FACULTAT DE FARMÀCIA

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DISSOCIACIÓ DELS RITMES CIRCADIARIS I TRACTAMENTS PER AL SEU ACOBLAMENT EN RATES SOTMESES A CICLES DE LLUM-FOSCOR DE 22 I 23 HORES

Montserrat Anglès Pujolràs

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IV. EXPERIMENTS

Tot i que l'ordre cronològic de la realització dels experiments no es correspon amb l'ordre amb el qual es presenten en la memòria, hem considerat oportú, per a una millor comprensió de la tesi, exposar aquest treball estructurant-lo en els següents apartats:

Apartat I: La dissociació en diverses variables fisiològiques
Apartat II: Influències ambientals durant l'alletament en la inducció de la dissociació
Apartat III: Manipulacions exògenes dels ritmes circadiaris del patró dissociat

Apartat I: **LA DISSOCIACIÓ EN DIVERSES VARIABLES FISIOLÒGIQUES**

EXPERIMENT 1: LA DISSOCIACIÓ EN L'ACTIVITAT MOTORA, LA TEMPERATURA I LA CONDUCTA DE BEGUDA, SOTA CICLES DE 22 I 23 HORES

INTRODUCCIÓ

En els mamífers, el nucli supraquiasmàtic de l'hipotàlem (NSQ) és l'estructura del sistema nerviós encarregada de generar i mantenir els ritmes de les diferents variables de l'organisme. Està format per dos nuclis localitzats bilateralment per sobre del quiasma òptic i laterals al tercer ventricle (Van den Pol, 1980). A cada un dels nuclis s'hi poden diferenciar dues parts: una part ventrolateral (VL) i una part dorsomedial (DM) (Moore *et al.*, 2002). La primera es caracteritza perquè el seu neurotransmissor (NT) principal és el pèptid intestinal vasoactiu (Card *et al.*, 1981) i perquè és una zona que rep aferències de la retina a través del tracte retino-hipotalàmic. Les neurones de la zona DM tenen la vasopressina com a NT principal i aquesta és una zona que rep aferències de la zona VL (Abrahamson i Moore, 2001). Actualment es creu que el sistema circadiari està format per un conjunt d'oscil·ladors que actuen de forma acoblada generant un ritme únic (Díez-Noguera, 1994); d'aquesta manera podem suposar que cada una de les parts del NSQ també està formada per un conjunt d'oscil·ladors.

En la natura, els organismes es troben sota condicions ambientals que varien periòdicament. L'alternança entre el dia i la nit, o dit d'altra manera, entre llum i foscor es converteix en el *zeitgeber* per excel·lència, ja que fa que els organismes encarrilin amb el medi ambient; és a dir, que manifestin els seus ritmes circadiaris amb el mateix període que l'entorn.

Si sotmetem un animal a cicles de llum-foscor de 22 o 23 hores el ritme d'activitat motora es dissocia en dos components: un component dependent de la llum (LDC) i un component, de naturalesa endògena, que va en curs lliure i que anomenem component no dependent de la llum (NLDC) (Campuzano *et al.*, 1998). S'ha demostrat que el LDC ve dirigit per la part VL del NSQ i que el NLDC per la regió DM (de la Iglesia *et al.*, 2004).

El fenomen de la dissociació no és comú a totes les espècies ja que, fins ara, no s'ha descrit ni en ratolins ni en hàmsters, per citar algunes de les espècies més utilitzades en el laboratori. Pel que fa a l'espècie humana, en condicions d'aïllament o de desincronització forçada, s'ha observat desincronització entre el ritme de son-vigília i el ritme de temperatura corporal (Czeisler *et al.*, 1999; Wever, 1979).

Fins ara, la dissociació de ritmes només s'havia estudiat en la variable activitat motora, però succeeix el mateix amb la resta de variables de l'organisme?

Per tal de donar resposta a aquesta pregunta vam emprar el model animal de dissociació per comprovar si altres variables, com la temperatura o la conducta de beguda també presentaven dissociació quan els animals eren sotmesos a aquestes condicions lumíniques.

OBJECTIU

L'objectiu d'aquest experiment és, d'una banda, observar si el fenomen de la dissociació es presenta en altres variables diferents de l'activitat motora (AM), comparant el ritme d'aquesta, amb el de temperatura (TEMP) i amb el de conducta de beguda (B), i per l'altra, esbrinar si hi ha diferències en la manifestació dels dos components del patró dissociat (LDC i NLDC), per part de les diverses variables, la qual cosa suposaria l'existència de desincronització interna. El nostre interès està també en deduir si els ritmes d'AM, de TEMP i de B vénen dirigits per la mateixa zona o per diferents parts del NSQ.

MATERIAL I MÈTODES

Per fer aquest experiment es van utilitzar 46 rates Wistar, de dos mesos d'edat a l'inici de l'experiment, distribuïdes en diferents grups en els quals la meitat dels animals eren mascles i l'altra meitat eren femelles. En funció del grup al qual pertanyien, van estar sotmesos a condicions d'il·luminació de T22 (LD 11:11), T23 (LD 11,5:11,5) o T24 (LD 12:12). Durant les etapes de llum, es van utilitzar dues làmpades fluorescents que projectaven una intensitat de 300 lux sobre les gàbies. Les etapes de foscor es van il·luminar amb una làmpada de llum vermella que produïa una intensitat inferior a 0,1 lux.

L'AM dels animals es va mesurar mitjançant actímetres de llum infraroja i es va enregistrar cada 5 o 15 minuts segons el grup d'animals (veure més endavant). El registre de TEMP dels animals es va dur a terme, cada 15 o 30 minuts, segons el grup d'animals, utilitzant sensors de temperatura (*Thermochron iButton*®, IDC SA Spain). Aquests dispositius es van implantar a través d'una petita incisió a la paret abdominal de l'animal, prèvia anestèsia amb una injecció ip de ketamina (50mg/kg) – xilacina (5mg/kg). La conducta de beguda es va enregistrar cada 5 minuts amb un aparell col·locat en l'abeurador i que, mitjançant un feix de llum infraroja, detecta cada vegada que l'animal beu.

Per conveniència, aquest experiment va ser realitzat en dues etapes. En la primera es van utilitzar 26 animals, dels quals 8 van ser sotmesos a T22, 8 a T23 i 10 a T24, durant 56 dies i després van estar 8 dies en foscor constant. En aquests animals, es van analitzar les variables AM i TEMP, durant tot l'experiment. La TEMP va ser enregistrada cada 30 minuts i l'AM cada 15 minuts. En la segona etapa, es van utilitzar 20 rates, 12 sotmeses a T22 i 8 a T24. Aquests animals, al cap de 22 dies de l'inici de l'experiment, van ser sotmesos a un canvi de fase (avançament de 5 hores) i el dia 65 se'ls va passar a condicions de DD. En aquests animals se'ls va mesurar l'AM i la B en LD i DD, i la TEMP únicament en LD. Les dades d'AM i de B en aquesta segona part van ser

acumulades cada 5 minuts i les de TEMP es van enregistrar cada 15 minuts.

Durant tot l'experiment, els animals van tenir accés lliure a aliment i a aigua.

Anàlisi de les dades

Tot l'experiment es va realitzar amb rates Wistar de la mateixa edat, i les condicions de manteniment i d'il·luminació també van ser les mateixes en les dues etapes. Atès que les condicions experimentals es repeteixen en ambdues parts, sempre que ha estat possible s'han analitzat i presentat els resultats de les dues etapes de l'experiment en conjunt. Les dades emprades en cada càlcul s'indiquen explícitament.

S'ha analitzat:

- **Període i percentatge de variança explicat pels ritmes:** Mitjançant el periodegrama de Sokolove i Bushell es van calcular els períodes i els percentatges de variança explicats pels ritmes d'AM, de TEMP i de B, per cada grup d'animals: T22 (n=20), T23 (n=8) i T24 (n=18), per l'etapa LD (23 cicles) i DD (8 cicles). Per a la realització d'aquest càlcul s'han utilitzat les dades d'ambdues parts de l'experiment.

- **Sortida de la fase d'activitat en DD, referida al darrer cicle LD:** Es va calcular la fase de sortida en DD, referida a l'últim cicle LD, de cada variable i per cada grup d'animals: T22 (n=19), T23 (n=8) i T24 (n=18). El procediment va ser el següent: es va dibuixar, per cada animal, una doble-gràfica a mòdul T i després es va projectar una línia recta per tal d'extrapolar l'inici de l'activitat des dels 10 últims cicles en DD cap a l'últim cicle en LD. L'agrupació de fases es va estudiar amb el test de Rayleigh, el qual indica la distribució temporal de les fases individuals al voltant del cicle T. Mitjançant aquest càlcul es pretenia observar l'existència de diferències entre grups d'animals i variables.

  Les dades emprades procedeixen de les dues etapes de l'experiment.

- **Efecte del canvi de fase:** Per comparar l'efecte del canvi de fase en les 3 variables estudiades es va fer un ajust de les dades a una sinusoide i es va determinar el moment de l'acrofase. Es van comptabilitzar els dies que tardava cada animal a tornar a estabilitzar la fase del ritme per cada variable i es van comparar els resultats obtinguts pels grups T22 (n=12) i T24 (n=8).

  En aquest cas, únicament es van utilitzar dades de l'etapa 2.

- **Relació entre les diferents variables en les quatre situacions que es presenten en la dissociació:** A l'hora d'estudiar la relació entre les diferents variables en la dissociació, hem de tenir en compte les 4 situacions (Figura IV-1) que es generen en base a la interacció entre l'activitat i el repòs del LDC i del NLDC, i en les quals la relació entre variables pot ser diferent:

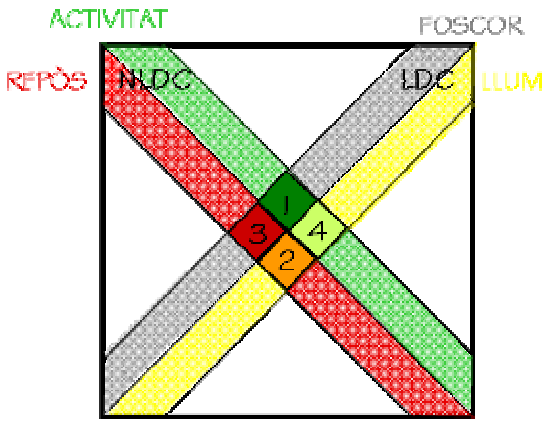

Figura IV-1. Esquema de les 4 situacions generades pel creuament dels dos components en un patró de dissociació.

Situació 1 (S1): Coincidència entre les fases d'activitat generades pel LDC i pel NLDC (doble nit).

Situació 2 (S2): Coincidència entre les fases de repòs generades pels dos components (doble dia).

Situació 3 (S3): Coincidència entre la fase d'activitat generada pel LDC i la fase de repòs generada pel NLDC (nit LDC - dia NLDC).

Situació 4 (S4): Coincidència entre la fase de repòs generada pel LDC i la fase d'activitat generada pel NLDC (dia LDC - nit NLDC).

Cal recordar que, degut al caràcter nocturn de l'espècie estudiada, durant la nit presentarà activitat i durant el dia, repòs.

Per cada una de les situacions es va calcular els valors respecte la mitjana de cada variable.

Per estudiar la relació entre TEMP i AM i entre B i AM de cada animal, es van expressar les dades de cada fase de TEMP i B com a desviació respecte la mitjana, i les dades d'AM es van transformar en percentil del màxim del total d'activitat (10 nivells d'AM). De la mateixa manera es va estudiar la relació entre TEMP i B, transformant, en aquest cas, els valors de B en percentil del màxim. Aquesta anàlisi es va fer pels grups d'animals sotmesos a T22, T24 i DD. Cal tenir en compte que, en les dues últimes condicions, únicament es generen dues situacions (nit i dia).

Anàlisi de l'espectre de potències d'activitat motora, de conducta de beguda i de temperatura a T24: Per cada animal del grup T24 de l'etapa 2 de l'experiment (n=8), es va calcular l'espectre de potències, mitjançant una anàlisi de Fourier (10 harmònics). Es va considerar el contingut de potència del primer harmònic (PCH1) com a component circadiari i els continguts de potència dels harmònics 4-10 (PCH4 - PCH10), com a components ultradiaris. Es va calcular la proporció entre el component circadiari i els components ultradiaris.

L'anàlisi de les dades es va fer mitjançant el programa El Temps© (A. Díez-Noguera, Universitat de Barcelona, 1998-2006, http://www.el-temps.com). L'anàlisi estadística es va realitzar mitjançant el programa SPSS® i va constar d'una ANOVA de diversos models lineals, considerant com a variables independents el sexe de la rata, l'etapa de l'experiment i el grup T.

RESULTATS

Període i percentatge de variança explicat pels ritmes: En l'etapa LD, per les tres variables, els animals del grup T22 (Figura IV-2) van mostrar, en els periodegrames, dos pics significatius corresponents als dos components de la dissociació. Els animals del grup T24 (Figura IV-4), a l'estar completament encarrilats al cicle LD, únicament van mostrar un pic, corresponent al LDC. El grup T23 (Figura IV-3) va esdevenir una situació intermitja entre les dues anteriors, per aquest motiu, els animals d'aquest grup o bé no van mostrar el NLDC o bé ho van fer d'una manera molt feble.

Tant en T22 com en T23, no es van observar diferències estadísticament significatives en el període del NLDC entre les tres variables estudiades, però sí que el període va ser diferent entre T22 i T23, tal i com s'esperava. Pel que fa als %V, els animals del grup T22 presenten, per l'AM un LDC més estable, mentre que per la TEMP, és el NLDC el que presenta un major valor de %V. Aquesta tendència es confirma al calcular la relació $\%V_{LDC}/\%V_{NLDC}$, que en aquests animals va ser major per la variable AM (1,23±0,10) que per la TEMP (1,03±0,22) ($p<0,05$; t-Student dades aparellades), la qual cosa no va succeir en el grup T23, que va tenir un valor de $\%V_{LDC}/\%V_{NLDC}$ per la variable AM de 2,93±0,32, i per la variable TEMP de 3,54±1,21. L'anàlisi estadística de la relació $\%V_{LDC}/\%V_{NLDC}$ entre AM i B va mostrar diferències significatives ($p<0,05$; t-Student dades aparellades), en el grup T22, a diferència de la comparació entre B i TEMP pels animals del mateix grup.

Per l'etapa DD, es va comparar el període i el %V del component en curs lliure per les tres variables i els tres grups. Es van trobar diferències estadísticament significatives en el període ($p<0,005$), essent el valor del grup T22 (24,43±0,04h) superior al valor del grup T24 (24,27±0,03h). El %V del ritme va resultar ser significativament diferent en funció de la variable estudiada ($p<0,005$), pels 3 grups; en aquest cas, la variable TEMP va mostrar els valors més alts (63,80±2,27), l'AM els valors intermitjos (34,43±1,01) i la B, els valors més baixos (22,97±1,39).

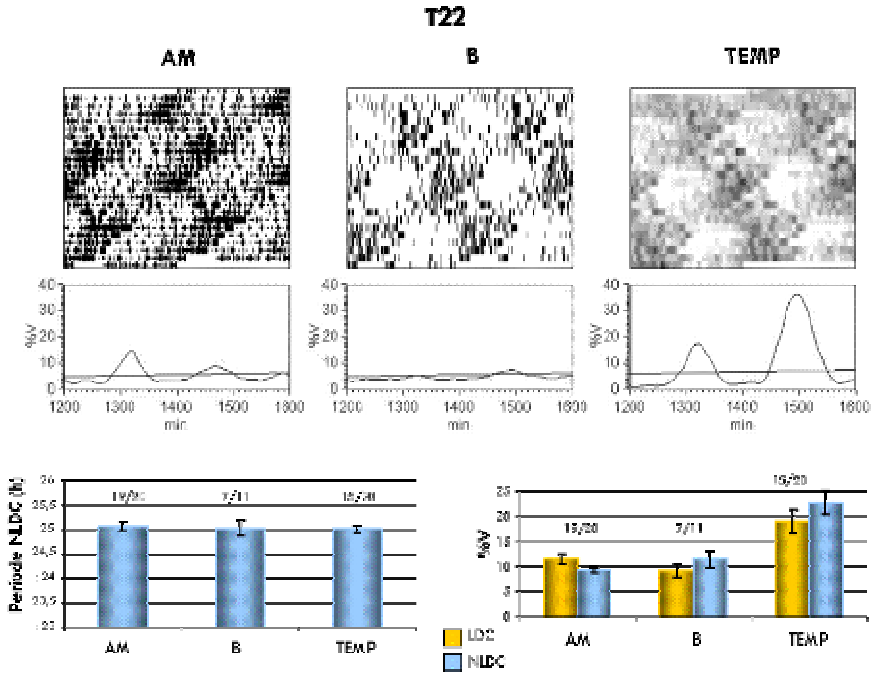

Figura IV-2. Part superior: Actogrames i periodegrames d'un animal representatiu del grup T22, per cada variable estudiada. Part inferior: Període del NLDC (mitjana ± error) (esquerra) i %V del LDC i del NLDC (dreta) per cada variable estudiada, pels animals del grup T22. A la part superior de cada barra s'indica el nombre d'animals sobre el total que s'han utilitzat per a cada càlcul (no s'han comptabilitzat les dades inferiors al nivell de significació, i s'han descartat les dades dels animals amb registres erronis).

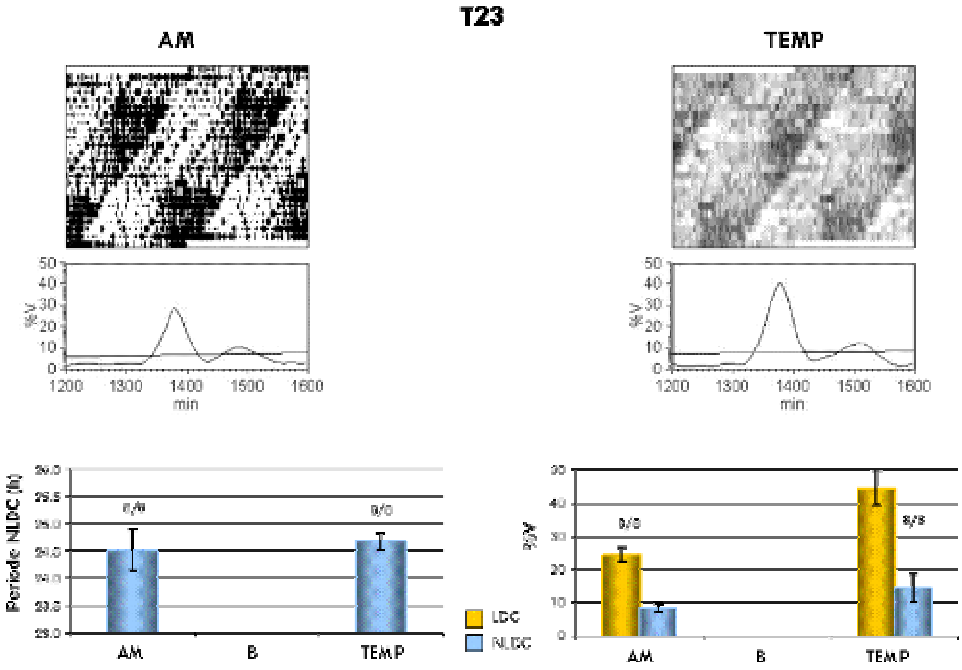

Figura IV-3. Part superior: Actogrames i periodegrames d'un animal representatiu del grup T23, per cada variable estudiada. Part inferior: Període del NLDC (mitjana ± error) (esquerra) i %V del LDC i del NLDC (dreta) per cada variable estudiada, pels animals del grup T23. A la part superior de cada barra s'indica el nombre d'animals sobre el total que s'han utilitzat per a cada càlcul (no s'han comptabilitzat les dades inferiors al nivell de significació, i s'han descartat les dades dels animals amb registres erronis).

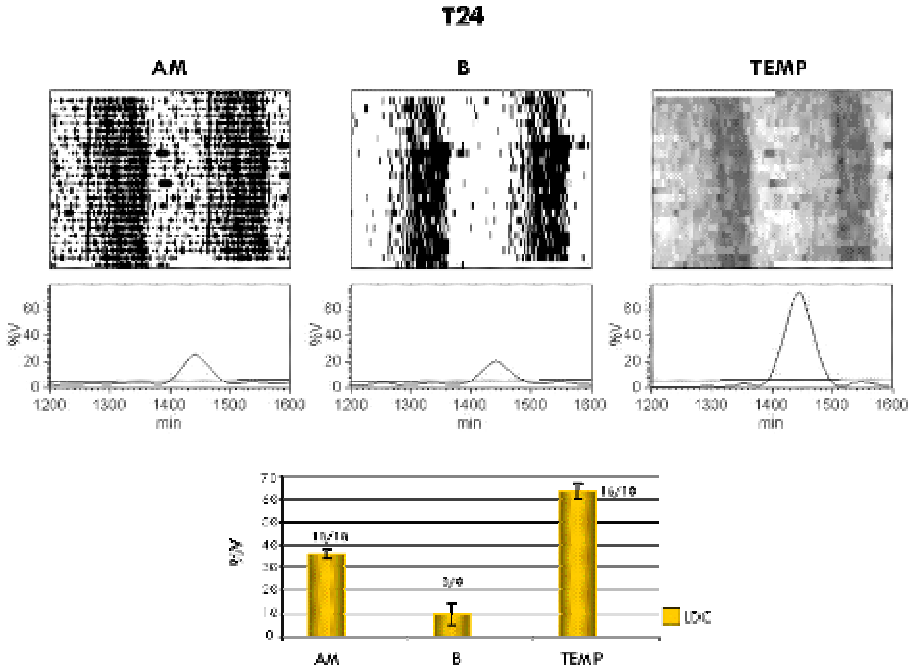

Figura IV-4. Part superior: Actogrames i periodegrames d'un animal representatiu del grup T24, per cada variable estudiada. Part inferior: %V del LDC per cada variable estudiada, pels animals del grup T24. A la part superior de cada barra s'indica el nombre d'animals sobre el total que s'han utilitzat per a cada càlcul (no s'han comptabilitzat les dades inferiors al nivell de significació, i s'han descartat les dades dels animals amb registres erronis).

Sortida de la fase d'activitat en DD, referida al darrer cicle LD: Per cada una de les variables estudiades es va calcular la fase de sortida en DD i, mitjançant un Test de Rayleigh, es va analitzar l'agrupació de fases a l'inici (I) i al final (F) de la fase d'activitat (Figura IV-5).

El grup d'animals sotmesos a cicles de 22 hores és el que va mostrar menor agrupació de fases, tant a l'inici com al final de la fase d'activitat, per les tres variables estudiades, en contraposició del grup T24 que va ser qui en va mostrar major agrupació.

Al comparar les agrupacions de fase en funció de la variable, s'observa que tots els tests resulten significatius, excepte per la variable TEMP en T22 I, T22 F i T23 F.

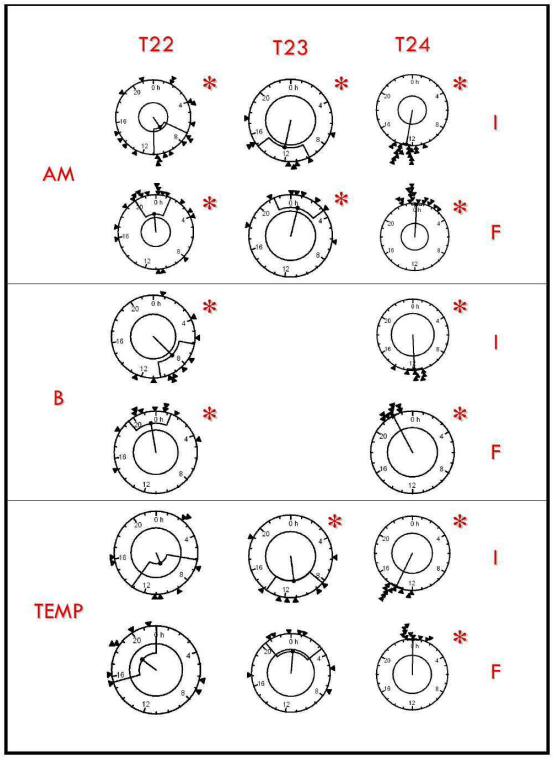

Figura IV-5. Test de Rayleigh pel conjunt d'animals de cada grup i per cada variable estudiada. El cercle interior marca la significació per una p=0,05. I, indica l'agrupació de fases de l'inici de la fase d'activitat; F, indica l'agrupació de fases del final de la fase d'activitat; *, assenyala els tests significatius.

Efecte del canvi de fase: En el grup T22, degut a la interferència dels dos components, no va ser possible comptabilitzar els dies que tardava cada animal a restablir el ritme de cada variable. En el grup T24 no es van observar diferències en el temps emprat, en cada una de les variables, per recuperar el ritme inicial, essent 8 dies el temps mitjà invertit per cada una d'elles.

Relació entre les diferents variables en les quatre situacions que presenta la dissociació: Es van calcular els valors mitjans per AM, B i TEMP en cada una de les 4 situacions, en funció del la interacció entre activitat i repòs dels dos components.

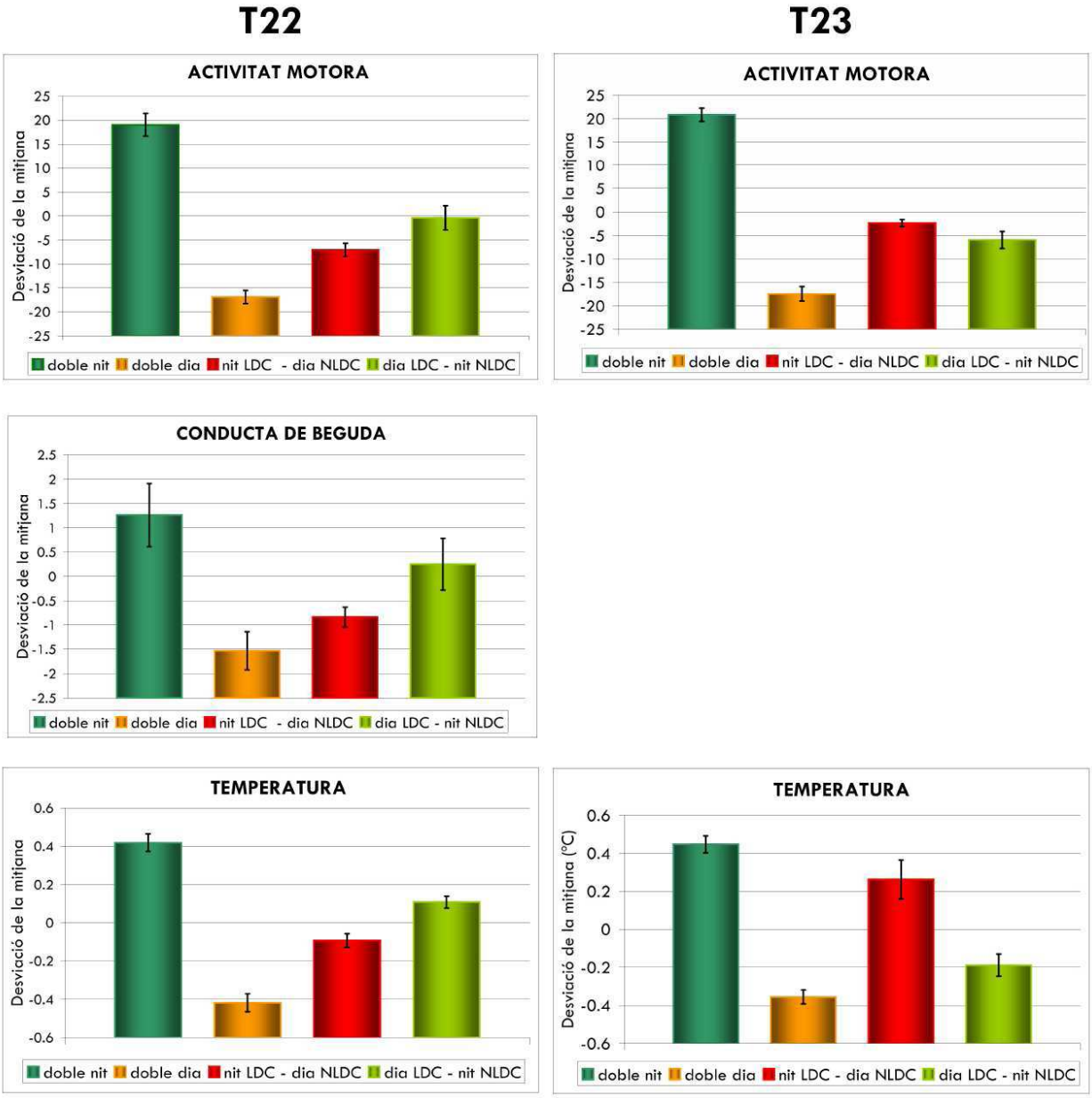

Figura IV-6. Valors mitjans (i error estàndard) d'activitat motora, conducta de beguda i temperatura per cadascuna de les 4 situacions analitzades en els animals sotmesos a T22 (esquerra) i T23 (dreta).

En el grup T22 (Figura IV-6, esquerra), per totes les variables estudiades, el valor de la mitjana en la situació 1 va resultar ser el més elevat, mentre que la situació 2 va ser la que presentava els valors més baixos. Les situacions 3 i 4 sempre van ser situacions intermitges. L'anàlisi estadística de les 4 situacions (model lineal amb mesures repetides) va mostrar diferències entre les S3 i S4 únicament per la variable TEMP, essent la S4 superior que la S3.

En el cas dels animals sotmesos a cicles de 23 hores (Figura IV-6, dreta), en ambdues variables, la S1 va ser la que va mostrar valors més alts, la S2 la que els va mostrar més baixos i S3 i S4 van mostrar valors intermitjos, no observant-se diferències estadísticament significatives amb un model lineal amb mesures repetides calculat entre aquestes dues últimes situacions (per l'AM, p=0,181 i per la TEMP, p=0,084). En el cas dels animals sotmesos a cicles de 24 hores, i també en les etapes de foscor constant (Figura IV-7), únicament es presenten dues situacions: la nit i el dia.

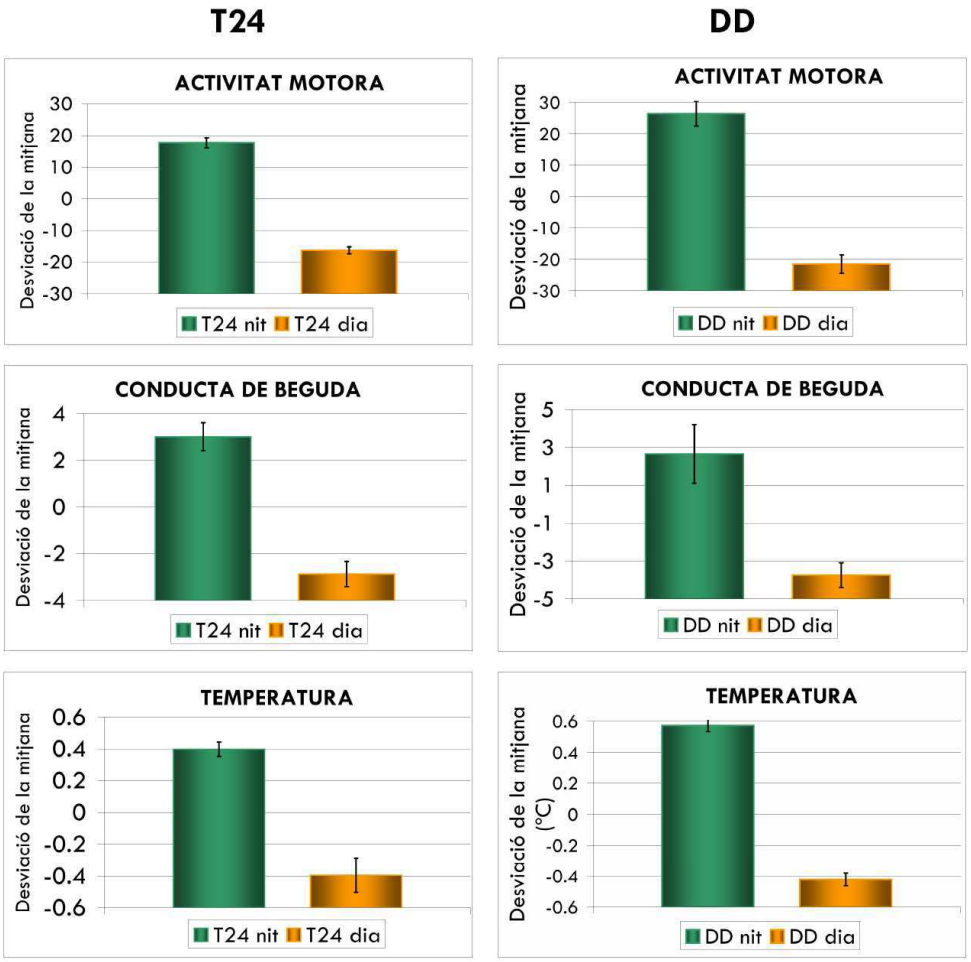

Figura IV-7. Valors mitjans (i error estàndard) d'activitat motora, conducta de beguda i temperatura per les 2 situacions analitzades en T24 (esquerra) i DD (dreta).

Tant en T24 com en DD, en les tres variables hi van haver diferències estadísticament significatives ($p<0,05$) entre la situació nit i la situació dia, tenint la primera, un valor superior de desviació respecte a la mitjana que la segona. Cap variable va mostrar diferències estadísticament significatives entre T24 i DD, al comparar la relació nit/dia.

Quan es van analitzar els nivells de B i TEMP en funció dels nivells d'AM (Figura IV-8), per cada una de les 4 situacions definides en base a la interacció del LDC i del NLDC, el perfil de la variable B (Figura IV-8, esquerra) va presentar clares diferències respecte al perfil de la TEMP. L'anàlisi estadística (models lineals mesures repetides) va mostrar diferències significatives entre S1-S2, S1-S3, S2-S4 i S3-S4 per a valors superiors a 0,5 del percentil d'AM, però per a valors inferiors a 0,5, la B va mostrar els mateixos nivells en les 4 situacions; és a partir d'aquest percentil quan la desviació respecte la mitjana d'aquesta variable comença a augmentar i les 4 situacions assoleixen nivells lleugerament diferents, essent la situació de doble dia la que presenta valors inferiors.

La TEMP (Figura IV-8, dreta), en canvi, quan es relaciona amb l'AM sempre presenta diferències ($p<0,05$) entre les 4 situacions, en tots els nivells, essent la S1, la que té un valor major, la S2 la que té el valor menor i les S3 i S4 les que tenen valors intermitjos, essent la S4 la que presenta un valor superior a S3.

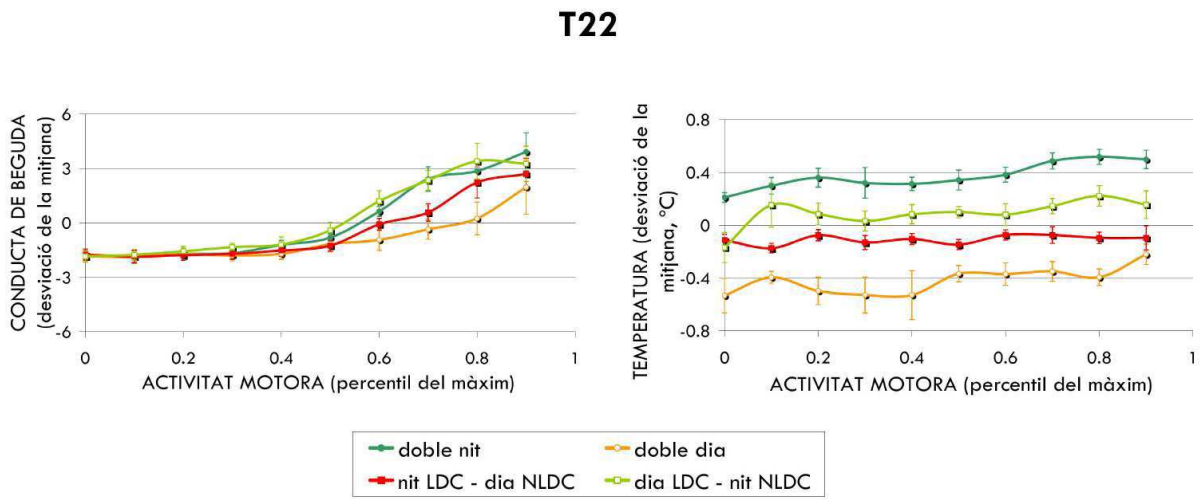

Figura IV-8. Relació entre els nivells mitjans de B (esquerra) i TEMP (dreta), en funció dels nivells d'AM (percentil del màxim), per les 4 situacions analitzades en els animals sotmesos a T22.

