UNIVERZITA KARLOVA V PRAZE

2. LÉKAŘSKÁ FAKULTA

Postgraduální doktorské studium biomedicíny

Obor: neurovědy

Kristýna Matějů

Vývoj světelné synchronizace cirkadiánního systému potkana v časné postnatální ontogenezi

Disertační práce

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Prohlášení

Prohlašuji, že jsem disertační práci vypracovala samostatně s použitím uvedených literárních pramenů.

v Praze dne Kristýna Matějů

Školicí pracoviště: Oddělení neurohumorálních regulací, Fyziologický ústav Akademie věd České republiky, v.v.i.

Školitelka: PharmDr. Alena Sumová, DSc.

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Podíl autorky na vzniku publikací které jsou podkaldem disertační práce:

Primární publikace

El Hennamy R., Matějů K., Bendová, Z., Sosniyenko S., Sumová A.: Maternal control of the fetal and neonatal rat suprachiasmatic nucleus. Journal of Biological Rhythms 2008, 23 (5), 435-444 (IF=3,868)

K.M. přispěla významně ke vzniku práce (podíl 50%). Samostatně vyhodnocovala záznamy pohybové aktivity pokusných zvířat (Fig.2) a spolu s R. El-H. stanovovala mRNA sledovaných genů metodou in situ hybridizace s radioaktivně značenou próbou. Rovněž se částečně podílela na vyhodnocování výsledků tohoto stanovení. Při přípravě článku se podílela na grafické úpravě obrázků.

Matějů K., Bendová Z., El-Hennamy R., Sládek M., Sosniyenko S., Sumová A.: Development of the light sensitivity of clock gene *Period1*, *Period2* and immediate-early gene *c-fos* within the rat suprachiasmatic nucleus. European Journal of Neuroscience, 2009, 29 (3), 490-501 (IF=3,675)

K.M. přispěla zásadně ke vzniku studie (podíl 80%). Samostatně prováděla většinu experimentů popsaných v článku, prováděla odběry mozků a připravovala mozkové řezy. Významnou měrou se podílela na stanovování mRNA sledovaných genů metodou in-situ hybridizace. K.M. připravovala samostatně preparáty pro emulsní autoradiografii a vyhodnocovala získaná data včetně statistického zhodnocení. Rovněž ve spolupráci se školitelkou připravila rukopis článku včetně grafické úpravy obrázků.

Kováčiková Z., Sládek M., Laurinová K., Bendová Z. Illnerová H., Sumová A.: Ontogenesis of photoperiodic entrainment of the molecular core clockwork in the rat suprachiasmatic nucleus. Brain Research 2005, 1064 (1-2), 83-89. (IF=2,296)

K.M. se významně podílela na vzniku článku (podíl 40%). Zabývala se zejména stanovením mRNA sledovaných genů metodou in situ hybridizace s radioaktivně značenou próbou a vyhodnocováním výsledků.

Matějů K., Bendová Z., Sumová A. Light sensitivity of expression of clock genes *Period1*, *Period2* and immediate-early gene *c-Fos* within retina of early postnatal rat. *manuscript v přípravě*

Podíl K.M. na vzniku studie byl zásadní (80%). Provedla většinu experimentů a rovněž připravovala ze získaného materiálu řezy. Stanovovala mRNA sledovaných genů pomocí in-situ hybridizace s radioaktivně značenou próbou a připravovala preparáty pro emulsní autoradiografii. Rovněž vyhodnocovala výsledky včetně statistického zhodnocení. K.M. pomáhala s přípravou rukopisu článku včetně grafické úpravy obrázků.

Souhrnné články

Sumová A., Bendová Z., Sládek M., El-Hennamy R., Matějů K., Polidarová L., Sosniyenko S., Illnerová H. Circadian molecular clocks tick along ontogenesis. Physiological Research 2008, 57, Suppl. 3, S139-S148 (IF=1,505)

Podíl K.M. na vzniku studie byl 20%.

Sumová A., Bendová Z., Sládek M., Kováčiková Z., El-Hennamy R., Laurinová K., Illnerová H.: The rat circadian clockwork and its photoperiodic entrainment during development. Chronobiology International 2006, 23 (1-2), 237-43 (IF=2,4)

Podíl K.M. na vzniku studie byl 20%.

Sumová A., Bendová Z., Sládek M., El-Hennamy R., Laurinová K., Jindráková Z., Illnerová H.: Setting the biological time in central and peripheral clocks during ontogenesis. FEBS Letters 2006, 580 (12), 2836-42 (IF=3,4)

Podíl K.M. na vzniku studie byl 20%.

Laurinová K., Sumová A.: Ontogenetický vývoj cirkadiánního systému savců. Československá fyziologie 2006; 55 (4), 96-102

Podíl K.M. na vzniku studie byl 90%.

Seznam použitých zkratek

AA-NAT - arylalkylamin N-acetyltransferasa

AVP – arginin-vasopresin

BDNF - brain derived neurotrophic factor

Bmal1 - brain and muscle Arnt-like protein 1

CaMKII - Ca^{2+}/calmodulin-dependentní proteinkinasa II

cAMP – cyklický adenosinmonofosfát

CCGs - clock controlled genes, geny ovládané hodinami

CK1ε/δ - kasein-kinasa1ε/δ

Clock - circadian locomotor output cycles kaput

CRE – Ca^{2+}/cAMP response element

CREB – Ca^{2+}/cAMP response element binding protein

Cry1, 2 - Cryptochrome1, Cryptochrome2

CT – circadian time

DD – dark-dark, konstantní tma

Dexras1 - dexamethasonem indukovaný RAS protein 1

dm - dorsomediální

E - den embryonálního (prenatálního) vývoje

ERK - extracellular signal-regulated kinase

GABA – kyselina γ-aminomáselná

GHT – geniculohypothalamický trakt

HIOMT - hydroxyindol-O-methyltransferasa

IGL – intergeniculate leaflet

LD – light-dark, světelný režim ve kterém se střídá světlá a tmavá fáze, např. při LD 12:12 se střídá 12 hod světla a 12 hod tmy

MAPK - mitogen-activated protein kinase

NMDA – N-methyl-D-aspartát

P – den postnatálního vývoje

PACAP - pituitary adenylate cyclase-activating polypeptide

Per1, 2, 3 - Period1, Period 2, Period 3

Rev-erbα - jaderný receptor NR1D1

RGCs - retinal ganglion cells, fotoreceptivní gangliové buňky sítnice

RHT – retinohypothalamický trakt

Rora - retinoic acid-related orphan receptor a

RORE - REV-ERBα/ROR response element

RPE – retinal pigment epithelium

SCN – suprachiasmatic nucleus, suprachiasmatická jádra hypothalamu

UTP – uridintrifosfát

VIP – vasoaktivní intestinální polypeptid

vl – ventrolaterální

OBSAH:

