EFECTE DE LES CONDICIONS AMBIENTALS EN EL DESENVOLUPAMENT DELS RITMES CIRCADIARIS

Maria Mercè Canal i Corretger
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Departament de Fisiologia-Divisió IV
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Departament de Fisiologia-Divisió IV

Facultat de Farmàcia
Av. Joan XXIII, s/n
08028 Barcelona
Tel. 93 402 45 05
Fax 93 403 59 01

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Directors:
DR. ANTONI DÍEZ NOGUERA
DR. JORDI VILAPLANA HORTENSI

Tutor:
DR. ALBERT FERRER PRATS

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Tel. 93 402 45 05
Fax 93 403 59 01

N'ANTONI DÍEZ i NOGUERA, Professor Titular de Fisiologia i EN JORDI VILAPLANA i HORTENSI, Professor Titular Interí de Fisiologia, ambdós del Departament de Fisiologia-Divisió IV de la Universitat de Barcelona,

INFORMEN:

Que la memòria titulada "Efecte de les condicions ambientals en el desenvolupament dels ritmes circadiaris" presentada per NA MARIA MERCÈ CANAL i CORRETGER per optar al títol de Doctor en Farmàcia, ha estat realitzada sota la nostra direcció al Departament de Fisiologia-Divisió IV i, considerant-la conclosa, n'autoritzen la seva presentació per ser jutjada pel tribunal corresponent.

I per tal que així consti, signem la present a Barcelona, el dia 26 de juliol de 2001.

Dr. Antoni Díez i Noguera

Dr. Jordi Vilaplana i Hortensi

Aquesta tesi ha estat subvencionada pels ajuts PB94-0927 de la *Dirección General de Investigación Científica y Técnica* del *Ministerio de Educación y Ciencia* i PM98-0186 de la *Dirección General de Enseñanza Superior e Investigación Científica* del *Ministerio de Educación y Cultura*. Durant la seva realització, l'autora ha gaudit d'una "Beca de formació en la recerca i la docència" de la Universitat de Barcelona, d'una "Beca per a estades curtes d'investigació a la República Federal Alemanya per a joves investigadors" del Servei d'Intercanvi Acadèmic del Govern Alemany i d'un "Ajut per a estades per a la recerca a fora de Catalunya" del Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya.

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A la meva família

“Des del principi hi hagué tambors que marcaven el ritme del món: el sonor onatge incessant a la platja; les quatre estacions, una rere l'altra; quan arriben les aus, quan se'n van, l'ós que passa hivernant el seu son hivernal. Insondable el perquè, però tot en el moment exacte. Observa el batec del cor en el teu canell, el redoblament precís del tambor de la vida; si perd el ritme, emmalalteixes.”

Jimalee Burton (cap cherokee)

GLOSSARI

τRC	*Tau Response Curve*
AM	Activitat Motora
BP	*Blood Pressure*
CRτ	Corba de Respostes de Tau
CRF	Corba de Respostes de Fase
DBP	*Diastolic Blood Pressure*
DD	*Constant Darkness*
FC	Freqüència Cardíaca
FIG	Feix Intergeniculat
GABA	*Gamma Amino Butiric Acid*
GHT	*Geniculohypothalamic tract*
GLN	*Geniculolateral Nucleus*
HR	*Heart Rate*
IGL	*Intergeniculate Leaflet*
LD	*Light-Dark cycles*
LEC	*Light Entrained Component*
LL	*Constant light*
MA	*Motor Activity*
mRNA	*messenger Ribonucleic Acid*
NLEC	*Non-light Entrained Component*
NPY	*Neuropeptide Y*
NSQ	Nuclis supraquiasmàtics
PA	Pressió Arterial
PRC	*Phase Response Curve*
RHT	*Retinohypothalamic Tract*
SBP	*Systolic Blood Pressure*
SCN	*Suprachiasmatic Nuclei*
SD	*Sprague-Dawley rats*
TGH	Tracte Geníiculohipotalàmic
TGR	*Transgenic hypertensive TGR(mRENs)27 rats*
TRH	Tracte retinohipotalàmic
VIP	*Vasointestinal Peptide*
VP	*Vasopressin*

INTRODUCCIÓ

1.- INTRODUCCIÓ

1.1.- ELS RITMES BIOLÒGICS

La Terra, per les voltes que dóna sobre el seu eix aproximadament cada 24 hores, sotmet a plantes i animals a uns ritmes diaris de llum i temperatura. La facilitat de trobar menjar i l'activitat dels depredadors es veuran, doncs, afectats per aquestes variacions periòdiques. La presència de ritmes de 24 hores en els éssers vius, però, no és deguda a un seguiment passiu de les condicions ambientals, sinó que l'origen és endogen. Així doncs, s'ha observat que quan un organisme s'aïlla de tota referència externa, la majoria de ritmes persisteixen.

Existeix una àmplia varietat de ritmes biològics, amb ritmes de període de menys d'un segon (per exemple l'electroencefalograma) fins a un any (com ara la hivernació). Els ritmes de període inferior a les 20 hores s'anomenen ultradiaris; els de període entre 20 i 28 hores circadiaris i finalment, els ritmes de període superior a les 28 hores s'anomenen infradiaris.

1.2.- FISIOLOGIA DEL SISTEMA CIRCADIARI

El sistema fisiològic responsable de mesurar el temps i de sincronitzar els processos interns dels organismes respecte dels esdeveniments diaris que se succeeixen en l'entorn, s'anomena sistema circadiari. A grans trets, el sistema circadiari dels mamífers està format per (Vegeu Fig.1):

- Rellotge intern o *pacemaker*: proporciona un marc temporal dins el qual els processos fisiològics i de comportament poden ser preparats per i relativament restringits a diferents hores del dia quan les condicions, tant internes com externes, estan en el seu punt òptim. El principal *pacemaker* dels mamífers són els nuclis supraquiasmàtics de l'hipotàlem.
- Vies aferents: contenen receptors que detecten canvis periòdics en l'entorn i transformen aquesta informació temporal en senyals que tenen un significat pels elements integrants del rellotge biològic. Les vies aferents, doncs, portaran aquests senyals fins al rellotge. Aquestes vies inclouen el tracte retinohipotalàmic i el tracte geniculohipotalàmic, entre d'altres.
- Vies eferents: transmeten la informació temporal procedent del rellotge cap a diversos sistemes efectors de l'organisme, com a conseqüència d'això es generaran

uns ritmes endògens, alguns dels quals podran ser observats des de l'exterior (ritme de son-vigília, cicle menstrual, etc.).

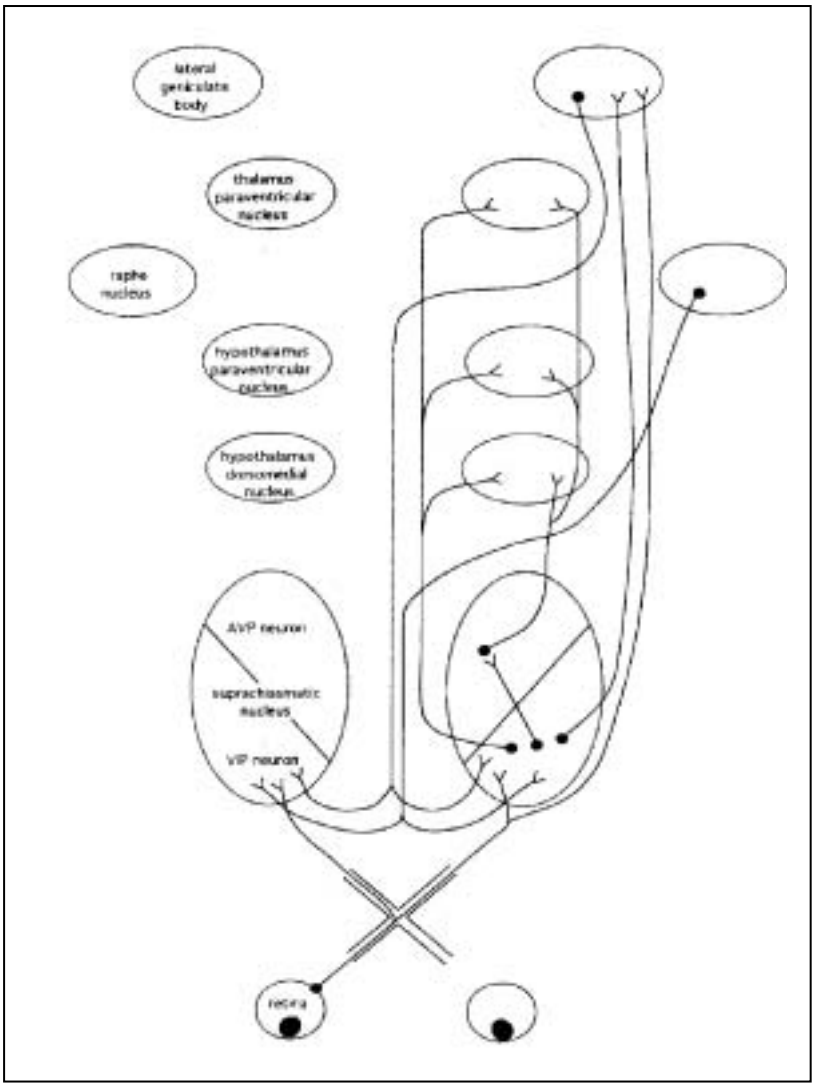

Fig.1.- Esquema de les aferències i eferències dels nuclis supraquiasmàtics.

1.2.1.- Rellotge intern: nucli supraquiasmàtic

El nucli supraquiasmàtic (NSQ) està format per dos nuclis simètrics i ovalats situats a la part inferior de l'hipotàlem, al costat de les parets inferiors del tercer ventricle i dorsalment al

quiasma òptic. A la rata els nuclis tenen una mida de 950x425x400 μm i contenen al voltant de 16.000 neurones molt petites (8-12 μm de diàmetre) que estan més agrupades que en d'altres zones del cervell (Van den Pol 1980). Els nuclis també contenen cèl·lules glials, que en la rata i en el hàmster estan en una proporció de 1 cada 3 neurones, aproximadament. Els dos nuclis estan interconnectats a través de molts circuits locals i funcionen com una sola estructura. Atenent a criteris anatòmics i neuroquímics, es poden diferenciar dues zones en el NSQ: una petita àrea rostral i una gran àrea caudal, que alhora conté una zona dorsomedial (amb neurones petites i que principalment contenen vasopressina) i una zona ventrolateral (amb neurones més grans i que majoritàriament contenen polipèptid actiu vasointestinal (VIP)). Els cossos neuronals acostumen a estar separats per cèl·lules glials, que envolten completament les unions sinàptiques en la zona ventrolateral (Güldner i Wolff 1996) i que es comuniquen entre sí mitjançant *gap juntions* (Van den Pol 1980, Welsh 1996). Els astròcits poden regular els nivells extracel·lulars de ions, especialment el potassi i el calci, a través de la seva resposta als neurotransmissors (Bowman i Kimelberg 1984, Usowicz et al. 1989). Les cèl·lules glials també poden secretar substàncies, com ara òxid nítric o àcid hialurònic, que influencien les neurones (Ding et al. 1994, Barbour et al. 1989, Van den Pol et al. 1992). La importància que tenen els astròcits en el funcionament del NSQ es reforça amb la troballa que les interrupcions funcionals de les *gap junctions* o bé del metabolisme glial són capaces d'interrompre la ritmicitat circadiària del NSQ (Prosser et al. 1994).

El paper del NSQ en els ritmes circadiaris ha estat àmpliament estudiat. Després d'estudis inicials en que es demostrava que el tracte retinohipotalàmic (TRH) acabava en el NSQ (Moore i Lenn 1972, Hendrickson et al. 1972) i dels estudis que demostraven que l'ablació del NSQ resultava en la pèrdua de la funció circadiària (Moore i Eichler 1972, Stephan i Zucker 1972), es va concloure que el NSQ és el rellotge biològic principal. Experiments més recents han corroborat aquesta afirmació: la demostració que el funcionament circadiari del NSQ es manté fins i tot quan aquest està aïllat, tant *in vivo* (Inouye i Kawamura 1979) com *in vitro* (Shibata i Moore 1988b, Meijer i Rietveld 1989) i també la descoberta que el transplantament de l'hipotàlem anterior fetal que conté el NSQ, en el tercer ventricle d'animals arrítmics amb el NSQ lesionat, restaurava la ritmicitat (Lehman et al. 1987) i que el nou ritme tenia el període del donant (Ralph et al. 1990). Per consegüent, tots aquests estudis indiquen que el NSQ està involucrat en la generació dels ritmes circadiaris i que, fins i tot quan manquen els estímuls ambientals externs, o bé en absència d'entrades d'altres parts del sistema nerviós central, les neurones del NSQ són capaces de mantenir un ritme circadiari. Malgrat que el NSQ és el rellotge principal dels mamífers, la capacitat de generar ritmes circadiaris no els està restringida, sinó que d'altres cèl·lules d'una gran varietat d'òrgans i organismes mostren ritmes circadiaris, com per exemple el *pacemaker* intrínsec de la retina de mamífers (Reme et al. 1991), neurones aïllades de la retina de *Aplysia* (Jacklet i Geronimo 1971), cèl·lules de la pineal d'ocells

(Takahashi i Menaker 1984), les algues unicel·lulars *Gonyaulax* i *Euglena* (Pittendrigh 1974) i plantes (de Mairan 1729).

Una de les funcions del NSQ és la relacionada amb l'encarrilament a estímuls ambientals. Com ja s'ha vist anteriorment, la informació sensorial provinent de la retina que pot influenciar l'encarrilament dels ritmes circadiaris, pot anar a parar directament en el NSQ a través del TRH, o indirectament a través del feix intergeniculat (FIG). Però el que és fonamental pels ritmes biològics en mamífers no és només la capacitat del NSQ de generar un ritme circadiari, sinó que també ho són les connexions neuronals que permeten que les cèl·lules del NSQ es comuniquin entre elles per tal de compartir la informació temporal i també, la capacitat de les neurones del NSQ d'influenciar les neurones de d'altres parts del cervell a través de les vies eferents del NSQ.

1.2.1.1.- Model multioscil·latori d'organització del rellotge biològic

Si hom mira els diversos ritmes circadiaris presents dins un individu, resultarà aparent que hi ha un ordre temporal altament específic en el sistema circadiari. Aquests ritmes ens deixen entreveure l'elaborat mecanisme que controla el temps en els processos fisiològics. La curiositat de saber l'estructura interna, o com a mínim els principis generals que regeixen l'organització del sistema circadiari, juntament amb els nous avanços tècnics han portat a molts investigadors a l'estudi del sistema circadiari a diversos nivells: molecular, cel·lular, d'òrgans i d'organismes, tant unicel·lulars com pluricel·lulars. La major part de resultats de tota aquesta recerca semblen indicar que fins i tot en organismes inferiors, la millor manera de descriure el sistema circadiari és com a contenidor de diversos oscil·ladors (Pittendrigh 1960, Rosenwasser i Adler 1986, Díez-Noguera 1994, Miller 1998, Honma et al. 1998). La presència de múltiples oscil·ladors sembla ben palesa quan s'observen casos d'*splitting* o de dissociació dels ritmes, casos on es poden presentar dos o més components de períodes diferents i amb una relació de fases inestable.

Tot i que la naturalesa d'aquests oscil·ladors és desconeguda, tot sembla indicar que es tractaria de neurones, ja que s'ha observat que quan neurones del NSQ són cultivades individualment, són capaces de generar descàrregues espontànies (Bos i Mirmiran 1990, Liu et al. 1997, Herzog et al. 1998, Honma et al. 1998) i d'expressar ritmes circadiaris de descàrrega amb fases independents (Welsh et al. 1995). Alhora, s'ha observat que la comunicació sinàptica i d'altres interaccions complexes són capaces de sincronitzar els ritmes circadiaris de neurones individuals del NSQ (Shirakawa et al. 2000) i per tant, són capaces de generar el ritme del rellotge circadiari (Mirmiran et al. 1995).

El model d'organització del sistema circadiari proposat pel nostre grup ("model de Barcelona") es fonamenta en el concepte d'intercomunicació en una població de múltiples oscil·ladors autònoms (Díez-Noguera 1994). La majoria de factors ambientals, incloent l'encarrilament dels ritmes, pot ser simulat en el model simplement controlant el grau d'intercomunicació entre els elements que el formen. A més, el model introdueix el concepte d'elements neutres (que possiblement serien les cèl·lules glials en sistemes reals) que escurçarien o allargarien el període en resposta a canvis en la intensitat d'il·luminació. S'ha proposat que un possible paper que tenen els astròcits (un tipus de cèl·lules glials) és la sincronització de les neurones del NSQ. El model de Barcelona permet també explicar fàcilment la generació de ritmes ultradiaris, la dissociació de ritmes circadiaris i l'*splitting*.

1.2.2.- Vies aferents

1.2.2.1.- Tracte retinohipotalàmic

El tracte retinohipotalàmic (TRH) és una projecció que parteix de la retina, és bilateral i acaba majoritàriament a la regió ventrolateral dels nuclis supraquiasmàtics (NSQ) (Hendrickson et al. 1972, Moore i Lenn 1972), tot i que addicionalment també té terminacions a l'àrea lateral hipotalàmica, a l'àrea anterior hipotalàmica i a l'àrea retroquiasmàtica (Johnson et al. 1988a). La variabilitat més gran entre espècies s'ha trobat en la simetria d'aquesta projecció (Moore 1973). Per exemple en les rates, el TRH és asimètric i aproximadament un 60% de les fibres provinents de cada ull travessa el quiasma òptic, mentre que en el hàmster la projecció és totalment simètrica. Sembla que diferències en la simetria estarien reflectint diferències en l'organització funcional del TRH (Stephan et al. 1982). Algunes cèl·lules retinals ganglionars que projecten cap al NSQ també ho fan cap al feix intergeniculat (Pickard 1985) i a més, sembla ser que els fotoreceptors que mitjancen l'encarrilament són diferents dels fotoreceptors clàssics de visió. Així doncs, s'ha observat per exemple que ratolins mutants homozigòtics *rd/rd*, en els que no es detecten bastons i que tenen molt pocs cons (Foster et al. 1993), són capaços d'encarrilar i de canviar la fase dels seus ritmes circadiaris (Ebihara i Tsuji 1980, Foster et al. 1991, Provencio et al. 1994), prefereixen la zona fosca d'una capsa de dos compartiments (Mrosovsky i Hampton 1997) i la seva producció de melatonina pot ser suprimida per la llum (Goto i Ebihara 1990, Lucas et al. 1999). Així doncs sembla que existeixen uns fotoreceptors a la retina diferents dels cons i dels bastons. Tot i que la naturalesa d'aquests fotoreceptors és encara desconeguda, recentment s'ha proposat en mamífers que aquests podrien contenir els criptocroms CRY1 i CRY2 com a fotopigments (Lucas et al. 1999). Aquests "nous" fotoreceptors formarien la base

d'una via a través de la qual es portaria informació general que no serviria per a la visió, sinó que estaria relacionada amb les respostes no visuals a la llum.

En treballs primerencs, es va observar que si se seccionaven totes les vies visuals i només es deixava el quiasma òptic, l'animal patia una pèrdua dels reflexes visuals i del comportament guiat visualment, però en canvi no es perdia la capacitat d'encarrilar visualment la funció circadiària (Klein i Moore 1979). Així doncs, aquests resultats indicaven que hi ha una separació de funcions en les vies fòtiques, de manera que una part fan de mitjanceres de la informació visual i una altra part s'encarreguen de l'encarrilament. El TRH és necessari i suficient per mitjançar l'encarrilament fòtic, ja que, com s'ha dit anteriorment, la seva projecció majoritària és cap al NSQ, la secció de les vies visuals més enllà del TRH no afecta l'encarrilament (Klein i Moore 1979) i per contra, la seva secció l'aboleix (Johnson et al. 1988b).

S'han descrit ritmes circadiaris en diferents nivells de la via d'entrada fòtica al NSQ. Els segments externs dels fotoreceptors retinals, per exemple, pateixen un desprendiment periòdic de la seva membrana fins i tot en condicions de foscor constant (Goldman et al. 1980, LaVail 1976). També s'ha detectat ritme circadiari de generació pròpia a la retina, demostrada per l'alliberament circadiari de melatonina de cultius de neurones retinals (Tosini i Menaker 1996). Aquest ritme és independent del ritme generat pel NSQ i podria estar modulant l'encarrilament del NSQ a la llum mitjançant la regulació de l'*input* retinal.

Una entrada serotoninèrgica també s'ha vist que influencia l'*input* fòtic cap al NSQ mitjançant receptors de serotonina situats en els àxons terminals del TRH a la retina (Pickard et al. 1996). Aquests processos podrien ser la base dels canvis de fase produïts per la llum.

1.2.2.2.- Feix intergeniculat-tracte geníulohipotalàmic

El feix intergeniculat (FIG) és anatòmicament i funcional una subdivisió diferent del complex geniculat lateral que rep innervació bilateral de la retina (Moore i Card 1994). El FIG es caracteritza per una població de neurones que contenen el neuropèptid Y (NPY) i que projecten, a través del tracte geníulohipotalàmic (TGH), cap al NSQ amb un patró que solapa l'entrada retinal i també consta d'una població de neurones que contenen encefalina que projecten cap al FIG contralateral (Card i Moore 1989). Totes les neurones del FIG també contenen àcid gamma-aminobutíric (GABA) (Moore i Speh 1993, Moore i Card 1994).

Actualment sembla establert que la presència del TGH és una característica comuna de la majoria de mamífers (Card i Moore 1984, Cassone et al. 1988, Harrington et al. 1985, Moore 1989) i que aquest tracte participa en l'encarrilament dels ritmes circadiaris de comportament (Albers i Ferris 1984, Albers et al. 1984, Harrington i Rusak 1988, Harrington i Rusak 1986, Pickard et al. 1987, Rusak et al. 1989). Així per exemple s'ha observat que estímuls que

indueixen activitat motora, produeixen canvis en la fase del ritme en curs lliure d'activitat motora, amb una corba de respostes de fase similar a la produïda per estimulació del TGH (Moore 1992a, Mrosovsky 1995). Aquest efecte sembla estar mitjançat pel FIG-TGH (Johnson 1989). Així doncs, aquestes observacions indiquen que el sistema FIG-TGH serveix per integrar informació fòtica i no fòtica per tal de modular la funció del rellotge biològic (Moore 1992b, Moore i Card 1994).

El solapament, en el NSQ, de les àrees de projecció del TRH i del TGH (Stopa et al. 1995) suggereix que el FIG està involucrat en la modulació de les respostes del sistema circadiari a la llum. Important és en aquest aspecte la troballa que el FIG i el NSQ estan innervats pel mateix grup de neurones ganglionars de la retina (Pickard 1985, Treep et al. 1995) i que les neurones retinorecipients del NSQ responen més a l'aplicació de neuropèptid Y (principal neurotransmissor del TRH) que les altres neurones (Shibata i Moore 1988c).

1.2.2.3.- Altres vies aferents

El NSQ rep entrades de diverses àrees addicionals, totes les quals probablement estan relacionades amb la modulació del funcionament del rellotge. Una de les entrades més importants prové de les neurones serotoninèrgiques provinents del nucli del rafe. Manipulacions en el sistema serotoninèrgic del cervell, per exemple per depleció química o per administració d'un antagonista, afecta la fase i el període dels ritmes circadiaris d'activitat motora de rates i hàmsters, tant si estan sotmesos a cicles de llum-foscor, com si estan sota condicions constants (Cutrera et al. 1994, Honma et al. 1979, Morin i Blanchard 1991), a més de modular els canvis de fase induïts per la llum (Bradbury et al. 1997, Glass et al. 1995, Pickard i Rea 1997b, Pickard et al. 1996, Weber et al. 1998). Així doncs, la modulació serotoninèrgica de la neurotransmissió del TRH s'aconsegueix via receptors postsinàptics de serotonina en les neurones del NSQ i també, via receptors presinàptics de serotonina en els àxons terminals provinents de la retina que es troben en el NSQ (Pickard i Rea 1997b, Pickard i Rea 1997a). Sembla ser, doncs, que la funció de la innervació de les neurones serotoninèrgiques del rafe cap al NSQ seria la de proporcionar una modulació retroalimentativa del funcionament del rellotge, similar a la produïda per la projecció FIG-TGH.

1.2.3.- Vies eferents

Demostrar la naturalesa de les unions entre el rellotge circadiari i el gran nombre de ritmes circadiaris fisiològics i de comportament que existeixen en l'organisme ha resultat ser una

tasca bastant difícil, especialment degut a la mida petita del NSQ i a la seva posició. Els dos llocs principals de projeccions originades en el NSQ són el mateix hipotàlem i diverses regions fora de l'hipotàlem (Watts 1991). Dins les eferències al propi hipotàlem s'inclouen: eferències al nucli paraventricular (relacionat amb els ritmes de les funcions hormonals i autonòmiques), eferències a l'àrea preòptica (relacionades amb la regulació de la temperatura, el balanç de fluids i la conducta sexual) i les eferències a l'àrea retroquiasmàtica (relacionades amb la regulació de l'estat conductual i la integració neocortical, la regulació autonòmica i el control sensorimotor). Pel que fa a les eferències fora de l'hipotàlem cal destacar les eferències al nucli paraventricular del tàlem (que intervé en la regulació de la locomoció), les eferències al sistema límbic (que intervé en la regulació de la memòria i del to afectiu) i les eferències al nucli geniculat lateral.

Estudis funcionals han confirmat en certa manera el paper de la vasopressina (VP) com a senyal del ritme circadiari per determinades àrees del NSQ a través de les seves projeccions eferents, però hi ha relativament poca evidència que el pèptid vasointestinal (VIP) tingui un paper semblant. Tot i això, com que hi ha diversos neurotransmissors que es localitzen en les mateixes neurones del NSQ que contenen VP i VIP, podria ser que l'alliberament d'un determinat còctel de transmissors fos més important en el paper de senyalització que tenen les vies eferents del NSQ, que la sola presència de VP o VIP (van Esseveldt et al. 2000).

S'ha proposat que la melatonina, la corticosterona i les gonadotropines podrien tenir un paper com a vies controladores neuroendocrines circadiàries. La més ben descrita d'aquestes vies és una via multisinàptica per la qual el NSQ controla la síntesi i secreció diàries de l'hormona pineal melatonina. La melatonina, mitjançant un procés de *feed-back*, pot inhibir la descàrrega neuronal del NSQ (Mason i Brooks 1988, Shibata et al. 1989) i pot encarrilar la fase del ritme (Cassone 1990, Kalsbeek et al. 1999, Lewy et al. 1992). El paper de la melatonina en el comportament circadiari canvia segons les espècies i per tant, no es pot donar cap vista global uniforme. Les que també estan ben descrites són les vies que controlen els ritmes plasmàtics de corticosterona (Buijs et al. 1999). Sembla que hi hagi incorporades diverses rutes de control: contactes sinàptics directes de neurones del NSQ amb neurones productores de corticoliberina del nucli paraventricular i també, entrades indirectes a aquestes neurones a través del nucli dorsomedial de l'hipotàlem. En ambdós casos, l'alliberament d'hormona adrenocorticotropa de la pituïtària pot ser controlat de manera rítmica, de manera que la producció i la secreció també seran rítmiques. A més també s'ha demostrat que hi ha una via multisinàptica a través del nucli paraventricular i la medul·la espinal cap a les glàndules adrenals (Buijs et al. 1998).

Tot i les variades projeccions del NSQ, encara no està del tot clar com pot aquest nucli regular l'organització temporal i l'estat del comportament d'un organisme. De tota manera, recentment s'han estudiat moltes possibles vies que hi podrien estar implicades mitjançant marcadors transneuronals retrògrads del NSQ de rata amb marcadors vírics (Buijs et al. 1999,

La Fleur et al. 2000, Ueyama et al. 1999, Scheer et al. 2001). Així s'han demostrat connexions del NSQ amb diversos sistemes fisiològics.