En T24 i en DD (Figures IV-9 i IV-10) únicament es presenten dues situacions: la nit i el dia. Pel que fa a la relació entre B i AM, en T24 (Figura IV-9, esquerra), a l'igual que succeïa en T22, és a partir d'un cert nivell d'AM que s'observen nivells diferents de B. Així, a partir del percentil 0,5 d'AM, la B de T24 nit passa a tenir valors diferents i més

alts que la mateixa variable en T24 dia. Calculant la regressió lineal s'observa que la tendència de la B, en les dues situacions, és a augmentar a mesura que ho fa l'AM.

En DD (Figura IV-9, dreta), els valors de B són molt similars entre DD nit i DD dia, tot i que la primera situació presenta valors lleugerament superiors que la segona. La diferència més destacable entre T24 i DD és el gran augment del nivell de B a partir del percentil 0,5 d'AM, que només succeeix en el grup T24. Quan es calcula la regressió lineal de tots els valors dels animals en DD, s'observen diferències estadísticament significatives ($p<0,0001$) entre DD nit i DD dia, essent valor del primer superior al valor del segon, tot i que ambdues situacions mostren augment de la B amb l'augment d'AM. L'anàlisi en funció del nivell d'AM no va mostrar diferències estadísticament significatives en els nivells de B entre els percentils 0,1 i 0,5 d'AM, però sí que se'n van trobar en els percentils 0,7, 0,8 i 0,9 d'AM ($p<0,05$).

Tant en T24 com en DD, les dues situacions van mostrar la tendència a augmentar els nivells de B amb l'augment de l'AM.

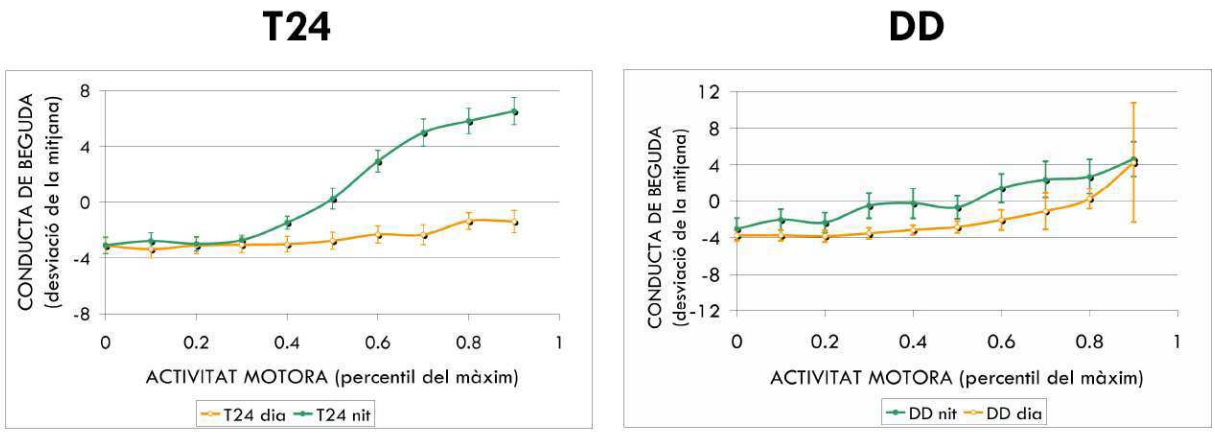

Figura IV-9. Relació entre els nivells mitjans de B (ordenades), i els nivells d'AM (abscisses) per les 2 situacions analitzades en els animals sotmesos a T24 (esquerra) i DD (dreta).

En l'anàlisi de la relació entre TEMP i AM en T24 i en DD (Figura IV-10), s'observen 2 nivells de TEMP (nit i dia) per cada percentil d'AM, essent, òbviament en ambdós casos, el valor de la nit superior al valor del dia. En T24, calculant una regressió lineal, ambdues situacions van mostrar una tendència positiva, mentre que en DD, tot i que la tendència de DD dia va resultar positiva, la tendència de DD nit va resultar negativa.

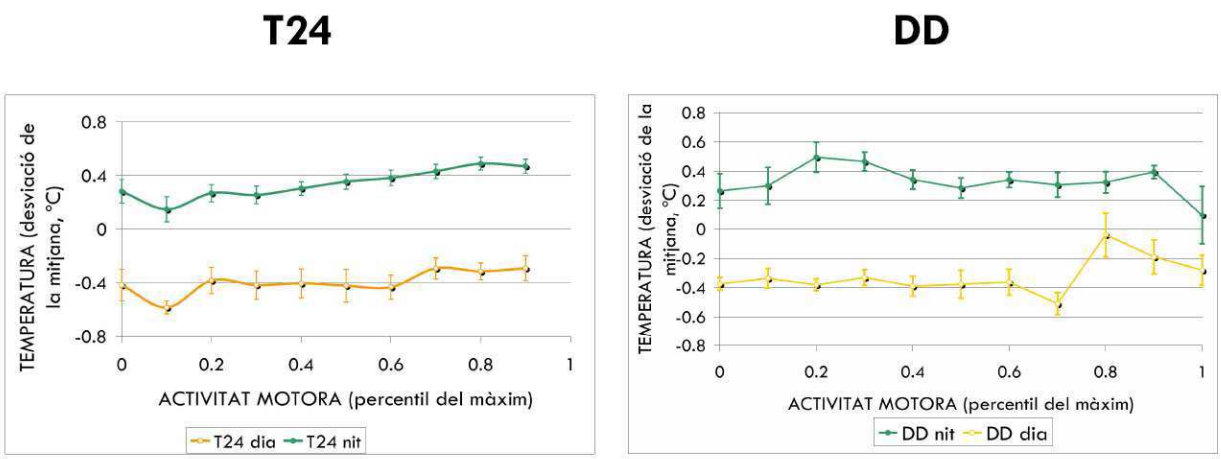

Figura IV-10. Relació entre els nivells mitjans de TEMP (ordenades), i els nivells d'AM (abscisses) per les 2 situacions analitzades en els animals sotmesos a T24 (esquerra) i DD (dreta).

Vam voler veure si, en T22 i T24, utilitzant la variable B enlloc d'AM, trobàvem els mateixos resultats (Figura IV-11). Efectivament, al relacionar TEMP amb B, transformant els valors d'aquesta última variable en percentil del màxim, vam trobar, en T22 (Figura IV-11, esquerra), diferències en les 4 situacions, a l'igual que succeïa al relacionar TEMP i AM, però ara es pot observar com la B no és una variable tan gradual com l'AM, ja que passa del percentil 0 al 0,5 directament. En T24 (Figura IV-11, dreta), la variable TEMP sempre presenta diferències entre les situacions de nit i dia, per tots els nivells de B, tenint sempre la situació de nit un valor més alt que la situació de dia.

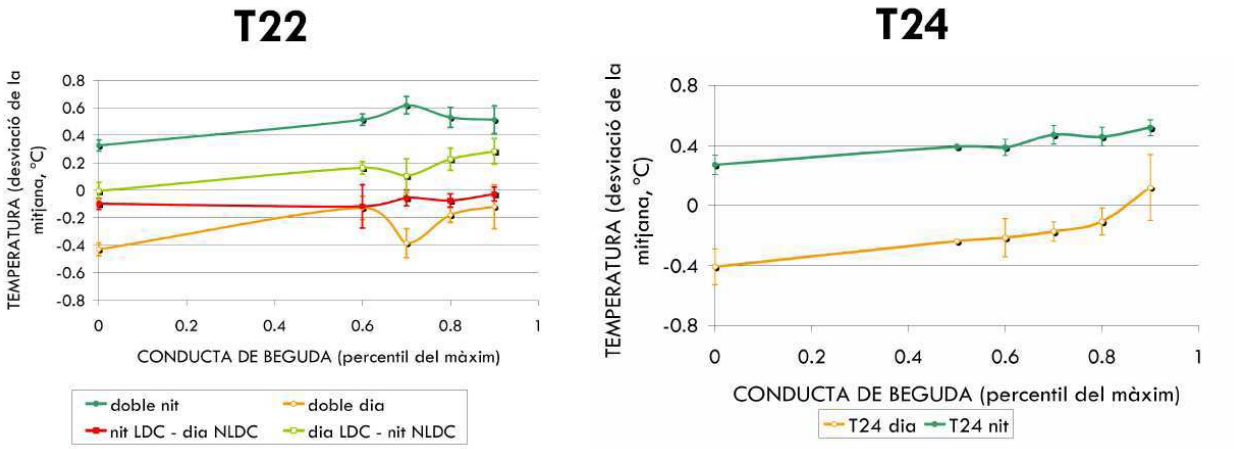

Figura IV-11. Relació entre els nivells mitjans de TEMP (ordenades), i els nivells de B (abscisses) per les situacions analitzades en els animals sotmesos a T22 (esquerra) i a T24 (dreta).

Anàlisi de l'espectre de potències d'activitat motora, de conducta de beguda i de temperatura a T24: L'anàlisi de l'espectre de potències (Figura IV-12) mostra que, en T24, la potència del primer harmònic és més alta en la variable TEMP, seguida de l'AM i la B; al calcular la relació PCH1/PCH4-10, dóna un valor més alt per la TEMP (13,58±3,28), seguit per l'AM (4,34±0,55) i la variable més ultradiària és la B (3,75±1,75).

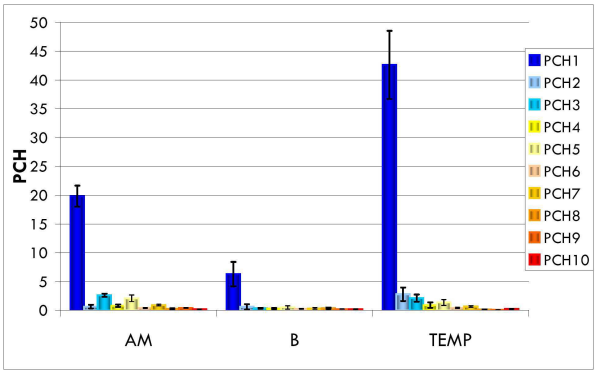

Figura IV-12. Espectre de potències amb 10 harmònics, per cada una de les variables. (PCH1= component circadiari; PCH4-PCH10 = components ultradiaris).

DISCUSSIÓ

El principal objectiu d'aquest experiment ha estat l'estudi de la relació entre els ritmes d'AM, de TEMP i de B de la rata sotmesa a condicions de dissociació forçada. D'una banda hem observat que la dissociació es presenta en altres variables diferents de l'AM. Els resultats obtinguts ens confirmen l'existència d'aquest fenomen en les variables B i TEMP, el que ens fa pensar que afecta tot el sistema temporal de l'organisme.

Un dels principals resultats del treball és la constatació de què els dos components de la dissociació es manifesten de manera diferent en funció de la variable estudiada. La variable que menys mostra una ritmicitat circadiària és la B. Suposem que aquest resultat és degut als valors baixos que presenta aquesta variable i a què probablement en el seu control hi intervinguin més mecanismes homeostàtics que no pas circadiaris. Això es posa de manifest al fer l'anàlisi de l'espectre de potències de cada variable en T24, en el qual s'observa que la variable que més components ultradiaris té és la B, mentre que la variable més circadiària és la TEMP.

En la B, els dos components mostren una importància similar si observem el percentatge de variança explicada determinada en el periodegrama. Per altra banda, la TEMP és la variable mitjançant la qual es detecta millor la ritmicitat, ja que amb ella sempre s'observen valors més alts de %V. Això ens fa pensar en una regulació més estricta d'aquesta variable i menys influïda per possibles alteracions exògenes.

Hem observat que a T22, la relació entre el %V dels dos components és inversa quan la variable estudiada és la TEMP o és l'AM. Així, i en proporció, amb la TEMP el NLDC és més estable respecte el LDC, mentre que amb l'AM, és el LDC qui és més estable. Això ens fa pensar que ambdues variables puguin tenir una regulació circadiària diferent, de manera que l'AM podria venir, majoritàriament regulada per

la part VL del NSQ, ja que l'activitat d'aquesta sembla estar relacionada amb la manifestació del LDC (de la Iglesia *et al.*, 2004), mentre que la TEMP vindria majoritàriament regulada per la part DM. Aquesta suposició vindria recolzada pel fet que a la part VL del NSQ és on arriben aferències des de la retina; per tant, tindria sentit que la variable més relacionada amb aquesta part del NSQ fos la que més reactivitat presentés als canvis de llum externs. De fet, l'AM presenta un pic reactiu en la transició llum-foscor que no s'observa en la TEMP (dades no mostrades en aquest treball).

Pel que fa a les fases de sortida del ritme en DD després d'haver passat per un cicle determinat, tant les fases d'inici d'activitat com les de final d'activitat, en els animals sotmesos prèviament a T24 estan més agrupades que a T23 i aquestes més que a T22. Això es deu a què a T22 es manifesten simultàniament els 2 components i, per tant, hi haurà animals en els que la fase de sortida dependrà més d'un component que de l'altre, donant lloc així, a una major dispersió de les fases. Els animals del grup T23 presenten una situació intermitja, entre T22 i T24, en la qual hi ha una part dels animals que no presenten el NLDC. Els animals del grup T24, a l'estar totalment encarrilats, totes les fases de sortida cap a DD provindran del mateix moment temporal. Quan observem els agrupaments de fase en els tres grups, mesurats en l'AM, la B o la TEMP, trobem que tant a T22 com a T23 l'agrupament de les fases és menys significatiu en la TEMP, el que novament ens fa pensar que aquesta variable ve més influïda pel NLDC que pel LDC.

En algunes espècies animals estudiades en el laboratori s'ha descrit (Refinetti, 1999) que la TEMP és superior en la fase d'activitat que en la de repòs, independentment dels nivells d'activitat que tingui l'animal, cosa que suggereix que la regulació circadiària de la TEMP és independent de l'AM de l'animal. Igualment altres autors (Scheer *et al.*, 2005) mostren que, mirant només el valor de la temperatura corporal al llarg d'un cicle circadiari quan l'activitat és 0, també hi ha una modulació circadiària, el que prova també la independència entre aquestes dues variables. Nosaltres observem el mateix en el grup T24. En canvi a T22, com que en els animals dissociats no hi ha només una fase d'activitat i una de repòs, sinó que n'hi ha dues de cada en funció dels dos components circadiaris, ens vam plantejar estudiar la relació entre AM, B i TEMP en les 4 situacions diferents que trobem en un animal dissociat, amb la idea de determinar la dependència o la independència entre aquestes tres variables en funció d'un component o altre. Així, quan els dos components circadiaris coincideixen en fase trobem la situació que denominem de doble nit i doble dia, i quan no coincideixen trobem les situacions que denominem nit d'un component - dia de l'altre, i a l'inrevés. El primer que vam fer va

ser estudiar els valors mitjans de les tres variables en les 4 fases, per cada T. Tal com esperàvem, els valors més alts corresponien a la doble nit i els més baixos al doble dia. Tot i així, el més interessant són els valors en les situacions no coincidents. Comparant aquestes dues situacions, veiem que en T22 i per la variable TEMP, les tres variables estudiades presenten un valor de la S4 superior a S3, mentre que en T23 succeeix el contrari. Això indica que els animals de T23, que mostren un valor més alt per la situació de nit LDC – dia NLDC que per la situació de dia LDC – nit NLDC, estan més sincronitzats al cicle LD que els del grup T22.

Quan observem els valors de TEMP en funció dels percentils d'AM o de B trobem que, en T22, el nivell d'AM influeix en el valor de la TEMP en cada una de les fases, però que les 4 línies siguin paral·leles i diferents indica una doble regulació de la TEMP. Aquesta variable presenta un ritme circadiari independent de l'AM, ja que quan l'AM és zero s'observen també 4 nivells de TEMP, en funció de la fase. Novament els valors superiors corresponen a la doble nit i els mínims al doble dia. Que els valors de les situacions on l'activitat dels dos components no és coincident quedin entre mig, mostra una veritable dissociació de la TEMP, independent de l'AM. Això indica que aquesta variable és regulada per ambdues parts del NSQ, però el fet que S4 sigui superior que S3 i que el %V sigui superior pel NLDC fa pensar que la TEMP ve més regulada per la part DM. L'anàlisi dels valors de B en funció del percentil d'AM mostra que la B no té una regulació circadiària independent de l'AM. Quan els nivells d'AM són baixos i també durant el dia, es manté una regulació homeostàtica, que podria ser deguda a necessitats metabòliques de l'animal, però a partir d'un cert nivell d'AM, la B en les 4 situacions augmenta i es diferencia. El mateix observem en T24, tot i que, en aquest cas, només hi ha dues situacions. En DD, en canvi, la B aniria més lligada a l'AM, disminuïnt les diferències entre el dia i la nit.

Per últim, vam voler estudiar si hi havia diferències en el temps de resincronització a un canvi de fase, entre l'AM, la TEMP i la B, el que indicaria una diferent inèrcia dels sistemes que regulen les diverses variables. En el cas de T22, no va ser possible fer aquesta anàlisi, ja que la presència dels dos components enmascara totalment el procés de resincronització, i en T24 no vam trobar diferències en el temps que tardava cada animal a restablir el ritme de cada variable.

De tots aquests resultats podem concloure que la dissociació no és un fenomen que únicament es presenta en l'activitat motora, sinó que també apareix en la temperatura i en la conducta de beguda. A més, els ritmes circadiaris d'activitat motora i de temperatura corporal a T22 poden estar desincronitzats. En els humans, la desincronització interna dels ritmes circadiaris és present en algunes patologies (malalties associades amb l'edat, depressions estacionals, *jet-lag*, canvis de

torn de treball, etc.). Sabent que el NSQ de les rates està format per dues parts diferenciables anatòmicament i neuroquímica, es pot pensar que en les persones succeeix el mateix. Els nostres resultats indiquen que la desincronització dels ritmes circadiaris dins un mateix individu podria ser causada per un desacoblament dels oscil·ladors neuronals dins el NSQ.

El model de dissociació emprat en aquest estudi ens pot permetre entendre el mecanisme del rellotge en els humans i, en un futur, provar diverses teràpies que ens ajudin a prevenir o revertir la desincronització de ritmes.

Atès que els estudis fets amb humans sota desincronització forçada mostren que la TEMP va estretament lligada al son REM (Czeisler *et al.*, 1999), vam optar per publicar el resultat més rellevant d'aquest experiment, que és la desincronització entre les variables AM i TEMP observada en els animals del grup T22, juntament amb els obtinguts en un estudi sobre les fases del son en animals dissociats realitzat a la Universitat de Washington. L'article conjunt "CIRCADIAN DESYNCHRONIZATION OF CORE BODY TEMPERATURE AND SLEEP STAGES IN THE RAT" que ha estat publicat a la revista Proceedings of the National Academy of Sciences of the United States of America es mostra a continuació.

Fruit de la col·laboració entre el nostre grup i el Grup de Cronobiologia de la Universitat de Washington, dirigit pel Dr. Horacio de la Iglesia, va sorgir l'article CIRCADIAN DESYNCHRONIZATION OF CORE BODY TEMPERATURE AND SLEEP STAGES IN THE RAT, publicat a la revista PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA.

Del treball realitzat, en el qual es van analitzar els ritmes d'activitat motora, de temperatura corporal i de les fases del son, únicament la part corresponent a l'anàlisi de temperatura i d'activitat motora formen part d'aquesta tesi.

ARTICLE

CIRCADIAN DESYNCHRONIZATION OF CORE BODY TEMPERATURE AND SLEEP STAGES IN THE RAT

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INTRODUCCIÓ

S'ha descrit que dins el NSQ hi ha dues subpoblacions d'oscil·ladors i que l'expressió genètica de cada una d'elles ve associada als dos components característics de la dissociació: la part VL es relaciona amb el LDC i la part DM amb el NLDC. Però, com afecta a la fisiologia de l'organisme el fet que ambdues parts del NSQ es dissociïn?

OBJECTIU

L'objectiu d'aquest treball va ser provar si la situació que genera el desacoblament entre les dues subpoblacions d'oscil·ladors neuronals dins el NSQ pot conduir a la desincronització de diferents processos fisiològics circadiaris.

MATERIAL I MÈTODES

Es van utilitzar rates Wistar mascles, que es van dividir en 2 grups: T22 i T24. A tots els animals se'ls va enregistrar l'AM i la TEMP; i a 4 rates del grup T22, a més, se'ls va enregistrar l'activitat electroencefalogràfica i electromiogràfica per tal d'avaluar el cicle son-vigília i les diferents fases del son.

Es va estudiar els ritmes de cada una de les variables i la relació entre AM i TEMP, en les 4 fases definides en base a la interacció entre el LDC i el NLDC.

RESULTATS

Sota condicions de T22, els ritmes d'AM, de son-vigília, i de SWS presenten dissociació, apareixent un ritme encarrilat pel cicle LD i un altre en curs lliure. Tot i que el ritme de TEMP també es presenta dissociat, en aquesta variable el NLDC presenta més estabilitat que el LDC. El PS només presenta un sol component, en curs lliure.

Per cada nivell d'AM s'han trobat 4 nivells de TEMP, que corresponen a cada una de les situacions que s'estableixen en la dissociació. La relació entre els nivells de

TEMP i els d'AM en T22, per cada una de les 4 situacions estudiades mostra que la situació de doble nit és la que presenta nivells més alts de TEMP, la situació de doble dia, la que els presenta més baixos i les situacions de dia d'un component i nit de l'altre manifesten nivells intermitjos, tenint la situació de dia LDC – nit NLDC un valor més alt que la situació de nit LDC – dia NLDC.

CONCLUSIÓ

Els resultats indiquen que en les situacions on el NSQ es dissocia funcionalment hi ha desincronització entre les diferents variables i que la dissociació de la TEMP és real ja que hi ha diferents nivells per cada nivell d'AM.

Això suggereix que els ritmes circadiaris de son-vigília i de SWS es poden desincronitzar dels ritmes de PS i de TEMP dins un mateix individu, i que les variables AM i SWS vénen controlades per ambdues parts (VL i DM) del NSQ, mentre que la temperatura i el PS, principalment, per la part DM.

Circadian desynchronization of core body temperature and sleep stages in the rat

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Proper functioning of the human circadian timing system is crucial to physical and mental health. Much of what we know about this system is based on experimental protocols that induce the desynchronization of behavioral and physiological rhythms within individual subjects, but the neural (or extraneural) substrates for such desynchronization are unknown. We have developed an animal model of human internal desynchrony in which rats are exposed to artificially short (22-h) light–dark cycles. Under these conditions, locomotor activity, sleep–wake, and slow-wave sleep (SWS) exhibit two rhythms within individual animals, one entrained to the 22-h light–dark cycle and the other free-running with a period >24 h ($\tau_{>24\ h}$). Whereas core body temperature showed two rhythms as well, further analysis indicates this variable oscillates more according to the $\tau_{>24\ h}$ rhythm than to the 22-h rhythm, and that this oscillation is due to an activity-independent circadian regulation. Paradoxical sleep (PS), on the other hand, shows only one free-running rhythm. Our results show that, similarly to humans, (*i*) circadian rhythms can be internally dissociated in a controlled and predictable manner in the rat and (*ii*) the circadian rhythms of sleep–wake and SWS can be desynchronized from the rhythms of PS and core body temperature within individual animals. This model now allows for a deeper understanding of the human timekeeping mechanism, for testing potential therapies for circadian dysrhythmias, and for studying the biology of PS and SWS states in a neurologically intact model.

suprachiasmatic

In mammals, a master circadian pacemaker localized within the hypothalamic suprachiasmatic nucleus (SCN) governs overt circadian rhythms of physiology and behavior. The SCN is constituted by a network of single-cell neuronal oscillators that regulates circadian rhythms through direct and indirect output pathways to brain regions controlling specific physiological and behavioral processes (1, 2). The SCN master regulation of circadian rhythms can potentially take place through control of circadian oscillators elsewhere in the brain and in virtually all peripheral tissues, which presumably act as local pacemakers for specific rhythmic modalities (3, 4).

Although the evidence clearly indicates that the circadian rhythms of locomotor activity, core body temperature (CBT), and sleep–wake share a common circadian pacemaker within the SCN (1, 2, 5), some features of these rhythmic modalities suggest that they might be differentially regulated. The first indication that the rhythms of CBT, rest–activity, and sleep structure could be independently regulated came from studies in humans that show "spontaneous internal desynchronization" (6, 7). Human subjects under temporal isolation sometimes exhibit a circadian rhythm of CBT with a near-24-h period, whereas their self-imposed rest–activity cycle (and associated sleep–wake cycle) oscillates with a considerably longer period (generally >30 h). Desynchronization between the rest–activity cycle and the CBT rhythm can be also experimentally induced through so-called "forced desynchrony protocols," in which the experimenter imposes a rest–activity cycle that is different from 24 h. Typically, in such studies the rhythms of CBT and other physiological variables including plasma melatonin and cortisol, sleep propensity, and rapid eye-movement sleep, also referred to as paradoxical sleep (PS), oscillate, out of synchrony with the imposed rest–activity cycle, with a period near 24 h (6, 7).

It is still a matter of controversy whether internal desynchronization of physiological and behavioral rhythms represents the activity of two independent oscillators and, if it does, whether these oscillators are anatomically identifiable. In fact, the anatomical basis of internal desynchronization, whether spontaneous or induced by forced desynchrony protocols, remains unknown and the lack of animal models of forced desynchronization has slowed progress toward determining the neural and molecular basis of circadian desynchrony. Here, we report an animal model of circadian desynchronization, in which the rhythms of CBT and PS can be dissociated from those of rest–activity, sleep–wake, and slow-wave sleep (SWS).

Results and Discussion

We recently developed an animal model of forced desynchrony: Rats exposed to 22-h light–dark (LD) cycles exhibit two stable locomotor activity rhythms with different period lengths in individual animals (8). We determined that one of these rhythms, with a period of 22 h ($T_{22\ h}$) and entrained to the LD cycle, is associated with the expression of clock genes in the ventrolateral (VL) SCN. The other rhythm, with a period longer than 24 h ($\tau_{>24\ h}$) and not entrained to the LD cycle, is associated with clock gene expression in the dorsomedial (DM) SCN (9). This finding suggests that the uncoupling of anatomically identifiable subpopulations of neuronal oscillators within the SCN itself could lead to the desynchronization of different circadian physiological and behavioral processes, similar to that observed in human subjects. To test this hypothesis, we monitored rhythms of locomotor activity, CBT, and electrocorticographic (ECoG) sleep–wake activity in forced desynchronized rats.

Adult male Wistar rats housed individually under a 22-h LD cycle (11 h of light:11 h of dark) were implanted under deep anesthesia during the light phase with an i.p. temperature sensor or with both an i.p. temperature sensor and ECoG electrodes. Animals were returned to their home cages where locomotor

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The authors declare no conflict of interest.

Abbreviations: SCN, suprachiasmatic nucleus; CBT, core body temperature; PS, paradoxical sleep; SWS, slow-wave sleep; LD, light–dark; VL, ventrolateral; DM, dorsomedial; ECoG, electrocorticographic.

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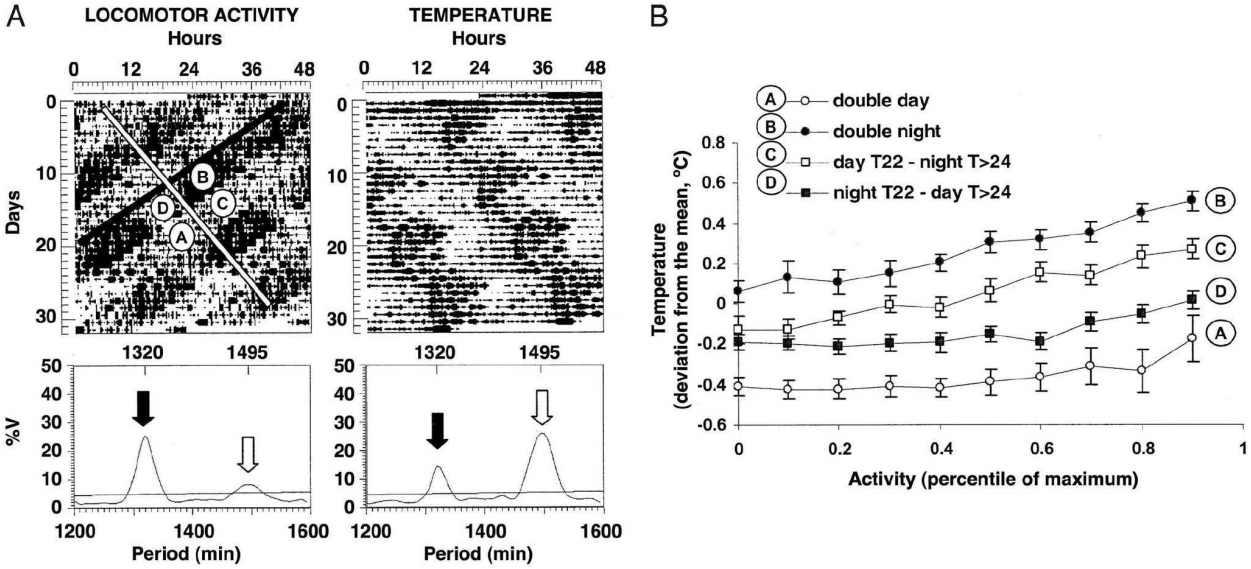

Fig. 1. Desynchronization of locomotor activity and CBT in the forced desynchronized rat. (*A*) (*Upper*) Double plotted actograms for motor activity and temperature of a representative forced desynchronized rat under a 22-h LD cycle. The white and black diagonal bars indicate the onset of the $\tau_{>24\ h}$ and the $T_{22\ h}$ locomotor activity rhythms, respectively. The circled letters represent the four phases (defined by the two activity rhythms) on which the analysis of CBT was performed. (*Lower*) χ^2 periodograms of the time series represented on the actograms. The analysis yielded statistically significant peaks for the $\tau_{>24\ h}$ (white arrow) and the $T_{22\ h}$ (black arrow) rhythms. The numbers on top indicate the period of the significant peaks in minutes. (*B*) Mean temperature levels (as deviation from the individual mean temperature) as a function of different levels of locomotor activity (as percentile of maximum values) for each of the four phases indicated in *A*. Each value represents the mean ± SE drawn from 16 animals with dual activity and dual CBT rhythms. General linear models with repeated measures yielded significant differences between phases A, B, C, and D in all possible compared pairs ($P < 0.001$) and linear regression analysis significant slopes within each phase ($P < 0.001$).

activity by infrared beam interruptions, and ECoG activity were recorded. At the end of the experiment, animals were killed and the temperature sensors were removed to acquire the temperature data. Fig. 1*A* depicts the rhythms of locomotor activity and temperature of a typical animal under a 22-h LD cycle. χ^2 periodogram analysis indicated two statistically significant rhythmic components with periods of 22 h and >24 h ($\tau_{>24\ h}$ = 25 h ± 5 min for all rats) for both locomotor activity and CBT. Sixteen animals (of 25 animals studied) showed this pattern of rhythmicity, with stable rhythms of locomotor activity and CBT for both $T_{22\ h}$ and $\tau_{>24\ h}$. Notably, six of the remaining nine animals showed statistically significant CBT rhythm only for the $\tau_{>24\ h}$ component despite the fact that they showed a statistically significant $T_{22\ h}$ locomotor activity rhythm (Table 1). In the 16 animals with dual locomotor activity rhythms and dual CBT rhythms, the percentage of variance of locomotor activity explained by $T_{22\ h}$ component in the periodogram (16 ± 1.46) was significantly higher than the percentage of variance explained by the $\tau_{>24\ h}$ component (10.2 ± 0.84; *t* test, $P < 0.005$). In contrast, the percentage of variance of CBT data explained by the $T_{22\ h}$ component (11.5 ± 1.38) was significantly smaller than that explained by the $\tau_{>24\ h}$ component (18.8 ± 2.2; *t* test, $P < 0.01$). This analysis indicates that, whereas the circadian oscillation of activity shows higher cycle-to-cycle phase stability under the $T_{22\ h}$ period, the circadian oscillation of temperature is more stable under the $\tau_{>24\ h}$ period. This, together with the fact that six animals showed solely a $\tau_{>24\ h}$ CBT rhythm despite having a significant $T_{22\ h}$ locomotor activity oscillation demonstrates that the circadian rhythm of CBT can be dissociated from rhythmic locomotor activity. The more robust oscillation of locomotor activity under a 22-h period may reflect the fact that this behavioral process is under stronger masking (10) by the LD cycle than CBT is, as it is clearly suggested by the reactive peak of activity after lights off (Fig. 2*B*).

Table 1. Most rats exposed to a 22-h LD cycle express dual locomotor activity rhythms and dual temperature rhythms

	CBT		
Locomotor activity	$T_{22\ h}$ component	$\tau_{>24\ h}$ component	Both components
$T_{22\ h}$ component	0	1	1
$\tau_{>24\ h}$ component	0	0	1
Both components	1	5	16

Number of animals that showed either both or only one of the $T_{22\ h}$ and $\tau_{>24\ h}$ components for locomotor activity and CBT.

Because in the animals with dual CBT rhythms, locomotor activity also oscillates both with 22- and >24-h periods, the oscillations of CBT with these respective periods could represent a true circadian modulation of heat-producing mechanisms or a by-product of activity-induced heat production. Accordingly, human subjects under a rest–activity (and respective dark and light phases) forced desynchrony protocol show both an endogenous free-running modulation of CBT but also behaviorally induced changes in CBT that are associated to the experimenter-imposed rest–activity cycle (11, 12). In animals, an imposed rest–activity cycle is not feasible and genuine circadian modulation of CBT must be statistically dissected from locomotor activity-induced rhythmic CBT (13).

Although a proportional increase of CBT with increased activity is present under all phases of the circadian cycle, a given level of activity may yield higher values of CBT at specific circadian phases. This activity-independent increase in temperature is interpreted as direct modulation of temperature control systems by a circadian pacemaker. In our forced desynchronized animals, the two overlapping rhythms of locomotor activity define a $T_{22\ h}$ night and day, and a $\tau_{>24\ h}$ subjective night and

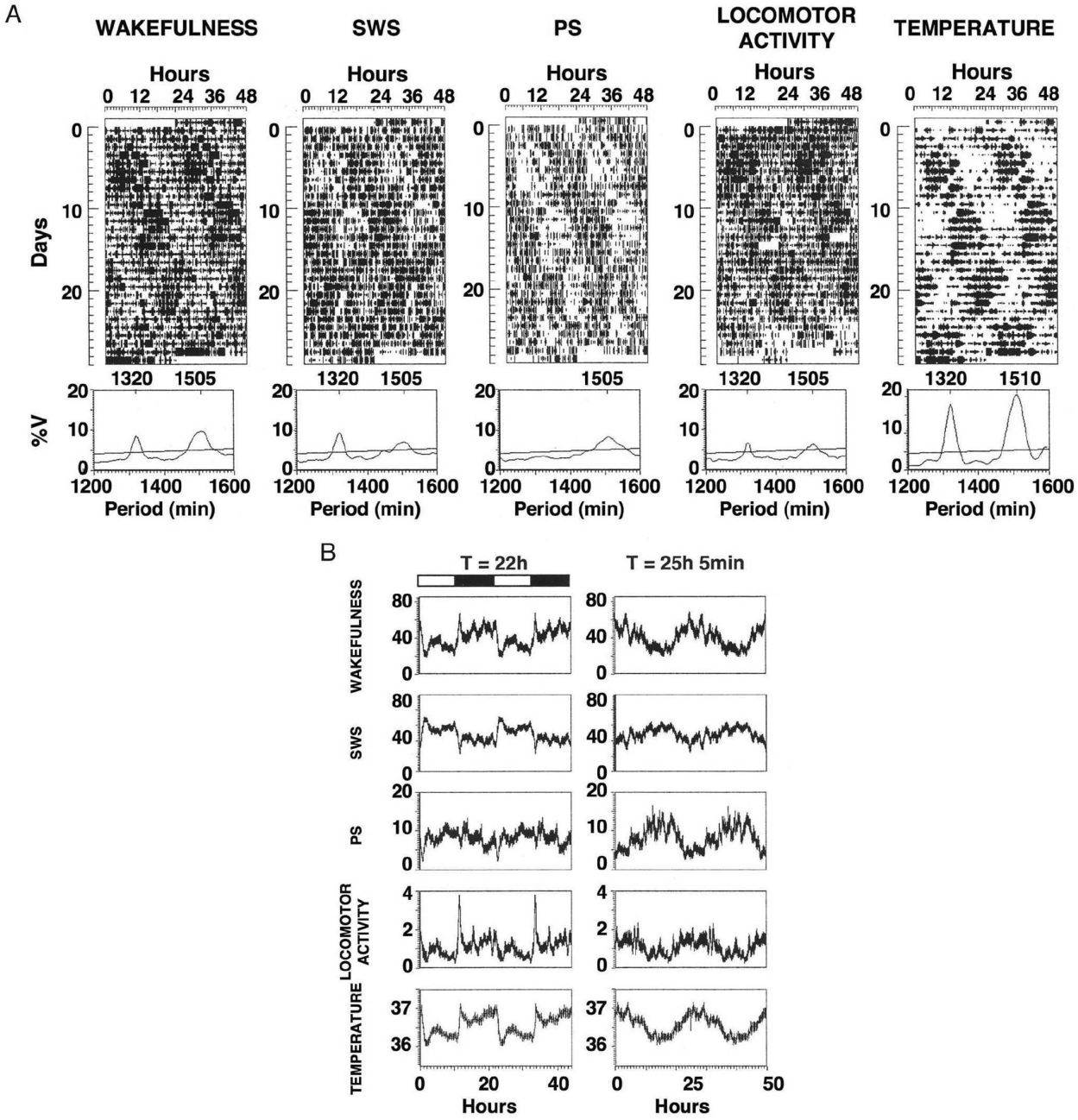

Fig. 2. Desynchronization of sleep stages in the forced desynchronized rat. (*A*) Double plotted actograms of wakefulness, SWS, PS, locomotor activity, and CBT of a representative rat, and their corresponding periodograms. (*B*) Circadian variation of all variables in the same animal shown in *A*, plotted in modulo of the two significant periods ($T_{22\ h}$ = 22 h; $\tau_{>24\ h}$ = 25 h, 5 min) obtained in the periodogram. Values represent the mean ± SE of the 10-min interval values for each successive cycle, smoothed by running averages of three data points. The dark and white horizontal bars on the left indicate the dark and light phases of the 22-h LD cycle, respectively.

subjective day, respectively. The relationship between activity levels and CBT at the phase representing "day" for both the $T_{22\ h}$ and $\tau_{>24\ h}$ rhythms (double day; Fig. 1*A*, phase A), which corresponds to the light phase for the $T_{22\ h}$ rhythm and the rest phase for the $\tau_{>24\ h}$ rhythm, yielded the lowest temperature values for any specific level of activity (Fig. 1*B*). The same relationship estimated at a phase representing "night" for both the $T_{22\ h}$ and $\tau_{>24\ h}$ rhythms (double night; Fig. 1*A*, phase B), which corresponds to the dark phase for the $T_{22\ h}$ rhythm and the active phase for the $\tau_{>24\ h}$ rhythm, yielded the highest temperature values for any specific level of activity. The relationship between temperature and activity in both conflicting phases, in which the lights are on ($T_{22\ h}$ day) but the $\tau_{>24\ h}$ rhythm is in subjective night (Fig. 1*A*, phase C), and vice versa, in which the

lights are off ($T_{22\ h}$ night) but the $\tau_{>24\ h}$ rhythm is in subjective day (Fig. 1*A*, phase D) yielded higher values of temperature than the double-day phase but lower than the double-night phase (Fig. 1*B*). Interestingly, phase C showed higher values than phase D (general linear models with repeated measures, $P < 0.001$ in all comparisons). Of note, the double-day and double-night curves were not statistically different from those for the day and night, respectively, of control animals housed under a 12:12 LD cycle (data not shown), suggesting that circadian regulation of CBT during the nonconflicting phases of the forced desynchrony protocol is similar to that of 24-h LD-synchronized animals. Our analysis also indicated that, in all four phases (Fig. 1, phases A–D), an increase in locomotor activity levels induced a proportional increase in temperature (linear regression, $P < 0.001$, in all four phases). Thus, CBT is both under circadian control and influenced by activity-induced heat.

