexarotene or UVI 3003

Systemic administration of Bexa or UVI modified the sleep-wake cycle, suggesting that the RXR might be involved in the modulation of the states of vigilance via the release of neurochemicals related to the sleep control. In support of this assumption, the technique of microdialysis was coupled for sampling molecules related to the sleep-wake cycle control

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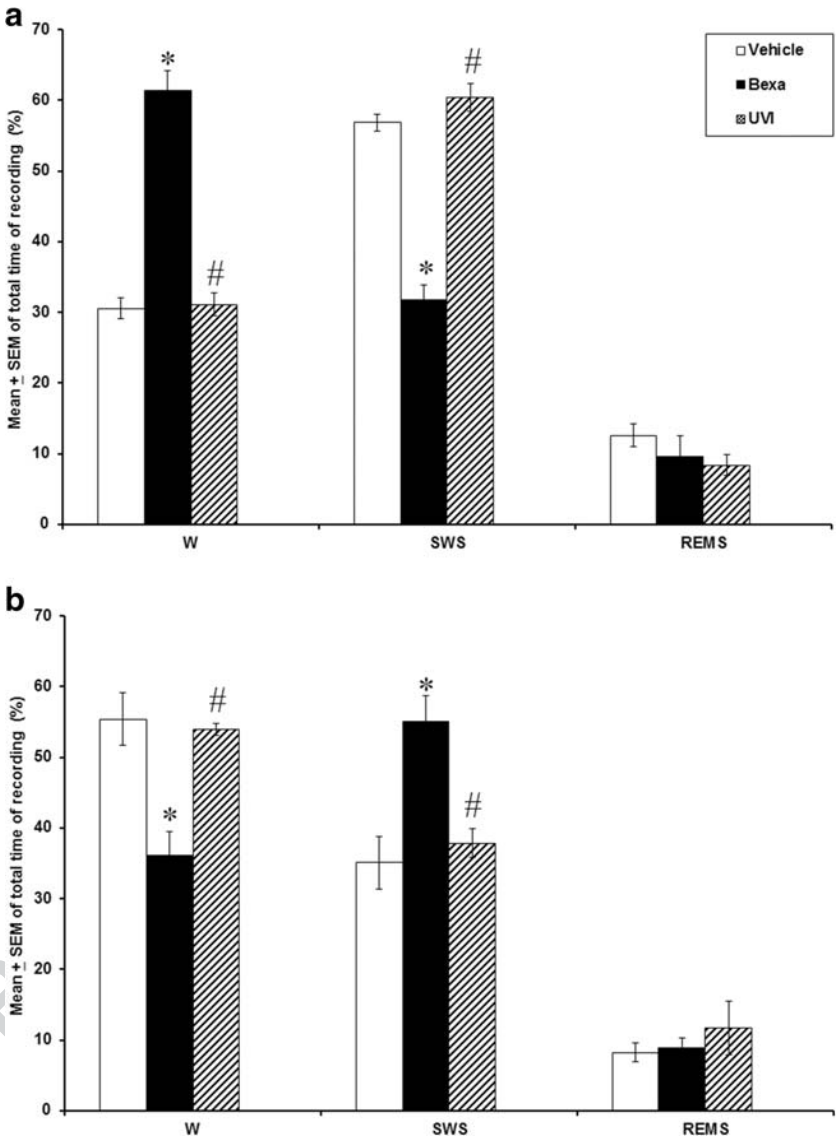

Fig. 2 Effects on total time (4 h) of wakefulness (W), slow-wave sleep (SWS), and rapid eye movement sleep (REMS) after 6 h of total sleep deprivation in rats that received a systemic application (i.p.) or either vehicle, bexarotene (Bexa), or UVI 3003 (UVI; 1 mM each compound). During the sleep rebound period, Bexa-treated animals showed an enhancement in W ($P < 0.0001$) with no changes in SWS ($P < 0.001$) whereas REMS was decreased ($P < 0.0001$). UVI induced opposite effects in the sleep rebound period in SWS and REMS. Bonferroni test showed differences in W (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), SWS (VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0003$), and REMS (VEH vs. Bexa, $P < 0.04$; VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.005$; *vs. control; #vs. Bexa)

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under the likely influence of Bexa or UVI. The total amount of the extracellular levels of DA, 5-HT, NE, EP, AD, and ACh was determined by the highly sensitive analytical technique HPLC which enables the quantification of low concentrations in small size samples (Hammarlund-Udenaes, 2017). The schematic illustration of the position of the probe into AcbC or basal forebrain is shown in Fig. 3 (panels a or b, respectively).

Next, we tested the ability of Bexa or UVI to modify the extracellular levels of DA, 5-HT, NE, EP, AD, and ACh. When challenged with Bexa at the start of the lights-on cycle, we found an increase in DA ($F_{(2,\ 21)} = 304.273$, $P < 0.0001$; Fig. 4, panel a), 5-HT ($F_{(2,\ 21)} = 69.859$, $P < 0.0001$; Fig. 4, panel b), but no significant changes were found in NE ($F_{(2,\ 21)} = 0.517$, $P = 0.6$; Fig. 4, panel c) but an increase was found in EP ($F_{(2,\ 21)} = 9.442$, $P < 0.001$; Fig. 4, panel d), AD ($F_{(2,\ 21)} = 174.966$, $P < 0.0001$; Fig. 4, panel e), and ACh ($F_{(2,\ 21)} = 30.588$, $P < 0.0001$; Fig. 4, panel f). Opposite results were found in UVI-treated rats in the sampled neurochemicals (Fig. 4, panels a–f). Further post hoc analysis via Bonferroni test showed significant differences among treatments for DA (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), 5-HT (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), EP (VEH vs. Bexa, $P < 0.0005$; Bexa vs. UVI, $P < 0.003$), AD (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), and ACh (VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$). The data suggest that RXR modulates the extracellular levels of neurochemicals linked to the sleep control if injected at the lights-on period.