1.3.- PROPIETATS DELS RELLOTGES BIOLÒGICS

Des que, fa més de 270 anys, de Mairan va demostrar que els ritmes circadiaris persisteixen fins i tot quan l'organisme és aïllat d'estímuls temporals, es va originar una tècnica que ha estat de considerable valor a l'hora d'estudiar les característiques dels rellotges circadiaris. El fet de treure un animal del seu entorn natural i posar-lo en una cambra on els nivells de llum, temperatura, menjar i soroll es mantenen constants, ha permès estudiar el comportament intrínsec d'un animal, ja que els esdeveniments que normalment se succeeixen en el medi ambient amaguen o "emmascaren" els ritmes circadiaris endògens.

1.3.1.- El rellotge en curs lliure

Un animal completament aïllat de qualsevol informació temporal externa, és a dir, envoltat per un entorn constant, es diu que es troba en curs lliure. El període d'un ritme d'un animal en curs lliure s'anomena tau (τ). El tau és característic de cada espècie i sol estar al voltant de les 24 hores en rosegadors i en humans.

El rellotge en curs lliure presenta diverses característiques:

- *Persistència en condicions constants:* Els ritmes endògens poden persistir durant una quantitat considerable de temps sense cap estímul ambiental. Fins i tot es poden criar generacions successives d'animals, sempre sotmesos a condicions constants.
- *Uniformitat:* La uniformitat es refereix a l'estabilitat del tau d'un rellotge. Alguns rellotges són posats a l'hora amb una impressionant precisió, mentre que d'altres tenen períodes menys estables que van canviant amb el temps.
- *Compensació de temperatura:* La uniformitat dels rellotges circadiaris ha fet que sorgissin mecanismes que compensen els canvis que produïria la temperatura ambiental. Aquests mecanismes són essencials, ja que si la periodicitat dels rellotges depengués de la temperatura, la seva utilitat com a mesuradors i indicadors del temps estaria limitada.
- *Postefectes:* El tau d'un ritme en condicions constants pot ser influenciat per les condicions prèvies (intensitat de llum, període del cicle extern, canvi de fase, etc.) en què un animal havia estat sotmès més de 100 dies enrere (Pittendrigh 1960).

- *Edat:* El període dels rellotges circadiaris acostuma a allargar-se a mesura que l'edat avança, de manera que el tau es va escurçant amb el temps (Pittendrigh 1974).
- *Base genètica:* Tot i el gran efecte que les condicions ambientals tenen sobre els rellotges biològics, cal no oblidar que les propietats dels rellotges determinades genèticament també tenen un gran pes.

La manifestació dels ritmes generats pel rellotge circadiari en condicions normals es veu influenciada per una sèrie de factors ambientals, el més important dels quals és la llum. Hi ha dos aspectes de la llum que cal considerar:

- *Efectes de la intensitat de llum:* Tot i que el valor del tau ve determinat genèticament, hi ha diversos factors ambientals que poden modificar-lo. D'entre aquests factors cal destacar la llum com a un dels més importants. L'estudi sistemàtic dels valors de tau de diverses espècies sota condicions constants d'il·luminació amb diferents intensitats va permetre que el 1960 Jürgen Aschoff enunciés unes regles generals conegudes com a "regles d'Aschoff" (Aschoff 1960):
  1) En animals nocturns el valor de tau augmenta quan s'incrementa la intensitat de llum, mentre que en animals diürns és al revés.
  2) En animals diürns la relació entre la fase d'activitat (α) i la fase de repòs (ρ) augmenta amb la intensitat de llum, mentre que en els nocturns disminueix.
  3) Sota condicions de foscor constant, els animals diürns tenen taus superiors a 24 hores, mentre que els nocturns és inferior a les 24 hores. Aquesta darrera regla però, té nombroses excepcions, com ara la de la rata, que té un tau proper a les 24.5 hores.

  Com s'ha vist, doncs, a mesura que augmenta la intensitat de llum, també ho fa el tau de l'animal. De tota manera, quan s'arriba a una certa intensitat, el que es produeix és una arritmicitat de l'animal, fet que s'ha comprovat en diverses espècies animals i en diversos ritmes circadiaris (Aschoff 1960). Aquesta arritmicitat s'hauria de considerar més com un desacoblament entre els oscil·ladors que formen el sistema circadiari (vegeu apartat 1.2.1.1), que no pas com una "aturada" de l'activitat del sistema circadiari.
- *Efectes de la longitud d'ona de la llum:* La troballa que el període d'algues unicel·lulars sotmeses a llum constant canviava depenent de la longitud d'ona de la llum (Roenneberg i Hastings 1988), va fer pensar que en mamífers potser també passaria. Efectivament, en rates es va observar que la supressió de melatonina induïda per la llum depèn de la longitud d'ona d'aquesta llum i de l'hora circadiària

en què aquesta és aplicada (un pols de llum verda a les 24 hores produïa una disminució més prolongada dels nivells plasmàtics de melatonina que un pols de llum vermella) (Honma et al. 1992) i que en hàmsters, sembla que existiria un sol tipus de fotoreceptors (amb la màxima sensibilitat a la zona del blau-verd) que mediarien canvis de fase induïts per la llum i també canvis en el període del rellotge circadiari (Boulos 1995).

Les diferències específiques d'espècie trobades en el tau en foscor constant, juntament amb les respostes específiques d'espècie del tau a la llum estarien reflectint una part de l'estratègia d'encarrilament de les espècies nocturnes i diürnes.

1.3.2.- Encarrilament per estímuls ambientals

Quan un organisme sotmès a condicions ambientals periòdiques manifesta un ritme circadiari que té el mateix període que l'entorn, direm que el ritme està encarrilat amb aquest entorn. El ritme extern no genera el ritme de l'organisme, sinó que el condueix o dirigeix (l'encarrila). El cicle extern (ambiental) s'anomena en aquest cas *Zeitgeber* o agent encarrilador. En el cas circadiari, el cicle de llum-foscor de 24 hores (LD) és el *Zeitgeber* més universal. Quan un organisme està encarrilat a un cicle LD de període T, el període del rellotge (τ) canviarà de τ a $\tau^* = T$. En aquest estat estable d'encarrilament, s'estableix una relació específica de fase (psi o ψ) entre el rellotge intern de l'organisme i el cicle de llum externa. Deixant a banda els cicles de llum-foscor, existeixen d'altres estímuls temporals capaços d'actuar com a *Zeitgeber*, com són la disponibilitat d'aliment, els senyals socials, l'activitat motora i la temperatura, entre d'altres.

La funció evolutiva de l'encarrilament és que els organismes funcionin sincrònics amb el seu entorn i per tant, s'aprofitin al màxim els recursos i es disminueixin les pèrdues energètiques de la manera més eficaç possible.

1.3.2.1.- Característiques de l'encarrilament

En ambients naturals existeixen diversos estímuls temporals. Per tal de separar els possibles *Zeitgebers* i per avaluar les seves accions, cal estudiar els organismes en ambients controlats. En aquestes condicions, es poden introduir i treure diversos estímuls. Per tal de poder dir que un determinat agent és realment un *Zeitgeber*, cal demostrar que és capaç d'encarrilar el ritme, cosa que es considera quan es compleixen els següents criteris:

- *Absència d'altres agents temporals:* El ritme circadiari estudiat ha d'estar en curs lliure i amb un període independent abans d'aplicar-hi l'estímul temporal i ha de tornar a anar en curs lliure un cop l'esmentat estímul es retira.
- *Control del període del ritme:* el període del ritme circadiari estudiat ha de ser el mateix que el del *Zeitgeber* i si canvia el període del *Zeitgeber* també ho ha de fer el del ritme.
- *Relació de fases estable:* hi ha d'haver una relació de fases estable i reproduïble entre el ritme estudiat i el *Zeitgeber*, és a dir, que el psi no pot variar per un període determinat d'aquest últim. De tota manera, si canvia el període d'un determinat *Zeitgeber* també canviarà la relació de fases amb aquest, ja que el nou cicle repercutirà en un punt de la corba de respostes de fase diferent (vegeu apartat següent).
- *Control de la fase del ritme:* quan es retira l'estímul temporal, el ritme ha de començar a anar en curs lliure amb una fase determinada pel cicle del *Zeitgeber*.

Una de les funcions més importants del sistema circadiari és doncs, assegurar que el comportament i els ajustos metabòlics interns estiguin apropiadament sincronitzats respecte els esdeveniments diaris en l'entorn. Aquestes relacions entre els ritmes circadiaris i els estímuls temporals ambientals maximitzen la supervivència de cadascuna de les espècies que viuen en aquest món. La relació de fases (psi o ψ) que s'estableix entre un *Zeitgeber* i un determinat ritme és molt característica, ja que depèn del període del *Zeitgeber* i del període natural del rellotge biològic que genera el ritme estudiat. Així doncs, per a un determinat període d'un *Zeitgeber*, l'estudi del psi (en termes de diferència de temps -hores- o de diferència d'angles de fase -graus-, entre l'inici del ritme biològic i l'inici del *Zeitgeber*), dóna idea d'una de les propietats fonamentals de tot rellotge i per tant, del funcionament d'aquest rellotge.

1.3.2.2.- Corbes de resposta de fase

Quan s'aplica un *Zeitgeber* no sempre es produeix el mateix efecte sobre la fase d'un ritme, sinó que depenent de l'hora subjectiva en què s'ha aplicat, es pot produir un avanç o un retard de fase i fins i tot, pot ser que la fase no canviï. Una Corba de Resposta de Fase (CRF) és una gràfica on es representen els canvis de fase en funció de la fase circadiària de l'estímul. D'estímuls n'hi ha de molt variats i van des d'un pols de llum (el més comú) fins a polsos de

temperatura, o bé la injecció de diversos fàrmacs o substàncies. L'any 1965 Jürgen Aschoff va dictaminar sis estratègies per calcular una CRF per un determinat estímul (Aschoff 1965):

1. S'aplica l'estímul a una determinada fase circadiària mentre l'oscil·lador va en curs lliure. L'avantatge d'aquest mètode és que el mateix individu serveix com a control seu. Hi ha, no obstant, diverses limitacions a aquest mètode. La primera d'elles és que per tal de conèixer en quina fase s'aplica l'estímul, hom hauria de ser capaç d'analitzar el ritme dia a dia mentre duri l'experiment. També hi ha problemes per analitzar el canvi de fase produït quan a més també hi ha hagut canvis en el període després de l'aplicació de l'estímul. Si el ritme presenta soroll de fons o bé és irregular, hi haurà problemes per decidir tan la fase en què es va aplicar l'estímul, com el canvi de fase produït.
2. L'estímul s'aplica a animals que, havent estat encarrilats, s'han posat recentment en curs lliure. Aquest és un bon mètode quan s'estudia un grup gran d'animals, ja que requereix pocs controls (animals que no rebran l'estímul) que es compararan amb els animals que sí han rebut l'estímul. Una altra avantatge és que després d'haver estat sotmesos a un règim fix, els post-efectes que se'n deriven seran els mateixos per tots els animals i a més seran predictibles. La fase del cicle de llum-foscor previ al curs lliure permet a l'experimentador de conèixer amb precisió la fase del ritme circadiari i l'hora exacta en què s'ha aplicat l'estímul. Finalment, aquest mètode permet a l'experimentador de triar l'hora que li és més convenient de donar l'estímul. Cal anar en compte, però, amb el canvi de fase que l'animal patirà només per haver-se-li canviat les condicions d'il·luminació.
3. L'estímul és un canvi d'una condició contínua a una altra (per exemple, passar de foscor constant a llum constant). Un problema que presenta aquest mètode és que pot ser que el període sigui diferent en una i altra condicions. Cal afegir que, donat que la relació entre la quantitat total d'activitat motora durant la fase d'activitat i la fase de repòs canvia segons la intensitat de llum, la fase aparent del ritme en curs lliure pot veure's influenciada, ja que la relació de fases entre l'inici i la fi de l'activitat estarà canviant.
4. La quarta estratègia utilitza el fenomen de la coordinació relativa: si un pols de llum es dóna a la mateixa hora cada dia amb una intensitat no gaire alta per tal que l'animal no pugui encarrilar, llavors s'observarà la coordinació relativa i el ritme mostrarà increments de període en determinades fases del dia subjectiu i decrements en altres fases. El canvi de fase del ritme relatiu al moment en què es dóna el senyal lluminós s'usa per determinar la CRF.

5. La cinquena estratègia és una combinació de la segona i la tercera: l'animal està sotmès a un determinat cicle de llum-foscor, després d'un canvi de fase en aquest cicle es deixa l'animal en curs lliure.
6. La CRF s'estima a partir dels angles de fase (ψ) assumits pel ritme en diversos cicles de diferent període de l'estímul. El problema d'aquest mètode és que no pot donar una CFR completa, ja que dependrà dels marges d'encarrilament de l'animal estudiat.

Les CRF tenen diversos usos: estudiar els mecanismes d'encarrilament d'un ritme respecte d'un determinat estímul i per tant, la forma de la corba pot donar idea de diverses estratègies ecològiques; si l'estímul és lluminós, podem obtenir informació del funcionament de les vies de transmissió de senyals fòtics; les CRF també s'usen en la investigació dels mecanismes de funcionament del rellotge biològic; com a marcadors de la fase de l'oscil·lador i com a indicador de l'amplitud dels oscil·ladors circadiaris (Johnson 1992).

Com s'ha dit anteriorment, l'estímul que més freqüentment es dóna per estudiar canvis de fase són polsos de llum. Això és degut al fet que la llum és el *Zeitgeber* més important a l'hora d'encarrilar oscil·ladors circadiaris i per tant, té un interès especial. Les CRF produïdes per polsos de llum normalment tenen totes les mateixes característiques: endarreriments de fase durant les primeres hores de la nit subjectiva, avanços de fase durant les últimes hores de la nit subjectiva i poc canvi de fase durant el dia subjectiu (Johnson 1992). Aquesta generalització es manté tan si l'animal és diürn com si és nocturn. La magnitud del canvi de fase produït en el rellotge dependrà dels límits d'encarrilament d'aquest, mentre que la magnitud del canvi de fase produït per la llum dependrà de la intensitat, de la durada i de la longitud d'ona de l'estímul lluminós aplicat, entre d'altres factors.

Existeixen però, d'altres estímuls diferents a la llum que també són capaços d'induir un canvi de fase en un determinat ritme. D'entre aquests cal destacar:

- *Temperatura:* a part del ritme de llum-foscor, el ritme diari d'alternància entre ambient calorós i ambient fred també pot tenir algun efecte significatiu. En espècies poiquilotèrmiques i heterotèrmiques és ben demostrada l'efectivitat de ritmes circadiaris d'alta i baixa temperatura a l'hora d'encarrilar (Erkert i Rothmund 1981, Tokura i Aschoff 1983, Underwood 1985). De tota manera, hi ha molt pocs estudis que examinin la temperatura com a possible estímul per canviar la fase del ritme d'un mamífer. Pel que fa a les rates, s'ha publicat la CRF per polsos de calor en rates Long-Evans (Francis i Coleman 1997), en les quals es produeix un avanç de fase

principalment durant el dia subjectiu i un endarreriment bàsicament durant la nit subjectiva.

- *Glucocorticoides:* s'ha observat que rates de laboratori sotmeses a una sola administració de l'hormona dexametasona responen amb una CRF característica (Horseman i Ehret 1982). Concretament, presenten avanços de fase durant la nit subjectiva i endarreriments durant el dia subjectiu.
- *Activitat motora:* un dels estímuls no fòtics més poderosos que es coneix actualment és l'activitat motora. Per exemple en hàmsters s'ha observat que quan es posa temporalment en una gàbia amb una roda, l'activitat induïda per la nova roda pot arribar a produir canvis de fase tant importants com els produïts per un pols de llum (Reebs et al. 1989). En el cas de l'activitat induïda per la roda, es produeixen avanços de fase durant el dia subjectiu i endarreriments durant la nit subjectiva. També s'ha observat que la magnitud del canvi de fase produït per la roda dependrà del nombre de voltes fetes per l'animal (Bobrzynska i Mrosovsky 1998).
- *Melatonina:* cada vegada més s'està usant melatonina exògena i agonistes de melatonina com a cronobiòtics per tractar i/o prevenir problemes de son, jet-lag i d'altres desordres circadiaris en humans. Aquest ús terapèutic es basa en la capacitat que té aquesta hormona de produir canvis de fase i per la seva participació en l'encarrilament, tant en humans com en animals (Redman 1997).

1.3.2.3.- Corbes de resposta de període

Dins del fenomen de l'encarrilament també s'inclou el fenomen de l'ajustament del període, que tot i ser molt important, ha estat insuficientment apreciat. Així, la precisió amb què els sistemes circadiaris són capaços de mantenir diverses constants d'angle de fase amb la rotació de la Terra és probablement el primer objectiu de la selecció natural del temps. Els sistemes circadiaris han d'haver desenvolupat maneres d'integrar la informació lluminosa que reben al llarg de diversos dies per tal de superar les respostes instantànies a l'atzar. Un possible mecanisme per a fer-ho, seria deixant que la velocitat del rellotge intern (que és inversament proporcional al tau) fos afectada per la llum de forma fase-depenent. Una "corba de resposta de velocitat" a la llum ha estat postulada per explicar l'encarrilament a cicles de llum dèbils i sinusoïdals (Swade 1969), l'encarrilament en general (Enright 1980) i per la dependència del tau de la intensitat de llum constant (Pittendrigh i Daan 1976a, Daan 1977).

Endarreriments de fase com a resposta a un sol pols de llum s'han associat sovint a increments de tau, mentre que avanços de fase s'han associat a decrements de tau (Pittendrigh i Daan 1976a). Alguns estímuls no fòtics responen de manera oposada (Mrosovsky 1993). Tot i

aquestes observacions, només s'han publicat quatre corbes de resposta de tau (CRτ) a polsos de llum: per tres espècies d'esquirols (Kramm i Kramm 1980, Pohl 1982) i pel talp camperol (Gerkema et al. 1993). A més, hi ha dades no publicades sobre l'esquirol nord-americà o ratllat (Vermij and Dijk).

Simulacions fetes en un model de sistema circadiari per Beersma i col·laboradors (Beersma et al. 1999) demostren que:

1. Si els sistemes circadiaris només poguessin encarrilar mitjançant canvis de fase, la seva precisió no es beneficiaria d'una CRF de gran amplitud, ja que en aquest cas l'organisme seria massa sensible a capricis de les variacions d'intensitat de llum diàries.
2. El mateix passaria si l'encarrilament hagués de dependre només d'un control de període.
3. La millor manera d'augmentar la precisió d'un determinat model de rellotge és si aquest respon a la llum no només amb canvis de fase, sinó també amb canvis de tau. Aquesta precisió millorada només s'esdevé quan els avanços de fase coincideixen amb escurçaments de tau i si els endarreriments ho fan amb allargaments de tau.

Així doncs, si un sistema circadiari rep un estímul lluminós inesperadament a una hora determinada del dia, aquest estímul serà interpretat com un error en la fase del ritme que per tant s'ajustarà, però també pot ser interpretat com un error en la velocitat del sistema, que també s'ajustarà. D'aquesta manera es millorarà la precisió del sistema i el resultat d'això serà que aquest haurà encarrilat quasi exactament a 24 hores.

1.3.2.4.- Marges d'encarrilament-dissociació-"splitting"

Una altra característica important d'un *Zeitgeber* per a una determinada espècie, és el seu marge d'encarrilament, els límits del qual són els períodes màxim i mínim capaços d'encarrilar un ritme concret. Així doncs, quan el *Zeitgeber* té un període massa curt o massa llarg, no serà capaç de sincronitzar un ritme al seu període, tot i que aquest mateix *Zeitgeber* pugui ser molt efectiu quan el seu període estigui dins els marges d'encarrilament. Així doncs, com a tall d'exemple, s'ha observat que en ratolins, el període del ritme de son-vigília es pot encarrilar a un període del *Zeitgeber* d'entre 21 i 28 hores (Aschoff 1978). Tot i això, alguns dels ratolins sotmesos a períodes de 21 i 22 hores presenten dos components en el ritme d'activitat motora: un component està encarrilat al cicle extern i per tant presenta un període de 21 o 22 hores, mentre que el segon component, incapaç d'encarrilar, va en curs lliure, amb un període proper a les 24 hores (Campuzano et al. 1999a). Aquesta dissociació del ritme en dos components també s'ha pogut observar en rates sotmeses a cicles llum-foscor de període inferior

a les 24 hores (Vilaplana et al. 1997, Campuzano et al. 1998) i s'ha trobat que depèn del període del cicle extern (Campuzano et al. 1998, Cambras et al. 2000), de l'accés a roda giratòria (Campuzano et al. 1999b, Cambras et al. 2000) i de la intensitat de llum durant la fase de llum del cicle (Cambras et al. 2000).

Cal no confondre, però, la dissociació del ritme en dos components, amb l'anomenat *splitting*. L'*splitting* va ser primer observat en hàmsters sotmesos a condicions de llum constant. En aquests, el ritme d'activitat motora es partia en dos o més components fàcilment distingibles. Cadascun d'aquests components pot anar en curs lliure amb un període diferent (Pittendrigh 1974). L'increment o decrement de llum no és l'únic estímul que pot provocar *splitting*, sinó que diverses intervencions fisiològiques, com ara la destrucció dels NSQ o canvis en els nivells hormonals, són igualment efectives.

1.4.- ONTOGÈNIA I DESENVOLUPAMENT DEL SISTEMA CIRCADIARI

L'ontogènia és la història de la vida dels organismes com a individus i inclou la seva construcció física a partir d'un ou fertilitzat, la maduració funcional del seu comportament i dels seus sistemes homeostàtic i reproductor i finalment, el declivi d'aquests sistemes amb l'edat.

1.4.1.- Desenvolupament del sistema circadiari

1.4.1.1.- Desenvolupament del nucli supraquiasmàtic

El cervell, incloent el NSQ, no està completament desenvolupat en el moment del naixement, almenys en rates. Mosko i Moore (Mosko i Moore 1978) han trobat que no hi ha una recuperació del funcionament del rellotge biològic després de l'ablació del NSQ durant el desenvolupament. Tampoc no hi ha una recuperació de la funció després de l'ablació del NSQ en neonats. El teixit fora de la zona lesionada, doncs, es comporta en els animals immadurs com en els adults, és a dir, que és incapaç de recuperar les funcions de rellotge biològic després de la destrucció del NSQ. Així, les cèl·lules hipotalàmiques estan determinades en el seu destí abans que el NSQ estigui completament desenvolupat i possiblement abans que les cèl·lules del NSQ hagin completat la seva divisió mitòtica final en l'epiteli germinal del tercer ventricle. Altman i Bayer (Altman i Bayer 1986) han apuntat la possibilitat que el destí de les cèl·lules precursores del

NSQ es determini ja abans de la migració per formar el nucli, és a dir, en l'epiteli germinal abans que el NSQ es formi.

El desenvolupament del NSQ consta de tres fases:

1.- Formació de neurones : l'hipotàlem es forma en tres onades de neurogènesi que van del dia embrionari 12 (E12) al E18 (Altman i Bayer 1986). Les neurones del NSQ es produeixen des del dia E14 al E17 (Altman i Bayer 1986, Seress 1985).

2.- Creixement de neurones i maduració.

3.- Formació de sinapsis o sinaptogènesi : la sinaptogènesi és un fet predominantment postnatal (Moore i Bernstein 1989). Hi ha poques sinapsis el dia E19, i les que es troben, són immadures. A partir del dia E21 fins al dia postnatal 2 (P2) hi ha un augment gradual de les sinapsis, seguit d'una taxa cada vegada més ràpida de sinaptogènesi des de P2 a P6, i particularment de P6 a P10 (Watts 1991). A P10 la densitat sinàptica està a nivells d'adult (aproximadament 25 sinapsis/10^3 mm^2). De tota manera, tot i que el nombre de sinapsis per unitat d'àrea de NSQ és la mateixa a P10 que en l'adult, el volum dels nuclis de neurones és significativament superior en adults.

L'organització de les eferències del NSQ en l'adult és molt extensa (Watts i Swanson 1987, Watts et al. 1987). No s'han fet estudis sobre el desenvolupament de les projeccions del NSQ, però se suposa que les connexions funcionals s'estableixen abans del dia P4. Les sinapsis que uniran el NSQ amb el quiasma òptic sobretot es donaran entre els dies P4 i P10. Els àxons de les cèl·lules ganglionars retinals sortiran de la retina i formaran els nervis òptics el dia E15 (Lund i Bunt 1976). El quiasma òptic i els tractes òptics primaris es formaran els dies E16 i E17, com també les projeccions al nucli terminal principal, el nucli geniculat lateral (Bunt et al. 1983).

Així doncs, el desenvolupament funcional del NSQ es pot dividir en dues etapes :

1.- El desenvolupament de la ritmicitat intrínseca : el primer ritme que es desenvolupa és el d'utilització de glucosa (Schwartz i Gainer 1977), que apareix en el NSQ a partir del dia E19. Aquest ritme està encarrilat al ritme matern per un mecanisme desconegut (Reppert 1985, Reppert i Schwartz 1986). El desenvolupament d'aquest ritme no depèn del ritme matern (Reppert i Schwartz 1986, Shibata i Moore 1988a), indicant que ve determinat genèticament. Aquest fet reflecteix l'activitat intrínseca de l'oscil·lador. El segon ritme que apareix és el de descàrrega de les neurones, present el dia E22, tot i que amb una baixa amplitud.

2.- Desenvolupament del NSQ com a rellotge circadiari: això passa quan ha format suficients connexions aferents, eferents i intrínseques com per a funcionar com una xarxa neuronal. S'arriba al nivell adult el dia P10 (Moore 1991).

1.4.1.2.- Desenvolupament del tracte retinohipotalàmic

En mamífers adults el TRH és la via necessària i suficient que té el sistema circadiari per transmetre els senyals fòtics al NSQ (Johnson et al. 1988b). Tant en rates com en hàmsters acabats de néixer, el TRH entra en el NSQ durant la primera setmana de vida, i el seu desenvolupament es completa al voltant del dia 10 (Lavialle i Servière 1995, Speh i Moore 1993).

Els àxons de les cèl·lules que estructuren el TRH es formen com a col·laterals de les fibres existents del quiasma òptic (Mason et al. 1977). D'aquesta manera, el TRH es desenvolupa com a resposta a un senyal, ja que l'hipotàlem adjacent al TRH en rates té projeccions al NSQ, altres àrees hipotalàmiques, àrea hipotalàmica lateral i àrea retroquiasmàtica (Johnson et al. 1988b).

El desenvolupament del TRH està molt lligat al NSQ, ja que si aquest últim es destrueix completament, el TRH no es formarà (Mosko i Moore 1979).

1.4.1.3.- Desenvolupament del feix intergeniculat

El desenvolupament del TGH requereix el desenvolupament de dos components: la projecció retinal cap el FIG i la projecció del FIG cap al NSQ.

Tant en visons (Peytevin et al. 1997), com en molts d'altres mamífers (Crabtree 1990, Cucchiaro i Guillery 1984), les fibres retinals que travessen el quiasma òptic ja han arribat tant a la part ventral com a la dorsal del NGL en el moment del naixement. Per tant, les projeccions de cèl·lules ganglionars que van cap al FIG s'estableixen abans del desenvolupament del TRH. La projecció que comunicarà el FIG amb el NSQ assolirà el nivell d'adult (en rata) el dia 10 després del naixement (Moore 1989).

El suposat emplaçament del FIG comença a ser aparent des del naixement en seccions de cervell tenyides amb violeta de cresil; en canvi, les fibres retinals del FIG no queden marcades amb toxina B del còlera fins al cap de cinc dies d'haver nascut l'animal (Peytevin et al. 1997).