Úvod

U většiny známých organismů byla prokázána existence biologických hodin, které řídí rytmy s periodou přibližně 24 hodin, tzv. cirkadiánní rytmy. Tyto rytmy lze pozorovat na různých úrovních, počínaje úrovní celého těla (rytmus v pohybové aktivitě, spánku a bdění, příjmu potravy), přes fyziologické funkce (jako např. rytmy v tělesné teplotě či tvorbě a uvolňování některých hormonů), až po molekulární a buněčnou úroveň (kde dochází k rytmickému přepisu některých genů a tvorbě proteinů). Cirkadiánní rytmy nejsou pouhou reakcí na změny vnějších podmínek, ale a jsou vrozené, tj. endogenní. V prostředí bez periodicky se opakujících podnětů běží s vlastní vnitřní periodou, jejíž délka je druhově specifická a zpravidla se nerovná zcela přesně 24 hodinám (např. u myši je to 23,6 hod, u potkana - podobně jako u člověka – cca 24,2 hod). Aby nedocházelo k předbíhání rytmů, jejichž perioda je kratší než 24 hodin, či naopak zpožďování rytmů s periodou delší než 24 hodin, jsou cirkadiánní hodiny s 24-hodinovým dnem pravidelně synchronizovány. Při synchronizaci dochází k posunu fáze rytmu tak, aby byla v souladu s fází rytmu synchronizátoru. Nejdůležitějším synchronizátorem cirkadiánních hodin je světlo, a to jak jednotlivé světelné záblesky (tzv. diskrétní synchronizace), tak i délka světlé části dne (tzv. kontinuální synchronizace). Světlo zvečera působí zpoždění rytmů s periodou kratší než 24 hodin, zatímco světlo zrána způsobuje předběhnutí rytmů s periodou delší než 24 hodin (Daan a Pittendrigh, 1976). Každodenní světelnou synchronizací je udržována perioda rytmů přesně 24 hodin. Kromě světla působí jako synchronizátory také signály nesvětelné povahy např. změny okolní teploty, pohybová aktivita nebo sociální podněty (přehledně viz Hastings et al., 1998).

V laboratorních podmínkách jsou pokusná zvířata obvykle chována při světelném režimu, kde se pravidelně střídá světlá a tmavá fáze (LD, light-dark). Při zkoumání endogenního mechanismu řídícího cirkadiánní rytmy je však nutno odstranit akutní „maskující“ vliv světla a proto bývají zvířata při experimentech převedena do konstantní tmy (DD, dark-dark). V DD se střídá tzv. subjektivní noc, tj. období kdy jsou noční zvířata aktivní a je možno světelným pulsem vyvolat posun fáze, a subjektivní den, kdy jsou noční zvířata neaktivní a světlo fázi cirkadiánních rytmů neovlivňuje.

1. Cirkadiánní systém savců, jeho funkce a vývoj

1.1 Cirkadiánní systém

Původní představy o cirkadiánním systému vycházely z předpokladu, že cirkadiánní rytmy jsou generovány jediným tzv. centrálním oscilátorem (pacemakerem, hodinami), který je u savců uložen v suprachiasmatických jádrech hypothalamu (Ralph et al., 1990; Klein et al., 1991). Novější studie však dokládají výskyt mnoha dalších oscilátorů v periferních orgánech a ukazují, že jde ve skutečnosti o mnohem komplikovanější systém se značným fyziologickým významem. Centrální hodiny v něm pravděpodobně fungují jako synchronizátor fází jednotlivých periferních oscilátorů.

Centrálními hodinami savců včetně člověka je párové jádro *nucleus suprachiasmaticus hypothalami* (SCN, suprachiasmatic nuclei). SCN je možno funkčně i morfologicky rozdělit na dvě části: část dorsomediální (dmSCN) a část ventrolaterální (vlSCN). DmSCN je tvořeno zejména neurony produkujícími arginin-vasopressin (AVP) a bývá funkčně spojováno se vznikem endogenní rytmicity. Neurony vlSCN produkují vasoaktivní intestinální polypeptid (VIP). Ventrální část SCN těsně přiléhá k *chiasma opticum* a hraje důležitou roli ve světelné synchronizaci cirkadiánních rytmů. Kromě toho jsou v SCN přítomny také další neuropeptidy a neuromodulátory, např. gastrin–releasing peptid, somatostatin, peptid histidin-isoleucin či substance P (přehled viz Ibata et al., 1999; van Esseveldt et al., 2000)

Informace o světle se do SCN dostává ze sítnice retinohypothalamickým traktem (RHT, *radix optica hypothalamica*). Hlavním mediátorem RHT je glutamát. Další nepřímá dráha, tzv. geniculo-hypothalamický trakt (GHT) vede ze sítnice přes *nucleus geniculatus lateralis thalami* (tzv. IGL, intergeniculate leaflet) a přivádí do SCN informace o světelných i nesvětelných podnětech. Hlavním mediátorem GHT je neuropeptid Y. SCN přijímají další podněty také serotoninergní dráhou z *raphe nuclei* a z dalších částí mozku.

Oscilace vznikající v buňkách SCN činností molekulárního mechanismu (viz kapitola 1.1.3.) jsou převáděny na rytmus v elektrické aktivitě neuronů a na rytmickou produkci neuropeptidů. Elektrická aktivita neuronů SCN je vyšší přes den a rytmus v elektrické aktivitě neuronů je přítomen i v případě že je jim v podmínkách *in vitro* zabráněno tvořit synapse. Za těchto okolností vykazuje každá buňka rytmus v elektrické aktivitě s fází odlišnou od okolních buněk a tento její rytmus se s nezměněnou fází

obnoví i po dočasném zablokování elektrické aktivity tetrodotoxinem (Welsh et al., 1995; Pennartz et al., 2002, Schwartz et al., 1987). Z výše uvedeného vyplývá, že centrální pacemaker je patrně *in vivo* složen z více nezávislých oscilátorů, které jsou navzájem synchronizovány dosud neznámým mechanismem.

Kromě elektrické aktivity neuronů se na přenosu rytmů z SCN do dalších částí mozku podílejí i mediátory rytmicky produkované buňkami SCN. Jedná se zejména o AVP, kyselinu γ-amminomáselnou (GABA), glutamát a další. V SCN je možno rozlišit několik subpopulací neuronů, které rytmicky produkují určitý mediátor, popř. jejich kombinaci. Tyto subpopulace hrají důležitou roli při cirkadiánní regulaci výlevu některých hormonů (souhrn viz Kalsbeek et al., 2006).

Výše popsané rytmy na úrovni SCN jsou přenášeny řadou nervových drah do dalších oblastí hypothalamu a také do dalších částí mozku (podrobnější anatomie viz Watts, 1991). Nervovými drahami z SCN je zprostředkována cirkadiánní regulace tvorby některých hormonů, např. melatoninu, kortikosteronu či gonadotropin-releasing faktoru. Melatonin (N-acetyl-5-hydroxytryptamin) je tvořen v epifýze ze serotoninu (5-hydroxytryptaminu) činností arylalkylamin N-acetyltransferasy (AA-NAT) a hydroxyindol-O-methyltransferasy (HIOMT). Aktivita AA-NAT je nízká přes den a vysoká v noci (Illnerová a Vaněček, 1980). Vysoká hladina melatoninu v plasmě tak působí jako významný signál, který informuje organismus nejen o denní době, ale i o délce noci a tím o aktuálním ročním období. Mediátorem, který se podílí na aktivaci nočního výlevu melatoninu je patrně glutamát produkovaný určitou subpopulací neuronů SCN, zatímco ranní pokles hladiny melatoninu řídí subpopulace buněk SCN produkující GABA (Kalsbeek et al., 2006). Do epifýzy se informace z SCN dostávají multisynaptickou drahou z dmSCN přes *nucleus paraventricularis hypothalami*, intermediolaterální sloupec šedé hmoty ve spinální míše a *ganglion cervicale superior*. *Nucleus paraventricularis* obsahuje mj. neurony produkující kortikotropin-releasing faktor a drahou z SCN je tak ovlivněna i hladina kortikosteronu v plasmě (Buijs et al., 1999). Nepřímo jsou signály z SCN ovlivněny i hladiny pohlavních hormonů drahou vedoucí do preoptické oblasti, kde se podílí na regulaci uvolňování gonadotropin-releasing faktoru (van der Beek et al., 1997).