Next, we analyzed if molecules related to the sleep modulation might be under the influence of RXR during the dark phase. To achieve this aim, Bexa or UVI was injected 1 h after the beginning of the lights-off period, and dialysates were collected across 4 h. This treatment regimen was highly

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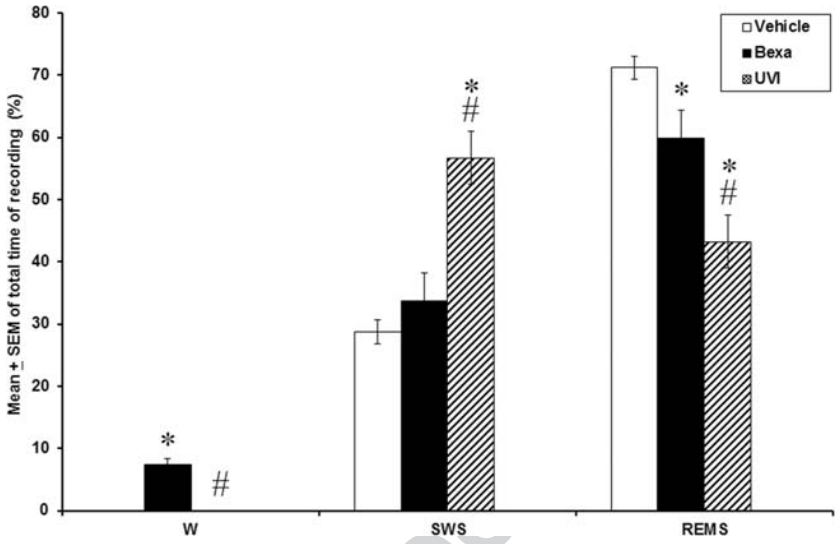

Fig. 3 Position of the microdialysis probe into the nucleus accumbens (AcbC) or basal forebrain. The schematic illustration from the rat brain atlas (Paxinos and Watson, 2005) shows the location of the microdialysis probe into the AcbC (coordinates $A = +1.2$, $L = +2.0$, and $H = -7.0$ mm; panel **a**) or into the basal forebrain (coordinates $A = -0.35$; $L = 2.0$; and $H = -7.5$ mm; panel b). AcbC, nucleus accumbens; LPO, lateral preoptic area. Scale bar 100 mm

effective for decreasing the contents of DA in Bexa-treated rats ($F_{(2,\ 21)} = 37.643$, $P < 0.0001$; Fig. 5, panel a), but enhanced 5-HT ($F_{(2,\ 21)} = 7.481$, $P < 0.003$; Fig. 5, panel b), and no significant changes were found for NE ($F_{(2,\ 21)} = 2.749$, $P > 0.08$; Fig. 5, panel c) and EP ($F_{(2,\ 21)} = 1.049$, $P = 0.3$; Fig. 5, panel d). Moreover, Bexa decreased AD ($F_{(2,\ 21)} = 257.340$, $P < 0.0001$; Fig. 5, panel e) and ACh contents ($F_{(2,\ 21)} = 380.604$, $P < 0.0001$; Fig. 5, panel f). Remarkably, UVI induced opposite effects in DA, 5-HT, AD, and ACh measurements (Fig. 5, panels a, b, e, and f, respectively). Further multiple comparisons by Bonferroni test showed significant differences among the experimental trials for DA (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), 5-HT (VEH vs. Bexa, $P < 0.001$; Bexa vs. UVI, $P < 0.009$), AD (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), and ACh (VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$). The microdialysis data (lights-on/lights-off period) suggest that RXR seems to modulate the extracellular levels of neurochemicals related to the sleep-wake cycle control in a time-dependent fashion.

c-Fos immunohistochemistry in the hypothalamus after administration of Bexarotene or UVI 3003

Because *c*-Fos has been used under different experimental paradigms as a functional marker of neuronal activity after diverse stimuli (Gallo et al., 2018; Jaworski et al., 2018; Kovács, 2008), in a subsequent experiment, we tested whether RXR might induce *c*-Fos expression in hypothalamus, a brain region linked to sleep modulation (Arrigoni et al., 2019; Jones, 2019). The schematic representations of rat brain sections at rostro-caudal planes used for the immunohistochemical study (according to the Rat Brain Atlas [Paxinos and Watson, 2005]) are shown in Fig. 6 (panel a). Compared with respective control (Fig. 6, panel b), Bexa applied at the beginning of the lights-on period enhanced *c*-Fos activity (Fig. 6, panel c) whereas UVI exerted opposite results (Fig. 6, panel c). In contrast to observed in the lights-on period, and compared with respective control (Fig. 6, panel e), the administration of RXR agonist at 1 h after the start of the dark phase did not induce difference in *c*-Fos immunoexpression in hypothalamus (Fig. 6, panel f). Similar results were observed in UVI-treated rats (Fig. 6, panel g). Next, we determined whether this pattern would be statistically different among experimental treatments, then the number of *c*-Fos expression in hypothalamus from VEH, Bexa, and UVI groups was counted. When treatments were applied at the beginning of the lights-on period, and as compared with respective control, Bexa increased the pattern expression of *c*-Fos in the hypothalamus while UVI induced an opposite effect ($F_{(2,\ 9)} = 18.302$, $P < 0.0007$; Fig. 6, panel h). Bonferroni test showed significant differences among experimental groups (VEH vs. Bexa, $P < 0.004$; VEH vs. UVI, $P < 0.05$; Bexa vs. UVI, $P < 0.0002$). Unlike the lights-on period, there were no significant differences in the number of positive *c*-Fos immunoreactivity in the dark phase after the pharmacological treatments ($F_{(2,\ 9)} = 0.510$, $P = 0.6$; Fig. 6, panel i). We conclude that *c*-Fos expression in hypothalamus reacted in a photoperiod-dependent fashion in the presence of RXR agonist/antagonist.

