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PARTICIPACIÓN DEL RECEPTOR A RYANODINA EN LA VÍA DE SALIDA DEL RELOJ CIRCÁDICO. IMPLICACIONES DE LA DINÁMICA DEL CALCIO INTRACELULAR

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CLARA ROSALVA MERCADO LÓPEZ

DIRECTOR DE TESIS: DR. RAÚL ANTONIO AGUILAR ROBLERO

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Índice General

Resumen

Estudios anteriores han aportado información sobre el mecanismo molecular del reloj circadiano y de cómo éste se sincroniza a las señales externas, pero se conoce muy poco acerca de los mecanismos de salida que vinculan al reloj molecular con la expresión de los ritmos circadianos. Se ha sugerido que la frecuencia de disparo espontáneo en las neuronas del NSQ funciona como una vía de salida. Nosotros proponemos que la unión entre la frecuencia de disparo y el reloj molecular podría ser la movilización del Ca^{2+} intracelular de los depósitos, a través de la actividad de los canales intracelulares de Ca^{2+} sensibles a ryanodina (RyR). Anteriormente, nuestro grupo de trabajo demostró que en las neuronas del NSQ los RyR muestran un ritmo circadiano endógeno. Por lo que el objetivo de este trabajo fue determinar si la manipulación farmacológica de los RyR altera de manera significativa la vía de salida del reloj circadiano.

En ratas, se midió el ritmo circadiano de la actividad locomotora e ingesta de agua en animales mantenidos en luz roja continua antes, durante y después de la administración de un activador (ryanodina 0.1 μM) o un inhibidor (ryanodina 100 μM) de los RyR. También se registró la frecuencia de disparo espontáneo y el potencial de membrana de las neuronas del NSQ mediante la técnica de parche perforado en rebanadas *in vitro,* en presencia de moduladores de los RyR. Los resultados mostraron que la activación de los RyR indujo el acortamiento significativo del periodo del ritmo circadiano de la conducta y el incremento en la frecuencia de disparo neuronal del NSQ; mientras que la inhibición de los RyR ocasionó la pérdida temporal del ritmo circadiano de la conducta y una disminución de la frecuencia de disparo neuronal en el NSQ. En conclusión, sugerimos que la actividad de los RyR es un elemento importante en la vía de salida del reloj circadiano molecular en las neuronas del NSQ para la expresión de los ritmos circadianos conductuales.

Abstract

Previous studies have contributed information of molecular circadian clock mechanism and how synchronizes to it external signals, but we don't know the mechanism of output that may be a link between the molecular clock and the expression of circadian rhythms. It has been suggest that spontaneous firing in SCN may be an output. We propose that the link between the firing rate and the molecular clock could be mobilization of intracellular Ca^{2+} stores, through from the activity of sensitive intracellular channels of Ca^{2+} to ryanodine (RyR). Since previously our group of investigation demonstrated that the RyR shows an endogenous circadian rhythm in the neurons of SCN. In the present study we further addressed if the pharmacological modulation of the RyR alters of significant way the output of circadian clock.

In rats, we measured the circadian rhythm of drinking and activity behaviors in dim red light before, during and after the administration of activator (0.1 μM ryanodine) or inhibitor (100 μM ryanodine) of the RyR. Also we measured the spontaneous firing frequency in SCN by the perforated-patch technique in acute brain slices *in vitro*, in presence of modulators of the RyR. The results showed that the activation of RyR induced a significant shortening of the endogenous period of circadian rhythm of behavior and the significant increment in the firing frequency of SCN, whereas the inhibition of these Ca^{2+} release channels, temporally disrupted the circadian rhythmicity of behavior and the decreased firing rate of SCN.

In conclusion, we suggested that activity of the RyR is a key element of the output pathway from the molecular circadian clock in SCN neurons to the overt behavioral expression of circadian rhythmicity.

Lista de Abreviaturas

aCSF	Artificial cerebrospinal fluid
AHP	Afterhyperpolarization potential
AMPc	Adenosine monophosphate cyclic
AMPA	alpha–amino-3-hydroxy-5-methyl-4-isoxazole propionate glutamate receptors
APV	DL-2-amino-5-phosphonopentanoic acid
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
Bic	Bicuculline
BK	Ca^{2+} -activated K^{+} channel
Bmal1	Brain and muscle aryl hydrocarbon receptor nuclear translocator gen
$\mathbf{[Ca^{2+}]_i}$	Concentración de Calcio intracelular
cADPR	Cyclic ADP-ribose
CaM	Calmodulin
CAMK	calcium/calmodulin kinase
CCGs	Clock controlled genes
CKIε	Casein Kinase Iε

CREB	cAMP response element binding
Cry	Cryptochrome gen
DBP	D-element binding proteín
DMSO	Dimetil sulfoxido
DNQX	6,7-dinitroquinoxaline-2,3(1H,4H)dione
E4bp4	Basic leucine zipper transcription factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
cGMP	Cyclic guanosine monophosphate
GRP	Gastrin-releasing peptide
IGL	Intergeniculate leaflet
IP_3R	Inositol 1,4,5-Triphosphate receptor
KCl	Cloruro de Potasio
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
NMDA	N-methyl D-aspartato glutamate receptors
NO	Nitric oxide

NOS Nitric oxide synthase

NSQ Núcleo supraquiasmático

PACAP Pituitary adenylate cyclase activating peptide

Per Period gen

PHI Peptide histidine isoleucine

PKG Protein kinase G

PRC Phase response curve

RGC Retinal ganglion cells

RHT Retinohypothalamic tract

Rya Ryanodina

RYR Canal intracelular de calcio sensible a ryanodina

SCN Suprachiasmatic nucleus

TTX Tetrodotoxin

VIP Vasoactive intestinal polypeptide

$\mathbf{V_{ahp}}$ Post-potencial hiperpolarizante

$\mathbf{V_{m}}$ Potencial de membrana

$\mathbf{V_{spk}}$ Potencial en el pico de disparo

$\mathbf{V_{thr}}$ Potencial en el umbral de disparo

I. INTRODUCCIÓN

1. Ritmos Circadianos

Las funciones de los seres vivos no se expresan de manera constante. Cuando se estudia un proceso orgánico en un tiempo suficientemente prolongado en cualquier ser vivo, desde organismos unicelulares hasta el hombre, se puede observar que se alternan periodos de máxima actividad con periodos de actividad escasa o nula. Cuando estas variaciones se presentan con cierta regularidad las denominamos **ritmos biológicos**. Hay una amplia gama de frecuencias que cubren los ritmos biológicos: hay ritmos que oscilan una vez por fracción de segundo (ejemplo, los ritmos en la actividad eléctrica neuronal), hasta los ritmos que oscilan una vez por año (ejemplo, la hibernación). Los ritmos que poseen una duración aproximada a la de un ciclo ambiental se les asigna el prefijo circa, que proviene del Latín *circa* (alrededor de, próximo a), por ejemplo, los ritmos cercanos al ciclo anual son **circanuales**; los ritmos próximos al ciclo de la marea son **circamareales**; los ritmos que se acercan al ciclo lunar son **circalunares**; los ritmos que se repiten con una frecuencia cercana a la del día son **circadianos** (Aschoff, 1981a).

Los **ritmos circadianos** son oscilaciones periódicas endógenas que se repiten aproximadamente cada veinticuatro horas y que pueden observarse en todos los niveles de organización de las especies estudiadas (Menaker, 1969; van Esseveldt et al, 2000). Cuando los ritmos circadianos se estudian aislados de señales de tiempo y en condiciones experimentales constantes, persisten y presentan una periodo cercano a las veinticuatro horas, entonces se dice que están en **libre curso**

u **oscilación espontánea** y su periodo endógeno (τ) presenta un intervalo entre las 22 y las 26 hrs (Aschoff, 1981b). Cuando los ritmos se estudian bajo condiciones ambientales cíclicas, el ritmo del periodo endógeno (τ) tiene la capacidad de ajustarse al periodo del ritmo ambiental (T), a este fenómeno lo llamamos sincronización. El ciclo externo que sincroniza al ritmo es llamado **zeitgeber** o **agente sincronizador** (Aschoff, 1981b).

La sincronización de los ritmos circadianos se estudia utilizando el protocolo denominado **Curva de Respuesta de Fase (PRC)** (Figura 1), en el cual se cuantifica el cambio de fase de un ritmo circadiano (conductual o fisiológico) de un organismo ante un estímulo (luminoso, químico, eléctrico o mecánico) aplicado a diferentes horas de su ciclo circadiano (Menaker, 1969). La PRC se divide en tres segmentos: A) La zona muerta, en la cual si se aplica un pulso del estímulo no hay cambios de fase; B) La zona de retrasos, el estímulo retrasa la fase (cambio de fase negativa); y C) La zona de avances, el estímulo adelanta la fase (cambio de fase positivo). Las horas e intensidad con que ocurren los cambios de fase depende de la especie estudiada (Menaker, 1969).

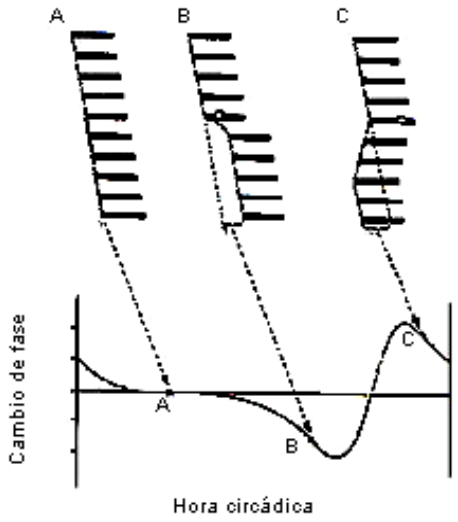

Figura 1. Curva de respuesta de fase. A zona muerta, B zona de retrasos, C zona de avances. El esquema de arriba representa la actividad del organismo observada antes y después del estímulo (Modificada de Moore-Ede, 1982).

2. Sistema Circadiano

Los ritmos circadianos son generados en el organismo de manera endógena por un sistema denominado “Sistema Circadiano”, que está constituido por tres componentes: a) El reloj u oscilador, el cual puede generar oscilaciones de manera constante en ausencia de señales cíclicas; b) Vías de entrada o de sincronización que transmiten las señales externas al reloj y c) Vías de salida, que transmiten la señal generada por el reloj hacia el resto del organismo.

a. Reloj u oscilador. El reloj biológico se define como un sistema orgánico capaz de generar un orden temporal en las actividades del organismo; debe tener la capacidad de oscilar con un periodo regular, además de usar dichas oscilaciones como una referencia temporal interna. Este sistema debe permitir la adecuada interacción del dominio temporal entre el organismo y su ambiente (Aschoff, 1981a). En diversos organismos se han identificado estructuras que funcionan como osciladores o relojes. Por ejemplo, en los moluscos las neuronas basales de la retina (Block y McMahon, 1984); en la polilla el ganglio cerebral (Aronson, 1993); en las aves y reptiles la glándula pineal (Takahashi et al, 1980; Menaker y Wisner, 1983); en los mamíferos el núcleo supraquiasmático (NSQ) del hipotálamo (Moore y Eichler, 1972; Stephan y Zucker, 1972).

En los mamíferos, el NSQ posee las características funcionales para ser considerado reloj biológico: sus oscilaciones son regulares, autosostenibles, cercanas a las veinticuatro horas, tiene la capacidad de sincronizarse a estímulos externos y puede imponer el periodo y la fase de sus oscilaciones a otros tejidos (Moore-Ede et al, 1982). Las evidencias experimentales

demuestran que el NSQ funciona de manera autónoma: A) En diversas especies estudiadas, la lesión bilateral del NSQ suprime el ritmo circadiano de algunos procesos conductuales y fisiológicos del organismo, como por ejemplo, la conducta de ingesta de agua (Stephan y Zucker, 1972) y de alimento (Nagai et al, 1978), el ciclo sueño-vigilia (Ibuka y Kawamura, 1975), la secreción de corticosteroides (Moore y Eichler, 1972), la actividad de la N-acetiltransferasa de la glándula pineal (Klein y Moore, 1979); por mencionar algunos. B) La estimulación eléctrica o química del NSQ induce cambios de fase en el ritmo circadiano de la conducta, similar a los provocados por la exposición a la luz (Rusak y Groos, 1982). C) El ritmo circadiano de la actividad eléctrica del NSQ persiste aún cuando se aísla esta estructura de sus conexiones neuronales y sinápticas (Inouye y Kawamura, 1979; Shibata et al, 1982; Welsh et al, 1995). D) El NSQ exhibe ritmicidad circadiana en su actividad metabólica; se ha reportado que la utilización de la 2-deoxiglucosa por las neuronas del NSQ incrementa durante el día y decae por la noche (Schwartz y Gainer, 1977; Schwartz et al, 1980). E) Finalmente, el transplante de NSQ fetal en animales lesionados induce la recuperación del ritmo circadiano de la conducta, además el tejido donador impone su periodo y su fase al organismo receptor (Druker-Colin et al, 1984; Aguilar-Roblero et al, 1986; Lehman et al, 1987; Aguilar-Roblero et al, 1992; 1994).

El NSQ es una estructura bilateral que se encuentra en la parte anterior ventral del hipotálamo, dorsal al quiasma óptico (Figura 2a y 2b). En ratas adultas el núcleo tiene un volumen de 0.136 mm^3, está compuesto por aproximadamente 16,000 neuronas de tamaño pequeño entre 8 y 12 μm de diámetro y empaquetadas en forma muy compacta (van den pol, 1980). Con base en sus diferencias morfológicas, el NSQ se puede dividir en una parte dorsomedial y otra ventrolateral

(Figura 2c) (van den pol, 1980). La parte dorsomedial contiene pequeñas neuronas alargadas, fuertemente empaquetadas, con núcleos largos y pocos organelos celulares. La parte ventrolateral se caracteriza por contener neuronas esféricas con densidad baja, que tienen un citoplasma con abundantes organelos (Leak et al, 1999; Leak y Moore, 2001). Los somas neuronales están separados por células de la glia, las cuales encierran completamente las uniones sinápticas dentro de la parte ventrolateral. La comunicación celular se lleva a cabo por medio de uniones comunicantes, transmisión sináptica o señales neurohumorales (van den pol, 1980; van den pol y Dudek, 1993).

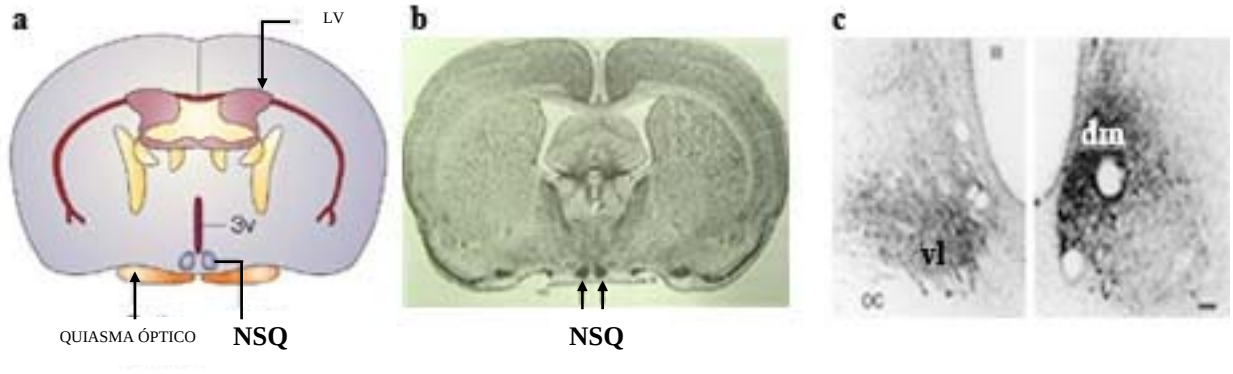

Figura 2. Ubicación del NSQ en cortes coronales de cerebro de rata. (a) Esquema que representa la localización del NSQ. (b) Corte histológico con tinción de Nissl. (c) Acercamiento a los NSQs con inmunohistoquímica a VIP (parte ventrolateral **vl**) y AVP (región dorsomedial **dm**). LV ventrículo lateral (b, Paxinos y Watso, 1998; c, Lisette et al, 2000).

Las diferencias morfológicas y de organización de las neuronas en las dos regiones del NSQ, sugieren que éstas tienen diferentes funciones en la generación y regulación del ritmo circadiano. Se ha detectado diferencias en el contenido neuropeptídico en las regiones del NSQ (van Esseveldt et al, 2000). Así, las neuronas de la región dorsomedial sintetizan arginina vasopresina (AVP), mientras que las neuronas de la región ventrolateral sintetizan el polipéptido intestinal

vasoactivo (VIP) (Vandesande et al, 1975; Sims et al, 1980; van den pol y Tsujimoto, 1985), el péptido isoleucina histidina (PHI) y el péptido liberador de la gastrina (GRP) (Card et al, 1988). Se ha reportado la presencia de otras sustancias en pequeñas proporciones en ambas regiones: somatostatina, sustancia P, angiotensina II, factor liberador de la corticotropina, encefalina, galanina, factor liberador de la tirotropina. La mayoría de las neuronas, si no es que todas, sintetizan GABA (ácido γ-amino butírico) el cual colocaliza con algunos de los neuropéptidos mencionados anteriormente (Moore y Speh, 1993).

b. Vías de entrada (Aferencias). La región ventrolateral del NSQ recibe la mayoría de las vías aferentes (van den pol, 1991): 1) El tracto retino-hipotalámico (RHT) proveniente de las células ganglionares de la retina (RGC), cuyos principales neurotransmisores son glutamato y el polipéptido hipofisiario activador de la adenilato ciclasa (PACAP) (Castel et al, 1993; Reppert y Weaver, 2002); 2) La vía genículo-hipotalámica proveniente de la hojuela intergeniculada (IGL) que secreta Neuropéptido Y (Albers y Ferris, 1984); 3) La vía que proviene de los núcleos dorsal y medial del núcleo del rafe mesencefálico que secreta serotonina (Cagampang e Inouye, 1994).

La parte dorsomedial también recibe algunas fibras del RHT y del tracto corticohipotalámico (proveniente del sistema límbico, núcleo septal medial, núcleo subículo anteroventral y áreas hipotalámicas adyacentes como el área preóptica, núcleo ventromedial, núcleo arcuato y área tuberal) (Moga y Moore, 1997; van Esseveldt et al, 2000).

La aferencia que se ha descrito más ampliamente es la que sincroniza al NSQ por estímulos luminosos, por medio del RHT. La cantidad de luz ambiental es detectada por fotorreceptores de la retina. Se ha demostrado que los fotorreceptores clásicos de la retina, conos y bastones, no son estrictamente necesarios para la sincronización a la luz, ya que los animales ciegos por degeneración congénita de los conos y los bastones aún son capaces de sincronizarse a la luz (Freedman et al, 1999). Trabajos recientes han demostrado que un tipo de células de las RGC que proyectan directamente al NSQ y a la IGL a través del RHT, contienen un fotopigmento llamado melanopsina, el cual es necesario para la adecuada sincronización de los ritmos a la luz (Reppert y Weaver, 2002). Con un estímulo luminoso, los axones de las RGC que se extienden al NSQ liberan glutamato y el PACAP para sincronizar al reloj circadiano (Figura 3) (Reppert y Weaver, 2002). La vía de señalización intracelular que se activa en las neuronas del NSQ depende del momento del ciclo en que se presenta el estímulo luminoso (Meijer y Schwartz, 2003). Esta respuesta se explicará en la sección de "Vías de señalización intracelular en el NSQ".

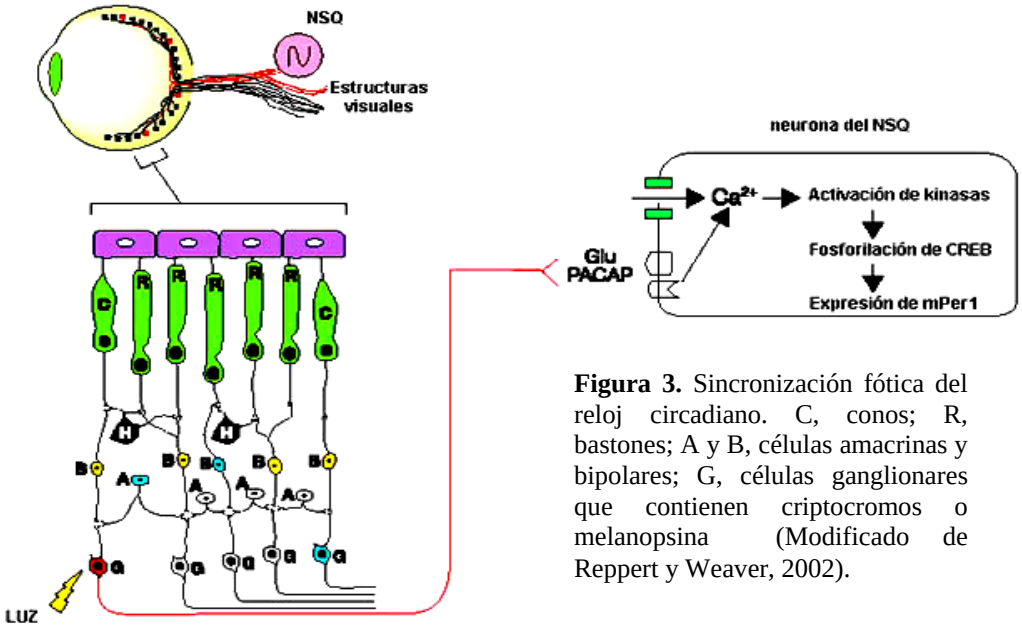

Figura 3. Sincronización fótica del reloj circadiano. C, conos; R, bastones; A y B, células amacrinas y bipolares; G, células ganglionares que contienen criptocromos o melanopsina (Modificado de Reppert y Weaver, 2002).

c. Vías de salida (Eferencias). Diversos trabajos han reportado que la información temporal generada por el NSQ se transmite al organismo para regular los diversos procesos fisiológicos y conductuales rítmicos como la temperatura del cuerpo, locomoción, ingesta de alimento y agua, sueño, secreción hormonal, utilización de oxígeno, producción de corticosterona, etc. Generalmente, se considera que la parte ventrolateral del NSQ recibe las vías de sincronización, mientras que la dorsomedial proyecta las fibras de salida para la transmisión de la señal de tiempo. Varias neuronas del NSQ proyectan principalmente a diferentes estructuras hipotalámicas para transmitir la información temporal a diferentes ejes funcionales, especialmente el eje hipotálamo-hipófisis para la secreción rítmica de hormonas (van den pol, 1980; 1991; Watts, 1991).

Las principales eferencias del NSQ que se han descrito son las siguientes: 1) Fibras que van a los núcleos hipotalámicos: zona subparaventricular, región tuberal lateral, ventromedial, dorsomedial, periventricular, premamilar, paraventricular (presumiblemente involucrado en el control de los ritmos de funciones hormonales y autonómicas), área preóptica (involucrada en la regulación de la temperatura, balance de fluidos y la conducta sexual), área retroquiasmática (desde la cual se envían señales a los hemisferios cerebrales para la regulación de la conducta); 2) Fibras que se dirigen al tronco encefálico (regulación autonómica) y a la médula espinal (control sensorial y motor); 3) Proyecciones que se dirigen al sistema límbico: núcleo medial, núcleo septal lateral, núcleo del lecho de la estría terminalis, órgano vasculoso de la lámina terminalis; 4) Vías que llegan al diencéfalo: núcleo paraventricular del tálamo, núcleo dorsal del tálamo, habénula lateral; 5) El tracto geníclulo-hipotalámico, que va hacía la IGL; y 6)

Proyecciones que van al núcleo del rafe: núcleo paratenial y gris periacueductual (van den pol, 1993; Ibata et al, 1999; van Esseveldt et al, 2000).

En la rata, los neurotransmisores del NSQ involucrados en la salida del reloj son esencialmente la AVP y el GABA (Watts y Swanson, 1987; Moore y Speh, 1993). Se ha propuesto que la AVP es un transmisor controlado por los genes reloj porque expresa un ritmo circadiano en su síntesis y liberación (Reppert, 1985; Murakami et al, 1991; Watanabe et al, 2000); y su gen promotor contiene la caja-E bajo el control directo de los genes reloj (Jin et al, 1999). El GABA es un neurotransmisor comúnmente inhibitorio en el Sistema Nervioso Central, el cual está ampliamente distribuido en el NSQ localizado mayormente en los somas neuronales (Moore y Speh, 1993) y terminales sinápticas (Decavel y van den pol, 1990; Castel et al, 1993). La concentración y la actividad del GABA, la actividad de la enzima GAD (ácido glutámico descarboxilasa) que convierte el glutamato en GABA y los niveles de mRNA del GABA muestran un ritmo circadiano en el NSQ, tanto en condiciones de luz y oscuridad, como en condiciones de oscuridad constante (van den pol y Dudek, 1993). La participación de este neurotransmisor como modulador es controversial; si se administra GABA a rebanadas que contengan al NSQ durante la noche tiene un efecto inhibitorio disminuyendo la frecuencia de disparo, mientras que si se administra durante el día actúa como un neurotransmisor excitatorio, incrementando la frecuencia de disparo (Wagner et al, 1997). Los autores atribuyen este efecto dual a la oscilación intracelular de la concentración de cloro.

3. Vías de señalización intracelular en el NSQ

Las neuronas del NSQ, cuando se extraen y se aíslan pueden generar ritmos circadianos autosostenibles en algunas de sus funciones, por ejemplo en su frecuencia de disparo (Inouye y Kawamura, 1979; Green y Gillette, 1982; Gross y Hendriks, 1982; Walsh et al, 1995; Ikeda et al, 2003), en el metabolismo de la glucosa (Schwartz y Gainer, 1977; Schwartz et al, 1980), en la secreción de neurotransmisores (Earnest y Sladek, 1986; 1987), en la expresión de los mRNA *Bmal1*, *Per* (1,2,3) y *Cry* (1,2) (Honma et al, 1998; Shearman et al, 2000; Reppert y Weaver, 2001; 2002), en la concentración de Ca^{2+} (Colwell, 2000; Ikeda et al, 2003), por señalar los más importantes.

Por lo anterior, cada neurona del NSQ funciona como un oscilador, el cual consta de varios componentes: a) Un reloj molecular, que funciona mediante mecanismos de asas de retroalimentación transcripción-traducción (Reppert y Weaver, 2001; 2002); b) Los componentes de entrada, que sincronizan al reloj molecular con las señales externas al oscilador (Hirota y Fukada, 2004); c) Los componentes de salida, que permiten la unión funcional entre el reloj circadiano intracelular y la expresión de los ritmos circadianos (Kuhlman y McMahon, 2006).

a) Reloj molecular. El mecanismo molecular que determina la operación del reloj circadiano está conformado por "genes reloj". En los análisis genéticos y moleculares se han identificado diversos genes reloj que forman asas de retroalimentación transcripción-traducción, que regulan la expresión del ritmo circadiano de los mRNAs y proteínas. En mamíferos, el aumento transcripcional de *Bmal1* y *Clock* es esencial para el funcionamiento básico del reloj, los

productos de estos genes BMAL1 y CLOCK respectivamente, forman heterodímeros BMAL1-CLOCK que se unen al elemento E-box CACGTG para activar la transcripción rítmica de dos familias de genes: *Per* (*mPer1, mPer2, mPer3*) y *Cry* (*mCry1, mCry2*). Los elementos *mPer* y *mCry* se traducen en el citoplasma, forman heterodímeros PER-CRY, los cuales son fosforilados por la caseína kinasa **ε** en el citosol y regresan al núcleo para actuar como reguladores negativos inhibiendo directamente su propia transcripción, desplazando al dímero BMAL1-CLOCK de su sitio de unión E-box (Figura 4). Alo cual reduce la transcripción de *Per* y *Cry* y, por consecuencia, también hay una reducción de la formación de dímeros PER-CRY con lo cual BMAL1-CLOCK vuelve a unirse al elemento E-box para iniciar nuevamente la transcripción de *Per* y *Cry* (Reppert y Weaver, 2002; Hirota y Fukada, 2004).

Aunque el asa de retroalimentación anteriormente mencionada es suficiente para oscilar por sí misma, a ésta se le sobrepone otra asa de retroalimentación, lo que le permite mayor estabilidad y precisión a la oscilación (Figura 4). El dímero BMAL1-CLOCK al unirse al promotor E-box induce la transcripción del gene *Rev-Erbα*, la proteína de este gene (REV-ERBα) inhibe la transcripción del gen *Bmal1*. Por lo tanto, cuando los complejos PER-CRY desplazan a BMAL1-CLOCK de su promotor E-box, la disminución en la transcripción de *Rev-Erbα* permite la transcripción de *Bmal1*, restableciendo los niveles de la proteína BMAL1, así se repite el ciclo (Reppert y Weaver, 2002; Hirota y Fukada, 2004). De estas dos asas de regulación, resulta la oscilación circadiana de *Bmal1* en antifase con la oscilación de *Per*. Para controlar los cambios circadianos conductuales y fisiológicos, las asas central y secundarias regulan la expresión de genes de salida CCGs (también llamados genes controlados por genes reloj). Por ejemplo, el

potenciador E-box regula la expresión genética de una variedad de factores como DBP (D-element binding protein), E4bp4 (Basic leucine zipper transcription factor), el neuropéptido vasopresina arginina (Jin et al, 1999) y la prokineticina 2 (Cheng et al, 2002).