1.4.2.- Desenvolupament dels ritmes circadiaris

L'estudi del desenvolupament dels ritmes circadiaris de la rata ha demostrat que diferents ritmes apareixen a edats diferents i que aquests tenen un període de maduració. La seqüència de maduració és la següent: 1) Ritmes associats a la imposició de menjar, com per exemple el guany de pes; 2) ritmes en la pineal i en el sistema nerviós simpàtic; 3) ritmes endocrins controlats per la pituïtària i 4) ritmes en conductes voluntàries, com ara la beguda, l'activitat motora i les característiques corticals del son. Aquesta seqüència, però, no ha de

reflectir necessàriament la completa maduració d'aquests ritmes, sinó que pot estar reflectint una successió de maduració general de les necessitats adaptatives i de regulació de l'organisme. Si resulta que la majoria de ritmes de l'organisme són generats per un únic rellotge circadiari central (probablement el NSQ), l'aparició seqüencial dels diversos ritmes reflectirà la maduració seqüencial de les vies aferents o dels mecanismes de control distals al rellotge central.

Ja en l'úter un mamífer està exposat a un ambient cíclic: la concentració de nutrients i hormones que creuen la placenta per anar cap al sistema sanguini del fetus reflecteixen la ritmicitat circadiària de la mare (Reppert et al. 1979). Un animal acabat de néixer desenvolupa gradualment ritmes circadiaris evidents en diverses funcions corporals a mesura que es van acoblant en els oscil·ladors interns durant les primeres setmanes de vida. La maduració dels ritmes circadiaris s'acostuma a caracteritzar per l'increment progressiu de l'amplitud del ritme fins que l'animal és adult (Davis 1981, Cambras i Díez-Noguera 1988) i a vegades també pot comportar modificacions del període endogen i de la forma o patró circadiari. Tant la funció del rellotge com l'expressió dels ritmes circadiaris evidents pot precedir la capacitat d'encarrilar a estímuls temporals ambientals.

1.4.3.- Factors que influencien el desenvolupament del sistema circadiari de la rata

1.4.3.1.- Influències maternes

El fet que el comportament maternal sigui inqüestionablement rítmic fa pensar en la possibilitat que la cria adapti la seva ritmicitat a la de la mare mentre dura el seu desenvolupament. S'ha vist que per una banda, la mare encarrila el fetus a les condicions externes a fi que les cries neixin en la fase adequada del cicle extern del ritme circadiari i per altra banda, la mare facilita aquest encarrilament fins que el sistema nerviós de les cries sigui prou madur com per permetre l'encarrilament fòtic.

Durant la vida fetal, hi ha diversos estudis en mamífers que demostren que el rellotge circadiari en el NSQ oscil·la en fase amb el cicle llum-foscor ambiental. Així doncs, es pot deduir que en l'encarrilament del NSQ fetal hi intervé la mare, que es comunicarà amb el fetus a través de senyals circadiaris. En el dinovè dia de gestació ja es pot detectar un ritme fetal d'activitat metabòlica en el NSQ en fase amb el ritme de la mare i amb el cicle d'il·luminació extern (Reppert i Schwartz 1983). Experiments amb mares cegues han demostrat que el fetus encarrila amb el ritme de la mare i en canvi no s'afecta directament per les condicions ambientals d'il·luminació, i experiments amb mares amb el NSQ lesionat han demostrat que

aquest encarrilament mare-fetus depèn de la integritat del NSQ matern (Davis i Gorski 1988, Honma et al. 1984a, b, Reppert i Schwartz 1986b).

Diversos estudis han intentat identificar el senyal/s matern/s que comuniquen la fase circadiària als fetus de rata. Com que l'úter és un ambient ric en hormones de la mare (prolactina, corticosterona, melatonina...), aquests estudis s'han dirigit a l'estudi d'hormones maternes com a senyal circadiari. De tota manera, la pinealectomia materna (que elimina pràcticament la producció de melatonina), l'extirpació de les glàndules adrenals maternes, les tiroides i paratiroides, la pituïtària o bé els ovaris (separadament) no aconsegueix eliminar la coordinació materna amb la fase circadiària del fetus (Reppert and Schwartz 1986a). Els ulls de la mare, possible font de senyals neurals i endocrins, tampoc no són necessaris per aquesta comunicació (Reppert and Schwartz 1983). Així doncs podríem pensar que, ja que el fetus està sotmès a una diversitat d'estímuls circadiaris provinents de la mare (tant hormonals com de comportament), caldria considerar la possibilitat que tota aquesta varietat de ritmes materns actuen conjuntament per encarrilar el rellotge circadiari del fetus. Per consegüent, una eliminació d'un d'aquests ritmes no seria suficient com per a interrompre l'encarrilament matern.

Però la influència que la mare exerceix sobre els ritmes circadiaris de la cria no només són presents durant la gestació, sinó que també apareixen durant l'alletament. Sembla ser que les influències maternes durant l'etapa postnatal mantenen o reforcen la coordinació de fase establerta prèviament. Concretament, en rates s'ha observat que la influència materna es manté fins que la cria desenvolupa el potencial necessari per a encarrilar directament amb el cicle de llum-foscor a través dels seus propis ulls. Aquesta efecte de la mare és més gran durant la primera setmana de vida.

1.4.3.2.- Influències ambientals

Tot i que, com hem vist anteriorment, el rellotge biològic de les cries és encarrilat pels ritmes materns, sembla ser que aquest encarrilament no és necessari per al desenvolupament normal del rellotge circadiari. S'ha observat que cries que tenien una mare amb el NSQ lesionat i van créixer sota condicions constants eren capaces d'expressar un ritme circadiari que semblava normal (Davis and Gorski 1988, Reppert and Schwartz 1986b). Per tal de veure si les condicions ambientals són capaces d'alterar el rellotge circadiari, només cal observar si aquestes poden modificar les propietats intrínseques d'aquest rellotge. Les dues propietats fonamentals dels rellotges biològics són el període i la capacitat que determinats senyals ambientals tenen de modificar la fase del ritme (valorada per la CRF). Per una banda, la troballa que ratolins que han crescut sota diferents cicles de llum-foscor durant les primeres setmanes de vida manifesten el mateix període en foscor constant (Davis and Menaker 1981), podria fer-nos pensar que les

condicions ambientals no afecten el desenvolupament del sistema circadiari; però per altra banda, el fet que rates que han crescut sota diferents condicions ambientals manifestin un ritme d'activitat motora diferent quan se sotmeten a condicions d'il·luminació constant (Cambras 1991), demostra que les coses no són tan simples com podria semblar a primer cop d'ull.

Sigui a través de la mare, o bé sigui a través d'estímuls ambientals, el que sembla ser avantatjós és encarrilar la cria durant el seu desenvolupament. Una raó per aquest encarrilament podria ser que la cria hagués d'estar sincronitzada a un ambient rítmic apropiat per tal de poder tenir un creixement i un desenvolupament normals.

1.5.- ESTUDI DELS RITMES CIRCADIARIS

En l'estudi dels ritmes circadiaris cal fer una distinció entre el "mecanisme" del rellotge i les "busques" d'aquest. La majoria dels ritmes circadiaris observats en una àmplia gamma de variables fisiològiques, bioquímiques o de comportament són anàlegs només a les busques del rellotge. És a dir, que si s'interfereix amb un d'aquests ritmes (per exemple lligant un animal perquè no es mogui i per tant no pugui mostrar el seu ritme d'activitat motora) el rellotge continuarà funcionant, tot i que no pugui marcar el temps. Un cop s'atura la interferència (es deixa anar l'animal), aquest es continuarà movent a partir de l'hora circadiària que li marqui el seu rellotge, el qual no s'ha aturat en cap moment.

Per tal d'estudiar les característiques d'un sistema circadiari se n'ha de poder enregistrar la seva sortida o *output*. L'ideal seria monitoritzar aquesta sortida directament; en aquest cas, només caldria estudiar un ritme que representés acuradament el comportament del rellotge. A la pràctica, però, trobar un ritme d'aquestes característiques és extremadament difícil i encara ho és més el poder-lo mesurar. La variable rítmica ideal hauria de ser mesurable dins un mateix individu (per tal de descartar un efecte poblacional), diverses vegades per cicle i durant molts cicles successius. Idealment també, la mesura d'aquesta variable de l'individu no hauria d'influenciar el seu comportament o fisiologia. A més, el procés de mesura de la variable hauria de ser automàtica, sinó caldria un gran nombre de persones treballant les 24 hores del dia.

Tot i que hi ha un gran nombre de ritmes, a continuació descriurem només els que s'han estudiat en aquest treball.

1.5.1.- Activitat motora

El ritme d'activitat motora és un ritme que s'adiu a la major part dels criteris esmentats per un ritme ideal: es pot enregistrar l'activitat motora d'un sol individu, el seu moviment és

espontani i lliure, el registre és continu, pot durar el temps que calgui (fins i tot anys) i es pot automatitzar. El ritme d'activitat motora d'un animal es pot mesurar mitjançant una roda giratòria, o bé sense roda, com per exemple mitjançant els actímetres de feixos d'infraroig. Aquest últim mètode és l'usat en el nostre laboratori per la seva facilitat de muntatge, d'instal·lació, pel poc espai i cost que suposa, i perquè l'animal està intacte (no ha sofert cap intervenció quirúrgica).

Una àmplia varietat d'activitats s'inclouen dins el títol general d'activitat motora (menjar, beure, córrer, bellugar-se, etc.), però tot i amb això, és evident que sigui quina sigui la causa d'un moviment, els animals es mouen rítmicament com a conseqüència dels seus propòsits adaptatius. Així doncs, els animals es mouran per canvis en la intensitat de llum o en el cicle de llum-foscor, per canvis en la temperatura, per olors, camp visual, etc. A part d'aquests estímuls externs, el ritme d'activitat motora també es veu afectat per la concentració d'hormones sexuals, la manca de menjar o d'aigua i per la mateixa ritmicitat circadiària. Així per exemple el patró circadiari d'activitat motora s'afectarà en gran manera per la gana i per altra banda, les respostes motores al menjar variaran en funció de l'hora del dia. Tot aquests factors doncs, interactuaran per donar lloc, al final, al ritme d'activitat motora mostrat per l'animal.

Està ben establert que en mamífers, el NSQ és qui dirigeix els ritmes circadiaris d'activitat motora que encarrilen al cicle extern de llum-foscor (Rusak and Zucker 1979), tot i això, més recentment s'ha trobat que d'altres àrees del cervell, com són el putamen caudat i l'escorça parietal, també estan implicades en la manifestació del ritme d'activitat motora en rates (Masubuchi and Honma 2000). El patró motor és característic d'una família, és a dir, que és hereditari tant en rates (Cambras and Díez-Noguera and Ribot 1988) com en ratolins (Díez-Noguera and Cambras 1989).

Pel que fa al desenvolupament del ritme d'activitat motora, s'ha observat que ocorre en el període postnatal i que consta de tres etapes: una primera on el ritme és bàsicament ultradiari, la segona on hi ha una disminució considerable dels nivells d'activitat motora diaris i la tercera, en la que aquests nivells recuperen els valors inicials i en la que el patró circadiari queda ben establert (Cambras and Díez-Noguera 1988)

1.5.2.- Pressió arterial i freqüència cardíaca

És ben demostrat que les funcions del sistema cardiovascular canvien amb un ritme circadiari. Per exemple, la pressió sanguínia i la freqüència cardíaca són més elevades durant la fase d'activitat que durant la fase de repòs. Fins i tot la incidència de malalties cardiovasculars, com ara l'isquèmia i l'infart de miocardi presenten variacions diürnes marcades (Rocco 1987, Muller 1999). Un dels factors limitants en l'estudi de la funció cardiovascular i concretament,

dels ritmes de pressió sanguínia i freqüència cardíaca, és la dificultat de mesurar aquestes variables en animals conscients, no estressats i que es puguin bellugar lliurement. L'aparició de la telemetria ha permès obtenir aquestes condicions. Així doncs s'ha observat que el ritme de les esmentades variables és controlat per un rellotge circadiari intern, ja que aquests ritmes són capaços d'encarrilar a un determinat cicle de llum-foscor i persisteixen en condicions de curs lliure (Takezawa 1994). Més concretament, els ritmes de pressió arterial i de freqüència cardíaca són controlats a nivell de NSQ, ja que quan aquests es lesionen el ritme circadiari desapareix (Sano 1995). Recentment s'ha posat en evidència la presència d'una via multisinàptica entre les neurones del NSQ i el cor i a més s'ha demostrat la independència del ritme de freqüència cardíaca respecte del ritme d'activitat motora (Scheer 2001).

1.5.2.1.- Regulació de la pressió arterial

Hi ha diversos mecanismes que ajuden a regular la pressió arterial: mecanismes de regulació ràpida (on intervé principalment el sistema nerviós), mecanismes de regulació a mig termini (on intervenen determinades hormones, com l'aldosterona) i mecanismes de regulació a llarg termini (on intervenen bàsicament el ronyó i el volum de líquid intravascular):

a) Mecanismes de regulació ràpida: Una de les funcions més importants del sistema nerviós en la de regulació la circulació és la seva capacitat per produir augments ràpids de la pressió. Hi ha tres mecanismes principals per augmentar la pressió: 1) la contracció de quasi totes les arterioles de l'organisme (incrementant d'aquesta forma la resistència perifèrica); 2) la contracció d'altres grans vasos de la circulació i en particular, les venes (fent que la sang es desplaci cap al cor i que per tant, augmenti directament el volum d'emplenat d'aquest òrgan i indirectament augmenti la força de contracció del miocardi, cosa que fa que es bombegi una quantitat major de sang), i 3) l'estimulació del propi cor a través del sistema nerviós autònom (que fa que incrementin tant la freqüència, com la força de bombeig del cor).

b) *Mecanismes de regulació a mig i llarg termini:* El sistema de ronyó-líquids corporals és un sistema de regulació de la pressió arterial molt simple capaç de controlar canvis lents de pressió arterial, sobre els quals el sistema nerviós perd progressivament la seva capacitat reguladora. L'augment de la pressió arterial té un efecte directe sobre els ronyons augmentant l'excreció renal de líquid extracel·lular, és a dir, augmentant la diüresi. Junt a aquest sistema

bàsic, s'hi ha afegit un mecanisme més refinat de regulació de pressió: el sistema renina-angiotensina.

La renina és un enzim alliberat per les cèl·lules juxtamedul·lars del ronyó quan disminueix la pressió arterial (Vegeu Fig.2). La major part de la renina sintetitzada passarà a circulació sistèmica, on interaccionarà amb una proteïna plasmàtica anomenada angiotensinogen per donar lloc a un pèptid conegut amb el nom d'angiotensina I. Aquest pèptid és un vasoconstrictor dèbil. En els petits vasos pulmonars l'enzim de conversió trencarà l'angiotensina I per formar angiotensina II. Aquest pèptid té diverses funcions: 1) és un potent vasoconstrictor, cosa que provoca que augmenti la resistència perifèrica; 2) fa augmentar la set i per tant, la ingesta d'aigua, cosa que fa augmentar la volèmia; 3) a nivell del sistema nerviós central provoca l'alliberació de vasopressina o hormona antidiurètica, que a part d'ocasionar una vasoconstricció, també controla la reabsorció d'aigua en el túbul distal del ronyó i per tant, controla també el volum intravascular i 4) és l'estimulador més important per la secreció d'aldosterona per les glàndules suprarenals, aquesta hormona produeix un augment de la reabsorció de sodi i d'aigua pels túbuls renals, de manera que es produeix una retenció d'aigua que farà incrementar la volèmia. Així doncs, per diverses vies s'augmentarà el volum de líquid intravascular, s'evitaran pèrdues d'aquest mateix líquid, s'augmentarà la resistència perifèrica i per tant, el què succeirà al final és que la pressió arterial augmentarà.

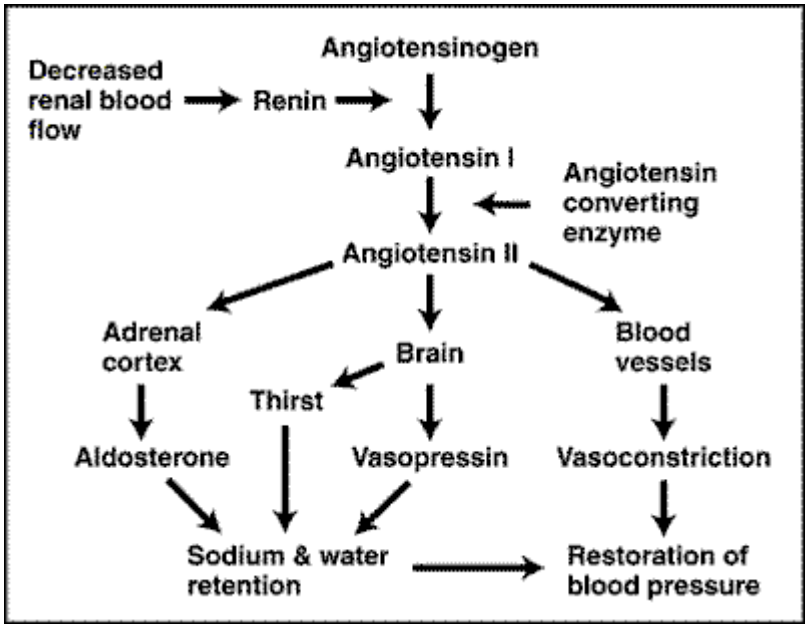

Fig.2.- Sistema renina-angiotensina.

OBJECTIUS

2.- OBJECTIUS

L'objectiu general de la tesi doctoral és l'estudi de l'efecte de les condicions ambientals d'il·luminació en què un animal ha crescut sobre la manifestació dels seus ritmes circadiaris. Per aconseguir aquest objectiu el treball s'ha estructurat en dues parts:

1. L'objectiu de la primera part del treball és estudiar els efectes de les condicions ambientals d'il·luminació rebudes durant les primeres setmanes de vida, en el desenvolupament i funcionalitat del sistema circadiari. Concretament, s'estudiarà, en primer lloc, si hi ha un període crític de dies després del naixement per al desenvolupament del sistema circadiari. D'altra banda s'estudiarà com les condicions d'il·luminació en què un animal s'ha criat poden afectar les respostes futures d'aquest animal a la llum, atenent al tau i a les respostes tònica i fàsica del sistema circadiari a la llum.

2. La segona part del treball es fixa en la soca trangènica de rates hipertenses TGR(mREN2)27 i en com les condicions ambientals d'il·luminació afecten el desenvolupament dels seus ritmes circadiaris. En concret, s'estudiarà en detall el desenvolupament dels ritmes circadiaris d'activitat motora, freqüència cardíaca i pressió sanguínia d'aquesta soca de rates, i també l'efecte de cicles de llum-foscor de període inferior a les 24 hores sobre els ritmes circadiaris de rates TGR joves i adultes.

2.- OBJECTIVES

The principal objective of the present thesis is to study the effect of the lighting conditions in which an animal was reared on the manifestation of its circadian rhythms. Therefore, the study has been divided into two parts:

1. *The objective of the first part is to study the effects of the environmental lighting conditions in which an animal was reared during the first postnatal weeks on the development and functioning of the circadian system. Specifically, the aim of this part is to look whether there is a critical period of days after birth for the development of the circadian system. We also want to examine whether the lighting conditions during lactation affect the future responses of the animal to light, examining the tau and the tonic and phasic responses of the circadian system to light.*

2. *The second part of the study focuses on the transgenic hypertensive strain of rats TGR(mREN2)27. This part aims to study how do lighting conditions affect the development of the circadian rhythms of these rats. Specifically, our objective is to study in depth the development of the circadian rhythms of motor activity, heart rate and blood pressure in this strain of rats, and our aim is also the study of the effect of light-dark cycles of a period inferior to 24 hours on the circadian rhythms of young and adult TGR rats.*

PART I

3.1.- OBJECTIUS

3.1.- OBJECTIUS

La primera part del present treball té com a objectiu principal l'estudi dels efectes de les condicions ambientals d'il·luminació durant les primeres setmanes de vida, en el desenvolupament i funcionalitat del sistema circadiari.

Per aconseguir aquest objectiu, s'han realitzat dos grups d'experiments que pretenen:

1.- Estudiar si hi ha una finestra crítica de dies després del naixement en què l'aplicació de llum tingui una major influència en el desenvolupament del sistema circadiari. És ben sabut que ja des de l'etapa fetal el comportament rítmic de la mare encarrila les cries. L'efecte de la mare durarà fins que el sistema circadiari de la cria estigui prou desenvolupat com per enfrontar-se i controlar els estímuls encarriladors de l'ambient, principalment la llum. De tota manera, tot i que la influència de la mare és important, les condicions ambientals en què la cria és mantinguda també són significatives, com per exemple en el desenvolupament del sistema visual, on l'experiència visual primerenca és crucial i només en un període concret de la vida de l'animal.

Pel que fa al desenvolupament del sistema circadiari, s'ha observat que rates que han crescut sota diverses condicions d'il·luminació manifesten un ritme circadiari d'activitat motora diferent en l'edat adulta. Per consegüent, estudiant la resposta a la llum d'aquests animals adults podrem esbrinar si els efectes de la llum sobre el desenvolupament del sistema circadiari són més marcats en uns determinats dies postnatals o no.

2.- Estudiar si les condicions d'il·luminació en què un animal ha crescut afectaran les seves respostes futures a la llum. S'ha demostrat que l'ambient lluminós en què ha crescut un animal és important pel desenvolupament del seu sistema circadiari, tot i això, no hi ha un consens alhora de determinar el grau d'aquesta importància. Així doncs, el nostre objectiu ha estat el de clarificar quin és el paper de la llum en el sistema circadiari quan els animals han crescut sota diverses condicions d'il·luminació.

3.1.- OBJECTIVES

The first part of the present work aims to study the effects of environmental lighting conditions during early postnatal weeks on the functioning and development of the circadian system. Therefore, two sets of experiments have been designed in order to:

1.- Study whether there is a critical period of postnatal days in which the application of light has a major effect on the development of the circadian system. It is well known that the rhythmic behaviour of a mother entrains the pup, starting as early as its foetal life. The effect of the mother will last until the circadian system of the pup is developed enough to cope with the ambient entraining stimuli, basically, the photic stimuli. Although the mother's influence is important, the environmental conditions under which a pup is reared are also of significance, for example in the development of the visual system, where the early visual experience is crucial during only a certain period of the animal's life.

Regarding the development of the circadian system, it has been observed that rats reared under different lighting conditions manifest a distinct circadian rhythm of motor activity under constant light as adults. Therefore, by studying the response to light of these adult animals, we will be able to know whether the effects of light on the developing circadian system are strengthened during certain postnatal days or not.

2.- Study whether the lighting conditions in which an animal has been reared affect its future responses to light. It has been demonstrated that the light environment in which a pup has been reared is of importance for the development of its circadian system, however, it appears that the degree of this importance is not so clear. Therefore, we wanted to clarify the role of light on the circadian system when the animals have been born under different lighting conditions. Consequently we studied three fundamental properties of the circadian clocks: the period of oscillation under different lighting environments, and their tonic and phasic responses to specific stimuli.

3.2.- EXPERIMENT 1

MANIFESTATION OF CIRCADIAN RHYTHM UNDER CONSTANT LIGHT DEPENDS ON LIGHTING CONDITIONS DURING LACTATION

***American journal of Physiology (Regulatory Integrative Comp. Physiol.41)*: R1039-R1046, 1997.**

Resum

Objectiu: Estudi de l'evolució del ritme d'activitat motora de rates sotmeses, durant l'alletament, a diverses condicions d'il·luminació. Influència de la ritmicitat de la mare en el desenvolupament d'aquest ritme.

Material i mètodes: Sis rates Wistar femella van arribar al nostre laboratori el dia 16 de gestació. El dia del naixement, les cries es van barrejar, de manera que cada mare alimentava 5 o 6 mascles i 5 o 6 femelles de diverses ventrades. Tres grups es van transferir a foscor constant i els altres es van sotmetre a il·luminació constant. Per cadascuna de les condicions d'il·luminació, una de les mares es va cegar per enucleació ocular per tal d'evitar la pèrdua del ritme circadiari d'activitat motora per efecte de la llum; una altra mare només tenia accés a menjar durant 3 hores al dia, per tal de sincronitzar el seu ritme a 24 hores i finalment, la tercera mare feia de control i per tant, degut a la llum constant, presentava arritmicitat. Les rates es van mantenir en aquestes condicions fins al deslletament (22 dies després del naixement). El dia del deslletament, es van agafar 4 mascles i 4 femelles de cadascun dels grups i es van posar en gàbies individuals amb actímetre d'infraroig, per tal d'enregistrar-ne la seva activitat motora. Totes les rates es van sotmetre a condicions d'il·luminació constant, amb accés a l'aigua i el menjar *ad libitum*. L'activitat motora es va enregistrar durant 55 dies.

Resultats: Tots els animals sotmesos a llum constant durant l'alletament manifesten un ritme circadiari d'activitat motora. Per contra, les rates sotmeses a foscor constant durant l'alletament esdevenen arrítmiques quan són sotmeses a llum constant. Aquestes conductes són independents de la ritmicitat de la mare.

Conclusions: Els resultats suggereixen que la ritmicitat circadiària de les mares no afecta el desenvolupament del ritme circadiari de les cries, però en canvi, les condicions d'il·luminació en què els animals han estat sotmesos durant l'alletament són crítiques pel desenvolupament del ritme circadiari.

Manifestation of circadian rhythm under constant light depends on lighting conditions during lactation

T. CAMBRAS, M. M. CANAL, A. TORRES, J. VILAPLANA, AND A. DÍEZ-NOGUERA
Unitat Fisiologia, Facultat Farmacia, Universitat de Barcelona, 08028 Barcelona, Spain

Cambras, T., M. M. Canal, A. Torres, J. Vilaplana, and A. Díez-Noguera. Manifestation of circadian rhythm under constant light depends on lighting conditions during lactation. *Am. J. Physiol.* 272 (*Regulatory Integrative Comp. Physiol.* 41): R1039–R1046, 1997.—Adult rats transferred to continuous illumination (LL) show a disruption of circadian rhythms, although the mechanisms underlying this effect are not yet well known. In previous experiments, we found that when rats were born and raised under LL they showed an ultradian pattern during the first 10 days after weaning, but afterward they generated a circadian rhythm that was maintained until adulthood. It was not clear whether this evolution was attributable to the influence of the rhythm of the mother or to the effect of constant light. Here, we have studied the motor activity rhythm of young rats maintained under LL after weaning, taking into account the conditions to which they were exposed during lactation [LL or continuous darkness (DD)]. To check the possible effect of the rhythm of the dam, on the day of delivery some of the dams were blinded, others were subjected to a restricted feeding schedule of 3 h/day, and the others were used as controls. For each rat, the period of the circadian rhythm and the percentage of variance explained by this rhythm were calculated. Results show that all rats maintained under LL during lactation expressed a circadian rhythm in their motor activity. However, rats maintained under DD during lactation did not. This effect did not seem to be dependent on the type of dam. These results suggest that the rhythm of the dams does not affect the manifestation of the rhythm of the pups and that the expression of circadian rhythmicity under constant bright light depends on the lighting conditions under which the animals were maintained during lactation, which could affect the development of the circadian pacemaker or the retina.

maturation

LIGHT HAS A STRONG EFFECT on the circadian system. Apart from the entraining capacity of light-dark (LD) cycles, exposure of animals to constant illumination (LL) is one of the conditions that reflects the effect of this factor on the manifestation of circadian rhythmicity. Splitting (2) and the presence of a large number of ultradian components (8) are the best-known consequences of the exposure of an animal to bright LL. This effect has been explained by considering the circadian system as a multioscillatory system in which light can inhibit the coupling among the different oscillators (1, 6). In the case of the rat, when adult rats are transferred to LL after LD or continuous darkness (DD), they lose the circadian rhythmicity after several days or weeks and manifest an arrhythmic pattern with a large number of ultradian components, with an unstable phase relationship (8, 11). In previous experiments, we found that when rats were born under LL, with the same light intensity as that which produces arhythmicity in adult rats, they showed an ultradian pattern in their motor activity for the first 10 days after weaning but later developed and maintained a circadian rhythm (3, 7). This circadian rhythm has a longer period than that of rats born and maintained under DD, as expected by Aschoff rules, and the stability of the waveform depends on light intensity (16).