Characterization of neurons expressing NeuN in hypothalamus after the application of bexarotene or UVI 3003

We assessed the impact of Bexa or UVI on the expression of NeuN in hypothalamus. Compared with the control group (Fig. 7, panel a), Bexa-treated animals showed higher NeuN expression (Fig. 7, panel b) whereas UVI caused a discrete activity of this neuronal marker (Fig. 7, panel c) when treatments were given at the beginning of the lights-on period.

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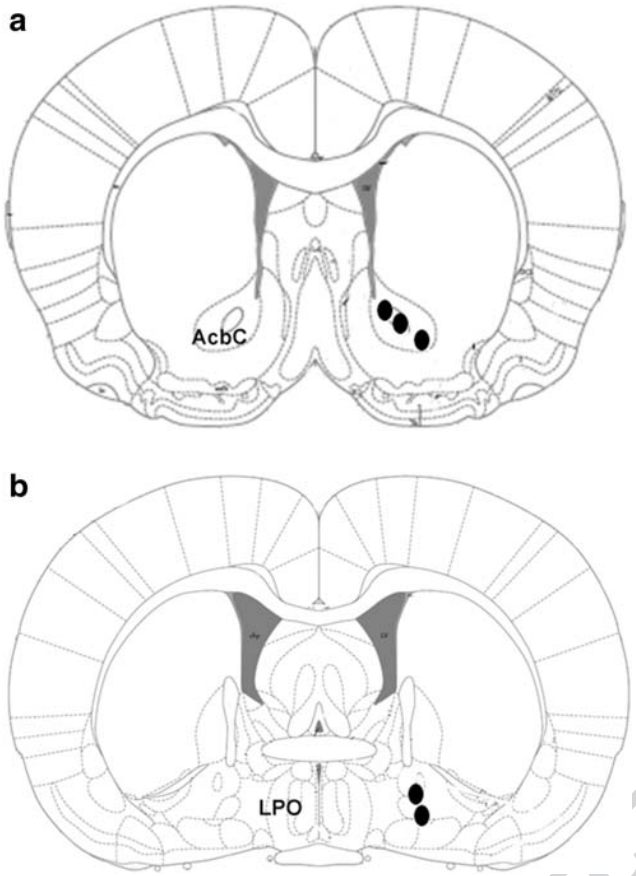

Fig. 4 Extracellular levels of dopamine (DA), serotonin (5-HT), norepinephrine (NE), epinephrine (EP), adenosine (AD), and acetylcholine (ACh) collected from either the AcbC or basal forebrain after treatment (i.p.) of either bexarotene (Bexa) or UVI 3003 (UVI; 1 mM each compound) during the lights-on period. When challenged with Bexa given at the beginning of the lights-on period, DA ($P < 0.0001$; panel **a**) and 5-HT ($P < 0.0001$; panel **b**) were enhanced and no significant changes were found in NE ($P = 0.6$; panel **c**). However, NE increased EP ($P < 0.001$; panel **d**), as well as AD ($P < 0.0001$; panel **e**) and ACh ($P < 0.0001$; panel **f**). UVI caused opposite effects in the levels of the neurochemicals. Bonferroni test showed differences for DA (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), 5-HT (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), EP (VEH vs. Bexa, $P < 0.0005$; Bexa vs. UVI, $P < 0.003$), AD (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), and ACh (VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$; *vs. control; #vs. Bexa)

When experimental challenges were applied at the start of the dark period, and compared with the respective control group (Fig. 7, panel d), we found that Bexa decreased the immunoreactivity to NeuN (Fig. 7, panel e) whereas UVI induced a higher expression of this neuronal marker (Fig. 7, panel f). Statistical analysis of number of NeuN-positive neurons showed that Bexa enhanced this neuronal marker whereas UVI decreased the immunoreactivity to NeuN when treatments were given at the start of the lights-on period ($F_{(2, 9)} = 130.614$, $P < 0.0001$; Fig. 7, panel g) Bonferroni test showed significant differences among experimental groups (VEH vs. Bexa, $P < 0.0001$; VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$). Remarkably, when treatments were applied at the lights-off period, NeuN expression was decreased in Bexa-treated rats whereas UVI caused an opposite result ($F_{(2, 9)} = 214.310$, $P < 0.0001$; Fig. 7, panel h). Bonferroni test showed significant differences among experimental groups (VEH vs. Bexa, $P < 0.0001$; VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$). These interesting results suggest that NeuN expression in hypothalamus after Bexa or UVI treatments showed a time-dependent effect.

Discussion

The present study provides the first detailed description of the pharmacological impact exerted by RXR agonist or antagonist in the sleep-wake cycle. Previous studies using bexarotene (Bexa) or UVI 3003 (UVI) have demonstrated their neurophysiological activity (Dheer et al., 2019; He et al., 2018; Martin et al., 2019; Simandi et al., 2018; Tu et al., 2018; Tunctan et al., 2018; Zhang et al., 2019), including a wider spectrum of positive outcomes, including glaucoma, cognitive, or motor improvements in animal models useful for the study of Alzheimer's and Parkinson's disease (Casali et al., 2018; McFarland et al., 2013; Simandi et al., 2018; Yu et al., 2015). However, in these studies, the role of retinoid X receptor (RXR) in sleep modulation was never described, and the role of this nuclear receptor in controlling the sleep-wake cycle and related neurochemicals remained therefore unclear. Here, we administered Bexa or UVI at either the beginning of the lights-on or the lights-off period, and sleep ad libitum, sleep homeostasis, extracellular levels of dopamine (DA), serotonin (5-HT), norepinephrine (NE), epinephrine (EP), adenosine (AD), and acetylcholine (ACh) collected from either the AcbC or basal forebrain were analyzed. In addition, *c*-Fos and NeuN expression in the hypothalamus was characterized in treated rats when compounds were given at the start of the lights-on or lights-off period. Our results showed that when given at the beginning of the lights-on period, Bexa increased wakefulness (W), decreased slow-wave sleep (SWS), and caused no changes in rapid eye movement sleep (REMS) whereas UVI caused opposite effects. On the other hand, when Bexa was injected at the start of the lights-off period, alertness was decreased and SWS was enhanced SWS whereas REMS remained with no changes. Complementarily, UVI showed no significant changes in the states of vigilance if administered at the beginning of the lights-off period. In addition, when sleep hemostasis was analyzed in response to the compounds, we found that during the sleep rebound period after total sleep deprivation, Bexa enhanced W but induced no changes in SWS while REMS was decreased. Interestingly, UVI induced contrary effects in the sleep rebound period.