The differences in activity-independent CBT indicate that CBT is truly oscillating according to two rhythms within individual animals, generating four basic levels of temperature for any given level of locomotor activity. This activity-independent, daily or circadian regulation of temperature has been previously described in several rodent species (13, 14), including the rat in which it relies on an intact SCN (15). Furthermore, our analysis clearly demonstrates that activity-independent CBT in the forced-desynchronized rat oscillates more robustly in accordance to a $\tau_{>24\ h}$ rhythm than to the entrained $T_{22\ h}$ rhythm. The activity-independent oscillation of CBT in the 22-h domain could represent the output of an oscillator and/or a masking effect of light on CBT. Light is known to produce a reduction in CBT that depends on the phase of the circadian cycle (15). On the other hand, the analysis of both locomotor activity rhythms (8, 16) and clock gene expression patterns (9) in the forced desynchronized rat has indicated that the $T_{22\ h}$ locomotor activity rhythm, associated with the $T_{22\ h}$ CBT rhythm here described, likely represents the output of a true, entrainable oscillator within the VL-SCN.

The robust $\tau_{>24\ h}$ circadian oscillation in CBT, independent of the imposed 22-h LD cycle, in our forced desynchronized rats is reminiscent of human CBT rhythms under similar forced desynchrony conditions. In the rat, this $\tau_{>24\ h}$ oscillation, as judged by the locomotor activity rhythm, is associated with the clock gene activity within the DM-SCN (9), and our results suggest that the expression of forced desynchronized rhythms of locomotor activity and CBT in humans could also be associated with uncoupling of dual oscillators within the SCN master circadian oscillator. Although humans do not exhibit two rhythms of rest–activity as our rats do, this may be a consequence of the experimenter-imposed forced rest–activity cycle. Notably, human subjects under spontaneous internal desynchronization do show evidence of two rest–activity periodicities, one that free-runs with a much longer than 24-h period and another that is in phase with the circa 24-h rhythm in CBT (17, 18).

Desynchronization of PS from the rest–activity cycle and its associated sleep–wake cycle is yet another signature of humans under forced desynchrony protocols. Under these circumstances, PS propensity increases shortly after the circadian minimum of CBT (12), a feature that is present also in spontaneously desynchronized human subjects (17, 19). The robust $\tau_{>24\ h}$ oscillation of CBT in the present study hinted to the possibility that this tight correlation between CBT circadian rhythmicity and PS may also be present in the forced desynchronized rat. We explored this possibility in four animals in which we performed long-term ECoG recordings and simultaneously monitored locomotor activity and CBT. Fig. 2 shows a representative animal. Whereas locomotor activity, CBT, wakefulness, and SWS presented dual $T_{22\ h}$ and $\tau_{>24\ h}$ rhythms, PS only showed a significant $\tau_{>24\ h}$ oscillation (Fig. 2*A*). The temporal profiles for slow-wave activity and the theta power were similar to the SWS and PS, respectively [supporting information (SI) Fig. 3]. The oscillation of SWS, slow-wave activity, and temperature in synchrony with the 22-h LD cycle could represent a masking phenomenon. Behavioral analysis (8, 16) indicates that the 22-h locomotor activity rhythm in the forced desynchronized rat represents an entrained rhythm that can predict the phase of the free-running rhythm when animals are released into constant darkness after desynchrony. Furthermore, the 22-h oscillation of clock gene expression within the VL-SCN in desynchronized animals persists under constant darkness conditions (9), although masking by the LD cycle could contribute to the expression rhythm of otherwise weak VL-SCN oscillators. These results suggest that the regulation of sleep stages and temperature in the 22-h domain here reported may emerge from the dual contribution of autonomous VL-SCN oscillators and masking processes.

Table 2. Desynchronization of sleep stages, CBT, and locomotor activity in the forced desynchronized rat

Variables	Period, h			
	Rat 14 (19 days)	Rat 16 (34 days)	Rat 21 (14 days)	Rat 23 (31 days)
Wakefulness				
$T_{22\ h}$	22	22.1	22.4	22
$\tau_{>24\ h}$	24.8	25.1	25.4	25.2
SWS				
$T_{22\ h}$	22	22	22.3	22
$\tau_{>24\ h}$	24.8	25.1	25.2	25.1
PS				
$T_{22\ h}$	NS	NS	NS	NS
$\tau_{>24\ h}$	24.9	25.1	25.7	25.2
Locomotor activity				
$T_{22\ h}$	21.9	22.1	22.2	22
$\tau_{>24\ h}$	24.8	25.1	25.3	25.2
CBT				
$T_{22\ h}$	22	22.1	22.2	22
$\tau_{>24\ h}$	25.1	25.1	25.5	25.2

For each animal, the periods indicated correspond to the statistically significant periods obtained by periodogram analysis. For each variable, periods are shown for the rhythmic component associated with the 22-h LD cycle ($T_{22\ h}$) or the free-running component ($\tau_{>24\ h}$). The days in parentheses indicate the duration of the study for each animal. NS, The variable did not show a statistically significant oscillation for that specific component.

The peak of PS propensity occurred during the nadir of the $\tau_{>24\ h}$ CBT rhythm (Fig. 2*B*). Although with lower amplitude, there was a progressive increase of PS propensity across the light phase of the 22-h LD cycle, a feature also observed in the scheduled sleep phase in human forced desynchrony protocols (12). The overall temporal distribution of sleep structure and activity was observed in all four animals studied by ECoG recordings (Table 2). Our results clearly demonstrate that the circadian timing of PS in the forced desynchronized rat is, as it is in humans, tightly associated to the free-running ($\tau_{>24\ h}$) CBT rhythm but not with the activity-induced CBT rhythm that results from the 22-h forced desynchrony protocol.

In summary, circadian rhythms can be uncoupled in a predictable and stable manner in our rat forced desynchrony model. Under these circumstances, the circadian regulation of CBT and PS shows the same properties as that in forced desynchronized humans. In the rat, the entrained and free-running locomotor activity rhythms are associated with the independent activities of the VL- and DM-SCN (9), respectively, and our present results strongly suggest that the free-running oscillation of CBT and PS may be also associated with the DM-SCN activity. We propose that, in humans, desynchronization of these rhythms may also be associated with the uncoupling of dual oscillators within the

hypothalamic master circadian clock. The association between the circadian rhythm of PS and the DM-SCN activity is particularly interesting. The SCN has been recognized for decades as the pacemaker for the sleep–wake cycle (5), and the output pathways that sustain this function are beginning to be mapped (20). However, so far there is no evidence that the SCN governs the timing of specific sleep stages independently. Our results point to a more protagonistic role of the SCN in the regulation of sleep stages, one in which SWS, which is more responsive to acute effects of light, would be governed by LD-associated clock gene expression of VL-SCN oscillators, and PS, typically under robust circadian control, would be governed by DM-SCN oscillators.

The VL-SCN and DM-SCN are recognized as areas that present different cytoarchitecture, chemoarchitecture, and topography of afferent and efferent connections (21), as well as different clock gene expression patterns (22, 23) and responses to light or abrupt phase shifts (refs. 24–26 and reviewed in ref. 27). Our findings add a new layer of complexity to this subregional organization, because they suggest that the VL- and DM-SCN independently control circadian rhythmicity of specific physiological and behavioral variables. Specifically, the circadian oscillation of CBT and PS in association with DM-SCN activity strongly suggests that this region controls, in a rather LD cycle-independent manner, these two rhythms, whereas the rhythms of locomotor activity and SWS are associated with the activity of either the VL- or DM-SCN.

Internal desynchronization of circadian rhythms is a common feature in most circadian pathologies, including those associated with aging, seasonal affective disorder, jet lag, nocturnal shift work, and work under non-24-h LD cycles (6, 28). Our findings in the forced desynchronized rat indicate that desynchronization of circadian rhythms within the same individual could emerge from uncoupling of neuronal oscillators within the SCN itself and may represent an entrée to explore potential treatments for these ailments.

Materials and Methods

Animals and Surgery. All experiments were approved by the Animal Care and Use Committee of the University of Washington and the University of Barcelona. Male Wistar rats, 2 months old on arrival, were purchased from Charles River [Raleigh, NC (for rats used at University of Washington); Les Oncines, France (for rats used at University of Barcelona)] and housed individually in transparent polycarbonate cages ($20 \times 25 \times 22$ cm) fitted with infrared beam detectors. Approximately one-half of the animals for temperature and activity recordings were studied at University of Barcelona and one-half at University of Washington. Given that no significant differences were seen between the two groups, the data were pooled. All sleep studies were performed at University of Washington.

Forced desynchronized animals were maintained under a symmetrical LD cycle of 11 h of light and 11 h of dark. Control animals were maintained under a 24-h symmetrical LD cycle. Light consisted of cool white light (100–300 lux) and darkness of dim red light (<1 lux). Locomotor activity was continuously monitored by means of a system with two crossed infrared beams and, after 10–15 days, once the rhythms were clearly visible, rats were anesthetized during the light phase of the LD cycle and implanted with i.p. temperature sensors (Thermochrone iButtons; Dallas Semiconductor, Dallas, TX) (29). Some of the rats were implanted with ECoG electrodes for sleep recording (see below). Temperature and motor activity were simultaneously detected and recorded in 15-min data bins.

ECoG electrodes were placed over the frontal and parietal cortices as previously described (30). The leads from the ECoG electrodes were routed to a Teflon pedestal, which was attached to the skull with dental cement. Animals were returned to their home cages where locomotor activity was monitored through infrared beam interruptions. After 5 days of recovery, the ECoG electrodes were connected to an amplifier through a wire attached to a swivel. ECoG signals (128-Hz sampling rate) were amplified, passed through filters, and digitized. Recordings lasted a minimum of 14 days and a maximum of 30 days.

Analysis of CBT and Locomotor Activity. CBT and activity were plotted as actograms to visualize rhythmic components. The χ^2 periodogram (31) was used to estimate the period of statistically significant oscillations in the circadian range.

To study the relationship between CBT and locomotor activity, within each animal temperature data points were expressed as deviation from the mean and activity data were transformed to a percentile of the maximum (10 levels of activity). For rats under a 22-h LD cycle, separate data sets were generated for each of the four phases outlined in Fig. 1: double day, double night, $T_{22\ h}$ day–$\tau_{>24\ h}$ subjective night, and $T_{22\ h}$ night–$\tau_{>24\ h}$ subjective day. For control (24-h LD cycle) rats, separate data sets were generated for the light and the dark phases. CBT (as a deviation from the individual's mean) was analyzed as a function of the different levels of activity (as a percentile of the individual's maximum activity) for each of these phases separately. Statistical analysis was carried out by means of general linear models with repeated measures to study the effect of the stages on body temperature, considering the levels of activity as an intersubject factor.

Analysis of Sleep Stages. The vigilance states of wakefulness, SWS, and PS were determined off-line in 10-s epochs by an operator blind to the circadian phase at which the recording was taken. Wakefulness was characterized by fast low-amplitude ECoG waves in coincidence with locomotor activity recorded through infrared beams. SWS was associated with slow high-amplitude ECoG waves and lack of locomotor activity. In contrast, PS is characterized by fast low-amplitude ECoG waves, appearance of theta ECoG (visualized through a fast Fourier transform), and lack of locomotor activity. Slow-wave activity (ECoG frequencies of 0.5–4.0 Hz) and theta (ECoG frequencies of 4.0–8.0 Hz) powers were calculated through fast Fourier transform, normalizing to the power calculated for SWS and PS episodes, respectively. The percentage of time spent in each state was calculated for every 10 min. The percentage data were plotted as actograms to visualize rhythmic components. The χ^2 periodogram was used to estimate the period of statistically significant oscillations in the circadian range.

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1. Moore RY, Leak RK (2001) in *Handbook of Behavioral Neurobiology: Circadian Clocks*, eds Takahashi JS, Turek F, Moore RY (Kluwer Academic/Plenum Publishers, New York), pp 141–179.
2. Klein DC, Moore RY, Reppert SM (1991) *Suprachiasmatic Nucleus. The Mind's Clock* (Oxford Univ Press, New York).
3. Schibler U, Ripperger J, Brown SA (2003) *J Biol Rhythms* 18:250–260.
4. Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, *et al.* (2004) *Proc Natl Acad Sci USA* 101:5339–5346.
5. Mistlberger RE (2005) *Brain Res Rev* 49:429–454.
6. Czeisler CA, Dijk DJ (2001) in *Handbook of Behavioral Neurobiology: Circadian Clocks*, eds Takahashi JS, Turek FW, Moore RY (Kluwer Academic/Plenum Publishers, New York), pp 531–569.

7. Lavie P (2001) *Annu Rev Psychol* 52:277–303.
8. Campuzano A, Vilaplana J, Cambras T, Diez-Noguera A (1998) *Physiol Behav* 63:171–176.
9. de la Iglesia HO, Cambras T, Schwartz WJ, Diez-Noguera A (2004) *Curr Biol* 14:796–800.
10. Mrosovsky N (1999) *Chronobiol Int* 16:415–429.
11. Hiddinga AE, Beersma DG, Van den Hoofdakker RH (1997) *J Sleep Res* 6:156–163.
12. Dijk DJ, Czeisler CA (1995) *J Neurosci* 15:3526–3538.
13. Refinetti R (1999) *Am J Physiol* 277:R1493–R1500.
14. Refinetti R (1994) *Physiol Behav* 56:829–831.
15. Scheer FA, Pirovano C, Van Someren EJ, Buijs RM (2005) *Neuroscience* 132:465–477.
16. Cambras T, Chiesa J, Araujo J, Diez-Noguera A (2004) *J Biol Rhythms* 19:216–225.
17. Czeisler CA, Weitzman E, Moore-Ede MC, Zimmerman JC, Knauer RS (1980) *Science* 210:1264–1267.
18. Wever RA (1979) *Circadian System of Man: Results of Experiments Under Temporal Isolation* (Springer-Verlag, New York).
19. Czeisler CA, Zimmerman JC, Ronda JM, Moore-Ede MC, Weitzman ED (1980) *Sleep* 2:329–346.
20. Saper CB, Scammell TE, Lu J (2005) *Nature* 437:1257–1263.
21. Moore RY, Speh JC, Leak RK (2002) *Cell Tissue Res* 309:89–98.
22. Yamaguchi S, Isejima H, Matsuo T, Okura R, Yagita K, Kobayashi M, Okamura H (2003) *Science* 302:1408–1412.
23. Yan L, Okamura H (2002) *Eur J Neurosci* 15:1153–1162.
24. Albus H, Vansteensel MJ, Michel S, Block GD, Meijer JH (2005) *Curr Biol* 15:886–893.
25. Nagano M, Adachi A, Nakahama K, Nakamura T, Tamada M, Meyer-Bernstein EL, Sehgal A, Shigeyoshi Y (2003) *J Neurosci* 23:6141–6151.
26. Nakamura W, Yamazaki S, Takasu NN, Mishima K, Block GD (2005) *J Neurosci* 25:5481–5487.
27. Antle MC, Silver R (2005) *Trends Neurosci* 28:145–151.
28. Waterhouse JM, Minors DS, Åkerstedt T, Reilly T, Atkinson G (2001) in *Handbook of Behavioral Neurobiology: Circadian Clocks*, eds Takahashi JS, Turek FW, Moore RY (Kluwer Academic/Plenum Publishers, New York), pp 571–601.
29. Davidson AJ, Aujard F, London B, Menaker M, Block GD (2003) *J Biol Rhythms* 18:430–432.
30. Kubota T, Kushikata T, Fang J, Krueger JM (2000) *Am J Physiol* 279:R404–R413.
31. Sokolove PG, Bushell WN (1978) *J Theor Biol* 72:131–160.

EXPERIMENT 2: SECRECIÓ DE MELATONINA EN T22

INTRODUCCIÓ

Se sap que la melatonina (MEL) és sintetizada a partir d'un aminoàcid essencial, el triptòfan, en els pinealòcits de la glàndula pineal majoritàriament, seguint un clar ritme circadiari dirigit pel NSQ. El cicle llum-foscor (LD) és el principal *zeitgeber*, de manera que durant la nit es produeix un pic de MEL, la durada del qual està relacionada amb la llargada de la nit, i durant el dia els nivells d'aquesta hormona són baixos, perquè la llum n'inhibeix la síntesi (Illnerová i Vanêcek, 1980; Middleton, 2006). De fet, la llum, en funció de com i quan aparegui, provoca un efecte diferent sobre la producció de MEL: un pols de llum durant l'inici del període de foscor n'inhibeix bruscament la síntesi (en rates, petits polsos de llum poden suprimir-ne la producció durant unes quantes hores (Vollrath *et al.*, 1989), amb la particularitat però, que si els animals són mantinguts en foscor la síntesi reapareix) i, polsos de llum al final del període de foscor també suprimeixen la síntesi de MEL (White *et al.*, 1985). Conseqüentment, aquesta hormona participa en la transmissió de la informació de la durada del fotoperíode, des de l'ambient a l'organisme (Reiter, 1993; Simonneaux i Ribelayga, 2003).

En individus sans i que segueixen un cicle de LD de 24 hores, el perfil del pic de MEL és perfectament reproduïble dia a dia i setmana a setmana (Arendt, 1988; Arendt, 2005), observant-se, en alguns estudis, diferències estacionals en el patró de MEL humana, havent-hi un petit avançament de fase a l'estiu (Bojkowski i Arendt, 1988) i un augment dels nivells i de la durada de la secreció a l'hivern (Kauppila *et al.*, 1987).

S'han desenvolupat diverses tècniques per determinar la concentració de MEL i dels seus metabòlits en els diversos fluids biològics (plasma, sèrum, saliva i orina): fluorometria, cromatografia de gasos, espectrometria de masses, cromatografia líquida d'alta pressió (*high-pressure liquid chromatography* - HPLC), microdiàlisi i immunoassaigs (*enzyme-linked immunosorbent assay* - ELISA i *radioimmunoassay* - RIA). D'entre totes elles, l'immunoassaig (el RIA en particular) ha estat una de les tècniques més utilitzades (Kennaway, 2002; Middleton, 2006), i és aquesta tècnica la que utilitzem en l'experiment que ens ocupa.

Se sap que sota condicions de T22 les rates presenten dissociació en el ritme d'activitat motora, però, quin patró té el ritme de l'hormona MEL quan els animals es troben sota aquest règim lumínic?

OBJECTIU

L'objectiu d'aquest experiment va ser determinar el ritme de MEL en rates sotmeses a cicles de LD de 22 hores, i poder especificar si la secreció d'aquesta hormona segueix majoritàriament un dels dos components que es presenten en la dissociació.

MATERIAL I MÈTODES

Animals i tractament dels grups

Un total de 24 rates Wistar, de dos mesos d'edat, es van utilitzar per a la realització d'aquest experiment. Quan van arribar al laboratori, els animals van ser col·locats, individualment, en gàbies transparents, de mides 25x25x12cm, situades dins una cabina aïllada de les influències externes, amb la temperatura i la humitat controlades. Durant tots els dies que va durar l'experiment, els animals van tenir accés lliure a la beguda i a l'aliment.

Els animals van estar un total de 40 dies en T22 (LD 11:11). En les etapes de llum, es van utilitzar dues làmpades fluorescents que projectaven una intensitat de 300 lux sobre les gàbies. Durant les etapes de foscor, es van il·luminar les cabines amb llum de neó vermella d'intensitat inferior a 0,1 lux.

El registre de l'activitat motora es va fer durant tot l'experiment, acumulant-se, les dades d'activitat motora, en intervals de 15 minuts.

Al voltant del dia 20 de l'experiment, quan els animals ja presentaven dissociació en el ritme d'activitat motora, es va procedir a canular la vena jugular dels 12 animals que millor mostraven ambdós components de la dissociació. Degut a les dificultats tècniques que presenta aquesta intervenció, únicament 9 rates van seguir el curs de l'experiment. Passats 3-4 dies, es va fer la primera sèrie d'extraccions de sang i al cap de 4-5 dies més, la segona. Després de cada extracció es va reposar el mateix volum amb solució fisiològica.

Cada sèrie consistia en extreure 200 µL de sang cada 4 hores circadiàries, cobrint tot el cicle de 22 hores, i coincidint amb les 4 situacions que es generen en un patró dissociat: S1: doble nit; S2: doble dia; S3: nit LDC-dia NLDC i S4: dia LDC-nit NLDC. En total, a cada animal se li van fer 14 extraccions, repartides en dos dies diferents, separats 4 dies. El primer dia es van fer les extraccions corresponents a les situacions 1 i 2, que són les que vam anomenar de components junts (J), ja que les fases dels dos components són coincidents; en la segona tanda d'extraccions es van fer les corresponents a les situacions 3 i 4, que vam anomenar de components separats (S), perquè les fases d'ambdós components són divergents. Vam utilitzar 2 kits de RIA que ens permetien analitzar mostres de 6-7 animals.

Anàlisi de les dades

Les mostres de sang es van centrifugar a 2300rpm, recollint-ne el plasma. Les concentracions de MEL es van obtenir processant, primer, el plasma per la tècnica de RIA (kit comercial d'IBL) i després, pel comptador gamma.

RESULTATS

Hem expressat els resultats obtinguts en forma de gràfica (Figura IV-13) en la qual s'observa el ritme de MEL en cada CT estudiat, per cada animal, quan els 2 components estan junts i quan estan separats.

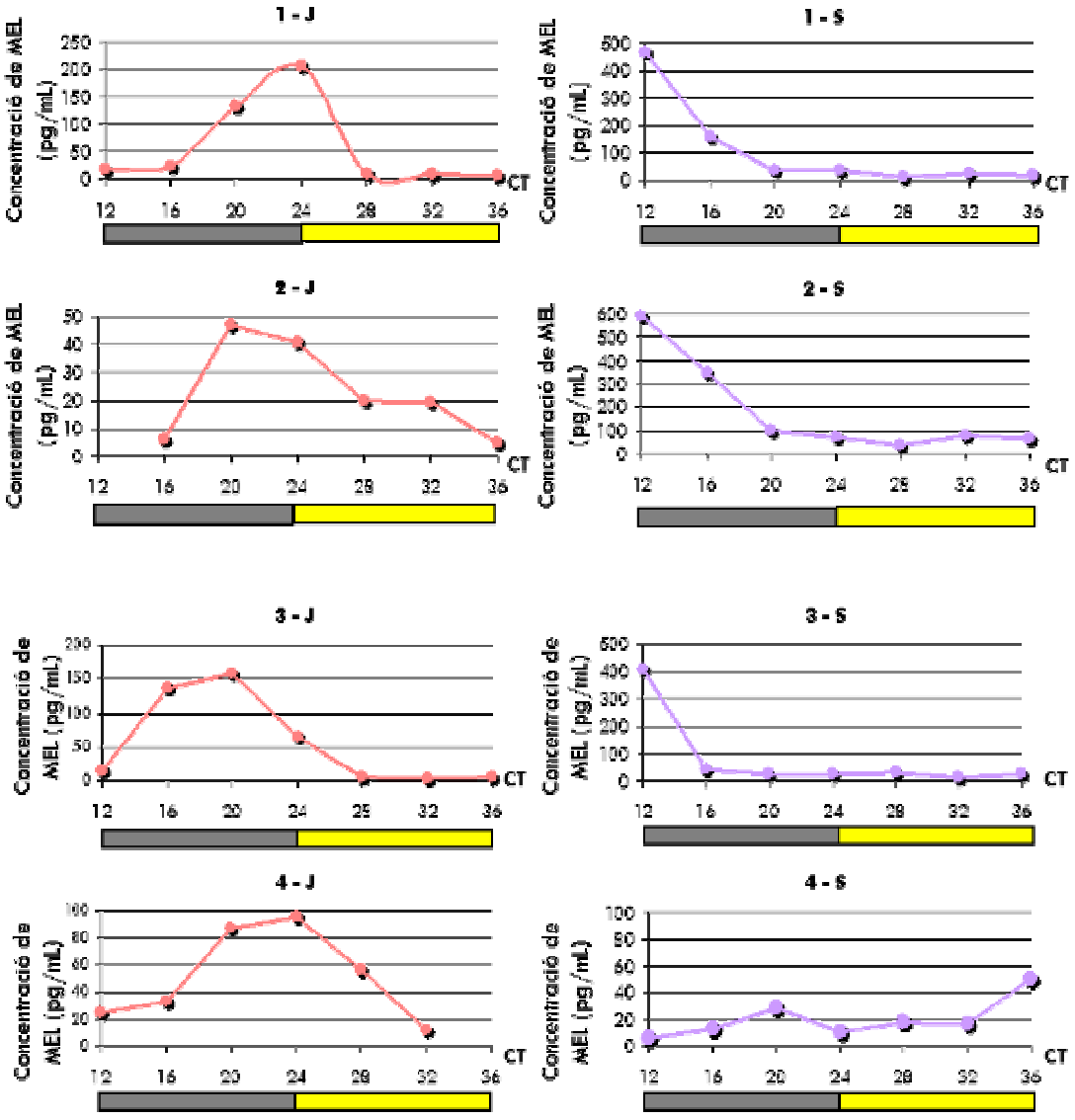

Figura IV-13. Concentració de MEL en plasma en diferents CTs, de quatre animals sotmesos a cicles de T22. J: dos components junts; S: dos components separats; barra grisa: foscor; barra groga: llum.

Els resultats indiquen que els nivells plasmàtics de MEL quan les fases dels dos components coincideixen, són semblants als que s'obtenen quan els animals estan sincronitzats a cicles de 24 hores: la secreció de MEL augmenta per la nit, produint-se'n un pic, i disminueix durant el dia. Quan els dos components estan separats, encara que segueix existint una inhibició de la MEL per causa de la llum, en la majoria d'animals s'observa un desplaçament de l'hora del pic de secreció, advertint-se, a més, quan

comparem J i S, un canvi en el patró d'aquest.

DISCUSSIÓ

Els resultats mostren que la secreció de MEL en animals sotmesos a T22 depèn de la relació de fases d'activitat i repòs deguda als dos components manifestos, cosa que ens fa pensar que la secreció de MEL no ve determinada per una part específica dels NSQ, sinó per les dues simultàniament.

Observem que quan la secreció de MEL es registra el dia en què els dos components són coincidents en fase, de manera que només hi ha una fase de repòs i una d'activitat, el ritme de MEL és similar al que s'observa en condicions de 24h de llum i foscor, havent-hi un pic de MEL cap al final de la nit. En canvi, quan la MEL es registra en el dia en què els dos components no són coincidents, és a dir, que l'animal es mou contínuament en les 24 hores, amb l'activitat induïda per un component o per l'altre, el patró rítmic de MEL canvia. En cap cas s'ha detectat MEL durant el dia, el qual mostra la inhibició de la llum sobre aquesta hormona fins i tot en animals dissociats, i d'altra banda, el pic de MEL canvia de fase, presentant-se en tres dels animals a l'inici de la foscor. Tampoc s'han detectat dos pics de MEL, el qual significa que no hi ha dissociació del ritme d'aquesta hormona. Tot això ens suggereix que la secreció de MEL ve regulada per tot el NSQ i no per una part en concret.

Cal comentar també la dificultat de la tècnica ja que suposa mantenir animals canulats durant almenys 10 dies, i treure un total de 14 mostres de sang en cada un d'ells.

Una vegada més, els resultats ens fan pensar que quan hi ha desincronització, aquesta afecta a tot l'organisme en el seu conjunt i que això pot tenir conseqüències negatives per la salut de l'individu.

Tot i així, després d'haver realitzat l'experiment, considerem que aquest no ens permet determinar amb fiabilitat el canvi de fase observat en el pic de MEL, com a conseqüència de la dissociació entre els dos components, i creiem que la tècnica idònia per repetir l'experiment seria la microdiàlisi. Aquesta tècnica (Liu i Borjigin, 2005) permet determinar, d'una manera contínua, la MEL durant dies seguits i seria el mètode més adient per una situació com la nostra en què la relació entre els dos components varia dia a dia.

Apartat II: **INFLUÈNCIES AMBIENTALS DURANT L'ALLETAMENT EN LA INDUCCIÓ DE LA DISSOCIACIÓ**

EXPERIMENT 3: EFECTE DE L'EXPOSICIÓ A CICLES T DURANT L'ALLETAMENT

ARTICLE

EXPOSURE TO T-CYCLES OF 22 AND 23H DURING LACTATION MODIFIES THE LATER DISSOCIATION OF MOTOR ACTIVITY AND TEMPERATURE RHYTHMS IN RATS

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INTRODUCCIÓ

Sota cicles circadiaris inferiors a 24h, les rates presenten dissociació d'AM i de TEMP. D'altra banda, les condicions ambientals en les primeres etapes de la vida poden afectar el desenvolupament i la manifestació dels ritmes circadiaris. Però, es presenta la dissociació si els animals són mantinguts des del naixement en condicions de LD de 22 i 23 hores?

OBJECTIU

L'objectiu d'aquest treball és estudiar si es produeix dissociació del sistema circadiari quan es sotmeten rates durant l'alletament a un cicle de període tal que, en el cas de ser imposat a rates adultes, els provocaria dissociació.

MATERIAL I MÈTODES

40 rates Wistar van ser mantingudes en cicles T (T22 o T23) des de l'alletament. En cada un dels grups la meitat dels animals havia nascut sota el corresponent cicle T i l'altra meitat, en T24.

Es va estudiar i comparar els ritmes d'AM i de TEMP dels dos grups d'animals en el corresponent cicle T i en DD.

RESULTATS

Els animals sotmesos a T23 des del naixement presenten una major amplitud del LDC, respecte a la resta d'animals del grup T23. En el grup T22, les condicions del naixement i l'alletament afecten més al NLDC i al grau de dissociació. Dins d'aquest grup, només els animals nascuts en T24 presenten desincronització entre AM i TEMP.

CONCLUSIÓ

Els resultats indiquen que el període del cicle LD durant l'alletament pot modificar la manifestació dels ritmes d'AM i de TEMP, i suggereixen que això pot ser degut a la modificació de l'acoblament entre les dues parts del NSQ.

EXPOSURE TO T-CYCLES OF 22 AND 23H DURING LACTATION MODIFIES THE LATER DISSOCIATION OF MOTOR ACTIVITY AND TEMPERATURE RHYTHMS IN RATS

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ABSTRACT

Early environmental conditions may affect the development and manifestation of circadian rhythms. We studied whether maintenance of rats under different T-cycles during lactation may influence the subsequent degree of dissociation of the circadian rhythms of motor activity and core body temperature. Two groups of 22 days-old Wistar rats were kept after weaning under T-cycles of 22h (T22) or 23 h (T23) for 70 days, and afterwards into constant darkness. Half of the animals in each group were born and reared under these experimental conditions while the other half were reared until weaning under 24h LD cycles (T24). As previously described, in rats transferred from T24 to T22 or T23, these animals show two circadian components in the motor activity and temperature, one entrained by light and the other free-running. In the case of T22 there is also desynchronization between temperature and motor activity. Results show that rats submitted to T23 from birth show higher stability of the 23h component, than those rats transferred from T24 to T23 after weaning. On the other hand, in T22 rats, those animals submitted to T22 from birth, as compared with rats born under T24 and later changed to T22, showed shorter values of the period of the non-light dependent component during T22, more aftereffects when transferred to DD and lack of desynchronization between motor activity and temperature. The results suggest that T-cycles in the early environment may modify the overt rhythms by modifying the internal coupling of the circadian pacemaker.

KEYWORDS: circadian rhythm, suprachiasmatic nucleus, dissociation, internal desynchronization, motor activity, temperature

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INTRODUCTION

In mammals, the major pacemaker for circadian rhythmicity is located in the suprachiasmatic nuclei (SCN) of the hypothalamus that drives circadian rhythms in physiological and behavioral functions (Klein et al., 1991). The SCN can be morphologically subdivided into the shell and the core (Moore et al., 2002). The core, mainly corresponding to the ventrolateral (VL) region, comprises neurons producing vasoactive intestinal polypeptide or gastrin-releasing peptide colocalized with gamma-aminobutyric acid (GABA), and receives visual and raphe afferents. The shell, mainly corresponding to the dorsomedial (DM) region contains a large population of arginine vasopressin-producing neurons and a smaller population of calretinin-producing neurons, also colocalized with GABA, and receives input from non-visual cortical and subcortical regions (Moore et al., 2002). Moreover, peripheral clocks exist with a genetical mechanism equivalent to that of the central pacemaker (Stratmann & Schibler, 2006). Usually, the two parts of the SCN are functionally coupled, generating a single rhythm in the overt rhythms, that, under constant conditions, has a period, tau, that is characteristic of the individuals due to its genetic background. The coupling between the two parts of the SCN seems to be mediated by GABA (Albus et al., 2005). Under cyclic environmental conditions, the animals adequate the endogenous period to be equal to that of the external cycle, that is, animals entrain to the cyclic outside world. The light-dark cycles are the most important regular environmental changes that animals are exposed to.

The SCN are already functional at birth, but to generate the overt rhythms, maturation is needed in the neural network and for full retinal activity (Wong, 1999). Although clock genes are already detected in rat SCN neurons before birth, there are no detectable oscillations in their expression, an activity which requires some time to be gradually develop (Vallone et al., 2007; Kováciková et al., 2006). The SCN develops from embryonic day 14 to 17, and synaptogenesis increases until postnatal day 10 (Moore, 1991). The emergence of the circadian rhythms in rodents seems to occur during the first 2-3 weeks after birth (Davis, 1981). For example, the rhythmic production of melatonin is first detected during the second week of life (Tamarkin et al., 1980). The mammalian circadian clock may initiate its function independently of the functional clock in the mother, although some studies report the importance of the mother to the entrainment of the pup to the environment (Vallone et al., 2007). Thus, apart from the genetic factors that define the functioning of the circadian clock, the early environment may affect the functioning of the circadian pacemaker as well as their overt rhythms.