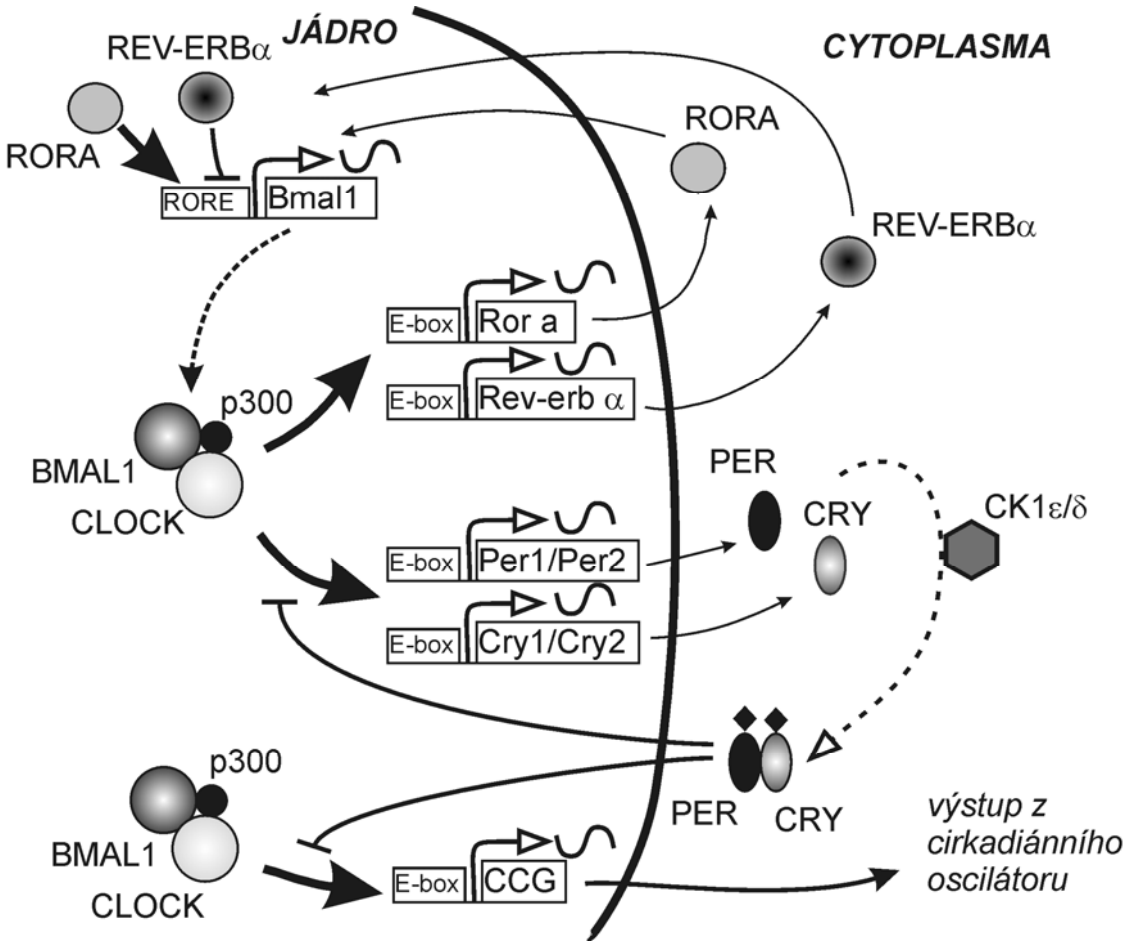
1.1.1 Molekulární mechanismus vzniku cirkadiánních rytmů

Vlastní mechanismus vzniku endogenních oscilací je založen na systému vzájemně propojených zpětnovazebných transkripčně-translačních smyček. Součástí

těchto smyček jsou tzv. hodinové geny, tj. geny jejichž mutace či vyřazení (knock-out) způsobí narušení až ztrátu cirkadiánní rytmicity. Proteiny kódované těmito geny vstupují do jádra a regulují transkripci vlastních genů. U savců byly jako hodinové označeny geny: *Clock* (circadian locomotor output cycles kaput), *Bmal1* (brain and muscle Arnt-like protein 1), *Period1* (*Per1*), *Period2* (*Per2*) a *Period3* (*Per3*), *Cryptochrome1* (*Cry1*) a *Cryptochrome2* (*Cry2*), *Rev-erbα* (jaderný receptor NR1D1), *Rora* (retinoic acid-related orphan receptor a) a *kasein-kinasa1ε/δ* (*CK1ε/δ*) (přehled viz Reppert a Weaver, 2001; Ko a Takahashi, 2006). Proteinové produkty hodinových genů *Clock* a *Bmal1* tvoří heterodimery CLOCK:BMAL1, které se v jádře v komplexu s histonacetylázou p300 vážou na E-boxy v promotorech genů skupiny *Per* a *Cry* a do promotoru *Rev-erbα* a *Rora* a aktivují tak jejich transkripci (Etchegaray et al., 2003; Sato et al., 2004). V cytoplasmě se následně tvoří proteiny PER1, 2, 3 a CRY1 a 2, které se spojují v homo i heterodimery a jsou transportovány do jádra. V jádře dimery PER:CRY narušují komplex CLOCK:BMAL1 – p300 a negativně tak ovlivňují vlastní transkripci. Protein REV-ERBα je rovněž transportován do jádra, kde se váže na tzv. RORE sekvence (REV-ERBα/ROR response element) a působí jako represor transkripce genu *Bmal1*. Tato represe snižuje dostupnost proteinu BMAL1, což vede k zpětnovazebné negativní regulaci vzniku REV-ERBα. Tato negativní zpětná vazba je patrně inhibována proteinem PER2, který tak působí jako pozitivní zpětnovazebný regulátor zvyšující dostupnost BMAL1 (Preitner et al., 2002; Ueda et al. 2002). Do stejného místa jako REV-ERBα se váže také protein RORA, který naopak působí jako pozitivní regulátor exprese *Bmal1* (Akashi a Takumi, 2005; Guillaumond et al., 2005). Výsledkem výše popsaných vzájemně propojených zpětnovazebných smyček (viz Obr. 1) jsou rytmické oscilace mRNA hodinových genů a jejich proteinových produktů. Exprese genů *Per*, *Cry* a *Rev-erbα* vrcholí během subjektivního dne a rytmus v jejich expresi je tak v protifázi k rytmu v expresi *Bmal1*, která vrcholí během noci. Výjimku tvoří gen *Clock*, který v SCN nevykazuje rytmus v expresi.

Důležitou roli při přesné koordinaci celého mechanismu hrají posttranslační modifikace proteinových produktů hodinových genů. Klíčovou roli hraje zejména rytmická aktivita kinasy CK1ε/δ, která fosforyluje proteiny PER a CRY a tím umožňuje jejich vstup do jádra (Akashi et al., 2002). Fosforylovaný protein PER2 se stává zároveň substrátem pro ubiquitin-ligasu, která připojením ubiquitinu označuje proteiny pro degradaci. Délka vnitřní periody organismu se zdá být závislá na rychlosti degradace PER2, resp. jeho fosforylace kinasou CK1ε/δ (Eide et al., 2005). Rovněž protein

BMAL1 podléhá rytmické posttranslační modifikaci. Když vytvoří komplex CLOCK:BMAL1 a iniciuje transkripci dalších hodinových genů, naváže se na něj malý protein SUMO, který mění jeho lokalizaci a označuje ho pro ubiquitinylaci a následnou degradaci (Cardone et al., 2005; Lee et al., 2008). Poruchy v mechanismu posttranslačních modifikací mohou způsobit poruchy cirkadiánní rytmicity, zejména změny v délce vnitřní periody cirkadiánních rytmů.