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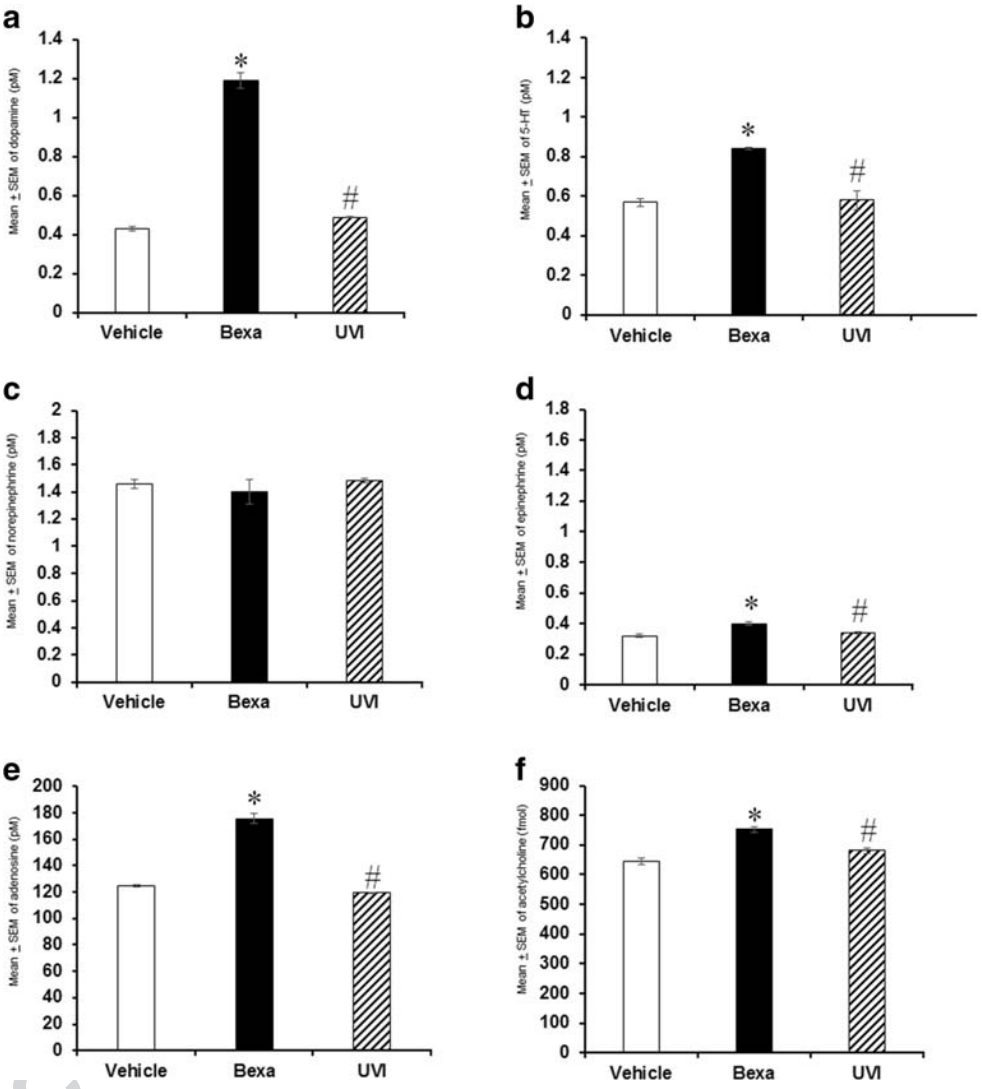

Fig. 5 Extracellular contents of dopamine (DA), serotonin (5-HT), norepinephrine (NE), epinephrine (EP), adenosine (AD), and acetylcholine (ACh) collected from either the AcbC or basal forebrain after application (i.p.) of either bexarotene (Bexa) or UVI 3003 (UVI; 1 mM each compound) during the lights-off period. Bexa decreased the contents of DA ($P < 0.0001$; panel **a**) but enhanced 5-HT ($P < 0.003$; panel **b**), and no significant differences were found in NE ($P = 0.3$; panel **c**) or EP ($P = 0.08$; panel **d**). However, Bexa decreased AD ($P < 0.0001$; panel **e**) and ACh ($P < 0.0001$; panel **f**). UVI induced opposite effects in the analyzed neurochemicals. Further multiple-comparison correction by Bonferroni test showed differences in DA (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), 5-HT (VEH vs. Bexa, $P < 0.001$; Bexa vs. UVI, $P < 0.009$), AD (VEH vs. Bexa, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$), and ACh (VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$; *vs. control; #vs. Bexa)

In regard to the neurochemical studies, when Bexa was given at the beginning of the lights-on period, we observed an increase in DA and 5-HT contents whereas no significant changes were found in NE. However, NE, AD, and ACh levels were increased in Bexa-treated animals. In addition, the UVI administration caused opposite effects in the extracellular levels of the neurochemicals analyzed. Remarkably, injection of Bexa during the lights-off period decreased the contents of DA but increased 5-HT while no significant differences were observed in NE or EP while a decrease for AD and ACh was observed. By contrast, UVI induced opposite effects in the extracellular levels of DA, 5-HT, AD, and ACh as determined using HPLC means.