Environmental conditions in some cases are able to influence the development of the rhythms. For example, in zebrafish LD exposure is required for the appearance of

rhythmic behaviour (Hurd & Cahill, 2002), since reducing the number of LD cycles during the first 4 days results in a decrease in amplitude of the free-running rhythms. In rats, we found out that the manifestation of the circadian rhythm under LL (Cambras et al., 1997) and the responses to light of the free-running rhythm under DD (Canal-Corretger et al., 2000; Canal-Corretger et al., 2001) were dependent on the quantity of light (number of days under LL) the animal received during lactation. Moreover it has been described that lighting conditions during development affect the structure of the SCN (Cambras et al., 2005; Ikeda et al., 2003). Thus, it seems that external time cues during the early developmental stage contribute to the development of the overt rhythms, and that the circadian pacemaker displays a dynamic plasticity adapting to the external conditions.

The circadian system is sometimes flexible in its functioning, and under certain abnormal external conditions, the overt rhythms can adopt nonstandard patterns. This is the case, for instance, with dissociation of circadian rhythms under light-dark cycles (LD) of 22 hours period (T22). Rats transferred to T22 show two simultaneous circadian rhythms in their motor activity expression: one, the Light Dependent Component (LDC), follows the same period as the external LD cycle, whereas the other, the Non-Light Dependent Component (NLDC), runs free with a period longer than 24 hours (Campuzano et al., 1998). Earlier studies have revealed that LDC coincides with the expression of clock genes in the ventrolateral region of the SCN, while the NLDC coincides with expression in the dorsomedial area (de la Iglesia et al., 2004). Recent studies indicate that under T22 dissociation, motor activity mainly follows the LDC while temperature and REM sleep the NLDC (Cambras et al., 2007)

The purpose of this experiment was to study whether the dissociation of the circadian system is influenced by the length of the period of the early cyclic environment. Specifically, taking into account that dissociation of the rat circadian pacemaker appears when animals are transferred from T24 to T22, we wondered whether the exposure of rats to LD cycles of T22 and T23 during lactation would affect the pattern of dissociation of the overt rhythms of motor activity and temperature.

MATERIALS AND METHODS

Experimental Procedures

10 pregnant Wistar rats (Charles River, France) arrived at our laboratory at the gestational age of 17 days. They were then housed in individual cages and located in three different isolated rooms with different light-dark (LD) conditions: 3 rats were placed in T22 conditions (11h light and 11h darkness; LD 11/11), another 3 into T23 (LD 11.5/11.5) and 4 more into T24 (LD 12/12). Light was provided by two fluorescent tubes

with an intensity of 300 lux at cage level; while during darkness, a dim red light with an intensity of less than 0.1 lux was used. The rats gave birth 3-4 days afterwards. When the pups were 22 days old (day 1 of the experiment) they were weaned and 40 were used for the experiment with the following distribution: 10 rats born under T22 and 10 born under T24 group were placed in a room under T22 conditions, while 10 rats born under T23 group and 10 under T24 were placed under T23 conditions. Groups were named according to the lighting conditions during lactation ($T22_L$, $T23_L$ or $T24_L$) and the T-cycles after weaning (T22 or T23). Thus, the $T24_L$-T22 group was born under T24 and transferred to T22 after weaning, while $T22_L$-T22, was always kept under T22. The same applies for $T24_L$-T23 and $T23_L$-T23. Males and females were equally distributed among the groups. 37 days after the beginning of the experiment, one temperature sensor was surgically implanted in 8 rats from $T24_L$-T22 and $T22_L$-T22 groups and 7 rats from $T24_L$-T23 and $T23_L$-T23 groups. The sensor was programmed to start recording 3 days later when the animals were recovered from surgery. 30 days later, all rats were transferred to DD conditions for 57 days more, until the end of the experiment. After 35 days under DD, a light pulse of 1 hour was applied to each rat on CT15 to test if the response of the circadian pacemaker to light could have been altered by exposure to T-cycles during lactation. CT15 was calculated by adding 3 circadian hours to CT12 (time of the beginning of the activity phase), which was estimated visually by two researchers. The light pulse was applied, by transferring each animal to a room next to the room where the experiment took place, that received a light intensity of 300 lux.

Animals were individually housed in transparent cages (25x25x15 cm) with food (Harlan Global Diet 2014) and tap water *ad libitum* in all conditions. Motor activity was recorded throughout the experiment by activity meters, which consisted of two crossed perpendicular infrared beams crossing the cage 7 cm above the floor. The number of movements were accumulated and recorded every 15 min. Body temperature was measured by data loggers (Thermochron®, iButton, IDC SA Spain) with a resolution of 0.125ºC implanted intraperitoneally to the animals under isofluorane anesthesia. This variable was sampled every 30 minutes during 42 days corresponding to the last 30 days under LD and the first 12 days under DD. At the end of the experiment, animals were sacrificed and the temperature sensors removed to acquire the data.

Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation of the University of Barcelona and the ethical and good practice standards for chronobiological research on animals (Touitou et al., 2006).

Data analysis

We analyzed separately the LD stage and the DD stage for each animal. For motor activity data we analyzed the LD in two parts. The first one (LD_0) corresponded to days 7-37 of the experiment and the other to days 40-70. Since temperature data were only recorded for the second stage, we will refer to this part as the LD stage. Circadian rhythms were studied by means of the chi-squared periodogram applied to data (Sokolove & Bushell, 1978). This provides the period of the most significant rhythm and the percentage of variance (%V) explained by this rhythm. %V is an indicator of the stability of the rhythm and was used as a marker of the importance of each rhythm. Since under T22 and T23 two components use to appear, one with the same period as the external cycle (LDC) and another free-runnning (NLDC), we calculated the ratio between the variances explained by the two components ($\%V_{LDC}/\%V_{NLDC}$) as an index of entrainability to the external cycle.

Since rats showed two simultaneous circadian rhythms, four different stages can be differentiated in their motor activity and temperature patterns according with the coincidence between the light (day) or the dark (night) phase of the external cycle with the rest ("day") or activity ("night") phases due to the NLDC (Cambras et al., 2007). Thus, we define situation 1 (S1) as the coincidence between the night of LDC and the night of NLDC (double night); situation 2 (S2) as the coincidence between the day of LDC and the day of NLDC (double day); situation 3 (S3) as the coincidence between the night of LDC and the day of NLDC and situation 4 (S4) as the coincidence between the day of LDC and the night of NLDC. For each stage, we calculated the mean motor activity and mean body temperature of each animal expressed as the deviation from the mean in each stage.

Once the animals were transferred to DD, data were analyzed into 5 stages of 11 days each, named as DD1, DD2, DD3, DD4 and DD5. The last two stages correspond to days after the light pulse. Since here the number of days was smaller than in the previous stage, we used the Lomb-Scargle's periodogram to estimate the period of DD stages, because of its higher accuracy. The evolution of the period throughout these stages was used to study the presence of aftereffects in the free-running period (tau) resulting from the previous LD conditions. The period for the temperature data was only available for DD1.

The phase shift in the motor activity rhythm after the light pulse at CT15 was calculated separately for the onsets and offsets of activity. On a graph, lines were drawn through the daily onsets (and offsets) for the 10 days before and after the treatment. The difference between the two eye-fitted lines before and after the light pulse was the phase shift value. This calculation was made by two independent researchers. Moreover, in order to ascertain

if the light pulse caused a period shift, we calculated the individual differences in the tau values between DD4 and DD3.

Also, the rhythm phase control exerted by the previous LD cycle was examined at the DD stage. To do so, actograms were plotted at modulo T (22 or 23 hours) and then, a line was drawn by extrapolating the onset of the activity of the first 10 days in DD to the last LD cycle. Then the grouping of the projected activity onsets of the individuals was studied with the Rayleigh z test (Batschelet, 1981), which indicates the temporal distribution of individual phases across the T cycle.

Statistical analysis was carried out with an ANOVA of several linear models and post-hoc comparisons with Bonferroni's correction. The dependent variables, each one tested in a different analysis, were: period of NLDC, %V of LDC ($\%V_{LDC}$) and NLDC ($\%V_{NLDC}$), the variable $\%V_{LDC}/\%V_{NLDC}$, the period of the endogenous rhythm, the phase delays in the onset and offset of activity after the light pulse at CT15, and the mean values of motor activity and temperature in each situation defined by the interaction of the two rhythms. The independent variables, depending on the model, could be the two measured variables (motor activity and temperature), the T-cycles (T22 or T23), the lighting conditions during lactation ($T22_L$, $T23_L$ or $T24_L$), the situations defined according to the interactions of the two components (S1, S2, S3 and S4 and for the analysis of the DD stage, the parts DD_1, DD_2, DD_3 -before light pulse- and DD_4, DD_5 –after light pulse.

Time series analyses were conducted by means of an integrated package of tools for chronobiology: "El Temps©" (Antoni Díez-Noguera, Universitat of Barcelona, 1998-2006, http://www.el-temps.com) and statistical analysis was carried out with SPSS® package.

RESULTS

Data corresponding to 2 rats (a male from $T24_L$-T22 and a female from $T22_L$-T22) were excluded from the analysis due to methodological problems. As expected, most of the rats showed two circadian components in their motor activity and temperature patterns when they were maintained under T22 and T23 cycles (Figure 1). The proportion of rats that showed two significant components in the periodogram are shown in table 1. It is noteworthy that very few animals showed the NLDC component as the only significant component, and this was always for temperature in T22 rats. Also it is worth to notice that the number of rats showing two peaks in the periodogram for the motor activity data was higher in the second than in the first part of the LD stage, except for the $T24_L$-T22 group, in which most of the animals were already dissociated since the beginning of the experiment. A chi-squared test between the frequencies of LD_0 and LD did not show significant differences, but in $T22_L$-T22

group it was close to significance ($p=0.057$). The same test showed statistical differences in the proportion of rats in LD_0 between $T24_L$-T22 and $T22_L$-T22 ($p=0.018$).

An ANOVA of general linear models for the period of NLDC in the LD stage considering as independent variables the two measured variables, the T-cycles (T22 or T23), and the lighting conditions during lactation ($T22_L$, $T23_L$ or $T24_L$) indicates that the period of NLDC depended on the T-cycles after weaning ($p<0.05$) and those during lactation ($p<0.05$), but not on the variable studied (temperature or motor activity). Post-hoc comparisons reveal that differences were due to the $T24_L$-T22 group, which had longer values of the period compared to the other groups (in all cases, $p<0.05$) (Figure 2).

In the same way, an ANOVA of general linear models for the $\%V_{LDC}$ indicated that this variable showed higher values while measured with temperature than with motor activity ($p<0.05$), that depended on the T-cycles ($p<0.001$), with T23 rats having higher values than T22 rats; and on the lighting conditions during lactation ($p<0.001$). Specific contrasts indicate that in T23 rats, $\%V_{LDC}$ was higher ($p<0.025$) in $T23_L$-T23 than in $T24_L$-T23, and in T22 rats, only for temperature data, the $\%V_{LDC}$ was higher for $T22_L$-T22 than for $T24_L$-T22 ($p<0.001$). (Figure 3).

The same linear model applied to the $\%V_{NLDC}$,,indicated that measured with temperature data, this variable had higher values than with motor activity ($p<0.001$). Post hoc comparisons reveal that differences among groups were due to the $T24_L$-T22 group, which showed higher values than the other 3 groups ($p<0.005$) in all comparisons (Figure 3).

We used the ratio $\%V_{LDC}/\%V_{NLDC}$ as an index of the entrainability of the system (Figure 4). The same linear model applied to this variable indicate that with motor activity data, this ratio showed higher values than with temperature data ($p<0.05$). Analyzing both variables separately, an ANOVA indicates that for motor activity data, $T24_L$-T22 rats had values significantly lower than rats kept under T23 ($p<0.05$), and that for temperature data, the values in $T24_L$-T22 were even lower, being significantly different from the other 3 groups ($p<0.01$ in all comparisons). It is worth to notice that only in $T24_L$-T22 and for temperature data this value is lower than 1.

The analysis of the mean motor activity and temperature in the 4 situations generated by the interaction of the two components indicate that there are differences depending on the situation (Figure 5). A linear model with repeated measures (the four situations) for each variable and for each group of rats, reveal that in all the groups the highest values correspond to S1 (double night), the lowest to S2 (double day) and the other two parts had intermediate values, being in all cases statistically significant different from S1 and S2 (in all cases $p<0.05$). However, S3 was

not different from S4 excepting in $T24_L$-T22 and for temperature data, where values in S4 were higher than in S3 ($p<0,05$) and in group $T24_L$-T22 were S3 had higher values than S4 ($p< 0.05$). To test if the mean values in each phase were affected by the lighting conditions, we also carried out t-tests, with Bonferroni's correction, considering the lighting conditions during lactation for the values of each variable, each stage and each group of rats. We found out that there were statistically significant differences in the values for temperature data of T22 rats in S3, where $T22_L$-T22 showed higher values than $T24_L$-T22($p<0.001$) and nearly in S4 where $T22_L$-T22 showed lower values than $T24_L$-T22($p=0.053$). Also motor activity for T23 rats showed in S1 higher values for $T23_L$-T23 than $T24_L$-T23 ($p<0.01$).

Once the animals were transferred to DD conditions they showed a free running rhythm (Figure 6), that the first days (DD1) had a different value among groups. An ANOVA of a general linear model considering the different stages under DD, the T-cycles and the conditions during lactation, indicated that this pattern was dependent on the T-cycles ($p<0.05$) and on the lighting conditions during lactation ($p<0.001$). Post-hoc tests, indicate that the group that had longer values of tau was $T24_L$-T22, being significantly different from $T22_L$-T22 ($p<0.01$) and $T23_L$-T23 ($p<0.01$). The evolution of tau through DD stages was analyzed separately for the 4 groups of animals (Figure 5). For each group the tau values increased throughout the stages, according to a significantly linear regression ($p<0.001$ for $T22_L$-T22; $p<0.01$ for $T24_L$-T22; $p<0.001$ for $T24_L$-T23; and $p<0.001$ for $T23_L$-T23). The slope of the regression was lower in the $T24_L$-T22 group. There were no differences between tau values in stages DD3, DD4 and DD5, indicating a stability of the period in these stages. Studying the four groups of rats separately, general linear models with repeated measures (DD1 to DD4) indicate that tau for the $T24_L$-T22 group in stages DD1 and DD2 was different than in DD4 ($p<0.01$); for $T22_L$-T22 and for $T24_L$-T23 only DD1 differ from DD4 ($p<0.01$) and for $T23_L$-T23 no differences were observed between stages.

As a result of the light pulse on CT15, changes in both phase and period were observed, but no differences between the groups were observed. The mean value of the phase shift (in both the onset and offset of motor activity) was a delay of 1.66 (e.s.: 0.09) hours (CT). The tau shift, calculated as the difference between the tau value of DD3 stage and the tau value of DD4, indicated a lengthening in all cases (mean: 6.22 min, e.s: 2.83 min).

The Rayleigh z-test was used to study the grouping of the temporal coincidence between the rhythm under LD and that under DD on the LD-DD transition. The results for both motor activity and temperature indicate statistically significant grouping of the phases in $T22_L$-T22 (motor activity: $r=0.705$; $p<0.05$; temperature: $r=0.606$; $p<0.05$)

and $T24_L$-T23 (motor activity: $r=0.670$; $p<0.05$; temperature: $r=0.746$; $p<0.05$) groups.

DISCUSSION

In this paper we proved that the capacity of the rat's circadian system to dissociate under T-cycles shorter than 24h is modified if animals are maintained under these conditions during lactation, and that these effects are different in rats are kept under T22 or T23.

In previous experiments (Madrid et al., 1992; Campuzano et al., 1998) it was found that when rats were maintained under LD cycles of T22 and T23, two simultaneous circadian rhythms were detected in their motor activity and food intake. More recently, it has been proved (de la Iglesia et al., 2004) that the dissociation was related to uncoupling between the ventrolateral and the dorsomedial parts of the SCN. The SCN uncoupling can induce desynchronization among different physiological variables, such as motor activity rhythm that shows a $\%V_{LDC}$ higher than $\%V_{NLDC}$, and the temperature or REM sleep rhythm, that showed a higher $\%V_{NLDC}$ and are supposed to be mainly driven by the DM region (Cambras et al., 2007). This resembles human internal desynchronization, since both motor activity and temperature data showed the same two significant circadian peaks, but the importance of each one was inverse comparing one or the other variable (Wever, 1979).

Taking into account the structure of the SCN, apart from its divisions into the core and the shell (Moore et al., 2002), the SCN comprises individual neuronal oscillators (Miller, 1998) that are coupled by neural connections to function as a pacemaker controlling effector systems like the rest-activity cycle, core body temperature, neuroendocrine function, or psychomotor performance (Moore, 1997). In previous work (Campuzano, 1998; Cambras, 2004) we found out that the motor activity pattern of dissociation is a gradual phenomenon in which the period and the %V of the NLDC changed proportionally as a function of the external period, this is as a difference between the spontaneous rhythm of the rat from the external cycle, in such a way that the shorter the period of the external cycle, the longer the period of the NLDC. Theoretically, in a multioscillatory system, different values of the endogenous period can be achieved by changing the degree of the internal coupling of the system. Thus considering the multioscillatory nature of the SCN, we interpret that in a dissociated circadian system the %V of a rhythm could indicate the strength of the output of the corresponding part of the SCN, and the period of the NLDC the degree of coupling of the two SCN regions, in the sense that the most coupled is the system, the shortest value has the period of the NLDC.

Until now, all the published experiments about dissociation under T22 were carried out under situations in which rats were born and raised under T24 and then transferred to other T-cycles. In these cases, animals manifest two circadian components in their overt rhythms, but the presence of the non-light dependent component was stronger in T22 than in T23, certainly due to the fact that T23 is closer to the endogenous period of the rat. T23 rats, as compared to T22, showed higher values of the ratio $\%V_{LDC}/\%V_{NLDC,}$ indicating a higher degree of entrainment, while the the period of NLDC showed shorter values (Campuzano et al., 1998). Obviously, the LDC is also influenced by masking responses to light, but as previously described (Cambras et al., 2004) we interpret the ratio between the %V of the two components as an index of the degree of adjustment of the rhythm manifestation to the LD cycle, without distinguishing if this is due to entrainment or masking. In this study, we found out that exposure to T-cycles during lactation modifies the expression of the rhythm dissociation. Since the manifestation of the NLDC is more pronounced in T22 than in T23 rats, it is comprehensible that T-cycles during lactation may have more effect in T22 rats. In the T23 group, rats born under T23, compared with those born under T24, have a higher $\%V_{LDC}$ than those born under T24, without differences in the manifestation of the NLDC, which can be interpreted as a stronger adaptation to the external LD cycle. However, for T22 rats, the lighting conditions during lactation did not imply differences in the LDC but in the manifestation of the NLDC, which suggests not a reinforcement of the external LD cycle, but a modification of the internal structure of the SCN. Since $T22_L$-T22 group manifested a shorter period than $T24_L$-T22, we may suggest that the degree of coupling between the two parts of the SCN might be modified by the environmental conditions during lactation. To explain why being exposed to LD cycles of different period during lactation induces strong coupling in the SCN, we can suggest that the LD cycle establishes the level of a hypothetical factor that determines entrainment. If the production (and elimination) of this factor were directly proportional to the illumination level, then we should not have found differences between groups, since all the cycles have the same percentage of light and darkness. However, if the production (and/or elimination) of this factor had an inertia through over time, the reached mean level would depend directly on the period of the oscillations, and this would explain the differences of coupling based on the periods of the LD cycles the animals were submitted to. This hypothetical factor remains to be identified.

It is noticeable that motor activity and temperature follow the same rhythmic pattern in all groups but $T24_L$-T22. If desynchronization between motor activity and temperature can be characterized by the ratio between the %V obtained by the

two components in each variable and also by the ratio between the mean values of this variables in S3 and S4 situations (Cambras et al, 2007), we can deduce that in animals $T22_L$-T22, temperature is not desynchronized from motor activity. If we consider a gradual process, desynchronization between the two rhythms should only appear when the two parts of the SCN are strongly dissociated, as it happens when rats are transferred from T24 to T22. In all the other groups, although they manifest two circadian rhythms in their physiological variables which imply that it is possible that the two parts of the SCN should be weakly dissociated, there is not desynchronization between motor activity and temperature. This indicates again that lighting conditions during lactation may reinforce the internal coupling of the circadian system.

Although one could think that the differences between the groups could be related to the number of days that the animals are under the T-cycles (animals born under T-cycles have been longer exposed to the external conditions) and consequently this derives in a better adaptation to the LD cycle, this not the case. Actually, the time exposure to T-cycles after weaning increases dissociation (the two rhythms take some time to be manifested when rats are transferred from T24 to T-cycles), while T-cycles during lactation seem to inhibit dissociation, understood as less manifestation of the NLDC or higher degree of entrainment. Thus, since rats from the $T24_L$-T22 group showed two components already in the first part of the LD stage, we believe that the conditions during lactation modify the rhythm manifestation in a different way than the "time" when the animal is transferred to T-cycles once adult. Therefore, the lactation period is a critical period for the development of the functionality of the circadian system. It must also be noted that the response to the light pulse did not differ between the groups; as it happens when the quantity of light received during lactation among groups is different (Canal-Corretger et al., 2001). It, therefore, seems that the retinal input or the sensibility of the SCN to light is not affected by the exposure to T-cycles. We interpret this fact to mean that, in this experiment, since all the rats received the same quantity of light throughout the experiment, a possible parametric effect of light (Aschoff, 1981) to explain changes in the period of NLDC of group $T24_L$-T22 is excluded.

For long, it has been known that the period of the free-running rhythm is history dependent (Pittendrigh & Daan, 1976). Aftereffects can be understood as a form of behavioral plasticity in which the free-running period of circadian behavior undergoes experience-dependent changes. We found out that aftereffects are less pronounced in $T24_L$-T22 rats than in the other three groups, which may indicate weaker adaptation to the LD cycle. It has been proposed that aftereffects may involve structures outside of the SCN, and may be

induced either by direct exposure to a non-24-h light cycle or by maternal effects (Aton et al., 2004). However, although maternal effects can not be excluded, we do not believe that differences between groups were due to the mother's rhythm, since animals were obtained from mothers with different rhythmic patterns. For example, of the three females under T22 conditions, one showed predominantly a free-running rhythm, the other the T22 rhythm and the third a mainly arrhythmic pattern (graphs not shown). Our results seem to indicate that the differences between groups are due to the direct exposure to T-cycles.

Although it has been postulated that early environment might have less effect on the free-running period of the animals than their genetic characteristics (Refinetti, 1998), we believe that the early exposure to abnormal conditions modifies the plasticity of the circadian pacemaker. If rats are exposed during lactation to constant light, the animal does not show the characteristic arrhythmicity under constant light once adult (Cambras et al., 1998), here if the rats are exposed to T22 during lactation shows higher degree of entrainment to the LD cycle, without desynchronization between motor activity and temperature. Thus it seems that during development, the young circadian system adapts to the environment in order to assure that the circadian rhythm is properly manifested. As we mentioned earlier (Canal-Corretger et al., 2003) the circadian system may be flexible enough during postnatal development to allow generation on the most suitable clock function for each environment. It has been proposed that an animal's ability to code for lengths of day derives from plasticity in the neuronal network of oscillating SCN neurons (Schaap et al., 2003). According to our results, the early exposure to a short T-cycle such as T22 can modify the coupling between the two parts of the SCN. It is true that under normal conditions animals will never be exposed to T-cycles but this situation results a good tool to study the plasticity of the circadian system and its ability to get adapted to external conditions.

ACKNOWLEDGEMENTS

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FIGURES

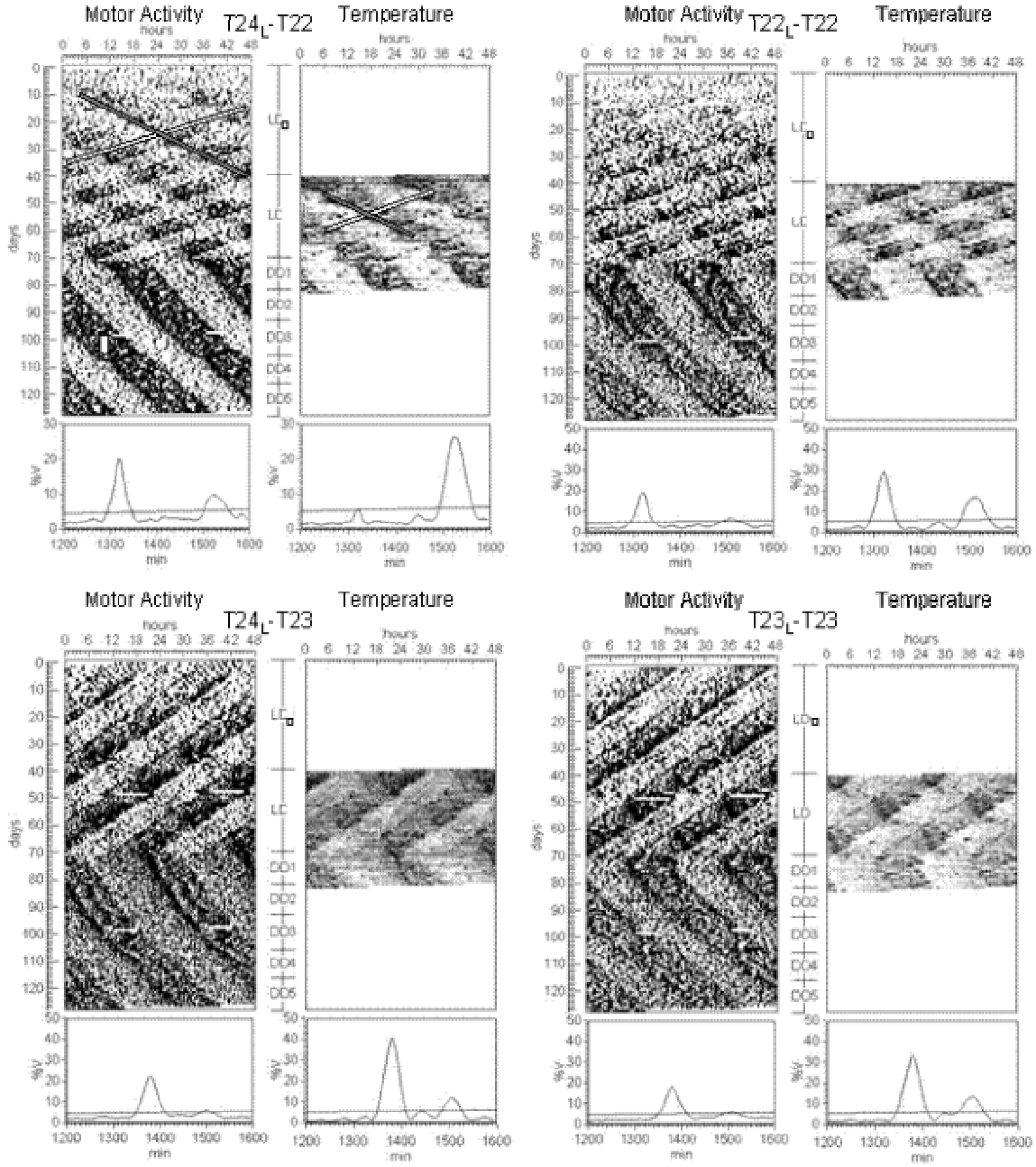

Figure 1: Double-plotted actograms (plotted at "modulo" 24h) and periodograms of a representative rat of each group. White bar corresponds to the onset of the LDC, while black bar to the onset of the NLDC. For each rat graphics on the left corresponds to motor activity data, while graphics on the right correspond to temperature. Periodograms correspond to the time series data between days 40-70 of the actograms (LD stage). Note the statistically significant peaks for the LDC and the NLDC in all the periodograms. Note the different relationship in the %V explained by each component between MA and TEMP of the $T24_L$-T22 group.

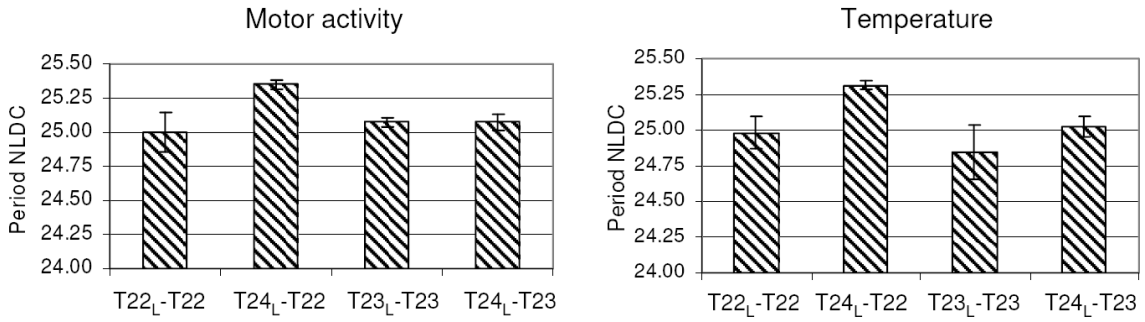

Figure 2: Mean values and standard errors of the NLDC period for motor activity and temperature rhythms in all groups of animals, during LD stage. The number of rats showing this rhythm significant for each group is indicated.

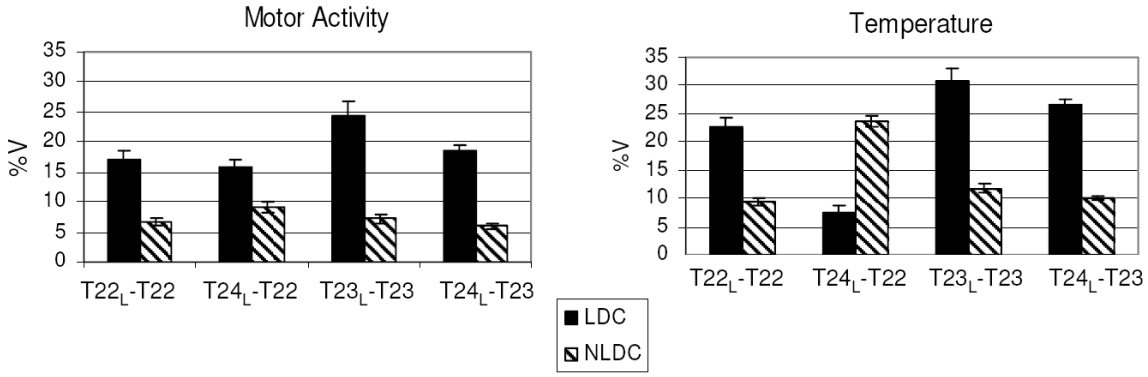

Figure 3: Mean values and standard errors of the percentage of variance (%V) explained by the LDC and the NLDC for motor activity and temperature rhythms for each group of rats. The number of rats showing each rhythm significant for each group is indicated. Note the inversion in the ratio of the %V of the two components in T22 groups for temperature.

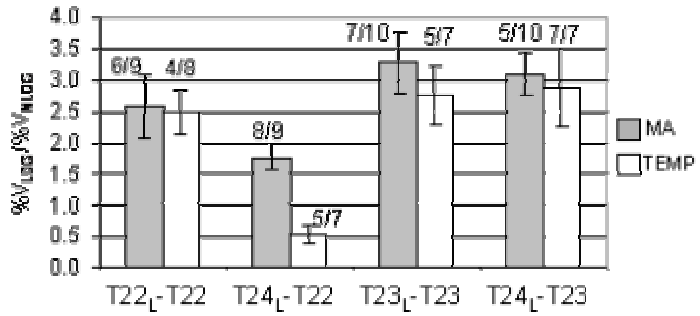

Figure 4: Mean values and standard errors of the ratio between $\%V_{LDC}$ and $\%V_{NLDC}$ ($\%V_{LDC}/\%V_{NLDC}$) of motor activity and temperature rhythms for each group of rats. The number of rats showing the two rhythm significant respecting to the total or rats in each group is indicated in the fraction. Note that values lower than 1 indicate lower stability of the LDC which only occurs for TEMP data of $T24_L$-T22.

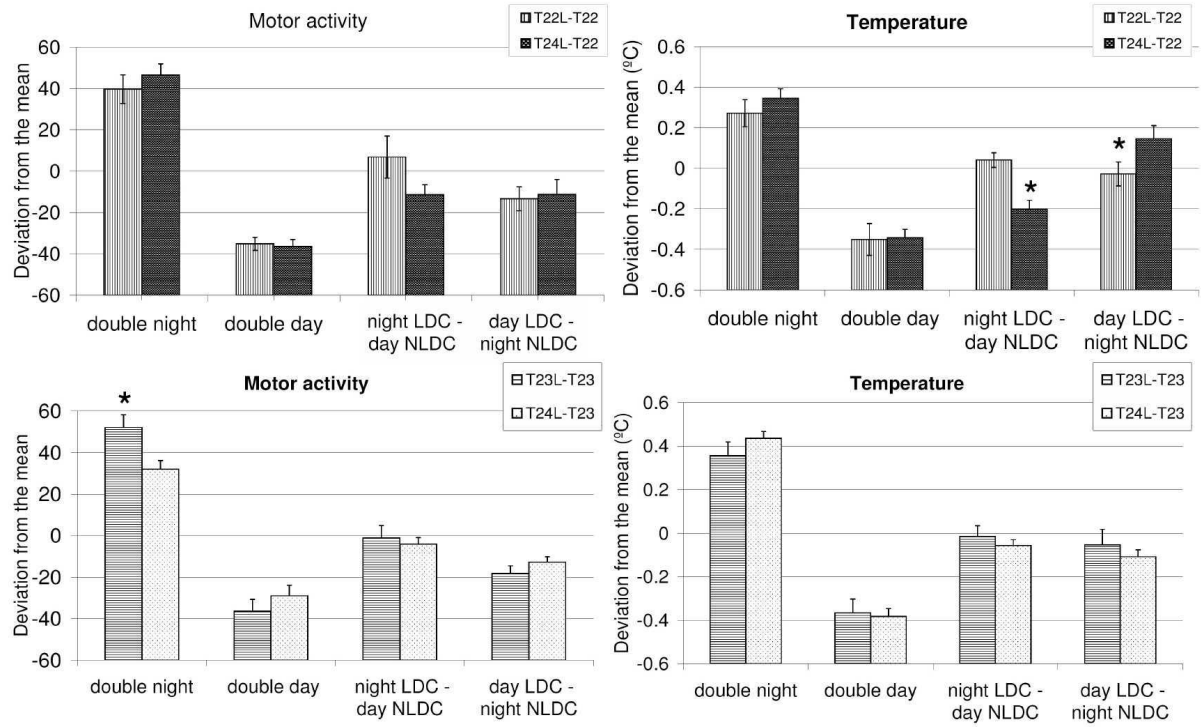

Figure 5: Deviation from the mean of motor activity and temperature in each one of the situations generated by the interaction between the two circadian components. Graphs at the top correspond to T22 groups, and those at the bottom to T23 groups. Asterisks indicate differences between the groups ($p<0.05$).

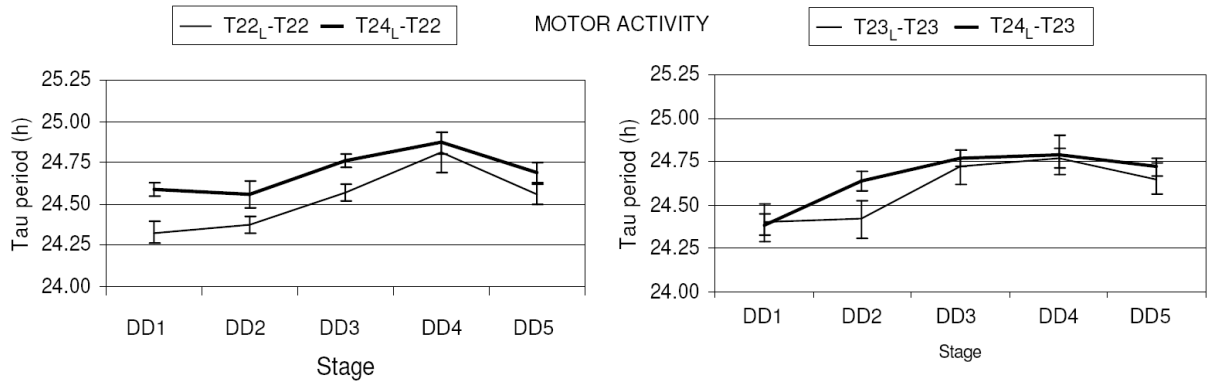

Figure 6: Mean values and standard errors of the free-running period of the motor activity rhythm in the DD stages for each group of rats. Note that at DD_1 the highest value is for $T24_L$-T22.