Obrázek 1: Schéma molekulárního mechanismu cirkadiánních hodin v buňkách SCN (podle Ko a Takahashi, 2006). Během noci dochází k expresi genu Bmal1, proteiny BMAL1 a CLOCK tvoří heterodimery a v komplexu s p300 na začátku dne aktivují transkripci hodinových genů Per, Cry, Rorα, Rev-erbα a také CCG (clock controlled genes, genů kontrolovaných hodinami). Proteiny PER a CRY jsou fosforylovány CK1ε/δ (kasein-kinasou 1ε/δ) a transportovány do jádra, kde negativní zpětnou vazbou ovlivňují CLOCK:BMAL1 iniciovanou transkripci a zároveň jsou označeny pro degradaci. Protein REV-ERBα slouží jako negativní a RORA jako pozitivní regulátor transkripce Bmal1. Snížení dostupnosti proteinu BMAL1 v průběhu subjektivního dne vede postupně ke snížení transkripce Per, Cry a Rev-Erbα, čímž dojde k přerušení negativní zpětné vazby a transkripce Bmal1 se během noci opět zvýší.

Činnost molekulárního mechanismu vytvářejícího cirkadiánní oscilace v SCN musí být nějakým způsobem převedena na rytmus v neuronální aktivitě a na produkci neuropeptidů. Výstup z cirkadiánního oscilátoru je zajišťován tzv. hodinami kontrolovanými geny (CCGs, clock controlled genes), resp. jejich proteinovými produkty. Jedná se o geny, které nejsou nutné pro vznik cirkadiánních oscilací, ale přesto jejich exprese vykazuje cirkadiánní rytmus. Mají totiž ve svém promotoru E-boxy, na které se vážou heterodimery CLOCK:BMAL1 a rytmicky tak spouští jejich transkripci. Mezi CCGs patří např. AVP a některé receptory a podjednotky iontových kanálů, díky čemuž je rytmicky řízena aktivita neuronů SCN (přehled viz Reppert a Weaver, 2001).

1.1.2 Úloha sítnice v cirkadiánním systému

Pro zajištění synchronizace cirkadiánních hodin světlem je u savců nezbytná světločivná funkce sítnice. Nedávno bylo zjištěno, že kromě tyčinek a čípků se v sítnici nacházejí ještě další fotoreceptivní buňky. Část gangliových buněk sítnice (RGCs, retinal ganglion cells) produkuje opsinu podobný fotopigment melanopsin a jejich axony tvoří podstatnou část RHT (Hattar et al., 2002; Berson et al., 2002). Předávají tak do SCN informaci o světle nezávisle na tyčinkách a čípcích a fungují jako tzv. „cirkadiánní fotoreceptory“ (přehled viz Morin a Allen, 2006).

Důležitou úlohu v adaptaci sítnice na měnící se světelné podmínky hraje rytmická produkce dopaminu a melatoninu v sítnici. Dopamin je ve dne produkován amakrinními buňkami a podílí se na adaptaci fotoreceptorů na světlo. Melatonin je syntetizován ve fotoreceptorové vrstvě zejména během noci a bývá spojován s adaptací fotoreceptorů na tmu. Jeho produkce je přes den negativně regulována dopaminem, zatímco během noci melatonin naopak snižuje produkci dopaminu (Doyle et al., 2002). Rytmus v produkci melatoninu přetrvává v *in vitro* podmínkách i v konstantní tmě s periodou přibližně 24hod, což dokládá, že sítnice funguje také jako cirkadiánní oscilátor (Tosini a Menaker, 1996). K rytmické expresi AA-NAT – enzymu, který se podílí na syntéze melatoninu – dochází i u zvířat s lézí SCN, takže produkce melatoninu v sítnici je patrně na SCN nezávislá (Sakamoto et al., 2000; přehled viz Tosini a Fukuhara, 2002).

V sítnici potkana dochází k expresi základních součástí molekulárního mechanismu cirkadiánních hodin - hodinových genů *Per1, Per2, Cry1, Cry2, Bmal1* a *Clock*. Dosavadní informace ale naznačují, že molekulární mechanismus cirkadiánních

hodin v sítnici je jiný než v SCN (přehled viz Tosini a Fukuhara, 2002; Tosini et al., 2008). V sítnici potkana byla popsána rytmická exprese hodinových genů *Cry1*, *Cry2*, *Per2* a *Clock*, ale nikoliv *Per1* a *Bmal1* (Namihira et al., 1999; 2001; Park a Kang, 2006). Jiné studie naopak ukázaly, že v sítnici myší jsou rytmicky exprimovány i geny *Per1* a *Bmal1* a exprese *Clock* rytmus nevykazuje (Ruan et al., 2006; Witkovsky et al., 2003). Po osvětlení byla pozorována indukce exprese hodinových genů *Per1* a *Per2* ale také *Bmal1* a *Clock* (Namihira et al., 1999; 2001). Výsledky studií exprese hodinových genů v sítnici potkanů a myší nejsou úplně konzistentní. Příčinou může být fakt, že sítnice je tvořena různými typy buněk, ve kterých může docházet k rytmické expresi jednoho či více hodinových genů, ale rytmy jsou navzájem fázově posunuty.

S ohledem na strukturu sítnice je obtížné zjistit, ve kterém typu buněk se cirkadiánní pacemaker nachází. Rytmická exprese *aa-nat* mRNA ve fotoreceptorové vrstvě sítnice je pravděpodobně – podobně jako CCGs v SCN - řízena heterodimerem CLOCK:BMAL1. Tosini *et al.* (2007) proto umístili cirkadiánní hodiny do fotoreceptorové vrstvy. Jiné práce naopak ukazují koordinovanou expresi hodinových genů ve vnitřních vrstvách sítnice, zejména v dopaminergních amakrinních buňkách (Ruan et al., 2006). Rytmická exprese hodinového genu *Per1* byla u myší pozorována ve vnitřní nukleární a gangliové vrstvě sítnice spíše než ve fotoreceptorech (Witkovsky et al., 2003). Cirkadiánní oscilátor v sítnici by se mohl podílet i na regulaci citlivosti cirkadiánního systému k vnějším světelným podnětům.

1.1.3 Světelná synchronizace na molekulární úrovni

Jak již bylo naznačeno v úvodu, cirkadiánní hodiny v SCN jsou citlivé k synchronizaci světelnými podněty pouze v době tzv. subjektivní noci. Dříve než byly známy hodinové geny savců, využívaly studie zkoumající vliv světla na SCN jako ukazatel neboli marker fáze cirkadiánních hodin časný raný gen *c-fos*. Exprese *c-fos* mRNA v SCN je zvýšená po osvětlení v první i druhé polovině subjektivní noci, tedy v době, kdy světlo působí fázové zpoždění, resp. předběhnutí cirkadiánních rytmů, ale nikoliv během subjektivního dne. K indukci exprese *c-fos* mRNA a následnému zvýšení produkce proteinu c-FOS dochází výhradně ve vlSCN, tedy v místě, které je v přímém spojení se sítnicí přes RHT (Kornhauser et al., 1990, Rusak et al., 1990, 1992).