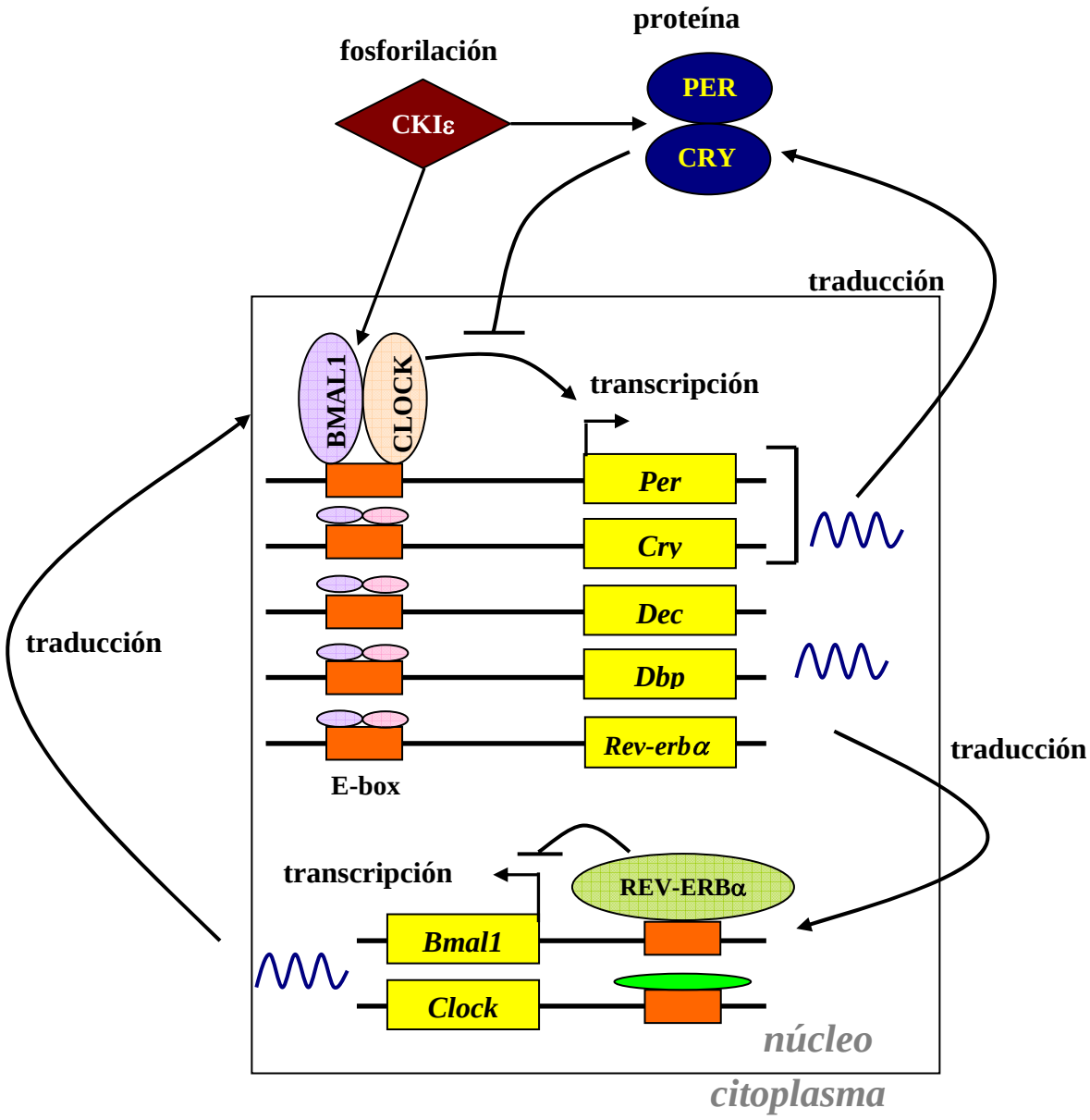

Figura 4. Mecanismo molecular del reloj circadiano en las neuronas del NSQ (Modificado de Hirota y Fukada, 2004).

b) Componentes de entrada que sincronizan al reloj molecular. Diversos autores han propuesto varias vías intracelulares que se activan por la estimulación de la luz para generar los cambios de fase en el reloj circadiano (Cermakian y Sassone-Corsi, 2002; Gillette y Mitchell, 2002; Golombek et al, 2003; Hirota y Fukada, 2004). Se ha propuesto que la señalización intracelular en el NSQ toma diferentes vías dependiendo de la hora en que se aplique el estímulo de sincronización, ya sea luminoso o con glutamato (Figura 5). Como se mencionó anteriormente, las terminales del RHT que forman los axones de las RGC liberan glutamato y PACAP en respuesta a la luz, la unión del glutamato con el receptor tipo NMDA (N-methyl D-aspartato) produce la entrada de Ca^{2+}. Si el estímulo se aplica durante la primera mitad de la noche, el Ca^{2+} que ingresa a la neurona activa directamente a los RyRs e induce la liberación de Ca^{2+} de los depósitos intracelulares. El $Ca^{2+}{}_i$ que entra al citosol se une a la calmodulina (CaM), y este complejo Ca^{2+}/CaM activa a la kinasa (CaMK-II) que fosforila la proteína de unión al CREB (cAMP response element binding). La interacción de CREB con el elemento de respuesta al AMPc (CRE) induce la expresión del gen *Per* para provocar un retraso de fase (Gillette y Mitchell, 2002; Hirota y Fukada, 2004). Otra ruta que se activa por el Ca^{2+} es por la vía de las proteínas kinasas activadas por mitógenos (MAPK), también llamadas kinasas reguladas por señales extracelulares (ERK) (Figura 5). Se ha reportado que la activación de la vía MAPK/ERK también participa en la fosforilación de CREB (Hirota y Fukada, 2004). Por otro lado, si el estímulo luminoso se aplica durante la segunda mitad de la noche, la entrada de Ca^{2+} extracelular activa la oxido nítrico sintetasa (NOS) que induce la síntesis de oxido nítrico (NO), el NO estimula la formación de (guanosina monofosfato cíclica) cGMP, el cual activa a la proteina

Kinasa G (PKG), y que actúa posiblemente sobre el gene *Per* para dar como resultado un avance de fase (Ding et al, 1997; 1998; Meijer y Schwartz, 2003).

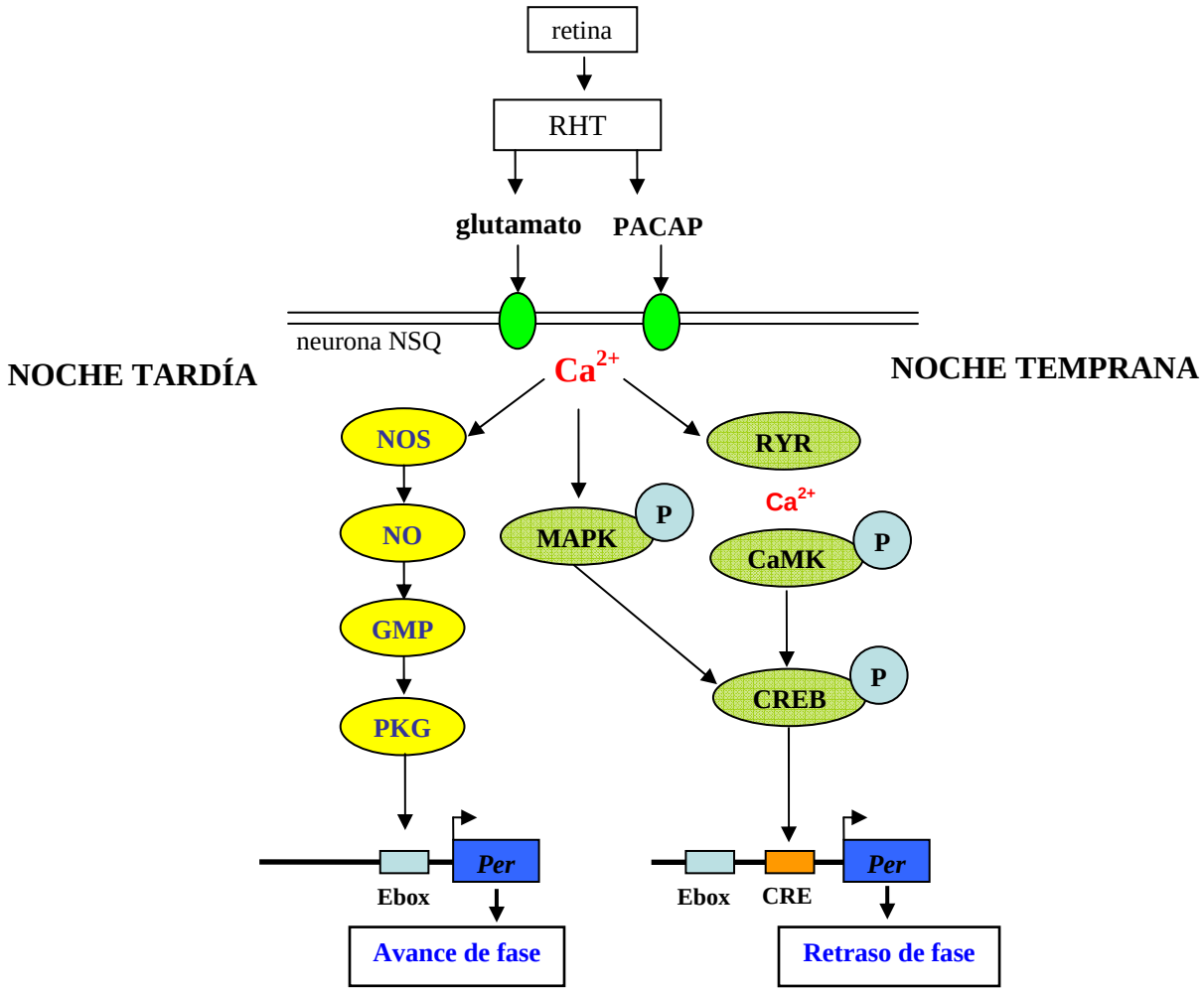

Figura 5. Vías intracelulares en las neuronas del NSQ que se activan por la estimulación de la luz para generar los cambios de fase en el reloj circadiano (Modificado de Hirota y Fukada, 2004).

c) Componentes de salida para la expresión del ritmo circadiano. En las neuronas del NSQ, Schwartz y colaboradores (1987) reportaron que los potenciales de acción dependientes de sodio participan en la sincronización y expresión del ritmo circadiano de la conducta de ingesta de

agua de las ratas. En su trabajo aplicaron tetrodotoxina (TTX, que es un bloqueador de los canales de sodio) a las neuronas del NSQ *in vivo*. Los animales privados de la vista que recibieron infusión de TTX presentaron conducta arrítmica durante el tratamiento, regresando a su periodo y fase inicial al terminar el tratamiento. Esta aproximación experimental demuestra que la actividad eléctrica neuronal dependiente de los canales de sodio en el NSQ, es un componente de salida para el reloj circadiano ya que al inhibirlos con la TTX se pierde el ritmo circadiano de la conducta. Diversos trabajos han registrado ritmos circadianos en la actividad eléctrica en el NSQ, con el pico de actividad durante el día y el valle durante la noche. Este ritmo en la frecuencia de disparo se mantiene aún cuando el NSQ es aislado de sus aferencias y por lo tanto no requiere de conexiones sinápticas o entradas humorales fuera del NSQ (Inouye y Kawamura, 1979; Welsh et al, 1995; Ikeda et al, 2003). Se ha reportado que el ritmo circadiano de la actividad eléctrica se ve afectado por manipulaciones genéticas del reloj molecular en el NSQ. Por ejemplo, la mutación *Tau* en el gen *Casein Kinasa Epsilon I* provoca el acortamiento del periodo del ritmo circadiano de la conducta y del ritmo en la frecuencia de disparo espontáneo en neuronas del NSQ (Liu et al, 1997). De forma parecida, la mutación de *Clock* ocasiona el alargamiento del periodo del ritmo circadiano de la conducta (Vitaterna et al, 1994) y del ritmo de la frecuencia de disparo (Herzog et al, 1998; Nakamura et al, 2002). Estos resultados sugieren que existe un mecanismo de unión entre el reloj molecular intracelular y la expresión de los ritmos circadianos, tanto de la actividad eléctrica de las neuronas del NSQ como de la actividad conductual del organismo.

La siguiente revisión presenta una visión fisiológica integral actualizada de la fisiología celular involucrada en la modulación de la frecuencia de disparo en las neuronas del reloj circadiano en mamíferos.

4. **Artículo: “Neuronal activity in the suprachiasmatic nuclei: Cellular and molecular mechanisms”**

5. Dinámica del $(Ca^{2+})_i$

El $(Ca^{2+})_i$ es una señal altamente versátil que opera en un amplio rango temporal para regular muchas funciones celulares. Por ejemplo, sólo en unos cuantos microsegundos activa la exocitosis, mientras que para activar la transcripción genética o la proliferación celular requiere de varias horas. El $(Ca^{2+})_i$ activa diversos efectores para las funciones celulares como: la excitabilidad membranal, exocitosis, contracción muscular, metabolismo, transcripción, fertilización, proliferación, etc (Figura 6). La célula en reposo tiene una $[Ca^{2+}]_i$ de 100 nM, cuando se activan muchos procesos celulares los niveles aumentan aproximadamente a los 1000 nM. Los niveles de la $[Ca^{2+}]_i$ están determinados por un balance entre los procesos que elevan la $[Ca^{2+}]_i$ (reacciones 'on') y los procesos para la terminación de la señal del Ca^{2+} (reacciones 'off') (Berridge et al, 2003).

El $(Ca^{2+})_i$ que se incorpora al citosol proviene del exterior y de los depósitos intracelulares. La incorporación de $(Ca^{2+})_i$ a los depósitos intracelulares y/o su salida al exterior es por medio de ATPasas de Ca^{2+} y transportadores. Del exterior, el Ca^{2+} se incorpora por medio de canales de Ca^{2+} dependientes de voltaje y de receptores ionotrópicos como el receptor a glutamato. Los principales depósitos intracelulares de Ca^{2+} son el retículo sarcoplásmico (células musculares), retículo endoplásmico y mitocondria, de los cuales los principales elementos que participan en la liberación de $(Ca^{2+})_i$ al citosol son: el intercambiador Na^+/Ca^{2+}, el receptor a inositol-1,4,5-trifosfato (IP_3R) y el receptor a ryanodina (RyR) (Berridge et al, 2003).

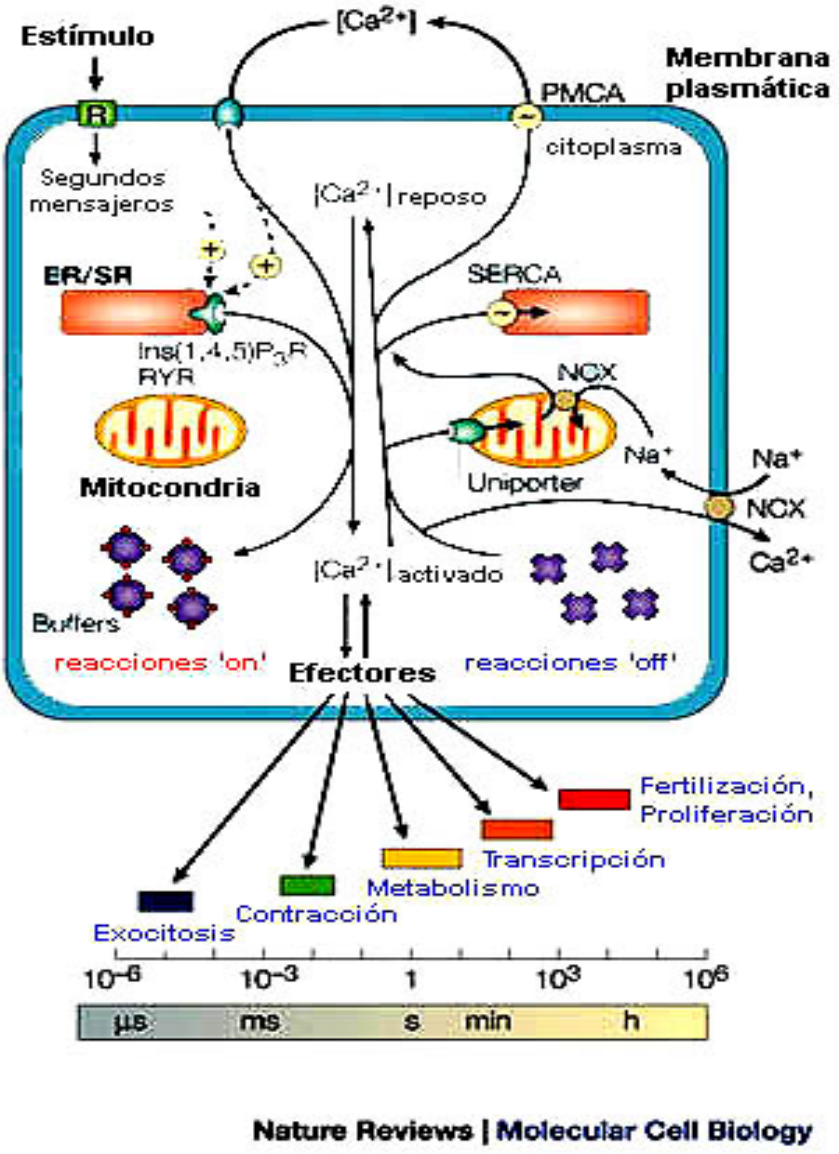

Figura 6. Dinámica del $(Ca^{2+})_i$. Las reacciones 'on' incorporan el Ca^{2+} al citosol, las reacciones 'off' retiran el Ca^{2+} del citosol. IP_3R ($Ins(1,4,5)P_3R$); RyR, receptor a ryanodina; PMCA, ATPasa de Ca^{2+} de la membrana plasmática; SERCA, ATPasa de Ca^{2+} del retículo sarco(endo)plásmico; NCX, intercambiador Na^+/Ca^{2+}. Tomado de Berridge et al, 2003.

6. Canal intracelular de Ca^{2+} sensible a ryanodina

Los receptores a ryanodina (RYRs) son canales intracelulares de Ca^{2+} que se encuentran en las membranas del retículo sarcoplásmico (SR) de las células musculares o en el retículo endoplasmático (ER) de los otros tipos celulares. Su función principal es la deliberar el Ca^{2+} hacia el citoplasma de la célula. En los mamíferos, el RYR es una proteína tetramérica con un peso molecular de 2.2 - 2.3 X 10^6 Daltons (figura 7A), cada monómero está constituido por aproximadamente 5000 amino ácidos y posee un peso molecular de 550 kDa. Se han clonado, secuenciado y caracterizado tres isoformas del receptor: 1) isoforma 1 (RYR-1) que se expresa mayormente en el músculo esquelético, 2) isoforma 2 (RYR-2) distribuida principalmente en el músculo cardiaco y en el cerebro, e 3) isoforma 3 (RYR-3) que se encuentra en pocas cantidades en páncreas, epitelio del pulmón, cerebro, músculo esquelético y liso. Las tres isoformas son codificadas por tres diferentes genes localizados en diferentes cromosomas (*ryr1, ryr2 y ryr3*) (Coronado et al, 1994; Williams et al, 2001; Wagenknecht y Samsó, 2002).

El RYR tiene dos componentes estructurales: a) un componente grande en forma de prisma cuadrado (280 X 280 X 120 Å) que se encuentra en la región citoplasmática del receptor y b) un componente transmembranal de menor tamaño que se proyecta en uno de sus lados en una estructura simétrica de cuatro lados (Figura 7B) (Wagenknecht y Samsó, 2002). El estudio de la estructura-función del receptor a ryanodina ha resultado muy complicado; primero, los canales son expresados en organelos intracelulares, por lo que no son fácilmente accesibles; segundo, es una proteína muy grande (constituye una décima parte del tamaño de un ribosoma), lo cual

complica su manipulación genética, así como la selección de los sitios a mutar. Actualmente se utiliza la técnica de microscopia por cryo-electron para determinar la estructura tridimensional del receptor (Wagenknecht y Samsó, 2002; Fill y Copello, 2002) (figura 7A).

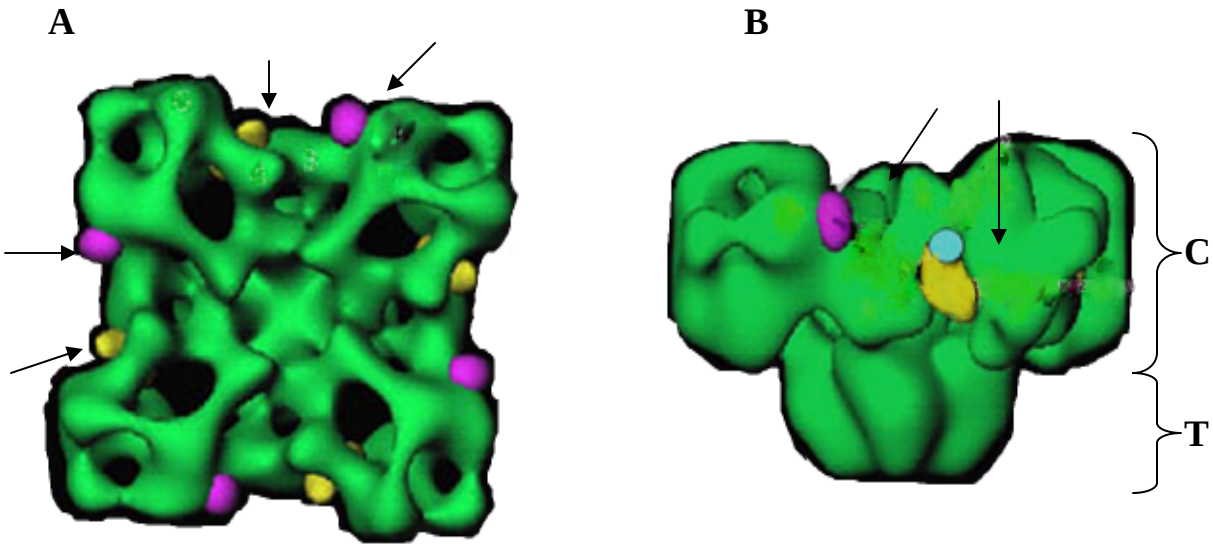

Figura 7. Receptor a ryanodina tipo 2. **A.** Esquema del homotetrámero visto desde la parte citoplásmica. **B.** Esquema del RyR con sus dos componentes estructurales T= transmembranal; C= citoplásmica. Las flechas indican el sitio de unión de diversas moléculas (Tomado de Wagenknecht y Samsó, 2002).

Diversas moléculas se unen al receptor y modifican su actividad, el efecto de éstas depende de su concentración, combinación con algún ion u otra molécula y el tipo de receptor al que se unan (RYR1, RYR2, RYR3). Algunos moduladores de la función del canal son: fosforilación, oxidación, Ca^{2+}, ATP, magnesio, ryanodina, cafeína, dantroleno, rojo de rutenio, etc (Zucchi y Ronca-Testoni, 1997; Shoshan-Barmatz y Ashlet, 1998; Williams et al, 2001; Fill y Copello, 2002).

a) [Ca^{2+}] citosólico: Se ha encontrado que los RYRs tienen sitios de unión para el Ca^{2+} y que el efecto sobre la probabilidad de apertura del canal depende de la [Ca^{2+}] citosólico (Williams et al, 2001). El Ca^{2+} a bajas concentraciones (1-10 μM) induce la liberación de Ca^{2+}, y a concentraciones altas (1-10 mM) inhibe la liberación de Ca^{2+} (Fill y Copello, 2002).

b) ATP: El trifosfato de adenosina (ATP) actúa como ligando en los RYRs. A concentraciones milimolar induce la liberación de Ca^{2+} (Shoshan-Barmatz y Ashley, 1998).

c) Magnesio: El magnesio en concentraciones milimolar cierra el canal del RYR y produce una fuerte inhibición en la liberación de Ca^{2+} (Coronado et al, 1994; Shoshan-Barmatz y Ashley, 1998).

d) Estado redox: Varios procesos redox modulan al RYR, los monómeros del receptor contienen de 80 a 100 residuos de cisteína, muchos de éstos son adecuados para la modificación por oxidantes. Recientemente se ha sugerido que el oxido nítrico (NO) podría modular la regulación redox en los RYR (Fill y Copello, 2002).

e) Cafeína: La cafeína en el rango milimolar estimula la liberación de Ca^{2+} y su efecto se potencia con el ATP y se inhibe con el Mg^{2+} y el rojo de rutenio. La cafeína incrementa la probabilidad de apertura de los canales sin cambiar su estado de conductancia. También estimula la unión de ryanodina al receptor incrementando la afinidad del receptor (Coronado et al, 1994; Shoshan-Barmatz y Ashlet, 1998).

f) Dantroleno y rojo de rutenio: El dantroleno es una droga que se usa como relajante muscular postsináptico, inhibe la liberación del Ca^{2+} a concentraciones entre 10 y 90 μM. El dantroleno reduce la tasa máxima de liberación de Ca^{2+} sin afectar la sensibilidad del Ca^{2+} en el receptor, su acción es más evidente en presencia de cafeína y nucleótidos de adenina (Zucchi y Ronca-Testoni, 1997). El rojo de rutenio también es un inhibidor de la liberación de Ca^{2+} en el SR del músculo esquelético y cardiaco, es muy efectivo a concentraciones entre 1 nM y 20 μM (Coronado et al, 1994; Zucchi y Ronca-Testoni, 1997).

g) Ryanodina: La ryanodina es un alcaloide que se encuentra en plantas del genero *Ryania*, las cuales crecen como arbustos o árboles delgados en bosques tropicales de Centro y Sur de América. Con este componente se identificó y caracterizó al receptor a ryanodina. El efecto sobre los RYRs depende de la concentración de ryanodina que se aplique, a concentraciones en el rango entre 0.01 y 1 μM induce la liberación de Ca^{2+}, a concentraciones en el rango entre 100 y 300 μM inhibe la liberación de Ca^{2+} y a concentraciones de 10 μM genera un estado de subconductancia en el canal (Coronado et al, 1994).

II. PLANTEAMIENTO DEL PROBLEMA

Los procesos celulares y moleculares que participan entre la expresión de los genes reloj y las propiedades electrofisiológicas, que determinan la expresión de la ritmicidad intrínseca en las neuronas del NSQ, han sido poco estudiados. Shibata y Moore (1988) propusieron que el $(Ca^{2+})_i$ libre en las neuronas del NSQ participa de manera importante tanto en el ritmo circadiano del índice de disparo unitario, como en el ritmo de la recaptura de la 2-deoxiglucosa. De acuerdo con estos autores, el ion Ca^{2+} podría ser un candidato como mensajero intracelular en el sistema circadiano. Se han detectado cambios rítmicos en la concentración de $(Ca^{2+})_i$ libre en las plantas del tabaco y Arabidopsis (Johnson et al, 1995; Wood et al, 2001), pero se conoce muy poco sobre la dinámica del $(Ca^{2+})_i$ en las neuronas del NSQ. Díaz-Muñoz y colaboradores (1999) mostraron que el número de receptores a ryanodina tipo 2 (RYR-2) se expresaba con un ritmo circadiano en las neuronas del NSQ, tanto en la condición de luz y oscuridad como en luz roja continua, mientras que los IP_3R no presentaban variación temporal en su expresión. Estos mismos autores también reportaron que la ryanodina en una concentración de 10 μM, administrada en rebanadas de cerebro de rata que contenían al NSQ inducía un avance de fase en el pico de la frecuencia de disparo de las neuronas del NSQ.

En cultivos organotípicos de cerebro de ratón que contenían al NSQ, Ikeda y colab. (2003) registraron de manera simultánea el ritmo circadiano de la $[Ca^{2+}]_i$ y la actividad eléctrica en neuronas del NSQ, usando una proteína fluorescente (camaleón) y un arreglo múltiple de eléctrodos respectivamente. Con estas técnicas se demostró que el pico de mayor $[Ca^{2+}]_i$ precede al pico de mayor actividad multiunitaria por 2-7 hr y que la aplicación de TTX suprime el ritmo

de la actividad eléctrica sin afectar el ritmo en la $[Ca^{2+}]_i$. El tratamiento con ryanodina (5 y 100 μM) y 8-bromo-cyclic ADP ribose (300 μM) sólo afectó la amplitud del ritmo del $[Ca^{2+}]_i$; mientras que la aplicación de nifedipina, un inhibidor de los canales de Ca^{2+} dependientes de voltaje, sólo disminuyó la amplitud del ritmo de la actividad eléctrica. En todos estos resultados el efecto solamente se observó durante la aplicación de los fármacos (Ikeda et al, 2003).

Con estos antecedentes se plantea que los RyR, modulando la $[Ca^{2+}]_i$, son parte de la vía de salida del reloj circadiano en el NSQ, y que forman parte de la unión entre el reloj molecular y la expresión de los ritmos circadianos de la conducta.

III. HIPÓTESIS GENERAL

Si los canales intracelulares de Ca^{2+} sensibles a ryanodina son parte de la vía de salida para transmitir la señal generada por el reloj molecular, entonces las manipulaciones farmacológicas sobre los RyRs modificarán la frecuencia de disparo neuronal y por lo tanto el ritmo circadiano de la conducta.

IV. OBJETIVO GENERAL

Analizar los efectos de la manipulación farmacológica de los RyRs sobre:

1) el ritmo circadiano de la conducta de ingesta de agua y locomoción en la rata,

2) las propiedades eléctricas de las neuronas del NSQ en rebanadas de cerebro de rata.

V. RESULTADOS

1) PARTICIPACIÓN DE LOS RYRS DEL NSQ SOBRE LA EXPRESIÓN DEL RITMO CIRCADIANO DE LA CONDUCTA

Hipótesis

Si los canales intracelulares de Ca^{2+} sensibles a ryanodina participan en la vía de salida del reloj molecular sobre la expresión del ritmo circadiano de la conducta, entonces la activación farmacológica de los RYRs en el NSQ deberá acortar el periodo del ritmo circadiano, mientras que la inhibición farmacológica de los RyRs provocará la pérdida del ritmo circadiano de la conducta durante el tratamiento farmacológico.

Objetivos

General

Analizar los efectos de la aplicación de ryanodina en el NSQ sobre el ritmo circadiano de la conducta de ingesta de agua y actividad locomotora en ratas.

Particulares

1. Estudiar el efecto de inducir la liberación de $(Ca^{2+})_i$ mediante la apertura farmacológica de los RyRs con ryanodina 0.1 μM.
2. Determinar el efecto de inhibir la liberación de $(Ca^{2+})_i$ mediante el bloqueo farmacológico de los RyRs con ryanodina 100 μM.

3. Comparar el efecto de la ryanodina 0.1 μM con el aumento de la excitabilidad membranal producido con una solución fisiológica aumentada en Cloruro de Potasio a 20 mM.
4. Comparar el efecto de la ryanodina 100 μM con la inhibición de los potenciales de acción producida con tetrodotoxina (1 μM).

Materiales y Métodos

Se utilizaron ratas macho de la cepa Wistar con un peso entre 250 y 300 g. Primero se acondicionaron una semana a un ciclo de luz/oscuridad 12:12 (la luz se prendía a las 8:00 hrs con 400 lux), en un cuarto sonoamortiguado a una temperatura de 22 ± 1° C. Posteriormente cada individuo se introdujo en una caja con agua y alimento *ad libitum.* Las cajas se colocaron en habitaciones cerradas en luz roja constante (50 lux), donde se monitoreo la conducta de ingesta de agua y locomoción de manera continua. El piso de la caja contenía una placa de aluminio conectado a tierra y la botella de agua estaba conectada a un sensor, cuando la rata tocaba al bebedero cerraba el circuito y generaba una señal eléctrica que se colectaba en un sistema de adquisición de datos automatizado (Omni-Alva UNAM-lab. Cronobiología v 1.1). La señal era integrada en intervalos de 5 minutos. Los registros obtenidos se almacenaron en una base de datos para los análisis posteriores.

Se registró la conducta de ingesta de agua y locomoción, y al quinceavo día de que se presentará un registro constante de la conducta se procedió a la implantación de la cánula para la administración de los diferentes tratamientos. Los animales fueron asignados de manera aleatoria a uno de los diferentes tratamientos: 1) líquido cefalorraquídeo artificial (aCSF) que contenía en mM: 140 NaCl, 3.5 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, y 5.0 HEPES (pH 7.2-7.3); 2) ryanodina 0.1 μM

para activar al RyR e inducir la liberación del Ca^{2+} de los depósitos intracelulares; y 3) ryanodina 100 μM para bloquear al RyR e inhibir la liberación de Ca^{2+}. Adicionalmente se formaron tres grupos: 4) TTX (1 μM), el cual bloquea la generación de los potenciales de acción por el bloqueo de los canales de Na^{+}; 5) KCl 20 mM, el cual permite la despolarización de la membrana; y 6) anisomysina (5 μg/μl), un inhibidor de la síntesis de proteínas. Todos los fármacos se administraron usando como vehículo aCSF durante 14 días. Después del tratamiento, los sujetos se registraron otros 15 a 20 días más.