TABLES

GROUP	COMPONENTS	MOTOR ACTIVITY LD1	MOTOR ACTIVITY LD	TEMPERATURE LD
T22L-T22	LDC + NLDC	2/9	6/9	4/8
	only LDC	7/9	3/9	3/8
	only NLDC			1/8
T24L-T22	LDC + NLDC	7/9	8/9	5/7
	only LDC	2/9	1/9	
	only NLDC			2/7
T23L-T23	LDC + NLDC	3/10	7/10	5/7
	only LDC	7/10	3/10	2/7
	only NLDC			
T24L-T23	LDC + NLDC	1/10	5/10	7/7
	only LDC	9/10	5/10	
	only NLDC			

TABLE 1: Number of animals respect to the total number of rats in each group that showed: only the LDC as significant peak, only the NLDC as significant peak or the two statistically significant peaks (LDC + NLDC) in the periodogram, during LD stage for motor activity and temperature data.

REFERENCES

Albus H, Vansteensel MJ, Michel S, Block GD, Meijer JH. (2005). A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. *Curr. Biol.* 15:886-893.

Aschoff J. (1981). Freerunning and entrained circadian rhythms. In: Handbood of behavioural neurobiology: Biological Rhythms. J. Aschoff ed, pp81:93, Plenum Press, New York.

Aton SJ, Block GD, Tei H, Yamazaki S, Herzog ED. (2004). Plasticity of circadian behavior and the suprachiasmatic nucleus following exposure to non-24-hour light cycles. *J. Biol. Rhythm.* 19(3):198-207.

Batschelet E. (1981). *Circular statistics in biology*. Academic Press, London, 371 pp.

Cambras T, Canal MM, Torres A, Vilaplana J, Díez-Noguera A. (1997). Manifestation of circadian rhythm under constant light depends on lighting condition during lactation. *Am. J. Physiol.* 276:1039-1046.

Cambras T, López L, Arias JL, Díez-Noguera A. (2005). Quantitative changes in neuronal and glial cells in the suprachiasmatic nucleus as a function of the lighting conditions during weaning. *Develop. Brain. Res.* 157:27-33.

Cambras T, Vilaplana J, Torres A, Canal M, Casamitjana N, Campuzano A, Díez-Noguera A. (1998). Constant bright light (LL) during lactation in rats prevents arhythmicity due to LL. *Physiol. Behav.* 63(5):875-882.

Cambras T, Weller JR, Anglès-Pujolràs M, Lee ML, Christopher A, Díez-Noguera A, Krueger J, de la Iglesia HO. (2007). Circadian desynchronization of core body temperature and sleep stages in the rat. *Proc. Natl. Acad. Sci. USA.* 104:7634-7639.

Campuzano A, Vilaplana J, Cambras T, Díez-Noguera A. (1998). Dissociation of the rat motor activity rhythm under T cycles shorter than 24 hours. *Physiol. Behav.* 63(2):171-176.

Canal-Corretger MM, Cambras T, Vilaplana J, Díez-Noguera A. (2000). Bright light during lactation alters the functioning of the circadian system of adult rats. *Am. J. Physiol. Regulatory. Integrative. Comp. Physiol.* 278:201-208.

Canal-Corretger MM, Vilaplana J, Cambras T, Díez-Noguera A. (2001). Functioning of the rat circadian system is modified by light applied in critical postnatal days. *Am. J. Physiol. Regulatory. Integrative. Comp. Physiol.* 280:1023-1030.

Davis FC. (1981). Ontogeny of circadian rhythms. In Aschoff J (ed). *Handbook of Behavior Neurobiology.* New York: Plenum Publishing Corp, pp. 257-274.

de la Iglesia HO, Cambras T, Schwartz WJ, Díez-Noguera A. (2004). Forced desynchronization of dual circadian oscillators within the rat suprachiasmatic nucleus. *Curr. Biol.* 14:796-800.

Hurd MW, Cahill GM. (2002). Entraining signals initiate behavioral circadian rhythmicity in larval zebrafish. *J. Biol. Rhythm.* 17:307-314.

Ikeda T, Iijima N, Munekawa K, Ishihara A, Ibata Y, Tanaka M. (2003). Functional retinal input stimulates expression of astroglial elements in the suprachiasmatic nucleus of postnatal developing rat. *Neurosci. Res.* 47:39-45.

Klein DC, Moore RY, Reppert SM. (1991). *Suprachiasmatic nucleus: the mind's clock.* New York: Oxford University Press.

Kováciková Z, Sládek M, Bendová Z, Illnerová H, Sumová A. (2006). Expression of clock and clock-driven genes in the rat suprachiasmatic nucleus during late fetal and early postnatal development. *J. Biol. Rhythm.* 21:140-148.

Madrid JA, Lax P, Vilaplana J, Cambras T, Díez-Noguera A. (1992). Presence of two differentiated circadian components in the eating and motor behaviour in the young rat. *J. Interdiscip. Cycle Res.* 23:211-212.

Miller JD. (1998). The SCN is comprised of a population of coupled oscillators. *Chronobiol. Int.* 15(5):489-511.

Moore RY. (1991). Development of the suprachiasmatic nucleus. In Klein DC, Moore RY, Reppert SM (eds). *Suprachiasmatic nucleus - the mind's clock* . New York: Oxford University Press, pp. 197-216.

Moore RY. (1997). Circadian rhythms: basic neurobiology and clinical applications. *Annu. Rev. Med.* 48:253-266.

Moore RY, Speh JC, Leak RK. (2002). Suprachiasmatic nucleus organization. *Cell. Tissue. Res.* 309:89-98.

Pittendrigh CS, Daan S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents: I. The stability of spontaneous frequency. *J. Comp. Physiol.* 106: 223-252.

Refinetti R. (1998). Influence of early environment on the circadian period of the tau-mutant hamster. *Behav. Gen.* 28(2):153-158.

Schaap J, Albus H, vanderLeest HT, Eilers PHC, Détári L, Meijer JH. (2003). Heterogeneity of rhythmic suprachiasmatic nucleus neurons: implications for circadian waveform and photoperiodic encoding. *Proc. Natl. Acad. Sci. USA.* 100(26):15994-15999.

Sokolove PG, Bushell WN. (1978). The chi square periodogram: its utility for analysis of circadian rhythms. *J. Theor. Biol.* 8:72(1):131-160.

Stratman M, Schibler U. (2006). Properties, entrainment, and physiological functions of mammalian peripheral oscillators. *J. Biol. Rhythms*: 21(6):494-506.

Tamarkin L, Reppert SM, Orloff DJ, Klein DC, Yellon SM, Goldman BD. (1980). Ontogeny of the pineal melatonin rhythm in the Syrian (*Mesocricetus auratus*) and Siverian (*Phodopus sungorus*) hamsters and in the rat. *Endocrinology.* 107:1061-1064.

Touitou Y., Smolensky M.H.. Portaluppi F (2006). Ethics standards and procedures of animal and human chronobiology research, Chronobiol. Int. 23:1083-96.

Vallone D, Lahiri K, Dickmeis T, Foulkes NS. (2007). Start the clock! Circadian rhythms and development. *Dev. Dyn.* 236:142-155.

Wever RA. (1979). The circadian system of man. Results of experiments under temporal isolation. Springer-Verlag, New York, pp. 43-58.

Wong RO. (1999). Retinal waves and visual system development. *Annu. Rev. Neurosci.* 22:29-47.

Apartat III: **MANIPULACIONS EXOGENES DELS RITMES CIRCADIARIS DEL PATRO DISSOCIAT**

EXPERIMENT 4: EFECTE DE L'ALIMENTACIÓ RESTRINGIDA

ARTICLE

MOTOR ACTIVITY RHYTHMS OF FORCED DESYNCHRONIZED RATS SUBMITTED TO RESTRICTED FEEDING

M. Anglès-Pujolràs, J.J. Chiesa, A. Díez-Noguera and T. Cambras

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Índex d'impacte: 2,445
Categoria: Behavioral sciences. Posició: 20/43

INTRODUCCIÓ

Tot i que per al rellotge circadiari la llum és el *zeitgeber* més potent, hi han altres estímuls, més febles, que poden produir encarrilament. Un d'ells és l'alimentació restringida a unes hores del dia. Es pot considerar que les rates sotmeses a cicles de LD de 22h presenten acoblament dèbil del NSQ. Podria l'alimentació restringida actuar com a *zeitgeber* en una situació de baix acoblament com és la dissociació?

OBJECTIU

L'objectiu va ser provar l'efecte d'un *zeitgeber* feble, com és l'alimentació restringida, sobre el ritme d'activitat motora de rates sotmeses a condicions de dissociació forçada, i veure si aquest *zeitgeber* tenia capacitat de modificar l'expressió d'un dels dos components, o de resincronitzar-los.

MATERIAL I MÈTODES

Es van emprar 40 rates Wistar i es van dividir en dos grups: T22 i T23. A 12 animals de cada grup (grup experimental) se'ls va sotmetre a cicles d'alimentació restringida (4h/dia). La resta d'animals (grup control) es van alimentar *ad libitum* durant tot l'experiment.

Es va analitzar i comparar el comportament rítmic de la variable activitat motora en l'etapa d'alimentació restringida i en l'etapa d'alimentació *ad libitum,* per cada un dels grups.

RESULTATS

Dins el grup experimental, en l'etapa d'alimentació restringida, els animals sotmesos a T22 van mostrar 3 components: a banda dels dos que són característics de la dissociació (LDC i NLDC), un altre component que depenia de l'aliment (FDC). Els animals de T23, en canvi, van mostrar únicament, el LDC i el FDC. Els animals control, tant de T22 com de T23, i els animals del grup experimental en l'etapa d'alimentació *ad*

libitum, tan sols van mostrar el LDC i el NLDC.

El grup experimental va mostrar activitat anticipadora a l'aliment durant les 2 hores anteriors a la ingesta d'aliment.

L'administració d'alimentació restringida no va modificar el període del NLDC en cap dels grups estudiats.

CONCLUSIÓ

Els resultats indiquen que l'alimentació restringida té un efecte feble sobre el sistema circadiari dissociat. La seva aplicació fa variar la distribució de l'AM afegint un tercer component, associat a l'alimentació restringida, al NLDC i al NLDC, generant d'aquesta manera, un patró encara més complex.

ELSEVIER

Physiology & Behavior 88 (2006) 30–38

PHYSIOLOGY & BEHAVIOR

Motor activity rhythms of forced desynchronized rats subjected to restricted feeding

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Abstract

Although light is the strongest zeitgeber for the circadian pacemaker, other stimuli can also produce entrainment. In the rat, periodic restricted feeding (RF) is a weak stimulus that may act as a zeitgeber. We tested the effect of RF on the motor activity rhythms of rats subjected to forced dissociation. In this situation two components, supposed to be related with the ventrolateral and dorsomedial areas of the suprachiasmatic nucleus, are detected in their motor activity. One component is entrained to the external light–dark cycle (Light Dependent Component, LDC) and thus has the same period, while the other has a period longer than 24 h (Non-Light Dependent Component, NLDC). This experiment examined whether RF can act on one or both of these two rhythms. Rats were maintained under the light–dark cycles of 22 h (T22) or 23 h (T23) for 44 days with food available for four hours per day. Afterwards the rats received food ad libitum, to test the effect of the previous RF condition. Results show that RF modifies the manifestation of the two initial rhythms, being this effect stronger under T23 than under T22. However RF does not affect the NLDC period. The results reveal that the animal can manifest simultaneously several rhythmic patterns.

Keywords: Restricted feeding; Suprachiasmatic nucleus; Motor activity; Circadian

1. Introduction

The suprachiasmatic nuclei of the hypothalamus (SCN) are considered to be the most important structure of the mammal circadian system, since they generate and coordinate the circadian rhythmicity of the organisms. The SCN generate the circadian expression in many behavioral and physiological variables such as locomotor activity, body temperature, plasma corticosterone and pineal melatonin [1]. Moreover, the circadian system allows synchronization with the environmental time cues. Light is the strongest zeitgeber since it shifts the clock in a circadian time-dependent way and produces the entrainment to the external light–dark cycle. However, this is not the only zeitgeber. Nonphotic stimuli such as increased locomotor activity, restricted feeding, social cues, and exposure to novelty can phase shift and/or entrain the circadian pacemaker of mammals [2]. One of the nonphotic stimuli that can affect the organization of activity, independently of the LD cycle, is restricted access to food. However, this is quite controversial. Although survival may depend on behavioral adaptation to periodic restricted feeding (RF), the extend to which RF acts as a zeitgeber varies among species [3–5]. Food availability may affect the circadian organization of daily rhythms [6]. Two behavioral activity components will result in case of a nocturnal animal submitted to an LD cycle, if food is only available during the day. One component is entrained by the feeding cycle, observed by anticipatory activity 2–3 h before the onset of the food availability, referred to as the food anticipatory activity (FAA) [7,8]. The other is the normal nocturnal activity entrained by the light–dark cycle. Although the rat prefers to feed at night, it will exhibit diurnal FAA if food is only available during the day [7]. Other variables such as serum corticosterone [9,10], free fatty acids, and core temperature are associated with FAA, and their values rise in anticipation of the daily meal. The SCN has been identified as the light-entrained pacemaker (LEP), whereas the mechanisms for the food-entrained oscillator (FEO) remain elusive. The FEO is thought to be a circadian oscillator independent of the SCN, because FAA is also present in animals with bilateral lesions of the SCN [11,12]. However, the precise

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anatomical location of the FEO remains to be identified [13,14,8]. Recent studies emphasize the importance of peripheral oscillators, placed outside the SCN, as possible components of the FEO. Studies of clock gene expression in mice and rats indicate that the phase of the genes in the SCN is not affected by the restricted feeding under LD cycle, while clock genes expressed in peripheral tissues are phased shifted with the feeding restriction [15]. Special attention has been given to the liver as a possible peripheral clock [13,8,15]. These peripheral organs interact with central nervous structures involved with ingestive behavior and energy balance. It has been suggested that FEO is a distributed system of interacting structures that require an integrative approach [16].

In the absence of other zeitgebers, for instance under constant lighting conditions, RF may entrain the circadian rhythm driven by the SCN. However, the entrainment depends on the species [17,7] on the closeness of the period of the RF cycle to the free-running period [12] and also on the duration of the RF [18].

All this seems to indicate that under an LD cycle, the SCN is not usually entrained by RF, but that, under certain circumstances, RF can act as a zeitgeber. It has been suggested that the coupling between the FEO and the light entrainable pacemaker (LEP) of the SCN is asymmetrical, as FEO is usually coupled to LEP, but FEO has a weak effect on the LEP. It is no clear how feeding activity affects the circadian pacemaker, although the two parts of the SCN, dorsomedial or ventrolateral, may not be equally affected by the feeding restriction [19].

When rats are exposed to T22 cycles, their motor activity rhythm can be dissociated and two simultaneous circadian rhythms appear in behavior: One, with a period equal to that of the external light–dark cycle (Light Dependent Component —LDC) that seems to be related to the function of the ventrolateral part of the SCN and the other, with a period of more than 24 h (Non-Light Dependent Component—NLDC), associated to the dorsomedial part [20]. This situation implies that the circadian clock is not as strongly coupled as it is under free-running or 24 h LD cycles and that the two main oscillator populations in the SCN can be functionally differentiated. Forced dissociation could provide an opportunity to study the effect of a weak zeitgeber, such as RF, on the motor behavior of the rat. This experiment was designed to test whether RF may affect the circadian pacemaker under the paradigm of forced dissociation by modifying one or the two circadian components which may correspond to the two anatomical parts of the SCN.

2. Material and methods

20 female and 20 male Wistar rats were used for the experiment. They were purchased from Charles River Laboratory (France) at the age of 3 months. When they arrived at our laboratory, they were housed individually in transparent cages measuring 25×25×12 cm and placed into two sound-proof rooms. During the first five days of the experiment, all the animals were kept under 12 h-light and 12 h-darkness cycles to synchronize their rhythms. Afterwards half of the rats were submitted to symmetrical cycles of 22 h: 11 h-light and 11 h-darkness (11:11 LD; T22) and the other half to an 11.5:11.5 LD cycle (T23) throughout the experiment. For each T, 6 males and 6 females were submitted to RF with a period of 24 h 12 min, with 4 h of food access per day (experimental rats), while 4 males and 4 females were maintained in the same room with food ad libitum (control rats). RF was applied by a researcher entering the room every 1452 min (24 h 12 min). A period different than 24 h was chosen as the periodicity of RF, to avoid potential coincidences with external and involuntary influences. RF was maintained from day 5 to day 66 (RF stage) excepting days 49–52 and 59–62, when rats were food deprived to test the endogenous character of the RF rhythm. Finally, the experiment ended with one stage of feeding ad libitum (aL stage) for a further 31 days. In all cases, lighting was provided indirectly by two fluorescent tubes with an intensity of 300 lux at cage level. Darkness was attained using a dim red light with an intensity of less than 0.1 lux. During the experiment, all the rats had free access to tap water, and control rats had free access to food. All rats were fed commercial rat chow (A04 for rat and mouse, Panlab).

The motor activity rhythm was recorded throughout the experiment by activity meters, which consisted of two crossed perpendicular infrared beams crossing the cage 7 cm above the floor. The number of movements was accumulated and recorded every 15 min.

2.1. Data analysis

The rhythmic behavior of the rats was analyzed separately for motor activity data corresponding to the two stages: stage 1 and stage 2. The first one corresponds to the time in which experimental rats were submitted to RF and the second, to aL. Control rats were used to test the effect of possible environmental factors apart from food during RF, and the effect of time (duration of the experiment) on the motor activity rhythms, and thus, had always free access to food.

We calculated the mean value of the motor activity of each animal in each stage, the food anticipatory activity (FAA) measured as the percentage of the total motor activity per cycle that occurs during the 2 h before the restricted food availability and also the activity during the 4 h of restricted feeding (4 h-RF), also calculated as a percentage of the total motor activity per cycle.

For each stage, the Sokolove–Bushell periodogram [23] was used to calculate the periods of the significant rhythms using the motor activity data smoothed by a moving average of 3 data points to reduce non-significant noise. To carry out the periodogram analysis, 42 days data were used in stage 1 for all rats. However for stage 2, 31 days data were used for T22 group and 22 days data for T23 group. In most of the rats 3 peaks were detected by the periodogram, one with the period of the external light–dark cycle (Light Dependent Component, LDC), another with the period of the RF cycle (Food Dependent Component FDC), and the third with a period that was not dependent on the light or on food availability (Non-Light Dependent Component, NLDC).

For each rat and stage we calculated the mean pattern of the three possible rhythms (LDC, NLDC, FDC). Thus, three mean rhythm profiles were built for the motor activity data of each animal, based on the period of each rhythm. Even if one of the rhythms was not statistically significant, we still used the value of its period to draw its profile. In the aL stage, the mean profiles were also constructed for the three components. In this case, as there was no RF, the waveform of FDC can be interpreted as the basal level of any periodicity in the time series.

For each waveform, the following variables were calculated: 1) the coefficient of correlation of the waveform with the original data series (r); 2) the area of the data oscillation around the mean (A), which has been calculated as the addition of the absolute values of the differences between the mean and each data of the waveform; 3) the value of A expressed as a percentage of the total area under the wave (%A), which can be considered to be the most indicative variable of the importance of one determined rhythm on the distribution of the motor activity data since it indicates a "degree of oscillation" of the rhythm; and 4) the difference between %A in RF and aL stages calculated for each animal, which indicates the gain or loss of the importance of a determinate rhythm when food is or is not available.

Statistical analysis of the period of the NLDC and the previously defined variables was carried out by ANOVA of several general linear models, considering each time a dependent variable and as independent variables the T cycle (T22 or T23), the sex of the rat, the stage of the experiment (RF vs aL) and the group (experimental vs control). Groups were also compared using the Student's t-test with Bonferroni's correction. Data were analyzed using an integrated package for chronobiology "El Temps" (A. Díez-Noguera, Universitat de Barcelona, 1999), and statistical analysis was carried out with the SPSS® package.

3. Results

Data corresponding to two control rats of the T22 group in stage 1, one control rat of the T23 group in stage 1 and one control rat of the T23 group in stage 2 were excluded from the analysis due to failures in the detection system.

Actograms of T22 rats (Fig. 1) show that in stage 1, three components in the motor activity rhythm can be observed in the experimental rats. In all rats in stage 2 and in control rats also in stage 1, the two components typically seen in a dissociated rat (LDC and NLDC) usually appeared. In T23 group in stage 1, only two components of the motor activity rhythm (LDC and FDC) were detected in all the experimental rats, although in some animals a weak NLDC was seen in the actogram, albeit not detected by the periodogram. Most of the control rats in RF stage and all the rats in aL stage showed dissociation, with LDC and NLDC.

The mean motor activity values depended on the stage ($p<0.01$). In both T22 and in T23 groups, activity was higher in stage 1 than in stage 2 ($p<0.05$) and in all groups males showed higher values than females ($p<0.001$).However, there were no significant differences between the control and the experimental or T22 and T23 groups.

The values of motor activity during the 4 h RF interval were not affected by the T cycle or sex. Only among the control rats, females showed higher values than males.

Experimental rats showed higher values of FAA than the control group ($p<0,001$), as measured by the percentage of activity during 2 h before RF, but there were no significant differences in this variable according to the T cycle or the sex of rats. When animals were food deprived, FAA was maintained for one or two cycles indicating the endogenous nature of this component.

The periodogram analysis gives the statistical significance of the three possible rhythms, in the RF and aL stages, with periods of 22 h (LDC), 24 h 12 min (FDC), and another period longer than 1452 min (NLDC). The number of rats that show a statistically significant rhythm in the periodogram as a ratio of the total number of rats is shown in Table 1.

The value of the period of the NLDC was not significantly different between the experimental and the control groups or between stages 1 and 2. The T cycle was the only significant factor determining this variable. T22 rats had a mean value of 1495 min, s.e.:1.73 min, while T23 rats had a value of 1482 min, s.e. 3.10 min.

As mentioned above, we built three rhythm profiles for each animal data and for each stage (Fig. 2) and for each rhythm we calculated four variables: r, A, %A and %A RF−%A aL. From its analysis, a global observation is that, in general, the distribution of the motor activity changes when RF is applied. The two representative components of the dissociation are manifested under ad libitum conditions. When RF is applied the new component, FDC, becomes the main factor governing the motor activity distribution, decreasing the importance of the other two components. In control rats, the variables studied did not change between the two stages. There were also differences in the variables of the waveform between T22 and T23, which also have different interpretations. These differences are due to the fact that under T23 the dissociation is weaker than under T22 and it does not appear in all the rats [21]. Consequently under T23 there is a situation where the NLDC is weak, and so, when RF is applied the NLDC practically disappears.

Concretely, the variable r (Fig. 3), which represents the stability of a rhythm, did not change in control rats between the RF and aL stages for no one of the rhythms under either T22 or T23. In these groups LDC was the component with the highest r value ($p<0.001$), being higher in T23 than in T22 rats. However, in the experimental rats r was different for each component ($p<0.001$) but also depended on the stage ($p<0.001$) and sex ($p<0.01$). Specifically, under T22 the r of the NLDC and the r of the LDC in experimental rats was higher in aL than in RF ($p<0.05$). Obviously the r of the FDC was higher under RF than under aL ($p<0.001$). In T23 experimental rats, r values were also higher in aL for the LDC ($p<0.05$) and for the NLDC ($p<0.001$) and lower in aL for the FDC ($p<0.001$). In this last group of rats the r value of the LDC was higher for females than for males ($p<0.001$).

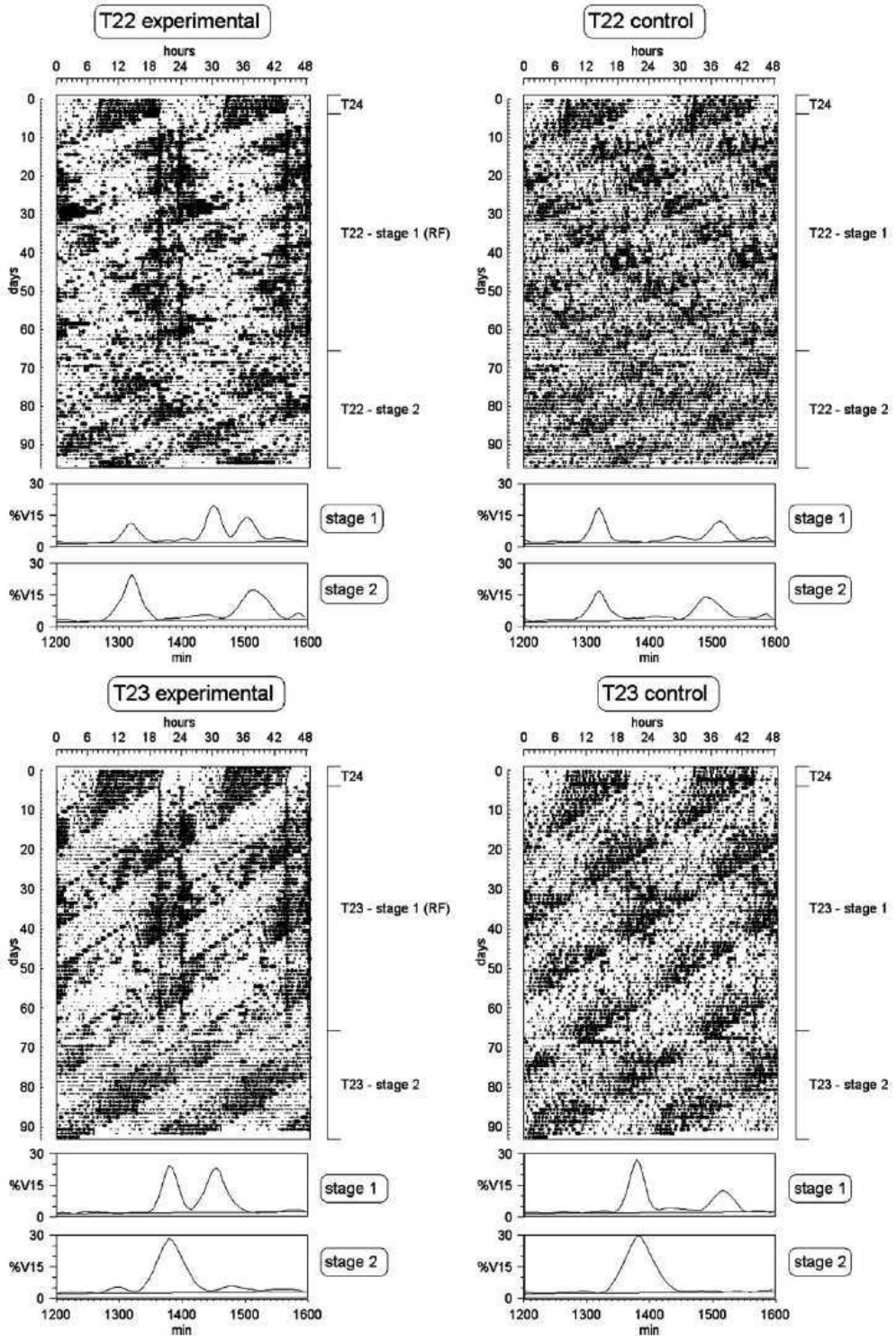

Fig. 1. Double-plotted actograms of a representative rat from each group, plotted at "modulo" 24 h 12 min (each row corresponds to one RF cycle). Stage 1 corresponds to the time when the experimental rats were submitted to restricted feeding and control rats had free access to food. In stage 2 all rats were fed ad libitum. The periodograms correspond to the data of the stage 1 (upper periodogram) and stage 2 (lower periodogram), and their time axes are expressed in min.

Table 1
Number of rats showing a statistically significant rhythm, $p<0.05$, out of the total number of rats for each group and stage of the experiment

		Stage 1			Stage 2		
		LDC	NLDC	FDC	LDC	NLDC	FDC
T22	Experimental	12/12	11/12	12/12	12/12	12/12	0/12
	Control	6/6	6/6	1/6	8/8	8/8	0/8
T23	Experimental	12/12	0/12	12/12	12/12	8/12	0/12
	Control	7/7	7/7	1/7	7/7	6/7	0/7

LDC: Light Dependent Component, period equal to 22 or 23 h; NLDC, Non-Light Dependent Component, period longer than 24.5 h and different for each rat; FDC, Food Dependent Component, period equal to 24 h 12 min. In stage 1 the experimental rats were submitted to restricted feeding.

Other variables obtained from the waveform were A and $\%A$. Both variables were related but since we consider $\%A$ the most indicative of the importance of the oscillation we will not show the results obtained with A and we will only mention those obtained from $\%A$ (Fig. 4). In control rats the highest value of this variable was for the LDC. There were no differences in the values of this variable for no one of the components between the two stages. The $\%A$ in the NLDC was sex-dependent in T22 control group ($p<0.001$) as was the $\%A$ of the FDC in T23 control group ($p<0.05$). Experimental T22 rats showed differences between RF and aL stages in the $\%A$ of the NLDC ($p<0.05$) which was higher in aL, and also in the $\%A$ of the FDC, which was higher in RF. In the T23 experimental group, the $\%A$ of the NLDC was higher in aL ($p<0.001$), as was the $\%A$ of the LDC ($p<0.001$). It is worth noting that sex differences in this variable of the experimental rats were found in T22 in the NLDC ($p<0,001$) and in the LDC ($p<0,05$), but in T23 only in the LDC ($p<0,001$), the females having higher values than males in all cases.

Finally, we calculated the differences between the changes of the degree of oscillation due to each one of the components when animals were transferred from RF to aL stage by calculating the variable ($\%A$ RF−$\%A$ aL) (Fig. 5). In T22 group, there were significant differences between the expression of the rhythms between these two stages in the experimental, but not in the control rats. After the Bonferroni correction, this value was non-zero for the FDC ($p<0.001$), but not for the other two components. In T23 group, the experimental rats showed non-zero values for all three components ($p<0.01$).

4. Discussion

The purpose of this experiment was to examine the effect of RF on the expression of one or both of the circadian rhythms expressed in the motor activity pattern. Taking into account that the food-entrainable pacemaker has a weak effect on the light entrainable pacemaker [22], we hypothesized that food restriction would have a greater effect on a non-strongly

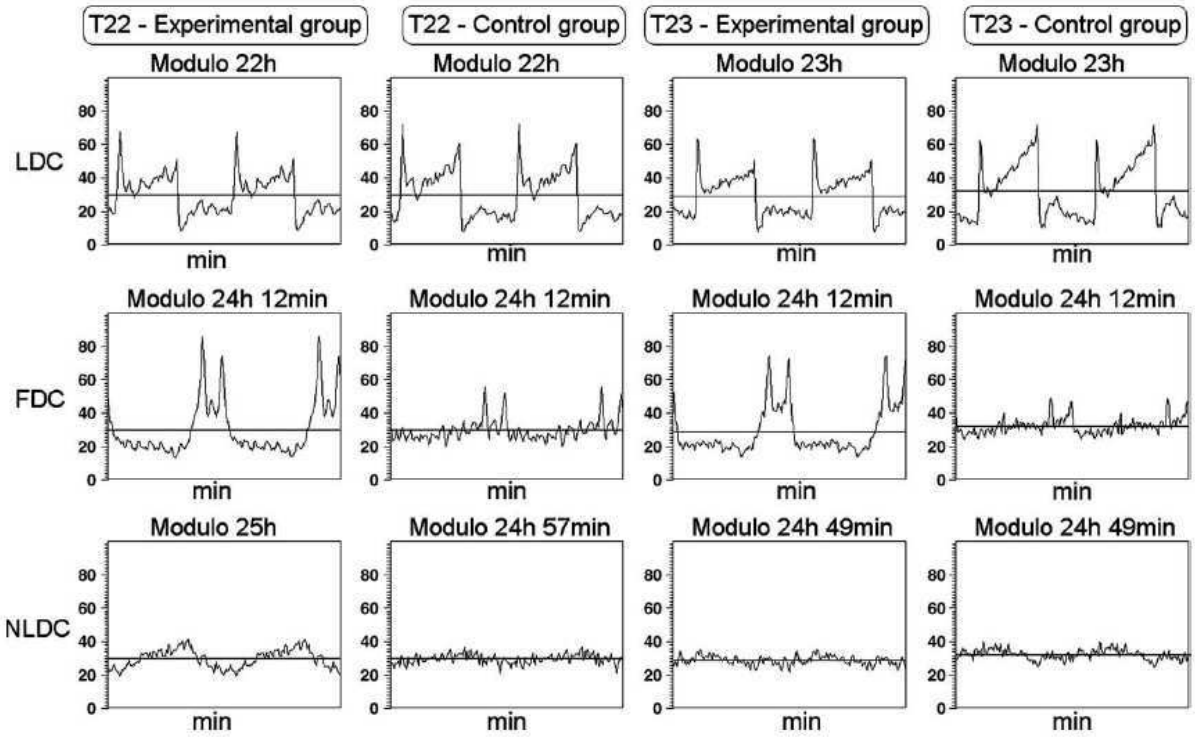

Fig. 2. Mean profiles of the motor activity of each group of rats plotted in terms of each one of the three rhythmic components. The profiles corresponding to the Light Dependent Component (LDC) are plotted at modulo 22 h (T22) or modulo 23 h (T23), those corresponding to the Food Dependent Component (FDC) are plotted at modulo 24 h 12 min, and those corresponding to the Non-Light Dependent Component (NLDC) are plotted at different modulo for each animal, since the period of this component differs between individuals. Note that all the graphs are double plotted.

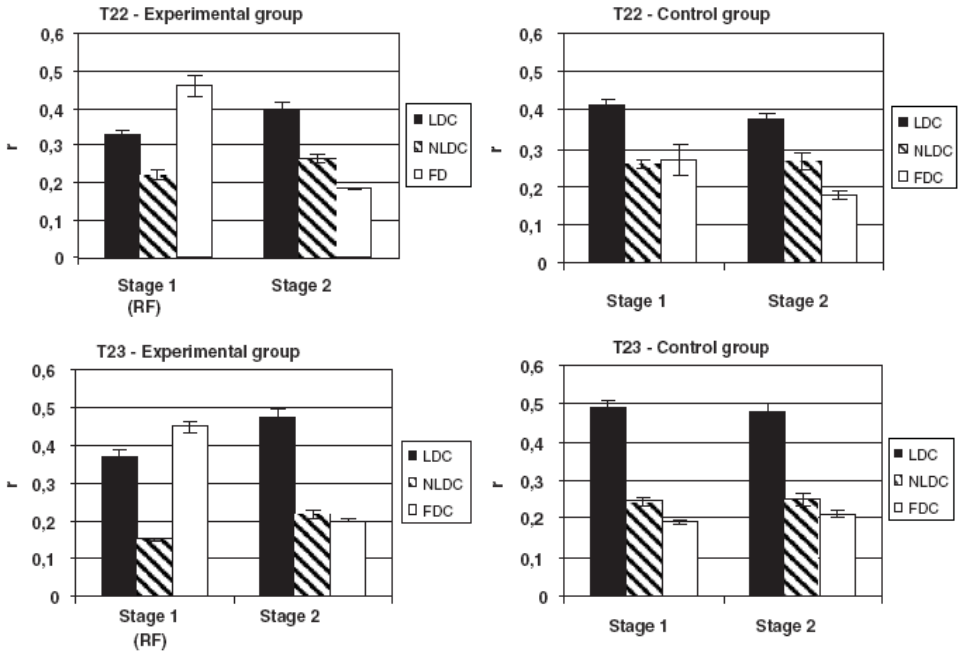

Fig. 3. Mean values of the variable *r* (and standard errors) of each rhythmic component for the two stages of the experiment (stage 1 and stage 2) and the different groups of rats. In stage 1 the experimental rats were submitted to restricted feeding (RF).

coupled circadian system than on a circadian system working as a whole. Our results indicate that although the circadian system is dissociated, RF has little effect on it, but it does influence the general behavior, specially under T23. The analysis of motor activity is important in understanding how an animal behaves when exposed simultaneously to two conflicting zeitgebers, neither of which is completely entrained by the circadian system. Our results indicate that the RF in dissociated rats modifies their general rhythmic behavior, being this effect stronger in T23 than in T22.

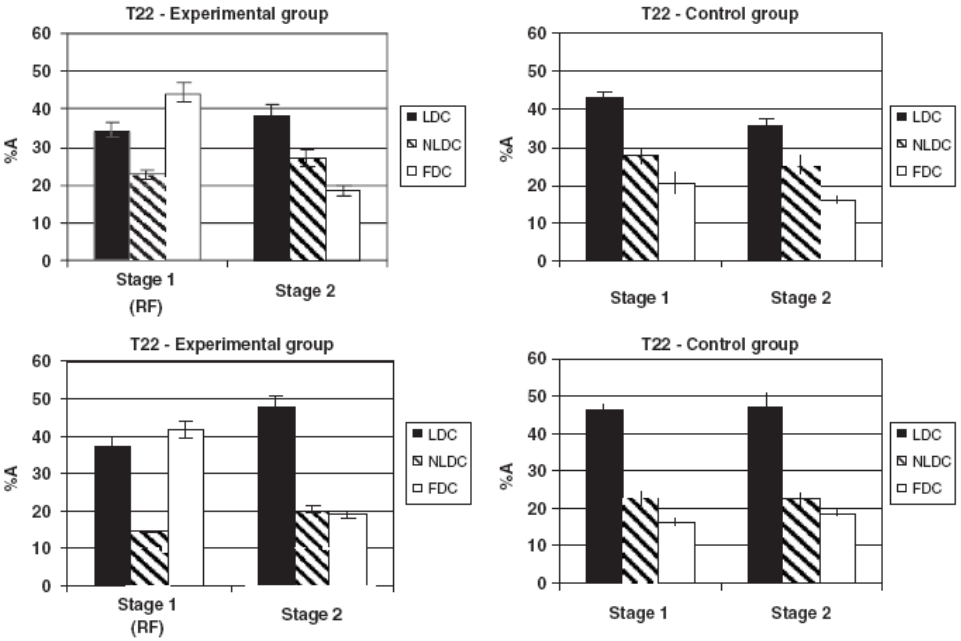

Fig. 4. Mean values of %*A* (and standard errors) of each rhythmic component for the two stages of the experiment (stage 1 and stage 2) and the different groups of rats. In stage 1 the experimental rats were submitted to restricted feeding (RF).