The results from the immunohistochemical experiment showed that Bexa enhanced *c*-Fos expression in hypothalamus while a contrary profile was found in UVI-treated rats when compounds were applied at the beginning of the lights-on period. Interestingly, no differences were found in *c*-Fos expression in hypothalamus in Bexa-treated rats as well as in animals that received UVI if drugs were given at the start of the lights-off period. Our findings indicate for the first time that RXR modifies the neuronal activity in hypothalamus as determined by *c*-Fos immunohistochemical approaches. In complementary study, we investigated whether RXR might modulate NeuN expression in hypothalamus of animals that received a systemic application of either Bexa or UVI at the beginning of the lights-on or lights-off period. NeuN activity in Bexa-treated animals showed higher NeuN immunohistochemical expression whereas UVI exerted a decrease in the activity of this neuronal marker. These findings were observed when treatments were given at the beginning of the lights-on period. Conversely, when drugs were applied at the start of the dark period, we found that Bexa decreased the expression of NeuN while UVI increased the expression of NeuN if treatments were given at the start of the lights-on period. Taking together, our results demonstrated that RXR modulates the neuronal activity in hypothalamus as studied using NeuN immunohistochemical procedures.

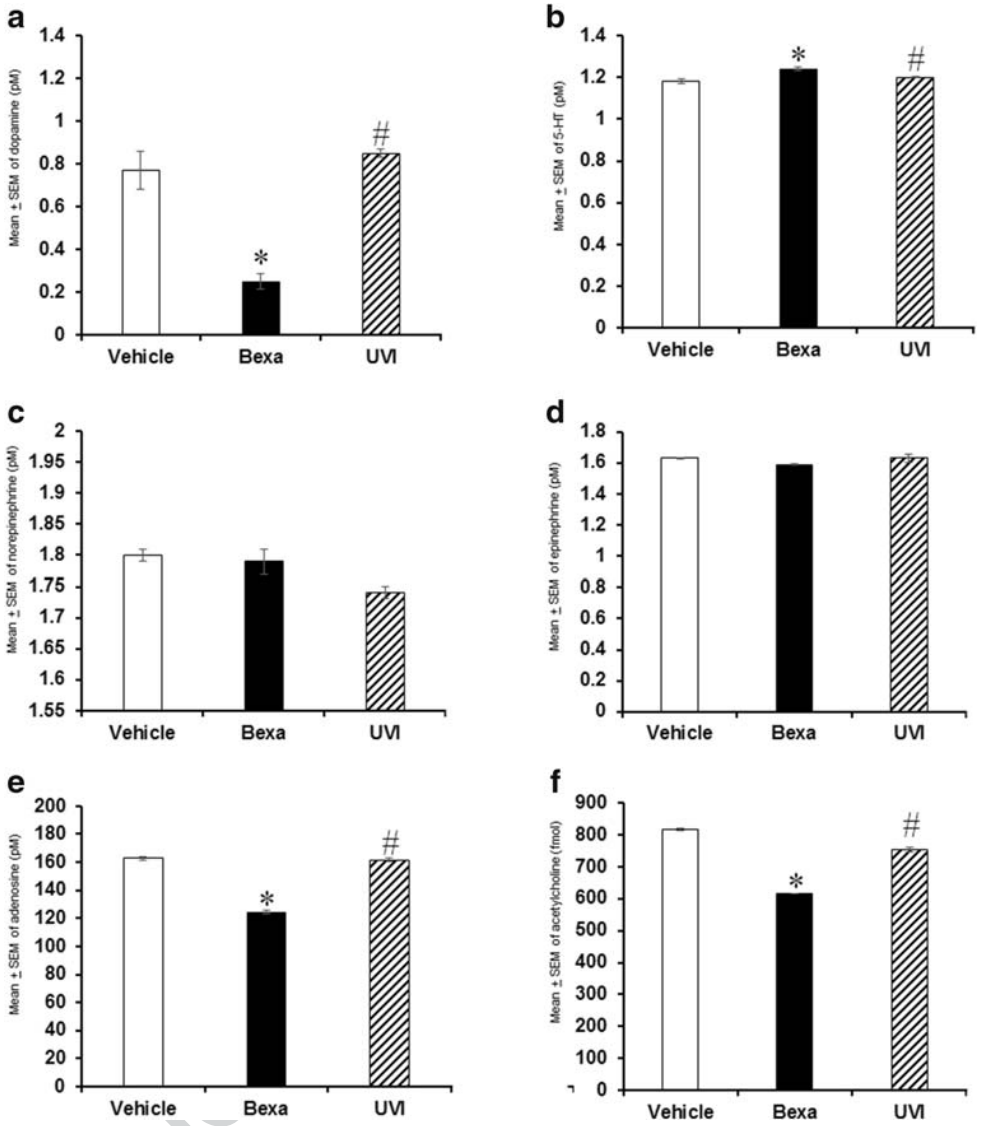

Fig. 6 The expression pattern of *c*-Fos in hypothalamus of rats that received bexarotene (Bexa) or UVI 3003 (UVI; 1 mM each compound) during either the lights-on or lights-off period. The dotted semicircle area in the schematic illustration from the rat brain atlas (Paxinos and Watson, 2005) shows the hypothalamus section taken for the immunohistochemical studies (coordinates −0.12 to −3.48 mm, from bregma according to the Rat Brain Atlas [Paxinos and Watson, 2005]; panel **a**). Compared with vehicle (panel **b**), Bexa (panel **c**) enhanced *c*-Fos expression in hypothalamus whereas UVI (panel **d**) caused opposite results when treatments were given at the beginning of the lights-on period. In comparison with vehicle (panel e), no differences were found in *c*-Fos expression in hypothalamus found in Bexa-treated rats (panel **f**) or animals that received UVI (panel **g**) when given at the start of the lights-off period. The relative expression of *c*-Fos over the hypothalamus showed an enhancement in rats that received Bexa (panel **h**) whereas UVI caused an opposite effect (panel **h**) when compounds were given at the lights-on period ($P < 0.0007$; panel **h**). Bonferroni test showed differences among groups (VEH vs. Bexa, $P < 0.004$; VEH vs. UVI, $P < 0.05$; Bexa vs. UVI, $P < 0.0002$). Lastly, no statistical differences were found in *c*-Fos expression in hypothalamus after pharmacological treatments of either Bexa or UVI during the lights-off period ($P = 0.6$; panel **i**; *vs. control; #vs. Bexa). 3V, third lateral ventricle; CPu, caudate putamen (striatum); och, optic chiasm. Scale bar 100 μm