Because development of the motor activity rhythm of the rat under LL is an appropriate experimental situation in which to study the transition from an ultradian to a circadian rhythm, probably reflecting the development of the circadian system, we planned to study this transition by considering the conditions under which rat pups were maintained before weaning. The ultradian rhythmicity present at the beginning of the record of rats born and kept under LL could be a reflection of an immature circadian system or attributable to the behavior of the dam, which could become arrhythmic under LL. To examine this, we studied the evolution of the motor activity rhythm of rats under LL; the different situations under which they had been maintained during lactation, such as lighting conditions and the rhythmic patterns of the dams, were taken into account.

MATERIAL AND METHODS

Six female Wistar rats (Charles River, France) were supplied to the laboratory by Iffa Credo on *day 16* of gestation. The rats were housed individually in transparent Makrolom cages (50 × 25 × 12 cm), under a 12:12-h LD cycle with light intensity between 270 and 300 lx. Rats were maintained under these conditions until delivery (5 days later). When pups were 1 day old, the litters were cross-fostered in such a way that each litter contained approximately the same number of males (between 5 and 6) and females (between 5 and 6), and each dam fed at least one pup of each original offspring. The day after delivery, three of the new litters were transferred to DD (0.5 lx of dim red light) and the other three to LL (270–300 lx). For each light condition, one of the dams was blinded by optical enucleation (B) to avoid the influence of light on the rhythm of the dam, another was allowed access to food for only 3 h/day (RF) to synchronize the rhythm of the dam, and the other was used as control (C). Rats were kept under these conditions until weaning.

Twenty-two days after birth the pups were weaned, and four males and four females from each litter were separated from the dams and isolated in individual cages (25 × 25 × 12 cm) with water and food ad libitum. From this day on, all the animals were subjected to LL, and their motor activity was recorded. The groups of animals considered in the experiment were *1) DD-C group*, maintained under DD during lactation, dam intact; *2) DD-B group*, maintained under DD during lactation, dam blinded; *3) DD-RF group*, maintained under DD during lactation, dam subjected to food restriction; *4) LL-C group*, maintained during lactation under LL, dam intact; *5) LL-B group*, maintained during lactation under LL, dam blinded; and *6) LL-RF group*, maintained during lactation under LL, dam subjected to food restriction. All the

groups included four males and four females. except *LL-RF*. All of the pups in *group LL-RF* died in the first 20 days except one male and one female. When they were 22 days old, these two animals weighed less than the animals in the other groups: therefore, they were not weaned until they were 32 days old. They were then transferred to individual cages, and motor activity was recorded as for the other groups. Because of failures in the detection system in the *DD-RF* group, the motor activity of one rat was not recorded. Thus this group included four males and three females.

Approximately every 10 days, the cages were cleaned and the rats were weighed. The motor activity of each rat was recorded by an optical detection system of two crossed perpendicular infrared beams, situated on a plane 3 cm above the floor of the cage. Data were automatically recorded every 15 min and saved on floppy disks for further analysis. The motor activity of the dams was recorded from *day 17* of pregnancy until the day of delivery under LD and from the day of delivery until the day of weaning under LL or DD, depending on the group. The motor activity of the pups was recorded individually from the day of weaning and for 55 days under LL as well as by crossed infrared beams.

Mathematical and statistical analysis. The period of the motor activity rhythm was determined by the periodogram of Sokolove and Bushell (14). For the dams, the period of the rhythm was determined using data for the first 15 days after delivery, because after this day no circadian rhythm was observed. The period of the rhythm of the pups was determined using data from *day 15* through *day 55* after weaning. Moreover, to study the evolution of the rhythm, a periodogram was applied using data from several stages of the record: from *day 1* to *day 11*, from *day 15* to *day 30*, and from *day 31* to *day 50*. The percentage of variance explained by the highest peak obtained in the periodogram was used as an indicator of the presence of the circadian rhythm in the mot activity data.

To study the evolution of the ultradian periodicities, sequential spectral analysis was carried out with 15 harmo ics for each daily data set. To test the effects of lighti conditions and those of the different types of dam, an analy of variance (ANOVA) was performed that considered t percentage of variance obtained in the periodogram as t dependent variable. The sex of the rat, lighting conditio during lactation (LL or DD), and type of dam (B, C, or R were the independent variables. The exact Fisher probabil test was applied to compare the number of animals th manifested circadian rhythms in two different groups. In t case the presence of the rhythm was a dichotomous varia and was considered present if the highest peak of t periodogram was statistically significant ($P < 0.05$).

RESULTS

The motor activity rhythm of the dams is shown Fig. 1. At the end of the lactation period, the rhythm the dam could not be detected clearly. Spectral analys shows that the circadian harmonic was most importa until *day 10* after delivery, when the pattern beca ultradian. Therefore, the period of the rhythm of t dams was calculated using the data from the day af delivery until 15 days afterward. The periodogr analysis applied to the motor activity data from t first 15 days after delivery shows a significant circadi rhythm in all cases. In the case of the dams subjected restricted feeding, the highest peak was near 24 h: 2 for *LL-RF* and 23 h 50 min for *DD-RF*, which could attributable to the phase shift of the rhythm unti

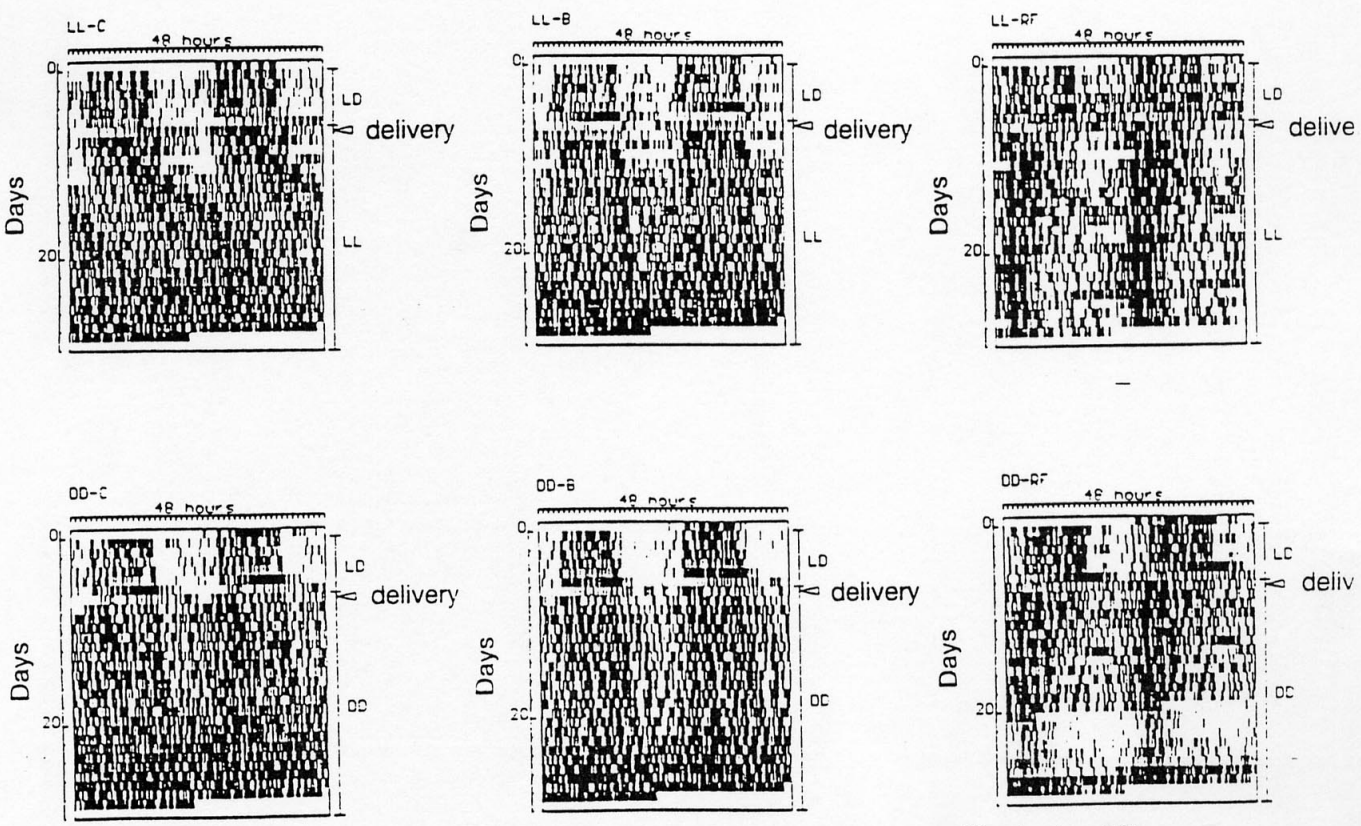

Fig. 1. Double-plotted actograms of the 6 dams (recorded with the litters). LL, continuous illumination: DD, continuous darkness: C, control: B, blinded: RF, food restriction: LD, light-dark.

stable phase relationship with the time of access to food is reached. The *DD-C* dam and the two blind rats showed a period slightly longer than 24 h (*DD-C*, 24 h 20 min; *DD-B*, 24 h 16 min; *LL-B*, 24 h 15 min). The *LL-C* dam, as expected, showed the longest period (25 h 55 min).

All pups that were maintained under LL during lactation (Fig. 2) developed clear circadian rhythms after weaning. Just after weaning, pups followed an ultradian pattern: the circadian rhythms appeared 10 days later and were maintained until the end of the experiment. On the other hand, all the rats that during lactation were kept under DD (Fig. 3) showed arhythmicity in their motor activity.

The periodogram analysis, calculated from *day 15* to *day 55* after weaning (Table 1), shows that all the 18 rats from the LL groups had a significant circadian rhythm, the mean period of which was 25 h 27 min (SE, 2.7 min). Most of the rats from the DD groups were arrhythmic, but in some cases the periodogram shows a significant peak (8 rats of 23). The number of animals with this peak depended on the lighting conditions during lactation ($P < 0.001$); this finding indicates that the circadian rhythm was more prevalent in LL rats than in DD rats. However, there were no differences in the number of rats that manifested a circadian rhythm based on the type of dam (see Table 1). Moreover, the percentage of variance of the motor activity data explained by the highest peak in the periodogram of the DD groups is significantly lower than that of the LL rats. ANOVA shows that the percentage of variance of the highest peak in the periodogram depends on lighting conditions ($P < 0.001$) and sex ($P < 0.05$); the females showed a higher percentage of variance than the males. To confirm these effects, we took into account that the RF group had fewer animals than the others and calculated an ANOVA excluding the values that corresponded to all the RF rats. This analysis shows no differences from the previous one: lighting conditions ($P < 0.0001$) and sex ($P < 0.05$), but not type of dam (control or blind), were statistically significant.

To study the presence of the circadian rhythm through the age of the rat, a periodogram analysis was carried out for the data corresponding to three different stages: *1) days 1–11, 2) days 15–30,* and *3) days 31–50.* We used this number of days because 15 cycles are enough to calculate reliably the period of a rhythm by using the periodogram of Sokolove and Bushell (14). However, because most LL rats develop a circadian rhythm after *day 10* (3, 7), for the first stage we used data corresponding to 11 days. The number of animals of the LL or DD group that showed circadian rhythmicity in these different stages is shown in Table 2. The exact probability test for the distribution of animals with significant rhythm between the two groups for each of these stages shows nonsignificance in the first stage, significance ($P < 0.05$) in the second stage, and significance ($P < 0.001$) in the third stage. This finding suggests that the differences between the two groups are not seen at the beginning of the record but appear later.

DISCUSSION

Our results show that the lighting conditions under which the rats are maintained during lactation influence the manifestation of the circadian rhythm under constant light. In other words, the rats exposed to LL during lactation develop a circadian rhythm under LL after weaning, but those exposed to DD during lactation do not. The rhythmicity of the dam seems to have insignificant influence on the manifestation of the rhythm of the pups.

All the groups in this study were formed by pups from six different litters, all of which came from pregnant rats maintained under LD. Thus we cannot interpret the differences in the manifestation of the rhythm as genetic differences or as differences caused by the conditions during gestation. It has been suggested that the rhythm of the mother plays a role in the synchronization of the rhythm of the pups (5), but the dam's rhythm does not seem to be important in the expression of the circadian rhythm of the pups. The effect of the synchrony of the mother cannot be evidenced in our experiment because of the lack of circadian rhythms in all the rats at the beginning of the record and during the whole record in the DD group. Moreover, the presence or absence of the circadian rhythm seems to be independent of the rhythm of the dam. All the dams show a circadian rhythm in their motor activity during at least the first 2 wk of lactation. However, because of the method we used, during the last days of the record no circadian rhythm could be detected. We cannot know if the dams became ultradian during the last days of lactation because they adapted their rhythms to those of the pups or if they maintained their own circadian rhythm although the movements of the pups masked their rhythm. In any case, the pups were maintained under a rhythmic environmental situation during the fetal state (LD conditions), during the first 15 days of lactation, and, in the case of RF litters, during the whole lactation period. In all cases, rats follow an ultradian pattern during the first week after weaning, and the power spectra during this first week are similar among the rats of all groups. Only later does the pattern become different, and this difference can be associated with the lighting conditions during lactation; however, we did not find differences associated with type of dam in the variables studied. The low survival rate of the rats in the RF-LL group deserves special mention. This may be attributable to stress caused by LL and RF. When both situations are applied at the same time, the dam probably is unable to feed the pups. However, as seen in the other groups, when RF or LL is applied separately, the nourishment and growth of the pups are not affected. We should take into account that there was only one dam under these conditions, and thus not enough data are available to speculate about the simultaneous effects of LL and RF. Furthermore, one dam of each type is not enough to permit conclusions about the effects of the external conditions on the circadian rhythm.

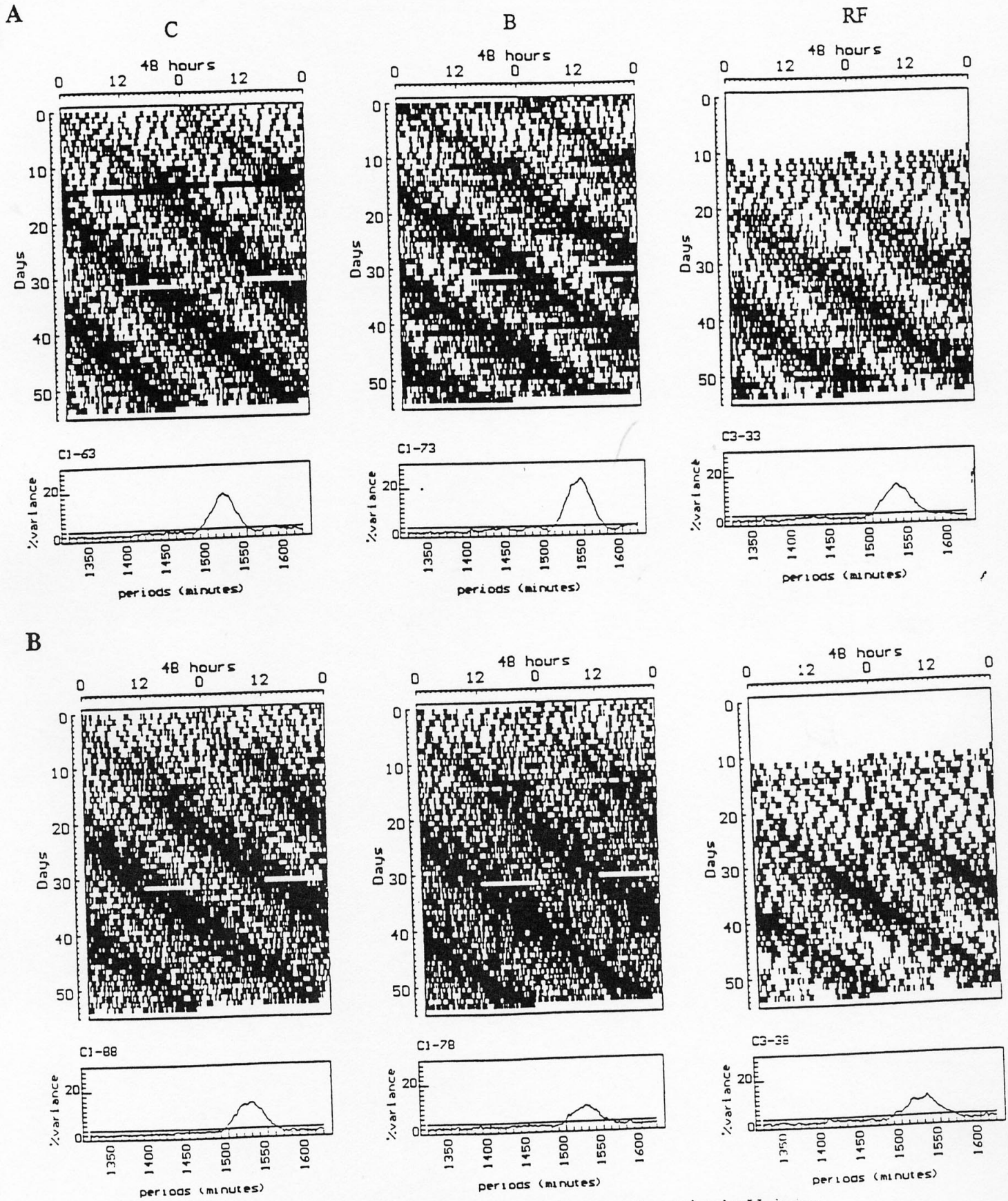

Fig. 2. Double-plotted actograms under LL of 1 representative rat from each group maintained under LL during lactation. The first day of the record corresponds to the day of weaning (22 days old). *A*: females; *B*: males. *Left*: animals from control dams (C); *center*: animals from blinded rats (B); and at *right*: animals from restricted-feeding rats (RF). Periodograms were carried out with data from *days 15* to *55*.

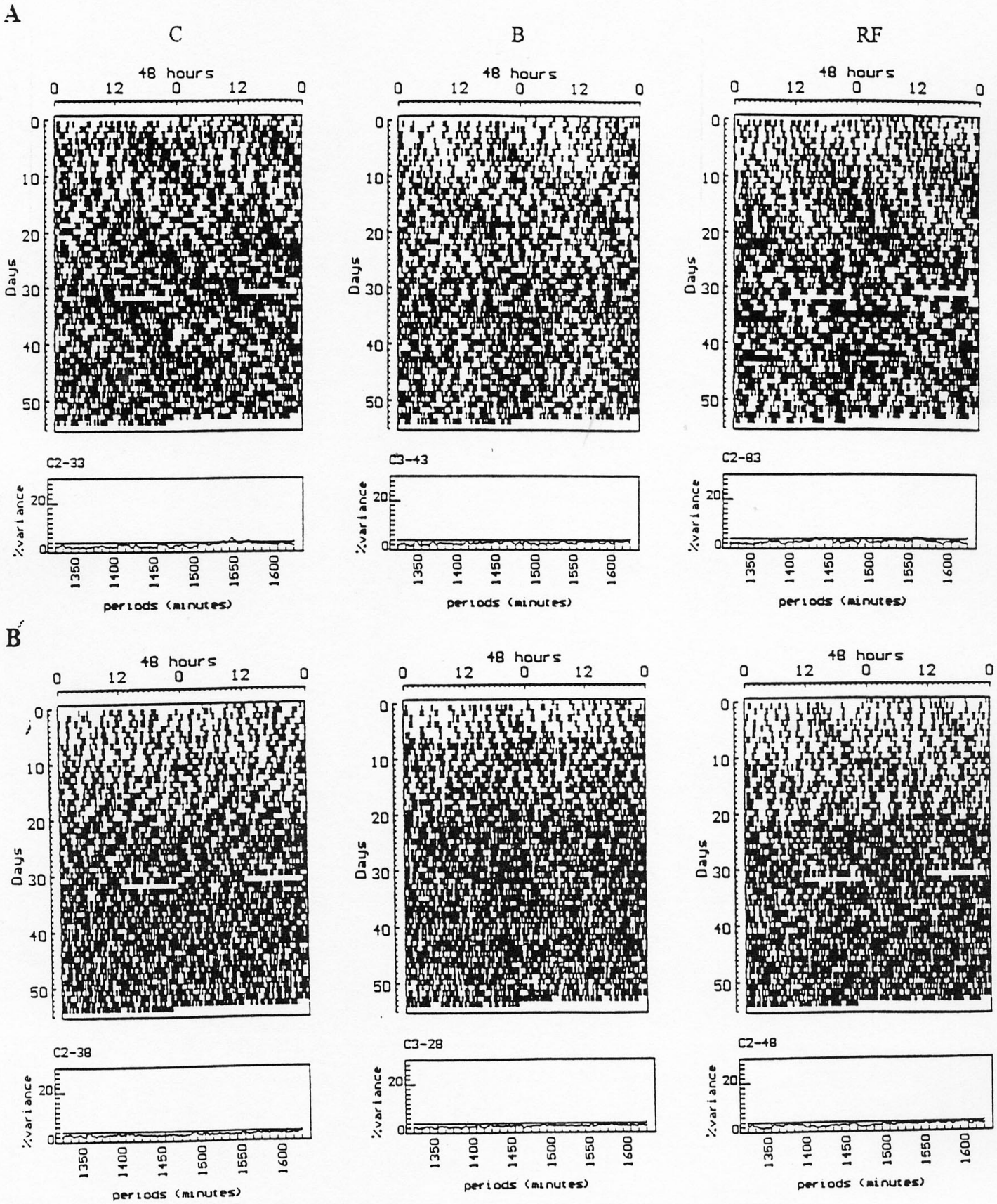

Fig. 3. Double-plotted actograms under LL of 1 representative rat from each group maintained under DD during lactation. The first day of the record corresponds to the day of weaning (22 days old). *A*, females; *B*, males. Periodograms were carried out with data from *days 15* to *55*.

Table 1. *Results obtained in periodogram analysis using data from day 15 to day 55 of the study*

Sex	Lactation in LL				Lactation in DD			
	τ	Mean ± SE	%Var	Mean ± SE	τ	Mean ± SE	%Var	Mean ± SE
				Control				
F	1.555	1.531 ± 8.3	20.9	17.6 ± 2.49	1.540	1.540	5.81	4.90 ± 0.45
	1.530		10.30		NS		4.26	
	1.520		18.71		NS		4.00	
	1.520		20.65		NS		5.55	
M	1.525	1.527 ± 4.3	6.45	10.10 ± 2.28	NS	1.555	4.90	5.26 ± 0.42
	1.520		6.45		1.555		6.45	
	1.540		11.61		NS		4.52	
	1.525		15.87		NS		5.16	
				Blinded				
F	1.535	1.531 ± 5.5	14.19	14.84 ± 3.6	1.530	1.481 ± 24.2	7.74	6.16 ± 0.6
	1.520		13.55		1.460		5.29	
	1.545		24.52		NS		5.16	
	1.525		7.10		1.455		6.45	
M	1.500	1.518 ± 6.3	5.81	10.16 ± 1.57	NS		3.87	4.29 ± 0.15
	1.525		13.29		NS		4.52	
	1.525		10.97		NS		4.26	
	1.525		10.58		NS		4.52	
				Restricted feeding				
F	1.530	1.530	15.48	15.48	1.575	1.565 ± 10	7.74	5.89 ± 0.96
					NS		4.52	
					F		F	
					1.555		5.42	
M	1.525	1.525	12.52	12.52	1.545	1.545	5.81	4.84 ± 0.56
					NS		5.16	
					NS		3.23	
					NS		5.16	

LL, constant illumination; DD, constant darkness; C, control; B, blind; RF, restricted feeding; τ, period corresponding to the highest peak obtained in the periodogram (in min); F, failure in the motor activity recording system; %Var, percentage of variance explained by the highest peak in the periodogram; NS, rhythm was not significant.

To explain the differences between the rhythmic patterns of rats born and raised under LL and DD, we can consider separately the effects of light in the retina and the circadian pacemaker. Although our experiment does not allow us to conclude in this respect, we can suggest two possibilities. Rats born under LL may not be as sensitive to light as the other group, and because of this they are less influenced by LL and do not become arrhythmic. However, it could also be argued that rats maintained under LL suffer retinal damage, which might produce insensitivity to constant light, and therefore they are less responsive to light and can generate a circadian rhythm. This would explain the differences in rhythm manifestations in the two groups. However, this last possibility does not seem probable because previous light history has been reported to strongly determine susceptibility to light damage to the retina (15); consequently, rats born and raised under bright light might have less retinal damage under LL than others born and raised under lower intensities. Thus the animals that are more susceptible to retinal damage would be those born and raised under DD. This contradiction suggests that although retinal damage could be present in one or both groups, the differences between the manifestation of the circadian rhythm in the two groups may be in the pacemaker itself. Perhaps the development of the circadian system depends on environmental conditions.

Table 2. *Distribution of number of rats that manifest a significant or no significant circadian rhythm in 3 different stages of the study*

	No. of Rats		
	Days 1–11	*Days 15–30*	*Days 31–55*
Lactation in LL			
S CR	11	18	17
NS CR	4	0	1
Lactation in DD			
S CR	12	14	6
NS CR	10	9	17

S CR, significant circadian rhythm; NS CR, no significant circadian rhythm. The total number of rats is not constant throughout the stages because there were failures in some activity recording systems on some days.

Adult rats become arrhythmic after several days under LL (8). Here, rats that were under DD during lactation show a pattern similar to that of adult rats and become arrhythmic under LL. This finding suggests that adaptation to the external lighting conditions takes place during lactation because the conditions before birth were presumed to be the same for all of the rats. This is expected because most of the neuronal connections in the circadian system are established during the first 2 wk after birth, i.e., synaptogen-

esis takes place between *day 7* and *day 10*, and some neurotransmitters reach the levels that correspond to those of adulthood during these days (12).

When only the manifestation of circadian rhythmicity is considered, the suprachiasmatic nucleus oscillates during the last days of gestation (13), although the circadian rhythm of most variables is not present until several days after weaning (4). Thus there must be a period during which the circadian system matures. The last part of the process of maturation could be manifested by the transition from an ultradian to a circadian pattern. This could be explained by the establishment of connections between the oscillators that form the circadian system. This phase is very clear in most of the rats, especially in those born and raised under LL, because after weaning they show an ultradian pattern and the circadian rhythm is developed later. However, the effect of light on the circadian system must be added to this evolution. Light is supposed to inhibit the coupling between the oscillators that drive the circadian system (6), and this effect might depend on the state of maturation of the circadian system. In the case of rats maintained under DD during lactation, when they are transferred to LL after they are weaned, the connections between the oscillators of the circadian system may already have been established. Thus light may inhibit this coupling, and an arrhythmic pattern is manifested. When the rats maintained under LL during lactation are taken into account, the inhibiting effect of the light on the coupling may be manifested as a delay in the establishment of the connections but not the prevention of it. This makes the pacemaker functionally adapted to the lighting conditions; therefore, a circadian rhythm appears after several days. This hypothesis fits with the manifestation of the rhythm during the different ages of the pups. All the pups show a motor activity pattern during the first week after weaning, that is predominantly ultradian, although in some pups the presence of a circadian rhythm can be detected. At these stages there are no differences between the two lighting conditions in the manifestation of the circadian rhythm, perhaps because most of the rats are young and the connections of the circadian system may not be completely developed. However, between *day 15* and *day 30*, differences caused by the manifestation of the circadian rhythm between the rats of the two lighting conditions are already present; most of the LL rats, but not the DD rats, show the circadian rhythm. From *day 30* to *day 50*, the differences caused by the manifestation of the circadian rhythm are much clearer. LL rats maintain the circadian rhythm, and DD rats lose it.