Se utilizó la cánula guía 26GA 5 mm (Plastic-one, Roanoke, VA) conectada a una bomba miniosmótica (Alzet modelo 2002) a través de un tubo de polyetileno (Tygon microbore tubing ID 0.020", wall 0.020", OD 0.060"), un extremo del tubo se insertó en la bomba pegándolo con poliacrilato y el otro extremo se conectó a la cánula guía. Los animales se anestesiaron con pentobarbital sódico con una dosis de 30 mg/kg, posteriormente las ratas se colocaron en un aparato estereotáxico, la cánula se colocó en un ángulo de 90° hacia el NSQ y se tomaron las siguientes coordenadas de acuerdo al atlas de Paxinos y Watson (1998): (AP) 0.4; (L) 0.0; (V) -8.5. La cánula se fijó con cemento dental al cráneo con cuatro tornillos de acero inoxidable. La cánula guía se conectó directamente a la bomba miniosmótica, por la cual se administró las diferentes soluciones (aCSF, ryanodina, TTX, KCl, anisomysina).

Al terminar el experimento se sacrificaron los animales con una sobredosis de pentobarbital, los sujetos fueron perfundidos por vía transcardiaca con solución de NaCl al 0.9 % seguida de formalina al 10 %. Se extrajo el cerebro de cada animal y se sumergió en solución de sacarosa, incrementando la concentración (10%, 20% y 30%). Los cerebros fueron seccionados en cortes coronales de 40 μm, se seleccionaron los cortes que presentaron a los NSQs y fueron procesados

para tinción de Nissl con violeta de cresilo. Las secciones fueron observadas en el microscopio para localizar la punta del inyector. Sólo los animales que mostraron el inyector en el NSQ se incluyeron en este estudio.

Los registros de conducta de ingesta de agua y locomoción se acoplaron en un solo actograma graficado en Double-Plot. El análisis para determinar el periodo se llevo a cabo mediante el periodograma de χ^2 (ver detalle en el artículo). Los periodogramas se graficaron en una matriz 3-dimensional, que es un procedimiento parecido al compressed sprectral array (Bickford et al, 1973), pero en lugar de utilizar la transformada de Fourier se utilizó el análisis χ^2 (ver detalle en el artículo).

Los parámetros de cada segmento experimental se compararon mediante el análisis bilateral de varianza por rangos de Friedman, seguida por la prueba post hoc de Dunnett T3 si se requería. Debido a la ausencia de ritmicidad inducida por algunos tratamientos, se utilizó la prueba Wilcoxon de calificación con signo, para comparar antes y después del tratamiento. Para hacer las comparaciones entre los grupos que presentaron ritmicidad circadiana (valor 1) vs los grupos arrítmicos (valor 0), se utilizó la prueba de Cochran Q. En todos los análisis el nivel de α fue de 0.05.

Resultados

De 56 animales implantados, en 32 se observó la punta del microinjector en la línea media entre los NSQs (Figura 8). De los 24 animales restantes, la punta del microinyector se localizó en la parte rostral (n=8) o caudal (n=10) al NSQ, en los otros 6 animales la punta del microinjector se encontró dentro y por debajo del NSQ y el daño sustancial en al menos uno de los núcleos fue

evidente. En la mayoría de estos animales, los tratamientos no tuvieron efectos notables: los animales con alguna lesión en el NSQ mostraron cambios de periodo o cambios de fase después de la implantación del microinyector, y en un sujeto que recibió tratamiento con TTX los patrones arrítmicos persistieron durante todo el tiempo que de registro. En 5 animales donde la punta del microinyector se localizó caudal al NSQ se observaron cambios en el periodo y pérdida transitoria de la ritmicidad. De los 32 sujetos que presentaron la punta del microinyector entre los NSQs, se seleccionó una n = 5 para cada uno de los grupos.

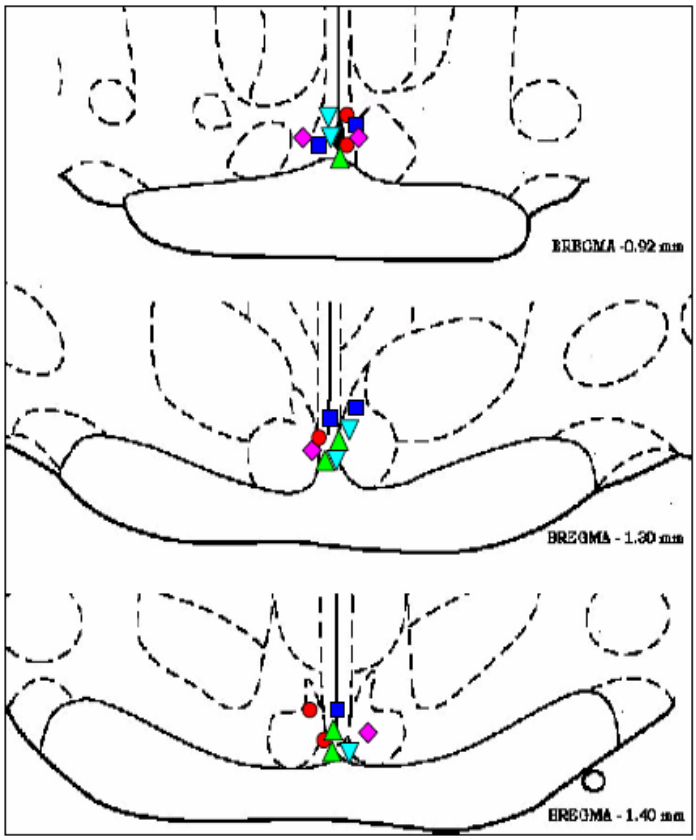

Figura 8. Ubicación de la punta del microinyector en el NSQ y sus alrededores. Los símbolos representan los grupos experimentales. ■ aCSF, ◆ 100 µM ryanodina, ▽ 0.1 µM ryanodina, ● 1 µM TTX, ▲ 20 mM KCl.

Durante el registro basal, todos los sujetos muestran claramente un ritmo circadiano con un periodo cercano a las 24 hrs. La perfusión continua de aCSF en los límites del NSQ no induce un cambio notable en el periodo o fase durante y después del tratamiento (Figura 9). El periodograma χ^2 (Figura 10) confirma que no hay efectos en el periodo durante o después de la infusión con aCSF con respecto a la basal. Los animales tratados con el activador de los RyRs (ryanodina 0.1 μM) conservan un ritmo circadiano claro, pero el periodo se ve ligeramente afectado mostrando un acortamiento, también se observó una reducción aparente en el intervalo de actividad durante y después de la administración de la ryanodina con respecto a la basal (Figura 9). El efecto en el periodo se puede observar claramente en el periodograma χ^2 (Figura 10). La administración de 20 mM KCl produce un efecto similar al inducido por la ryanodina 0.1 μM, se observó un acortamiento del periodo y una compresión en el intervalo de la actividad durante y después del tratamiento. El bloqueo de los RyR con ryanodina 100 μM provocó una alteración del ritmo conductual debido a la desorganización de los patrones diarios de la actividad durante la administración del fármaco (Figura 9). Debido a la ausencia de ritmicidad inducida por la ryanodina 100 μM se utilizó la prueba de signos de Wilcoxon para comparar antes y después del tratamiento ($Z= -1.3$, n.s.). Después del termino de la aplicación de la ryanodina, el ritmo circadiano reaparece y el inicio de la actividad se ajusta a la línea proyectada del registro basal. Los efectos en el periodo del ritmo fueron evidentes en el periodograma χ^2 (Figura 10). Con la administración de TTX se observó también una clara desorganización del ritmo circadiano y después de la administración del fármaco el ritmo se restauró. Estos efectos fueron claramente observados en el periodograma χ^2 (Figura 10). En todos los casos, excepto uno, la fase del ritmo restaurado (después de la actividad) se ajusto a la línea proyectada del

inicio de la actividad desde el registro basal. Finalmente, la ryanodina 100 μM bloquea parcialmente la liberación de Ca^{2+} del retículo endoplásmico hacia el citoplasma y esto podría afectar la síntesis de proteínas, por lo que se estudio el efecto de la inhibición de la síntesis de proteínas en el NSQ sobre la expresión del ritmo circadiano de la conducta. La administración de anisomycina 5 μg/μl alteró el ritmo circadiano de la conducta y su efecto, aparentemente persiste 2 semanas después del tratamiento.

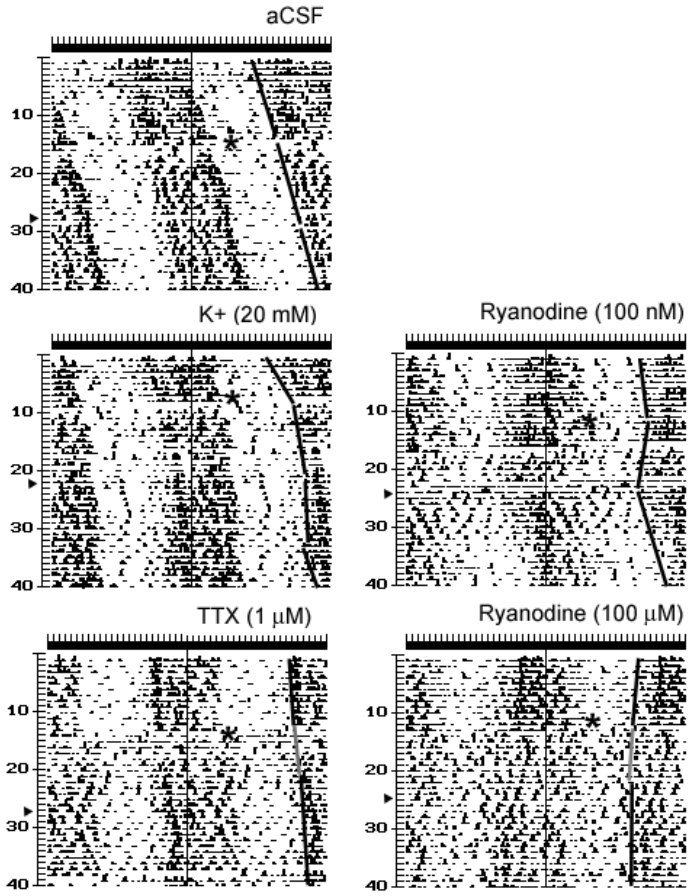

Figura 9. Actogramas graficados en Double-Plot de la suma de la actividad de ingesta de agua y locomoción de 5 animales que recibieron tratamiento.

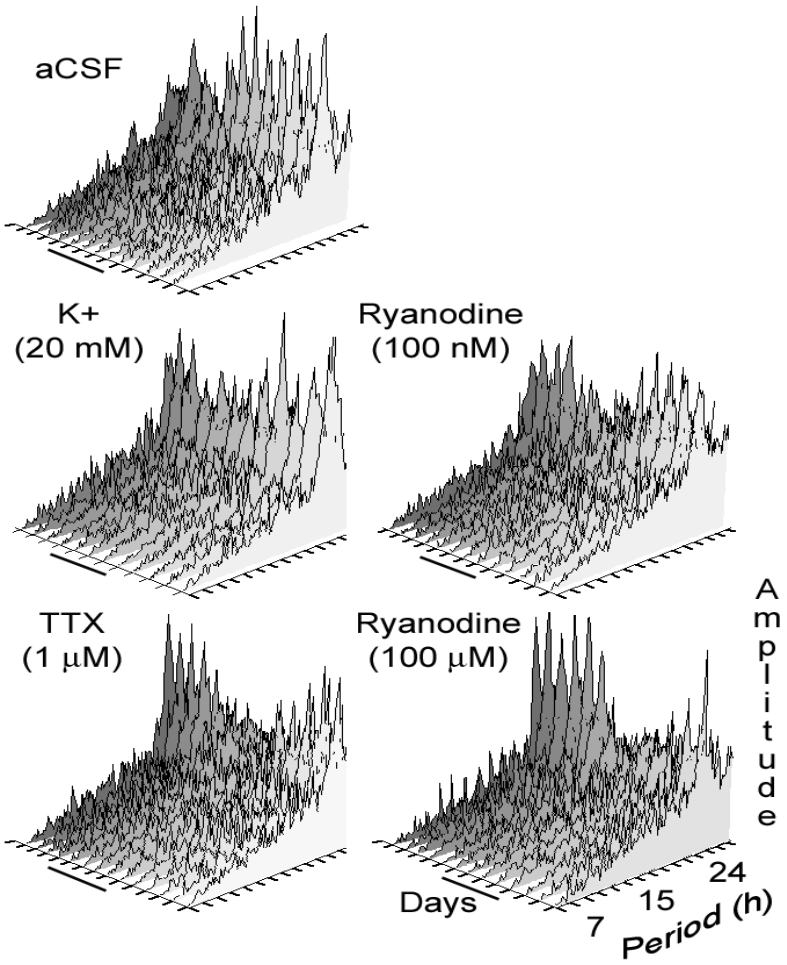

Figura 10. Periodograma χ^2 de los datos mostrados en la figura 9. La línea del eje z indica los días de registro, el eje x el periodo del ritmo y el eje y la amplitud.

En el siguiente artículo se explica de manera más detallada los resultados y en la Tabla 1 se muestran los valores obtenidos en cada uno de los grupos experimentales antes, durante y después del tratamiento. También se discute de manera detallada los resultados obtenidos de estos experimentos.

Artículo: "Ryanodine-sensitive intracellular Ca^{2+} channels in rat suprachiasmatic nuclei are requiered for circadian clock control of behavior"

2) PARTICIPACIÓN DE LOS RYRS SOBRE LA FRECUENCIA DE DISPARO DE NEURONAS DEL NSQ

Hipótesis

Si la frecuencia de disparo en neuronas del NSQ es modulado por la vía de los RYR que movilizan $(Ca^{2+})_i$, entonces la activación farmacológica de los RYRs aumentará la frecuencia de disparo de las neuronas del NSQ, mientras que la inhibición disminuirá la frecuencia de disparo.

Objetivos

General

Analizar los efectos de la aplicación de ryanodina en rebanadas de cerebro que contienen al NSQ sobre la frecuencia de disparo espontáneo en neuronas del NSQ *in vitro*.

Particulares

1. Evaluar el efecto de inducir la liberación de $(Ca^{2+})_i$ por la activación de los RYR, con el tratamiento de ryanodina 0.1 μM y cafeína 1 mM.
2. Analizar el efecto de reducir la liberación de $(Ca^{2+})_i$ por la inhibición de los RYR, mediante el tratamiento de ryanodina 80 μM y dantroleno 10 μM.
3. Comparar los efectos de la ryanodina 0.1 μM con el resultado de manipular la excitabilidad membranal, con una solución fisiológica aumentada en Cloruro de Potasio a 20 mM.
4. Comparar los efectos de la ryanodina 80 μM con el resultado de inhibir los potenciales de acción con tetrodotoxina (1 μM).

Materiales y Métodos

Se utilizaron ratas macho Wistar con un peso de entre 90 y 110 g. Los animales se mantuvieron en cajas con libre acceso al agua y al alimento, en un cuarto sonoamortiguado a una temperatura de 22 ± 1° C, en un ciclo de luz-oscuridad 12:12 (encendido de la luz a las 6:00 con 400 lux), por lo menos una semana antes del experimento. Para los experimentos electrofisiológicos realizados en la fase de oscuridad, un grupo de animales se mantuvo con un ciclo invertido de luz-oscuridad 12:12 (encendido de la luz a las 22:00 con 400 lux), por lo menos tres semanas antes del experimento. Todos los experimentos se llevaron a cabo acorde a los lineamientos publicados en el diario oficial NOM-062-200-1999, para el uso de animales de laboratorio.

Al grupo de animales utilizado para los registros durante la fase de luz, se les anestesió profundamente con éter entre ZT 03-05 (horas después del encendido de la luz) y al grupo de animales para los experimentos realizados durante la fase de oscuridad se les anestesió entre ZT 11-12 (antes del apagado de las luces) para evitar los cambios de fase inducidos por la luz. Los cerebros fueron extraídos rápidamente y colocados en una solución fisiológica (aCSF) oxigenada a 4ºC. Se hicieron rebanadas del cerebro en secciones coronales de 250 μm, sólo se utilizaron las rebanadas que contenían al NSQ. La rebanada se colocó en una cámara de registro con perfusión continua de aCSF oxigenada a temperatura ambiente. La solución de aCSF contenía en mM: 126 NaCl; 2.5 KCl; 1.2 NaH_2PO_4; 4 $MgCl_2$; 2.4 $CaCl_2$; 26 $NaHCO_3$; 10 glucosa; 0.2 de ácido ascórbico; 0.2 de Tiourea. El pH se ajusto a 7.4 con NaOH y la osmolaridad a 330 mOsm/l, la solución se filtró y se oxigenó con una mezcla de gas 95% O_2 / 5% CO_2. Las neuronas del NSQ

se visualizaron mediante microscopia de Nomarski con iluminación infrarroja a 600x, por medio de un microscopio Nikon Eclipse 600.

Los registros se hicieron por medio de la técnica de parche perforado durante la fase de luz (ZT04-10) o durante la fase de oscuridad (ZT17-22). Para la perforación de la membrana se utilizó amfotericina 10 mg/ml disuelta en dimetil sulfoxido (DMSO). Los electrodos de registro fueron llenados con una solución que contenía KCl (150 mM), con un pH de 7.2 a 300 mOsm/l y amfotericina. Una vez obtenido un buen sello (2 GΩ) se dejó de 2 a 8 minutos para conseguir el parche perforado. Los registros se hicieron en configuración de fijación de corriente con un amplificador Axoclamp 200A. Se hizo un registro basal de la actividad espontánea durante diez minutos, posteriormente se administró el fármaco en la aCSF y se continuó registrando la actividad espontánea. Cada neurona recibió sólo uno de los siguientes tratamientos: 1) aCSF; 2) Activadores de los RyRs: ryanodina 0.1 μM ó cafeína 1 mM; 3) Inhibidores de los RyRs: ryanodina 80 μM ó dantroleno 10 μM.

La frecuencia de disparo se estimó con el inverso de la mediana del intervalo interespiga. El disparo regular (rítmico) se consideró en aquellas neuronas que mostraron una distribución Gaussiana estrecha en el histograma del intervalo interespiga (Figura 11A), mientras que el disparo irregular (arrítmico) se consideró en aquellas neuronas que mostraron una distribución sesgada (Figura 11B) (Groos y Hendricks, 1979; Shibata et al, 1984; Thomson et al, 1984).

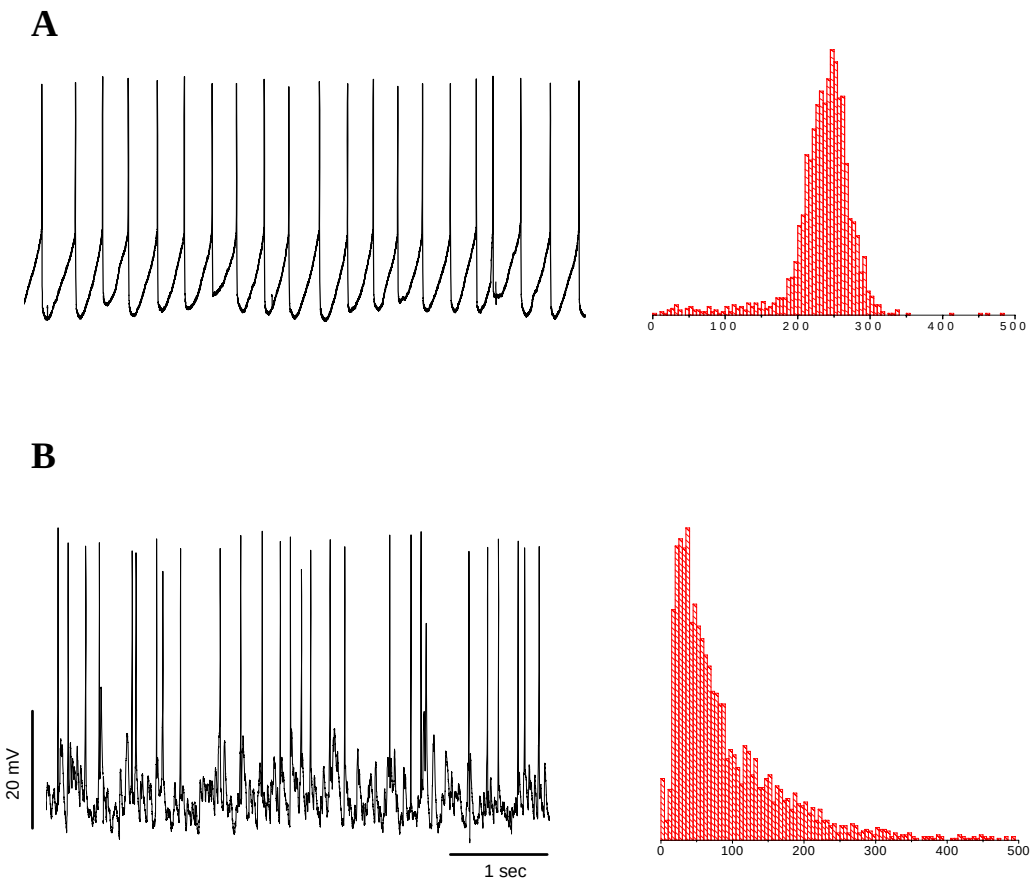

Figura 11. Clasificación de la frecuencia de disparo. A) Regular, con distribución Gaussiana estrecha, B) Irregular, distribución Gaussiana sesgada.

Para medir el potencial de membrana y la duración del potencial de acción se alinearon 20 segmentos consecutivos tomados 20 mseg antes del potencial de acción y hasta 20 mseg antes del siguiente potencial de acción. Se trazó el perfil promedio para medir el potencial de membrana en los siguientes puntos: 20 mseg antes del disparo (V_m); en el umbral del disparo (V_{thr}); en el pico del disparo (V_{spk}) y en el punto más bajo del post-potencial hiperpolarizante (V_{ahp}). La duración del potencial de acción se midió hasta el V_{thr}; el tiempo de subida del potencial de acción (τ_{spk}) se midió de V_{thr} a V_{spk}, y el tiempo de decaimiento del potencial de

acción (τ_{ahp}) se midió de V_{spk} al 66% del decaimiento de V_{spk} a V_{ahp}. El análisis estadístico de los parámetros anteriormente mencionados se midió con una prueba de t pareada (antes y después del tratamiento). Para la recolección de los datos se utilizó una PC compatible con una tarjeta digital de adquisición de datos en un programa elaborado en ambiente LabView. Los registros fueron analizados en el programa MiniAnalysis v6 y Origin 6.0. La comparación estadística de la frecuencia de disparo antes y después del tratamiento se hizo con la prueba de signos de Wilcoxon. En todos los análisis el nivel de α fue de 0.05.

Para determinar un posible efecto de la ryanodina en la comunicación sináptica del NSQ, se diseñaron experimentos donde se utilizaron fármacos para bloquear los receptores GABA A, AMPA y NMDA. Después del registro basal del patrón de disparo espontáneo a las neuronas se les aplicó: bicuculina 10 μM (bloqueador de los receptores GABA A), 6,7-Dinitroquinoxalina-2,3(1H,4H)-diona 10 μM (DNQX bloqueador de los receptores a glutamato AMPA) y Ácido DL-2-Amino-5-fosfonopentanoico 50 μM (APV bloqueador de los receptores a glutamato NMDA).

Resultados

En total se registraron 76 neuronas en el NSQ entre ZT 4 y ZT10. El 38.2 % (29 neuronas) mostraron frecuencia regular (rítmico) 5.0 ± 0.6 Hz (Figura 12). La mayoría de estas neuronas (25) se registraron en la modalidad de parche perforado, 2 fueron registradas en cell attached y 2 en modo whole cell. El potencial de membrana en estas neuronas de disparo regular fue en promedio de –52.1 ± 2.0 mV. El 61.8% (47 neuronas) presentaron un patrón irregular de

disparo (arrítmico) (Figura 12). Las neuronas arrítmicas registradas en modo whole cell (n=20) presentaron una frecuencia de 1.3 ± 0.3 Hz, el potencial de reposo fue de –54.8 ± 1.8. La frecuencia de las neuronas arrítmicas registradas en modo parche perforado (n=27) fue de 2.0 ± 0.2 Hz. Y su potencial en reposo fue de -46.3 ± 1.4 mV. En 24 neuronas que no recibieron algún tipo de tratamiento la frecuencia de disparo espontáneo se mantuvo estable durante 40 min, el cual fue la duración promedio de los experimentos farmacológicos. Su frecuencia de disparo fue de 3.8 ± 0.6 Hz entre los 5 y 10 min de registro y 4.5 ± 0.9 Hz entre los 35 y 40 min de registro. Los efectos de los fármacos que modulan los RYRs se estudiaron en 52 neuronas: cafeína n=12; dantroleno n=21; ryanodina 0.1 μM n=12; ryanodina 80 μM; n=7. Los efectos de los diferentes fármacos aparecieron alrededor de los 3 minutos después de administrada la solución y se mantuvo estable después de los 6 minutos. El efecto de las drogas persistió aún después de 15 minutos después de reemplazar la solución extracelular por aCSF nuevo.

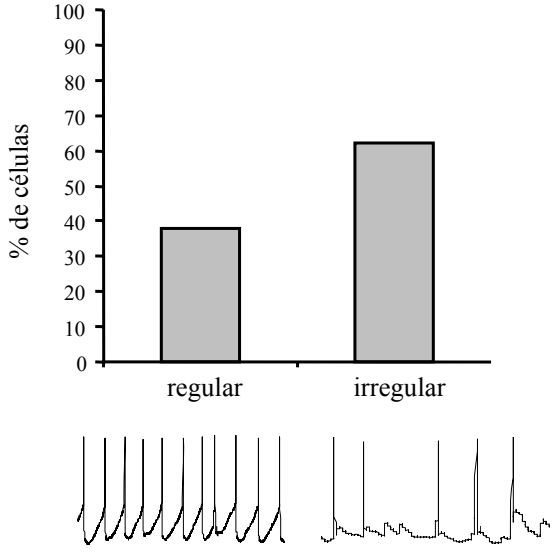

Figura 12. Porcentaje de neuronas que presentaron patrón de disparo regular e irregular.

La liberación de Ca^{2+} de los depósitos intracelulares mediante la activación de los RYRs con la ryanodina 0.1 μM provocó un incremento en la frecuencia de disparo espontáneo en 7 de 12 neuronas de 1.5 ± 0.7 Hz a 4.0 ± 1.0 Hz ($p < 0.05$, prueba de Wilcoxon). Otro efecto de la ryanodina 0.1 μM fue la disminución en V_{ahp} de -52.8 ± 1.7 mV a -44.0 ± 3.4 mV ($t = 2.98$ $p < 0.05$) y un incremento en la duración del potencial de acción de 5.3 ± 1.1 mseg a 11.0 ± 2.3 mseg ($t = -3$, $p < 0.05$), el cual se debió a un aumento en τ_{spk} y τ_{ahp}. Las 5 neuronas restantes no presentaron cambios significativos en su frecuencia de disparo (3.2 ± 0.7 Hz antes y 3.3 ± 0.8 Hz después del tratamiento). De las 12 neuronas que recibieron cafeína 1 mM, en 7 se observó un aumento en su frecuencia de disparo de 1.1 ± 0.3 a 3.0 ± 0.9 Hz ($p>0.05$, prueba de Wilcoxon), ningún otro parámetro cambió durante el tratamiento. En las 5 neuronas restantes no se presentó un cambio significativo en la frecuencia de disparo (3.5 ± 1.4 antes y 3.0 ± 1.4 Hz después del tratamiento) (Figura 13).

El bloqueo de los RYRs con la ryanodina 80 μM indujo una disminución significativa en la frecuencia de disparo espontáneo en todas las neuronas registradas (n=7) de 5.6 ± 1.5 Hz a 0.9 ± 0.4 ($p<0.05$, prueba de Wilcoxon). Otros efectos por el tratamiento con la ryanodina a altas concentraciones fue una disminución en: la amplitud de V_{spk} de 6.3 ± 4.1 mV a -5.8 ± 4.3 ($t = 2.8$, $p< 0.05$); en la amplitud de V_{ahp} de -54.6 ± 2.2 mV a -43.8 ± 2.7 mV ($t = -3.7$, $p < 0.05$) y el cambio en el patrón de disparo de rítmico a arrítmico. Por otro lado, en 13 de las 21 neuronas que recibieron dantroleno 10 μM, disminuyó la tasa de la frecuencia de disparo espontáneo de 2.4 ± 0.6 Hz a 0.7 ± 0.2 Hz (Figura 13). Otros efectos del dantroleno fue el incremento en V_m, en V_{thr} y en V_{ahp}; así como una disminución en la amplitud del potencial de acción. En el resto de

las 8 neuronas no se observaron efectos significativos en la frecuencia de disparo (ver Tabla 1 del siguiente artículo).

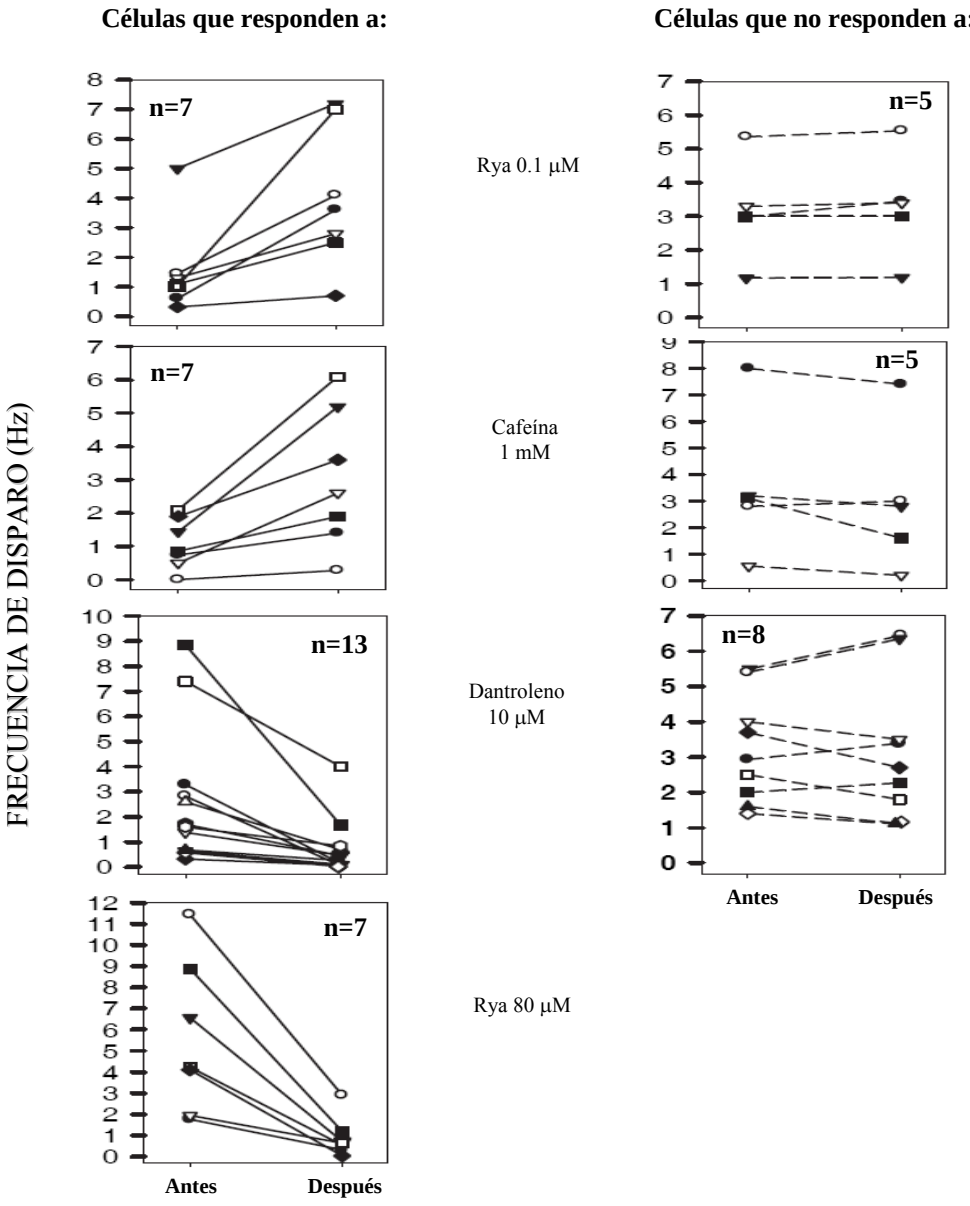

Figura 13. Respuesta farmacológica de los diferentes tratamientos en la frecuencia de disparo de neuronas del NSQ. Las gráficas de la izquierda corresponden al grupo de neuronas que respondieron de manera significativa a la activación o inhibición de los RyRs. Las gráficas de la derecha representan a las neuronas que no cambiaron significativamente su frecuencia de disparo.