The RXR is a critical member of the superfamily of nuclear receptors that requires heterodimerization with peroxisome proliferator-activated receptors (PPAR) for binding to specific DNA sequences and activating genes to modulate multiple neurophysiological functions (Krężel et al., 2019; Kojetin et al., 2015; Lefebvre et al., 2010;; Schierle and Merk, 2019; Watanabe and Kakuta, 2018; Fig. 8). Thorough understanding of the functional role of RXR in the control of a diversity neurobiological functions is crucial to determine the relevance of this nuclear receptor in controlling additional neurobiological variables, including the sleep-wake cycle (Dheer et al., 2019; Guleria et al., 2013; Kojetin et al., 2015; Lefebvre et al., 2010; Loera-Valencia et al., 2019; Martin et al., 2019; Martínez et al., 2019; Mengeling et al., 2018; Simandi et al., 2018; Schierle and Merk, 2019).

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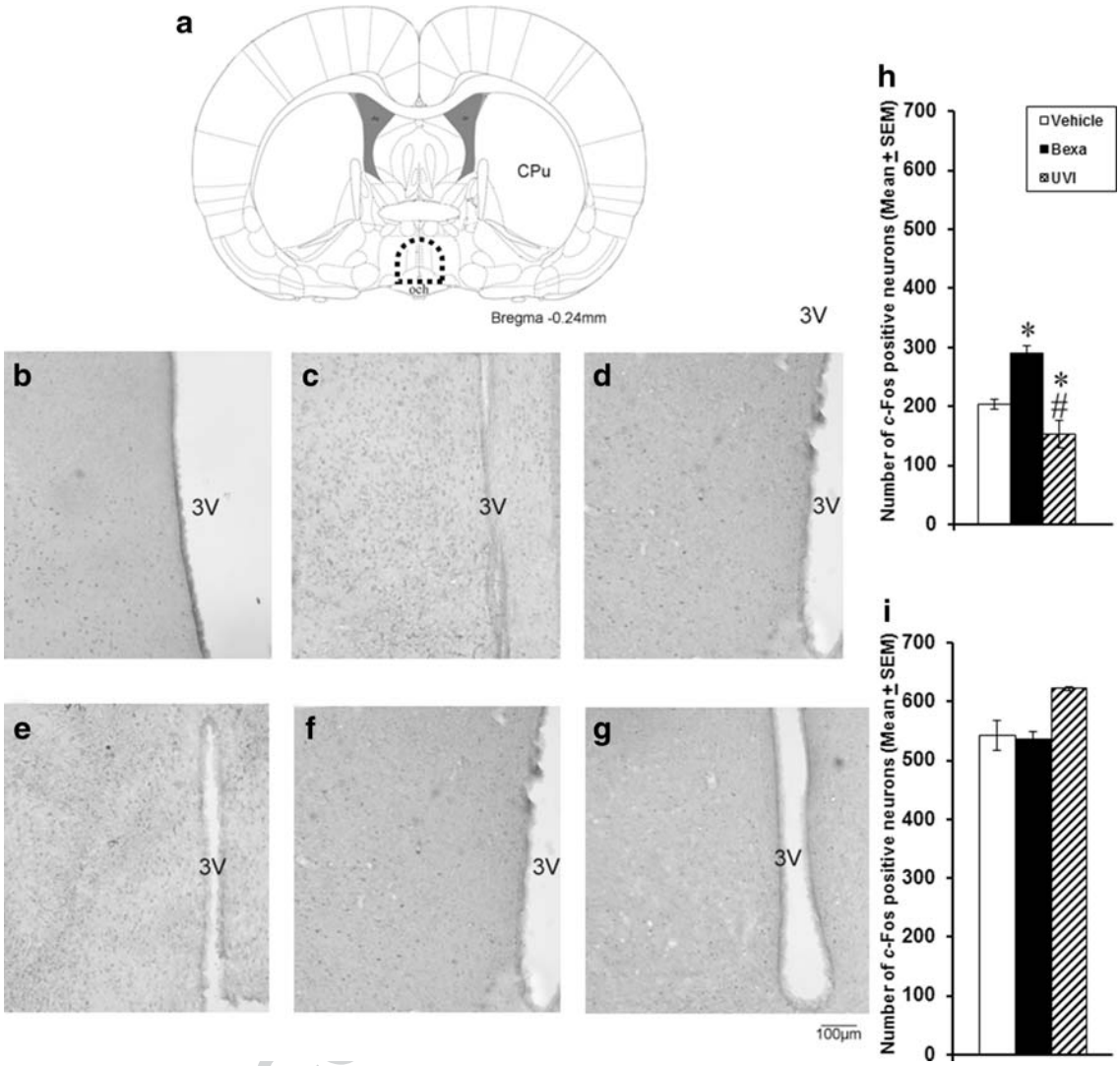