In this experiment, as in other reports (17), we found differences attributable to sex in the manifestation of the circadian rhythm. Females showed a higher percentage of variance of the circadian rhythm than males. This difference suggested more importance of the rhythm; however, we cannot suggest a convincing explanation for this. The differences attributable to sex could be related to the different morphology of the suprachiasmatic nucleus (9, 10), but they also could be caused by different sensitivity to light.

In conclusion, this experiment suggests that the external lighting conditions under which the animals are born and raised determine the effect of light on the circadian system. However, further experiments should be carried out to check whether these conditions could produce any stable alteration on the function of the circadian system or the retina itself.

Perspectives

The present experiment shows that the manifestation of the circadian rhythmicity under constant bright light depends on the lighting conditions during lactation. This suggests that the effect of LL, or perhaps that of other factors that influence the circadian rhythmicity of adult rats, depends on the state of maturation of the circadian system. The circadian system of young rats may be adapted to the external (lighting) conditions, generating a circadian rhythm even under certain circumstances that, if applied to an adult rat, could cause arhythmicity.

Further studies should attempt to determine whether this adaptation lasts for the whole life of the animal or is just a transitory effect and whether this adaptation involves changes in the morphology or neurochemistry of the suprachiasmatic nucleus. As a speculation, we can also question the influences of continuous light during the first days of life in humans. For instance, this is the case of preterm infants, who are mostly maintained under constant conditions in incubators. Whether these conditions could produce changes in the circadian manifestation when the infants become adults, and the extent of this change remain to be determined.

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Address for reprint requests: T. Cambras, Unitat de Fisiologia, Facultat de Farmacia, 08028 Barcelona, Spain (E-mail: cambras@farmacia.far.ub.es).

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3.3.- EXPERIMENT 2

BRIGHT LIGHT DURING LACTATION ALTERS THE FUNCTIONING OF THE CIRCADIAN SYSTEM OF ADULT RATS

***American journal of Physiology (Regulatory Integrative Comp. Physiol.278)*: R201-R208, 2000.**

Resum

Objectiu: Les rates criades en llum constant durant l'alletament, al contrari que les rates criades sota condicions de llum-foscor o foscor constant, manifesten un ritme circadiari d'activitat motora quan són sotmeses a llum constant. L'objectiu del present experiment és esbrinar si la prevenció de l'arritmicitat produïda en rates exposades a llum constant durant l'alletament és deguda a la quantitat total de llum rebuda (nombre de dies), o bé a l'exposició a llum constant durant un període crític de l'alletament.

Material i mètodes: Dotze rates Wistar femella van arribar el dia 16 de gestació. El dia del naixement es van barrejar les cries, de manera que van quedar 5 mascles i 5 femelles de diferents ventrades per grup. Els grups es van sotmetre a diferent nombre de dies de foscor constant i de llum constant al llarg del període d'alletament, que va durar 24 dies. El dia del deslletament cadascuna de les cries es va col·locar en una gàbia individual amb un actímetre de feixos d'infraroig, per tal d'enregistrar-ne la seva activitat motora. Totes les rates van ser sotmeses llavors a llum constant durant 55 dies i es van estudiar les característiques del ritme circadiari d'activitat motora. Després foren passades a foscor constant, per estudiar-ne el seu ritme en curs lliure i finalment, per tal d'estudiar la resposta dels animals a un estímul lluminós, els dies 103 i 131 de l'experiment van rebre un pols de llum d'una hora de durada i d'uns 350 lux d'intensitat a les hores circadiàries 15 (CT15) i 22 (CT22), respectivament.

Resultats: Les rates que van rebre menys de 12 dies de llum constant durant l'alletament, al contrari que les que en van rebre més de 12, són majoritàriament arrítmiques sota condicions de llum constant i tenen un endarreriment de fase més important després del pols de llum a CT 15. Els dies concrets en què es va rebre la llum constant durant l'alletament també afecten aquestes variables.

Conclusions: L'expressió del ritme circadiari depèn tant del nombre, com del dia concret en què els animals han estat sotmesos a llum constant durant l'alletament. Sembla doncs, que hi ha un període crític de dies durant les primeres setmanes de vida, durant els quals si els animals són sotmesos a llum constant es podria influenciar el desenvolupament del sistema circadiari, modificant-lo estructural o funcionalment.

Am. J. Physiol. Regulatory Integrative Comp. Physiol.
278: R201–R208, 2000.

Bright light during lactation alters the functioning of the circadian system of adult rats

M. M. CANAL-CORRETGER, T. CAMBRAS, J. VILAPLANA, AND A. DÍEZ-NOGUERA
Departament de Fisiologia-Divisió IV, Facultat de Farmàcia,
Universitat de Barcelona, 08028 Barcelona, Spain

Canal-Corretger, M. M., T. Cambras, J. Vilaplana, and A. Díez-Noguera. Bright light during lactation alters the functioning of the circadian system of adult rats. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* 278: R201–R208, 2000.—To examine the role of light in the maturation of the circadian pacemaker, twelve groups of rats were raised in different conditions of exposure to constant bright light (LL) during lactation: both duration and timing of LL were varied. We studied the motor activity rhythm of the rats after weaning, first under LL and then under constant darkness (DD). In DD, two light pulses [at circadian time 15 (CT15) and CT22] were applied to test the response of the pacemaker. Greater exposure to LL days during lactation increased the number of rhythmic animals and the amplitude of their motor activity rhythm in the LL stage and decreased the phase delay due to the light pulse at CT15. The timing of LL during lactation affected these variables too. Because the response of the adult to light depended on both the number and timing of LL days during lactation, the exposure to light at early stages may influence the development of the circadian system by modifying it structurally or functionally.

development; motor activity

THE CIRCADIAN SYSTEM PROVIDES organisms with temporal organization. In mammals, the circadian system consists mainly of the suprachiasmatic nuclei (SCN) of the hypothalamus but also of structures such as the retina (26). The circadian system generates circadian rhythms, synchronizes them to environmental factors such as light, and transmits this rhythmic pattern to physiological processes and behavior. As light-dark alternation is the main zeitgeber for the circadian system, many physiological variables under natural lighting conditions show a 24-h rhythm that synchronizes to the external zeitgeber. Under constant conditions, such as constant darkness (DD), daily rhythms usually deviate slightly from 24 h. The period of this free-running rhythm, which is very stable, is known as τ and varies according to species and individuals. However, under constant bright light (LL), the circadian rhythm of most animals is not that established, because splitting (4, 8) and many ultradian components in the pattern of motor activity rhythm appear. In rats, after a long exposure to LL, the circadian periodicity disappears, as do rhythms of motor activity (9, 15, 18), plasma melatonin (18), sexual hormones (34), and body temperature (9, 11, 15).

Previous experiments indicate that the arrhythmicity under LL can be prevented: rats reared under LL during all their lactation period showed, after weaning, a circadian rhythm of motor activity under LL that emerged from an ultradian pattern (5, 6). The period of this rhythm under LL is much longer than that under DD and lasts most of the life span of the animal, especially in females (7). These results reveal the influence of the lighting conditions during lactation, when the circadian system matures. This is not surprising, taking into account that although SCN neurons are already formed in the embryo (1) and are rhythmic before birth (25), various studies demonstrate that the general maturation of the SCN is postnatal. It develops rapidly from the time when the neurons are formed until postnatal *day 10* (P10), and more slowly to adulthood. The synaptogenesis increases until P10, when the number of synapses per unit of SCN area is the same as in adulthood. However, the volume of the SCN grows between P10 and adulthood, which may be due to the extension of some dendritic processes (see Ref. 21 for review). Moreover, the pathways that bring light information to the SCN are present at P4 but develop until P10 (19, 29). Thus, in the early life of the rat, during lactation, the SCN reaches its full outgrowth, which suggests that its functionality might be modified at distinct development stages by the effect of external factors such as light.

The purpose of this experiment was to find out whether the prevention of arrhythmicity due to LL exposure during the lactation period was related to the amount of light (no. of days in LL) that the animal had received during this period or whether it was due to the effect of LL during some critical days of development. We therefore subjected rats to a fixed number of days of constant bright light during the lactation stage. In the adult rats, the motor activity pattern under LL and under DD and also the phase shifts induced by a light pulse were studied.

MATERIALS AND METHODS

Twelve pregnant Wistar rats (Criffa, France) were brought to our laboratory on *day 16* of gestation. The rats were housed in individual transparent Makrolom cages (50 × 25 × 12 cm) under a 12:12-h light-dark (LD) cycle (with a light intensity of ~300 lx). They remained in these conditions until delivery, 5 days later. When all the pups were born, they were cross-

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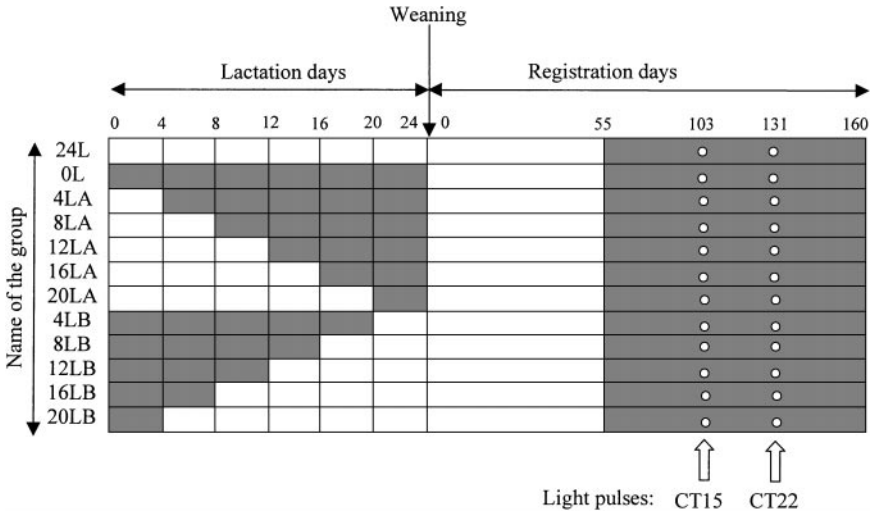

Fig. 1. Scheme of the groups of rats and the lighting conditions during the experiment. The first 24 days correspond to the lactation stage, and the remaining days correspond to the days when motor activity was registered. Open areas, constant light (LL); shaded areas, constant darkness (DD). CT15 and CT22 represent the 2 circadian times when a light pulse was applied. See MATERIALS AND METHODS for descriptions of groups.

fostered so that each dam fed one group of rats, made up of five males and five females [except *groups 12LA* and *8LB* (see Fig. 1), which each had 6 males and 4 females] belonging to different litters.

The new litters (each one was an experimental group of pups) were subjected to DD (<0.1 lx of dim red light) or LL (~300 lx) for a different number of days during lactation, which lasted 24 days. Thus pups of each group were called after the number (0, 4, 8, 12, 16, 20, or 24) and timing (A groups, LL close to birth; B groups, LL close to day of weaning) of those LL days during lactation (see Fig. 1). For instance, *group 4LA* remained under LL the first 4 days of lactation and then was under DD until *day 24*; *group 8LB* remained under DD the first 16 days of lactation and then was under LL the last 8 days of lactation.

At 25 days old (*day 1* of the experiment), the pups were weaned and isolated in individual cages (25 × 25 × 12 cm). From this day on, and until the end of the experiment, motor activity was detected by means of an actimeter using two crossed perpendicular infrared beams situated on a plane 3 cm above the floor of the cage. Motor activity counts were automatically recorded every 15 min in a personal computer by means of a data-acquisition system developed in our department.

Rats were fed commercial chow (Rodent Toxicology Diet, B&K Universal) and tap water ad libitum. Approximately every 10 days, the cages were cleaned, and until experimental *day 78* the rats were weighed.

After weaning, all the rats were maintained under LL for 55 days to examine the appearance of a circadian rhythm under this condition. They were then all shifted to DD, to study the free-running rhythm. Rats remained under DD until *day 160* of the experiment. The DD situation was also used to test the responsiveness of the circadian system of each animal to a light pulse. Thus, on *day 103* of the experiment, all the rats received a 1-h light pulse of a mean intensity of 345 lx, at circadian time 15 (CT15); on *day 131*, a second light pulse of the same intensity and duration was given at CT22. The phase shifts were calculated in both cases.

To determine the exact hour (local time) when the light pulses were to be applied, the time of activity onset (or CT12) was independently estimated from the actograms for each animal by four investigators by visual estimation of the rest-activity transition; mean values were used. CT15 was the result of adding 3 circadian hours to CT12; for CT22, we added 10 circadian hours to CT12.

Mathematical and statistical analysis. The circadian rhythm was separately studied for the LL stage and for the DD stage. To determine the presence of motor activity rhythm and its period, we used Sokolove and Bushell's periodogram (28), with a low global level of significance ($P = 0.01$) to reject spurious peaks.

In the LL stage, the rhythm was determined using data from *day 15* to *day 55*; for the DD stage, data were from *day 74* to *day 102*. The first days of each stage were excluded to ensure a stable motor activity pattern in the analyzed data. An animal was considered to be rhythmic only when the period of its circadian rhythm in the periodogram was statistically significant. The percentage of variance explained (PVE) by the highest peak (significant or not) obtained in the periodogram was used as an indicator of the importance of the motor activity rhythm. Moreover, in the LL stage, a Fourier analysis was applied to the data, using the period of the highest peak of the periodogram of each rat as the period of the fundamental harmonic. The amplitude of the first harmonic, and the sum of the power content of the first five harmonics (PC5H), also showed the importance of the circadian rhythm.

Phase shift responses due to a 1-h light pulse in the activity rhythm were determined by drawing eye-fitted lines through the daily onsets and offsets (calculated separately) of activity for the 10 days before and the 10 days after treatment. The phase shifts resulted from the difference between the two lines at the day after the light pulse. These phase shifts were calculated independently by four researchers, and the mean values for each pulse were used for further statistical analyses.

Statistical analysis was carried out by ANOVA of several linear models (Systat). In all the models, the independent variables were sex, number of days under LL (considered categorical), and five other variables used to estimate differences within the groups that had 4, 8, 12, 16, or 20 days of light, depending on the timing of the LL days in the lactation stage (A and B groups). In this way, we tested the influence of the different number of LL days and determined whether the timing of the LL days in the lactation period was significant. The dependent variables were body weight increase; period, PVE, amplitude, and PC5H in the LL stage; period and PVE in the DD stage; and the delays and advances in the onset and offset occurring after the light pulses at CT15 and CT22, respectively. Each dependent variable was analyzed in a separate model.

Moreover, to determine whether the variables studied had a linear relation with the number of LL days, we applied the above model, but using the number of days under LL during the lactation as a quantitative independent variable.

Graphs and calculations were carried out using the integrated package for chronobiology analysis, El Temps (A. Díez-Noguera, Universitat de Barcelona, 1999).

RESULTS

The double-plotted actograms (see Fig. 2) show the general traits of the activity of the rats. At first sight, three distinct patterns of motor activity can be differentiated in the LL stage, independently of the group: some rats (e.g., Fig. 2*A*) developed a clear circadian rhythm; other rats (e.g., Fig. 2*C*) showed an initially weak circadian rhythm, which gradually became completely arrhythmic; finally, a third group of rats (e.g., Fig. 2, *B* and *D*) did not show a circadian rhythm of motor activity. In the DD stage, all the rats manifested a clear circadian rhythm, a phase delay after the light pulse at CT15 and a phase advance after the light pulse at CT22.

On studying the manifestation of rhythmicity under LL, it can be seen that the number of rhythmic rats (rats with a statistically significant peak in the periodogram) and nonrhythmic rats differs between groups, depending on the number of LL days during lactation. The rats from groups subjected to less than 12 LL days

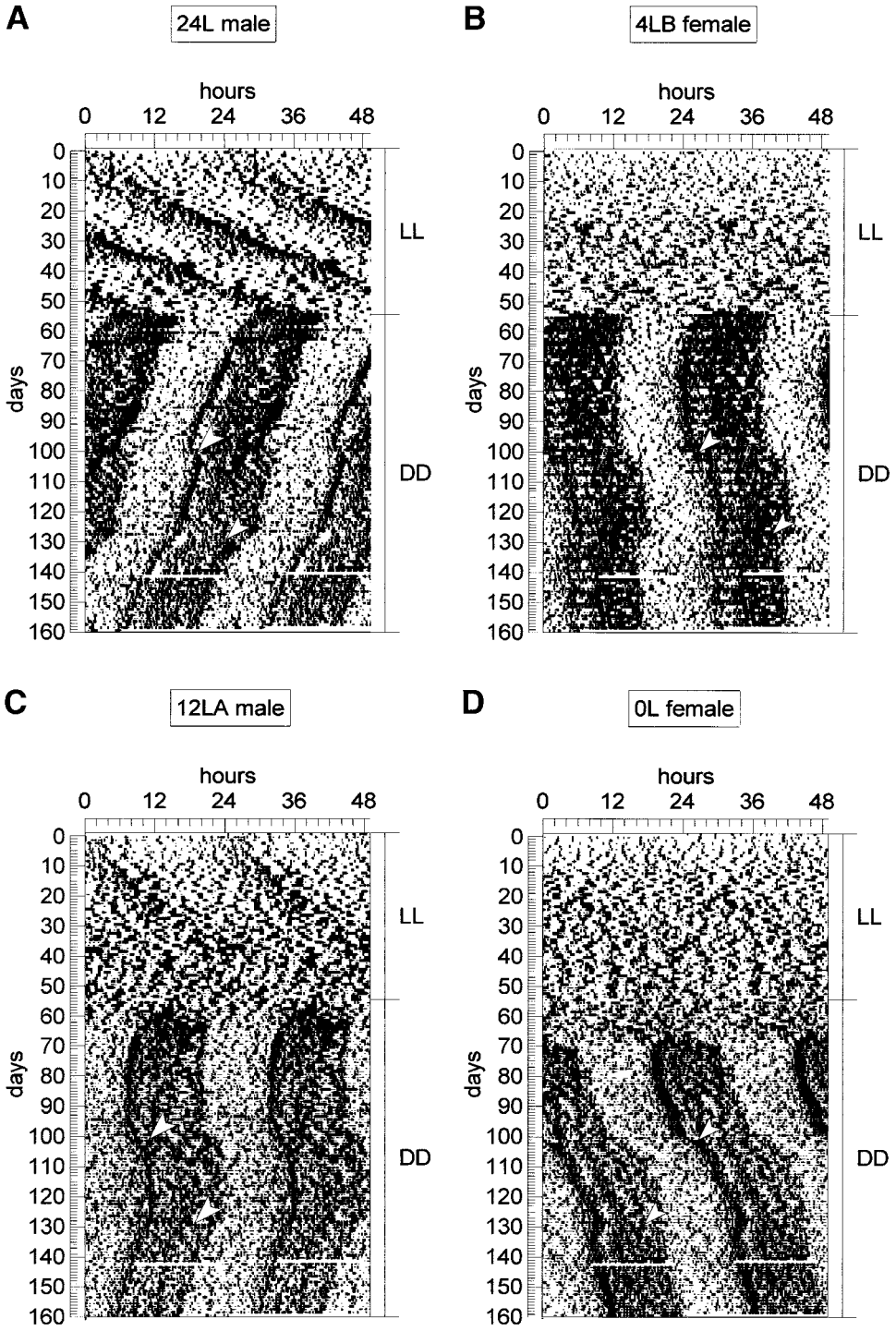

Fig. 2. Double-plotted actograms representing motor activity rhythm of 4 representative rats. Arrowheads indicate 1-h light pulses, the first at CT15 and the second at CT22. See MATERIALS AND METHODS for descriptions of groups.

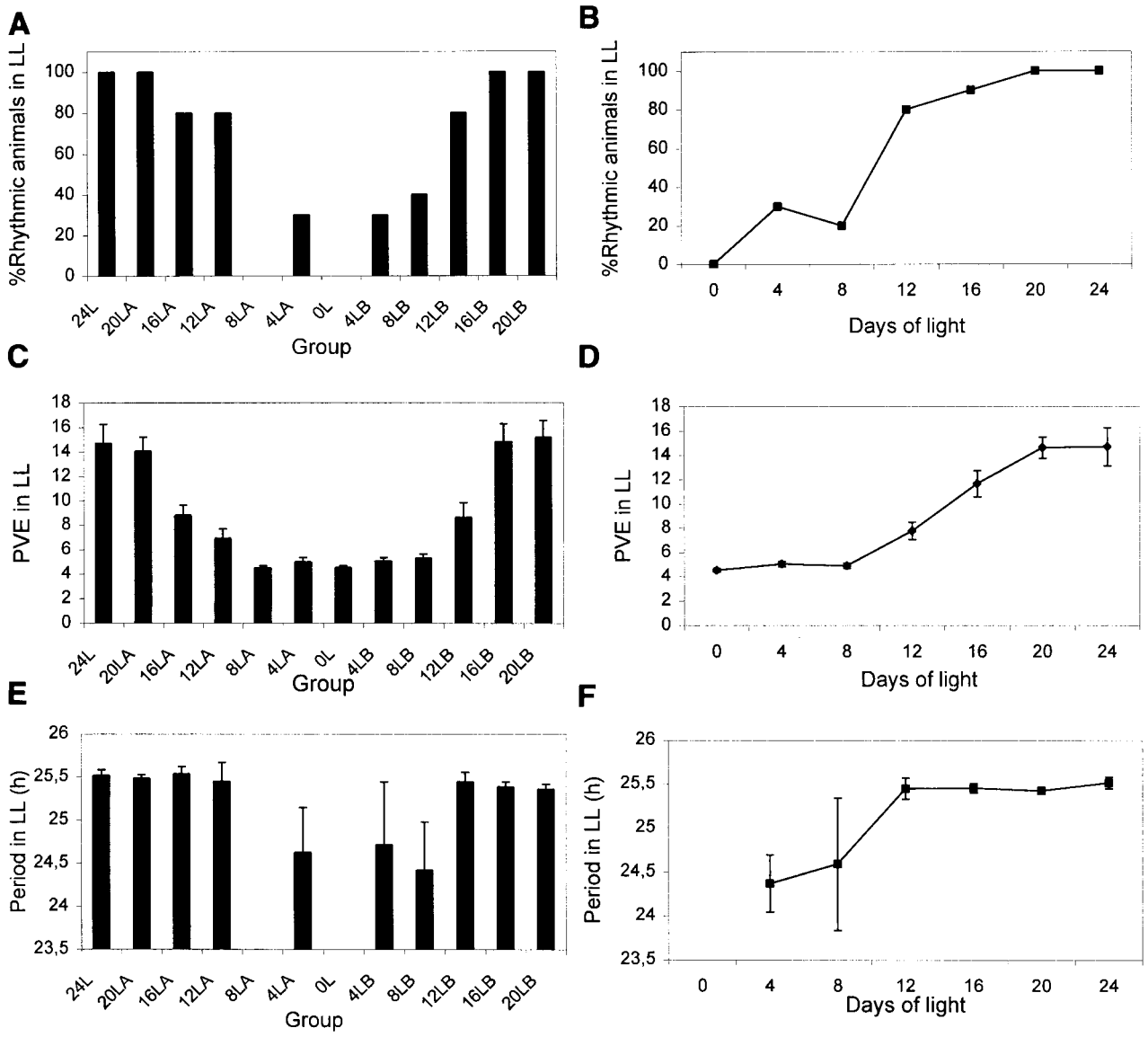

Fig. 3. Variables studied in LL stage (means ± SE). *A* and *B*: percentage of animals with significant rhythm. *C* and *D*: percentage of variance explained by the rhythm (PVE). *E* and *F*: period of motor activity rhythm. *Left*: value of variable for each group of rats. *Right*: variable related to no. of days under LL. See MATERIALS AND METHODS for descriptions of groups.

were mainly arrhythmic (8 rhythmic, 42 arrhythmic), whereas rats that received 12 or more LL days were mainly rhythmic (64 rhythmic, 6 arrhythmic) (Fig. 3, *A* and *B*).

The PVE of the highest peak in the periodogram of the LL-stage data was different for each group. It is related to the number of days under LL during the lactation period ($P < 0.001$) but does not depend on the sex of the rat. Thus, the higher the number of LL days, the higher the PVE. We also found differences between *groups 16LA* and *16LB*; the latter had a higher PVE ($P < 0.001$) (see Fig. 3, *C* and *D*). Amplitude and PC5H behave like the PVE, that is, they rise with an increasing number of LL days during lactation and do not depend on the sex of the animal, and there are also differences between *groups 16LA* and *16LB* (data not shown).

The period of the rhythm under LL did not depend on sex, but it did depend on the number of days of LL during lactation (more days of light imply a longer period under LL) (see Fig. 3, *E* and *F*). It should be borne in mind that for the study of this variable, we only included the values of the rats whose rhythm was statistically significant. As a result, there were few rhythmic rats (8 of 50) in the groups that had 8 or fewer days of LL during lactation. Thus care must be taken when interpreting the correlation of this variable with the number of LL days, because there were no differences between groups with 12 or more LL days during lactation (64 rhythmic rats of 70). The mean value of the period in LL for all of the rhythmic animals was 25 h, 21 ± 2.75 min (mean ± SE).

When transferred to DD all rats generated a stable circadian rhythm. Most of the rats acquired this stable rhythm immediately after being moved to DD (Fig. 2, *A* and *B*), but in some of the rats that were arrhythmic in the last days of the LL stage (Fig. 2, *C* and *D*), the

appearance of their rhythm under DD was delayed for more than 3 days after the lights were switched off.

Under DD, period values of the motor activity rhythm (24 h, 39 ± 0.69 min) were not dependent on sex or on the number of days of LL during the lactation stage (see Fig. 4, *A* and *B*). However, the animals that received more than 12 LL days during the lactation showed a longer period (24 h, 42 ± 0.94 min) than the others (24 h, 38 ± 0.91 min); the difference was statistically significant (Student's *t*-test, $P < 0.05$). PVE under DD

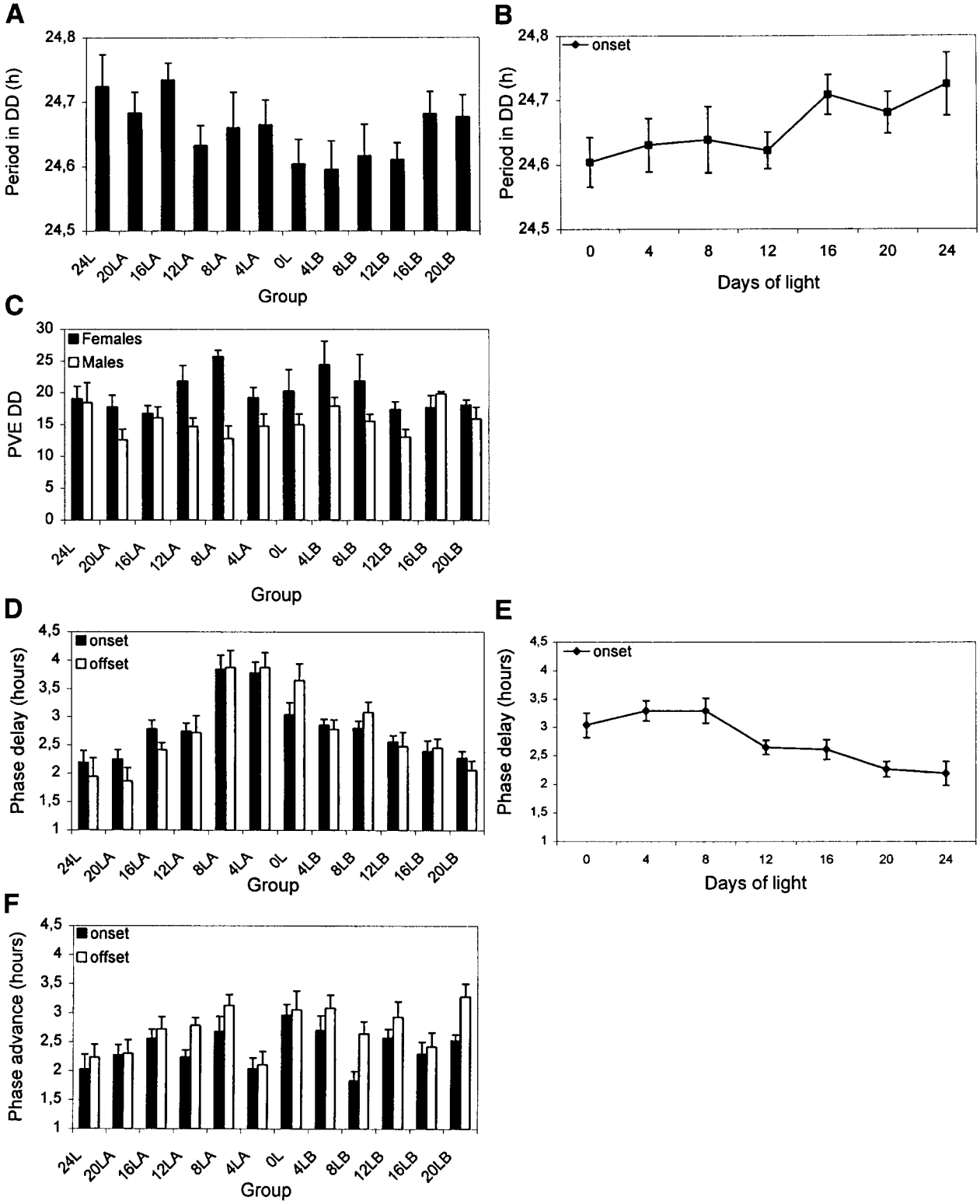

Fig. 4. Variables studied in DD stage (mean ± SE). *A* and *B*: period of motor activity rhythm. *C*: PVE. *D* and *E*: phase delays after light pulse at CT15. *F*: phase advances after light pulse at CT22. *Left*: value of variable for each group of rats. *Right*: variable related to no. of days under LL (graph is missing when no correlation was found).

only depends on the sex of the animal ($P < 0.001$); the females had a higher PVE than the males (see Fig. 4*C*).