Po osvětlení v noci je v SCN uvolněn z nervových zakončení RHT glutamát, který se váže na NMDA receptory neuronů SCN a spouští tak systém signálních drah uvnitř buňky (viz Obr. 2; přehledně viz Hirota a Fukada, 2004). Dochází ke zvýšení

intracelulární koncentrace Ca^{2+} a aktivaci NO synthasy (Ding et al., 1994), což vede k aktivaci Ca2+/calmodulin-dependentní proteinkinasy II (CaMKII) a MAPKinasové (mitogen-activated protein kinase) dráhy (Obrietan et al., 1998; Dziema et al., 2003). Aktivací této signální dráhy dochází k fosforylaci kinasy ERK (extracellular signal-regulated kinase) a jejímu vstupu do jádra, kde fosforyluje DNA-vazebný Ca^{2+}/cAMP response element binding protein (CREB). Fosforylace CREB, který se váže na Ca^{2+}/cAMP response element (CRE) sekvenci v promotorech hodinových genů *Per1*, *Per2* a časného raného genu *c-Fos* vede k aktivaci histonacetylas a tím ke změnám ve struktuře chromatinu usnadňujícím iniciaci transkripce (Crosio et al., 2000; Naruse et al., 2004). Aktivace transkripce *Per* genů prostřednictvím CLOCK:BMAL1 heterodimeru popsaná v předchozí kapitole, je na CRE pravděpodobně nezávislá (Trávníčková-Bendová et al., 2002). Další signální dráha zahrnuje zvýšení produkce cyklického guanosinmonofosfátu NO-dependentní guanylátcyklasou a aktivaci cGMP-dependentní proteinkinasy II, která se pravděpodobně podílí zejména na aktivaci transkripce *Per2* (Oster et al., 2003).

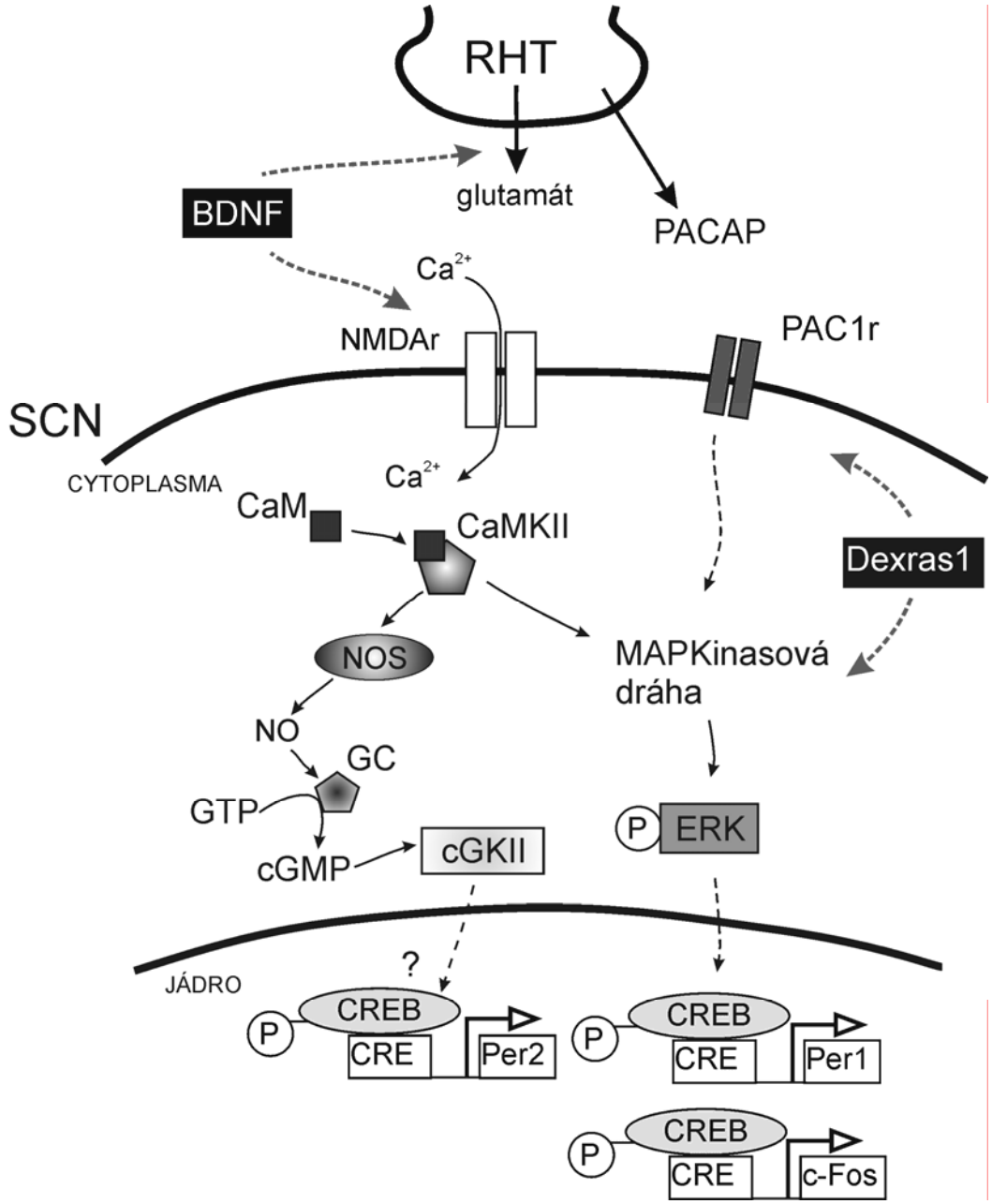
Část melanopsin-produkujících RGCs, které vedou informaci o světle ze sítnice do SCN (viz kapitola 1.1.2), produkuje také signální peptid PACAP (pituitary adenylate cyclase-activating polypeptide). PACAP funguje jako modulátor glutamátergní signalizace v SCN (Hannibal et al., 2000; Chen et al., 1999). Váže se na receptory PAC1 spřažené s G-proteiny, pravděpodobně aktivuje MAPKinasovou dráhu a může se tak podílet na světelné indukci hodinových genů *Per* (Nielsen et al., 2001; Butcher et al., 2005).

Aplikace světelného pulsu během první a druhé poloviny subjektivní noci indukuje v SCN expresi hodinového genu *Per1*. Exprese hodinového genu *Per2* je světlem indukována převážně v první polovině subjektivní noci (Miyake et al., 2000). Hodinové geny *Per1* a *Per2* jsou považovány za komponenty molekulárního mechanismu cirkadiánních hodin nezbytné pro synchronizaci cirkadiánních rytmů světelnými podněty (Albrecht et al., 2001). Ke zvýšení hladiny *Per1* a *Per2* mRNA, resp. proteinů PER1 a PER2 po světelném pulsu dochází zejména ve vlSCN (Yan a Silver, 2004). Odtud je zřejmě informace předávána do dmSCN a ovlivňuje výstupy z oscilátoru.

Exprese hodinových genů *Per1* a *Per2* je citlivá na světlo pouze v době, kdy je jejich spontánní exprese v SCN nízká. Mechanismus regulace neboli „vrátkování“ citlivosti ke světlu je tak zakódován v samotném molekulárním základu cirkadiánních

hodin. Některé studie spojují mechanismus regulace citlivosti SCN ke světlu s peptidem BDNF (brain derived neurotrophic factor) a proteinem Dexras1 (dexamethasonem indukovaný RAS protein 1). Vzhledem k tomu, že receptory pro BDNF se nacházejí na nervových zakončeních RHT v blízkosti neuronů SCN produkujících BDNF, je pravděpodobné, že BDNF zvyšuje citlivost cirkadiánních hodin v SCN ke světelným podnětům (Liang et al., 1998a; Liang et al., 2000). Ke zvýšení citlivosti může docházet jak presynapticky - zvýšením výlevu glutamátu z RHT, tak postsynapticky – ovlivněním citlivosti NMDA a AMPA receptorů na neuronech SCN (Michel et al., 2006). *Bdnf* i *Dexras1* mRNA vykazují v SCN cirkadiánní rytmus a produkce BDNF a Dexras1 v SCN vrcholí v době subjektivní noci (Liang et al., 1998b; Takahashi et al., 2003). Dexras1 moduluje citlivost cirkadiánních hodin ke světelným i nesvětelným podnětům působením na signální dráhy, zejména na MAPKinasovou dráhu a na signalizaci přes $G_{i/o}$ skupinu G-proteinů (Cheng et al., 2006).