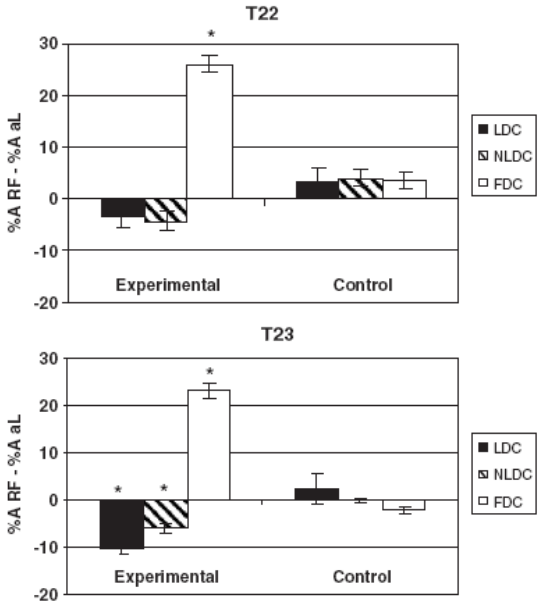

Fig. 5. Mean differences (and standard errors) of the values of the variable %*A* between stage 1 and stage 2 for the animals of each group (variable %*A* RF − %*A* aL). Take into account that only experimental, but not control rats, were submitted to restricted feeding during RF stage. Asterisks indicate those values statistically significant different from 0.

First, we would comment on the variables used to determine the motor activity rhythm. Typically, a rhythm is analyzed by the periodogram [23,24], which provides the variable Q_p used to indicate the importance of the rhythm. We used the periodogram to determine the number of significant rhythms and their period. However, this method is not adequate for our data, since it does not reflect the amplitude of the oscillation, but merely the stability and constancy of the pattern. Moreover, the motor activity pattern of these rats was very complex since 3 simultaneous rhythms had to be detected in one single variable. To our knowledge it is the first case of the detection of the three significant simultaneous rhythms, in the circadian range, in the rat's motor activity. Furthermore, one of these 3 rhythms, the NLDC, although visible in the actogram, is not always detected by the periodogram analysis. All this makes it difficult to quantify and compare rhythmic patterns. This complexity leads us to suggest that an alternative method should be used. To this end, throughout the waveform, we calculated the degree of oscillation around the mean for a specific modulo, the %*A*. The idea is to consider that any rhythm can influence the motor activity of the rat even if it is not statistically significant. The question is how much this rhythm contributes to the distribution of the motor activity. We have considered the oscillation of motor activity for each animal according to three possible rhythms with periods equal to the LD cycle, the RF cycle and the NLDC, which is close to the free-running rhythm of a rat. Thus, for the analysis of a complex pattern, such those with more than a single rhythm, %*A* is a reliable indicator of the amplitude of the oscillation of the motor activity generated by each of the components. In addition, *r*, the correlation between the waveform and the rough data, is a normalized variable that reflects the stability of the rhythm.

As anticipated [21], control rats and experimental rats with access to food ad libitum show two rhythmic components. However, when food was restricted, an extra circadian component is generated. In some control rats a weak FDC was also detected, which may have been due to the entrance of the researcher in the experimental room or because of the smell or the noise induced by the activity of the other rats. It is worth noting that the FDC in the control, but not in the experimental rats, was more evident in females, indicating a greater sensitivity to environmental stimuli. Females have more robust circadian rhythms than males [21], but when all three rhythms are manifested, the sex determines the rhythm generated by the SCN, this is both the NLDC and the LDC in T22 and only the LDC in T23. Sexually morphological differences have been reported in the SCN [25,26] and also in its rhythmic manifestation [21]. Our results indicate that females are always more rhythmic than males in whatever the component they express if this is generated by the SCN. In a rhythm generated by peripheral oscillators such as FDC no sex differences are found. We trust that this observation lends additional validity to our analytical methods.

It is worth to notice that the mean motor activity does not differ between the animals submitted to a different T or between the experimental and the control group. Indeed, the mean motor activity is higher in stage 1 than in stage 2. However, although this suggests that RF and all the external disturbances involved increase activity, we consider that the decreases in motor activity in stage 2 could be due to age, or to the time the animals have been under the conditions of the experiment. Thus, considering that motor activity does not change among the different groups of rats, we can ascertain that the motor activity pattern of an animal is distributed according to two possibilities: a) the distribution of the motor activity occurs according to a sort of 'homeostatic hypothesis' in such a way that an animal has a fixed amount of calories to expend in a cycle and must therefore divide its activity into a given number of activity bouts (i.e. different possible rhythms). Thus, if one component is strongly manifested because it is externally reinforced (i.e. RF cycle, that it is crucial for the survival of the animals), then, the other components (i.e. LD cycle) will be less manifested. b) The distribution of the motor activity expresses the action of the different groups of oscillators that make up a circadian multioscillatory system [27]. Our results could be explained taking both possibilities into account. The animal will distribute its activity in different components (without greatly modifying the total activity) according to the response to external stimuli, but also, the number of components and the degree of distribution of the motor activity in each component may be dependent on the groups of functional oscillators.

In a previous paper [20] we demonstrated that when the rats were submitted to T22 and two components appeared, the LDC was associated with the rhythmic expression of *per1* in the ventrolateral SCN and the NLDC with that of the dorsomedial part. In the present study, moreover, we detected an additional

component generated by RF, with a significant anticipatory activity preceding feeding in experimental animals. This anticipatory activity is generally attributed to the action of peripheral oscillators outside the SCN [8,13]. The anticipatory activity is advantageous since it represents one of the major adaptive features for a cyclic environment. According to the present results, FAA does not depend on T, nor on the number of rhythmic components present in the motor activity pattern. FAA is an output of the FEO outside the SCN. Taking all this into account, we can infer that the motor activity pattern is a reflection of the three groups of oscillators mentioned above, in such a way that the stability and the amplitude of the rhythm indicate a higher number and synchrony of oscillators involved in the generation of that component. Therefore, when we study the difference of the %*A* of each one of the components between RF and aL stages, we also study the loss (or gain) of the importance of a given rhythm due to the imposition of RF. When the FDC is present, the amplitude and stability of both LDC and NLDC decrease. However under T22, the difference of the %*A* between RF and aL is not significant for LDC or NLDC, which indicates that, contrary to what we expected, a dissociated system is less influenced by the FEO. However, under T23, when RF was applied the rhythm induced by both the LDC and NLDC were less evident, suggesting that the functioning of the oscillators of the SCN have been modified. In T23 the NLDC is always less apparent than in T22 [21], and both, the period of the LDC and of the NLDC, are closer to the period of the FDC than under T22 rats. All this may imply that the oscillators belonging to the different parts of the SCN are more susceptible to the effects of the FEO in T23 than in T22.

Kalsbeek et al. [19] found that confronting an animal with two simultaneously entraining signals applied in opposite phase (feeding during the light period) affects the normal activity of SCN neurons, and so the VIP neurons entrain to the daytime feeding and the VP containing neurons remain entrained to the LD cycle. They suggested that VP release from SCN terminals represents the output of the circadian pacemaker, while VIP neurons in the SCN integrate and convey entraining signals from the environment to the pacemaker neurons, and that the RF-induced changes in VIP phase are not sufficient to change the phase of the VP pacemaker neurons, which remain entrained to the LD cycle. These authors also suggest that the most prominent effect of the RF is a feeding-related decline of the increased extracellular VP levels during the light period, indicating that RF information reaches the SCN. Just looking at the animal's behavior, our results do not reveal differences in the degree of changes of LDC and NLDC due to the application of RF, indicating that these two rhythms are, proportionally, equally affected by RF.

Our approach to the study of the different groups of oscillators that drive the motor activity is quite new, since we have examined the manifestation of the circadian system inducing three different and simultaneous rhythms in the circadian range, two of them due to a determined zeitgeber. Moreover, it demonstrates the physiology of the circadian system as sexual differences are detected in the SCN driven components but not in those generated by peripheral oscillators. It also shows that the FAA is independent of the SCN. This indicates the value of careful behavioral analysis when the rhythmic pattern is generated by different systems. We consider that the distribution of the circadian system throughout the whole organism leads to the flexibility needed for adaptation to different stimuli, in such a way that the physiology and behavior of an animal can be adapted to internal stimuli (homeostatic or hormonal status), or external stimuli such as food availability. This adaptability is obtained by coupling or uncoupling the different oscillators that conform the whole structure of the circadian system. The degree of coupling among the oscillators determines the patterns of motor activity. It has been suggested [13] that the clock, presumably within the SCN, is synchronized by the predictable presence of meal. Our results are also consistent with the view that the SCN output can be modified by the RF, but only when the rhythms of the animal (LDC and NLDC) are close to the period of the FDC.

For the maintenance of the temporal order within the organism, two processes appear essential: phase-setting with the environment and interaction of the oscillators within the organism. Food availability is an important conditioner, since the survival of the organism depends on it. When the animal must deal with conflicting zeitgebers, as it is in this case the circadian system must be flexible enough to adapt behavior to external circumstances. The fact that the circadian system is distributed in the oscillators in the SCN and in the peripheral tissues driven by different zeitgebers, allows more flexible behavior which may improve survival.

Motor activity is the result of the expression of the multioscillatory system's action with capacity to adapt to different zeitgebers, but also of the masking effect of the self zeitgebers which contributes to a reorganization of the animal's activity. In any case the presence of rhythmic external stimuli contributes to enhance rhythmic behavior. The most contributing external agent is light, but the food anticipatory activity has also a considerable physiological impact on the animal since a restructure of the motor activity pattern is acquired. In the present case, the activity due to the availability of food decreases the manifestation of the other two rhythms.

In humans, desynchronization of the circadian rhythms is frequent in our society due to the disruption between the internal rhythms and the environment. For instance after a rapid journey across time zones, or on shift-work [28–30]. Potential treatment strategies to facilitate re-entrainment and adaptation to the LD cycle are being investigated. One such strategy is the use of fixed mealtimes. Our results also indicate that oscillators in the periphery can alter the expression of the overt rhythm of the SCN (as observed in T23) and helps entrainment by light. However, our results also suggest that when light and food are in conflict, the behavior of the organism may follow various rhythms simultaneously with periods other than in normal conditions (as under T22). In such circumstances, feeding may generate another rhythm, thus inducing a more complex pattern. So, we conclude that the period of the feeding rhythm must be compared with the spontaneous rhythms of the organism especially when the circadian system is not strongly coupled.

Acknowledgements

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References

[1] Klein DC, Moore RY, Reppert SM. Suprachiasmatic nucleus. The mind's clock. New York: Oxford University Press; 1991.
[2] Mrosovsky N. Locomotor activity and non-photic influences on circadian clocks. Biol Rev 1996;71:343–72.
[3] Holmes MM, Mistlberger RE. Food anticipatory activity and photic entrainment in food-restricted BALB/c mice. Physiol Behav 2000; 68:655–66.
[4] Mistlberger RE. Effects of scheduled food and water access on circadian rhythms of hamsters in constant light, dark, and light-dark. Physiol Behav 1993;53:509–16.
[5] Coleman GJ, Harper S, Clarke JD, Armstrong S. Evidence for a separate meal-associated oscillator in the rat. Physiol Behav 1982;29(1):107–15.
[6] Boulos Z, Terman M. Food availability and daily biological rhythms. Neurosci Biobehav 1980;4:119–31.
[7] Mistlberger RE. Circadian food-anticipatory activity: formal models and physiological mechanisms. Neurosci Biobehav 1994;18(2):171–95.
[8] Davidson AJ, Poole AS, Yamazaki S, Menaker M. Is the food-entrainable circadian oscillator in the digestive system? Genes Brain Behav 2003; 2:32–9.
[9] Krieger DT. Regulation of circadian periodicity of plasma corticosteroid concentrations and of body temperature by time of food presentation. In: Suda M, Hayaishi O, Nakagawa H, editors. Biological rhythms and their central mechanism. New York: Elsevier/North Holland Biomedical; 1979. p. 247–59.
[10] Escobar C, Díaz-Muñoz M, Encinas F, Aguilar-Roblero R. Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. Am J Physiol Regul Integr Comp Physiol 1998;274:1309–16.
[11] Stephan FK, Swann JM, Sisk CL. Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions. Behav Neural Biol 1979;25(4):545–54.
[12] Stephan FK. Circadian rhythm dissociation induced by periodic feeding in rats with suprachiasmatic lesions. Behav Brain Res 1983;7:81–98.
[13] Stephan FK. The 'other' circadian system: food as zeitgeber. J Biol Rhythm 2002;17(4):284–92.
[14] Stephan FK. Broken circadian clocks: a clock gene mutation and entrainment by feeding. Am J Physiol Regul Integr Comp Physiol 2003; 285(1):32–3.
[15] Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 2000;14:2950–61.
[16] Angeles-Castellanos M, Aguilar-Robledo R, Escobar C. c-Fos expression in hypothalamic nuclei of food-entrained rats. Am J Physiol-Reg I 2004;286:158–65.
[17] Castillo MR, Hochstetler KJ, Tavernier RJ, Greene DM, Bult-Ito A. Entrainment of the master circadian clock by scheduled feeding. Am J Physiol Regul Integr Comp Physiol 2004;287(3):551–5.
[18] Cambras T, Vilaplana J, Díez-Noguera A. Effects of long-term restricted feeding on motor activity rhythm in the rat. Am J Physiol 1993;265: 467–73.
[19] Kalsbeek A, van Heerikhuize JJ, Wortel J, Buijs RM. Restricted daytime feeding modifies suprachiasmatic nucleus vasopressin release in rats. J Biol Rhythm 1998;13(1):18–29.
[20] de la Iglesia HO, Cambras T, Schwartz WJ, Díez-Noguera A. Forced desynchronization of dual circadian oscillators within the rat suprachiasmatic nucleus. Curr Biol 2004;14:796–800.
[21] Campuzano A, Vilaplana J, Cambras T, Díez-Noguera A. Dissociation of the rat motor activity rhythm under T cycles shorter than 24 hours. Physiol Behav 1998;63(2):171–6.
[22] Stephan FK. Coupling between feeding- and light-entrainable circadian pacemakers in the rat. Physiol Behav 1986;38:537–44.
[23] Sokolove PG, Bushell WN. The chi square periodogram: its utility for analysis of circadian rhythms. J Theor Biol 1978;8:72(1):131–60.
[24] Refinetti R. Non-stationary time series and the robustness of circadian rhythms. J Theor Biol 2004;227(4):571–81.
[25] Güldner FH. Numbers of neurons and astroglial cells in the suprachiasmatic nucleus of male and female rats. Exp Brain Res 1983;50(2–3):373–6.
[26] Cambras T, López L, Arias JL, Díez-Noguera A. Quantitative changes in neuronal and glial cells in the suprachiasmatic nucleus as a function of the lighting conditions during weaning. Dev Brain Res 2005;157:27–33.
[27] Díez-Noguera A. A functional model of the circadian system based on the degree of intercommunication in a complex system. Am J Physiol 1994;267:1118–35.
[28] Gander PH, Myhre G, Graeber RC, Andersen HT, Lauber JK. Adjustments of sleep and the circadian temperature rhythm after flights across nine time zones. Aviat Space Environ Med 1989;60:733–43.
[29] Weibel L, Spiegel K, Follenius M, Ehrhart J, Branderberger G. Internal dissociation of the circadian markers of the cortisol rhythms in night workers. Am J Physiol 1996;270:E608–13.
[30] Waterhouse J, Reilly T, Adkinson G. Jet-lag. Lancet 1997;350:1611–6.

EXPERIMENT 5: EFECTE DE LA MELATONINA I EL DIAZEPAM

ARTICLE

EFFECT OF MELATONIN AND DIAZEPAM ON THE DISSOCIATED CIRCADIAN RHYTHM IN RATS

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INTRODUCCIÓ

Tot i que el cicle extern de LD és el principal *zeitgeber*, altres factors, entre ells substàncies farmacològiques, poden actuar sobre el sistema circadiari.

Se sap que al NSQ hi ha receptors per a la MEL i el DZP (GABA), i per tant, hi poden actuar directament. Però aquestes dues substàncies funcionen de manera diferent en les dues àrees del NSQ? Poden, la MEL i el DZP, acoblar un NSQ funcionalment dissociat?

OBJECTIU

L'objectiu va ser estudiar l'efecte de l'administració de MEL i DZP en aigua de beguda sobre els dos components d'AM de rates amb el sistema circadiari dissociat.

MATERIAL I MÈTODES

Es van emprar un total de 45 rates Wistar mascle, i es van sotmetre a cicles de LD de 22 hores. A un grup d'animals se'ls va extirpar la glàndula pineal amb l'objectiu de descartar qualsevol possible interacció entre el DZP i aquest òrgan i a un altre grup, els ganglis cervicals superiors, per tal d'eliminar el ritme circadiari endogen de MEL. A la resta (grup control) se'ls va mantenir intactes. Els tractaments utilitzats van ser: MEL, DZP i solució aquosa d'etanol (vehicle) en aigua de beguda

RESULTATS

Els resultats van mostrar que el tractament amb MEL o amb DZP augmenta la manifestació del LDC en detriment de la del NLDC, i que la MEL, a més, actua sobre el NLDC escurçant-ne el període.

CONCLUSIÓ

Els resultats obtinguts indiquen que tant la MEL com el DZP afavoreixen l'encarrilament al cicle extern de LD. Això suggereix que ambdues substàncies poden induir l'acoblament del NSQ dissociat i que la MEL exerceix la seva acció directament sobre la part DM del NSQ, ja que aquesta hormona modifica el període del NLDC.

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Effect of melatonin and diazepam on the dissociated circadian rhythm in rats

Abstract: The main structures involved in the circadian system in mammals are the suprachiasmatic nuclei (SCN) of the hypothalamus. The SCN contain multiple autonomous single-cell circadian oscillators that are coupled among themselves, generating a single rhythm. However, under determined circumstances, the oscillators may uncouple and generate several rhythmic patterns. Rats exposed to an artificially established 22-h light–dark cycle (T22) express two stable circadian rhythms in their motor activity that reflect the separate activities of two groups of oscillators in the morphologically well-defined ventrolateral and dorsomedial SCN subdivisions. In the experiments described in this paper, we studied the effect of melatonin and diazepam (DZP) administration in drinking water on the dissociated components of rat motor activity exposed to T22, to deduce the possible mechanism of these drugs on the circadian system. In order to suppress the endogenous circadian rhythm of melatonin, in some of the rats the pineal gland or the superior cervical ganglia were removed. The results show that melatonin or DZP treatment increased the manifestation of the light-dependent component to the detriment of the manifestation of the non-light-dependent component and that melatonin, but not DZP, shortens the period of the non-light-dependent component. These findings suggest that both DZP and melatonin favor entrainment to external light, and that melatonin could also act on the SCN, producing changes in the period of the circadian cycle.

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Key words: circadian rhythms, diazepam, dissociation, entrainment, GABA receptor, melatonin, suprachiasmatic nuclei

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Introduction

The mammalian hypothalamic suprachiasmatic nuclei (SCN) is the site of an endogenous, self-sustained circadian timekeeper which provides temporal information for a wide range of physiological processes and behaviors [1]. This master clock is entrained to the light–dark cycle (LD) because light information from the retina reaches the SCN through a direct and an indirect neural pathway. The SCN is composed of multiple, single-cell circadian oscillators [2] which, when synchronized, lead to entrainment of circadian outputs that ultimately regulate overt rhythms. To date, it remains unknown as to how individual SCN cells are coupled to each other to generate a unique circadian rhythm.

Under certain external conditions, the SCN can be functionally dissociated. When rats are subjected to a 22-h LD cycle, the simultaneous expression of two circadian activity rhythms with different time lengths is observed [3, 4]. One of these rhythms is entrained by light and oscillates with a period similar to the external cycle (22 h) and is driven by the ventrolateral SCN region. We term this rhythm the 'light-dependent component' (LDC). The other component, not entrained, runs free with a period which is longer than 24 h and distinct for each animal, and is driven by the dorsomedial SCN region. This component was termed the 'non-light-dependent component' (NLDC).

Although the environmental LD cycle is the major zeitgeber, other nonphotic cues, including pharmacological substances such as melatonin [5] and benzodiazepines [6], can also entrain the circadian system. The SCN influences the synthesis of melatonin. Melatonin is released primarily from the pineal gland following the circadian rhythm with high levels at night and low levels during the day [7]. The SCN is connected to the pineal gland by a multisynaptic pathway which successively includes neurons on the paraventricular nucleus of the hypothalamus (PVN), sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord, and noradrenergic sympathetic neurons of the superior cervical ganglion (SCG) [8–10]. The daily rhythm of melatonin is considered to be a circadian mediator used by the endogenous SCN clock as the circadian messenger. In addition, pineal melatonin can modulate the clock function through a direct action on G-protein-coupled MT1 and MT2 melatonin receptors in the SCN [11, 12].

The major neurotransmitter at intra-SCN synapses is γ-aminobutiric acid (GABA). Nearly all neurons in the SCN contain glutamic acid decarboxylase, the GABA-synthesizing enzyme [13, 14]. Postsynaptic $GABA_A$

receptors connect the local synaptic circuitry [15] and could contribute to the synchronization of the SCN firing-rate rhythm [16]. GABA and benzodiazepine (positive allosteric $GABA_A$ receptor modulator) can phase-shift the circadian clock, whereas GABA antagonists block these effects and the light-induced phase shift [17].

Melatonin and benzodiazepines are pharmacologically and chronobiologically related substances. Melatonin, through activation of its different receptor subtypes, can differentially modulate the function of $GABA_A$ receptors [18]. In addition, it has been proposed that some effects of exogenous melatonin in adult rodents (sedative, analgesic, anticonvulsive, anxiolitic) could be related to its interaction with the GABAergic system [19, 20]. On the other hand, the benzodiazepine, diazepam (DZP), that has a GABAergic effect on the SCN, is involved in the modulation of the endogenous melatonin synthesis via the classic pathway SCN–PVN–SCG–pineal [21] and has a direct effect on the pineal gland [22, 23]. Although melatonin and DZP have direct effects on SCN, it is unknown whether they act differentially on the dorsomedial and ventrolateral areas, or whether they couple the two parts of the functionally dissociated SCN. We hypothesized that the dissociation model would be appropriate to observe these effects.

The present study addresses the testing of the consequences produced by the continuous administration of melatonin or DZP on the dissociated motor activity pattern in rats. In order to examine the effect of melatonin, we administered indoleamine in drinking water to intact rats and to rats submitted to a ganglionectomy to eliminate the endogenous circadian melatonin rhythm. The same procedure was followed for DZP but we introduced a pinealectomized group to discard possible interaction between DZP and the pineal gland. The results throw more light on the mechanism that these nonphotic cues use to entrain or modify the SCN-coupling mechanisms.

Material and methods

Animals and experimental design

A total of 45 male Wistar rats, supplied by Charles River, France, were used for the experiment. They reached our laboratory at 22 days of age and were housed individually in $22 \times 22 \times 15$ cm transparent cages in a sound-proof room, with food and water available ad libitum. Animals were maintained in a 24-h light–dark cycle (LD) with 12 h light and 12 h darkness for 4 days. Then, on day 1 of the experiment, they were transferred to a 22-h LD cycle (T22) with 300 lx indirect white light for 11 h alternating with <0.5 lx red light for 11 h for 24 days (stage 1). These light conditions were maintained during the entire experiment. On day 25, the rats were randomly distributed into three groups and one of them was submitted to pinealectomy, another to ganglionectomy and a third group remained intact. The animals were maintained under these conditions for 30 days (stage 2). On day 55, they started receiving a pharmacological treatment via drinking water (melatonin, DZP or vehicle) for 30 days more (stage 3). Finally, the treatment was discontinued and the animals again received tap water for 30 days (stage 4) (Fig. 1). Motor activity was

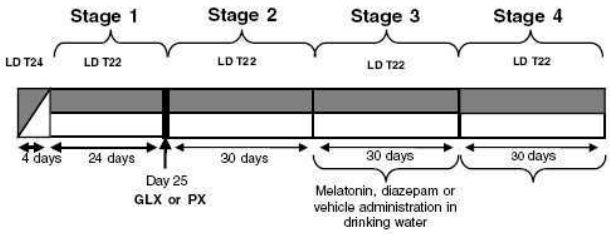

Fig. 1. Scheme of the experimental design. GX, ganglionectomy; PX, pinealectomy.

detected throughout the entire experiment by means of an activity meter with two infrared beams that crossed perpendicularly 7 cm above the floor of the cage. The number of movements was accumulated and recorded every 15 min.

Pinealectomy

A group of eight animals were anesthetized by intraperitoneal administration of ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight) and subjected to surgery according to the method of Hoffman and Reiter [24]. Briefly, the anesthetized animal was placed in a stereotaxical apparatus for small animals (Kopf Instruments, Tujunga, CA, USA) and a sagittal opening was made in the scalp. The skin and muscles were pushed aside in order to expose the lambda suture. By means of a circular drill, a disc-shaped perforation was made around the lambda and the disc-shaped piece of bone was delicately removed. Thereafter, the pineal gland (which is located just below the posterior venous sinus confluence) was removed with a fine forceps. After a brief period of hemostasis, the skull was closed by returning the disc-shaped bone and the scalp was sutured.

Superior cervical ganglionectomy

A group of 19 rats were anesthetized as described above and subjected to surgery according to the method of Reiter [25]. Briefly, through a ventral incision in the neck, their muscles were exposed and retracted, and each SCG was identified at the bifurcation of the common carotid artery into its internal and external branches. The ganglia were totally removed from both sides. The success of the surgery was checked by observing the ptosis of both eyelids.

Treatments

We used melatonin (Sigma-Aldrich, Madrid, Spain) at a dose of 1 mg/kg body weight/day and DZP (Almirall-Prodesfarma, Barcelona, Spain) at a dose of 2.5 mg/kg body weight/day. Both drugs were dissolved in 0.1% ethanol aqueous solution, vehicle (VEH), and administered in drinking water for 30 days. The volume of the water drunk by each rat was measured, the drug concentration was modified accordingly, and the actual doses were calculated on the basis of the volume consumed. The actual doses were 0.92 ± 0.20 mg/kg body weight/day for

Table 1. Number of rats used for the experiment

	Melatonin	Diazepam	Vehicle
CONTROLS	7	7	4
GX	7	8	4
PX	–	8	–

melatonin and 2.20 ± 0.49 mg/kgbody weight/day for DZP.

Data analysis

Data were analyzed in four stages, according to the design of the experiment (Fig. 1 and Table 1). For each stage, periodogram of Sokolove and Bushell [26] was applied to 20-day data to determine the period of the statistically significant motor activity rhythm as well as the percentage of variance (PV) explained by these rhythms. This PV enables quantification of the stability of a specific rhythm on the whole motor activity pattern. To study the balance of the two components, LDC and NLDC, we defined an index relating the percentages of the variance of them, applying the formula: $PV_{LDC}/(PV_{LDC} + PV_{NLDC})$, where PV_{LDC} is the percentage of the variance of the light-entrained component and PV_{NLCD} is the percentage of the variance of the non-light-entrained component. We refer to this coefficient as the LDC ratio. This ratio indicates the importance of LDC with regard to NLDC.

Moreover, for each rat and each stage, we calculated the mean rhythm profile on the basis of the periods of the two components, 22 h and the period of NLDC. Then, for each rhythm profile, we calculated the value of the area of the data around the mean (A) obtained as the sum of the absolute value of the differences between the mean and each datum of the mean profile. This value is expressed as a percentage of the total area under the wave (%A) which can be considered to be the variable most indicative of the degree of oscillation of the motor activity according to a determinate rhythm. Calculations were carried out by means of an integrated program for chronobiology 'El Temps' (A. Díez-Noguera, Universitat de Barcelona, 1999).

Statistical analysis was carried out by an ANOVA on various lineal models and multiple-comparison post hoc with Bonferroni correction. NLDC period, LDC ratio, the percentage of variance explained by each component (PV_{LDC} and PV_{NLDC}) and the %A calculated also for each component ($\%A_{LDC}$ and $\%A_{NLDC}$) were used as dependent variables while the stage, surgery and treatment were the independent variables. In certain cases, we applied a lineal model to paired data corresponding to the difference of the variables between the stages (stage 1–2, 2–3 and 3–4) and using surgeries and treatment as independent variables.

Results

The observation of the double plotted actograms (Fig. 2) shows that in all the rats, two circadian components are clearly visible in the four stages of the experiment. One of the components, LDC, is entrained by light with a period equal to the external cycle (T22 h), whereas the other, NLDC, is not entrained and free-runs with a period longer than 24 h ($\tau > 24$ h). However, the Sokolove–Bushell periodograms indicate that the manifestation of the two

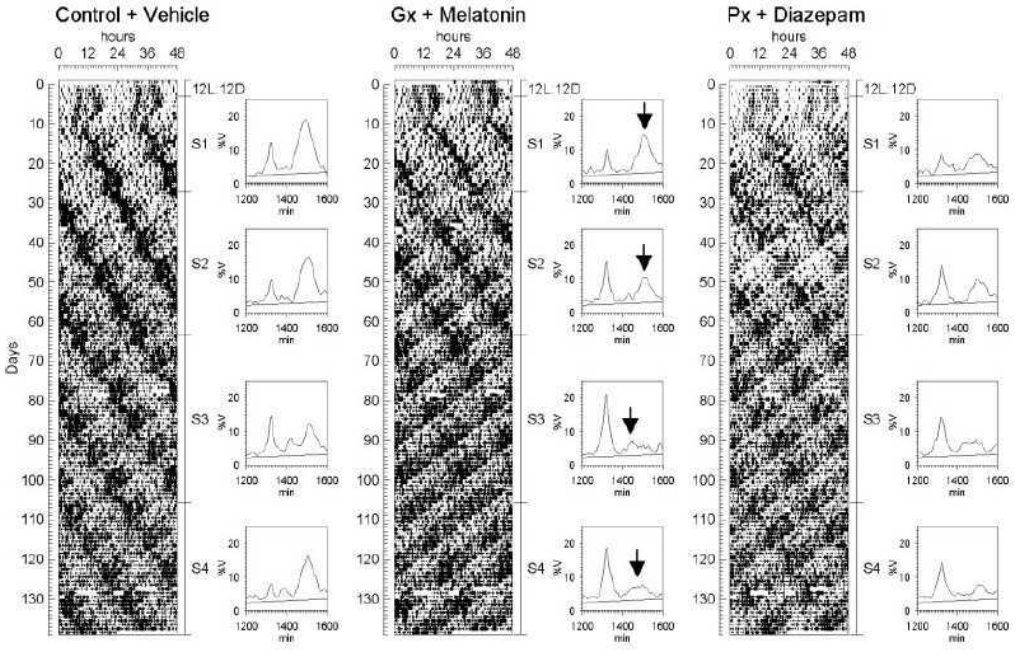

Fig. 2. Double-plotted actograms, plotted at modulo T 24, and Sokolove–Bushell periodograms corresponding to each one of the four stages (see experimental design) of three rats from a representative group submitted in T22 cycle. We can observe the dissociated motor activity rhythm into two components, represented by the two peaks in the periodogram. Note: the arrows indicating the shortening of the NLDC period by melatonin effect.

components is not the same throughout the stages in the different groups of rats. To test this effect, we analyzed the period of the NLDC, and the relative manifestation of each of the two components.

First, we analyzed the rhythms of the rats at the beginning of the experiment (stage 1) and found that in this stage there were no statistically significant differences between groups in the period of the NLDC or in the variance expressed by any of them. This confirmed that at the beginning of the experiment there were no differences among the groups of animals.

Analyzing only the differences between stage 1 and stage 2, we discovered that the period of the NLDC lengthens significantly in all the groups ($P < 0.001$) (Fig. 3). A paired-data analysis reveals that there were no statistically significant differences between GX, PX or CONTROL rats. We believe that this increase is due to the time that the rats have been under T22.

An ANOVA with data corresponding to stages 2, 3 and 4 indicates that the period of NLDC depends on the stage, with the longest values of this variable ($P < 0.001$) occurring in the second stage; on the treatment, with the rats treated with melatonin being those that show the shortest values ($P < 0.005$); but not on the surgery. Data analysis between stages 2 and 3 reveals that animals treated with melatonin show a statistically significant decrease of the period of NLDC ($P < 0.0001$) with a mean value of 30 min (SE 6.17 min), and that animals treated with DZP ($P < 0.01$) show a decrease with a mean value of 12.38 min (SE 4.26 min). However an ANOVA with paired data did not show statistically significant difference between DZP and VEH rats, and only the treatment with melatonin was significantly different from the other two treatments. Surgery had no statistically significant effects on these values. These differences because of the treatment, thus generated in stage 3, are maintained during stage 4 (without treatment), as there are no statistically significant differences between stages 3 and 4.

An ANOVA with data corresponding to stages 2, 3 and 4 (Fig. 4), indicates that the LDC ratio depends on the stage, with the highest values of this variable occurring in stage 3 ($P < 0.001$); on the treatment, rats treated with VEH showing lower values than those treated with melatonin ($P < 0.05$) and than those treated with DZP ($P < 0.001$); but also on the surgery, GX rats showing higher values than CONTROL rats ($P < 0.05$).

To test whether the LDC ratio increase is due to an increase of the LDC or to a decrease of the NLDC, we also

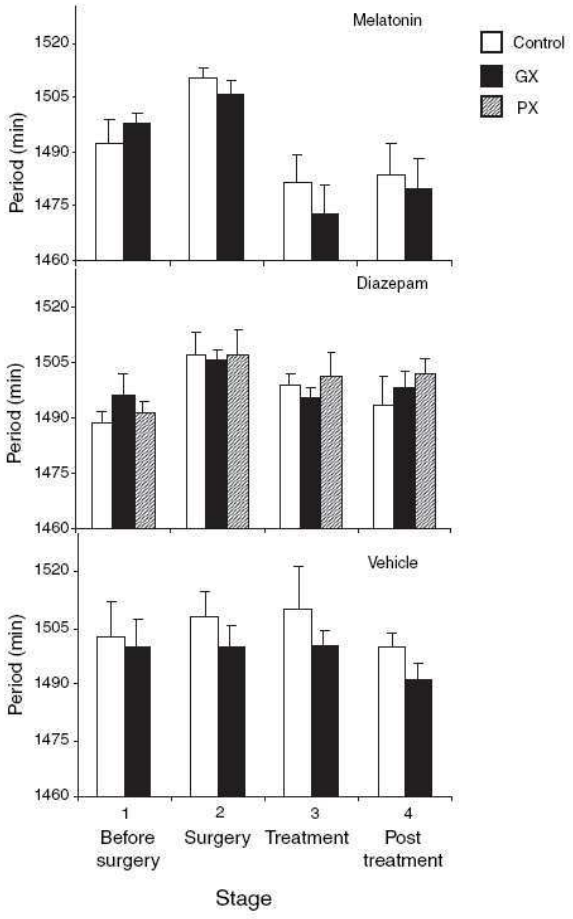

Fig. 3. Mean and standard error of the period of the NLDC.