After the light pulse at CT15, a phase delay (mean ± SE = 2 h, 51 ± 4 min) was observed in all the animals, in both onset and offset of motor activity rhythm. A correlation between the phase delay and the number of LL days was found (see Fig. 4, *D* and *E*): more LL days during the lactation stage implies a smaller phase delay ($P < 0.05$). Differences can also be seen between *groups 4LA* and *4LB* ($P < 0.005$) as well as between *groups 8LA* and *8LB* ($P < 0.005$): the ones that started the lactation period under LL (*groups 4LA* and *8LA*) have greater phase shifts than their corresponding couple. Anyway, no significant differences were found between the phase delays calculated using the onset or the offset of the rhythm, and so only the onset is shown in Fig. 4*E*.

Light pulse given at CT22 (see Fig. 4*F*) caused a phase advance (mean ± SE = 2 h, 42 ± 4 min) of the motor activity rhythm in all the rats, but there was no correlation with the number of LL days during lactation. In this case, there were differences between the values of the phase advance calculated using the onset and the offset ($P < 0.001$).

At *day 50* of the experiment we found no differences in body weight due to the lighting conditions during lactation; differences were only related to the sex of the animal.

DISCUSSION

Constant light provokes the loss of circadian rhythmicity in adult rats in some variables such as motor activity (9, 15, 18). However, in past experiments we observed that adult rats exhibited a circadian rhythm of motor activity under LL if previously subjected to LL throughout their entire lactation period (5–7). Therefore it appears that, at least in rats, the lighting environment at the age when the circadian system is developing plays a critical role in the manifestation of the rhythm of the adult animal. In the present study, we have not only corroborated the former results but have also demonstrated that the number of LL days is critical; in particular, 12 or more days of LL during the lactation period seem to be needed to elicit a circadian rhythm of motor activity under LL in adult rats. Rats that had fewer than 12 LL days during lactation were mainly arrhythmic and showed a lower amplitude of rhythm under LL. The present study also suggests that there is a critical stage during the lactation in which light affects the circadian system.

Taking into account that the circadian system is formed by coupled individual oscillators (3, 10, 20, 35), the arrhythmicity observed in some adult rats subjected to LL could be interpreted as the loss of the coupling between the oscillators. In our experiment this can explain why most of the rats that are arrhythmic under LL, although they all developed a circadian rhythm under DD, take some time after lights off to manifest the endogenous rhythm. Probably the absence of light forced the oscillators to couple and to oscillate synchronically. In the case of rats that are rhythmic under LL, we may assume that if light is given when the oscillators are establishing their coupling, this coupling will become strong enough to generate and maintain a circadian rhythmicity. We can thus suggest that in this last group, the pacemaker is robust enough to endure the effects of light, and therefore their oscillators remain coupled.

Although the nature of the oscillators remains unknown, some hypotheses regard neurons as the principal candidates and suggest that the glial cells could be responsible for the coupling among these oscillators (35). Hence, as the complete morphological and functional maturation of these cells, as well as the synaptogenesis and the development of the SCN afferences [i.e., from the optic chiasm (retina) and the intergeniculate leaflet] take place during lactation (see Ref. 21 for review), this stage becomes decisive for the normal development of the circadian system. In fact, our findings indicate that LL during lactation results in a more robust pacemaker, which is less susceptible to the inhibiting effects of light on the manifestation of the circadian rhythm. More precisely, greater exposure to LL during lactation implies *1*) fewer arrhythmic adult animals under LL, *2*) higher amplitude of the circadian rhythm under LL, and *3*) smaller phase shifts.

Despite the differences between groups in the phase shift after a light pulse, the values of the phase advances and delays (~3 h each) observed here fit with the values found previously by Honma et al. (16) in Wistar albino rats, taking into account that in our experiment the duration and intensity of the light pulse were higher. In fact, Gander et al. (14) demonstrated that the higher the duration of the light pulse, the higher the phase shift.

In this experiment we have found sexual differences in the manifestation of the rhythm in the DD stage (in PVE), but not in the LL stage. This lack of sexual differentiation in the rhythm under LL seems to disagree with our previous experiments (5), in which the PVE of females was significantly higher than that of males. We trust that the reason for this disagreement is that in the present experiment the number of rats per group was smaller than in the previous one and those sexual differences in the rhythm could not be detected. Under DD, as the rats are older and consequently sexual maturation has been achieved, the sexual differences in the manifestation of the rhythm should be more pronounced and, thus, detected.

The motor activity rhythm is influenced not only by the number of LL days during lactation but also by the timing of these days. This hypothesis is supported by the differences found between A and B groups (e.g., differences between *groups 16LA* and *16LB* in PVE, amplitude, and PC5H of the rhythm in the LL stage). If the development of the circadian system is a continuous process, the effect of a certain number of days of LL may change, according to the stage of development at which it is applied. Because *groups 4LA*, *8LA*, and *0L* have a similar behavior, it can be assumed that light applied the first 8 days of the rat's life does not affect the later expression of the circadian pacemaker. We

may consider that before postnatal *day 8*, the circadian system is too immature to perceive, transmit, or manage the surrounding light information. Likewise, as *groups 20LA* and *4LB* compared with *groups 24L* and *0L*, respectively, show a similar pattern in the manifestation of the rhythm, we may assume that from 20 days of age the development of the circadian system is not influenced either by darkness or by light. Therefore, a window of effectiveness of light on the biological clock could be placed between *day 8* and *day 20* after birth.

Actually, in rats, the synaptogenesis between the SCN cells and the expansion of the geniculohypothalamic tract (GHT) take place between postnatal *day 4* (P4) and P10 (22), and the number of projections of the retinohypothalamic tract (RHT) to the SCN increases gradually from P1, achieving the adult pattern between P10 and P15 (29). Also, on P20 the vasoactive intestinal peptide mRNA signals produced by the SCN neurons (2) and the number of neuropeptide Y-immunoreactive fibers originating from the GHT (33) reach their adult stage. This indicates that before P20 the main events of the development of the circadian system take place, and thus this period may be more influenced by light.

However, light is not the only zeitgeber for the biological clock at this early age. Honma et al. (17), through the use of restricted feeding as a zeitgeber to the mother and by analyzing the pups' locomotor pattern, suggested that the first postnatal week is ineffective for maternal entrainment and that the critical period extends until the end of the second postnatal week. In a similar way, Takahashi et al. (32) proposed that for the blinded pups to be entrained by a foster mother, nursing had to start before 10 days of age and be continued for more than 10 days. Thus there is general agreement that the critical period for sensitivity and ability to adapt to external factors may be located, at least in rats, in the middle of the lactation stage.

The retina is a component of the circadian system that plays an important role in the sensitivity and ability of the circadian pacemaker to adapt to the external environment, as it is the only photoreceptor organ in mammals (loss of the eyes blocks all the circadian responses to light) (23). The retinal input may be important for a normal morphological formation of the SCN during development (31). In fact, the SCN of the hereditary microphthalmic rat (the retina of which is seen as a cyst and lacks the optic nerve) has a shorter length, lower total volume, and fewer neurons than the SCN of normal rats, but this does not prevent it from generating circadian and ultradian rhythms (30). Therefore, if the pacemaker itself is altered, the sensitivity of the circadian system to light may also be affected. Moreover, Foster and co-workers (13, 24) found that despite extensive damage of their visual photoreceptors and loss of visual function, *rd/rd* mice (mice whose rods and cones suffer a massive degeneration) still showed circadian responses to light that are indistinguishable from those of mice with normal retinas. On the other hand, *rdta* mice, whose rods degenerate during ontogeny because of a fusion gene integrated in the genome (12), showed 2.5-fold greater shifts than wild-type mice, at irradiances that produce saturating phase shifts in the wild-type mice. The only difference between the two strains of retina-degenerated mice is the time at which the onset of rod ablation takes place (1 wk earlier in *rdta* mice than in *rd/rd* mice). It has been suggested (27) that the earlier loss of rods in the *rdta* mice alters the amplitude of clock responses to light but does not change the sensitivity of the clock to light, possibly because the loss of photoreceptors occurs when the retina and/or its central projections are still plastic enough to permit some reorganization. In our experiment, we have observed, on the one hand, that both the manifestation of the rhythm under LL and the value of the phase shifts vary depending on the lighting conditions during lactation. These two variables are related to both the functionality of the pacemaker and its sensitivity to light. On the other hand, the rhythm manifested in the DD stage (a variable that permits one to study exclusively the effect of light on the functionality of the clock) does not seem to depend on the lighting conditions during lactation. A possible effect of light, however, appears in the period under DD, as two levels of this variable (groups with less or groups with more than 12 LL days during lactation) can be differentiated. On the basis of our results, we cannot know whether a change in the sensitivity of the circadian system to light, an increase in the circadian responses to light due to a change in the functioning of the circadian system, or both have occurred. Thus further clarifying experiments that would test separately these two effects of light on the pacemaker are needed to find out which is responsible for our findings.

It is clear that the lighting conditions to which newborn animals are exposed are of great significance, as they will affect the circadian system and condition the further adaptation of the adult animal to the external conditions in which it lives. Therefore, it is crucial for the organism to adapt to the coming circumstances as early as possible, while the system is still sufficiently plastic.

Perspectives

The present experiment shows the importance of lighting conditions during the development of the circadian system. This suggests that some responses, and probably the functionality of the circadian system of the adult animal, depend on specific environmental conditions during the early stages of life. This has several implications for further lines of research. First, we wonder whether the biological clock of species other than rats, including humans, would respond in the same way to LL during lactation. It may also be questioned whether zeitgebers other than light, applied at early stages, may also be able to modify the functioning of the circadian system in adulthood. If this were the case, would the responses of the circadian system be similar to those generated by light? Moreover, the way constant light affects the final structure of the circadian pacemaker when applied during the first days of light also remains to be elucidated. Light may

induce changes in the distribution and secretion of some neurotransmitters in the SCN or in its afferences. Light could also act on the connection between SCN cells, for instance, by influencing synaptogenesis or the development of glial cells. Further experiments that will determine the structure of the SCN, the distribution of the neurotransmitters, and its afferences under different lighting conditions may answer some of these questions.

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Address for reprint requests and other correspondence: A. Díez-Noguera, Departament de Fisiologia-Divisió IV, Facultat de Farmàcia, Av. Joan XXIII, s/n, 08028 Barcelona, Spain (E-mail: andiez@farmacia.far.ub.es).

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3.4.- EXPERIMENT 3

FUNCTIONING OF THE RAT CIRCADIAN SYSTEM IS MODIFIED BY LIGHT APPLIED IN CRITICAL POSTNATAL DAYS

***American journal of Physiology (Regulatory Integrative Comp. Physiol.280)*: R1023-R1030, 2001.**

Resum

Objectiu: La manifestació del ritme circadiari d'activitat motora sota condicions de llum constant en rates adultes depèn del nombre de dies en què les rates han estat sotmeses a llum constant durant l'alletament. Però no només el nombre de dies és important, sinó que un mateix nombre de dies no té el mateix efecte segons si està situat al principi o bé al final de l'alletament. El present experiment té com a objectiu determinar l'efecte puntual de la llum durant el desenvolupament del sistema circadiari.

Material i mètodes: Onze rates Wistar femella van arribar al nostre laboratori el dia 16 de la gestació i es van col·locar sota condicions de llum constant. El dia del naixement es van barrejar les cries. Cadascun dels grups estava format per la mare i les cries. Aquests grups es van sotmetre a condicions de foscor constant, excepte per 4, 8 o 12 dies de llum constant, començant en diversos moments de l'alletament segons el grup. Al cap de 25 dies, les cries es van separar de les mares i es van col·locar en gàbies individuals sota condicions de llum constant. A partir del deslletament es va començar a enregistrar l'activitat motora d'aquestes rates mitjançant actímetres de feixos d'infraroig. Després de 55 dies, es van passar totes les rates a condicions de foscor constant per tal d'estudiar-ne el seu ritme en curs lliure. Trenta-quatre dies més tard, es va donar un pols de llum d'una hora de durada i d'uns 350 lux d'intensitat a l'hora circadiària 15 (CT15), amb l'objectiu d'estudiar la resposta del *pacemaker* a la llum.

Resultats: Com més dies de llum constant han rebut els animals durant l'alletament, més manifest és el ritme en llum constant i menor és el canvi de fase induït pel pols de llum. Aquestes respostes s'accentuen quan les rates han estat exposades a llum constant al voltant del dia 12 després del naixement. S'ha construït un model matemàtic per descriure la variació de les respostes del sistema circadiari a la llum respecte el moment en què els animals reben llum durant l'alletament. Hi ha un període crític de sensibilitat a la llum situat entre els dies 10 i 20 després del naixement.

Conclusions: Al voltant del dia 16 després del naixement hi ha un període de dies durant el qual el sistema circadiari seria més sensible a la llum, pel què fa a la posterior protecció front l'arritmicitat induïda per la llum constant. Això permetria que l'animal s'adaptés a les condicions d'il·luminació específiques de l'entorn en el qual neix.

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Functioning of the rat circadian system is modified by light applied in critical postnatal days

M. M. CANAL-CORRETGER, J. VILAPLANA, T. CAMBRAS, AND A. DÍEZ-NOGUERA
Department de Fisiologia-Divisió IV, Facultat de Farmàcia,
Universitat de Barcelona, 08028 Barcelona, Spain

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Canal-Corretger, M. M., J. Vilaplana, T. Cambras, and A. Díez-Noguera. Functioning of the rat circadian system is modified by light applied in critical postnatal days. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R1023–R1030, 2001.—Lighting conditions influence biological clocks. The present experiment was designed to test the presence of a critical window of days during the lactation stage of the rat in which light has a decisive role on the development of the circadian system. Rats were exposed to 4, 8, or 12 days of constant light (LL) during the first days of life. Their circadian rhythm was later studied under LL and constant darkness. The response to a light pulse was also examined. Results show that the greater the number of LL days during lactation, the stronger the rhythm under LL and the smaller the phase shift due to the light pulse. These responses are enhanced when rats are exposed to LL days around postnatal *day 12*. A mathematical model was built to explain the responses of the circadian system with respect to the timing of LL during lactation, and we deduced that between postnatal *days 10* to *20* there is a critical period of sensitivity to light; consequently, exposure to LL during this time modifies the circadian organization of the motor activity.

circadian rhythm; light sensitivity

THE CIRCADIAN SYSTEM is formed by a network of structures that control the circadian rhythms of organisms. In mammals, it is located basically in the brain. It comprises structures such as the suprachiasmatic nuclei (SCN) of the hypothalamus (the main circadian pacemaker in mammals), the retinohypothalamic tract (RHT) (necessary and sufficient for photic entrainment), the geniculohypothalamic tract (GHT) (pathway that brings photic and nonphotic information to the SCN), and the retina (the only known photoreceptor organ in mammals).

The development and maturation of these structures start during gestation and progressively continue until an adult pattern is attained (normally 2 or 3 wk after birth). For example (see Ref. 18 for review), between embryonic *day 17* (*E17*) and postnatal *day 10* (*P10*), the SCN gradually enlarges and takes on an adult appearance; between *E20* and *P10,* there is a gradual maturation of neuronal morphology such that the distinctive separation of neuron types into the SCN subdivisions is evident by *P6* and completely developed by *P10*. At *E19* there are few synapses, but their number increases until *P10*, when the synaptic density in the SCN reaches adult levels. Speh and Moore (23) found that the RHT projection to the SCN and adjacent areas first appears as scattered varicosities in the ventral part of the SCN at *P1* and gradually increases until the adult pattern is reached at approximately *P10*. The ganglion cell projections to the intergeniculate leaflet (IGL) are present before the development of the RHT, and, in the rat, the GHT reaches an adult pattern on *P10* (19).

Therefore, during the first weeks of life, although the adult pattern is not completely developed and the definitive structures are still not established, some external factors, such as environmental light conditions, may play a decisive role in the future organization of the circadian system. For instance, it has been shown that short cycles (i.e., of 4 h) have a different effect on the circadian rhythm manifestation in young and adult rats (25). Similarly, rats that are subjected to constant light (LL) throughout their lactation period manifest a circadian rhythm of motor activity under LL in adulthood, whereas adult rats reared under constant darkness (DD) or a light-dark cycle during the lactation period show arrhythmicity under LL (3). Likewise, we recently observed (5) that the expression of the circadian rhythm of motor activity under LL in adult rats depends on the number of days of LL to which rats are exposed during lactation. In the same experiment, we also found that the same number of LL days did not appear to have the same effect depending on their timing during lactation: applied in the first part or in the last part of lactation, they produced distinct responses of the circadian system to light. We then proposed that there may be a critical period of sensitivity of the circadian system to light during the development of rats.

The present experiment aims to verify the hypothesis that there are some critical days during the early life of rats in which exposition to LL influences the

Address for reprint requests and other correspondence: A. Díez-Noguera, Departament de Fisiologia-Divisió IV, Facultat de Farmàcia, Av. Joan XXIII, s/n, 08028 Barcelona, Spain (E-mail: andiez@farmacia.far.ub.es).

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further functioning of the circadian system, and it also aims to time this critical period. The identification of such a stage may provide further information about the adaptive response of the circadian system to environmental conditions.

MATERIAL AND METHODS

Eleven pregnant Wistar rats, supplied by Criffa (Barcelona), arrived at our laboratory on *day 16* of gestation and were then subjected to LL (~300 lx of intensity). After delivery, 7 days later, all the pups were cross-fostered and then placed into a transparent Makrolom cage of 50 × 25 × 12 cm. From the day of birth and throughout the lactation period, each dam and her pups were kept under DD (~0.1 lx of dim red light), except for a determined number of days in which they were submitted to LL (~300 lx of intensity) (Fig. 1). The groups of rats were named according to the number of LL days during lactation and their timing, that is to say, "number-L-number". The first number indicates the number of LL days during the lactation period (4, 8, or 12 days), and the second number corresponds to the postnatal day on which LL started. For example, group 12L0 received 12 LL days during lactation, starting on the day of birth, and group 4L16 received 4 LL days during lactation, starting on *day 16* after birth. In a more general way, all the groups that had 12, 8, and 4 days of LL during lactation are referred to as 12L, 8L, and 4L groups, respectively.

On *day 25* after birth, the pups were weaned and placed in individual transparent Makrolom cages (25 × 25 × 12 cm). A register of their motor activity was started. From now on, the time of the experiment will be expressed as recording days. The activity was individually measured by means of an activity meter with two crossed perpendicular infrared beams situated on a plane 7 cm above the floor of the cage. Movements produced in successive intervals of 15 min were automatically recorded in a personal computer and stored for further analyses. The total number of registered rats per group varied from 6 to 11 (Fig. 1).

From the day of weaning (*day 1* of the recording period), all the rats were subjected to LL for 55 days to study the evolution of the motor activity rhythm under such conditions. On *day 56*, the rats were transferred to DD to observe their free-running rhythm. Thirty-four days later, a light pulse of 1 h of duration and a mean intensity of 350 lx was given to all the rats at circadian time (CT) 15 to study the response of the pacemaker to light. CT15 was calculated by adding 3 circadian hours to CT12 (time of the beginning of the activity phase), which was estimated visually by four investigators, independently, from the double-plotted actograms. Mean values were used.

Throughout the experiment, rats had free access to food (Rodent Toxicology Diet, B&K Universal) and tap water. Approximately every 7 days, the cages were cleaned and, until *day 73* of the register, the rats were weighed.

Mathematical and statistical analysis. In both the LL and DD stages, the period of the circadian rhythm was calculated by means of Sokolove and Bushell's periodogram (22), with a high global level of significance ($P = 0.01$ with Bonferroni correction) to reject spurious peaks. The percentage of variance (PV) explained by the highest peak in the periodogram was used as an indicator of the importance of the motor activity rhythm.

Due to some registration problems (which did not affect the lighting conditions), only data from *days 15* to *35* (in the LL stage) and from *days 64* to *89* (in the DD stage) were considered for the statistical analysis.

The phase shift after the light pulse at CT15 was calculated separately by six researchers for the onsets and offsets of activity. 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. The mean value obtained by the researchers was used for the statistical analysis.

Graphs and calculations were carried out using the integrated package for chronobiology analysis "El Temps" (A. Díez-Noguera, Barcelona 1999, Universitat de Barcelona).

ANOVA of several linear models (Systat) was carried out. The dependent variables for all the models were period and PV of the rhythm in the LL stage, period and PV of the rhythm in the DD stage, and the phase delay in the onset and offset of activity after the light pulse at CT15. Four types of linear models, which differ in their independent variables,

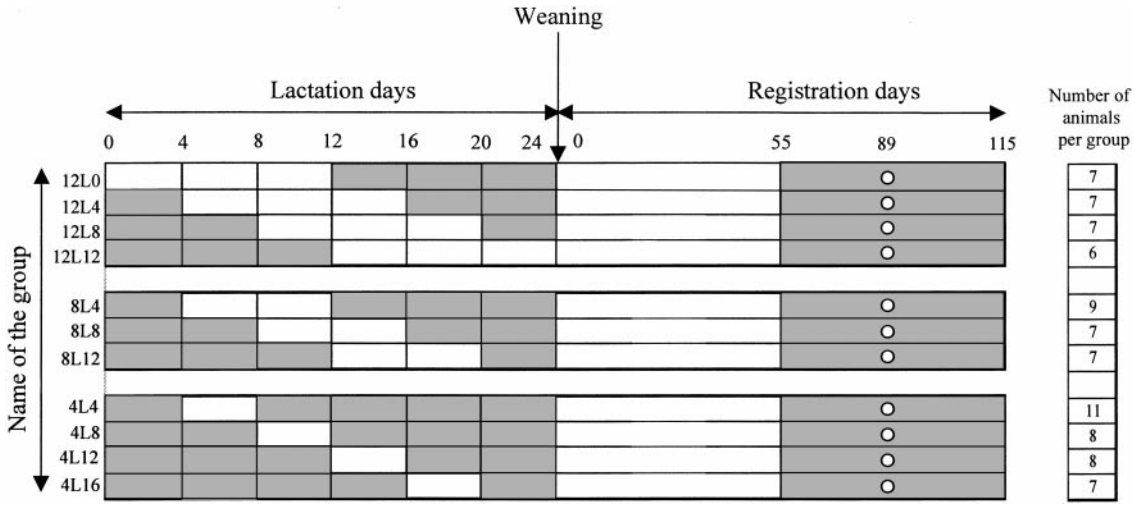

Fig. 1. Scheme of the rat groups and the lighting conditions during the experiment. The first 24 days correspond to the lactation stage and the rest to the days when motor activity was registered. Open areas indicate constant light (LL), and filled areas constant darkness (DD). On *day 89* of registration, a 1-h light pulse was applied at circadian time (CT) 15.

were calculated. In the first model, the independent variable was the number of LL days during the lactation stage (4, 8, or 12). The second, third, and fourth models were built to test the influence of the timing of the same number of LL days during the lactation stage. Thus these models correspond to 12L, 8L, and 4L groups, respectively, and in each case the independent variable was the day on which LL started. The four models were analyzed in two ways: first, by considering the independent variable as qualitative to test differences between groups and, second, by considering the independent variable as quantitative to test a linear regression. When comparing two groups of data, a Student's *t*-test was used.

RESULTS

At first sight and in general, we can observe a distinct manifestation of the motor activity rhythm of the rats under LL or DD (Fig. 2). Under LL, rats showed distinct motor activity patterns: some rats had a clear circadian rhythm, with a mean tau of 25.45 h (SE 0.56 h), whereas some others had an arrhythmic pattern. Under DD, all the rats showed a similar circadian rhythm with a mean period of 24.53 h (SE 0.14 h). No statistically significant differences were found in the period values between groups, either in LL or DD. Therefore, the period of the free-running rhythm after weaning was not significantly affected either by the number of days under LL during lactation or by the initial day of LL during lactation begins. The actograms also show that all the rats responded to the light pulse at CT15 with a phase delay.

Regarding the PV explained by the circadian rhythm in the LL and DD stages, differences were found related to the number of LL days to which the animals were submitted during lactation; however, such differences change depending on the stage we consider. Analysis of the rhythm in the LL stage showed that the greater the number of LL days during lactation, the more consistent the rhythm in the LL stage is (12L groups > 8L groups > 4L groups, $P < 0.05$, Fig. 3*Aa*). In contrast, in the DD stage, this tendency was inverted: the more LL days during lactation, the lower the manifestation of the circadian rhythm in the DD stage (12L groups < 8L groups < 4L groups, $P < 0.01$, Fig. 3*Ab*).

The initial day of LL during lactation also influenced the PV explained by the circadian rhythm, but only in the LL stage. In the case of 12L groups, we found several statistically significant differences depending on the LL onset (Fig. 3*Ba*): group 12L8 had the highest PV values (Student's *t*-test, $P < 0.05$), whereas groups 12L0 and 12L12 had similar values. In the 8L group, a statistically significant correlation ($P < 0.05$) was observed between the PV and the initial day of LL. Because the sign of the regression coefficient is positive, then the earlier LL starts, the lower the PV (Fig. 3*Ca*). Finally, in 4L groups, no statistically significant positive correlation between the beginning of the LL stage during lactation and the PV was observed (Fig. 3*Da*). Among these groups, group 4L4 had the lowest PV values (Student's *t*-test, $P < 0.05$, Fig. 3*Da*).