Kromě okamžitého vlivu světelných pulsů na expresi hodinových genů *Per1* a *Per2*, dochází vlivem změny délky dne, tj. fotoperiody, ke změnám v profilech exprese hodinových genů *Per1*, *Cry1* a *Bmal1* v SCN (přehled viz Sumová et al., 2003).



Obrázek 2: Schematické znázornění signálních drah světelné synchronizace v SCN s vyznačenými místy regulačního působení BDNF a Dexras1 (šedé šipky). Použité zkratky: BDNF = brain derived neurotrophic factor, CaM = calmodulin, CaMII = calmodulin-dependentní proteinkinasa II, cGKII = cGMP-dependentní proteinkinasa II, cGMP = cyklický guanosinmonofosfát, CRE = Ca2+/cAMP response element, CREB = Ca2+/cAMP response element binding protein, Dexras1 = dexamethasone-induced RAS protein 1, ERK = extracellular signal-regulated kinase, GC = guanylátcyklasa, GTP = guanosintrifosfát, MAPK = mitogen-activated protein kinase, NMDAr = N-methyl-D-aspartátový receptor, NOS = NO-synthasa, PACAP = pituitary adenylate cyclase-activating polypeptide, PAC1r = receptor pro PACAP (zpracováno podle Hirota a Fukada (2004) a prací uvedených v textu)

1.1.4 Vývoj cirkadiánního systému

Mláďata primátů včetně člověka a také dalších velkých savců se rodí poměrně dobře vyvinutá a některé fyziologické rytmy jsou u nich patrné ještě v prenatálním období vývoje. U novorozených dětí však ihned po porodu převažuje u příjmu potravy či spánku a bdění spíše rytmicita s periodou výrazně kratší než 24 hodin (ultradiánní). Cirkadiánní rytmicita začíná převládat teprve postupně během prvního měsíce života (Lohr a Sigmund, 1999). Mláďata hlodavců se rodí méně vyvinutá a měřitelné cirkadiánní rytmy se objevují převážně až po narození. Z tohoto důvodu jsou potkani a myši v časných stádiích postnatálního vývoje vhodným objektem pro výzkum vývoje cirkadiánní rytmicity. V předkládané práci byl modelovým zvířetem laboratorní potkan a proto je souhrn poznatků o vývoji cirkadiánního systému omezen na tento živočišný druh.

Březost trvá u potkanů 22-23 dnů. SCN je tvořeno mezi 13. a 16. dnem embryonálního vývoje a vlSCN vzniká dříve než dmSCN (přehled viz Weinert, 2005). Brzy po zformování SCN, 19. den embryonálního vývoje, byl pozorován rozdíl mezi denní a noční metabolickou aktivitou buněk SCN (Reppert a Schwarz, 1984). 20. den embryonálního vývoje však ještě nedochází k rytmické expresi genu *Avp*, protože hladiny heteronukleární RNA *Avp* jsou během 24-hodinového cyklu konstantní (Kováčiková et al., 2006). 21. den embryonálního vývoje byly zaznamenány první rozdíly v hladině *Avp* mRNA v SCN mezi dnem a nocí (Reppert a Uhl, 1987). Neuronální aktivita v SCN potkana je vyšší během dne a nižší během noci ode dne porodu. Tento rytmus se postupně vyvíjí a u mláďat starých dva týdny dosahuje stejné úrovně jako u dospělých potkanů (Shibata a Moore, 1987).

Exprese hodinových genů *Per1*, *Per2*, *Cry1*, *Bmal1* a *Clock* byla v SCN potkana detekována již 19. den embryonálního vývoje, v této době však ještě nebyl detekován cirkadiánní rytmus v jejich expresi (Sládek et al., 2004). Denní rytmy v hladině *Per1* a *Per2* mRNA v SCN potkana začínají být patrné až 1. den po narození. Exprese je prokazatelně rytmická u 2-denních mláďat a dále se vyvíjí. První náznaky rytmu v expresi *Cry1* mRNA jsou patrné druhý den po narození (Kováčiková et al., 2006) a u 3-denních mláďat je rytmus již prokazatelný a rovněž se s věkem dále vyvíjí (Sládek et al., 2004). Hladina *Bmal1* mRNA v SCN je 19. a 20. den embryonálního vývoje vyšší než u ostatních hodinových genů a její rytmické změny jsou patrné od 1. dne po narození, přičemž teprve u 2-denních mláďat se začíná objevovat typický 24-hod profil,

který je v protifázi k profilům *Per* a *Cry* mRNA (Kováčiková et al., 2006). Amplituda rytmu v expresi všech sledovaných hodinových genů se dále zvětšuje mezi 3. a 10. postnatálním dnem (Sládek et al., 2004). Stejně jako u dospělých potkanů není exprese hodinového genu *Clock* v SCN během prenatálního a časného postnatálního vývoje rytmická (Sládek et al., 2004; Kováčiková et al., 2006). Z výše uvedených údajů je patrné že jednotlivé části molekulárního mechanismu cirkadiánních hodin se vyvíjejí postupně a převážně během časného postnatálního období (viz Tab. 1).

1.1.4.1 Synchronizace světlem během ontogeneze

Reakce vyvíjejícího se cirkadiánního systému na světelné podněty je kromě dosažení zralosti jednotlivých částí molekulárního mechanismu závislá i na vývoji drah, kterými je informace o světle přenášena ze sítnice do SCN. Od 18. dne embryonálního vývoje začíná být ve vnitřní vrstvě neuroblastů sítnice potkana tvořen cirkadiánní fotopigment melanopsin. V období okolo porodu buňky obsahující melanopsin migrují do vrstvy gangliových buněk a v časné postnatální ontogenezi vytvářejí dendritickou síť ve vnitřní plexiformní vrstvě (Fahrenkrug et al., 2004). Fotoreceptory, které se účastní přenosu vizuálních podnětů, se u mláďat vyvíjejí později a svůj vývoj dokončují až během třetího týdne postnatálního života. Projekce RHT, tvořeného z větší části vlákny RGCs, do SCN a přilehlých oblastí se začíná objevovat 1. den po narození v podobě izolovaných vláken vedoucích na ventrální okraj SCN. Dospělé úrovně inervace je dosaženo přibližně 10. den postnatálního vývoje (Speh a Moore, 1993). Oči se potkanům otevírají okolo 14. a 15. dne života. Mláďata však i přesto do určité míry vnímají světlo ještě před tímto důležitým vývojovým mezníkem (Duncan et al., 1986). Již 1. den po porodu byl pozorován vliv světelného pulsu na zvýšení exprese časného raného genu *c-fos* v SCN a to jak během subjektivní noci, tak během subjektivního dne (Leard et al., 1994). Produkce proteinu c-FOS v SCN přestává být indukovatelná světelnými podněty během subjektivního dne až 10. den po narození (Bendová et al., 2004). Je pravděpodobné, že vývoj diskrétní (světelné) a kontinuální (fotoperiodické) synchronizace souvisí s vývojem „vrátkového“ mechanismu, který vymezuje dobu citlivosti ke světlu.