Fig. 7 The expression pattern of NeuN in hypothalamus of rats that received bexarotene (Bexa) or UVI 3003 (UVI; 1 mM each compound) during either the lights-on or lights-off period. Compared with vehicle (panel **a**), Bexa (panel **b**) enhanced *c*-Fos expression in hypothalamus whereas UVI (panel **c**) caused opposite results when experimental challenges were given at the start of the lights-on period. In contrast, in comparison with vehicle (panel **d**), NeuN expression in hypothalamus was decreased in Bexa-treated rats (panel **e**) while UVI enhanced NeuN activity (panel **f**) when treatments were given at the beginning of the dark phase. The relative expression of NeuN over the hypothalamus showed an enhancement in rats that received Bexa (panel **g**) while UVI caused a contrary effect (panel **g**) when compounds were given at the lights-on period ($P < 0.0001$). Bonferroni test showed differences among groups (VEH vs. Bexa, $P < 0.0001$; VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$). In addition, statistical differences were found in NeuN expression in hypothalamus after treatments given at the beginning of the lights-off period ($P < 0.0001$; panel **h**). Bonferroni test showed differences among groups (VEH vs. Bexa, $P < 0.0001$; VEH vs. UVI, $P < 0.0001$; Bexa vs. UVI, $P < 0.0001$; *vs. control; #vs. Bexa). 3V, third lateral ventricle. Scale bar 100 μm

The role of PPAR in sleep modulation, as heterodimer partner of RXR, has been already studied. For example, previous pharmacological experiments have indicated that PPARα participates in the control of the sleep-wake cycle since applications of synthetic agonist (Wy14643) of PPARα in rats enhanced W and decreased SWS and REMS whereas the injection of the antagonist (MK-886) promoted opposite effects. Moreover, it has been demonstrated that injections of Wy14643 enhanced DA, NE, 5-HT, and AD levels collected from AcbC. In line with the idea that PPARα participates in sleep control, the administration of natural (oleoylethanolamide) or synthetic (Wy14643) ligand of PPARα after 6 h of TSD showed a decrease in the sleep rebound, suggesting that PPARα seems to modulate sleep homeostasis (Mijangos-Moreno et al., 2016; Murillo-Rodríguez et al., 2016; Murillo-Rodriguez, 2017). Since it was reasonable to argue that PPARα might participate in the control of the sleep-wake cycle (Mijangos-Moreno et al., 2016; Murillo-Rodríguez et al., 2016; Murillo-Rodriguez, 2017), then RXR was, therefore, an ideal candidate to study its modulatory properties in sleep. Moreover, the role on sleep of RXR tested by the pharmacological administration of Bexa

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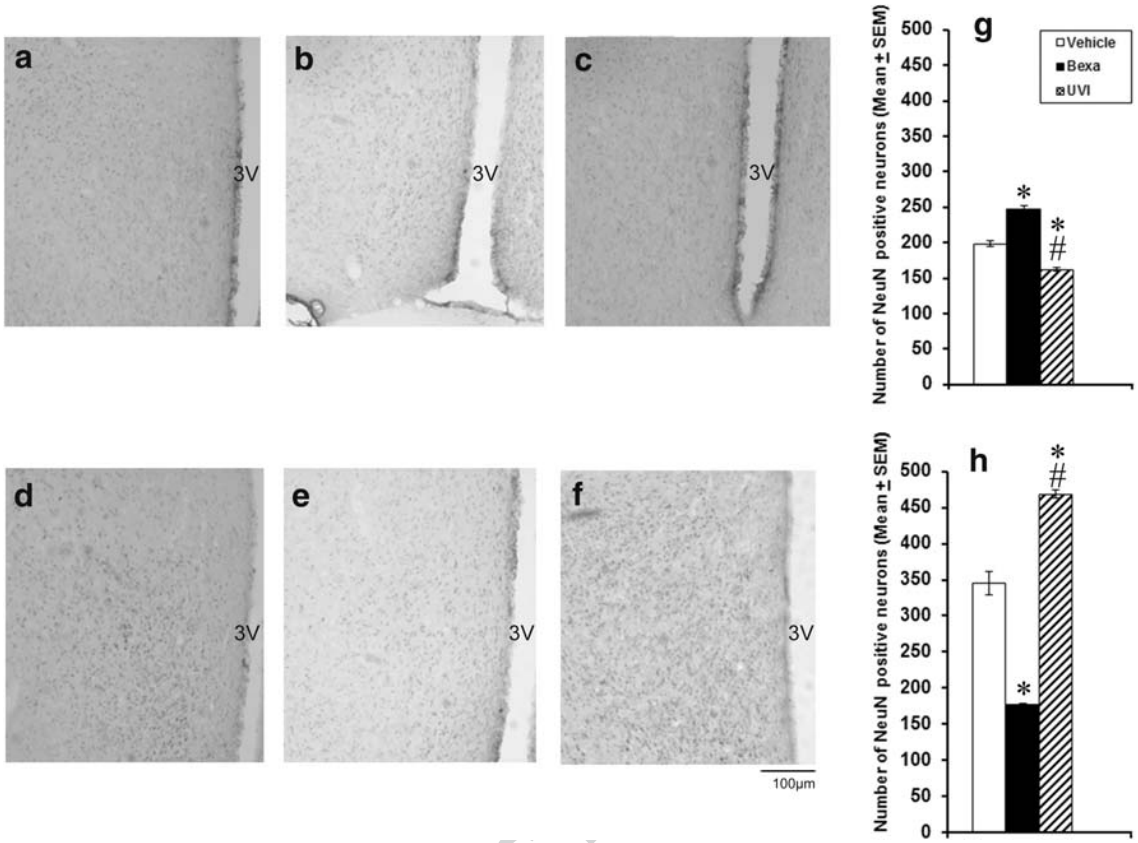

Fig. 8 Putative nuclear mechanism of action for RXR in sleep modulation. PPAR and RXR form a heterodimer, which is activated by their respective ligands for PPAR and RXR. Then, the activated PPAR/RXR heterodimer is translocated into the nucleus for the regulation of downstream target genes related to sleep/neurochemical/*c*-Fos/NeuN control /Q21

or UVI was unknown. To overcome this limitation, in the present study, we applied Bexa or UVI at either the start of the lights-on or lights-off period, and sleep, sleep homeostasis, neurochemicals linked to sleep control and the expression of *c*-Fos and NeuN were analyzed.