The application of a light pulse at CT15 induced phase delays in all the rats, as expected, with slight differences in their magnitude depending on the groups (Fig. 2). The animals subjected to longer LL during lactation responded with shorter phase shifts, both in the onset and offset of the motor activity profile ($P < 0.01$, Fig. 3*Ac*). In the three groups (4, 8, and 12 days under LL), the offsets were longer than the onset delays (Student's *t*-test, $P < 0.001$), and these values were positively correlated when all the animals were considered individually ($P < 0.01$). An analysis of the light-induced phase shifts within each group shows several statistically significant differences in the offsets but not in the onsets. In the 12L group, the offset phase shifts were shorter in those animals that started the LL stage later during lactation ($P < 0.01$, Fig. 3*Bc*). In the 8L group, no differences ($P > 0.05$) were found in the light-induced phase shifts (onset and offset), whereas in the 4L group, the higher offset phase shifts

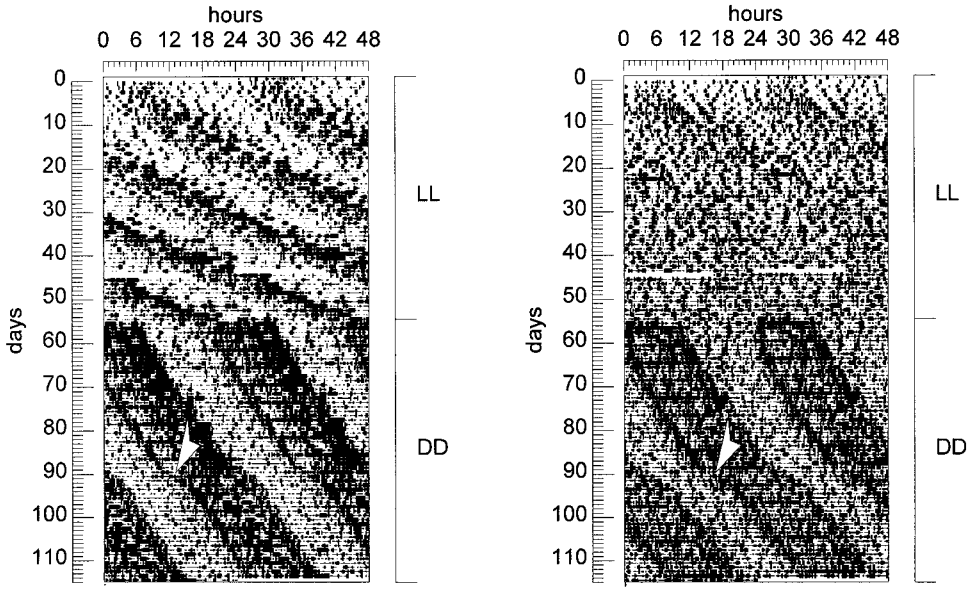

Fig. 2. Double-plotted actograms showing the motor activity rhythm of 2 representative rats. In the ordinates, days after weaning are represented. The arrowhead indicates the 1-h light pulse at CT15.

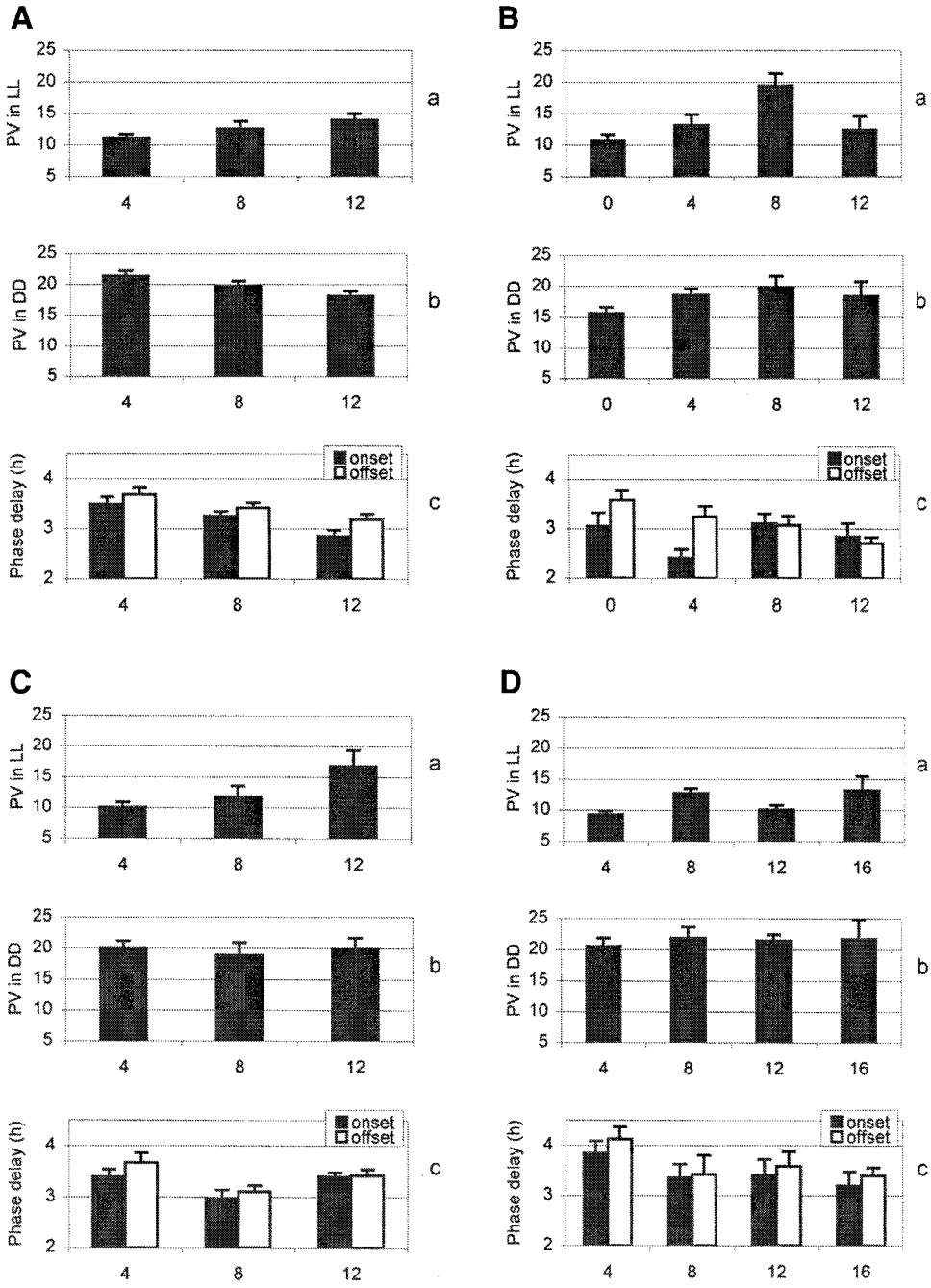

Fig. 3. *A*: variables studied depending on the number of LL days during the lactation stage (4, 8, or 12) (mean ± SE). *B*: variables studied for the 12L groups (groups that had 12 LL days during the lactation stage) depending on the day LL started (0, 4, 8, or 12 after birth) (mean ± SE). *C*: variables studied for the 8L groups (groups that had 8 LL days during the lactation stage) depending on the day LL started (4, 8, or 12 after birth) (mean ± SE). *D*: variables studied for the 4L groups (groups that had 4 LL days during the lactation stage) depending on the day LL started (4, 8, 12, or 16 after birth). In *A*, *B*, *C*, and *D*, the variables studied are: *a*, the percentage of variance (PV) explained by the circadian rhythm of motor activity in the LL stage; *b*, the PV explained by the circadian rhythm of motor activity in the DD stage; and *c*, the phase delay (in hours) after the light pulse at CT15 in the DD stage.

observed corresponded to the animals subjected to continuous light starting on *P4* (Student's *t*-test, $P < 0.05$, Fig. 3*Dc*).

No other factors were observed to affect any other dependent variable.

DISCUSSION

The current experiment was designed based on the hypothesis that the lighting conditions during the early days of the rat's life may affect the further functioning of the circadian pacemaker. We consider the expression of the circadian rhythm of motor activity of the adult rat under LL as an indicator, because it has been widely described that adult rats become arrhythmic when submitted to constant bright light (7, 9, 11, 12, 24). We found that rats reared under LL develop a stable circadian rhythm under LL and that this rhythm is maintained throughout the lifespan of the animal (4). Specifically, we observed that the development of the adult's circadian rhythm under LL depended on the length of the exposure to LL during lactation (5) and that at least 12 days under LL during lactation were needed for it to develop.

With the present experiment, we aimed to identify whether there is a critical interval of time in the developmental stage of the rat in which LL might

modify the further expression of the circadian system. As 12 LL days during lactation were found to be the minimum number of days needed for an adult submitted to LL to develop a circadian rhythm, newborn rats were kept under LL for only 12 or fewer days during the first 24 days of life; light treatments started on different days depending on the group. Thus, when interpreting the results, we must take into consideration that as the number of LL days is small, the effects will also be small, but if, even at this level, differences between groups are noticeable, then the presence of a critical window is validated.

Our results confirm that there is an effect of the number of LL days during the lactation stage on the responses of the circadian rhythm of the adult rat to light: more LL days produced a marked manifestation of the rhythm under LL (higher PV values) and smaller phase shifts due to the light pulse. Second, our findings show for the first time, that the same number of LL days has a distinct effect on the circadian system depending on their timing, as can be seen from the differences encountered within 12L, 8L, and 4L groups. For instance, in our previous experiment (5), we observed that the manifestation of the motor activity rhythm under LL and the phase shift after the light pulse of adult rats that had been submitted to 4 LL days starting on *P0* or on *P20* did not differ from the group that was submitted to DD throughout its whole lactation stage. However, in this study we observed that the rats that received 4 LL days after *P8* differed in the expression of rhythm and in phase shifts as adults from those that received LL days on *P4* (group 4L4). Group 4L4 had the least-marked rhythm in LL and showed the longest phase shift induced by the light pulse; therefore, we suggest that these rats had the weakest circadian pacemaker. Following the same reasoning, the 12L8 group had the most robust pacemaker, as it had the most marked rhythm in LL, together with one of the shortest phase shift values in response to a light pulse.

The distinct functioning of the circadian pacemaker or distinct sensitivities of adult rats to light may explain the various manifestations of motor activity rhythm under LL and the differing responses to the light pulse under DD between groups. Hamsters neonatally treated with monosodium glutamate, which induces acute degeneration of the retina, optic nerve, visual pathways, and some areas of the brain, will phase shift after a light pulse in the same way as untreated counterparts (6). Moreover, mutant *rd/rd* mice, which experience a massive degeneration of rods and cones during development, show the same phase shift in response to a light pulse as wild-type mice (10). This indicates that alterations in the retina are not sufficient to induce changes in the response of the circadian pacemaker to light. However, as we detected differences in the phase shift between groups, we hypothesize that the circadian pacemaker differs between groups, although alterations in the retina and visual pathways must not be excluded. Moreover, *rdta* mice, whose rods degenerate during ontogeny because of a fusion gene integrated in the genome, show greater shifts than wild-type mice at irradiances that produce saturating phase shifts in the latter (14). The explanation proposed was a varying amplitude of the clock response to light and not a distinct clock sensitivity to light. Retina and clock alterations are not excludable, both affect the processing of light information and the manifestation of the motor activity rhythm. It is worth noting that the only difference between *rd/rd* and *rdta* mice is that, in the latter, retina degeneration occurs 1 wk earlier. This observation supports the idea that the effect of altered retinal signals on the circadian system depends on the stage of the development of the animal. In the present experiment, we were unable to elucidate whether the distinct responses to light and the rhythm expression of rats are due to alterations in the retina or in the SCN; however, it is clear that LL during the first days of life of rats affects their circadian organization of the motor activity. Taking into account that it is not known what photoreceptors are responsible for regulating the circadian photosensitivity, up to now it is impossible to know whether the state of the retina of our animals is functionally equivalent to any retinal dystrophy, especially considering that the different groups of rats receive a different number of days, at different ages, of LL.

To estimate the changes in rat sensitivity to light as a function of age (days after birth), we pooled the results of a previous experiment (5) that studied the effect of the number of LL days during lactation with the results obtained in this study. The composite analysis includes data from 204 rats, males and females, thus making the conclusions more reliable. We took the PV value of the adult in LL as an indicator of the response of the circadian system to light. There are two aspects of time to be examined: the duration of LL exposure and the time at which light is applied. If it is assumed that the effect of light has a cumulative effect through the time (see mathematical description in APPENDIX), then we suggest that these two aspects do not correspond with two distinct variables but to a single one. Therefore, the expression calculated is the value of PV as a function of time. This curve is crucial because it indicates the evolution of the instantaneous sensitivity of the animal to light during lactation. The curve of the pooled data from the two experiments is shown in Fig. 4. It can be seen that the maximum of the curve is around *day 16* after birth, indicating that the period of highest sensitivity to light is between *P10* and *P20*. Integrating the curve around *P16*, one can calculate that an interval of 6 days is enough to generate, in an adult rat under LL, a PV >8% (equivalent to a significant circadian rhythm), whereas around *P6,* a 10-day interval is necessary. It is worth noting that the ANOVA of the linear model used to calculate the function shows that the model used is statistically highly significant ($P < 0.001$). This significance can also be appreciated by the visual inspection of the fiducial limits of the function: it is clearly visible that a straight line cannot be traced inside the 95% confidence band,

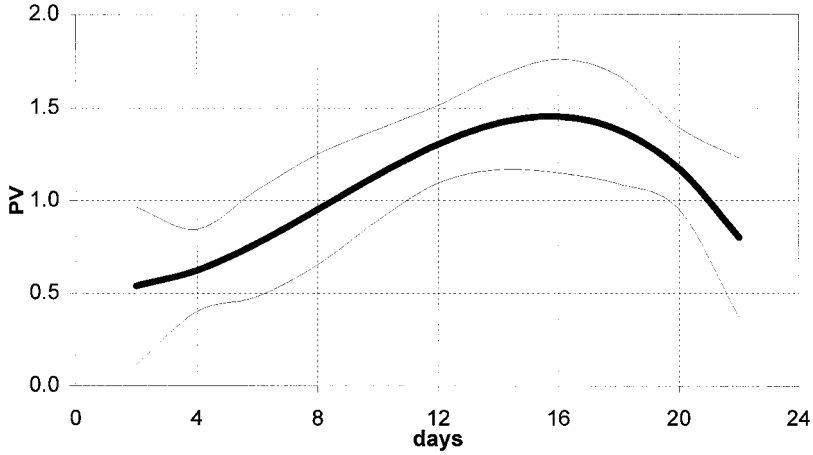

Fig. 4. Sensitivity to light is plotted vs. time. Time is expressed as days after birth, and sensitivity (see MATERIAL AND METHODS for explanation) is represented as the instantaneous contribution to the PV explained by the circadian rhythm in adults under LL. The thick line represents expected values according to the estimated polynomial function (see APPENDIX), and the thin lines are the 95% fiducial limits of the estimation.

thus demonstrating the presence of a window of sensitivity to light during lactation.

The same statistical process was applied to the phase shifts induced by a light pulse at CT15 (separately for the onset and the offset of motor activity). Although the calculated functions are not statistically significant, it is worth noting that the shorter phase shifts (the minimum of the curve) occurred between *P10* and *P20*, just when the specific sensitivity to light reaches its maximum. In both experiments, we observed that the rats with the most consistent rhythm under LL were those with shorter phase shifts after a light pulse under DD.

As we mentioned above, although we cannot disregard the possibility that our results are due to alterations in the circadian photosensitivity, we may also consider that the different light treatment affects the circadian pacemaker itself. Considering that the circadian clock is formed by several groups of coupled oscillators (2, 8, 17, 26), the motor activity arrhythmicity of adult animals under LL can be explained by an uncoupling effect of light on these oscillators. When light is present from the day of birth, the animal adapts accordingly, and the manifestation of the circadian rhythm is permitted even under LL. This may lead to a stronger circadian pacemaker that is not easily affected by environmental light. Externally, this strong clock may manifest a marked circadian rhythm under LL and a low response to a light pulse in darkness. It has been proposed that glial cells are responsible for the coupling between oscillators (26), therefore the mechanisms that regulate the connection between such cells and neurons may be those altered by light. The effect of light on the circadian system may be, in part, mediated by glutamate, which is the main neurotransmitter of the RHT. We suggest that if a rat is submitted to LL when the RHT is developing its connections, the levels of glutamate will be modified, and therefore the intracellular calcium levels of both neurons and astrocytes will be altered. This could modify the functioning and the coupling between neurons and astrocytes, because changes in intracellular calcium levels during early development have been shown to alter the rate of neurite outgrowth (16), synapse formation (20), neural migration (13), and neural phenotype (15).

We conclude that the lighting conditions under which a rat is reared during its first days of life are decisive for the future response of the animal to light. Light has several effects on the circadian organization of the motor activity depending on the stage of maturation of the rat. From the first embryonic stages and until an animal reaches an adult pattern some weeks after birth, a long process of maturation of the nervous system takes place. The SCN are completely developed by *P10*. Likewise, the pathways that carry environmental information to the SCN develop postnatally: the RHT projection to the SCN and adjacent areas and the GHT also reach an adult pattern also on approximately *P10* (19, 23). From the curve calculated, we can observe that the sensitivity of the circadian system to light increases throughout the lactation period, reaching its maximum at about *P16*, which coincides with the time in which nearly all the significant structures of the circadian system (SCN, RHT, GHT, pineal gland) have already developed and reached their adult pattern. Thus it appears that the circadian system must be developed before it can be influenced and molded by external factors such as light. The internal and external influences that the animal receives during the maturation period are of great importance for the complete development of the nervous system. From studies of the development of the visual cortex (see Ref. 1 for review), it is known that although the developmental processes that lead to the functional pattern of connections that underlie normal vision are genetically determined, they can be interfered with or impaired by abnormal visual experience in the "sensitive period" of the first few months of life. Environment influences can cause large-scale changes in the anatomy and/or functional response properties of neurons that can even persist throughout the lifetime of an animal. For instance, experiments on the presence and type of orientation-selective cells in the primary visual cortex of kittens reared under different visual environments (see Ref. 21 for review) have shown that sensory experience plays a decisive role in the future appearance and orientation of these cells. The effect of environmen-

tal conditions is only effective on kittens during the critical period of their development and not on adult cats, because during the critical period the neural circuitry responsible for orientation detector cells in the primary visual cortex is in a "plastic" state, i.e., open to environmental molding and responsive to use and/or disuse. Likewise, we could expect a plastic state of the circadian system between *P10* and *P20*, during which the circadian system would be predisposed to be instructed and shaped by experience, and, therefore, the best input pathways would be selected.

Perspectives

Here we report that the sensitivity of the circadian system to light changes in the first few days of life. This finding may help us to understand the response of the circadian system to environmental factors. This system generates rhythmicity in the various functions of the organism, which allows synchronization to the environment. Thus the circadian rhythm needs to be manifested, whatever the conditions of the environment. This implies that the development of the circadian system may depend on the environment, although the functioning of the system is expected to be similar in all adult animals, which would guarantee adaptation.

Because light is the main zeitgeber, the threshold of the sensitivity of mammals to light may depend on the basal level of light intensity. However, several questions remain; for instance, how does light affect the developing circadian system? Are all the structures of the circadian system (retina, visual pathways, and the circadian pacemaker itself) affected by early exposure to LL? Moreover, might stimuli other than light influence the development of the circadian system? Our findings indicate that the external conditions during ontogeny may be decisive, because brain structures are plastic and external inputs from the environment can act on the connections between nerve cells and modify them structurally and/or functionally. These modifications lead to the acquisition of the mature pattern of the brain.

APPENDIX A

Using the same notation as Sokolove and Bushell (22), the PV can be derived from Q_p following the formula

$$PV = 100\,\frac{SS_{\bar{X}}}{SS_X} = 100\,\frac{K\sum_{h=1}^{P}(\bar{X}_h - \bar{\bar{X}})^2}{\sum_{i=1}^{N}(X_i - \bar{\bar{X}})^2} = \frac{100}{N}\,\frac{K\sum_{h=1}^{P}(\bar{X}_h - \bar{\bar{X}})^2}{N^{-1}\sum_{i=1}^{N}(X_i - \bar{\bar{X}})^2} = \frac{100}{N}\,Q_p$$

Data must be arranged in a matrix of K rows and P columns for each T ($N = K \cdot P$).

APPENDIX B

To quantify the effect induced by light during lactation, we used several variables measured in the adult rat (PV explained by the rhythm or phase shifts). In accordance with our experimental hypothesis, these variables (effects) are dependent on the specific time of light application. We consider the "instantaneous" effect of light as a continuous function of time, but as the nature of this function is unknown, we will use a third-order polynomial expansion of it

$$f(t) \approx b_0 + b_1 t + b_2 t^2 + b_3 t^3 = \sum_{i=0}^{3} b_i t^i \qquad (1)$$

In real situations, light is not applied just for an instant but during an interval of time (days), consequently, the powers of the instantaneous values of t in *Eq. 1* must be substituted by the integrals I_i, corresponding to the distinct intervals during which the animals received light. The integrals are defined as

$$I_{i,a-b} = \int_a^b t^i dt = \left[\frac{t^{i+1}}{i+1}\right]_a^b = \frac{b^{i+1} - a^{i+1}}{i+1} \qquad (2)$$

where a and b are the initial and final time values corresponding to the interval. Therefore, we can rewrite $f(t)$ as

$$f(t) \approx b_0 + b_1 I_1 + b_2 I_2 + b_3 I_3 = \sum_{i=0}^{3} b_i I_i \qquad (3)$$

This function was used to calculate the coefficients b_i, for the polynomial linear regression analysis. Once the coefficients were calculated, one can deduce a function $f_d(t)$ to express the effect of the application of light for an interval of length d as a function of time (the time of application). If time in the function represents the initial day of the interval, the final day will be $t+d$, and the function will be expressed as

$$f_d(t) = \sum_{i=0}^{4} b_i I_{i,t\sim t+d} = \sum_{i=0}^{4} b_i \frac{(t+d)^{i+1} - t^{i+1}}{i+1} \qquad (4)$$

The maximum of the function $f(t)$ is estimated by solving the first derivative of *Eq. 1*

$$b_1 + 2b_2 t + 3b_3 t^2 = 0 \qquad (5)$$

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ADDENDUM

The estimated polynomial function represented in Fig.4 is the following:

$$PV = 0.546 - 0.0309t + 0.0149t^2 - 0.000592t^3$$

PV is the percentage of variance explained by the circadian rhythm of motor activity in adult rats under LL, and t is time expressed as days after birth.

3.5.- EXPERIMENT 4

THE ENTRAINMENT OF THE MOTOR ACTIVITY CIRCADIAN RHYTHM OF THE RAT TO LIGHT-DARK CYCLES DEPENDS ON THE LIGHTING CONDITIONS DURING LACTATION

Resum

Objectiu: Per tal d'examinar quin és l'efecte de les condicions d'il·luminació en què una rata ha crescut, sobre el funcionament del sistema circadiari, el nostre objectiu és estudiar les característiques de l'encarrilament de 3 grups de rates que s'han criat sota diferents condicions d'il·luminació durant l'alletament. En cadascun dels grups es mirarà el valor de psi, que indica la relació de fase amb la que el *pacemaker* circadiari sincronitza amb el cicle extern i per tant es pot considerar una propietat fonamental del *pacemaker* circadiari.

Material i mètodes: Quinze rates Wistar femella van arribar al nostre laboratori el dia 15 de gestació. El dia del naixement, les cries es van barrejar, de manera que cada mare alimentava grups de 17 cries provinents de diverses ventrades. Cinc grups es van transferir a foscor constant (DD), 5 a llum constant (LL) i 5 a cicles simètrics de llum-foscor (LD) de 24 hores de període. El dia del deslletament (24 dies després del naixement) les cries es van separar de la mare i es van posar en gàbies individuals amb accés a l'aigua i al menjar *ad libitum* i l'activitat motora es va enregistrar mitjançant actímetre d'infraroig. Set cries (4 mascles i 3 femelles, o bé 3 mascles i 4 femelles) de cadascun dels grups es van posar sota cicles LD de 22h de període (grup T22). Unes altres 21 cries es van sotmetre a cicles LD de 23h de període (grup T23). De la mateixa manera es van formar 4 grups més, que es van sotmetre a cicles LD de 24, 25, 26 o 27 hores de període (grups T24, T25, T26 i T27, respectivament). Al cap de 52 dies en aquestes condicions, les rates es van passar a condicions de DD per tal d'estudiar-ne el seu ritme en curs lliure.

Resultats: En tots els cicles LD estudiats, les rates que s'han criat sota condicions de DD manifesten un ritme més fort que les rates que s'han criat en LD o LL. Pel què fa a la fase d'encarrilament o psi, en els cicles LD de menys de 25h de període, les rates DD tenen els valors més baixos (un cop apagada la llum, la fase alfa s'inicia abans), mentre que per períodes superiors a les 25 hores, totes les rates comencen a moure's abans que s'apagui la llum i les que s'han criat sota cicles LD són les que comencen més aviat. A més a més, la major part de les rates T22 i T23 manifesten un ritme d'activitat motora dissociat en dos components, un d'encarrilat pel cicle LD extern i l'altre de no encarrilat. Les característiques d'aquesta dissociació són independents de les condicions d'il·luminació en què aquestes rates s'han criat.Les característiques del ritme en curs lliure (DD) d'aquestes rates depenen exclusivament del període del cicle LD previ.

Conclusions: El psi depèn de les condicions d'il·luminació rebudes durant l'alletament. Això indica que les condicions d'il·luminació en què una rata ha crescut modifiquen les propietats del seu sistema circadiari.

THE ENTRAINMENT CHARACTERISTICS OF THE MOTOR ACTIVITY RHYTHM OF THE RAT TO LIGHT-DARK CYCLES DEPEND ON THE LIGHTING CONDITIONS DURING LACTATION

ABSTRACT

A rat reared under constant light (LL), when compared with a rat reared under constant darkness (DD), manifests a circadian rhythm of motor activity under LL and has a lower phase shift after a light pulse under DD. The aim of the present experiment was to test whether the lighting conditions in which a rat has been reared could also affect the phase relation (or psi) of the entrained rhythm to a specific *Zeitgeber*. Therefore we reared three groups of rats under different lighting conditions (DD, LL, 24h-period light dark cycles) and then placed them under 22h-, 23h-, 24h-, 25h-, 26h- or 27h-period LD symmetric cycles to study the entrainment characteristics of their circadian rhythm of motor activity. Results show that when the period of the LD cycle was shorter than 25 hours, the DD rats had lower values (started earlier to move) than the other groups, whereas for periods longer than 25 hours, LD rats were the ones whose onset of activity was earlier. We also observed that, independently of the period of the LD cycle, the DD rats showed a stronger rhythm than the LD and LL rats. Therefore the psi, one fundamental property of the circadian pacemakers, depends on the lighting conditions in which a rat has been reared. This suggests that early light history can modify the properties of the circadian system.

INTRODUCTION

The ontogeny of the circadian system may be conditioned by the environment experienced during development. In particular, the phase angle relative to the light cycle in silkmoths (Truman 1973), the free-running period (Barrett and Page 1989) and the sensitivity to phase shifting by light pulses (Page and Barrett 1989) of the cockroach depend on the environment in which an individual was reared. Regarding mammals, early experiments rearing rats under constant light (LL) or constant darkness (DD) showed that these conditions did not disrupt the circadian rhythm of plasma corticosteroid levels permanently (Krieger 1973) and only the electroshock seizure threshold differed between the two groups of rats (Meisami and Timiras 1970). More recent experiments performed in our laboratory revealed that rats reared under LL showed a circadian rhythm of motor activity when placed under LL and a phase shift after a light pulse in DD shorter than rats raised under DD (Cambras et al. 1997; Canal-Corretger et al. 2000).

According to Daan and Pittendrigh (Daan and Pittendrigh 1976), there are two reliable measurable pacemaker properties: the free-running period and the Phase Response Curve (PRC) for some standard perturbation. PRC is commonly calculated by applying the stimulus (pulse) while the animal is free-running and measuring the phase shift induced. This method is equivalent to estimating the PRC from the phase angle (psi) assumed by the rhythm to stimulus cycles of a different period (Eskin 1971). PRCs are useful to analyze the mechanisms of entrainment, either by measuring a stimulus pulse or by calculating the psi (Pittendrigh and Minis 1964; Pittendrigh 1966), and so they are helpful to understand the functioning of the pacemaker.