den	vývoj cirkadiánního systému laboratorního potkana
E13-E16	formování SCN
E18	začíná tvorba melanopsinu v buňkách sítnice (Fahrenkrug et al., 2004)
E19	v SCN zjištěn rytmus v metabolické aktivitě (Reppert a Schwarz, 1984)
E19-E22	v SCN detekována exprese hodinových genů: *Per1, Per2, Cry1, Bmal1, Clock* (Sládek et al., 2004, Kováčiková et al., 2006)
P0	den porodu v SCN zjištěn náznak rytmu v neuronální aktivitě (Shibata a Moore, 1987) první projekce RHT do SCN (Speh a Moore, 1993)
P1	detekován náznak rytmické exprese hodinových genů *Per1*, *Per2* a *Bmal1* (Kováčiková et al., 2006) exprese časného raného genu c-fos v SCN je citlivá na světlo během subjektivního dne i subjektivní noci (Leard et al., 1994)
P2	Detekována rytmická exprese *Per1*, *Per2* a *Bmal1*; náznak rytmu u *Cry1* (Kováčiková et al., 2006)
P3	detekován rytmus v expresi *Cry1* u ostatních hodinových genů se zvětšuje amplituda rytmu (Sládek et al., 2004)
P10	pozorován rytmus v pohybové aktivitě dále se zvyšuje amplituda rytmu v expresi hodinových genů (Sládek et al., 2004) produkce proteinu c-FOS v SCN je indukovatelná světelným pulsem pouze v době subjektivní noci (Bendová et al., 2004)
P14-P15	otevírání očí

Tabulka 1: Shrnutí vývoje cirkadiánního systému laboratorního potkana v prenatální a časné postnatální ontogenezi. E = den embryonálního vývoje; P=den postnatálního vývoje

1.1.4.2 Synchronizace mateřskými nesvětelnými podněty během ontogeneze

U savců dostává *fetus* informace o vnějších světelných podmínkách již před narozením. Děje se tak zprostředkovaně přes cirkadiánní systém matky. Pokusy s lézemi SCN u potkaních samic v různých stádiích březosti ukázaly, že mateřská

synchronizace se uplatňuje zhruba od okamžiku, kdy je zformováno embryonální SCN. U potomků samic, kterým bylo odstraněno SCN v časných fázích březosti, nedochází ke ztrátě cirkadiánní rytmicity, je však narušena synchronizace s vnějším dnem (Shibata a Moore, 1988). Podobně dysfunkce cirkadiánních hodin matky vlivem mutace hodinových genů neovlivní vývoj cirkadiánního systému jejích mláďat (Jud a Albrecht, 2006). SCN matky se tedy přímo nepodílí na vývoji cirkadiánních rytmů mláďat, ale slouží jako významný synchronizátor cirkadiánních rytmů celého vrhu.

Signály, které během prenatálního období přijímá embryonální cirkadiánní systém od matky, jsou povahy neuronální i humorální. Důležitým signálem je zřejmě noční výlev melatoninu. Melatonin přechází přes placentu a pro mláďata by tak mohl být „signálem noci“. Jako „signál dne“ by mohl sloužit dopamin. Melatonin a dopamin tak zřejmě působí v embryonálním cirkadiánním systému jako dva vzájemně se doplňující signály (Weaver et al., 1992; Weaver a Reppert, 1995). Experimenty z poslední doby ukazují, že by dalším důležitým faktorem, synchronizujícím cirkadiánní hodiny mláděte v prenatálním období, mohl být matčin rytmus v příjmu potravy (Ohta et al., 2008)

Vliv matky na cirkadiánní systém mláděte pokračuje i po narození. Nejsilnější je v prvních dnech až týdnech postnatálního vývoje a pak postupně slábne. Mláďata hlodavců jsou zřejmě významně synchronizována rytmem v krmení a tedy i rytmickou přítomností, resp. nepřítomností, matky v hnízdě. Potkaní samice krmí mláďata převážně během dne a v noci je opouští, aby si sama opatřila potravu. Nepřítomnost matky v době krmení, tedy přes den, je pro mláďata silným stresujícím signálem, který ovlivňuje fázi rytmů v expresi hodinových genů *Per1* a *Per2* v jejich SCN (Ohta et al., 2003). Obecně lze říci, že cirkadiánní systém mláděte je v prvních dnech života citlivý převážně k nesvětelným podnětům zprostředkovaným mateřskou péčí a teprve později ke světelným signálům. Vliv matky v prenatální i postnatální ontogenezi napomáhá synchronizaci cirkadiánního systému mláděte s vnějšími podmínkami v době, kdy jeho cirkadiánní systém není ještě plně vyvinut.

2. Cíle práce

Cíle předkládané práce lze rozdělit do dvou tématických okruhů:

A) Vývoj cirkadiánních hodin a synchronizační vliv matky v prenatálním a časném postnatálním období

Cílem práce bylo zjistit kdy začíná molekulární mechanismus cirkadiánních hodin v SCN mláděte fungovat nezávisle na matce a do jaké míry jsou cirkadiánní hodiny mláděte ovlivňovány mateřskými signály během prenatálního a časného postnatálního období.

B) Vývoj citlivosti cirkadiánního systému potkana ke světelným podnětům

Cílem práce bylo odhalit kdy a jak v průběhu časné postnatální ontogeneze začíná být exprese hodinových genů *Per1* a *Per2* a časného raného genu *c-fos* v SCN mláděte citlivá na světelné podněty. Speciálním cílem pak bylo objasnit, kdy začínají cirkadiánní hodiny regulovat neboli „vrátkovat“ svou citlivost ke světelným podnětům. Také jsme se pokusili zmapovat vliv světelných podnětů aplikovaných během denní i noční doby na expresi hodinových genů *Per1* a *Per2* a časného raného genu *c-fos* v sítnici potkaního mláděte. Dalším cílem práce bylo zjistit, kdy během časné postnatální ontogeneze začíná být denní profil exprese hodinových genů modulován délkou světlé části dne, tzv. fotoperiodou.

3. Seznam publikací

Vývoj cirkadiánních hodin a synchronizační vliv matky v prenatálním a časném postnatálním období

El Hennamy R., Matějů K., Bendová, Z., Sosniyenko S., Sumová A.: Maternal control of the fetal and neonatal rat suprachiasmatic nucleus. Journal of Biological Rhythms 2008, 23 (5), 435-444 (*IF=3,868*)

Vývoj citlivosti cirkadiánního systému ke světelným podnětům

Matějů K., Bendová Z., El-Hennamy R., Sládek M., Sosniyenko S., Sumová A.: Development of the light sensitivity of clock gene *Period1*, *Period2* and immediate-early gene *c-fos* within the rat suprachiasmatic nucleus. European Journal of Neuroscience, 2009, 29 (3), 490-501 (*IF=3,673*)

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Souhrnné články

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Laurinová K., Sumová A.: Ontogenetický vývoj cirkadiánního systému savců. Československá fyziologie 2006; 55 (4), 96-102

4. Přehled použitých metod

4.1 Experimentální zvířata

K experimentům byla použita mláďata a dospělí potkani (*Rattus norwegicus*) kmene Wistar (BioTest s.r.o., Konárovice, ČR). Zvířata byla chována při teplotě 23 ± 2 °C s volným přístupem k vodě a potravě. Osvětlení bylo zajištěno 40W fluorescenčními zářivkami a intenzita světla byla podle umístění chovné nádoby 50-200lux. Světelné režimy a průběh experimentů jsou podrobně popsány v jednotlivých článcích. Všechny experimenty byly provedeny v souladu se zákonem na ochranu zvířat proti týrání (č.42084/2003-1020).