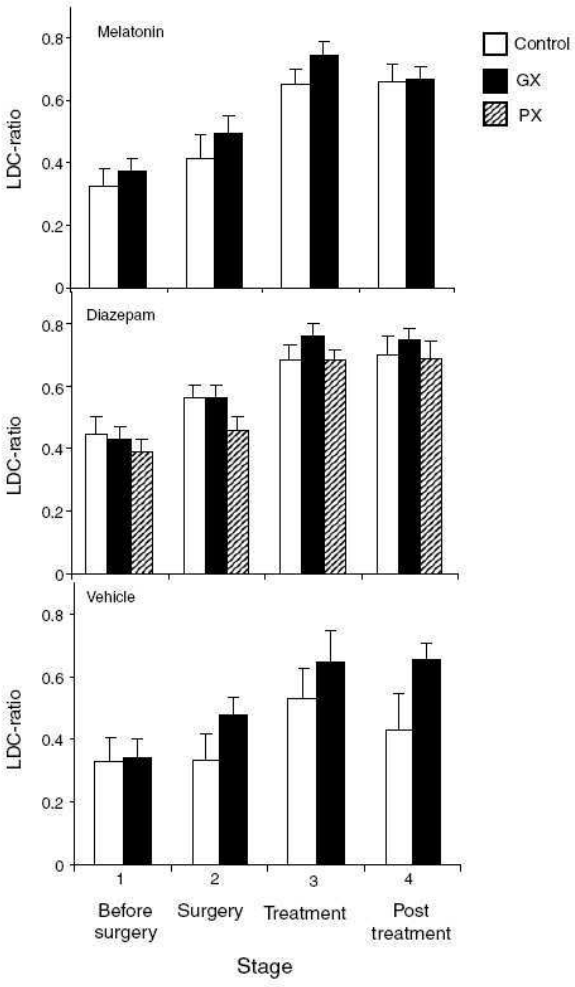

Fig. 4. Mean and standard error of the LDC ratio.

carried out a separate ANOVA with these two variables. The results show changes in both variables (figures not shown). Concretely, PV_{LDC} depends on stage, the highest values being in stage 3 ($P < 0.001$) which is different from stages 2 and 4; on the surgery with CONTROL rats showing lower values than GX ($P < 0.001$) and PX rats ($P < 0.05$); and on the treatment with DZP rats showing highest values than VEH rats ($P < 0.005$).

The PV_{NLDC} was also stage-dependent ($P < 0.0001$), but here the highest values correspond to stage 2, which is significantly different statistically from stages 3 and 4 (in both cases $P < 0.0001$), and on the treatment ($P < 0.0001$) with the highest values corresponding to animals treated with VEH, which is different from DZP ($P < 0.001$) and melatonin ($P < 0.05$). Surgery had no effect on this variable.

Finally, we studied the degree of oscillation for each component by means of the ANOVA of %A. These variables changed in a similar way to that of PV. $\%A_{LDC}$ shows the highest values in the groups that received pharmacological treatment (Fig. 5). Thus, rats that received DZP had higher values ($P < 0.01$) than those that received the VEH as well as rats treated with melatonin ($P < 0.05$). Surgery also has an effect on this variable, with CONTROL rats having lower values ($P < 0.05$). When we studied the interaction of treatment and surgery, we found that this variable had highest values in the group of ganglionectomized rats treated with DZP.

ANOVA of the $\%A_{NLDC}$ reveals that this variable decreases from stage 1 to stage 3 in all the groups. This variable also depends on the treatment ($P < 0.001$), with the lowest values of this variable in rats treated with the drugs, although the differences were only statistically significant between DZP and VEH groups ($P < 0.05$). Moreover, there is an interaction between the treatment and the surgery. Only in rats treated with DZP CONTROL rats had lower values than GX rats ($P < 0.05$) and PX rats ($P < 0.001$).

Discussion

Motor activity dissociation under T22 can be regarded as a model of an internal uncoupling of the SCN into ventrolateral and dorsomedial areas [4]. We used this model as a tool to study the coupling effect as well as the differential effect that melatonin or DZP have on the dissociated rhythms.

In order to evaluate the direct action of the exogenous drugs on the dissociated periods, we believed it important to administer those drugs in a way that does not by itself induce entrainment and to eliminate the non-specific disturbance of the animal associated with the daily injection. Slotten et al. [27] have demonstrated that melatonin administration via timed access to drinking water is an efficient way to entrain free-running activity rhythms in rats. Our experiment chose the same way for drug administration but allowed an unrestricted access to the water containing the drugs. As we found that drinking behavior follows the same pattern as locomotor activity (TC, ADN unpublished data), we consider this form of administration to be the most appropriate. Our rationale for this is that the same animal could reinforce one component or another according to their self-dissociated drinking rhythm. Regarding the possible ethanol effect used as VEH, the

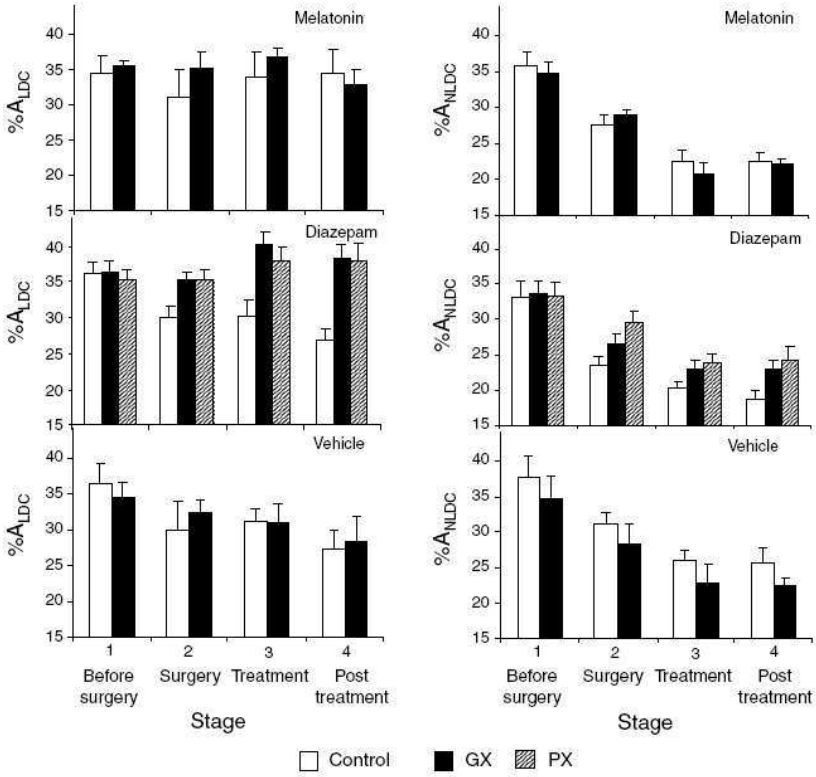

Fig. 5. Mean and standard error of the %A of LDC and NLDC.

small concentration of the 0.1% ethanol solution used as VEH is unlikely to have any effect on the locomotor activity, as it has been described that the 2% ethanol has no direct effect on the motor activity rhythm [28].

One of noteworthy results of this experiment was the shortening of the period of the NLDC. The chronobiotic properties of melatonin were extensively studied. In rats and hamsters with free-running circadian rhythms, pharmacological doses of exogenous melatonin are capable of synchronizing the circadian rhythms of locomotor activity [29–31]. Nevertheless, there are no evidences that melatonin shortens the free-running rhythm. On the contrary, however, there are some reports that show a lengthening of the free-running period after melatonin treatment [32]. Our results suggest a different effect of melatonin treatment when administered in a dissociated system from results described previously by other authors. The effects of melatonin on the NLDC period were pharmacological. Based on data from the literature, high doses of melatonin have to be used independent of the form of administration to obtain entrainment of rat activity rhythm [5, 27]. At the moment, it is unknown as to why high doses of exogenous melatonin are necessary to exert entrainment effects. The suggestion that melatonin receptors are less sensitive to exogenous than endogenous melatonin [33] cannot be an explanation because chronobiotic effects are also obtained in pinealectomized or ganglionectomized rats where the levels of endogenous melatonin are null or very low [34]. The finding that CONTROL and GX rats shorten their NLDC period during melatonin administration in the same way indicates that endogenous melatonin is not necessary for the effect on NLDC period of exogenous melatonin.

As melatonin treatment affects the period of the free-running component and as this period is an intrinsic property of the central oscillator, we can suppose that the action of melatonin is exercised directly on the SCN. Although the melatonin receptors are thoroughly distributed in both the ventrolateral and in the dorsomedial regions of the suprachiasmatic nuclei [12, 35], the effect observed on the NLDC period might indicate a predominant action of melatonin on the dorsomedial region, as this region drives the NLDC [4]. We could hypothesize, then, that melatonin promotes the coupling of the oscillators in the dorsomedial part producing a shortening of the NLDC period and making it closer to the period of the external light cycle and therefore more easily entrained. Further studies will be required to clarify whether melatonin receptors are differently distributed in each part of dissociated rat SCN and how this distribution would correspond to a specific function.

On the other hand, we analyzed the manifestation of the dissociation by means of the analysis of percentage of variance explained by each component, which mainly reflects the stability of the rhythms, and also by means of the %A which indicates the quantity of motor activity that oscillates around the mean. The more stable and the higher amplitude a rhythm has, the higher values for PV and for %A, respectively. In this case, the more entrained the animal is to the external T22 cycle, the higher are the values of these variables. For the LDC, we found that PX or GX rats, compared with CONTROL rats, showed higher LDC expression, as indicated by the LDC ratio and the $\%A_{LDC}$. However, this effect is noticeable only in stage 3, when the treatment is applied. This could be explained by assuming that the decrease of the endogenous circulating melatonin, as consequence of the surgeries, produces a reduction of the internal coupling of the circadian system. This reduction makes the system more sensitive to the effect of the drugs, which can thus more easily manifest the external cycle. However, we also found that the %A of the LDC has higher values for GX rats especially when they receive treatment with DZP. This indicates that when an animal is treated with DZP, PX is not equivalent to GX. These results are not easily interpreted, because DZP acts on the pineal gland [22, 23] and on the superior cervical ganglia [36], and also perhaps because GX can alter other systems apart from the secretion of melatonin.

The administration of both drugs, melatonin or DZP improved the entrainment of the activity rhythm to the external cycle. In fact, an increase of the PV_{LDC} takes place to the detriment of PV_{NLDC}, after the treatment with these substances. However, this effect was stronger in DZP treated rats, and this indicates that the LDC is reinforced when the animal is treated with GABAergic substances like DZP.

It is known that the phase shift in the motor activity rhythm induced by light pulses can be blocked by agents that alter the GABAergic neurotransmission. In addition, it has been demonstrated that the DZP has an effect on the circadian system through the interaction with $GABA_A$-benzodiazepine receptor increase of the conductance of a chloride ionophore associated with this complex [17]. On the other hand, the DZP also has a direct effect on the pineal gland, producing a delay in the secretion of melatonin [22, 23]. Nevertheless, the results obtained in the present experiments with the GX and PX animals and DZP treated suggest that the main action is exercised directly on the SCN and not via the pineal gland. Moreover, the absence of the endogenous melatonin rhythm in these groups seems to favor the GABAergic action on coupling. These results suggest that the stimulation of the GABAergic receptors would improve the coupling of the SCN oscillators. This finding agrees with a recent report suggesting that GABAergic transmission is critically involved in communication between oscillators in the dorsal and ventral SCN, and that resetting of the dorsal SCN to new lighting conditions is dependent in part on a GABA input from the ventral SCN [37]. The data presented here suggest that the DZP treatment, through the GABAergic transmission, is able to promote internal coupling in functionally dissociated SCN, facilitating the entrainment to the external light cycles. It is worth noting that although DZP increases the entrainment it does not affect the period of NLDC, as melatonin does. These results may suggest that melatonin and DZP have differential effects on dorsal or ventral SCN and their treatment in dissociated circadian systems promote entrainment with the external light cycle.

The dissociation phenomenon may not be exclusive to laboratory animals. In humans, some pathologies such as insomnia, and some cases of depression, as well as jet-lag can also be explained by desynchronization [38]. For this reason, the dissociation model developed in our laboratory

constitutes a good tool to study the coupling mechanism between the suprachiasmatic nucleus oscillators and the pharmacological and environmental factors capable of modifying it. This knowledge will contribute to clarify the entrainment mechanisms in the circadian system, for improving the quality of the human life. Thus, our findings on the effects of melatonin and DZP on dissociated activity rhythms may have physiologically and pharmacologically significant implications. Further studies are required to establish the mechanisms operating on entrainment by pharmacological zeitgebers.

Acknowledgments

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References

1. Klein DC, Moore RY, Reppert SM. Suprachiasmatic Nucleus: The Mind's Clock. Oxford University Press, New York, 1991.
2. Welsh DK, Logothetis DE, Meister M et al. Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 1995; **14**:697–706.
3. Campuzano A, Vilaplana J, Cambras T et al. Dissociation of the rat motor activity rhythm under T cycles shorter than 24 hours. Physiol Behav 1998; **63**:171–176.
4. De La Iglesia HO, Cambras T, Schwartz WJ et al. Forced desynchronization of dual circadian oscillators within the rat suprachiasmatic nucleus. Curr Biol 2004; **14**:796–800.
5. Cassone VM, Chesworth MJ, Armstrong SM. Dose-dependent entrainment of rat circadian rhythms by daily injection of melatonin. J Biol Rhythms 1986; **1**:219–229.
6. Ralph MR, Menaker M. Effects of diazepam on circadian phase advances and delays. Brain Res 1986; **372**:405–408.
7. Bartness TJ, Goldman BD. Mammalian pineal melatonin: a clock for all seasons. Experentia 1989; **45**:939–945.
8. Reiter RJ. The melatonin rhythm: both a clock and a calendar. Experientia 1993; **49**:654–664.
9. Larsen PJ, Enquist LW, Card JP. Characterization of the multisynaptic neuronal control of the rat pineal gland using viral transneuronal tracing. Eur J Neurosci 1998; **10**:128–145.
10. Teclemariam-Mesbah R, Ter Horst GJ, Postema F et al. Anatomical demonstration of the suprachiasmatic nucleus-pineal pathway. J Comp Neurol 1999; **406**:171–182.
11. Vanecek J, Pavlik A, Illnerova H. Hypothalamic melatonin receptor sites revealed by autoradiography. Brain Res 1987; **435**:359–362.
12. Dubocovich ML, Rivera-Bermudez MA et al. Molecular pharmacology, regulation and function of mammalian melatonin receptors. Front Biosci 2003; **8**:d1093–108.
13. Moore RY, Speh JC. GABA is the principal neurotransmitter of the circadian system. Neurosci Lett 1993; **150**:112–116.
14. Belenky MA, Sagiv N et al. Presynaptic and postsynaptic GABAA receptors in rat suprachiasmatic nucleus. Neuroscience 2003; **118**:909–923.
15. Jiang ZG, Yang Y, Liu ZP et al. Membrane properties and synaptic inputs of suprachiasmatic nucleus neurons in rat brain slices. J Physiol 1997; **499**(Pt 1):141–159.
16. Liu C, Reppert SM. GABA synchronizes clock cells within the suprachiasmatic circadian clock. Neuron 2000;**25**:123–128.
17. Ralph MR, Menaker M. GABA regulation of circadian responses to light. I. Involvement of GABAA-benzodiazepine and GABAB receptors. J Neurosci 1989; **9**:2858–2865.
18. Wan Q, Man HY, Liu F et al. Differential modulation of GABAA receptor function by Mel1a and Mel1b receptors. Nat Neurosci 1999; **2**:401–403.
19. Golombek DA, Pevet P, Cardinali DP. Melatonin effects on behavior: possible mediation by the central GABAergic system. Neurosci Biobehav Rev 1996; **20**:403–412.
20. Atsmon J, Oaknin S, Laudon M et al. Reciprocal effects of chronic diazepam and melatonin on brain melatonin and benzodiazepine binding sites. J Pineal Res 1996; **20**:65–71.
21. Kalsbeek A, Garidou ML, Palm IF et al. Melatonin sees the light: blocking GABA-ergic transmission in the paraventricular nucleus induces daytime secretion of melatonin. Eur J Neurosci 2000; **12**:3146–3154.
22. Wakabayashi H, Shimada K, Satoh T. Effects of diazepam administration on melatonin synthesis in the rat pineal gland in vivo. Chem Pharm Bull 1991; **39**:2674–2676.
23. Djeridane Y, Touitou Y. Effects of diazepam and its metabolites on nocturnal melatonin secretion in the rat pineal and Harderian glands. A comparative in vivo and in vitro study. Chronobiol Int 2003; **20**:285–297.
24. Hoffman RA, Reiter RJ. Rapid pinealectomy in hamsters and other small rodents. Anat Rec 1965; **153**:1922.
25. Reiter RJ. Morphological studies on the reproductive organs of blinded male hamsters and the effects of pinealectomy or superior cervical ganglionectomy. Anat Rec 1968; **160**:13–23.
26. Sokolove PG, Bushell WN. The chi square periodogram: its utility for analysis of circadian rhythms. J Theor Biol 1978; **72**:131–160.
27. Slotten HA, Pitrosky B, Pevet P. Influence of the mode of daily melatonin administration on entrainment of rat circadian rhythms. J Biol Rhythms 1999; **14**:347–353.
28. Subramanian P, Subbaraj R. Diazepam modulates the period of locomotor rhythm in mice (*Mus booduga*) and attenuates light-induced phase advances. Pharmacol Biochem Behav 1996; **54**:393–398.
29. Redman J, Armstrong S, Ng KT. Free-running activity rhythms in the rat: entrainment by melatonin. Science 1983; **219**:1089–1091.
30. Armstrong SM, Chessworth MJ. Melatonin phase-shifts a mammalian circadian clock. In: Fundamentals and Clinics in Pineal Research. Trentini GP, De Gaetani C, Pévet P, eds. Raven Press, New York, 1987; pp. 195–198.
31. Pévet P, Bothorel B, Slotten H et al. The chronobiotic properties of melatonin. Cell Tissue Res 2002; **309**:183–191.
32. Armstrong SM, Redman J. Melatonin administration: effects on rodent circadian rhythms. In: Photoperiodism, Melatonin and the Pineal. Ciba Foundation Symposium 117. Evered D, Clarke S, eds. Pitman, London, 1985; pp. 188–207.
33. Warren WS, Hodges DB, Cassone VM. Pinealectomized rats entrain and phase-shift to melatonin injections in a dose-dependent manner. J Biol Rhythms 1993; **8**:233–245.
34. Garidou ML, Bartol I, Calgari C et al. In vivo observation of a non-noradrenergic regulation of arylalkylamine N-acetyltransferase gene expression in the rat pineal complex. Neuroscience 2001; **105**:721–729.

35. SONG CK, BARTNESS TJ, PETERSEN SL et al. SCN cells expressing mt1 receptor mRNA coexpress AVP mRNA in Syrian and Siberian hamsters. Adv Exp Med Biol 1999; **460**:229–232.
36. AGUAYO LG, CISTERNAS C, TAPIA JC et al. Effects of benzodiazepine receptor ligands on isolated rat superior cervical ganglia neurons. Pharmacology 1996; **52**:371–376.
37. ALBUS H, VANSTEENSEL MJ, MICHEL S et al. A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. Curr Biol 2005; **15**:886–893.
38. TUREK FW, DUGOVIC C, ZEE PC. Current understanding of the circadian clock and the clinical implications for neurological disorders. Arch Neurol 2001; **58**:1781–1787.

EXPERIMENT 6: EFECTE DEL XOC ELECTROCONVULSIU

ARTICLE

EFFECTS OF ELECTROCONVULSIVE SHOCK ON THE RAT'S MOTOR ACTIVITY AND TEMPERATURE CIRCADIAN RHYTHMS

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INTRODUCCIÓ

S'ha descrit que en la depressió es pot produir desincronització dels ritmes circadiaris, i que la TEC resulta eficaç en el tractament d'aquesta malaltia. Però, l'administració de XECs afecta directament el sistema circadiari? Els XECs poden ser capaços d'acoblar dos ritmes circadiaris desincronitzats, o de modificar, almenys, algun dels dos components de la dissociació?

OBJECTIU

L'objectiu va ser estudiar l'efecte de l'administració aguda i crònica del XEC en els ritmes circadiaris d'AM i de TEMP, en rates amb el sistema circadiari dissociat (sotmeses a T22) i en rates control, amb un sol ritme circadiari (mantingudes en DD).

MATERIAL I MÈTODES

Es van realitzar 3 experiments en el transcurs dels quals es va enregistrar l'AM i la TEMP de rates Wistar per tal de determinar el funcionament del sistema circadiari. En el primer experiment es van analitzar els efectes de l'aplicació aguda d'un XEC a diferents moments del dia subjectiu sobre la fase i el període dels ritmes circadiaris. En el segon i el tercer experiments es van provar els efectes de l'aplicació crònica del XEC quan els animals estan en condicions de DD i T22, respectivament.

RESULTATS

L'aplicació aguda del XEC no modifica ni la fase ni el període dels ritmes circadiaris. L'aplicació crònica, en canvi, produeix un increment dels valors d'AM i de TEMP, alhora que fa disminuir l'amplitud dels ritmes, però no afecta el període ni la fase del ritme en curs lliure.

CONCLUSIÓ

Els resultats suggereixen que el XEC no modifica el funcionament del rellotge circadiari, però sí que n'altera els sistemes efectors d'AM i de TEMP.

EFFECTS OF ELECTROCONVULSIVE SHOCK ON RAT MOTOR ACTIVITY AND TEMPERATURE CIRCADIAN RHYTHMS

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ABSTRACT

The hypothetical relationship between circadian rhythms alterations and depression has prompted studies that examine the resultant effects of various antidepressants. Electroconvulsive therapy (ECT) exerts significant antidepressant effects that have been modelled in the laboratory via the use of electroconvulsive shock (ECS) in rats. We report here the effects of acute and chronic ECS administration on the temperature and motor activity circadian rhythms of rats. We carried out three experiments. In the first, we analyzed the effects of acute ECS, while in the other two ECS was daily applied to rats for 3 weeks: respectively, under dim red light, and under light-dark cycles of 22 hours, that implies dissociation in the circadian system. Results show that acute ECS does not modify the circadian rhythms. Chronic administration of ECS increases the levels of motor activity and temperature but it does not modify the functioning of the circadian pacemaker.

KEYWORDS: electroconvulsive therapy, circadian rhythm, motor activity, temperature, suprachiasmatic nucleus, electroconvulsive shock

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INTRODUCTION

In mammals, circadian rhythms are driven by a central pacemaker located in the suprachiasmatic nucleus (SCN). The SCN generates the genetically determined, endogenous periodicity which, under constant environment, is slightly different from 24h. To entrain an organism to 24h light-dark cycle, the pacemaker requires regular environmental signals. In animals, such signals are mainly provided by light information, which is transmitted to the SCN by retinal ganglion cells (Hattar et al, 2003). The SCN also receives input from other brain regions such as the intergeniculate leaflet of the thalamus and the raphe nucleus (Morin, 2007). The latter's main neurotransmitter is serotonin, which provides nonphotic input to the SCN (Moore et al, 2002). The circadian pacemaker is responsible for coordinating physiologic rhythms within the body. This is accomplished by a set of direct and indirect projections from the SCN onto other regions of the brain. Under normal conditions most of an organism's rhythms exhibit the same circadian period, thus reflecting an internal temporal order. However, humans kept under isolation (Wever, 1979) or under forced desynchronization (Czeisler et al, 1999), show a divergence between the timing of body rhythms and the light-dark cycle and exhibit rhythms with different periods between temperature and activity. It has been suggested that this rhythmic disturbance can have profound effects on mood, sleep and health (Wirz-Justice, 2006) and may attend jet-lag, shift work or even depression (Dunlap et al, 2004). We have proposed that submitting rats to T22, situation that induces two circadian components in the overt rhythms (Campuzano et al, 1998), may prove an effective model for human desynchronization. Circadian rhythm alterations have also been described in mood disorders. Depressive patients frequently experience sleep disturbances, as well as alterations in hormonal secretion (e.g., cortisol or melatonin) and in temperature circadian rhythms (Ford & Kamerow, 1989; Rubin et al, 1992; Wirz-Justice, 2000). Such changes are mainly characterized by a reduction in rhythm amplitude, which has been shown to normalize during remission. It has also suggested that unavoidable disturbances in circadian rhythms can trigger depressive episodes in humans (Wirz-Justice, 2003). It is noticeable that an antidepressant, such as agomelatine (a melatonergic receptor agonist and 5HT2c receptor antagonist) resynchronizes human circadian rhythms in healthy volunteers and depressed patients (Duval et al, 2006), as well as rats, which when administered agomelatine daily were entrained to a 24h cycle (Guardiola-Lemaitre, 2005). Taken together, this suggests that circadian abnormalities may play a role in the pathogenesis of depression. However, how circadian rhythms are related to depression still remains unknown. The SCN involves some of the same

neurotransmitters thought to be important in depression, such as serotonin (Moore & Speh, 2004; Carlsson et al, 1980; Lambert et al, 2002; Wirz-Justice & Richter, 1979; Kennaway, 1997) and gamma-aminobutyric acid (GABA) (Moore et al, 2002).

Electroconvulsive therapy (ECT) is a well-established, effective, and quick treatment for depression. Indeed, it may be considered a first-line treatment in cases of severe depression, depression characterized by specific symptoms (psychosis or catatonia), or suicidal tendencies. The intensity of ECT typically administered to patients induces a self-sustained after-discharge of cortical neurons that produces electroconvulsive seizures. The beneficial effects from ECT may stem not from the convulsion itself, but rather from the anticonvulsant effects of the seizure that result from enhanced transmission of inhibitory neurotransmitters and neuropeptides (Eitan & Lerer, 2006).

Electroconvulsive shock (ECS) has been widely used as an animal model of ECT and has, moreover, helped elucidate the therapeutic profile and side effects of ECT (Green & Nutt, 1987). Although ECS or ECT mainly affects hippocampus and neocortex (Mc Donald et al, 2000), the exact mechanisms are not yet well known. The mechanism underlying ECS is thought to involve a large number or neurotransmitters systems (Eitan & Lerer, 2006; Newman et al, 1998; Dremencov et al, 2003). In addition, ECT not only acutely increases plasma prolactin (Swartz, 1997; Markianos et al, 2002), but also exerts an effect on neuronal plasticity and neurogenesis (Altar et al, 2004). It also reportedly increases the amplitude of the core body temperature in depressed subjects (Szuba et al, 1997). However, data on ECS's effects on circadian rhythms remain scare, with an attendant lack of systematic studies.

In this study, we directly examined the effects of ECS on motor activity and temperature circadian rhythms in rats. To this end, we carried out three experiments. In the first, we tested the acute effects of ECS administration during different times of the subjective day of the animal, while in the second we evaluated the effects of chronic ECS treatment on circadian rhythms. We also hypothesized whether ECS would exert different effects on an altered circadian system. Specifically, we conducted a third experiment examining the effects of chronic ECS in rats submitted to T22 light-dark cycles, which implies dissociation in the rat's circadian rhythms.

EXPERIMENTAL PROCEDURES

Animals and experimental conditions

Forty-four male Wistar rats (Charles River, France) were used in these experiments. The animals arrived at the laboratory at age 4 weeks and were individually housed in transparent cages (25x25x12cm), maintained in three different isolated rooms (one for each experiment, see

below), with access to food and water *ad libitum*. The motor activity rhythm was detected by activity meters having two crossed, perpendicular infrared beams crossing the cage 7cm above the floor. The number of movements was recorded every 15min. Temperature was measured (with an accuracy of 0.125ºC) via data loggers (Thermochron®) implanted intraperitoneally in the animals following administration of isofluorane anesthesia. To avoid alterations in the animal's rhythm, the surgery was carried out just prior to the start of experiments. Data loggers were programmed to start recording on different days depending on the experiment, but always with a sampling period of 30 minutes. At the end of the experiments, animals were sacrificed and the temperature sensors were removed to acquire the data.

Electroconvulsive shock administration:

Following attachment of saline-soaked earclips, ECS consisted of a 1s, 100 pulses/s, 90 mA stimulus of 0.5ms square-wave pulses delivered using a UGO Basile ECT unit, model 57800-001. At these settings, all animals had generalized tonic-clonic seizures with hind limb extension. ECS administrations were performed in an adjacent room without altering the lighting conditions of the experimental room.

All the procedures used in the experiments were approved by the Animal Care and Use Committee of the University of Barcelona.

Experimental procedure

Experiment 1: Phase and period changes of motor activity and temperature rhythms as induced by ECS applied at different circadian time points.

In this case, activity and temperature in eight rats were continuously recorded through the entire experimental period. Animals were maintained under constant dim red light (0.40-2.20 lux). This light intensity not only allowed animals to maintain a robust circadian rhythm, but also permitted researchers to handle them. Each animal received one ECS every 7-10 days at different circadian time (CT) points, with CT12 serving as the onset of the active phase for rats. The CTs assayed were as follows: CT0, CT4, CT8, CT12 and CT20. The day before ECS administration, a line intersecting the rhythm onset was drawn to extrapolate the CT12 occurring the following day. The corresponding number of circadian hours was then added or subtracted to CT12 in order to obtain the exact time of each specific CT. At the end of the experiment, the CTs at which animals received ECS were revised, and in some cases corrected, based on the entire motor activity and temperature plot. Thus, more CTs than initially envisaged were obtained.

Data analysis: Phase and period shifts produced by a single ECS were analyzed. To determine the former, we first calculated the mean period of the endogenous rhythm (tau) for each animal by means of a chi-squared periodogram (29), which was applied to all days of the experimental period. We then conducted cosinor analysis for each animal and variable (motor activity and temperature) data, with a period equal to tau for 6 days before and 6 days after ECS application, thereby obtaining the corresponding acrophases. The value of one acrophase was subtracted from the next and the phase shift value was plotted according to the CT. In this way, we obtained the phase response curves (PRC) for ECS vis-à-vis motor activity and temperature rhythm. In similar fashion, we calculated the period-response curve (tauRC), which measures the period change based on the CT applied by the ECS. To this end, we calculated a periodogram for each animal and variable with the data that corresponded to 6 days before and 6 days after each ECS application. The tau value from a single stage was then subtracted from the next, thereby obtaining the tau shift value for each CT. These shifts were plotted based on the corresponding CT and the tauRC was subsequently drawn.
Statistical analysis for motor activity and temperature was carried out to calculate the differences from 0 in each CT, by means of a one-sample *t*-test, and to determine the differences between the various CTs by means of ANOVAs. In all cases, Bonferroni's correction was applied.

Experiment 2: Effects of ECS chronic administration on the free running rhythms of motor activity and temperature.

We used 16 male Wistar rats divided in two groups: experimental (n=8) and control (n=8). Lighting conditions were the same as in experiment 1. After 30 days (pre-ECS stage), the experimental group received a near daily ECS administration. Each rat received 4-6 ECS per week for 3 weeks, always an ECS per day between 11 and 12 a.m., without any alterations to the lighting conditions. At this stage, control rats were handled in the same way as the experimental rats, albeit without receiving any electrical current. Afterwards, rats were kept under the identical conditions (post-ECS stage) in order to determine motor activity and temperature rhythms following ECS application. Motor activity was recorded for all rats throughout the entire experiment, while temperature was recorded only for 6 experimental and 5 control rats during the ECS and post-ECS stages.

Data analysis: To calculate the periods and percentages of variance (%V) explained by the corresponding rhythm, we used a periodogram (Sokolove & Bushell, 1978) with data from 18 days from every stage for each animal. Motor activity and temperature data were analyzed

separately. The mean period and the mean %V were then calculated for each group (experimental and control), each variable (motor activity and temperature) and each stage (pre-ECS, ECS and post-ECS). In addition, the daily mean values for motor activity and temperature, based on the tau period, were calculated for each rat and for each day the experiment lasted. To determine variations in motor activity and temperature rhythm expression, we carried out a power spectrum analysis to those data sets corresponding to the length of the endogenous period (tau). The power content for the first spectrum harmonic (PCH1) reflects the amplitude of the free-running period. Moreover, a 24h mean profile for ECS stage was calculated for each animal and variable, thereby yielding the mean value of these curves. Using the individual 24h mean profile, we calculated the area under the curve for 4 hours after ECS administration. This is referred to as area percentage beneath the 24h profile (4h-MA and 4h-TEMP for motor activity and temperature, respectively). Statistical analysis (ANOVA) involved several linear models, taking into account period and %V as dependent variables, and the stage of the experiment (pre-ECS, ECS and post-ECS) and group (experimental and control) as independent variables. Groups were also compared using the Student's *t*-test with Bonferroni's correction.

Experiment 3: Study of the chronic ECS application on the rat's dissociated circadian rhythms vis-à-vis motor activity and temperature.

In this case, 20 male Wistar rats (10 experimental and 10 control) were submitted to a 22-hour period light-dark cycle (T22, 11h-light and 11h-dark) throughout the entire experiment. During the light phase, indirect light was provided by two fluorescent tubes (300 lux at cage level), while during darkness, a dim red light (0.40-2.20 lux) was used. Animals were maintained under these conditions for 30 days (pre-ECS stage). Afterwards, experimental rats received 4-6 ECS per week for 3 weeks (ECS stage), always an ECS per day between 11 and 12 a.m. Control rats were similarly handled, albeit without electrical current. Finally, rats remained thus for 24 more days to test the rhythm following ECS administration (post-ECS stage). Motor activity was recorded for all the rats throughout the entire experiment. Temperature was recorded only for 6 experimental and 5 control rats during the pre-ECS and ECS stages.

Data analysis: Under T22, two significant circadian rhythms were observed regarding motor activity and temperature: one exhibiting a period equal to 22h (light-dependent component, LDC) and another with a period greater than 24h (non-light-dependent component, NLDC). To calculate the periods and %V of the two rhythms ($\%V_{LDC}$ and $\%V_{NLDC}$), the periodogram

(Sokolove & Bushell, 1978) was applied to each individual animal data (19 days data for each stage). The ratio "$\%V_{LDC}$ / $\%V_{NLDC}$" was calculated for each animal and used as an indicator of the adaptation to the LD cycle. The daily means (based on 24h sections) of motor activity and temperature were calculated for each animal as well as for each day of the experiment. In addition, a 24h mean individual profile for motor activity and temperature was calculated for the ECS stage, and the mean values of the profiles, as well as the variables 4h-MA and 4h-TEMP, were obtained as described in Experiment 2.

In all three experiments, data were analyzed using the integrated package for chronobiology "El Temps" (A. Díez-Noguera, Universitat de Barcelona, 1999), while statistical analysis was carried out using the SPSS® package.

RESULTS

Experiment 1: All the rats in Experiment 1 exhibited a robust free-running circadian rhythm under the described conditions, with a mean period of 24 hours 40 minutes. Calculation of both PRC and tauRC as regards motor activity and temperature indicated that ECS administration exerted very little effects on the period and phase of either temperature or motor activity rhythms (Figure 1). When we calculated whether the changes produced by ECS in the various CTs were different from 0 (after making a Bonferroni's correction to the level of significance), we discovered that only one point (CT16) in the tau response curve of motor activity resulted in a lengthening of the period statistically significant (different) from 0. In regards to differences based on the various CTs, ANOVA detected only statistically significant differences ($p<0.05$) in tau changes related to motor activity rhythm. Post-hoc comparisons indicated that CT20 differed from CT8 and CT16, and that CT22 differed from CT4, CT8, CT12, CT14, and CT16.

Experiment 2: All rats in Experiment 2 exhibited a clear free-running circadian rhythm (Figure 2), with the same mean period noted in Experiment 1. However, in terms of motor activity data, one experimental and one control rat displayed a second statistically significant peak within a 24 h period (period of the ECS application) in the periodogram. This was also true for temperature data in 4 experimental rats. The period of motor activity rhythm increased throughout the experiment, but it was only significantly different between pre and post-ECS stages in the control group (Student's *t*-test with Bonferroni's correction $p<0.008$). The mean value of the tau period did not differ between experimental and control groups, nor between periods obtained in terms of temperature or MA data (Figure. 3).

The %V of the motor activity rhythm in both control and experimental groups was significantly higher in the pre-ECS compared to the post-ECS stage ($p<0.008$). Differences in this variable between experimental and control groups ocurred only in the ECS stage, where the %V in the ECS stage of the experimental group displayed lower values than in the other stages ($p<0.008$). Temperature data for the experimental groups revealed %V values in the ECS stage lower than in the post-ECS stage ($p<0.008$), while no differences were found for controls (Figure 3).

The evolution of the motor activity PCH1 in both experimental and control groups (Figure 4) showed that when ECS is applied, PCH1 decreases in the former, but not in the latter. This decrease is particularly evident during the first week of the ECS stage, although later the value of the PCH1 increases slowly, while still remaining lower than in the control group. This alteration in the circadian rhythm is even visible to the naked eye in actograms (Figure 2), where an interruption in the circadian rhythm between days 33 to 38 can be clearly observed. During post-ECS, there were no differences in the PCH1 between the two groups. The PCH1 for temperature data consistently displayed higher values than for motor activity, although no differences were detected between the experimental and control groups.

The mean motor activity (per cycle) for motor activity and temperature (Figure 5) increased during the ECS-stage in the experimental group, but not in the control group. When the mean value of the 24h profile was compared between the two groups, the experimental group exhibited higher values than controls in terms of motor activity, but not in temperature ($p<0.005$). The variables MA-4h and TEMP-4h were not statistically different between the experimental and control groups; thus, these results are not shown.