Para determinar si los efectos de la ryanodina sobre la frecuencia de disparo también ocurrían en la noche subjetiva, se probó los efectos de ambas dosis de la ryanodina en la frecuencia de disparo en 13 neuronas durante la noche subjetiva de ZT17 a ZT22. En 7 de las 10 neuronas tratadas con ryanodina 0.1 μM, la frecuencia de disparo se incrementó de 1.9 ± 0.4 Hz a 3.9 ± 0.4 Hz ($p < 0.05$, prueba de Wilcoxon). Por otro lado, las 3 neuronas que recibieron el tratamiento con ryanodina 80 μM disminuyeron su frecuencia de disparo espontáneo en 34%, 58% y 90% de sus valores basales.

Se administró bicuculina, DNQX y APV en 28 neuronas en conjunto a la ryanodina (Figura 14). En presencia de los bloqueadores, en 15 de 21 neuronas tratadas con la ryanodina 0.1 μM aumentó la tasa de frecuencia de disparo de 1.1 ± 0.3 a 3.1 ± 0.6 Hz ($p < 0.05$, prueba de Wilcoxon). La administración de DNQX y APV no afectó la frecuencia de disparo en el NSQ en ninguno de los casos en que los bloqueadores se aplicarán antes o después de la ryanodina. En contraste, 5 de las 14 neuronas con bicuculina incrementó la tasa de la frecuencia de disparo un 78.6 % con respecto al control cuando se administró antes de la ryanodina. En 7 neuronas tratadas con la ryanodina 80 μM se observó una disminución significativa de la frecuencia de disparo de 6.3 ± 1.6 Hz a 3.3 ± 1.0 Hz después de la administración de los bloqueadores. La aplicación de DNQX y APV no alteró la tasa de disparo espontáneo en ninguna neurona, sólo en 4 neuronas con bicuculina incrementó la tasa de la frecuencia de disparo un 49% con respecto a la basal.

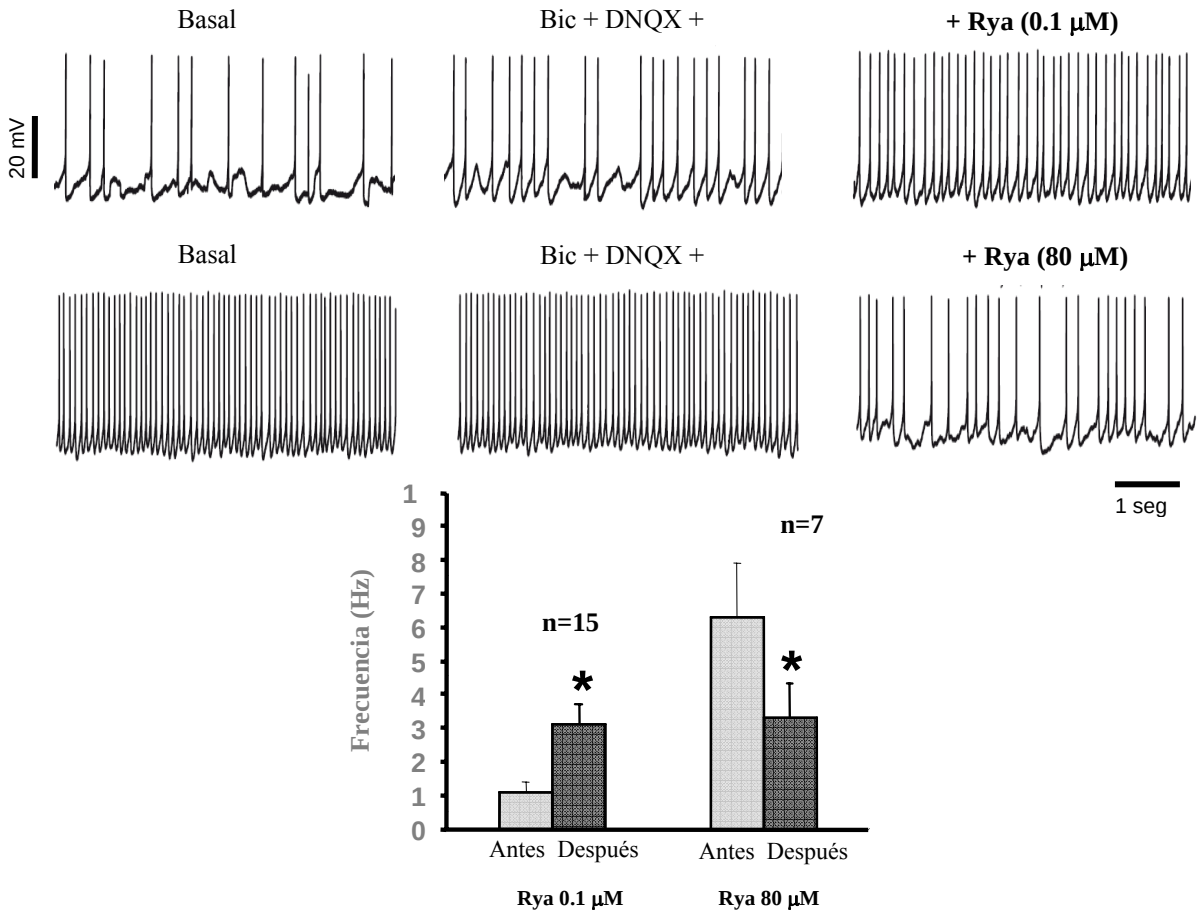

Figura 14. Efecto de la manipulación farmacológica de los RyRs, sobre la frecuencia de disparo de neuronas del NSQ en presencia de bloqueadores: Bicuculina 10 μM (Bic), DNQX 10 μM y APV 50 μM. Ryanodina (Rya). * $p < 0.05$ prueba de Wilcoxon.

En el siguiente artículo se explica de manera más detallada los resultados y en la Tabla 1 se muestran los valores obtenidos en cada uno de los grupos experimentales. También se discute de manera detallada los resultados obtenidos de estos experimentos.

Artículo: "Ryanodine receptor Ca^{2+} release channels are an output pathway for the circadian clock in the rat suprachiasmatic nuclei.

VI. DISCUSIÓN GENERAL

La homeostasis de Ca^{2+} es un elemento importante para la regulación de las vías de señalización intracelular como: el metabolismo, liberación de neurotransmisores, transcripción, excitabilidad, etc. En el NSQ se ha reportado que los niveles de $(Ca^{2+})_i$ y el número de RyRs presentan un ritmo circadiano y se ha propuesto que el $(Ca^{2+})_i$ podría ser un elemento importante para la modulación de la expresión de los ritmos circadianos. Los experimentos reunidos en la presente tesis aportan evidencia directa de que los RyRs, forman parte de la vía de salida del reloj molecular para la expresión de los ritmos circadianos. De los resultados obtenidos en este proyecto podemos identificar tres efectos principales por la manipulación farmacológica de los RyRs: 1) La alteración en la expresión del ritmo circadiano de la conducta, es transitoria; 2) Hay modulación circadiana de la frecuencia de disparo y potencial de membrana, y 3) Los efectos no son mediados por transmisión sináptica.

La manipulación farmacológica directa sobre la excitabilidad membranal y los RyRs afecta de forma transitoria el ritmo circadiano de la conducta, pero sin afectar el mecanismo propio del reloj, lo cual se puede observar por la restauración del periodo y fase del ritmo de la conducta a sus niveles basales después de la administración de la ryanodina.

Cuando se administró la ryanodina a altas concentraciones (80, 100 μM), las cuales bloquean a los RyRs, se observó una disminución en la frecuencia de disparo y la pérdida temporal del ritmo circadiano de la conducta, lo que sugiere que el efecto se debe a la disminución en la liberación

de Ca^{2+} por el bloqueo de los RyRs. El efecto fue parecido con la aplicación de TTX (un bloqueador de los canales de Na^{+}) a las neuronas del NSQ: una pérdida temporal del ritmo circadiano de la conducta y una disminución en la frecuencia de disparo espontáneo de las neuronas. La aplicación de la ryanodina a dosis bajas (0.1 μM), la cual activa a los RYRs, provocó el aumento en la frecuencia de disparo neuronal espontáneo del NSQ, un acortamiento del periodo y la compresión del intervalo de actividad en el ritmo circadiano de la conducta. Lo que sugiere que el efecto se debió a la liberación de Ca^{2+} por la apertura de los RyRs. La administración de la solución fisiológica con un incremento en KCl a 20 mM tuvo el mismo efecto que la ryanodina 0.1 μM. Estos datos indicarían que la movilización de $(Ca^{2+})_i$ por medio de los RyRs podrían actuar sobre la excitabilidad membranal para modular la salida de la expresión del ritmo circadiano.

Para determinar los efectos de la inhibición de la síntesis de proteínas sobre la expresión conductual del ritmo circadiano, se administró anisomicina 5 μg/μl sobre el NSQ in vivo. La anisomicina interrumpió la expresión del ritmo circadiano de la conducta, pero a diferencia de la ryanodina 100 μM, sus efectos persistieron aún tres semanas después de la aplicación de la anisomicina. Posiblemente la persistencia del patrón arrítmico de la conducta durante y después de la anisomicina podría reflejar un severo daño en el reloj molecular, que involucra la síntesis de proteínas. Por lo que, la ryanodina a altas concentraciones sólo afecta de manera transitoria el ritmo circadiano de la conducta, sin alterar el mecanismo propio del reloj molecular.

Con los datos obtenidos en este trabajo, nosotros proponemos que la modulación farmacológica de los RyRs induce cambios en el movimiento del Ca^{2+} de los reservorios intracelulares hacia el citoplasma y viceversa. Este movimiento de Ca^{2+} podría modular la frecuencia de disparo espontáneo a través de varías vías: 1) Activación o inhibición de segundos mensajeros que modulen canales iónicos de la membrana (Nitabach et al, 2002; Lundkvist et al, 2005); 2) Modulación del potencial de membrana por cambios en las corrientes de potasio (Itri et al, 2005). De manera indirecta el Ca^{2+} se puede unir a la calmodulina para activar de forma rítmica las proteínas kinasas que fosforilan canales iónicos voltaje-dependientes como es el caso de los canales de calcio tipo L o canales de potasio, de esta manera modular la actividad eléctrica de las neuronas del NSQ (Shibata y Moore, 1988; Walsh et al, 1995).

Se ha observado que en las neuronas del NSQ hay variación circadiana en el potencial de membrana, durante la fase del día el potencial de membrana está más despolarizado, mientras que durante la fase de oscuridad el potencial de membrana está menos despolarizado (de Jeu et al, 1998). Esta despolarización rítmica es acompañada por una disminución en la conductancia membranal (Jiang et al, 1997; de Jeu et al, 1998). Estos cambios son mediados principalmente por cambios en las corrientes del ion potasio en las neuronas del NSQ (Kuhlman y McMahon, 2006). Además se ha descrito que un tipo de canal de potasio activado por Ca^{2+} (BK) es regulado de manera circadiana y contribuye a la salida de la frecuencia de disparo (Meredith et al, 2006). Las corrientes del BK se elevan durante la fase nocturna, así como sus niveles de mRNA. Tanto el bloqueo farmacológico de las corrientes de BK, como la modificación genética del canal, alteran el ritmo circadiano de la frecuencia de disparo (Pitts y McMahon, 2006). Se propone que

la elevación de las corrientes de BK durante la noche contribuye a la disminución de la frecuencia de disparo del NSQ, por la alteración del post-potencial hiperpolarizante (AHP) (Cloues y Sather, 2003), sin embargo es necesario el estudio más profundo de estos canales BK en el NSQ para determinar su participación precisa en la salida del reloj.

El bloqueo de la actividad sináptica no interfirió con el aumento o disminución de la frecuencia de disparo inducida por la ryanodina a 0.1 o 80 μM respectivamente. Estos resultados indican que la ryanodina modula el patrón de disparo de las neuronas del NSQ por la movilización directa del $(Ca^{2+})_i$ intracelular, mediante los RyR y no por efecto de la transmisión sináptica.

En conjunto, los datos presentados en esta tesis apoyan la hipótesis de que la modulación circadiana de los RyRs es parte de la vía de salida del oscilador molecular para la expresión de los ritmos circadianos.

VII. CONCLUSIONES

- La activación farmacológica de los RyR en neuronas del NSQ, con ryanodina a bajas concentraciones (0.1 μM) acortó el periodo y comprimió el intervalo de actividad del ritmo circadiano de la conducta. Este efecto fue parecido cuando se aplicó un estímulo despolarizante en la membrana con KCl.
- La inhibición farmacológica de los RyR con ryanodina a altas concentraciones (100 μM) en neuronas del NSQ provocó la pérdida momentánea del ritmo circadiano de la conducta. Este efecto fue parecido cuando se administró un inhibidor de los canales de sodio (TTX).
- Los efectos de las manipulaciones farmacológicas de los RyRs, sobre el ritmo circadiano de la conducta fueron transitorios, es decir, después del tratamiento los parámetros del ritmo (periodo y fase) retornaron a sus niveles basales.
- La liberación de Ca^{2+} al citoplasma inducida por la activación farmacológica de los RyR ocasionó un aumento en la frecuencia de disparo en las neuronas del NSQ, mientras que el bloqueo farmacológico de los RyRs provocó una disminución en la frecuencia de disparo. Estos efectos fueron parecidos cuando se administró KCl y TTX respectivamente.
- Los efectos producidos por la ryanodina sobre la frecuencia de disparo neuronal del NSQ, se debió a la liberación de Ca^{2+} por los RyRs y no dependen de la transmisión sináptica.
- Con los resultados de este trabajo se propone un modelo representado en la Figura 15 en donde los RyRs, modulando la homeostasis del $(Ca^{2+})_i$, de alguna forma alteran de

manera indirecta o directa, la excitabilidad membranal de las neuronas del NSQ, al cambiar la actividad eléctrica neuronal se modifica la expresión del ritmo circadiano de la conducta. Finalmente los RyR podrían ser parte importante de los elementos en la transmisión de las oscilaciones circadianas de las asas de retroalimentación transcripción-traducción de los genes reloj hacia la excitabilidad membranal en las neuronas del NSQ.

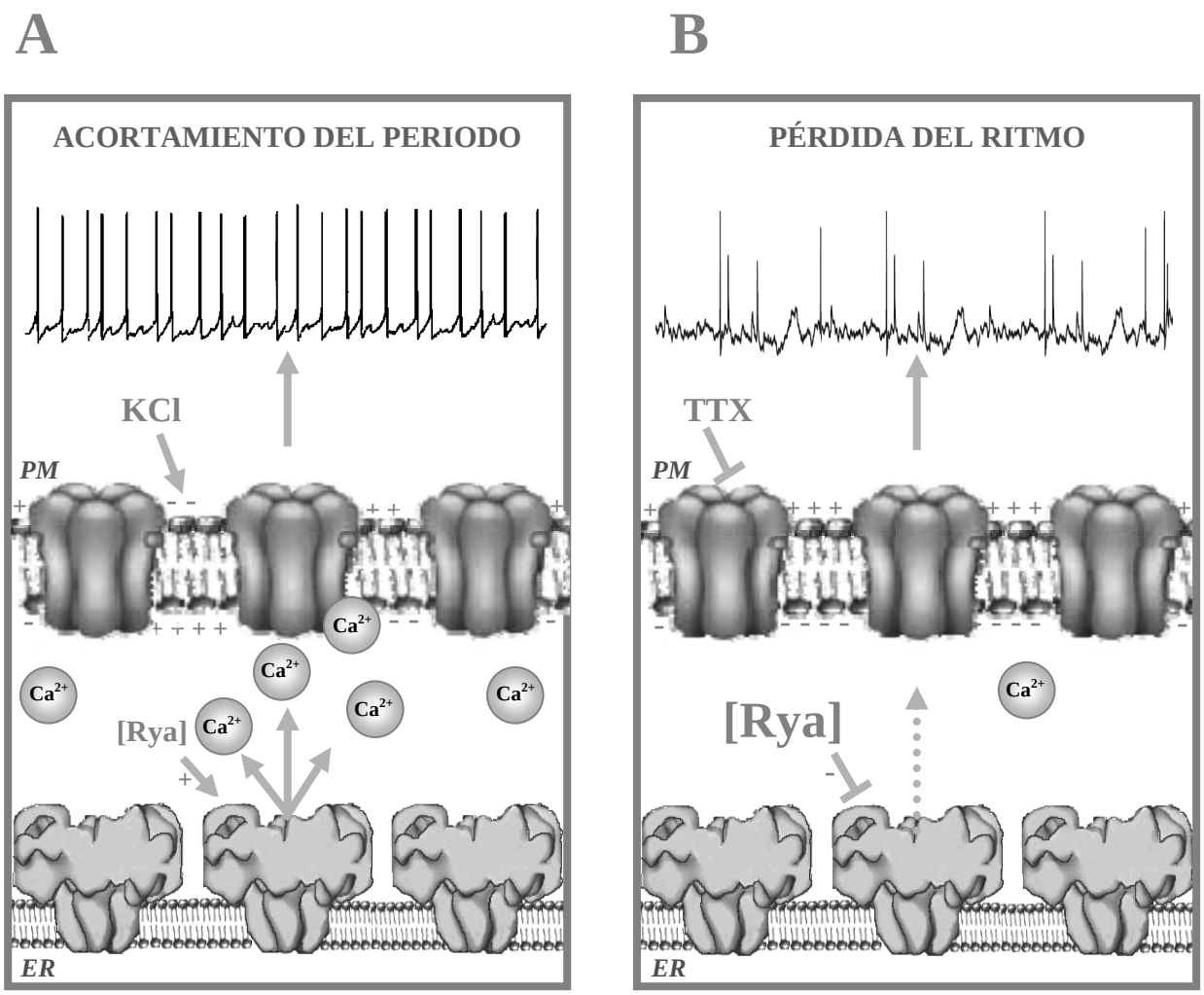

Figura 15. Representación esquemática de los eventos intracelulares, por los cuales la actividad de los RyR influye en las propiedades electrofisiológicas de las neuronas del NSQ y la expresión del ritmo circadiano conductual.
Panel A. Activación de los RyR localizados en las endomembranas del retículo endoplasmático (ER) por las concentraciones bajas de ryanodina (0.1 μM, [Rya]), seguido de un aumento en el Ca^{2+} citoplásmico. Las concentraciones elevadas de Ca^{2+} promueven un aumento en la excitabilidad eléctrica en la membrana plasmática (PM) por la modulación de diversos canales. El aumento en la actividad eléctrica se ve reflejado en un aumento en la frecuencia de disparo, y éste a su vez provoca un acortamiento del periodo en el ritmo circadiano de la conducta de ingesta de agua, alimento o locomoción. Efectos similares se observan por la aplicación de un estímulo despolarizante con KCl 20 mM.
Panel B. La inhibición de los RyR por las concentraciones altas de ryanodina (80-100 μM, [Rya]), bloquea la liberación de Ca^{2+} por los depósitos internos (ER). Las concentraciones bajas de Ca^{2+} provoca una disminución en la actividad eléctrica de las neuronas del NSQ, el cual se ve reflejada en una disminución en la frecuencia de disparo y la pérdida en el ritmo circadiano de la conducta de ingesta de agua, alimento o locomoción. Efectos similares se observan por la aplicación de un inhibidor de la actividad de los canales de sodio, tal como la TTX.

VIII. PERSPECTIVAS

Los resultados de esta tesis sugieren que la homeostasis de Ca^{2+} es un factor importante en la señalización intracelular del oscilador circadiano; consecuentemente, resulta necesario caracterizar las oscilaciones de Ca^{2+} en las células del NSQ, y determinar si estas oscilaciones son diferentes en la fase de luz a la fase de oscuridad. Aunque no se ha encontrado modulación circadiana por parte de los IP_3R en el cerebro de rata (Díaz-Muñoz et al, 1999), se ha demostrado en otros sistemas la coordinación entre los RyRs y los IP_3R (Morales-Tlalpan et al, 2005), por lo que es necesario determinar la contribución de los RyR y los receptores a inositol (1,4,5)-trifosfato (IP_3R) en la regulación circadiana de la concentración de Ca^{2+} citoplasmático. A continuación se describe los avances que se han obtenido del proyecto.

MOVILIZACIÓN CIRCADIANA DEL $[Ca^{2+}]i$ EN CULTIVO PRIMARIO DE CÉLULAS DEL NSQ

Hipótesis

Si la movilización de Ca^{2+} intracelular a través de los RYRs tiene modulación circadiana, entonces las manipulaciones farmacológicas que se realicen sobre los RYR, en las células de cultivo primario del NSQ, tendrán efectos diferentes en el día y en la noche sobre la movilización de Ca^{2+} intracelular en las células del NSQ.

Objetivos

General

Determinar la contribución temporal de los canales intracelulares de Ca^{2+} sensibles a ryanodina (RyR) sobre las oscilaciones de Ca^{2+} en cultivo primario de células del NSQ.

Particulares

1. Constatar la identidad de las células del cultivo primario, mediante anticuerpos fluorescentes de AVP y VIP.
2. Caracterizar las oscilaciones del Ca^{2+} intracelular mediante fluorescencia, en cultivo primario de células del NSQ.
3. Determinar si existe interacción entre los RyRs y los IP_3R que contribuya en la regulación circadiana del Ca^{2+} en neuronas del NSQ.
4. Medir las fluctuaciones espontáneas de Ca^{2+} intracelular a la mitad de la fase de luz y a la mitad de la fase de oscuridad en células del NSQ.
5. Analizar el efecto de la activación de los RYRs con ryanodina 0.1 μM, cADPR 5 μM, sobre la movilización de Ca^{2+} intracelular en células del NSQ.
6. Determinar la localización circadiana de los RyRs y su colocalización con los IP_3R en el cultivo de células del NSQ.

Materiales y Métodos

Para obtener el cultivo primario de células del NSQ se sacrificaron ratas Wistar (10-15 días de nacidas). Previo a la disección, los sujetos se mantuvieron en ciclos de luz-oscuridad 12:12 (encendido de la luz a las 6:00 con 400 lux), con la temperatura regulada a 22º ± 1ºC y con libre acceso al agua y alimento. En todos los casos la disección se realizó entre las ZT4 y ZT6 para evitar cambios de fase en los ritmos circadianos.

El procedimiento para obtener los cultivos primarios es una modificación de los protocolos descritos por van den pol et al, (1992); Ren y Millar (2003); Morales-Tlalpan et al, (2005). Los animales se sacrificaron por decapitación y rápidamente se les extrajo el cerebro, el cual se colocó en una solución fisiológica (aCSF) oxigenada. Para obtener los NSQs, cada cerebro se rebanó de forma coronal en bloques de 400 μm a nivel del quiasma óptico bajo condiciones asépticas. Las rebanadas se colocaron en la aCSF oxigenada y fueron examinadas en un microscopio estereoscópico para la extracción de los NSQs. Para la disociación del tejido se utilizó tripsina al 0.25%. Las células se mantuvieron en Medio Basal de Eagle (BME) y cada tercer día se cambió el medio a la misma hora para evitar cambios de fase. Todos los experimentos que se muestran en este trabajo se han obtenido de cultivos de entre 2 a 3 días. La aCSF contenía en mM: 150 de NaCl, 1 de KCl, 1 de $MgCl_2$, 1.8 de $CaCl_2$, 4 de glucosa, 10 de HEPES. El pH se ajustó a 7.4 con NaOH. Para el medio de cultivo se utilizó 50% de GIBCO Medio Basal Eagle, suplementado con: 25% BBS Hanks, 25% de suero de caballo, 5 mg/ml de glucosa, 1:100 de Glutamax, 2 g/l de NaH_2CO_3, 10 mM de Hepes, 1:100 de PSN GIBCO (Antibiotic mixture)

Para identificar el tipo celular que se estaba estudiando, se determinó por inmunofluoresencia neuronas positivas a AVP y el VIP. Para identificar las células que no correspondieran a ninguno de estos dos anticuerpos también se marcaron los núcleos con DAPI (excitación 358 nm / emisión 461 nm). Los anticuerpos anti-VIP y anti-VP se diluyeron a una concentración 1:1000. Para detectar el anticuerpo primario las células se incubaron con el anticuerpo secundario (Conejo anti-cabra IgG-Rojo Texas o conejo anti-cabra IgG (P-L) Fluoresceína-ICT-conjugado), en una dilución 1:1000. El amortiguador de fosfatos (PBS) estaba conformado en mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 2 K_2HPO_4, el pH de está solución se ajusto a 7.4 con HCl .

Para la medición de Ca^{2+} intracelular se siguió el protocolo descrito por Morales-Tlalpan et al (2005). Se utilizó el indicador Fluo4-AM mezclado con 20% de ácido plurónico y aCSF (para facilitar la captación del indicador).

Para determinar la localización de los receptores se utilizó diferentes marcadores fluorescentes. Los IP_3R y RyR se marcaron utilizando los fluoróforos FL-Heparina (excitación 488 nm/ emisión 530/560 nm) y BODIPY Tx-Red Ryanodina (excitación 568/ emisión 600/630) respectivamente. También se marcaron los núcleos con DAPI (excitación 358 nm / emisión 461 nm).

La medición de las fluctuaciones de Ca^{2+} y las inmunofluorescencias se obtuvieron utilizando un Microscopio Confocal Zeiss LSM 510 META (Laser Scanning Microscopy) invertido (Axiovert 200). Las observaciones se hicieron con los objetivos 25X (Plan-Apochromat N.A. 0.8), 63X (Plan-Apochromat N.A. 1.4) y 100X (α Plan-Fluor N.A. 1.45). La señal fluorescente se logró excitando al indicador a 506 nm y la emisión se colectó a 526 nm. La pérdida de fluorescencia se minimizó al reducir la potencia del láser de Argón al 5 %. La velocidad de captura de imágenes

osciló entre 1-2 imágenes por segundo. Se utilizaron los laser Argon (488 nM); DPSS (561 nM); HeNe (633 nM); Multifotónico coherent-XR entonable (705-980 nM). Después de su captura las imágenes se procesaron en el Software Zeiss LSM 510 META v4.2 y se analizaron en OriginPro 7.5. Para la evaluación de la intensidad de la fluorescencia, se seleccionaron diversas regiones en toda la célula.

Resultados preliminares

Para todos los experimentos se utilizaron cultivos de 2 a 3 días de cultivo. Primero se hicieron cultivos primarios de células para determinar el porcentaje de tipo celular positivas a AVP y VIP. Se cuantificó el total de células positivas a DAPI (figura 16), y se comparó con las células positivas a AVP o VIP. Se observaron 4 cubreobjetos de 12 mm, en los cuales en promedio el 48.59 % de las células observadas fueron positivas a AVP (figura 16A). De otros 4 cubreobjetos el 41.22 % de las células fueron positivas a VIP (figura 16B).

El protocolo que se siguió para el registro del $(Ca^{2+})_i$ libre fue el siguiente: registro basal, registro con tratamiento (ryanodina 100 μM ó cADPR 5 μM ADP ribosa cíclica). De las 10 células tratadas con ryanodina, 7 respondieron al tratamiento y en 3 no se observó efecto. De las 3 células tratadas con cADPR sólo en 2 se observó el efecto de la solución. Se mostrará el resultado del registro de las 9 células que respondieron a los tratamientos. Se analizaron diversos puntos en toda la célula y se observó que en promedio de las 9 células, el 35% de la célula entera responde a la ryanodina o al cADPR (Figura 17).

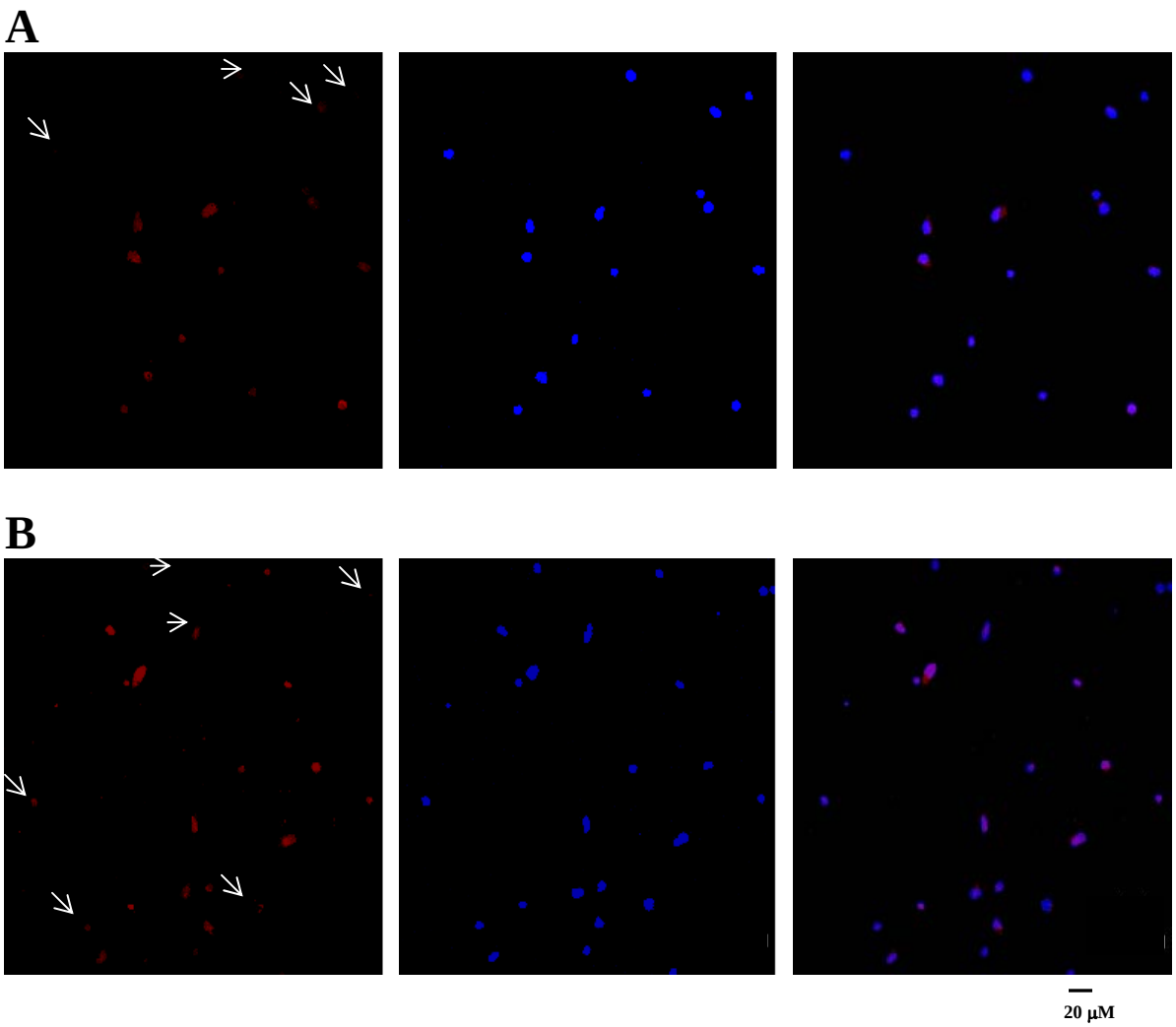

Figura 16. Inmunofluorescencia con VP y VIP en células de cultivo primario del NSQ. En color rojo se observan las células positivas a AVP (A) y VIP (B), en azul todas las células marcadas con DAPI (marcador de núcleos). Las flechas indican algunas de las células que no fueron positivas a VP o VIP. Imágenes tomadas con el objetivo de 25X.