Although it has been known that RXR modulates many neurobiological processes (Bartzokis, 2014; Casali et al., 2018; Clemens et al., 2018; Fanaee-Danesh et al., 2019; Geller et al., 2019; Guleria et al., 2013; Hebert et al., 2017; Loera-Valencia et al., 2019; Martin et al., 2019; McFarland et al., 2013; Mengeling et al., 2018; Schierle and Merk, 2019; Simandi et al., 2018; Tu et al., 2018; Watanabe and Kakuta, 2018), the mechanism of action is not fully understood. Thus, our observations that Bexa or UVI modified the sleep architecture and sleep homeostasis might be linked with the changes found in the neurochemical studies. However, there are non-exclusive explanations for these findings. First, the microdialysis data suggest that RXR plays a crucial role for modulating the extracellular levels of neurochemicals related to the sleep-wake cycle. Although the results indicated that Bexa and UVI controlled DA, 5-HT, NE, EP, AD, and ACh contents during lights-on or lights-off period, it is likely that Bexa or UVI could engage other neurobiological substrates rather than the neurochemicals analyzed. In this regard, Kitaoka et al. (2011) reported that chronic administration of retinoic acid receptor antagonist, LE540 (30 mg/kg/day), during 1 or 4 weeks decreased delta power during sleep as well as attenuation of alertness. In addition, they found a significant decrease in the expression of dopamine D1 receptor in the striatum of treated animals as addressed by Western blot means.

Second, the intense activation of neurons have been linked to the activity of immediate expression genes, including *c*-Fos since this gene is related to multiple signal cascades that are involved in neurobiological processes and to mapping neuronal activity (Farivar et al., 2004; Gallo et al., 2018; Huang et al., 2019; Jaworski et al., 2018; Kovács, 2008; Yang et al., 2019). In our study, we analyzed the *c*-Fos expression in a brain area related to sleep modulation, the hypothalamus (Arrigoni et al., 2019; Jones, 2019), and we found that Bexa or UVI showed differential function activity. We surmise that different brain areas could be under the influence of Bexa or UVI as determined by changes in the levels of *c*-Fos marker. Consistent with this view, ablation of RXRγ in mice leads to *c*-Fos expression in dorsal caudate putamen (Krzyzosiak et al., 2010). Due to the large number of brain nuclei related to sleep control (Eban-Rothschild et al., 2018; Iovino et al., 2019), identifying specific areas may reveal novel pathways for

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understanding the relevant role of RXR in modulation of the states of vigilance. Importantly, we found that Bexa enhanced W during the lights-on period whereas UVI increased SWS during the lights-off period suggesting that the hypothalamus is reacting to compounds in a different pattern and depending on the time of the day as reported by others (Barros et al., 2015). We hypothesize that the expression of *c*-Fos in the hypothalamus after Bexa or UVI administrations might represent a nuclear mechanism of action for sleep modulation and seems to be depending on the time of administration of compounds.

There is an assumption that NeuN is linked to neuronal functional state (Duan et al., 2016; Gusel'nikova and Korzhevskiy 2015). However, NeuN also may not be expressed at uniform levels within a specific brain region (Hight et al., 2010), meaning that a likely limitation of our study is that NeuN expression could be detected in a non-uniform fashion within the hypothalamus. In addition, it is worthy to highlight that our results showed a time-of-day expression of NeuN after treatments. The activity dependency of NeuN in our experiments is consistent with the previous observations (Geoghegan and Carter, 2008; Hight et al., 2010). Analyzing the role of NeuN in the activity of RXR could shed light on the neuromolecular basis of the sleep-wake cycle modulation by RXR. Indeed, further studies could provide a robust data linked to the mechanism of action of RXR and NeuN expression. We suspect that NeuN expression in animals under treatment of Bexa or UVI is beyond than simple neuronal marker activity and it will require to be analyzed as a possible key participant in sleep modulation since this field has been completely unnoticed.

Finally, our understanding of the pharmacological effects of RXR isoforms is, in some cases, limited to in vitro assays. The elucidation of the neurobiological role of RXR isoforms has become an essential step in understanding how the RXR receptors modulate behaviors such as the sleep-wake cycle. In this regard, limited evidence is available about the role of RXR isoforms in the sleep control, sleep homeostasis regulation, as well as the modulation of neurochemicals and *c*-Fos or NeuN expression. The indirect evidence shows that the three PPAR isoforms display circadian rhythmicity in mouse tissues (Chen and Yang 2014), whereas PPARα knockout mice displays an increase in sleepiness (Kondo et al., 2020). Thus, further studies are required to fully elucidate the functions and potential mechanisms associated with RXR isoforms in sleep modulation.

In conclusion, by using Bexa or UVI, RXR activity modified the sleep-wake cycle and sleep homeostasis dependent on the light/dark cycle. Our results also clearly showed that Bexa or UVI modulated the extracellular levels of neurochemicals linked to sleep control whereas the expression of *c*-Fos and NeuN in hypothalamus showed different effects if treatments were given during either the lights-on or lights-off period. Our data allow us to speculate that RXR might play a functional role in the modulation of the sleep-wake cycle.

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Compliance with ethical standards

The experimental protocols were approved by the Research and Ethics Committee of Universidad Anáhuac Mayab (Mérida, Yucatán, México), and the protocols met the requirements of Animal Welfare including the Mexican Standards Related to Use and Management of Laboratory Animals (DOF. NOM-062-Z00-1999), the National Institutes of Health (NIH Publication No. 80-23, revised 1996), and the ARRIVE (Animal Research: Reporting of in vivo Experiments) guidelines, the commonly accepted "3Rs" Guidelines.

Conflict of interest The authors declare that they have no conflict of interest.

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