Experiment 3: As animals were submitted to light-dark cycles of 22 hours (T22), actograms display two circadian components (LDC and NLDC) (Figure 6) for both motor activity and temperature. In the ECS stage, however, the addition of a (third) rhythm induced by ECS administration, made it difficult to draw conclusions about the overall pattern. Consequently, we only compared the period and the %V before and after ECS administration. Since temperature data was not available for the post-ECS stage, only MA data was used for these calculations. The values of the NLDC period and that of the variable $\%V_{LDC}/\%V_{NLDC}$ were higher in the control than in the experimental group ($p<0.005$). In experimental rats, however, no differences were found before or after ECS application. Since we do not consider these results relevant, graphs are not shown. It is noticeable that the period or phase of the NLDC does not change throughout the stages.

Analysis of the daily mean motor activity indicated that it increased as a consequence of ECS administration, later returning to the previous values, which was also true of temperature (Figure 7). As in Experiment 2, a mean profile based on a period of 24 hours was calculated for the ECS stage and the mean value for each rat was calculated. For both temperature and activity, experimental had higher values than control rats ($p<0.05$), while MA-4h and TEMP-4h were not different between experimental and control group.

DISCUSSION

The overall conclusion of these three experiments, which examined the effects of ECS on the circadian rhythm, is that overt rhythms in both motor activity and temperature are modified by the administration of a daily ECS, leaving the circadian pacemaker unaffected.

From our first experiment, we deduced that a single ECS, applied at any time point, does not affect the circadian rhythm, nor does it exert any real effect on the daily manifestation of either variables. This seems consistent with the fact that repeated administrations of ECS are more likely to impact the therapeutic mechanisms of ECT than would a single ECS (Eitan & Lerer, 2006). Other experiments show that convulsive seizures produced phase shifts in rat circadian rhythms (Quigg et al, 2001). However, before concluding that a specific stimulus affects the circadian pacemaker, one must establish that its effects are evident for a entire group of animals. Moreover, these effects should resemble a phase response curve with that includes a dead zone, as well as zones with phase advances and delays (Johnson, 1992), which this has not thus far been encountered. Although it appears that the resulting PRC and tauRC have a coherent shape, these changes are not different from 0. Thus, we have concluded that the effects on the circadian pacemaker, if any, are insignificant. Slight alterations in overt rhythms, which are reflected by minor changes in phase or tau, are most likely due to day-to-day fluctuations in the effector system, rather than in the brain pacemaker. Our results are consistent with previous experiments in the sense that the rat circadian system appears little affected by non-photic stimuli (Canal-Corretger et al, 2003).

This lack of effect became even more apparent when we carried out the second experiment, in which an undisturbed free running rhythm remained unaffected by ECS treatment. In this case, daily ECS application modified the levels of motor activity although not the levels of temperature, and the overt rhythm of the two variables. Moreover, neither the period nor the phase of the free-running rhythms were affected. During the ECS administration, there was a noticeable loss of circadian rhythmicity, which was stronger in the initial days of the ECS stage. During this time, some of the rats were

arrhythmic. After a few days, however, the circadian rhythm recovered the same phase and period as before ECS administration, indicating no effect on the circadian clock.

In the third experiment, the most interesting result was the fact that ECS produced a strong disturbance in the circadian structure. Unlike Experiment 2, here not only motor activity but also temperature levels increased because of ECS, rendering the circadian pattern disorganized. This may stem the fact that ECS affects dissociated rats more than free-running rats, perhaps because of stress mechanisms. Since stress produces temperature increases (Meerlo et al, 1997), it could be argued that dissociated animals are more sensitive to stress. In fact, rhythm dissociations reportedly alter mood in humans (Wirz-Justice, 2003). It is worth noting that alterations in motor activity and temperature levels are due not only to reactivity to ECS, since the variables 4h-MA and 4h-TEMP do not differ between experimental and control groups, but also to the general levels of these two variables.

The SCN can be entrained by external stimuli other than light, such as serotonergic innervations from the raphe (Cutrera et al, 1994; Morin, 2007). ECT involves massive discharge encompassing large areas of the brain (Kety, 1974). Indeed, there are several pathways by which ECT can affect circadian rhythms. ECT induces transcriptional changes primarily in the locus coeruleus (Conti et al, 2007), whose main neurotransmitter is norepinephrine. The locus coeruleus is a well-known stress response modulator since it receives input from other neurotransmitter systems providing information about homeostasis (e.g. serotonin, GABA, corticotropine releasing factor, dopamine and Glutamate). Moreover, GABA is produced by most, if not all, the SCN neurons (Moore et al, 2002). It has been demonstrated that GABA concentrations in the occipital cortex of depressed patients are lower and that they increases following ECT treatment (Sanacora et al, 2006). Some, or all, of these pathways may be responsible for alterations in overt rhythms. The disturbance in the motor activity rhythm following ECS administration may stem from the effects of ECS on areas of the brain that control motor activity.

However, the results of the present experiment clearly show that the robust changes in overt temperature and activity rhythms are not mirrored by changes in pacemaker function; i.e., either period or phase are affected by ECS administration. This not only agrees with the fact that ECT does not greatly alter the hypothalamus area (Ji et al, 1998), but also suggests that cortex inputs do not significantly affect the circadian system. Therefore, changes in temperature and activity are apparently caused by peripheral alterations to the circadian timing system, which masks the output of the unaffected pacemaker. One must be careful when drawing conclusions about the state of this internal clock when

overt rhythms are measured, since changes in amplitude do not necessarily reflect alterations in the circadian pacemaker. While some experiments reveal changes stemming from stress or depression, evident in measurements of overt circadian rhythms (Meerlo et al, 1996; Ushijima et al, 2006), more careful analysis (Meerlo et el, 1997) indicates that these alterations occur only in the overt rhythms rather than in the pacemaker itself. Our experiments show similar results, and indicate that extensive recordings of circadian rhythms, before, during and after a manipulation represent the only reliable way to detect real changes in a pacemaker. In any case, this does not invalidate the hypothesis contending that a relationship between circadian rhythms and depression exists, or the fact that ECT re-establishes the amplitude of the circadian rhythms in depressed patients (Szuba et al, 1997). While ECS does not affect the entire circadian pacemaker, it does induce reversible alterations in the system output. Moreover, we believe that the antidepressant effects of ECT do not affect the circadian pacemaker. Instead, we content that, via effectors systems, it helps re-establish the altered overt circadian rhythms observed in depression to their previous norms. This suggests the importance of carrying out careful studies when addressing the functionality of the circadian system in animal models of depression.

AUTHOR DISCLOSURES

The authors do not have any conflicts to disclose.

CONTRIBUTORS

MAP, TC, VS and MU designed the study. MAP and TC wrote the protocol, carried out the experimental procedure, made the analysis and wrote the manuscript. VS, MU, JMM managed part of the literature searches and the discussion of the manuscript. ADN contributed to the experimental procedure and discussion. All authors contributed to and have approved the final manuscript.

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FIGURES

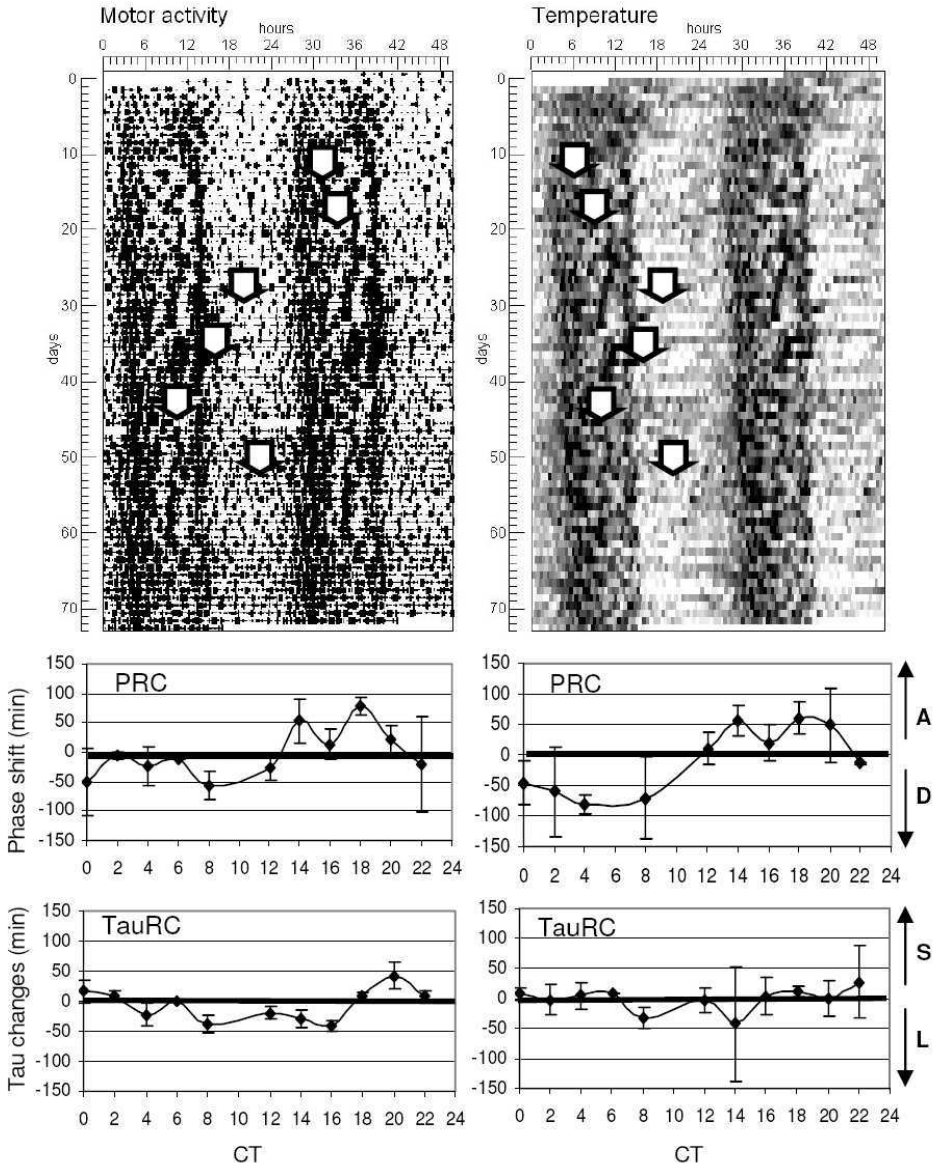

Figure 1. Top: Double-plot actograms of a representative rat from Experiment 1, plotted at "modulo" 24h 40min (each row represents one cycle), which corresponds to the rhythm value for that animal. The graphs on the left correspond to the motor activity variable, while the graphs on the right reflect temperature. Each mark coincides with an ECS application.

Bottom: Mean phase response curves (PRC) and tau response curves (tauRC) for the two variables analyzed: motor activity (left) and body temperature (right). Positive phase shifts indicate an advance (A) in PRC and shortening of the period (S) in tauRC, whereas phase delays (D) and lengthening of the period (L) are negative.

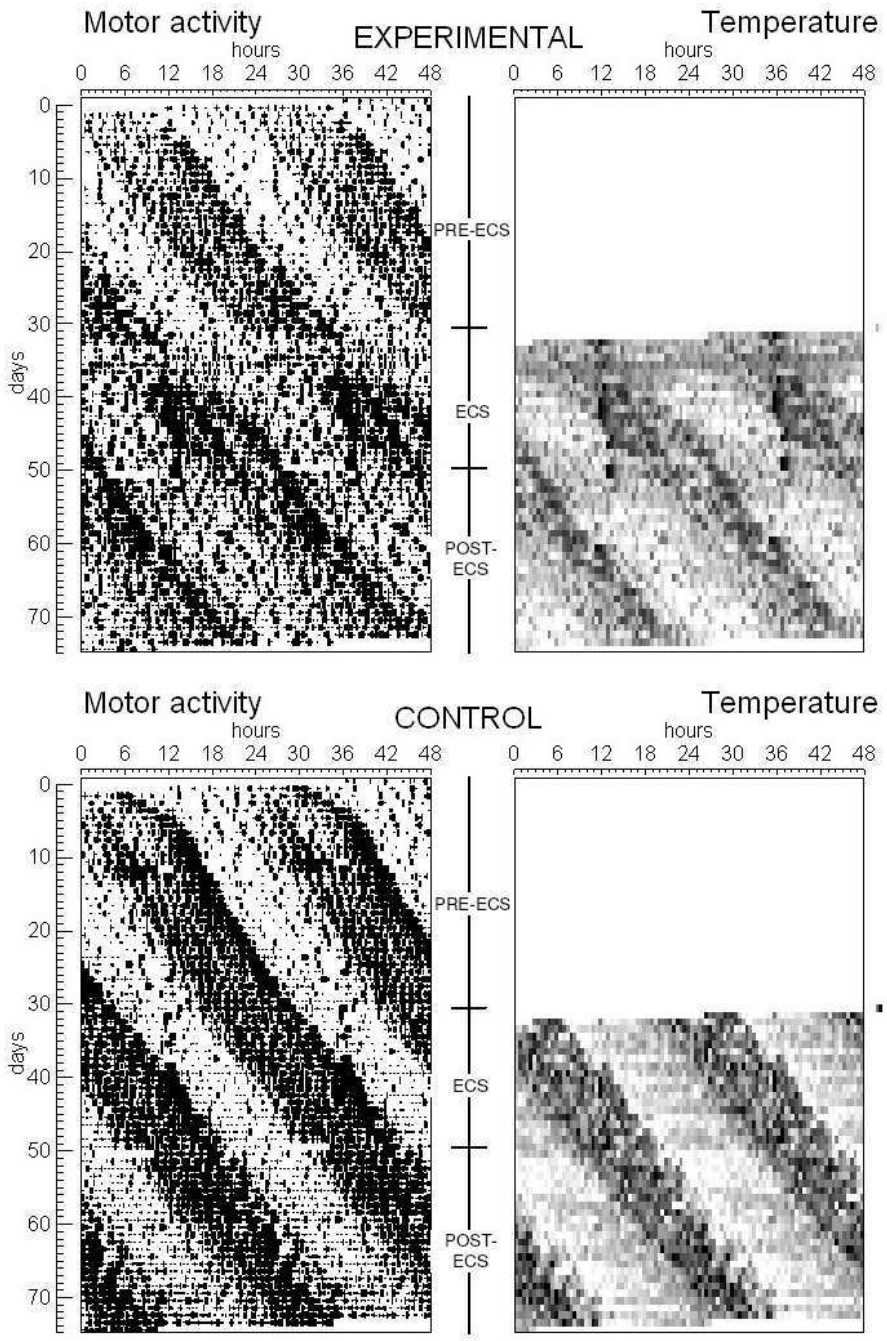

Figure 2. Double-plot showing the free-running activity and temperature patterns of a representative rat from each group (experimental and control) from Experiment 2, plotted at "modulo" 24h. ECS corresponds to the time when experimental rats were submitted to ECS administration and control rats were submitted to handling. Pre-ECS and post-ECS correspond to the previous and follow-on stage of ECS administration, respectively.

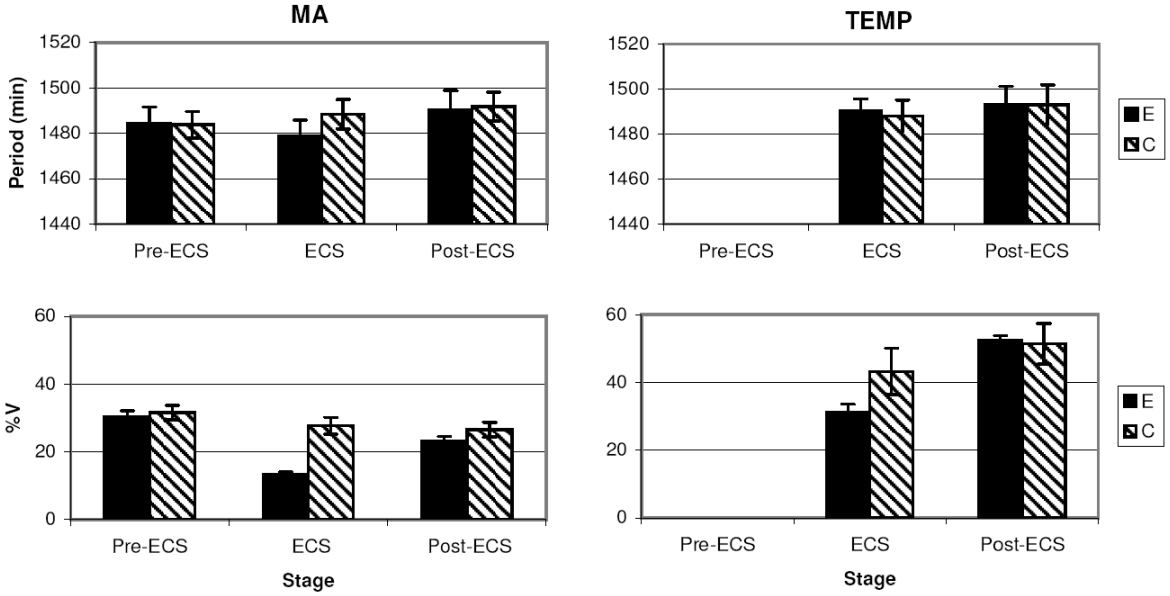

Figure 3. Mean values and standard errors for the free-running circadian periods and the percentage of variance explained by these for activity (MA) and temperature (TEMP) rhythms in the experimental (E) and control (C) animals from Experiment 2. The variables were calculated over 18-day intervals before (pre-ECS), during (ECS) and after (post-ECS) treatment.

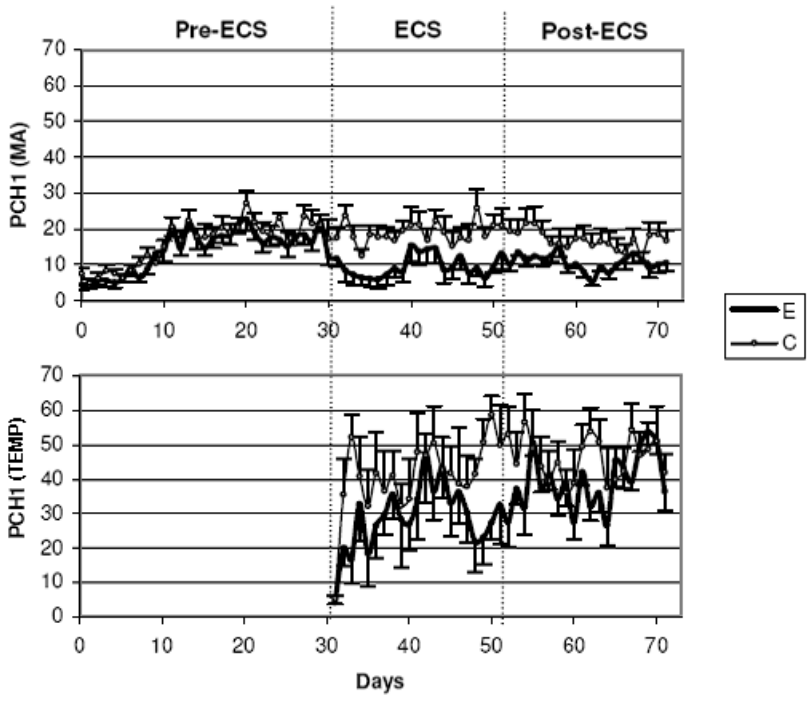

Figure 4. Evolution through all three stages (pre-ECS, ECS and post-ECS) of the power content of the first harmonic of the spectrum (PCH1) for both motor activity (MA) and temperature (TEMP) circadian rhythms in experimental (E) and control (C) groups of free-running animals (Experiment 2).

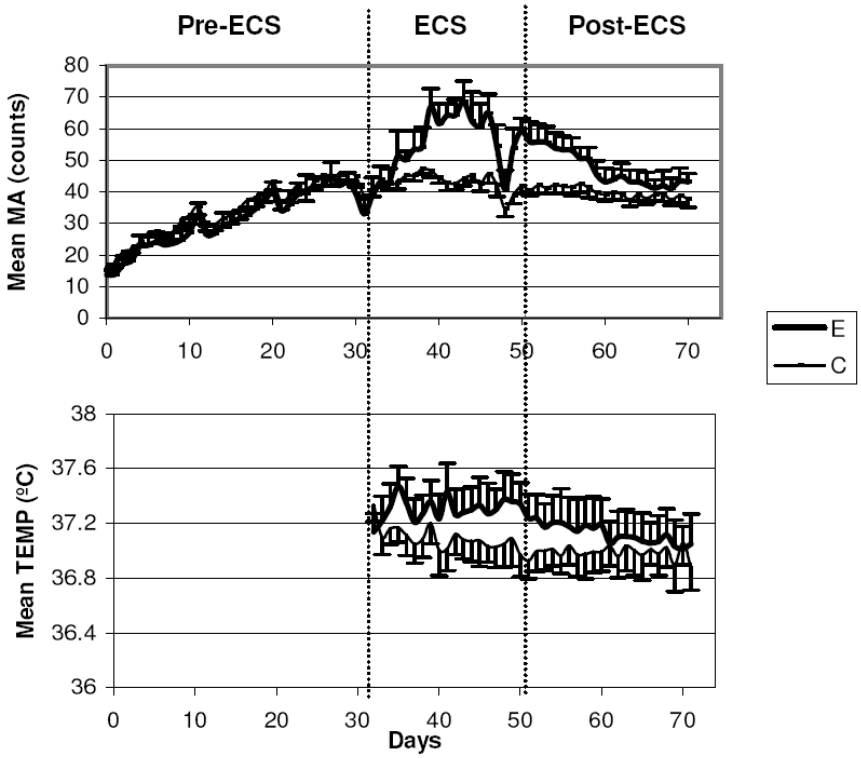

Figure 5. Mean values for motor activity (MA) and temperature (TEMP) circadian rhythms per cycle through all three stages (pre-ECS, ECS and post-ECS) for both experimental (E) and control (C) groups of free-running animals (Experiment 2).

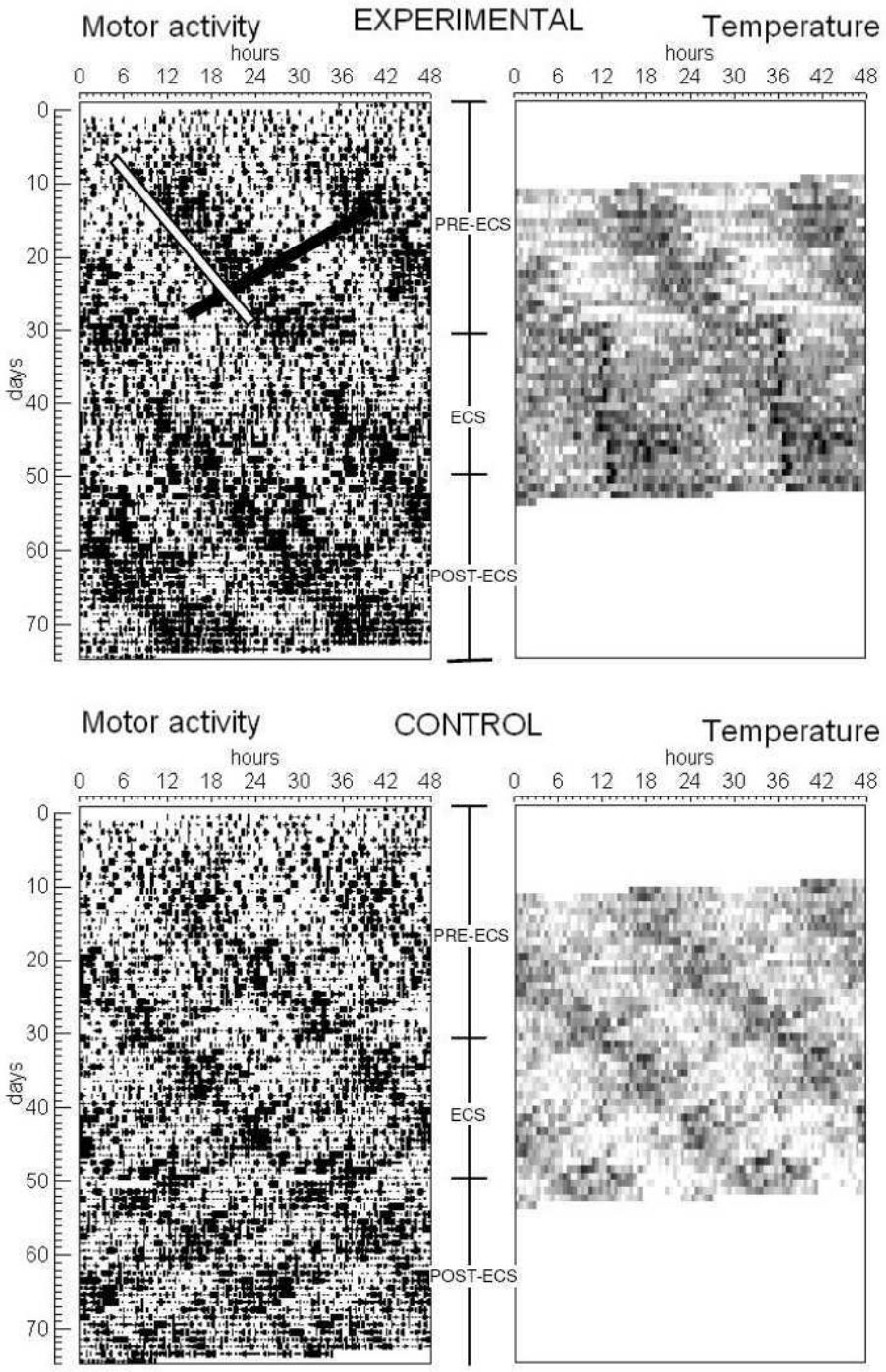

Figure 6. Double-plot showing the dissociation pattern of a representative rat from each group of Experiment 3, plotted at "modulo" 24 h. ECS corresponds to the time when experimental rats were submitted to ECS administration and control rats were submitted to handling. Pre-ECS and post-ECS correspond to the previous and follow-on stages of ECS administration, respectively. The black bar corresponds to the onset of the light-dependent component, the white bar to the onset of the non-light-dependent component.

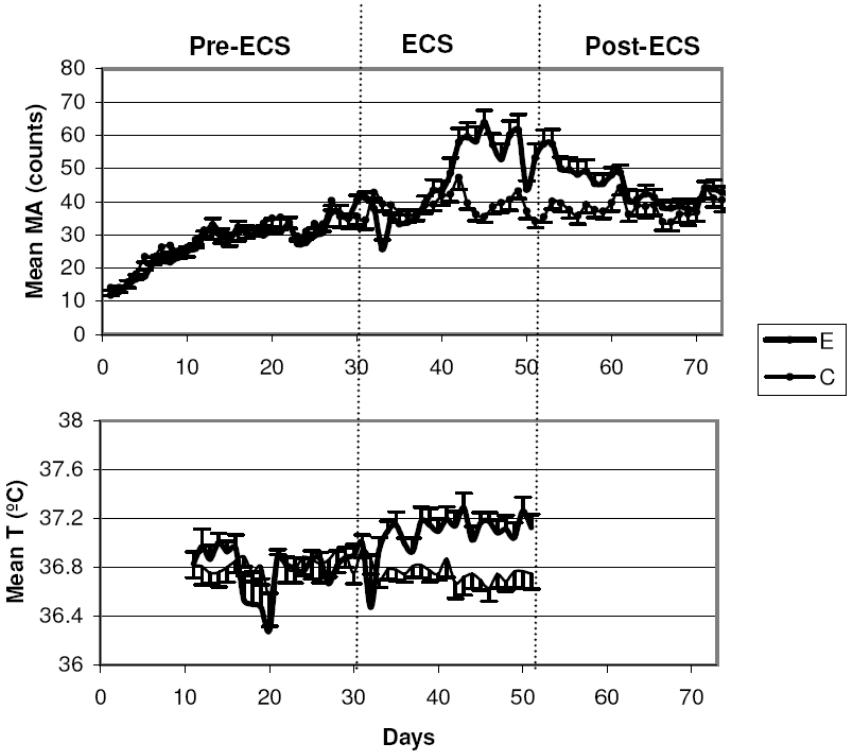

Figure 7. Mean values of motor activity (MA) and temperature (T) circadian rhythms per cycle through all three stages (pre-ECS, ECS and post-ECS) for both experimental (E) and control (C) groups of dissociated animals (Experiment 3).

REFERENCES

Altar, C.A., Laeng, P., Jurata, L.W., Brockman, J.A., Lemire, A., Bullard, J., Bukhman, Y.V., Young, T.A., Charles, V., Palfreyman, M.G. (2004) Electroconvulsive seizures regulate gene expression of distinct neurotrophic signaling pathways. J Neurosci. 24(11):2667-2677.

Campuzano, A., Vilaplana, J., Cambras, T., Díez-Noguera, A. (1998) Dissociation of the rat motor activity rhythm under T cycles shorter than 24 hours. Physiol. Behav. 63(2):171-176.

Canal-Corretger, M.M., Cambras, T., Díez-Noguera, A. (2003) Tau and phase response curves for non-photic stimuli in blinded rats. Biol. Rhythm. Res. 34(1):91-99.

Carlsson, A., Svenbnerhold, L., Winblad, B. (1980) Seasonal and circadian monoamine variations in human brains examined post mortem. Acta. Psychiatr. Scand. 61(280):75-85.

Conti, B., Maier, R., Barr, A.M., Morale, M.C., Lu, X., Sanna, P.P., Bilbe, G., Hoyer, D., Bartfai, T. (2007) Region-specific transcriptional changes following the three antidepressant treatments electro convulsive therapy, sleep deprivation and fluoxetine. Mol. Psychiatry. 12:167-189.

Cutrera, R.A., Kalsbeek, A., Pevet, P. (1994) Specific destruction of the serotoninergic afferents to the suprachiasmatic nuclei prevents triazolam-induced phase advances of hamster activity rhythms. Behav. Brain. Res. 62(1):21-28.

Czeisler, C.A., Duffy, J.F., Shanahan, T.L., Brown, E.N., Mitchell, J.F., Rimmer, D.W., Ronda, J.M., Silva, E.J., Allan, J.S., Emens, J.S., Dijk, D.J., Kronauer, R.E. (1999) Stability, precision and near-24-hour period of the human circadian pacemaker. Science. 284:2177-2181.

Dremenkov, E., Gur, E., Lerer, B., Newman, M.E. (2003) Effects of chronica antidepressants and electroconvulsive shock on serotonergic neurotransmission in the rat hippocampus. Prog. Neuro-Psychopharmacol. Biol. Psychiatry. 27:729-739.

Dunlap, J.C., Loros, J.J., DeCoursey, P.J. (2004) The relevance of circadian rhythms for human welfare. In: Chronobiology. Biological timekeeping. 325-356.

Duval, F., Lebowitz, B.D., Macher, J.P. (2006) Pharmacological aspects. Treatments in depression. In: Macher, J.P., Crocq, M.A.. Dialogues. Clin. Neurosci.: Depression. 8:191-206.

Eitan, R., Lerer, B. (2006) Nonpharmacological, somatic treatments of depression: electroconvulsive therapy and novel brain stimulation modalities. Dialogues. Clin. Neurosci. Depression. 241- 253.

Ford, D.E., Kamerow, D.B. (1989) Epidemiologic study of sleep disturbances and psychiatric disorders. An opportunity for prevention? J.A.M.A. 262(11):1479-1484.

Green, A.R., Nutt, D.J. (1987) Psychopharmacology of repeated seizures: possible relevance to the mechanisms of action of ECT. In: Iversen, L.L., Snyder, S.H., editors. Handbook of Psychopharmacology. New York, Plenum Press. 375-419.

Guardiola-Lemaitre, B. (2005) Agonistes et antagonistes des récepteurs mélatoninergiques: effets pharmacologiques et perspectives thérapeutiques. Ann. Pharm. Fr. 63:385-400.

Hattar, S., Lucas, R.J., Mrosovsky, N., Thompson, S., Douglas, R.H., Hankins, M.W., Lem, J., Biel, M., Hofmann, F., Foster, R.G. and Yau, K.W. (2003) Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. Nature. 3,424(6944):76-81.

Ji, R.R., Schlaepfer, T.E., Aizenman, C.D., Epstein, C.M., Qiu, D., Huang, J.C., Rupp, F. (1998) Repetitive transcranial magnetic stimulation activates specific regions in rat brain. Proc.. Natl. Acad. Sci. USA. 95:15635-15640.

Johnson, C.H. (1992) Phase response curves: what can they tell us about circadian clocks? In: Hiroshige, T., Honma, K., editors. Circadian clocks from cell to human. Sapporo, Hokkaido, Univ Press. 209-219.

Kennaway, D.J. (1997) Light, neurotransmitters and the suprachiasmatic nucleus control of pineal melatonin production in the rat. Biol. Signals. Recept. 6:247-254.

Kety, S. (1974) Effects of repeated electroconvulsive shock on brain catecholamines. In: Fink, M., Kety, S., McGaugh, J.W.T.A., editors. Psychobiology of convulsive therapy. Washington, DC. Winston and sons.

Lambert, G.W., Reid, C., Kaye, D.M., Jennings, G.L., Esler, M.D. (2002) Effect of sunlight and season on serotonin turnover in the brain. Lancet. 360: 1840-1842.

Markianos, M., Hatzimanolis, J., Lykouras, L. (2002) Relationship between prolactin responses to ECT and dopaminergic and serotonergic responsivity in depressed patients. Eur. Arch. Psychiatry. Clin. Neurosci. 252:166-171.

Mc Donald, W.M., Vaughn McCall, W., Epstein, C.M. (2000) Electroconvulsive therapy: sixty years of progress and a comparison with transcranial magnetic stimulation and vagal nerve stimulation. Neuropsychopharmacology: the fifth generation of progress. 1097-1108.

Meerlo, P., de Boer, S.F., Koolhaas, J.M., Daan, S., Van den Hoofdakker, R.H. (1996) Changes in daily rhythms of body temperature and activity after a single social defeat in rats. Physiol. Behav. 59(4/5):735-739.

Meerlo, P., van den Hoofdakker, R.H., Koolhaas, J.M., Daan, S. (1997) Stress-induced changes in circadian rhythms of body temperature and activity in rats are not caused by pacemaker changes. J. Biol. Rhythms. 12(1): 80-92.

Moore, R.Y., Speh, J.C. (2004) Serotonin innervation of the primate suprachiasmatic nucleus. Brain. Res. 1010:169-173.

Moore, R.Y., Speh, J.C., Leak, R.K. (2002) Suprachiasmatic nucleus organization. Cell. Tissue. Res. 309(1):89-98.

Morin, L.P. (2007) SCN organization reconsidered. J. Biol. Rhythms. 22(1):3-13.

Newman, M.E., Shapira, B., Lerer, B. (1998) Evaluation of central serotonergic function in affective and related disorders by the fenfluramine challenge test: a critical review. Int. J. Neuropsychopharmacol. Biol. Psychiatry. 27:729-739.

Quigg, M., Straume, M., Smith, T., Menaker, M., Bertram, E.H. (2001) Seizures induce phase shifts of rat circadian rhythms. Brain. Res. 913:165-169.

Rubin, R.T., Heist, E.K., McGeoy, S.S., Hanada, K., Lesser, I.M. (1992) Neuroendocrine aspects of primary endogenous depression. XI. Serum melatonin measures in patients and matched control subjects. Arch. Gen. Psychiatry. 49(7):558-567.

Sanacora, G., Fenton, L.R., Fasula, M.K., Rothman, D.L., Levin, Y., Krystal, J.H., Mason, G.F. (2006) Cortical gamma-aminobutyric acid concentration in depressed patients receiving cognitive behavioral therapy. Biol. Psychiatry. 59(3):284-286.

Sokolove, P.G., Bushell, W.N. (1978) The chi square periodogram: its utility for analysis of circadian rhythms. J. Theor. Biol. 8:72(1):131-160.

Swartz, C.M. (1997) Related neuroendocrine effects of electroconvulsive therapy (ECT). Psychopharmacol. Bull. 33: 265-271.

Szuba, M.P., Guze, B.H., Baxter, L.R. Jr. (1997) Electroconvulsive therapy increases circadian amplitude and lowers core body temperature in depressed subjects. Biol. Psychiatry. 15;42(12):1130-1137.

Ushijima, K., Morikawa, T., To, H., Higuchi, S., Ohdo, S. (2006) Chronobiological disturbances with hyperthermia and hypercortisolism induced by chronic mild stress in rats. Behav. Brain. Res. 173:326-330.

Wever, R.A. (1979) The circadian system of man. Results of experiments under temporal isolation. Springer-Verlag, New York.

Wirz-Justice, A. (2000) Biological rhythms in mood disorders. Neuropsychopharmacology: The fifth generation of progress.

Wirz-Justice, A. (2003) Chronobiology and mood disorders. Dialogues. Clin. Neurosci. 5(4):315-323.

Wirz-Justice, A. (2006) Biological rhythm disturbances in mood disorders. Int. Clin. Psychopharmacol. 21(1):11-15.

Wirz-Justice, A., Richter, R. (1979) Seasonality in biochemical determinations: a source of variance and a clue to the temporal incidence of affective illness. Psychiatr. Res. 1:53-60.