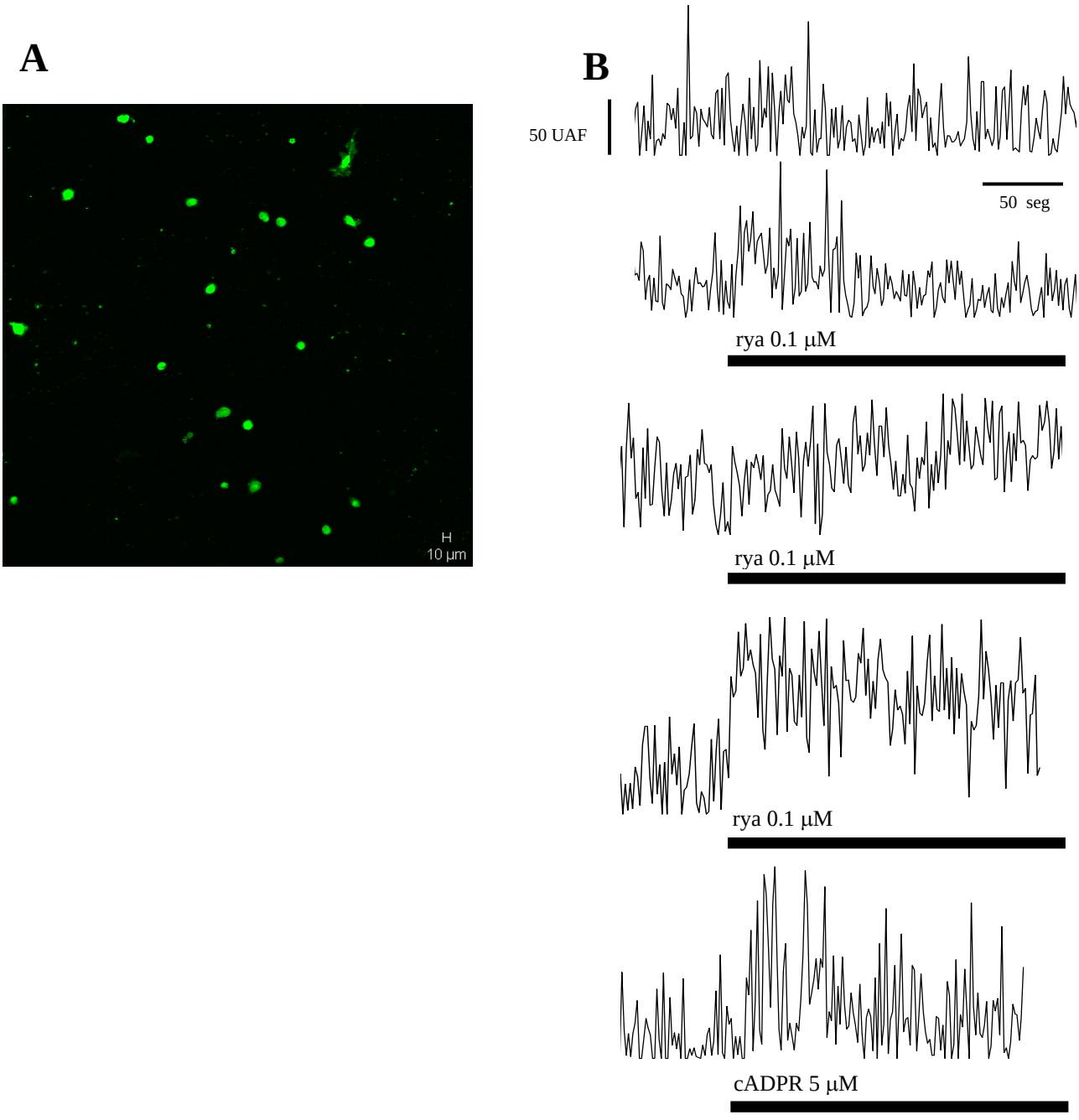

Figura 2. Medición del $(Ca^{2+})_i$ en células del NSQ. A) Cultivo primario de células del NSQ cargadas con Fluo-4, objetivo de 25x. B) Fluctuaciones del $(Ca^{2+})_i$ libre en diferentes regiones de una célula del NSQ, se administró ryanodina a 0.1 µM o cADPR 5 µM. UAF Unidades arbitrarias de fluorescencia.

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10

Neuronal activity in the suprachiasmatic nuclei: Cellular and molecular mechanisms

Raúl Aguilar-Roblero[1], Javier Alamilla[1], Clara Mercado[1]
Vania Carmona-Alcocer[1] and Christopher S. Colwell[2]
Departamento de Neurobiología Cellular y Molecular, Instituto de Neurobiología, UNAM, Querétaro, QRO., México

1. Abstract

Humans and other organisms exhibit daily rhythms in their behavior and physiology. In mammals, the part of the nervous system responsible for most circadian behavior can be localized to a bilaterally paired structure in the hypothalamus known as the suprachiasmatic nucleus (SCN). Many neurons in the SCN are intrinsic oscillators that continue to generate near 24-hour rhythms in electrical activity, secretion, and gene expression when isolated from the rest of the

Correspondence/Reprint request: Dr. Raúl Aguilar-Roblero, Apdo., Postal 70-253, México D.F. 04510 México
E-mail: raguilar@ifc.unam.mx

organism. Individual SCN neurons contain a molecular feedback loop that drives these rhythms. However, there is also evidence suggesting that membrane excitability and/or synaptic transmission may be required for generation of the molecular oscillations. Thus clarifying the ionic mechanisms responsible for the generation of rhythms in electrical activity in SCN neurons is an important step to understanding the generation and output of circadian oscillations.

The present chapter aims to provide an integrated perspective on our current knowledge on the cellular physiology involved in the modulation of firing rate in the neurons of the mammalian circadian clock. To date, a number of ionic membrane channels have been studied in SCN neurons. Channels of particular interest include a persistent sodium current, the fast delayed rectifier potassium currents, and those involved in the afterhyperpolarization potential which is characteristic of regular firing neurons. The firing rate in SCN neurons is also modulated by intracellular calcium mobilized through ryanodine-receptor intracellular calcium channel and voltage-sensitive currents. In the present chapter, we will summarize our current knowledge on the role of the SCN in the generation and regulation of circadian rhythms. In addition, we will review SCN cell physiology, with emphasis on the mechanisms involved in the generation and modulation of the spontaneous action potential firing rate, which characterize SCN neuronal activity.

2. Evidence that the SCN is a circadian clock

At many levels, SCN is the most comprehensively characterized circadian clock in mammals and other vertebrates. The first evidence suggesting the role of the SCN in the generation of circadian rhythms came from the description of the retino-hypothalamic tract (RHT) and the finding of a disruption of behavioral and physiological circadian rhythms after SCN complete ablation [1,2]. In the organism, it is possible to demonstrate that SCN neurons exhibit rhythms in metabolism [3] and electrical [4] *in vivo*. These rhythms in electrical activity continue when the SCN are removed from the organism and maintained *in vitro* [5, 6, 7]. Even single SCN neurons maintained in culture are capable of maintaining rhythms in electrical activity [8]. Transplantation of fetal SCN into adult SCN-lesioned animals restores circadian rhythms [9, 10, 11, 12, 13], and the restored rhythm has the period of the donor tissue [14, 15]. This body of evidence, briefly described here, provides a compelling argument that the SCN is the site of the biological clock in mammals [16].

3. SCN circuitry

The SCN are a two neuronal clusters located in the anterior hypothalamus, ventrolateral to the optic recess of the third ventricle and dorsal to the optic

chiasm. Each cluster comprises from 8,000 to 10,000 neurons. These neurons do not form a homogenous cell population but instead are organized into distinct regions whose functions are still not understood. Anatomical evidence supports the broad separation of the SCN into distinct ventrolateral (core) and dorsomedial (shell) subdivisions [17]. Neurons in the core are innervated by visual inputs and, in many cases, express the neuropeptide vasoactive intestinal peptide (VIP) or gastrin releasing peptide (GRP). These retino-recipient cells must then communicate light information to the rest of the SCN. The cells in the dorsal SCN appear to exhibit more robust rhythmicity in gene expression compared to the ventral retino-recipient cells [18, 19]. *In vivo* electrophysiological studies have even suggested that the retino-recipient neurons in the SCN may not express rhythms in action potential frequency [20, 21]. Similarly, in the hamster, Silver and colleagues have identified a calbindin positive cell population [22] that is photically regulated but not electrically rhythmic [23]. This distinct cell population may function to "gate" the photic response of the SCN. Yet other ventral SCN neurons must be rhythmic as the bisection of the SCN slice revealed a robust neural activity rhythm that could be recorded from the isolated ventral region [24].

4. SCN electrophysiology

Most SCN neurons show a clear circadian rhythm in spontaneous firing rate, which peaks in the middle of the day, both *in vivo* and *in vitro*. In anaesthetized animals, rhythms in neuronal activity can be recorded, but the absolute firing rate is affected by the use of anesthesia. In freely moving animals, spontaneous neuronal activity can be recorded from small groups of neurons (multiple unit activity or MUA). The size and impedance of the electrode determines the number of neurons recorded and thus the firing rate reported varies depending on this and the duration of the bin used to integrate the neuronal activity. In hypothalamic slices kept *in vitro*, single neurons are easily recorded and the firing rate varies from about 0.5 Hz to 12 Hz (Figure 1). From the persistence of spontaneous neuronal firing in all these conditions, SCN neurons must have a powerful self-sustained firing mechanism. Long-term recording from dissociated SCN neurons cultured on a multi-electrode array clearly demonstrated that circadian modulation of the spontaneous firing rate is inherent to most SCN neurons [8].

Three types of firing pattern were described from *in vitro* extracellular recordings in SCN neurons [25] (Figure 2): Type I neurons (or rhythmic) shows regular firing at high frequencies, interspike intervals from these neurons has a normal and narrow distribution characteristic from pacemaker activity; type II neuronal pattern (or arrhythmic) is irregular and ranges from low to middle frequency, interspike interval shows a skewed distribution, which

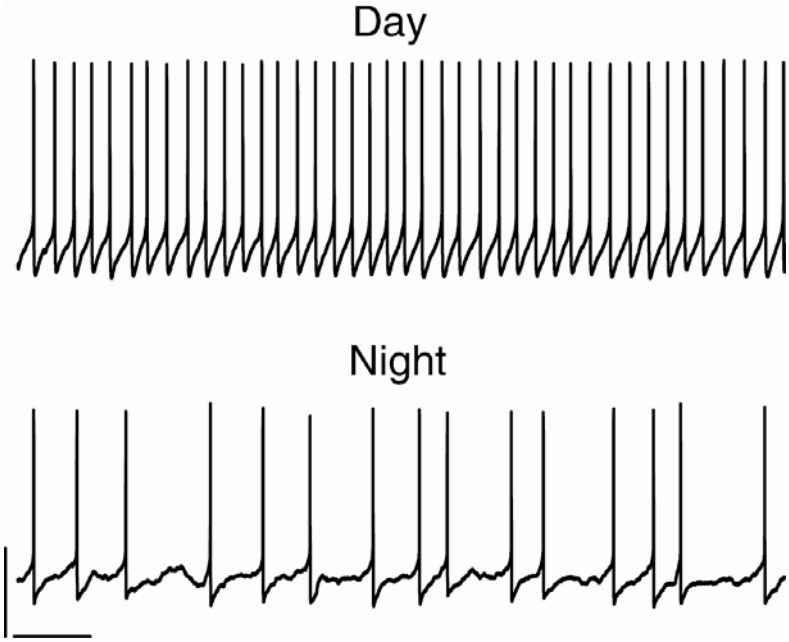

Figure 1. SCN neurons recorded *in vitro* at two times of the circadian cycle. Calibration bars 1 sec, 30 mV.

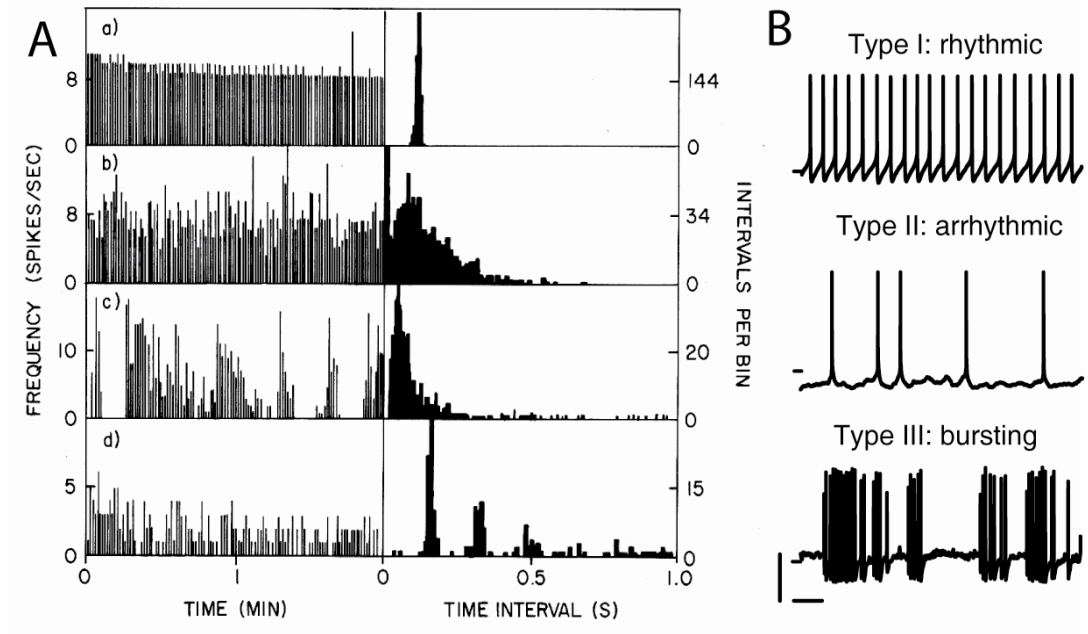

Figure 2. Firing patterns in SCN neurons. A, Firing frequency (left) and interspike (right) histograms from 4 extracellular recorded neurons illustrating regular (a), irregular (b) and bursting (c) patterns according to the frequency histogram; the harmonic pattern (d) is only recognizable with the interspike histogram. B, SCN firing patterns from neurons recorded in perforated patch-whole cell configuration. Calibration bars 500 ms, 30 mV.

has been fitted to Poisson models; type III neurons (or bursting) show middle rate firing bursts interrupted by silent intervals, interspike interval distribution is best fitted by log normal or Poisson models. We have described a fourth type of firing pattern only evident in the interspike interval distribution, this pattern is characterized by three to four decreasing harmonic inserted within a Poisson distribution, these neurons show irregular or bursting patterns [26, 27, 21].

Neurons in the SCN are among the smallest in the mammalian central nervous system and, consequently, the electrical properties from these neurons have not been extensively studied. In particular, there were only a few studies using conventional intracellular recording techniques from SCN neurons *in vitro* [28, 6, 29, 30]. More recently, the number of reports increased in direct relation to the technical advances to maintain the SCN *in vitro* (acute slices, dissociated neurons, slice cultures), and improvements in the recording procedures for whole cell and perforated patch clamp [31]. A summary of the published values describing the membrane properties of these neurons is provided in Table 1.

Table 1. Electrical properties from SCN neuron from selected reports. WC, whole cell recording, PP, perforated patch recording; SFR, spike firing frequency (in Hertz); Vm, membrane potential; Rm, membrane resistance; SPK_A, spike amplitude (in mV); Trh, spike threshold; AHP_A, amplitude of the after hyperpolarized potential.

Reference	Mode	Time (h)	SFR (Hz)	Vm (mV)	Rm (GΩ)	SPK_A (mV)	Trh (mV)	AHP_A (mV)
32	WC	-	-	-50±2	1.6±.0.3	-	-	-
25	WC	-	3.0±0.3	-56±0.04	1.3±0.5	74.7±1.2	-40.6±0.4	21.3±0.5
31	WC-long	4-10	3.1±0.4	-56.1±1.1	1.3±0.07	-	-	-
		12-24	3.1±0.5	-56.4±0.5	1.3±0.06	-	-	-
	WC-short	4-10	5.3 ±0.6	-54.3±1.1	1.8±0.2	-	-	-
		12-24	2.6±0.6	-56.2±0.8	1.0±0.1	-	-	-
39	WC	-	-	-54.9±0.7	1.2±0.05	74.0±2.0	-38.0±1.0	-
42	PP	4-8	8.3±0.6	-43±1	2.3±0.1	-	-	-
		13-20	2.5±0.5	-55±1	1.3±0.2	-	-	-
36	WC	-	< 3	-41.3±1.9	-	82.8±1.7	-53.8±1.6	13±1.5
			> 6	-44.0±2.3	-	77.7±3.6	-58.1±2	14±2.2
37		day	2.6±0.3	-60.0±1.0	1.2±0.1	-	-	-
35	PP	4-8	4.3±0.6	-56.0±2.0	-	-	-	-
		16-20	2.0±0.7	-61.0±3.0	-	-	-	-
40	WC	5-7	8.2±3.8 d	-67.0±5.0	-	93±10	-55±3.9	26±6.1
90	PP	Rhythmic	5.0±0.6	-50.1±5.0	-	70±10	-39.0±1	14±2.3
		Arrhythmic	2.02±0.2	-45.4±1.3	-	65±12	-40.6±2	6±2.1

Because SCN neurons are spontaneously active, the membrane potential during the interspike interval is not steady but instead is continually changing. Immediately after an action potential is generated, the membrane potential is at its most hyperpolarized. After which point, membrane depolarization steadily builds until reaching the firing threshold and a new action potential occurs. Both the interspike interval and the action potential itself involves a particular set of cell membrane channels, which control specific membrane currents in a selective and timed manner. Thus, the depolarizing shift in the membrane potential during the interspike interval results from a net outward current, which results from the balance of potassium (K^+) outward current and sodium (Na^+) and calcium (Ca^{2+}) inward currents (Figure 3). The identified outward K^+ currents involved in the interspike interval in SCN neurons are the fast delayed rectifier (fDR) [32], the transient outward current (I_A) [33], and at least three Ca^{2+} regulated currents ($K_{(Ca)}$) related to the after-hyperpolarizing potential

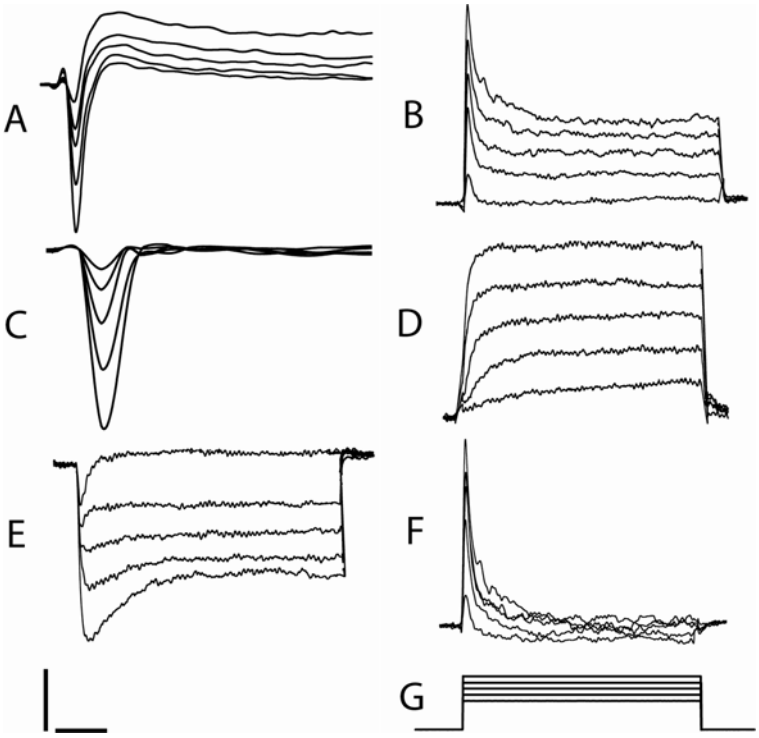

Figure 3. Ionic currents from SCN neurons; total and inward currents are shown on the left, outward currents are shown on the right; for each panel the values of the calibration bars are indicated in parenthesis. A, Global currents (2.5 ms, 500 pA). B, Global potassium currents (50 ms, 200 pA). C, TTX-sensitive sodium currents (1.3 ms, 400 pA). D, Persistent potassium current (50 ms, 200 pA). E, Global calcium currents (50 ms, 100 pA). F, Transient outward current -I_A (50 ms, 200 pA). G, Voltage steps protocol, the holding potential was at -70 mV, 250 ms square pulses at 10 mV steps from -30 to +30 mV. Specific currents were blocked by the use of TTX (Na^+), cadmium, nickel and barium (Ca^{2+}) and TEA (K^+).

(AHP): the big conductance current (BK) iberotoxin-sensitive [34], the small conductance current (SK) apamine-sensitive [35], an iberotoxin- and apamine-insensitive $K_{(Ca)}$ current [36], and a barium-sensitive K^+ current ($K_{(Ba)}$) [37]. On the other hand, the inward currents identified in SCN neurons are the mixed cationic inward current which activates at hyperpolarized membrane values (I_h) [38]; two different persistent Na^+ currents, one subthreshold TTX-sensitive current [39] and a background tetrodotoxin (TTX)-insensitive Na^+ current [40]; and several voltage dependent Ca^{2+} currents [41] including L- [42], N-, P/Q- and R- types [36].

Our current understanding of dynamics of ionic currents during the firing cycle in SCN neurons is yet incomplete. From an oversimplified view, it starts with the fast rising depolarizing phase of the action potential (Figure 4). As with all electrically active neurons, when the membrane potential reaches the threshold necessary to open a critical number of voltage dependent Na^+ channels necessary to induce a self-regenerating process, which is only limited by the inactivation of the channel. As the Na^+ enters the cell, the membrane depolarize and L-type Ca^{2+} channels open and Ca^{2+} enters contributing to further membrane depolarization. Simultaneously, different K^+ channels open, including the slow and fast K^+ rectifying channels. The outward K^+ current eventually counterbalance the inward Na^+ and Ca^{2+} currents and the depolarization ends close to the Nearst´s equilibrium potential of Na^+ (E_{Na}), and

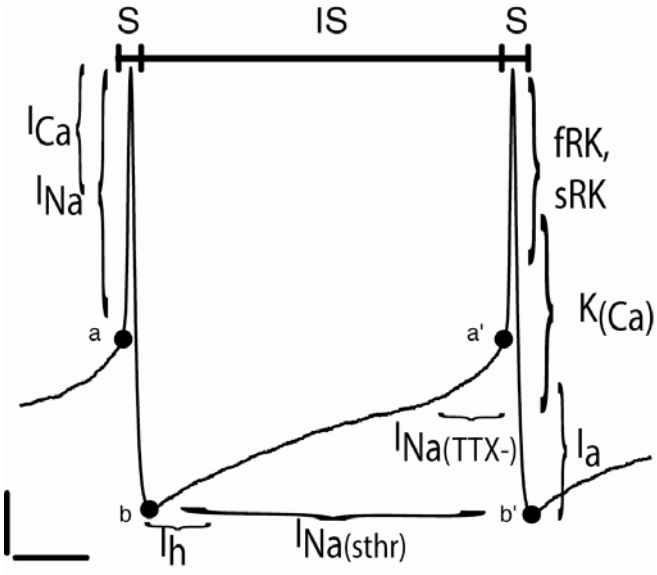

Figure 4. Characteristic action potentials from a rhythmic SCN neuron. The filled circles indicate the threshold potential (a, a') and the maximal after hyperpolarization (b, b'). The spikes (S) and interspike (IS) intervals are indicated. The domain of each ionic conductance during the action potential is indicated by the brackets. See text for further details. Calibration bars 20 ms and 10 mV.

then the membrane goes into the fast repolarization phase of the action potential. At this time $K_{(Ca)}$ are activated, and the outward K^+ current continues further polarizing the membrane to even more negative values, the so-called after hyperpolarization potential. Such hyperpolarization is important to the drift in the membrane potential during the interspike interval, since enables the activation of the transient outward current (I_A) and the mixed cationic inward current (I_H). Thus the membrane potential slowly depolarize towards the threshold potential activating the subthreshold TTX-sensitive current ($I_{Na\ (sthr)}$) and the background TTX-insensitive current ($I_{Na(TTX-)}$) , which seem to be mainly responsible to eventually reach the spike threshold potential. The role of voltage dependent Ca^{2+} currents in this process is not yet clear.

The circadian modulation of SCN neuronal firing rate is essential for the expression of circadian rhythms. Neuronal firing rate encodes information used by the nervous system to control different effectors systems, that is to say that this is the language of the brain used to communicate with the rest of the body. Therefore, the time measure by the circadian clock must be eventually translated into neuronal firing rate or at least a change in neuronal excitability, in order to be a meaningful signal to the rest of the organism. To address the circadian modulation of SCN neuronal firing is useful to first analyze the molecular mechanisms of the circadian clock in SCN cells.

5. Clock genes and the molecular circadian oscillator

Previous studies have identified the basic molecular elements are responsible for the generation of circadian oscillations in SCN neurons [43, 44]. Such molecular core include the *Period* genes Per_1 and Per_2; the *Cryptochrome* genes Cry_1 and $Cry_{2;}$; the transcription factor genes *Clock* and *Bmal*1; and the repressor factors *Rev-Erb-*α, Dec_1 and Dec_2. These elements interact through transcription-translation feedback loops (Figure 5). Present evidence indicates that CLOCK and BMAL-1 proteins form a heterodimer which act as a transcriptional factor binding to the promoter sequence E box [45, 46] to induce the transcription of *Per1, Per2* [47]*, Cry1, Cry2,*[48] Dec_1, *Dec*2 [49,50] *and Reb-erb*α [51] genes. Although the exact sequence of the following events is still under study, after its transduction PER and CRY protein are phosphorylated by casein kinases δ and ξ, form heterodimers and are translocated to the nucleus [52, 53]. Once in the nucleus, PER/CRY dimmers bind to the CLOCK/BMAL-1 complex displacing it from the E-box and thus inhibit its own transcription [54, 55] as well as those of *Reb-erb-*α [56] and *Dec* [49] genes. It is now clear that when phosphorylated PER proteins are not degraded as fast as the dephosphorylated one, and this affect the rate of dimerization with CRY proteins and its translocation to the nucleus, which has been suggested to determine the stability and period of the oscillation within the circadian range [57, 58].

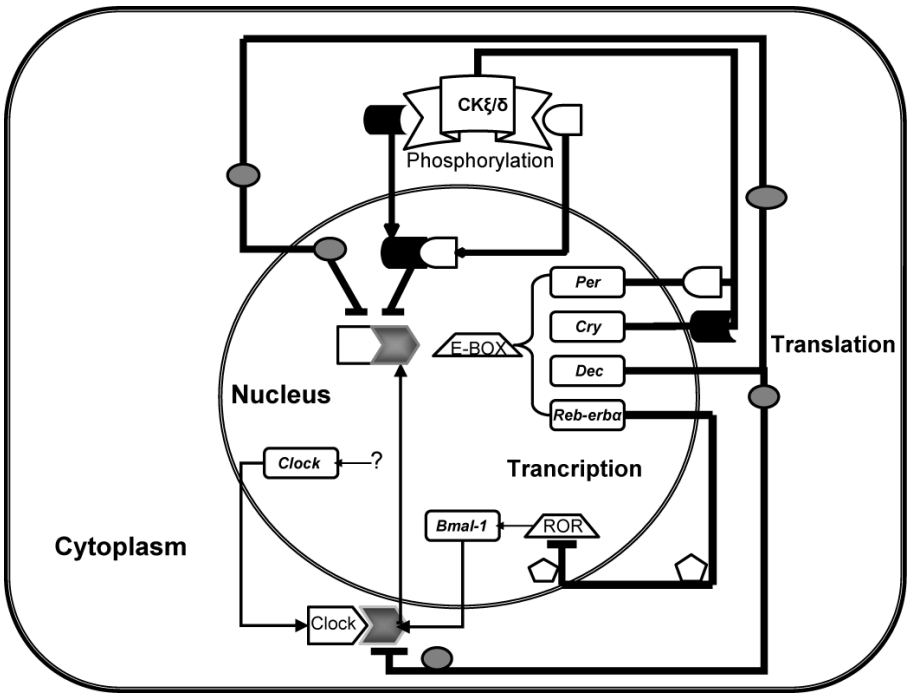

Figure 5. Transcription/translation feedback loops. Pointed arrows represent transcription promoters, T shaped connectors represent repressor factors. PER ( ); CRY ( ); DEC ( ); REV-ERBα ( ); BMAL-1 ( ); CLOCK ( ).

The negative feedback loop is necessary to sustain the circadian oscillation, as indicated by the disruption of circadian rhythms in mutants lacking any of the *Per, Cry, or Clock genes* [59, 60, 61]. Other elements like *Reb-erbα* and the *Dec* genes seem to contribute to stabilize the oscillation but the basic oscillation will continue in their absence. Transcription of *Reb-erb α* is induced by CLOCK/BMAL1 binding to the E Box in the regulatory sequence of the gene [51], in turn REB-ERB α protein inhibits the transcription of *Bmal1* either by acting as a suppressor or by displacing the RORα or RORβ promoters from the RORE sequence that regulates the *Bmal*1 gene. When PER/CRY remove CLOCK/ BMAL1 from the Ebox enhancer, *Reb-erbα* transcription decrease will eventually allow the increase in *Bmal1* transcription, which results in a more stable oscillation. Thus REB-ERB α may function as a link between the positive and negative limbs involved in the feedback loops of *Bmal*1/*Clock* and *Per/Cry* genes [62]. On the other hand, Dec_1 and Dec_2 genes are induced by CLOCK, BMAL1 in a similar way as *Per* and *Cry* genes, and PER and CRY suppress *Dec* genes [50]. In turn DEC_1 and DEC_2 proteins suppress their own expression either by binding to BMAL1 or by directly binding to the E-Box promoter in both cases displacing CLOCK/BMAL1 from the E-Box [63. 64]. DEC proteins also suppress transcription of *Per* and *Cry* genes [49].