For a given species, changes in the response to a specific stimulus, and therefore of the PRC, can reflect changes in the mechanisms used by circadian pacemakers to entrain to an external cycle (Johnson 1992). However, changes in the PRC to a light stimulus can either reflect modifications in the functioning of the

pacemaker, or in the functioning of the photoreceptor system, as long-term exposure to light causes severe damage on the photoreceptor cells of the retina (Birch and Jacobs 1977; Lemmon and Anderson 1979a; Lemmon and Anderson 1979b; Williams et al. 1985; Penn et al. 1989). Thus, the aim of the present experiment was to clarify the role of early lighting conditions in the functioning of the circadian clock of the rat. To this end, three groups were reared under distinct lighting environments. After lactation, they were placed under several light-dark cycles of a different period, and entrainment characteristics, and specially the psi, were studied and compared to elucidate whether the functioning of the circadian pacemaker differed between the three groups.

MATERIALS AND METHODS

Fifteen pregnant Wistar rats from Criffa (France) on the 15th day of gestation were used. Each rat was kept in an individual transparent Makrolom® cage of 50x25x12 cm under a light-dark cycle (LD 12:12h). After delivery (6 days later), the pups were cross-fostered to avoid family influences. Each dam fed between 8 and 9 pups, half of which were males. Five dams with their respective pups were left under constant light (white light of around 300 lux, LL group); five were placed under constant darkness (dim red light of around 0.5 lux, DD group); and the remaining dams were placed under LD 12:12 cycles (LD group) for 24 days.

On the day of weaning (day 0 of the registration period), pups were separated from the dam and placed in individual Makrolom® cages of 25x25x12 cm. Seven pups (3 males+4 females, or 4 males+3 females) of each group were then placed under a symmetric 22h-period LD cycle (11:11h, T22 group). Likewise, 7 other pups of each group were placed under symmetric 23h-period LD cycles (T23 group). Four more groups were sorted in the same way, but with an external 24h-, 25h-, 26h- or 27h-period LD cycle (T24, T25, T26 and T27 groups, respectively). The rats were reared in these conditions until day 52 of the registration period. Meanwhile, the motor activity was registered by an activity-meter with crossed-infrared beams situated on a plane 7 cm above the cage floor. At this stage of the experiment (LD stage), the entrainment characteristics of each group were studied. On day 52, all rats were placed into constant darkness (DD stage) to study their free-running rhythm.

Throughout the experiment, rats were maintained under a controlled ambient temperature of 21ºC (±1ºC) with tap water and food *ad libitum* (Standard Diet, Prolabor, Spain).

To calculate the period of the motor activity rhythm in both the LD and the DD stages, the Lomb&Scargle periodogram (VanDongen et al. 1999; Ruf 1999) was obtained. The data used ranged from days 10 to 50 of the registration period in the LD stage and from days 52 to 82 in the DD stage. In addition, the power content of the first harmonic (PC1H, with a period equal to the period of the LD cycle in the LD stage, and a period equal to tau in the DD stage) was computed through a Fourier's analysis. In the LD stage, some rats of the T22 and T23 groups showed two significant peaks in the periodogram. Therefore, when comparing these groups with the others, only the period and PC of the peak with the same period as the external LD cycle were taken into account.

Entrainment characteristics in the LD stage were studied with the value of psi. The mean wave form, which had previously been smoothed ±6 hours to avoid reactive peaks produced by the onset or the offset of light, was used. To calculate the psi, the distance (in minutes) between lights off and the onset of activity (time when the motor activity was above the median) was measured for each rat. When psi was positive, the activity started after the lights went off, whereas negative values indicated that the activity began before the lights were turned off.

In the DD stage, the phase of the free-running rhythm in relation to the last light-dark cycle was calculated for each animal by estimating an eye-fitted line to the onset of the free-running rhythm and by extrapolating this line for the last LD cycle. The randomness of the animal phase

distribution in each group was tested by the Rayleigh z-test (Batschelet 1981).

For the statistical analysis, ANOVA was used. In the LD stage, the sex of the animal, the period of the external LD cycle (22, 23, 24, 25, 26 or 27 hours) and the lighting conditions during lactation (DD, LD or LL) were taken as the independent variables. The dependent variables were, one at each time, the period, the PC1H and the value of psi. In the DD stage, the independent variables were the sex of the animal, the lighting conditions during lactation and the period of the previous LD cycle and; the dependent variables were, one at each time, the period and the PC1H.

Graphs and results were obtained using the integrated package for chronobiology "El Temps"© (A. Díez-Noguera, Barcelona 1999, Universitat de Barcelona).

RESULTS

LD stage

In Figure 1, the double-plotted actograms show the entrainment characteristics at each T cycle of a representative animal. Although the data of all the rats from the three groups were used for the mathematical and statistical analyses, only the double-plotted actograms of males of the LL group are shown, as there are no differences between these actograms and those of females, or between the rats of the LD and DD groups.

In the LD stage, one rat of the LL group placed under T22 cycles free-ran with a period of 24.9 hours. Regarding the rest of the rats, 18 of 21 rats of the T22 group (6/7 of the DD group, 6/7 of the LD group, and 6/7 of the LL group), and 18 of 21 of the T23 group (7/7 of the DD group, 6/7 of the LD group, and 5/7 of the LL group) manifested a dissociation in the circadian rhythm of motor activity (with two significant peaks in the periodogram), whereas all the animals of the other groups showed a period equal to that of the LD cycle. No differences in the rate of dissociation were observed between the LL, LD and DD groups. The entrainment to the LD cycle was corroborated by the phase angle between the onset of the free-running activity rhythm in the DD stage and the last LD cycle, since the Rayleigh's z-test revealed significant clustering of the 7 data points for all groups ($p<0.05$) except for the T22 group (Fig.2). Here, no differences between the LL, LD and DD groups were found.

PC1H also varied ($p<0.05$) according to the period of the LD cycle: the closer the external period to the tau of the rat, the higher the PC1H. No significant differences due to the sex were found in the PC1H ($p>0.05$). PC1H depended on the lighting conditions during lactation ($p<0.01$): the DD group had higher values than the LD group, and the latter had higher values than the LL group (Fig.3).

Figure 5 shows the smoothed mean wave forms of the LL, LD and DD groups under each LD cycle. The onset of light causes a reactive peak of activity, which is visible with the mean wave form. To avoid the error due to this reactive peak in the determination of the activity onset (the first step in the calculation of the psi value), the mean wave form was smoothed ±6 hours, thus the rest-activity transition became constant and gradual. This way, differences in psi due to entrainment to the period of the LD cycle were found ($p<0.01$): in T22 rats the motor activity started around two hours after lights off (mean±se = 2.0±0.2 hours) and psi values progressively decreased with the increase in the external period, such that in T27 rats, the motor activity started around 3 hours before the lights were switched off (mean±se = -3.1±0.3 hours) (Fig.4,5). Statistically significant differences ($p<0.05$) were also observed in the values of psi due to the lighting conditions during lactation: for LD cycles of a period shorter than 25 hours, the LL group showed higher values than the LD group, while the DD group had the lowest values; for LD cycles with a period longer than 25 hours, the DD group showed the highest values, followed by the LL group and finally, the LD group (Fig.4).

DD stage

All the rats showed a free-running rhythm of motor activity after being

submitted to constant darkness (Fig.1). As expected because of the after-effects, the period of the free-running rhythm depended on the previous lighting conditions ($p<0.01$), and thus it increased with that of the previous LD cycle (Fig.6a). No significant differences due to the lighting conditions during lactation were found in the period or PC1H in the DD stage. PC1H varied according to the sex of the animal (females showed higher values than males, $p<0.01$), and to the previous period of the LD cycle (PC1H tended to decrease as the period of the previous LD cycle increased, $p<0.01$) (Fig.6b).

DISCUSSION

The aim of this study was to determine whether external environmental stimuli like light applied early in development affects the circadian clock. We would like to highlight that psi values, i.e. the phase of the entrained rhythm, vary according to the lighting conditions in which the animal was raised. According to Daan and Pittendrigh (Daan and Pittendrigh 1976), there are two pacemaker properties that can be easily measured: the free-running period and the PRC for some standard perturbation. No differences due to the lighting conditions during lactation were found in the free-running period, but this coincides with the findings of Davis et al., who showed that the free-running period of the circadian pacemaker that underlies locomotor activity/rest rhythmicity is not influenced by the environmental cycle under which a mouse had been raised (Davis and Menaker 1981). However, the phase of the entrained rhythm and so the PRC differed between LL and DD groups, suggesting that the circadian pacemaker of these rats differ owing to the light environment in which they were raised, as it was previously demonstrated that this rhythm was independent of that of the dam (Cambras et al. 1997).

In the LD stage, the PC1H of the entrained rhythm of LL rats was significantly lower than that of DD rats. Previous observations showed that rats submitted to a low-intensity 23h-LD cycle showed a lower amplitude and PC1H of the rhythm than rats submitted to a high-intensity 23h-LD cycle (Cambras et al. 2000). Hence, the rats reared under LL may be less sensitive to light and thus show lower amplitude and PC1H of the circadian rhythm. However, the same experiment revealed (unpublished results) that the psi of the entrained rhythm of rats submitted to high-intensity LD cycle was indistinguishable to that of the group submitted to a low-intensity LD cycle, which had been reared in the same lighting conditions. This suggests that the psi value is independent of light intensity and that it only depends on the functioning of the pacemaker, expressed with the PRC. This supports the hypothesis that the functioning of the circadian pacemaker of LL rats differs from that of DD rats.

It seems that the effect of light applied at the early age extends further than the retinal level. Exposure to LL rapidly affects the posterior pituitary morphology (Weiss et al. 1995), modifies the optic synapses within the suprachiasmatic nuclei (SCN, principal pacemaker in mammals) (Güldner et al. 1997), increases the overall responsiveness of the SCN neurons to melatonin (Yu et al. 1993), alters the SCN rhythmicity (Yu et al. 1993), and induces dramatic changes in glial fibrillary acidic protein both in the SCN and the intergeniculate leaflet (Moriya et al. 2000). Hence, together with alterations in the retina, light may also modify other parts of the circadian system, which can be reflected in the locomotor behavior. Moreover, light can alter the circadian system in both young and adult rats, but only the light applied during the first postnatal weeks seem to modify the locomotor behavior (Cambras 1998). As to the retinal function, it has been stated that the illumination level in which a rat is born and raised determines its subsequent susceptibility to damage by white light (Terman et al. 1991). The via which brings light information to the SCN is the retinohypothalamic tract (RHT) (Moore 1973). In the rat, the fibers of the RHT enter the SCN between postnatal days 3 and 7, and reach the adult pattern by the second postnatal week (Moore 1991). Some studies on the development of the visual cortex (see

Barlow and Smith for review) have demonstrated that in the early stages of development, until the neural circuitry is definitely established, the system is plastic enough to be influenced by the surrounding environment. In addition, there seems to be a period of days around the second postnatal week (postnatal day 16) in which the rat is more sensitive to light, as rats that received light during this period of time have afterwards a stronger circadian rhythm of motor activity under LL (Canal-Corretger et al. 2001).

Taking all this into account, it appears that light can affect the circadian system, both at the retinal and at the pacemaker level. Nevertheless, these modifications may alter the output of the circadian rhythm (specifically, the entrainment characteristics and the response to a light pulse) only when light has been applied during development.

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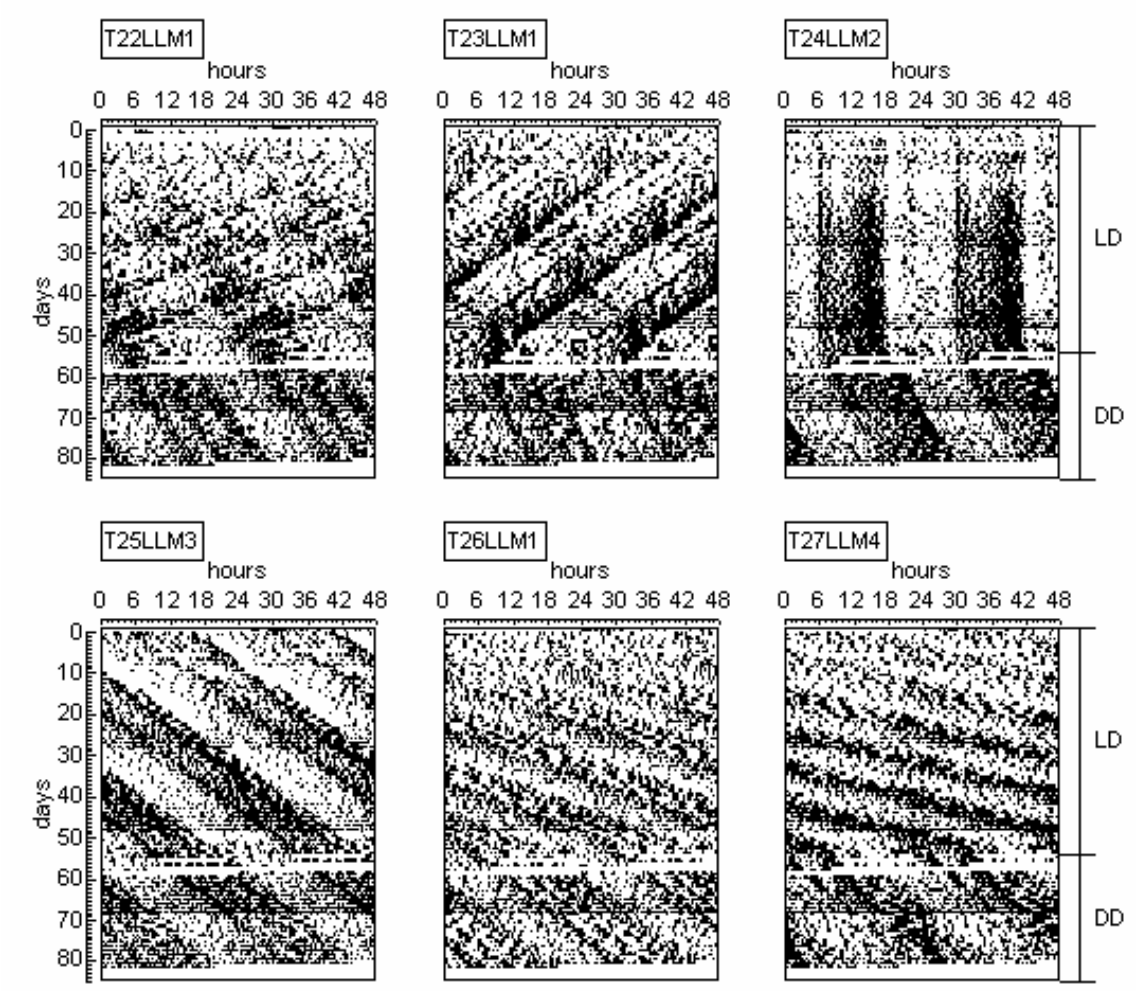


Fig. 1.- Double-plotted actograms at modulo 24 hours representing the motor activity rhythm of a representative animal under T22, T23, T24, T25, T26 or T27 LD cycles (see Materials and Methods for further explanation). The horizontal axis indicates the time of the day in hours. The axis on the left represents the registration days, day 0 is the day of weaning. The axis on the right shows the lighting conditions of each stage of the experiment. Blank spaces are missing data due to technical problems.

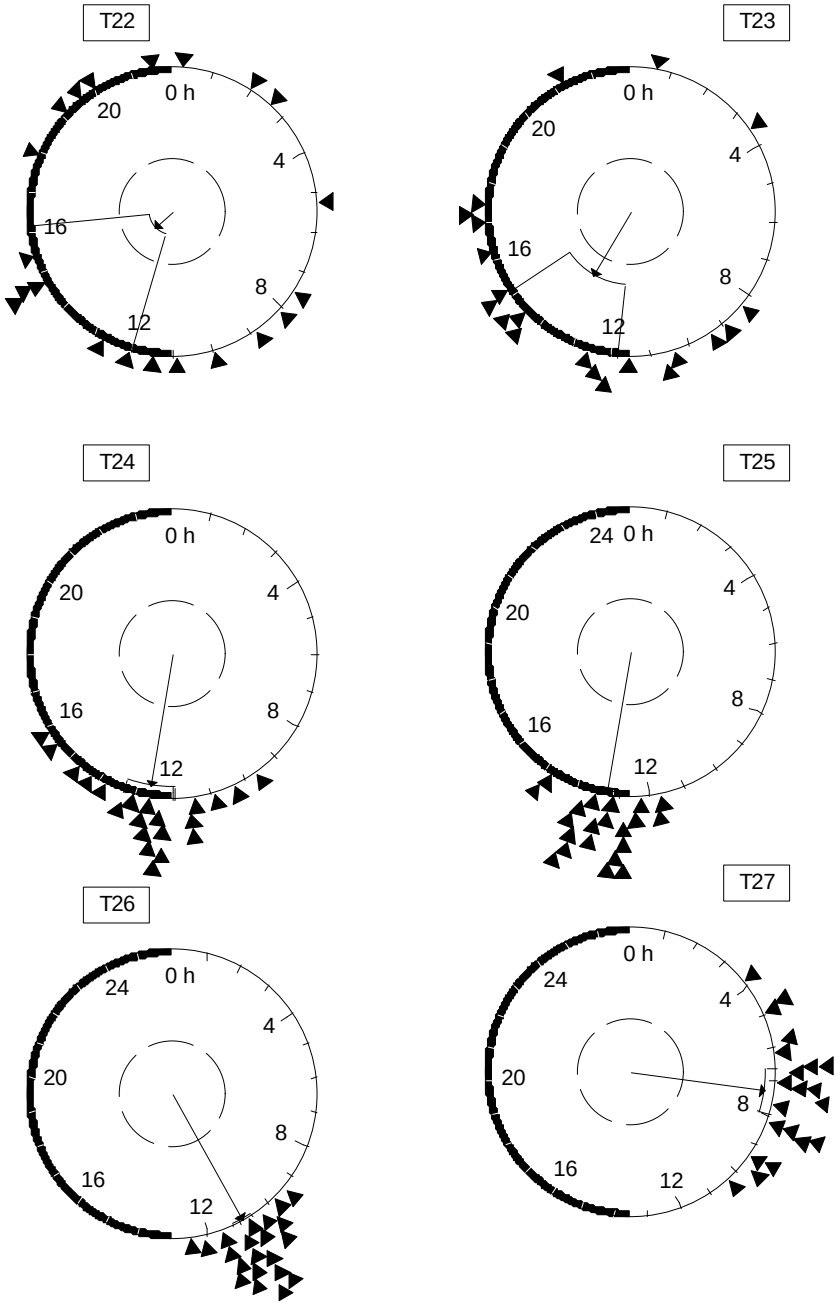


Fig. 2.- Phases of the activity onset of the free-running component after exposure to LD cycles. Black areas correspond to the dark phase of the last LD cycle. Each black triangle represents the phase of the activity onset of one animal. The dotted circle is the threshold for p=0.05, according to the Rayleigh's z-test. The arrow shows the mean phase of the activity for one group of animals.

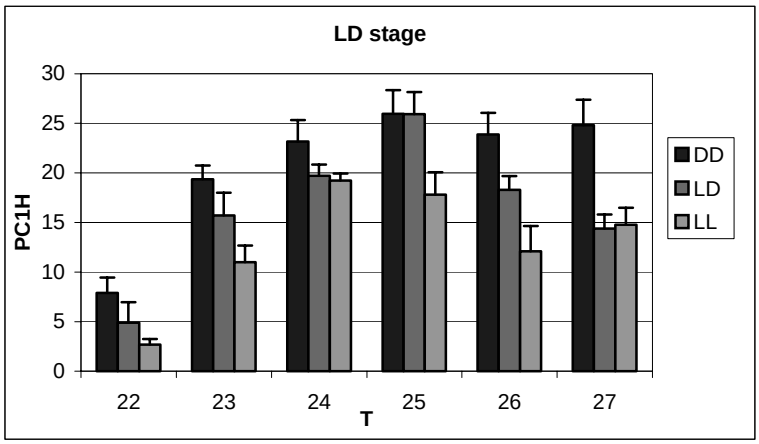


Fig. 3.- Power content of the first harmonic of the circadian rhythm of motor activity, for each period of the LD cycle, and for each group of rats, during the LD stage. Error bars show the standard error.

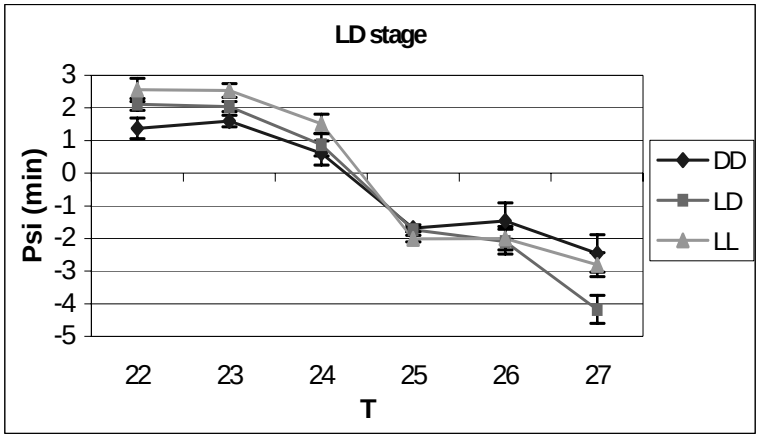


Fig. 4.- Value of psi for each period of the LD cycle, and for each group of rats, during the LD stage. Error bars show the standard error. See text for more details.

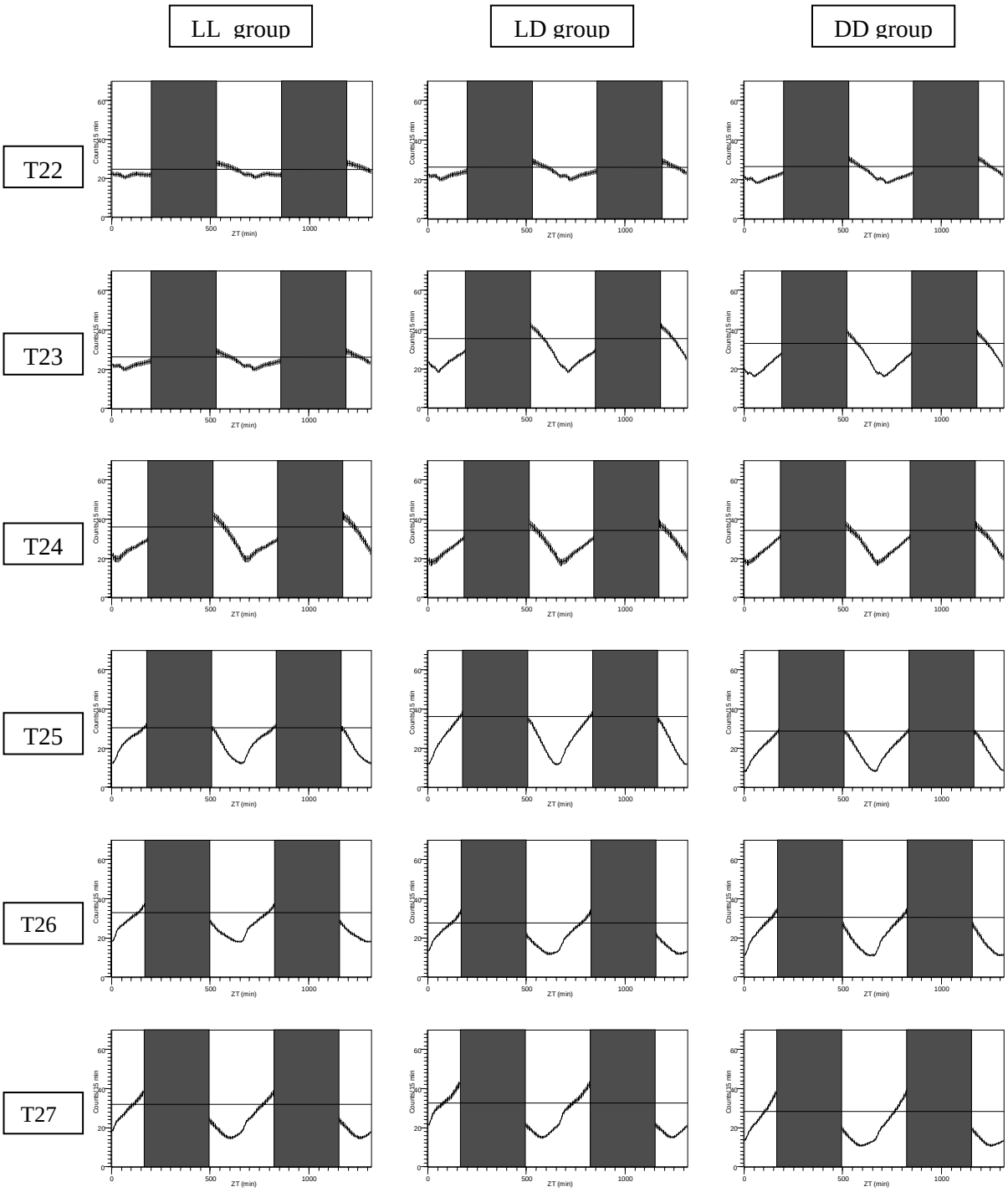
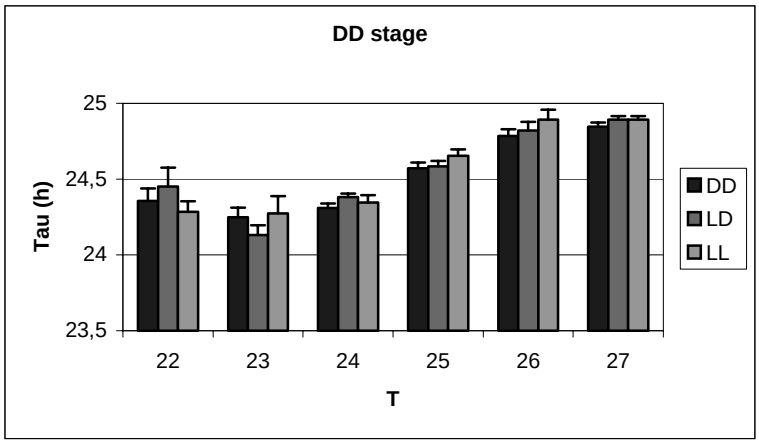


Fig. 5.- Mean wave forms for each group of rats in the LD stage. The vertical axis represents arbitrary units of motor activity, and the horizontal axis represents the *Zeitgeber* time. The white area indicates light and the shadowed area indicates darkness. Error bars show the standard error.



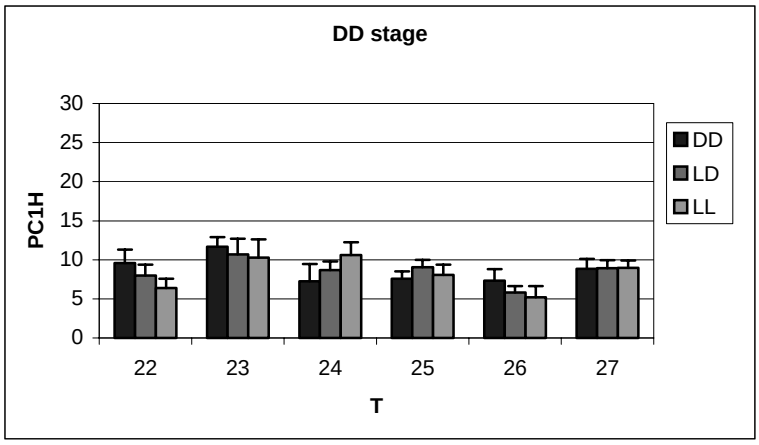


Fig. 6.- a.- Period of the free-running rhythm of motor activity in the DD stage, for each group of rats, and depending on the period of the previous LD cycle. Error bars show the standard error.

b.- Power content of the first harmonic of the motor activity rhythm in the DD stage, for each group of rats, and depending on the period of the previous LD cycle. Error bars show the standard error.

3.6.- EXPERIMENT 5