6. Output pathways from the molecular oscillator

As previously mentioned, the output pathways from the molecular oscillator must regulate neuronal membrane excitability. At present time, we do not understand how the molecular oscillator is linked to the membrane and metabolic processes within SCN neurons. One possibility is that the transcription of the genes coding for membrane channels may themselves be modulated on a circadian time scale. These genes could be under regulation from the core oscillator proteins acting directly on E-box, PAS, D-box or bHLH elements present in the regulatory sequences. The gene coding for the neuropeptide vasopressin gene provides one particularly well studied example. The expression of this gene exhibits a circadian oscillation driven, in part, by E-box enhancers in its promoter region [65]. The use of microarrays to analyze mRNA indicates that many genes exhibit circadian expression and that the identity of the genes regulated by the circadian system varies in a tissue-specific manner. In the nervous system, the identified genes are related to synthesis of neurotransmitter, neuroactive peptides, ionic channels and receptors, as well to regulation of the redox state, the energy balance and neurosecretion (table 2) [66]. In *Drosophila*, circadian rhythms in mRNA coding for a regulatory protein associated with Ca^{2+}-sensitive K^+ channels have been described [67, 68]. Furthermore, in mammalian cardiac tissue, diurnal variation in the expression of genes coding for two K^+ channels (Kv1.5 and Kv4.2) has been described [69]. Therefore, although the half-life of these membrane proteins is not well described, it is quite possible that transcriptional rhythms may play a role in driving the rhythms in electrical activity.

That being said, post-translational modifications of the channels are perhaps the most likely explanation for the circadian variation in the electrical activity of SCN neurons. Outside of the SCN, numerous studies have demonstrated the importance of kinase/phosphatase activity in mediating short-term changes in channel function that alter electrical excitability [70]. In chick photoreceptors, circadian oscillations in cone cGMP-gated channels have been well described [71]. Within the SCN, there is evidence for a daily rhythm in the transcription of AC1 [72]. In addition, diurnal variations for levels of cAMP and PKA activity have been shown with peaks during the late night/early day [73]. Many ion channels possess phosphorylation sites that can be phosphorylated by PKA and other kinases. Thus, circadian patterns of phosphorylation are likely to drive circadian rhythms in the membrane properties of SCN neurons.

Whether the mechanism driving the membrane rhythms involves transcription or post-translational regulation, the eventual targets can be conceptually divided into two independent sets of ionic mechanisms [74]. The first set of currents would be responsible for the regulation of the interspike

Table 2. Selected examples from genes showing circadian transcription (75, 76).

Gene	Description	Acrophase
Cytoskeleton Control		
K-alpha-1	Tubulin, alpha, ubiquitous	12:00
Tubb 5	Tubulin, beta 5	21:49
Pin	Dynein, cytoplasmic, Light polypeptide	12:00
Tgoln2	Trans-golgi network protein 2	07:54
Metabolism regulation		
Ndufv1	NADH dehydrogenase flavoprotein 1	11:00
Cox6a1	Cytochrome c oxidase, subunit VI a, polypeptide 1	21:16
Ar6Ip2	ADP-ribosylation-like factor 6 interacting protein 2	14:57
G6pt1	Gucose-6-phosphatase, transport protein 1	15:03
Ca^{++} Regulation		
Cacng6	Calcium channel, voltage-dependent, gamma subunit 6	19:10
Chp-pending	Calcium binding protein P22	18:30
Calm1	Calmodulin 1	17:40
Calm2	Calmodulin 2	05:40
G Protein Regulation		
Gpr27	G protein-coupled receptor 27	18:52
Dexras1	RAS, dexamethasoneinduced 1	15:00
Csnk1a1	Casein kinase 1, alpha 1	06:19
Calb2	Calbindin 2	20:57
Receptor Regulation		
Chrnb2	Cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)	09:44
Gad1	Glutamic acid decarboxylase 1	15:47
Cck	Cholecystokinin	15:56
Gabarap11	GABA(A) receptorassociated protein like 1	05:10
Via(avpr1a)	Aginine vasopressin receptor 1A	22:20
Oprs1	Opioid receptor, sigma 1	01:27
Penk1	Preproenkephalin 1	01:51

(basal) membrane potential relative to the spike threshold, which involves tonic, voltage-independent "leak" channels. The second set of currents would be responsible for the translation of the membrane potential into action potentials, which involves fast gated, voltage-dependent channels.

SCN neurons recorded during the day show a depolarization of about 10 mV in basal membrane potential and a decrease in the overall membrane conductance with respect to the night [77, 78] due to a decrease in outward K^+ currents [79]. A daily decrease in membrane K^+ conductance would drive the membrane away from the K^+ equilibrium potential and closer to the threshold to spike generation. Such a mechanism would move the cell to a more depolarized state and contribute to the higher firing rate during the day [74]. The Na^+ persistence currents are also important to play a role in the excitatory drive during the interspike interval to the spike threshold potential [40], but so far no evidence of circadian modulation of these currents has been provided. During the day, L-type Ca^{2+} channels are involved in oscillations of the basal membrane potential that drive the membrane potential towards the spike threshold [42]. These currents do not appear to be required for the expression of rhythmicity in physiological conditions [72].

Using whole–cell patch electrophysiological techniques, we provided the first description of a circadian modulation of an fDR K^+ current in SCN neurons [32]. These currents activate only at depolarized membrane potentials, have rapid activation, and deactivation kinetics. In the SCN, the fDR current begins to activate at –20 mV and most neurons exhibit half-activation voltages around 6 mV. Once activated, the fast kinetics of this current allows neurons to quickly repolarize after generation of APs but do not alter the spike threshold or action potential height [80]. Previous studies have found these currents in cell populations with high firing rates in sensory [81] and motor circuits [82]. The presence of this current should allow SCN neurons to discharge at higher rates during the day without adaptation. The fDR current is produced by channels of the Kv3 family and we found that at least two members of this family are expressed in the SCN (Kv3.1 and 3.2). The fDR current is sensitive to both 4-aminopyridine (4-AP, 0.5 mM) and TEA (1 mM), giving us pharmacological tools to investigate the contribution of this current to the frequency of action potential generation in the SCN. Using perforated patch recording techniques, we found that acutely blocking the fDR with 0.5 mM 4-AP or 1 mM TEA significantly decreases the firing rate of SCN neurons during the day so that the day/night difference in spontaneous activity was eliminated. This is a characteristic feature of the fDR because the blockade of most K^+ currents would be expected to increase the firing rate. Finally, using extracellular recordings of rhythms in multi–unit activity, we found that longer-term application of 4-AP (0.5 mM) prevented expression of diurnal rhythms in electrical activity recorded from SCN tissue. We conclude that the

fDR K^+ current is necessary for the circadian modulation of electrical activity in SCN neurons, and represents an important part of the ionic basis for the generation of rhythmic output.

Other K^+ channel under circadian regulation is the large conductance Ca^{2+}-activated K^+ channel (BK). Such channel is expressed at higher levels during the night than in the day. In Kcnma1$^{-/-}$ knock out mice which lack BK channels, show disrupted behavioral circadian rhythms and a high SCN neuronal firing rate during the night, close or up to the spike rate characteristic from daytime recorded SCN neurons; in spite of circadian expression of clock genes such as *Bmal-1* and *mPer2* [83,34]. Previous evidence clearly indicates that circadian modulation of BK is also involved in the circadian modulation of SCN neuronal excitability. Other Ca^{2+}-activated K^+ channels present in neurons from the SCN includes SK [35] and apamine/iblerotoxin-insensitive channels [36], these channels are involved in the generation of the after-hyperpolarization potential and contribute to the rhythmic firing pattern from the SCN, although their circadian modulation has not been yet demonstrated.

Free cytoplasmic Ca^{2+} rhythm mobilized from intracellular Ca^{2+} storages or driven by voltage-sensitive calcium channels may be one of the first transmission elements linking the molecular oscillator to the circadian modulation of SCN neurons physiology, and particularly the modulation of the firing rate. Intracellular Ca^{2+} homeostasis in the SCN is under circadian control [84, 85, 86] and its manipulation affects expression of overt circadian rhythms [87, 88, 89]. In organotypic cultures, SCN circadian rhythms in cytoplasmic Ca^{2+} levels and multiple neurons firing rate can be dissociated. Thus, blockade of the electrical by TTX did not affect the Ca^{2+} rhythm [85], while both rhythms

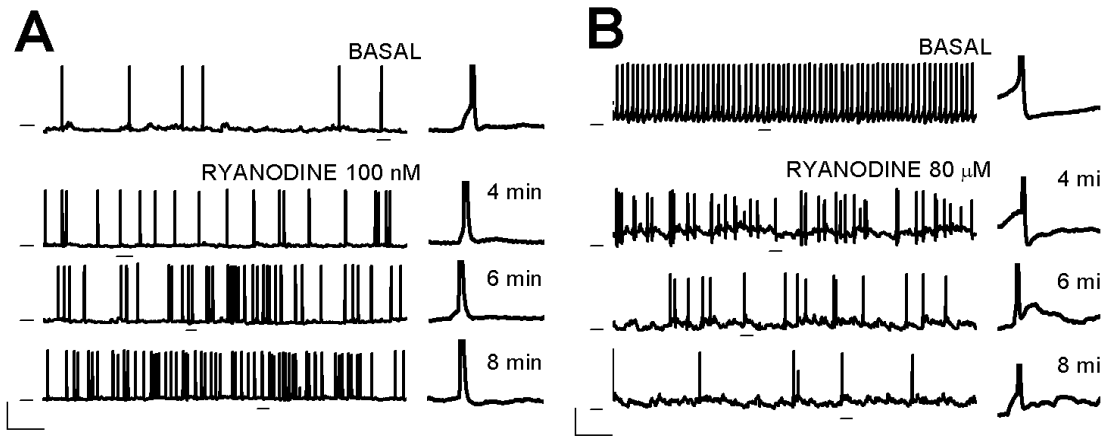

Figure 6. Effect of different doses of ryanodine on the spontaneous firing frequency from SCN neurons recorded in hypothalamic slices. A) opening and B) closing the ryanodine-sensitive intracellular Ca^{2+} channel. On each panel calibration bars indicate 30 mV and 1000 ms (left) and 10 mV and 60 ms (right).

were abolished by closing the RyR-Ca^{2+} channel with high doses of ryanodine. In contrast, inhibition of voltage-gated Ca^{2+} channels by nifedipine did not affected any of the rhythms [86].

In SCN neurons recorded from brain slices by perforated patch [90], it has been shown that pharmacological opening of the RyR-Ca^{2+} channel, with either caffeine or a low dose of ryanodione, increases its firing frequency, whereas the closure of the channel by dantrolene or a high dose of ryanodine decreased firing rate (Figure 6). The modulation of the RyR-Ca^{2+} channel seems to be in the output pathway from the oscillator to the neuron excitability, since the pharmacological gating overrides the circadian control of the firing rate as indicated by similar results obtained at either midday or midnight. These effects are not mediated by synaptic transmission, since blockade of $GABA_A$, AMPA and NMDA receptors did not prevent the excitatory or inhibitory effects induced by either dose of ryanodine on SCN firing. Furthermore, intracellular Ca^{2+} imaging analysis from cultured SCN neurons has confirmed that the ryanodine doses used to open and close the RyR-Ca^{2+} channel actually increase or decrease the cytoplasmic concentration of free Ca^{2+} (Figure, 7; unpublished observations).

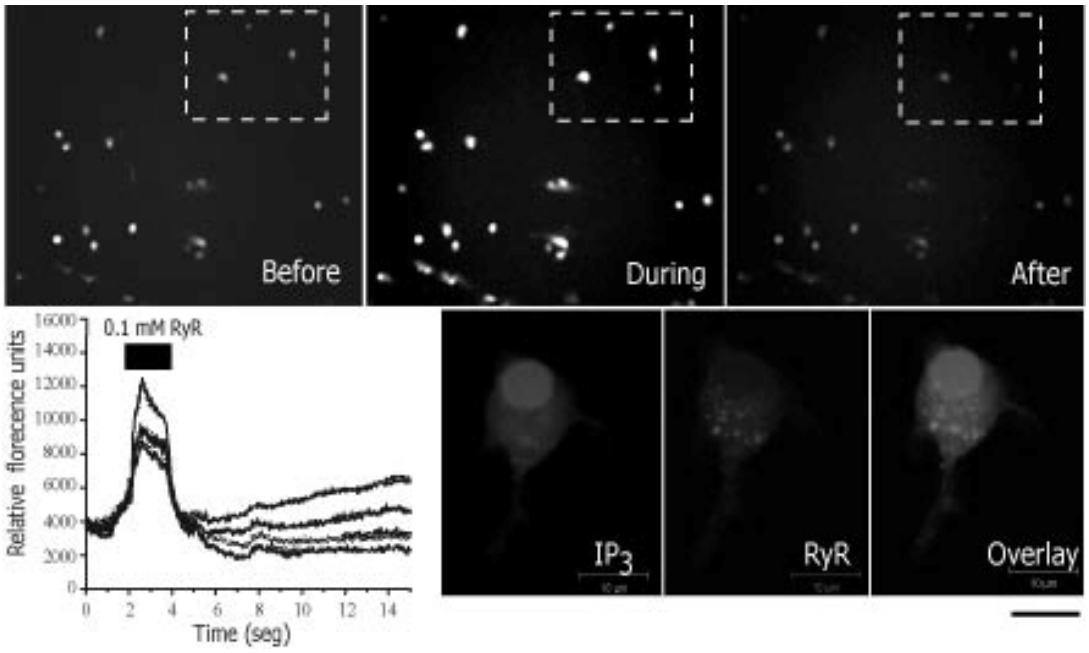

Figure 7. Top pannels, Ca^{2+} release from ryanodine-sensitive intracellular Ca^{2+} channels induced by a brief pulse of 100 nM ryanodine applied to dissociated cultured SCN neurons. Bottom left, quantification of the fluorescence from the neurons from the inset. Bottom right, immunofluorescence from IP_3- and ryanodine-sensitive (RyR) intracellular Ca^{2+} channels in the same SCN neuron. (Courtesy from Mauricio Díaz-Muñoz).

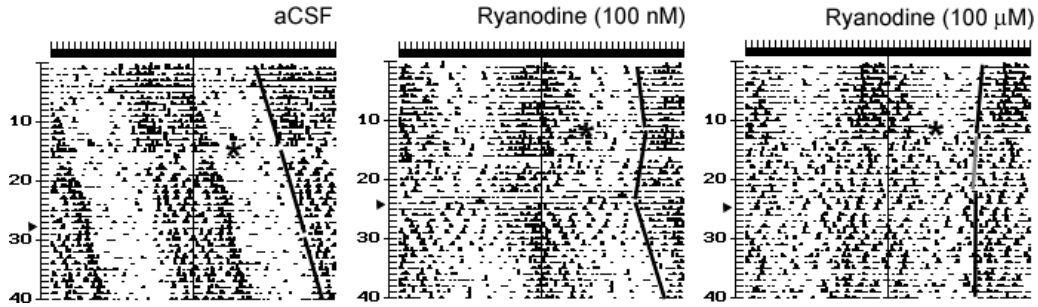

Figure 8. Double plotted actograms of general activity at 15 min bins from rats receiving either: aCSF (A); Ryanodine 100 nM or Ryanodine 100 μM. The asterisk indicates the time of osmotic minipump implantation. The arrowhead indicates the expected day of drug withdrawal. The black lines on the left part of each double plot indicate the time of activity onset at circadian time 12 (CT 12).

Chronic administration of different doses of ryanodine directly into the SCN has an effect on behavioral circadian rhythms from rats under continuous recording ((unpublished). Activation of RyRs by ryanodine 100 nM leads to shortening of endogenous period, whereas inhibition of the RyRs by ryanodine 100 μM disrupts circadian rhythmicity (Figure 8). Since the rhythm expression returned to basal values of period and phase after the treatment, the changes in overt rhythm induced by both doses of ryanodine involve the output rather than the clock mechanism. Overall, these data are also consistent with the hypothesis that the cytoplasmic Ca^{2+} rhythm may be one of the key elements linking the molecular oscillator to the circadian modulation of the firing rate in SCN neurons.

7. Conclusions

The ability to generate intrinsic circadian rhythms in electrical activity appears to be a unique property of mammalian SCN neurons and one essential to the function of the circadian timing system. Recently some progress has been made in understanding the currents and intracellular signaling pathways responsible for the circadian modulation of electrical activity in SCN neurons. This progress represents an important step toward the goal of understanding the ionic basis for the generation of circadian output. Future studies will need to provide a physiological explanation for the rhythm in spontaneous neural activity in SCN neurons, understand how intrinsic membrane currents vary between cell populations within the SCN, and understand how these membrane currents are regulated by the core molecular mechanisms that drive circadian oscillations within individual neurons. In order to address these issues, the field will need to utilize a variety of behavioral physiological, anatomical, molecular techniques. By developing a mechanistic-based understanding of

how electrical activity rhythms are generated within the circadian timing system, we believe that it will be possible to develop new therapies and disease management strategies that can be applied to a range of psychiatric and neurological disorders in which dysfunction in circadian timing are part of the symptoms.

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Ryanodine-Sensitive Intracellular Ca2+ Channels in Rat Suprachiasmatic Nuclei Are Required for Circadian Clock Control of Behavior

Clara Mercado, Mauricio Díaz-Muñoz, Javier Alamilla, Karla Valderrama, Verónica Morales-Tlalpan and Raúl Aguilar-Roblero

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Ryanodine-Sensitive Intracellular Ca^{2+} Channels in Rat Suprachiasmatic Nuclei Are Required for Circadian Clock Control of Behavior

Clara Mercado,[*,†] Mauricio Díaz-Muñoz,[†] Javier Alamilla,[*] Karla Valderrama,[*] Verónica Morales-Tlalpan,[†] and Raúl Aguilar-Roblero[*,1]

[*]*Departamento de Neurociencias, Instituto de Fisiología Celular, Distrito Federal, México,*
[†]*Departamento de Neurobiología Celular y Molecular, Instituto de Neurobiología, Querétaro, México, Universidad Nacional Autónoma de México*

Abstract Electrophysiological and calcium mobilization experiments have suggested that the intracellular calcium release channel ryanodine receptors (RyRs) are involved in the circadian rhythmicity of the suprachiasmatic nucleus (SCN). In the present report the authors provide behavioral evidence that RyRs play a specific and major role in the output of the molecular circadian clock in SCN neurons. They measured the circadian rhythm of drinking and locomotor behaviors in dim red light before, during, and after administration of an activator (ryanodine 0.1 μM) or an inhibitor (ryanodine 100 μM) of the RyRs. Drugs were delivered directly into the SCN by cannulas connected to osmotic minipumps. Control treatments included administration of artificial cerebrospinal fluid, KCl (20 mM), tetrodotoxin (1 μM), and anysomicin (5 μg/μl). Activation of RyRs induced a significant shortening of the endogenous period, whereas inhibition of these Ca^{2+} release channels disrupted the circadian rhythmicity. After the pharmacological treatments the period of rhythmicity returned to basal values and the phase of activity onset was predicted from a line projected from the activity onset of basal recordings. These results indicate that changes in overt rhythms induced by both doses of ryanodine did not involve an alteration in the clock mechanism. The authors conclude that circadian modulation of RyRs is a key element of the output pathway from the molecular circadian clock in SCN neurons in rats.

Key words circadian timing system, suprachiasmatic nucleus, ryanodine receptor, intracellular calcium, circadian output, behavioral regulation

In mammals, endogenous circadian rhythms are generated within the suprachiasmatic nucleus (SCN) localized in the ventral hypothalamus (Moore, 1983). Functional studies have demonstrated that SCN neurons are able to generate 24-h rhythms of metabolic and electrical activities when they are isolated or in cultured conditions (Inouye and Kawamura, 1982; Welsh et al., 1995). The timing system in each SCN neuron consists of 3 components (Reppert and Weaver, 2002): 1) a synchronization input pathway that entrains the clock to environmental signals, 2) a pacemaker (molecular clock) underlying feedback

1. To whom all correspondence should be addressed: Dr. Raúl Aguilar-Roblero, Neurociencias, IFC/UNAM, Apdo. Postal 70-253, México D.F., 04510 México; e-mail raguilar@ifc.unam.mx.

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regulation of clock genes that generate circadian rhythmicity, and 3) an output pathway that coordinates the metabolic, endocrine, and behavioral expression of the circadian rhythms. The mechanisms involved in the SCN synchronization by light are well documented. Specialized retinal ganglion cells form the retinohypothalamic tract that projects to the ventral region of the SCN (Hannibal et al., 2000; Hannibal, 2002). Upon photic stimulation glutamate is released to initiate 2nd messenger cascades that lead to molecular regulation of clock genes (Gillette and Mitchell, 2002).

In contrast, the information about the output of the circadian clock is scarce. One of the few established facts is that the SCN electrophysiological activity plays a major role in the output of the circadian pacemaker (Schwartz et al., 1987; Meredith et al., 2006; Aguilar-Roblero et al., 2007). The neuronal excitability is then linked to the rhythmic variations of metabolic activity, hormones levels, physiological processes, and behavioral responses, including the circadian rhythm of drinking and eating. Schwartz et al. (1987) demonstrated that blocking of the electrical properties of the SCN by tetrodotoxin (TTX) administration prevented the circadian expression of drinking behavior. Upon removal of TTX, the rhythm of drinking water reappeared with the same phase and period displayed prior to toxin administration. This experiment suggested that the clock continues running despite that its electrical output is silenced; therefore, the rhythmic electrical activity of SCN neurons is an output of the clock and not a component of the pacemaker machinery itself.

Calcium-binding proteins and intracellular calcium are involved in most of the relevant aspects of SCN physiology. Ca^{2+} entry by *N*-methyl D-aspartate (NMDA) receptor is necessary for light entrainment (Meijer and Schwartz, 2003), and calbindin-containing neurons form a core region that lack rhythmic properties (Kriegsfeld et al., 2004; Jobst et al., 2004; Hamada et al., 2003). Ca^{2+}-activated K^+ channels of large conductance (BK) have been suggested as important for the pacemaker output (Colwell, 2006; Meredith et al., 2006). We have shown that Ca^{2+} mobilization through the intracellular calcium channel activated by ryanodine (RyR) in the SCN is involved in the output pathway between the molecular circadian clock and the electrical output of the SCN. Previous work by our laboratory reported that the RyRs showed a circadian rhythm in [^{3}H]ryanodine binding in membranes of SCN cells (Díaz-Muñoz et al., 1999) and pharmacological manipulation of RyRs modulated the firing frequency in SCN neurons (Aguilar-Roblero et al., 2007).

In the present study we further addressed the role of RyRs in the output of SCN rhythmicity by testing in vivo whether its pharmacological manipulation affects the circadian expression of locomotion and drinking behavior. The transient alteration of behavioral rhythms by the pharmacological activation (0.1 µM ryanodine) and inhibition (100 µm ryanodine) of RyR without long-lasting effects on behavioral rhythmicity, and the return to basal values of period and phase of activity onset after cessation of treatment, confirm that this intracellular Ca^{2+}-release channel participates in the output pathways linking the molecular oscillator in the SCN to the overt behavioral expression of circadian rhythmicity.

MATERIAL AND METHODS

Male Wistar rats weighing 250 to 300 g were housed under 12:12 h light/dark cycle (lights-on at 08:00 h, 400 lux) in a sound-attenuated room with regulated temperature (22 ± 1 °C) for at least 1 week before starting the experiment. Animals had continuous access to food and water. All surgical procedures were conducted in antiseptic conditions and under general anesthesia with pentobarbital (30 mg/kg of body weight, intraperitoneal). At the end of the procedure all animals received benzatinic penicillin (800,000 IU). All other procedures were conducted according to the guidelines for use of experimental animals from the Universidad Nacional Autónoma de México in accordance with national laws (NOM-062-200-1999).

Rats were continuously recorded under constant red light (50 lux, DD) for drinking behavior and locomotion as follows. Animals were individually housed in Plexiglas cages equipped with contact sensors in the water spout and infrared beams in the sides of the cage. Each time the sensors were touched or 2 successive light beams were interrupted, a normalized electric square pulse was generated and added to the respective event counter in a microprocessor. The counters were read at 5-min intervals and reset to 0, and the data from all independent sensors and channels were stored in a computer database for post hoc plotting and analysis.

The animals were randomly assigned for treatment to 1 of the following groups: 1) artificial cerebrospinal fluid (aCSF) containing in mM: 140 NaCl, 3.5 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, and 5.0 HEPES (pH

7.25); 2) ryanodine 0.1 μM to activate the RyR and induce Ca^{2+}release from its intracellular stores; and 3) ryanodine 100 μM to inhibit the RyR and prevent Ca^{2+} release from its intracellular deposits. Additional groups used as controls were 4) TTX 1 μM, which prevents generation of action potentials by blocking Na^{+} channels; 5) KCl 20 mM, which leads to membrane depolarization—the osmolarity of the solution was kept constant by decreasing NaCl to 120 mM; and 6) anisomycin (5 μg/μl), an inhibitor of protein synthesis, was used to discriminate whether the effects of blocking Ca^{2+} release involved alteration in protein synthesis. All drugs and KCl were administered in a CSF vehicle at a rate of 0.5 μl/h by means of an activated osmotic minipump (Alzet model 2002; Durect Corporation, Cupertino, CA) implanted subcutaneously between the scapular blades and filled with the corresponding treatment. The minipump was connected by Tygon tubing to a 26-ga injector (Plastic One, Roanoke, VA) stereotaxically implanted in the midline at the upper edge of the SCN (AP 0.4; L 0.0; V –8.5, according to Paxinos and Watson, 1998). The experiment consisted of 7 days of habituation to the environmental conditions, and the next 15 days were recorded as the experimental baseline. At the end of the baseline the animals were anesthetized between CT02 and CT10 under red light (50 lux) and the surgery to implant the injector and the minipump was performed; 15 to 20 min after surgery, animals were returned to their recording chamber until recovery was complete. The recording continued for at least 30 more days. All treatments took place during the first 14 days after implantation of the minipump, which corresponds to the estimated time of ejection from the reservoir according to the manufacturer. The remaining 16 to 24 days of recording were used to determine whether or not the parameters under study returned to baseline levels.

At the end of the recordings the animals were killed with an overdose of pentobarbital and transcardially perfused with 0.9% NaCl solution followed by 10% formalin. The brain from each animal was then extracted and immersed in sucrose solutions of increasing concentration (10%, 20%, and 30%) until it sank. The anterior hypothalamus was sectioned at 40 μm in the coronal plane and sections 200 μm apart were processed for Nissl staining with cresyl violet. The sections were inspected in a microscope to locate the tip of the injector. Only those animals that showed the injector in the SCN were included in this study.

In control and all experimental groups the data of drinking and locomotor recordings were similar with only slight differences in onset or intensity; therefore, we decided to add the values of both parameters at 15-min time intervals. Actograms from experimental data were double plotted. Complete recordings were analyzed by reiterative χ^2 periodogram analysis of 8 day segments at 3-day intervals. That is, for a recording lasting 45 days the 1st periodogram was performed from the 1st to 8th days of recording, the next one from the 3rd to the 10th day, and so on to the 38th to 45th days. Each successive periodogram was plotted displaced from the previous one to simulate a 3-dimensional array. This procedure is similar to the compress spectral array (Bickford et al., 1973), but instead of the fast Fourier analysis we used the χ^2 analysis. The procedure reveals the time course of the change in amplitude of periodic components throughout the analyzed time span. The effect of the different treatments on the period of rhythmicity was estimated from periodograms obtained from the last 8 days of each segment of the experiment (baseline, treatment, and recovery); the period of rhythmicity was read from the peak in the range from 20 to 28 h that reach an α level of 0.0001; arrythmicity was considered to occur when the amplitude of the peaks did not reach an α level of 0.01.

The activity onset was considered as the point of inflexion in the slope of the cumulative plot of activity for each 24-h segment; the activity onset of each segment of the experiment was identified by visual inspection of the actogram and a line was fitted to successive activity onsets. The value of linear regression fitting of the activity onsets during recovery to the projected line generated from the activity onsets during the basal recording was used to estimate whether the phase was the same before and after the treatments. We also estimated the phase differences in activity onset occurring in the transitions from the baseline to the treatment, and from the treatment to the recovery. For animals in which treatment prevented the expression of overt rhythmicity, we estimated the phase difference in activity onset between the baseline projected to the end of the treatment and the corresponding recovery (Schwartz et al., 1987).

The parameters from each experimental segment were compared by Friedman ANOVA followed by the Dunnett T3 post hoc test when necessary. Due to the absence of rhythmicity induced by some treatments we use the Wilcoxon sign rank test to compare before and after drug administration when rhythmicity returned, and the Cochran Q test to compare between groups showing circadian rhythmicity (score

Table 1. Effect of different treatments on circadian parameters.

	τ (*h:min ± min*)			ϕ (*r*)		
Group	*Basal Mean ± SEM*	*Treatment Mean ± SEM*	*Recovery Mean ± SEM*	*Basal Mean ± SEM*	*Treatment Mean ± SEM*	*Recovery Mean ± SEM*
aCSF	24:10 ± 00:02	24:10 ± 00:03	24:14 ± 00.03	0.95 ± 0.02	0.92 ± 0.05	0.92 ± 0.02
Rya 0.1	24:14 ± 00:02	**23:59 ± 00:12**[a]	24:16 ± 00:02	0.94 ± 0.02	**0.63 ± 0.06**[b]	0.89 ± 0.05
KCl	24:18 ± 00:09	**24:07 ± 00:03**[a]	24:15 ± 00:02	0.96 ± 0.02	**0.73 ± 0.03**[b]	0.89 ± 0.02
Rya 100	24:09 ± 00:06	—	24:09 ± 00:03	0.95 ± 0.03	—	0.87 ± 0.05
TTX	24:13 ± 00:03	—	24:09 ± 00:06	0.95 ± 0.01	—	0.88 ± 0.04
Any	24:15 ± 00:04	—	—	0.94 ± 0.01	—	—

τ = free running period estimated from the χ^2 periodogram; ϕ = phase of the activity onset; aCSF = artificial cerebrospinal fluid; Rya = ryanodine; KCl = 20 mM KCl; TTX = tetrodotoxin. The *r* value refers to the fitting of activity onsets during each experimental phase to the line adjusted from the basal recording. Post hoc comparisons were made with Dunnett T3 test; superscript letters indicate significant differences with respect to the control ([a] $p < 0.05$; [b] $p < 0.01$). Bold face text indicates data with significant differences with the control.

1) versus those arrhythmic groups (score 0). In all analyses, the α level was set at 0.05.

RESULTS

From 56 implanted rats at the beginning of the experiment, only 32 had the microinjector tip within the SCN boundaries. From the remaining 24 animals the microinjector tip was found rostral ($n = 8$) or caudal ($n = 10$) to the SCN; in the other 6 animals the microinjector was found within or below the SCN and substantial damage to at least one of the nuclei was evident. In most of these animals the treatments had no noticeable effects; in the animals bearing an SCN lesion there were changes of period, phase shifts after implantation of the microinjector, and in 1 subject receiving TTX the arrhythmic pattern persisted through the remainder of the recording. In 5 animals in which the microinjector was placed caudal to the SCN we observed changes in period and transient loss of rhythmicity in 2 TTX-treated subjects. All other results are summarized in Table 1.

All subjects showed clear free-running circadian rhythmicity during the baseline recording. Continuous infusion of aCSF into the SCN vicinity did not induced noticeable changes in period or phase during and after the treatment (Fig. 1A). Dynamical χ^2 periodogram (Fig. 1B) confirmed no effects on the period during or after the aCSF infusion with respect to the baseline 24:10 ± 2 (mean [h:min] ± SEM [min]) and 24:10 ± 2.6 and 24:14 ± 2.6, respectively (Friedman $\chi^2 = 3.8$, NS; Table 1). Furthermore, the *r* value of activity onset after the aCFS fitted to the lines projected from the basal recordings was 0.92 ± 0.02 (Friedman $\chi^2 = 4.8$. NS; Table 1).

Animals treated with the activator dose of ryanodine (100 nM) maintained clear circadian rhythms, but the period was slightly shortened and the interval of activity apparently was compressed during and after the treatment with respect to the baseline (Fig. 1C). The effect on the period was clearly seen in the χ^2 periodogram: 24:14 ± 2.40 (mean [h:min] ± SEM [min]) during baseline; 23:59 ± 12.2 during treatment, and 24:16 ± 2.10 during recovery (Fig. 1D; Friedman $\chi^2 = 8.6$, $p = 0.014$). The *r* value of activity onset after 100 nM ryanodine fitted to the lines projected from the basal recordings was 0.89 ± 0.05 (Table 1; Friedman $\chi^2 = 8.4$, $p = 0.015$). Administration of 20 mM KCl produced a very similar effect to the one induced by a 0.1 μM ryanodine, that is, shortening of period and compression of the activity interval during and after the treatment. The period estimated by the χ^2 periodogram was 24:18 ± 9.0 during the baseline, 24:07 ± 3.00 during the treatment, and 24:15 ± 1.6 during recovery (Friedman $\chi^2 = 8.7$, $p = 0.013$). The *r* value of activity onset after high potassium treatment fitted to the lines projected from the basal recordings was 0.89 ± 0.02 (Table 1; Friedman $\chi^2 = 10$, $p = 0.007$).

Rats treated with 100 μM ryanodine showed disruption of behavioral rhythms by disorganization of the daily pattern of activity during the period of administration of the drug (Fig. 1E). After withdrawal of ryanodine the rhythm reappeared and the activity onset adjusted to a line projected from the activity onsets from the baseline recording. The effects on the period of the rhythm were evident in the χ^2 periodogram (Fig. 1F): baseline 24:09 ± 5.60, recovery 24:09 ± 3.40. Because of the absence of rhythmicity induced by ryanodine 100 μM we use the Wilcoxon sign rank test to compare before and after drug administration ($Z = -1.3$, NS); the Cochran test was used to compare all 3 groups coding 1 for circadian rhythm and 0 for arrhythmcity ($Q = 10$, $p = 0.007$). The *r* value of activity onsets after the treatment fitted to the line projection from the basal recordings was 0.87 ± 0.05 (Table 1; Wilcoxon sign rank test $Z = -2.02$, $p = 0.053$). Similar alterations were

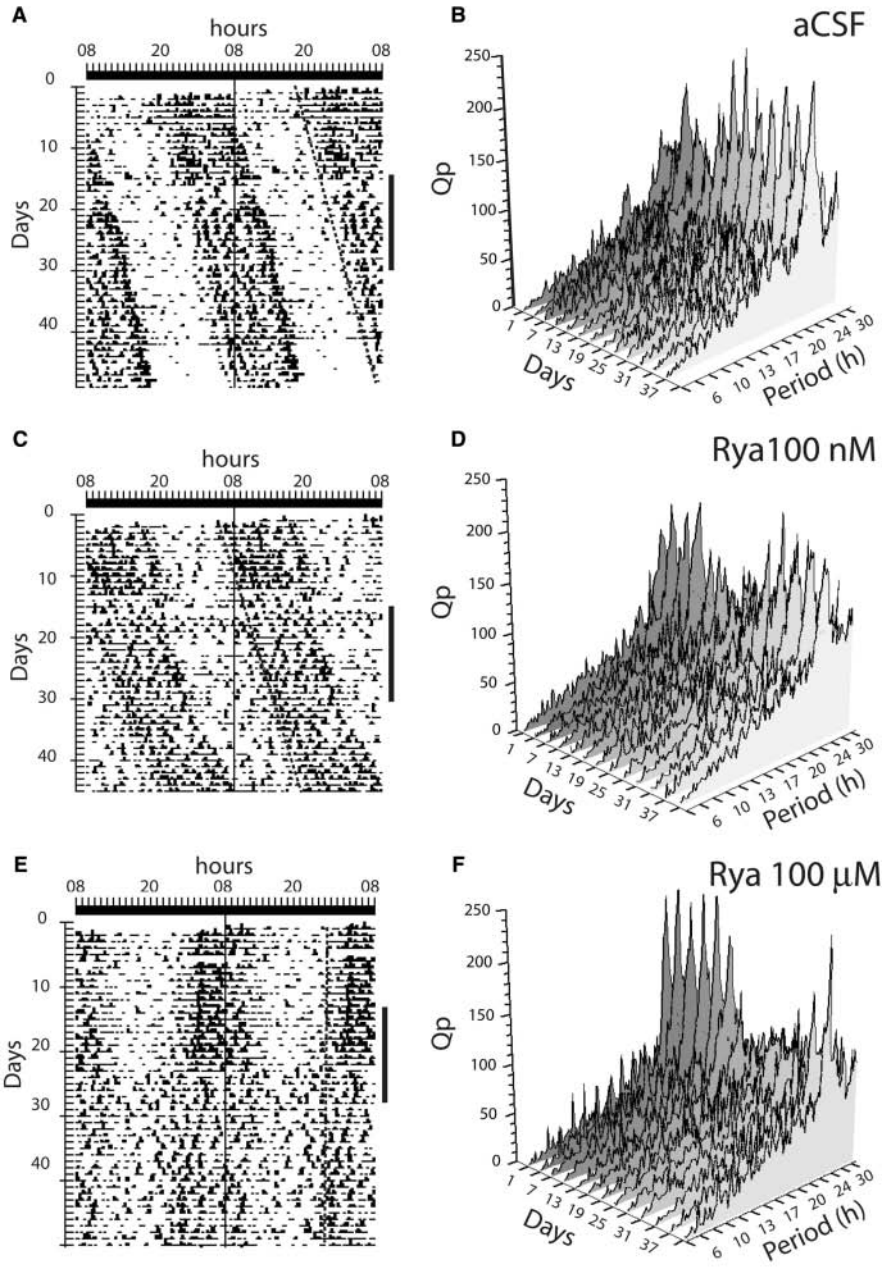

Figure 1. Actogram and the corresponding χ^2 periodogram to exemplify the effect of artificial cerebrospinal fluid (aCSF) and 2 doses of ryanodine on the circadian rhythms of rats. aCSF administration into the SCN (A, B) did not affect the expression of the circadian rhythm. Ryanodine 0.1 µM (C, D) produced shortening of the period of the rhythm, which disappeared shortly after the treatment. Ryanodine 100 µM (E, F) produced disorganization of the daily pattern of activity, which disappears from 7 to 12 days after the end of the treatment. (A, C, and E) Double plotted actograms at 15-min bins. The vertical lines on the right of the actograms indicate the duration of the treatment, from the implant of the injector and the minipump to the nominal duration of activity of the osmotic pump. The dashed lines on the actogram indicate the time of activity onset (CT 12) adjusted during the basal recording and projected to the recordings during and after the treatment. (B, D, and F) Dynamical χ^2 periodograms from 8 consecutive days were obtained at 3-day intervals from the beginning to the end of the recording. Each gray tone indicates a segment of the recordings (basal = dark; treatment = medium; recovery = light). The *y* axis indicates the Qp value.

observed during TTX administration, which also induced clear disruption of the circadian rhythm. After the drug was withdrawn the rhythm was restored. These effects were clearly observed in the dynamical χ^2 periodogram (baseline 24:13 ± 2.80, recovery 24:09 ± 5.60; Wilcoxon sign rank test $Z = -1.8$, NS; Cochran $Q = 8$, $p = 0.018$). In all but 1 case, the phase of the restored rhythm was predicted by projecting of the activity onset from the baseline recording to the days after treatment. The r value of activity onsets after the treatment fitted to the line projection from the basal recordings was 0.80 ± 0.08 (Table 1; Wilcoxon sign rank test $Z = -1.83$, $p = 0.068$). Finally, because 100 µM of ryanodine partially prevents the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm and this may impair protein synthesis, we studied the effect of inhibiting protein synthesis in the SCN area on the expression of behavioral rhythms. Administration of anisomycin 5 µg/µl disrupted the circadian rhythmicity, and this effect apparently persists after 2 weeks of drug withdrawal. Dynamical χ^2 periodogram confirmed the lack of rhythmicity during and after drug treatment (Cochran $Q = 10$, $p > 0.007$).

DISCUSSION

The present data are consistent with the hypothesis that RyRs are an output pathway from the molecular oscillator to the expression of overt circadian rhythms. When applied directly into the SCN both doses of ryanodine transiently change the

expression of behavioral circadian rhythms but did not affect the circadian clock itself; as indicated by the restoration to baseline levels of the period and the phase of the rhythm shortly after withdrawal of ryanodine. We have previously shown that a low dose of ryanodine (0.1 μM), which activates the RyRs, increases SCN spontaneous neuronal firing rate secondary to release of intracellular Ca^{2+} by opening of the RyRs, whereas a higher dose of ryanodine (100 μM), which blocks the RyRs, decreases the SCN spontaneous firing rate as a consequence of a diminishing of intracellular Ca^{2+} release by closing the RyRs, (Chu et al., 1990; Aguilar-Roblero et al., 2007).

As previously reported by Schwartz et al. (1987), we found that manipulating SCN excitability with either 1 μM TTX or 20 mM KCl modifies behavioral circadian rhythms without affecting the clock mechanism. Thus, a nonspecific increase of cellular excitability produced by high K^+ membrane depolarization induces shortening of the endogenous period and compression of the activity interval of behavioral circadian rhythms, whereas depression of excitability by blockade of Na^+ channels by TTX completely abolishes expression of circadian rhythms. These effects were transitory and the period returned to basal values with the phase predicted from the basal recording after ceasing high K^+ or TTX administration. The effects of high K^+ may also involve nonspecific behavioral feedback on the clock. Although TTX does not seem to affect the molecular clock in SCN organotypic cultures from *mPer$_1$-luc* mouse, its effects on behavioral rhythmicity may be due to disruption of the synchrony between individual neuronal oscillators (Yamaguchi et al., 2003). Altogether previous results are consistent with the hypothesis that RyRs are regulated by the molecular circadian oscillator in SCN neurons, and thus Ca^{2+} mobilized from the endoplasmic deposits modulates the neuronal firing frequency and eventually regulates the circadian expression of behavior (Fig. 2).

It is worth noting that the effects of 100 μM ryanodine persisted beyond the administration time in 4 of the 5 subjects, but in all cases the rhythm was restored by the 2nd week after the administration ceased. This long-lasting effect may be related to some other effect on intracellular Ca^{2+} homeostasis, including inhibition of protein synthesis. Nevertheless, when the rhythmic pattern was recovered the phase of the rhythm was the one predicted from the baseline rhythm; this observation is consistent with the hypothesis that the time-keeping mechanism

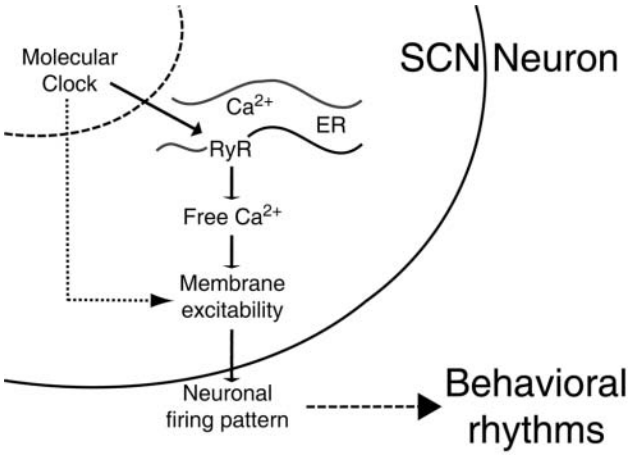

Figure 2. Schematic model of an output pathway from the molecular circadian clock to expression of behavioral rhythms. The core circadian clock drives the sensitivity and conductance of ryanodine receptors (RyR; Diaz-Muñoz et al., 1999), which in turn generate a rhythm in free cytosolic Ca^{2+} (Colwell, 2000; Ikeda et al., 2003), which in turn modulates the rhythm in neuronal excitability and spontaneous firing rate (Aguilar-Roblero et al., 2007). Once the rhythm is encoded into a neuronal firing rate it is transmitted to the neuronal effectors involved in behavioral expression (present data).

was functioning during this treatment. Previous interpretation is further supported by the observation that anisomycin (a general protein synthesis inhibitor) disrupted expression of circadian rhythmicity, but, in contrast to 100 μM ryanodine, its effects persisted for the remainder of the experiment. The persistence of an arrhythmic pattern of behavior during and after the anisomycin may reflect severe impairment on the molecular oscillator involving protein synthesis. In SCN organotypic cultures from the *mPer$_1$-luc* mouse, it has been shown that inhibition of protein synthesis by cycloheximide administration stops the molecular clock, which restarts at the same phase in all neurons after 3 h of washout from the medium (Yamaguchi et al., 2003). In the present study the onset of rhythmicity after 100 μM ryanodine administration showed different phases among the animals and, as we have previously stated, were better predicted by the phase of activity onset previous to the drug administration.

Different Ca^{2+} pools have specific roles in regulating the spatiotemporal neuronal responses to Ca^{2+} signaling (Finkbeiner and Greenberg, 1998; Rizzuto, 2001). In the SCN there are reports on the role of intracellular Ca^{2+} as a signal involved in resetting the clock, maintaining the molecular oscillation, and

transmitting the rhythmicity from the clock itself to the efferents involved in the expression of the overt rhythmicity. Thus, manipulation of calmodulin phase shifts metabolic and electrical rhythms in SCN neurons (Shibata et al., 1987), while RyRs mediate light-induced phase delays during early subjective night (Ding et al., 1998). Cytoplasmic free Ca^{2+} levels display circadian oscillations in the SCN, being higher during the light phase than in the night (Colwell, 2000; Ikeda et al., 2003). It has been suggested that such intracellular Ca^{2+} rhythm may modulate extracellular Ca^{2+} influx in response to light pulses during the night (S. Michel, personal communication, August 26, 2008; Michel et al., 2002). Díaz-Muñoz et al. (1999) demonstrated a circadian rhythm of activity from RyR type 2 in SCN neurons, and pharmacological modulation of the RyR modifies the spontaneous firing rate frequency in SCN neurons (Aguilar-Roblero et al., 2007). Previous evidence indicates that cytosolic Ca^{2+} mobilized through these channels is a direct link between the core oscillator and the SCN electrical activity rhythm, as previously suggested from the independence between voltage-sensitive Na^{+} and Ca^{2+} membrane currents and the intracellular Ca^{2+} rhythm, and the decrease in amplitude of both rhythms by RyRs blockers (Aguilar-Roblero et al., 2007; Ikeda et al., 2003). Finally, Nitabach et al. (2002) had shown that neuronal electrical activity is necessary for expression of the molecular oscillator and suggested that the coupling of these events could involve Ca^{2+} entry through voltage-dependent calcium channels and some yet unidentified calcium signal pathway. Lundkvist et al. (2005) and Nahm et al. (2005) had extended this observation to the rodent SCN and confirmed the role of voltage-dependent calcium channels in maintaining the molecular clock oscillations; more recently Harrishing et al. (2007) provided evidence on a role for intracellular Ca^{2+} signaling regulating intrinsic cellular oscillations in *Drosophila* in vivo.

Present data extend to the expression of behavioral overt rhythmicity, the evidence supporting the hypothesis that circadian modulation of the RyRs is part of the output pathway from the molecular oscillator. The participation of Ca^{2+} handling proteins and its relation to the RyRs in the output pathway from the molecular oscillator remains to be explored. Further studies are also needed to dissect other roles of Ca^{2+} signaling in the clock mechanism and resetting, as well as studies aimed to unravel the specific targets of Ca^{2+} signaling to modulate neuronal excitability in the SCN.

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Ryanodine receptor Ca^{2+}-release channels are an output pathway for the circadian clock in the rat suprachiasmatic nuclei

Raúl Aguilar-Roblero, Clara Mercado, Javier Alamilla, Antonio Laville and Mauricio Díaz-Muñoz[1]
Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apdo. Postal 70–253, México D.F. 04510, Mexico
[1]Departamento de Neurobiología Celular y Molecular, Instituto de Neurobiología, Universidad Nacional Autónoma de México

Keywords: circadian clock, clock output, electrophysiology, intracellular calcium, neuronal excitability, SCN

Abstract

Ryanodine-sensitive intracellular Ca^{2+} channels (RyRs) are present in suprachiasmatic nuclei (SCN) neurons, but the functions served by these channels are not known. Here we addressed whether mobilization of intracellular Ca^{2+} stores through the RyRs may be a link between the molecular clock and the firing rate in SCN neurons. Activation of the RyRs by administration of either 1 mM caffeine or 100 nM ryanodine increased the firing frequency, whereas inhibition of RyRs by 10 μM dantrolene or 80 μM ryanodine decreased firing rate. Similar results were obtained in experiments conducted at either midday or midnight. Furthermore, these effects were not mediated by synaptic transmission as blockade of GABA A, AMPA and NMDA receptors did not prevent the excitatory or inhibitory effects induced by either dose of ryanodine on SCN firing. We conclude that gating of RyRs is a key element of the intricate output pathway from the circadian clock within SCN neurons in rats.

Introduction

Circadian rhythms in mammals are generated by the suprachiasmatic nuclei (SCN) in the anterior hypothalamus (Klein *et al*., 1991). The molecular circadian oscillator in SCN neurons consists of a self-regulated transcription–translation loop (see Reppert & Weaver, 2002 for a review) among a group of genes known as 'clock genes'. Entrainment of rhythmicity to the light–dark cycle depends on the retinal input to SCN neurons (the retinohypothalamic tract; RHT) from a subset of retinal ganglion neurons expressing melanopsin (Rollag *et al*., 2003; Morin & Allen, 2006). Both excitatory amino acids and the pituitary adenylate cyclase activating polypeptide seem to be involved in the synaptic transmission in the RHT (Hannibal, 2002; Morin & Allen, 2006). Recently, the role of voltage-gated Ca^{2+} channels (particularly the T-type Ca^{2+} current) associated with the glutamatergic response has been clearly demonstrated (Kim *et al*., 2005). Although the specific details are still under study, the signalling pathway within SCN neurons in response to light involves a variety of parameters, such as increased cytoplasmic Ca^{2+}, synthesis of nitric oxide, activation of MAPK and CaMK-II, and CREB phosphorylation, which lead to change in the expression of clock genes (Meijer & Schwartz, 2003). Much less is known about the output pathways from the molecular oscillator. However, it is clear that it must regulate neuronal membrane excitability and thus induce the circadian pattern in the firing rate characteristic of the SCN.

Although calcium-dependent synaptic transmission is not essential to sustain the clock mechanism in SCN neurons (Bouskila & Dudek, 1993), intracellular calcium homeostasis in the SCN is under circadian control (Díaz-Muñoz *et al*., 1999; Colwell, 2000; Ikeda *et al*., 2003) and its manipulation affects expression of overt circadian rhythms (Prosser *et al*., 1992; Shibata & Moore, 1994; Biello *et al*., 1997). Previously, we showed a circadian rhythm in ^{3}[H]-ryanodine binding that was specific to the SCN but not in other brain areas. The peak of the rhythm occurred at circadian time 07.00 and was due to an increase in the protein expression of the neuronal ryanodine receptor (RyR) type 2 in the SCN during the middle of the day (Díaz-Muñoz *et al*., 1999). More recently, organotypic cultures from the SCN expressed circadian rhythms in cytoplasmic calcium levels ($[Ca^{2+}]i$) and electrical firing rate, which could be dissociated by tetrodotoxin applied to the medium. In this condition, the blockade of the electrical activity rhythm did not affect the $[Ca^{2+}]i$ rhythm. The cytoplasmic Ca^{2+} rhythm was damped by treatment with negative modulation of the RyR such as ryanodine (5 and 100 μM) and 8-bromo-cyclic ADP ribose (300 μM), but was not affected by nifedipine, an inhibitor of voltage-gated Ca^{2+} channels (Ikeda *et al*., 2003). Altogether, these data strongly suggest that the $[Ca^{2+}]i$ circadian rhythm is mainly related to the mobilization of intracellular Ca^{2+} stores through the ryanodine-sensitive Ca^{2+} channels, and are also consistent with the hypothesis that the cytoplasmic Ca^{2+} rhythm may be one of the first transmission elements linking the molecular oscillator to the circadian modulation of the firing rate in SCN neurons.

In the present study we addressed the previous hypothesis by testing the effects of pharmacological activation and inhibition of ryanodine-sensitive Ca^{2+} channels on SCN neuronal membrane potential and spontaneous firing frequency as measured by the perforated-patch technique in acute brain slices *in vitro*. If firing rate in SCN neurons is modulated via the intracellular Ca^{2+} mobilized through ryanodine-

Correspondence: Dr Raúl Aguilar-Roblero, as above.
E-mail: raguilar@ifc.unam.mx

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sensitive Ca^{2+} channels, we expect that its pharmacological opening would increase SCN neuronal firing while its closure would decrease the firing rate. Present results confirm this hypothesis, and further support the notion that intracellular Ca^{2+} mobilization through the ryanodine-sensitive Ca^{2+} channels is part of the output pathway linking the molecular oscillator to the expression of circadian rhythms in the SCN.

Materials and methods

Male Wistar rats were housed under a 12 : 12 h light : dark cycle (lights on at 06.00 h; 400 lux) in a sound-attenuated room with regulated temperature (22° ± 1 °C) for at least 1 week before starting the experiment. For electrophysiological recordings made during subjective midnight the animals were maintained in a reversed 12 : 12 h light : dark cycle (lights on at 22.00 h; 400 lux) for at least 3 weeks before the experiment. Animals had continuous access to food and water. Rats used to prepare brain slices were deeply anaesthetized with ether before extraction of the brain. All the procedures were conducted according to the guidelines for use of experimental animals from the Universidad Nacional Autónoma de México in accordance to national laws on the matter (NOM-062-200- 1999).

Slice preparation

Male Wistar rats weighing between 100 and 120 g were deeply anaesthetized with ether between Zeitgeber time (ZT) 03 and 05 (ZT 0 is lights on) and the brain was quickly removed and placed in ice-cold extraction solution (low-Ca^{2+} aCSF) containing (in mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 4; $CaCl_2$, 0.5; $NaHCO_3$, 26; and glucose, 10; pH 7.38, 330 mOsm/L, oxygenated with 95% O_2 and 5% CO_2 gas mixture. To avoid phase shifts induced by light, the brain slices used during subjective night were prepared between ZT 11 and 12 (before lights off). Coronal sections of 250 μm were obtained using a vibratome (Pelco) and the slices containing the SCN were transferred to fresh low-Ca^{2+} aCSF under continuous oxygenation at room temperature and kept in this condition until use. One slice was then placed in the recording chamber and continuously superfused with oxygenated aCSF at room temperature. The bath solution was identical to the extraction solution except that $CaCl_2$ was increased to 2.4 mM, $MgCl_2$ reduced to 1.3 mM and the pH adjusted to 7.38 at room temperature. SCN neurons were visualized and the recording electrodes were positioned by infrared Nomarski microscopy at 600× using a Nikon Eclipse 600 with Dage MTI video camera and monitor.

Patch-clamp recording

Recordings were made at room temperature (20–25 °C) using the whole-cell or perforated-patch technique at different moments of the circadian cycle, either between ZT 05 and ZT 10 (subjective day) or between ZT 17 and ZT 22 (subjective night). For whole-cell recordings electrodes were filled with a solution containing (in mM): KH_2PO_4, 115; $MgCl_2$, 2; HEPES, 10; EGTA, 0.5; Na_2ATP, 2; Na_2GTP, 0.2; pH 7.2, 275 mOsm/L, as previously reported by Vergara *et al*. (2003). In the case of whole-cell recordings, the membrane was disrupted by gentle suction. For perforated-patch recordings amphotericin was dissolved in dimethyl sulfoxide, 10 mg/mL, and diluted before recording to a final concentration of 80–150 μg/mL in an electrode-filling solution consisting of 150 mM KCl, pH 7.2, 300 mOsm/L. Once a good seal (>2 GΩ) was obtained between the electrode and the neuron, we waited between 2 and 8 min in order to obtain a perforated patch. Recordings were made in a current-clamp configuration with an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA, USA). On-line data collection was performed using a PC compatible with a digital acquisition board (DAQ; National Instruments) using a custom-made program in the LabView environment.

After at least 5 min of basal recording of spontaneous activity, drugs were applied by changing the extracellular solution to one containing the drug for testing, and the neuronal activity was recorded again. Each recorded neuron received only one of the following treatments: controls with aCSF; opening of the ryanodine-sensitive Ca^{2+} channels with 100 nM ryanodine or 1 mM caffeine; closure of these channels with 80 μM ryanodine or 10 μM dantrolene. Only one cell from each hypothalamic slice was recorded after administration of the drugs. Statistical comparison before and after treatment was made with the Wilcoxon test and the α level was set at 0.05. All drugs were purchased from Sigma (St Louis, MO, USA). Digitized data were stored on disk as ASCII or binary files, and were imported for analysis into commercial graphing (Origin, Microcal) and analysis (MiniAnalysis, Synaptosoft) software. Spike frequency was estimated from the inverse of the median interspike interval. Regular (rhythmic) firing was considered to occur in those neurons showing a narrow Gaussian distribution in the interspike interval histogram, while irregular (arrhythmic) firing was considered to occur in those neurons with a skewed distribution (Groos & Hendricks, 1979; Shibata *et al*., 1984; Thomson *et al*., 1984). In order to measure the membrane potential and the duration of the action potential, 20 consecutive segments starting 20 ms before an action potential and ending 20 ms before the next one were aligned, and the average profile was plotted to measure the membrane potential at the following instants: 20 ms before the spike (*V*m); at the threshold of the spike (*V*thr); at the peak of the spike (*V*spk); and at the lowest point of the afterhyperpolarization potential (*V*ahp). The duration of the action potential was measured at the level of *V*thr; the rising time of the action potential (τ_{spk}) was measured from *V*thr to *V*spk, and the decay time of the action potential (τ_{ahp}) was measured from *V*spk to the 66% decay from *V*spk to *V*ahp. Statistical comparison before and after the treatment was made with a paired *t*-test; the α level was set at 0.05.

In order to determine whether effects of ryanodine treatment on firing rate are a consequence of its effects on synaptic activity, in some experiments during subjective day we also blocked GABA A, AMPA and NMDA receptors. After recording the basal firing pattern, neurons received ryanodine (either 100 nM or 80 μM) and a cocktail containing DL-2-amino-5-phosphonopentanoic acid (AP-V), 50 μM, to block NMDA glutamate receptors; 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX), 10 μM, to block AMPA glutamate receptors; and bicuculline, 10 μM, to block GABA A receptors. AP-V, DNQX and bicuculline were always applied in sequence, but they were applied in a counterbalanced design with respect to the dose of ryanodine. Recording procedures and drug administration were performed between ZT 05 and ZT 10 as described in the previous paragraphs.

Results

Basal recordings

A total of 76 neurons were recorded within the SCN boundaries between ZT 5 and ZT 10; 22 neurons were recorded in whole-cell, 52 were recorded in perforated-patch and two were recorded in cell-attached mode. Twenty-nine neurons (38.2%) showed regular (rhythmic) firing at a rate of 5.0 ± 0.6 Hz. Most rhythmic neurons (25) were recorded in the perforated-patch, two were recorded in cell-attached

and the remaining two in whole-cell mode. In these regular firing neurons the pacemaker membrane potential ranged from −52.1 ± 2.0 mV at *V*ahp to −39.0 ± 1.0 mV at the spike threshold potential. The remaining 47 (61.8%) neurons fired with an irregular (arrhythmic) pattern. Arrhythmic neurons recorded in whole-cell mode ($n = 20$) fired at a rate of 1.3 ± 0.3 Hz, the resting potential was −54.8 ± 1.8 and the firing threshold was −46.2 ± 1.3 mV. Arrhythmic neurons recorded in the perforated-patch mode ($n = 27$) fired at a rate of 2.0 ± 0.2 Hz. These neurons showed a *V*m of −46.3 ± 1.4 mV and *V*thr was −40.6 ± 2.1 mV. In 24 neurons which did not received any treatment the spontaneous firing remained stable for at least 40 min, which was the average duration of the pharmacological experiments. Firing rates of these neurons were 3.8 ± 0.6 Hz between the 5th and 10th min of recording and 4.5 ± 0.9 Hz between the 35th and 40th min of recording (Table 1).

The effects of drugs affecting the gating of the intracellular Ca^{2+} channel sensitive to ryanodine were studied in 52 neurons as follows: caffeine, $n = 12$; dantrolene, $n = 21$; 100 nM ryanodine, $n = 12$; and 80 μM ryanodine, $n = 7$. The main results are summarized in Table 1. The effects of the different drugs started ∼3 min after their addition to the extracellular solution and were stable after 6 min. Drug effects were not washed out even 15 min after replacement of the extracellular solution with fresh aCSF.

Effect of activating the RyRs

Ryanodine at 100 nM induced an increase in the spontaneous firing rate in seven of 12 neurons (Figs 1A and 2A), from 1.5 ± 0.7 to 4.0 ± 1.0 Hz ($P < 0.05$, Wilcoxon test). All responding neurons had an arrhythmic firing pattern prior to the ryanodine administration. Other effects of this activatory dose of ryanodine included (Table 1) a decrease in *V*ahp from −52.8 ± 1.7 to −44.0 ± 3.4 mV ($t = 2.98$, $P < 0.05$) and an increase in the duration of the action potential from 5.3 ± 1.1 to 11.0 ± 2.3 ms ($t = -3$, $P < 0.05$), which was due to an increase in both the τ_{spk} and τ_{ahp}. In the remaining five rhythmically firing neurons no significant changes in firing frequency were found from before (3.2 ± 0.7 Hz) to after (3.3 ± 0.8 Hz) the treatment (Fig. 2B). From the 12 neurons treated with 1 mM caffeine we found increased spontaneous firing rates in seven neurons (Fig. 2C), from 1.1 ± 0.3 to 3.0 ± 0.9 Hz ($P > 0.05$, Wilcoxon test); no other parameter was changed by the treatment. In the remaining five neurons we did found no significant change in the firing rate (Fig. 2D) from 3.5 ± 1.4 before to 3.0 ± 1.4 Hz after the treatment. Most neurons responding to caffeine ($n = 6$) showed an arrhythmic firing pattern; the remaining neuron was rhythmic.

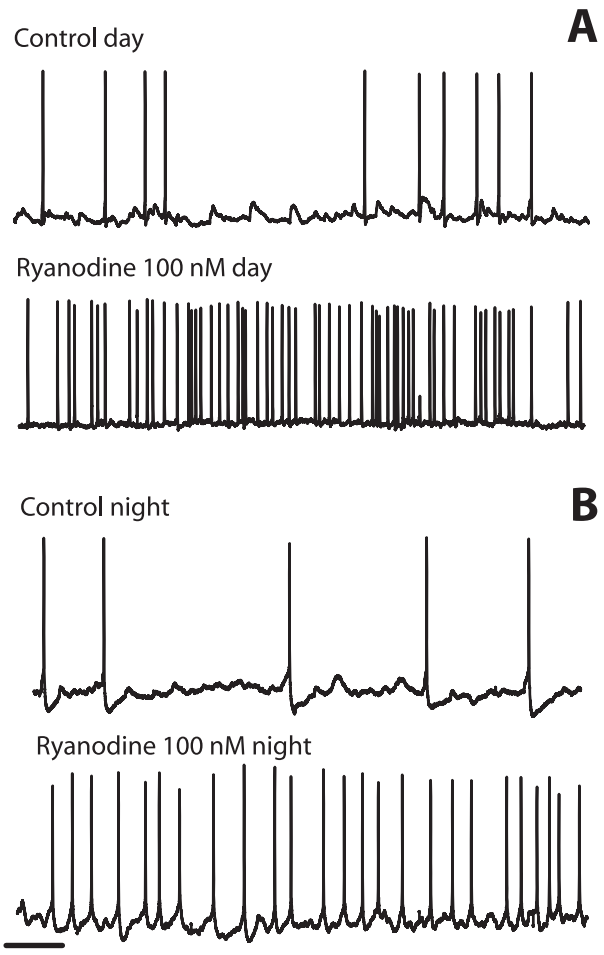

FIG. 1. Firing rate from two SCN neurons treated with 100 nM ryanodine at either (A) subjective day or (B) subjective night; at both times pharmacologically opening the RyRs led to an increase in spontaneous firing rate. Calibration bars, 10 mV and 1.0 s.

Effect of inhibiting the RyRs

Ryanodine, 80 μM, induced a decrease in the firing rate in all the seven (rhythmic) neurons tested (Figs 3A and 4C), from 5.6 ± 1.5 to 0.9 ± 0.4 Hz ($P < 0.05$, Wilcoxon test). Other effects of this inhibitory dose of ryanodine (Fig. 5A, Table 1) were a decrease in the value of *V*spk from 6.3 ± 4.1 to −5.8 ± 4.3 mV ($t = 2.8$, $P < 0.05$), a

TABLE 1. Effect of different drugs acting on the RyRs on the electrical activity from SCN neurons

	*V*m (mV)		Firing rate (Hz)		Spike duration (ms)		AHP (mV)	
Treatment	Before	After (↑/↓)	Before	After (↑/↓)	Before	After (↑/↓)	Before	After (↑/↓)
Control	−45.4 ± 1.3	−46.4 ± 1.7	3.8 ± 0.6	4.5 ± 0.9	4.5 ± 0.6	4.9 ± 0.8	−53.6 ± 1.0	−55.0 ± 1.4
Caffeine 1 mM	−48.5 ± 2.3	−49.0 ± 1.9	1.1 ± 0.3	3.0 ± 0.9*↑	5.1 ± 1.5	4.8 ± 0.9	−54.0 ± 2.4	−51.0 ± 1.9
Ryanodine 100 nM								
Day	−46.3 ± 2.9	−41.3 ± 3.2	1.5 ± 0.7	4.0 ± 1.0*↑	5.3 ± 1.1	11.0 ± 2.3*↑	−52.8 ± 1.7	−44.0 ± 3.4*↓
Night	−46.1 ± 0.5	−47.6 ± 0.6	1.9 ± 0.4	3.9 ± 0.4*↑	4.5 ± 0.1	5.4 ± 0.1*↑	−55.0 ± 0.3	−52.2 ± 0.2*↓
Dantrolene 10 μM	−47.3 ± 1.0	−54.3 ± 0.8*↑	2.4 ± 0.6	0.7 ± 0.2*↓	3.8 ± 0.8	3.6 ± 1.4	−52.0 ± 4.4	−61.7 ± 3.1*↑
Ryanodine 80 μM								
Day	−42.0 ± 1.9	−41.7 ± 1.8	5.6 ± 1.5	0.9 ± 0.4*↓	3.9 ± 1.2	4.2 ± 0.9	−54.6 ± 2.2	−43.8 ± 2.7*↓
Night	−44.0 ± 0.5	−45.0 ± 0.4	3.9 ± 1.4	2.4 ± 0.8*↓	4.0 ± 0.1	4.58 ± 0.1*↑	−56.1 ± 0.4	−48.9 ± 0.6*↓

Values are mean ± SEM. *$P < 0.05$, *t*-test, compared to baseline before treatment (↑, increase; and ↓, decrease).

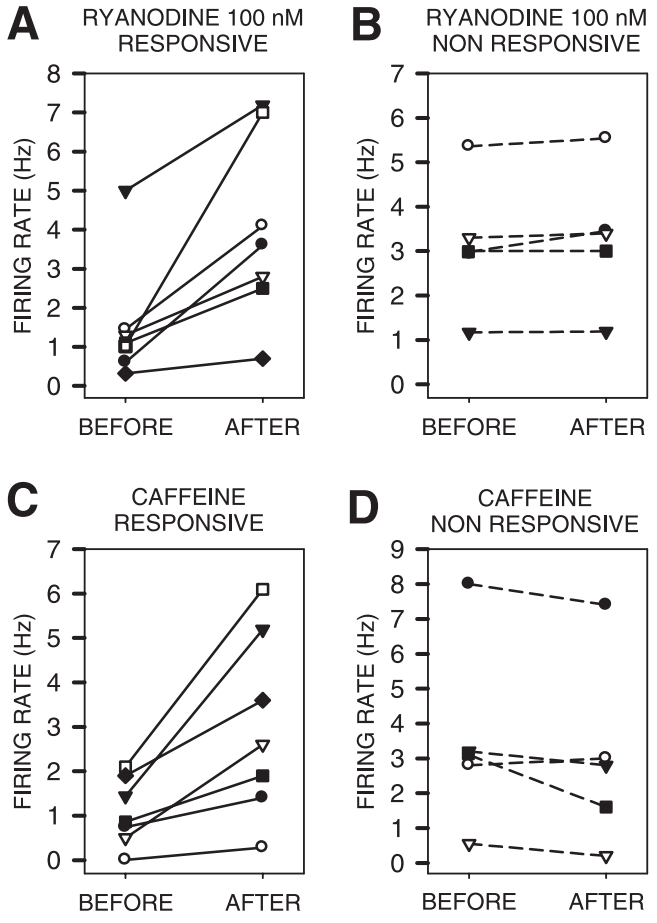

FIG. 2. Pharmacological opening the RyRs with (A) 1 mM caffeine or (C) 100 nM ryanodine increased basal firing rate in 58% of neurons tested; (B and D, respectively) the remaining neurons did not respond. The basal firing frequency partially predicted the responsiveness to either treatment.

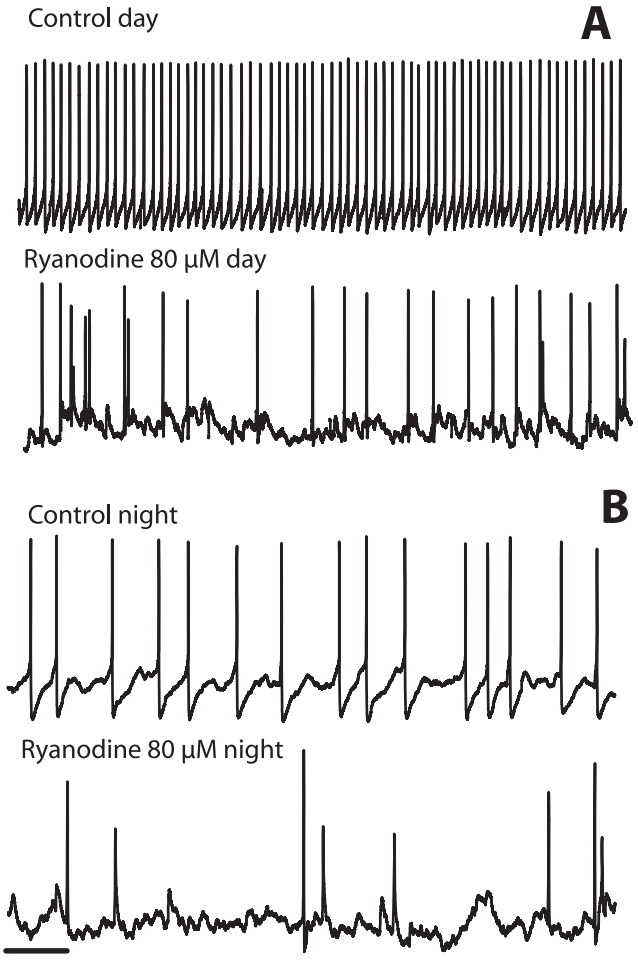

FIG. 3. Firing rate from two SCN neurons treated with 80 μM ryanodine at either (A) subjective day or (B) subjective night; at both times pharmacologically closing the RyRs led to a decrease in spontaneous firing rate. Calibration bars, 10 mV and 1.0 s.

decrease in the value of *V*ahp from −54.6 ± 2.2 to −43.8 ± 2.7 mV ($t = -3.7$, $P < 0.05$) and a change in the firing pattern from rhythmic to arrhythmic. On the other hand, in 14 (six arrhythmic and eight rhythmic) of 22 neurons receiving 10 μM dantrolene we found a decrease in the spontaneous firing rate (Fig. 4A), from 2.4 ± 0.6 to 0.7 ± 0.2 Hz. Other effects of dantrolene were (Fig. 5A, Table 1) to induce increases in *V*m from −47.3 ± 1.0 to −54.3 ± 0.8 mV ($t = 3.3$, $P < 0.05$), *V*thr from −36.0 ± 0.9 to −46.3 ± 1.3 mV ($t = 4.3$, $P < 0.05$) and *V*ahp from −52.0 ± 4.4 to −61.7 ± 3.1 mV ($t = 5.4$, $P < 0.05$), as well as a decrease in the V_{spk} from −5.7 ± 9.5 mV to −17.5 ± 10.5 mV ($t = 5.9$, $P < 0.05$). In the remaining eight (arrhythmic) neurons no significant effect was observed in firing frequency (Fig. 4B), which was 3.4 ± 0.8 before and 4.1 ± 1.4 Hz after treatment.

Effects of ryanodine during subjective night

In order to assess whether the effects of ryanodine on the firing frequency also occurred during the subjective night, the effects of both doses of ryanodine on SCN firing frequency were tested in 14 neurons during subjective night from ZT 17 to ZT 22 (one from each animal maintained in a shifted light:dark cycle as described in Materials and Methods). Consistent with the results obtained during the subjective day, in seven out of 10 neurons treated with 100 nM ryanodine, the neuronal firing frequency increased (Fig. 1B and 5B) from 1.9 ± 0.4 to 3.9 ± 0.4 Hz ($P < 0.05$, Wilcoxon test). On the other hand, all four neurons treated with 80 μM ryanodine decreased their firing rate(Fig. 3B and 5B) from 3.9 ± 1.4 to 2.4 ± 0.8 Hz ($P < 0.05$, Wilcoxon test).

Blockade of SCN synaptic activity and the effects of ryanodine

Bicuculline, DNQX and APV were applied to 28 neurons in a counterbalanced design with respect to ryanodine as follows: seven neurons received 100 nM ryanodine before the synaptic blockers, and 14 neurons received 100 nM ryanodine and seven 80 μM ryanodine after the administration of the synaptic blockers. In the presence of the receptor blockers 15 of 21 neurons treated with 100 nM ryanodine increased their firing rate (Fig. 6) from 1.1 ± 0.3 to 3.1 ± 0.6 Hz ($P < 0.05$, Wilcoxon test). Likewise, all seven neurons treated with 80 μM ryanodine in the presence of the blockers decreased their firing rate from 6.3 ± 1.6 to 3.3 ± 1.0 Hz (Fig. 7). Administration of DNQX and APV had no effect on SCN firing frequency, regardless of whether they were applied before or after ryanodine. In contrast, when administered before ryanodine (21 neurons), bicuculline increased the firing rate in nine arrhythmic neurons which became rhythmic, decreased firing rate in six neurons (four arrhythmic and two rhythmic), and did not changed the firing rate in the remaining six rhythmic neurons. It is worth noting that 100 nM ryanodine further increased the firing rate in eight of the nine neurons which had already increased their firing following application of bicuculline.

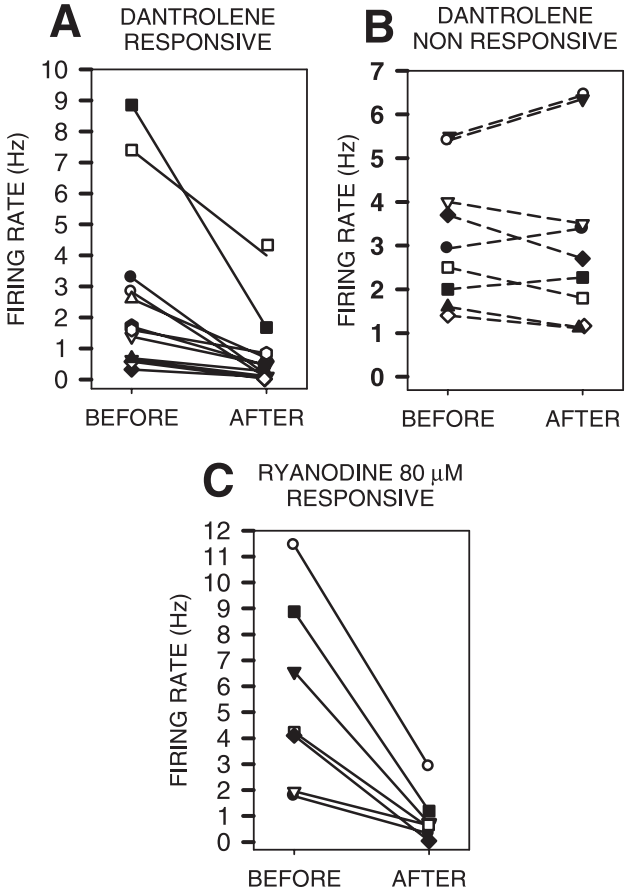

FIG. 4. Pharmacological closing of the RyRs with 10 μM dantrolene (A) decreased basal firing rate in 64% of neurons tested; (B) the remaining neurons did not respond. (C) We selected only rhythmic neurons to be treated with the inhibitory dose of ryanodine (80 μM) and all neurons decreased their firing rate.

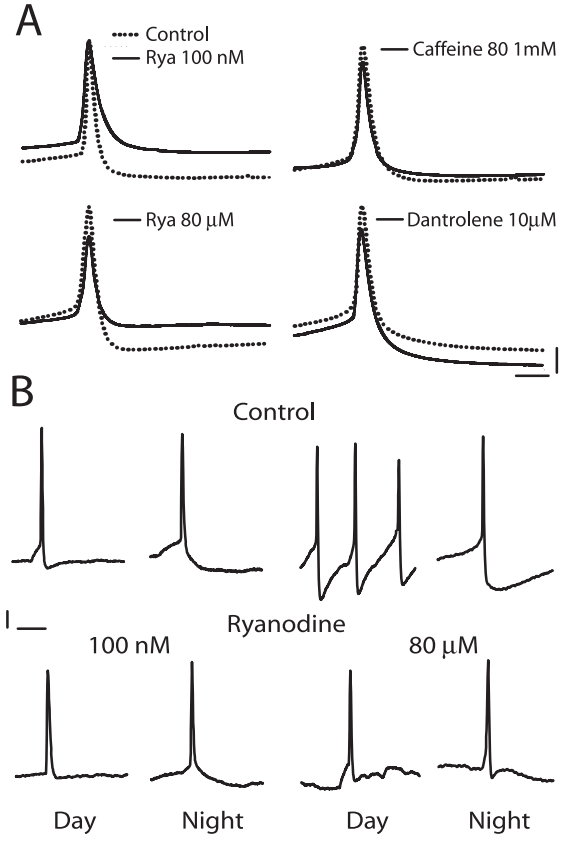

FIG. 5. Effects of different treatments on the characteristics of the action potentials. (A) Mean value ($n = 5$ neurons) of action potentials from each treatment and its control. (B) Original traces from action potentials before and after treatment with each dose of ryanodine (either 100 nM or 80 μM) administered during the day or night. Calibration bars, 10 mV, 4 ms (A), 10 mV, 25 ms (B).

Discussion

Regulation of Ca^{2+} concentration in the cytoplasm occurs by a fine-tuned set of counterbalanced mechanisms collectively known as intracellular calcium homeostasis. Intracellular calcium stores in the endoplasmic reticulum are a key component in the dynamics of $[Ca^{2+}]i$ and release from these stores depends mainly on high-conductance Ca^{2+} channels from the endoplasmic reticulum, termed RyRs and inositol (1,4,5)-triphosphate receptors (IP_3Rs) respectively (Meldolesi & Pozzan, 1998; Verkhratsky, 2005). Intracellular Ca^{2+} is an important element in diverse intracellular signalling pathways including modulation of neuronal excitability (Carafoli *et al*., 2001; Rizzuto, 2001). In the present work we tested the hypothesis that the pharmacological manipulation of the RyRs is able to modulate the neuronal firing rate in the SCN.

As predicted by our hypothesis, pharmacological manipulation of ryanodine-sensitive intracellular Ca^{2+} channels in SCN neurons *in vitro* modify their firing rate. Thus, opening the RyRs by administration of either 100 nM ryanodine or 1 mM caffeine increased the firing frequency, without effects on the firing threshold, while closing of RyRs by administration of 80 μM ryanodine or 10 μM dantrolene decreased the firing rate. These effects were induced both during daytime (ZT 4–10) and nighttime (ZT 17–22), which clearly indicates that pharmacological opening or closing of the RyRs overrides the control of the SCN neuronal firing rate by the molecular clock; this suggests that RyRs are functionally located in the pathway from the molecular clock to the membrane excitability.

Blockade of $GABA_A$ receptors with 10 μM bicuculline and activation of RyRs with 100 nM ryanodine in the SCN had similar effects, which were an increase in the spontaneous firing frequency and a change from an arrhythmic to a rhythmic firing pattern (Kononenko & Dudek, 2004). Therefore, we decided to address whether synaptic activity (either GABAergic or glutamatergic) was involved in the effects of the different doses of ryanodine on the SCN. Blockade of synaptic activity did not prevent the increase or the decrease in spontaneous firing rate induced by ryanodine at 100 nM or 80 μM, respectively. This finding indicates that ryanodine modulates the SCN firing rate by acting directly on Ca^{2+} mobilization through RyRs rather than affecting overall synaptic transmission within the SCN. Furthermore, present results confirm previous observations by Kononenko & Dudek (2003) that bicuculline increases the firing rate and changes the firing pattern in some SCN neurons; they also indicate that there is no contribution from glutamatergic synaptic activity to the spontaneous firing rate in SCN neurons.

As shown in Table 1, the neuronal responsiveness to the treatments that induced opening of the RyRs was related to basal firing frequency. Thus increases in firing rate induced by activation of the RyRs were found in neurons with a low mean firing frequency, while the opposite was found in neurons responding to drugs closing the RyRs. Nevertheless, Figs 2 and 4 also show that responsive and nonresponsive neurons showed a wide range of firing rates, suggesting that other

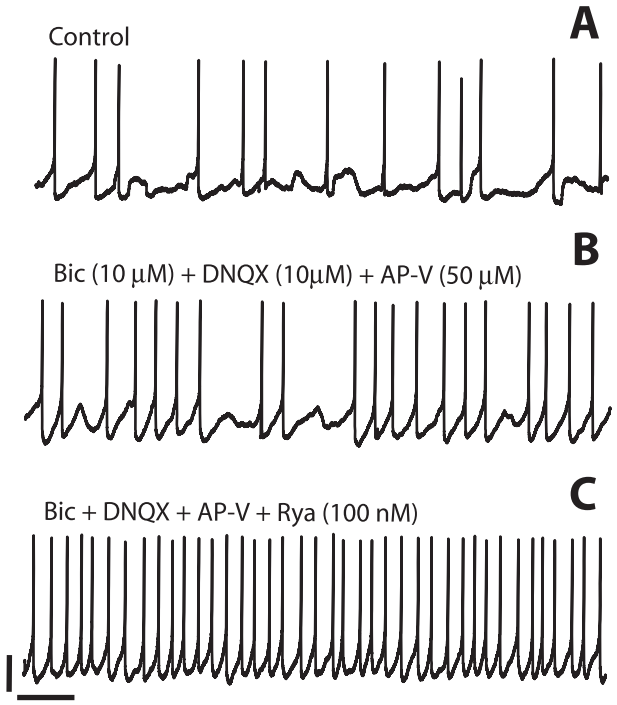

FIG. 6. Firing rate from an SCN neuron (A) before and (B) after treatments with a cocktail containing 10 μM bicuculline (Bic), 10 μM DNQX and 50 μM AP-V, and (C) the previous cocktail with 100 nM ryanodine (Rya) added. Calibration bars, 10 mV and 1.0 s.

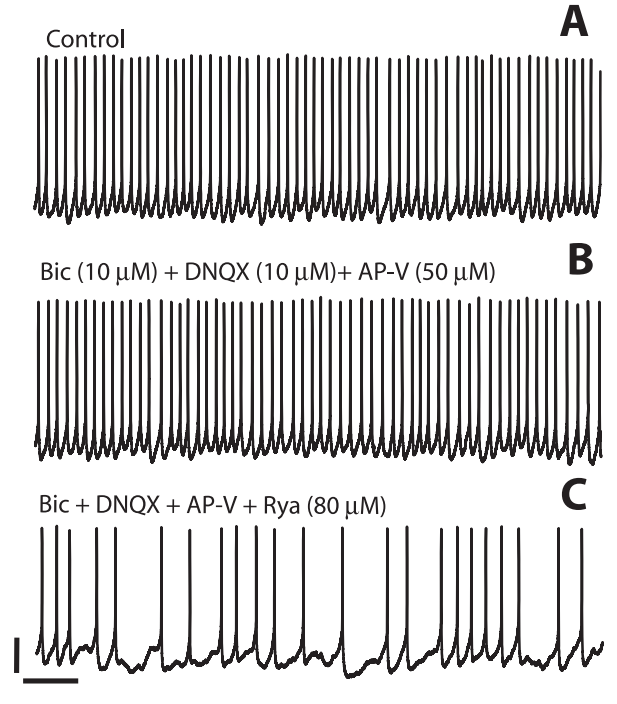

FIG. 7. Firing rate from an SCN neuron (A) before and (B) after treatments with a cocktail containing 10 μM bicuculline (Bic), 10 μM DNQX and 50 μM AP-V, and (C) the previous cocktail with 80 μM ryanodine (Rya) added. Calibration bars, 10 mV and 1.0 s.

factors are involved in neuronal responsiveness to ryanodine. Activation of RyRs by caffeine or 100 nM ryanodine increased firing rates in neurons with irregular firing patterns, whereas neurons which did not respond already showed a rhythmic pattern before the treatment. On the other hand, inhibition of RyRs with dantrolene decreased firing rate, mostly in neurons showing a regular firing pattern before treatment, whereas those neurons which did not respond to dantrolene already had an irregular pattern. Based on these observations, we applied 80 μM ryanodine only to those neurons showing a regular firing pattern before treatment, and found both a decrease in the firing rate and a change to an irregular firing pattern in 100% of the tested neurons.

Although caffeine at μM doses is also an antagonist of adenosine receptors (Fredholm *et al*., 1999), which are present in the SCN (Watanabe *et al*., 1996; Antle *et al*., 2001; Elliot *et al*., 2001), *in vitro* studies on the effect of agonists and antagonists to A_1 and A_{2A} adenosine receptors suggest that caffeine will not by itself have any effect on the spontaneous SCN activity, but rather would prevent the effects induced by activation of presynaptic A_1 adenosine receptors (Chen & van den Pol, 1997; Hallworth *et al*., 2002). The effects of caffeine (1 mM) found in this study are mainly due to its effects on the RyRs, as 100 nM of ryanodine induced a similar increase in firing rate. Nevertheless, the differential effects on the spike duration and the value of the afterhyperpolarization potential (AHP) found between 1 mM caffeine and 100 nM ryanodine indicates that the present dose of caffeine may involve other mechanisms of action.

Further studies are needed to characterize the ionic currents involved in the effects previously described, but those involved in AHP which is characteristic of regular firing neurons are of particular interest. The role of ryanodine-sensitive intracellular Ca^{2+} stores on the generation of the AHP in SCN neurons was discounted in a previous study (Cloues & Sather, 2003). However, the dose of ryanodine used (10 μM) did not inhibit the RyRs, but rather induced a subconductance state characterized by a long-term open state of the channel, which led to an eventual depletion of internal calcium stores (Chu *et al*., 1990). The use of 80 μM ryanodine to effectively block RyRs used in this study clearly abolished the AHP of rhythmic SCN neurons and indicates AHP modulation by intracellular Ca^{2+} mobilized through RyRs. Similar dependence on RyR activity was observed in the onset of plateau potentials and wind-up in spinal motoneurons (Mejía-Gervacio *et al*., 2004) On the other hand, the role of Ca^{2+} inward current through L- and R-type voltage gated Ca^{2+} channels on driving the pacemaker firing rate is still controversial: from studies in brain slices it has been suggested that modulation of these channels regulates AHP and thus the firing rate (Pennartz *et al*., 2002; Cloues & Sather, 2003), whereas studies from dissociated SCN neurons indicate only a minor contribution from inward Ca^{2+} currents in these processes (Jackson *et al*., 2004). Present data are consistent with a role for intracellular Ca^{2+} mobilization through RyRs, acting in concert with plasmatic membrane Ca^{2+} currents in regulating both the AHP and the firing rate in SCN neurons, probably via large- and small-conductance Ca^{2+}-dependent K^+ (K_{Ca}) channels (i.e. BKCa- and SKCa-type, respectively), and apamine- and iberotoxin-insensitive K_{Ca} channels (Cloues & Sather, 2003; Teshima *et al*., 2003). However, inactivation of L- and R-type Ca^{2+} channels by intracellular calcium concentration (reviewed in Eckert & Chad, 1984) can also play a role in the firing rate as well as the dynamics of the action potential. Furthermore, the role of intracellular Ca^{2+} in the firing-rate modulation of SCN is not a simple one, as indicated by the increase in the duration of the action potential and the decrease in I_{AHP} induced by 100 nM ryanodine. These results suggest the modulation of other currents besides those involved in the AHP; this could be related to the fact that, in addition to directly modulating the activity of Ca^{2+}-sensitive channels, such as Ca^{2+}-activated K^+ and Cl^- channels (Faber & Sah, 2003 and Frings *et al*., 2000) and inactivation of L- and R-type Ca^{2+}

channels (Eckert & Chad, 1984), cytoplasmic calcium can also regulate the gating of plasma membrane ion channels by influencing the equilibrium between phosphorylated and dephosphorylated channels via Ca^{2+}–calmodulin kinases and phosphatases (Kortvely & Gulya, 2004). In addition, cytoplasmic calcium may also alter physical properties of phospholipids which influence membrane fluidity (Simkiss, 1998) and, in the longer term, activate transcriptional programs (Konur & Ghosh, 2005).

The role of intracellular Ca^{2+} in the regulation of circadian rhythms in SCN neurons has been previously suggested by independent groups. Glutamate and serotonin can elicit $[Ca^{2+}]$i oscillations in SCN neurons and glia even in the absence of extracellular Ca^{2+}, suggesting that the stimulus-induced Ca^{2+} mobilization derives mainly from intracellular stores (Van den Pol *et al*., 1992). Díaz-Muñoz *et al*. (1999) demonstrated circadian regulation of type 2 RyRs in SNC neurons, and Ding *et al*. (1998) showed that light- and glutamate-induced phase delays during early subjective night are dependent on RyR activity. Colwell (2000) suggested that cytoplasmic Ca^{2+} rhythms were driven by voltage-sensitive Ca^{2+} channels. Recently two groups reported a role for transmembrane Ca^{2+} flux to sustain the rhythmic clock gene expression in the SCN (Lundkvist *et al*., 2005; Sang-Soep *et al*., 2005). Intracellular calcium dynamics involve both Ca^{2+} influx through plasma membrane Ca^{2+}-dependent channels and Ca^{2+} efflux from internal deposits (Carafoli *et al*., 2001). Accordingly, Ikeda *et al*. (2003) blocked or decreased the amplitude in the neuronal activity rhythm without affecting the rhythm of cytoplasmic Ca^{2+} concentration. The independence between voltage-sensitive Na^{+} and Ca^{2+} membrane currents and the intracellular Ca^{2+} rhythm, and the decrease in amplitude of both rhythms by RyR blockers, suggests that Ca^{2+} mobilized through these channels might directly link the core oscillator with the electrical activity rhythm. However, Honma & Honma (2003) have suggested that cytosolic Ca^{2+} might only indirectly regulate the firing rate in SCN neurons due to the 4-h phase delay between the intracellular Ca^{2+} and the electrical firing rhythms in the same neuron found by Ikeda *et al.* (2003). Present data demonstrate that the SCN neuronal firing rate can be regulated by intracellular Ca^{2+} mobilized through RyRs. It remains to be established whether the circadian variation in the number of RyRs present in SCN neurons is the only process under regulation of the clock core, or whether other elements involved the gating of RyRs are also circadian-regulated, such as metabolic signals, regulatory proteins and plasmatic membrane channels interacting with the endoplasmic reticulum (Fill & Copello, 2002; Meissner, 2002; Butanda-Ochoa *et al*., 2003). Any of these elements is likely to also be under control of the clock genes, and therefore to be a part of their output pathway. On the other hand, although we did not find circadian modulation in inositol (1,4,5)-triphosphate receptors (IP_3Rs) in the rat brain (Díaz-Muñoz *et al*., 1999), coordination between IP_3Rs and RyRs has been demonstrated in other systems (Morales-Tlalpan *et al*., 2005), and it is necessary to evaluate its contribution to the regulation of cytoplasmic Ca^{2+} in SCN neurons. Finally, intracellular Ca^{2+} is able to control neuronal excitability at many levels (Berridge, 1998; Morikawa *et al*., 2003) and, as pointed out by Honma & Honma (2003), it is not possible to rule out *a priori* the contribution of these processes on the clock output.

In conclusion, we propose that RyRs are key elements in the transmission of the circadian oscillation from the transcription–translation loop of clock genes to the membrane excitability in the SCN neurons, which in turn would send a circadian modulated firing pattern to other brain areas involved in behavioural rhythm expression. Intracellular Ca^{2+} mobilization through RyRs may affect neuronal excitability, directly through Ca^{2+}-modulated plasma membrane channels and indirectly as a second messenger activating Ca^{2+}-dependent protein kinases and phosphatases regulating a variety of cellular processes converging at the cell membrane. In the long term, intracellular calcium fluctuations may impact on membrane potential by controlling, at the transcriptional level, the synthesis of new ion channels and receptors. Further studies are needed to continue unraveling the role of other elements of cellular Ca^{2+}homeostasis in this process.

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Abbreviations

$[Ca^{2+}]$i, cytoplasmic calcium levels; AHP, afterhyperpolarization potential; AP-V, DL-2-amino-5-phosphonopentanoic acid; DNQX, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione; RyR, ryanodine receptor; SCN, suprachiasmatic nuclei; τ_{ahp}, decay time of the action potential; τ_{spk}, rise time of the action potential; *V*ahp, membrane potential at the lowest point of the afterhyperpolarization; *V*m, membrane potential 20 ms before the spike; *V*spk, membrane potential at the peak of the spike; *V*thr, membrane potential at the threshold of the spike; ZT, Zeitgeber time.

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