4.2 In situ hybridizace s radioaktivně značenou RNA sondou

Ke stanovení mRNA na koronálních řezech obsahujících SCN či oko byla použita metoda *in situ* hybridizace s ^{35}S – značenou RNA sondou. K přípravě prób pomocí *in vitro* transkripce bylo použito α-^{35}S-UTP (MP-Biosciences, USA; Izotop s.r.o., Maďarsko). Metoda je podrobně popsána v předložených článcích. Hybridizační signál v SCN byl detekován na autoradiografickém filmu. V případě nutnosti rozlišení lokalizace hybridizačního signálu na buněčné úrovni (rozlišení subpopulací buněk SCN a sítnice) byla skla po ukončení expozice filmu následně pokryta autoradiografickou emulsí. Relativní množství mRNA bylo na autoradiografickém filmu nebo emulsním preparátu stanoveno jako relativní optická densita.

4.3 Sledování pohybové aktivity zvířat

Pro sledování pohybové aktivity pokusných zvířat bylo použit systém CAMS (Circadian Activity Monitoring System, INSERM, Francie). Tento systém využívá ke snímání pohybu zvířat detektory infračerveného záření. Získaná data jsou ukládána do průmyslového počítače vybaveného softwarem umožňujícím sledovat probíhající záznamy z jednotlivých detektorů v reálném čase (tzv. aktogramy) a zároveň jednotlivé záznamy ukládat ve formě souborů. Tyto soubory je pak možno analyzovat pomocí programu Actiview (Mini Mitter Copany, Inc. Bend, Oregon USA).

5. Souhrn výsledků a diskuse

5.1 Vývoj cirkadiánních hodin a synchronizační vliv matky v prenatálním a časném postnatálním období

Související články:

Sumová A., Bendová Z., Sládek M., El-Hennamy R., Laurinová K., Jindráková Z., Illnerová H.: Setting the biological time in central and peripheral clocks during ontogenesis. FEBS Letters 2006, 580 (12), 2836-2842

El Hennamy R., Matějů K., Bendová, Z., Sosniyenko S., Sumová A.: Maternal control of the fetal and neonatal rat suprachiasmatic nucleus. Journal of Biological Rhythms 2008, 23 (5), 435-444.

Molekulární mechanismus cirkadiánních hodin v SCN laboratorního potkana je v prenatálním období značně nezralý. Hodinové geny jsou v SCN mláďat exprimovány již před narozením, ale cirkadiánní rytmus v jejich expresi se objevuje až v prvních dnech po narození a dále se vyvíjí během časné postnatální ontogeneze. Fáze vyvíjejících se cirkadiánních rytmů v expresi hodinových i hodinami řízených genů se shoduje s fází cirkadiánních hodin matky (Sládek et al., 2004; Kováčiková et al., 2006). Předpokládá se, že během fetálního vývoje hraje rytmická signalizace řízená z SCN matky zásadní roli při vývoji synchronizace nezralých cirkadiánních hodin s vnějším dnem (Shibata a Moore, 1988; Jud a Albrecht, 2006). Položili jsme si proto otázku, do jaké míry a ve kterém vývojovém stádiu je vyvíjející molekulární mechanismus cirkadiánních hodin v SCN během prenatálního a časného postnatálního vývoje seřizován cirkadiánními hodinami v SCN matky.

V první fázi experimentu byl vypracován protokol pro fázový posun cirkadiánních hodin v SCN dospělých zvířat, který byl později aplikován březím samicím tak, aby bylo možno sledovat jeho vliv na fázi fetálních hodin. Potkanům chovaným na světelném režimu s 12-ti hodinami světla a 12-ti hodinami tmy (LD12:12) byl zpožděn začátek a konec tmavé fáze světelného režimu o 6 hodin a poté byli následující cyklus vypuštěni do konstantní tmy, ve které byli chováni do konce experimentu (viz **El-Hennamy et al., 2008**). Monitorování pohybové aktivity ukázalo, že k fázovému zpoždění dochází během tří dnů následujících po posunu. Stejně tak byly během tří dnů fázově opožděny i profily v expresi hodinových genů *Per1*, *Per2*,

hodinami kontrolovaného genu *Avp* a časného raného genu *c-fos* v SCN v porovnání s profily v SCN kontrolních zvířat. Prokázali jsme tak, že cirkadiánní hodiny v SCN dospělého jedince jsou seřízeny během tří dnů po aplikaci posunu světelného režimu.

Abychom zjistili, zda fázový posun cirkadiánních hodin v SCN březí samice může seřídit cirkadiánní hodiny v SCN fétu, aplikovali jsme shora popsaný fázový posun světelného režimu březím samicím ve 20. dnu březosti (tj. 20. den embryonálního vývoje mláďat, E20) a sledovali jeho vliv na profily exprese genů *Avp* a *c-fos* v SCN během prvního postnatálního dne (P0-1), tj. 3. den po posunu. Zjistili jsme, že profily v expresi *c-fos* a *Avp* v SCN mláďat vystavených posunu světelného režimu v E20 nebyly v porovnání s profily u stejně starých mláďat z kontrolní skupiny, která nebyla fázovému posunu vystavena, nijak ovlivněny. Pokud jsme však porovnávali fáze profilů v expresi genů *Per1* a *Per2* v SCN u 3-denních a 6-denních potkaních mláďat, tj. za 5 a 8 dnů po posunu, prokázali jsme jejich významné fázové opoždění po vystavení posunu světelného režimu v E20. Velikost fázového posunu v P3 a P6 se přitom významně nelišila a byla přibližně stejná s posunem u dospělých zvířat. Nepřítomnost fázového posunu v P0-1 tak byla zřejmě zapříčiněna skutečností, že interval mezi E20 a P0-1 (tj. 3 dny) byl příliš krátký na to, aby mohlo dojít k seřízení fetálního SCN signály z SCN matky. Interval mezi E20 a P3 (tj. 5 dní) byl pro seřízení SCN mateřskou synchronizací již dostačující.

Ovlivnění cirkadiánních hodin mláděte posunem světelného režimu v E20 se projevilo až v časném postnatálním období, tedy v době, kdy je SCN mláděte již zralejší a je schopno samo generovat cirkadiánní rytmy v expresi hodinových genů. Zajímalo nás tedy, zda neschopnost seřídit cirkadiánní hodiny v SCN během intervalu E20 a P0-1 není způsobena nezralostí fetálního SCN. V dalším experimentu jsme vystavili březí samice posunu světelného režimu již 18. den březosti (E18) a sledovali jsme jak tento posun ovlivní SCN mláďat v P0-P1, tj. po pěti dnech vývoje *in utero*. Profily exprese *c-fos* a *Avp* v SCN novorozených mláďat vystavených v E18 posunu světelného režimu byly signifikantně fázově opožděny oproti profilům stejně starých mláďat z kontrolní skupiny. Prokázali jsme tak, že cirkadiánní hodiny jsou ve fetálním SCN schopny reagovat na mateřské signály fázovým posunem během pěti dnů počínaje již od E18. Mateřské signály tak mohou ovlivňovat cirkadiánní pacemaker v SCN mláďat během velmi časného vývojového stádia, kdy vyvíjející se cirkadiánní hodiny samy ještě nejsou schopny vytvářet synchronní rytmy v expresi hodinových genů.

Prokázali jsme tak, že vyvíjející se cirkadiánní hodiny mláděte nejsou v prenatálním období synchronizovány světelným režimem paralelně s hodinami matky. Zatímco u dospělých zvířat se změna světelného režimu projevila na úrovni cirkadiánního pacemakeru v SCN již po třech dnech, u vyvíjejícího se fétu se tato změna projevila v SCN až po pěti dnech. Výsledky naznačují, že fetální SCN nepřijímá samostatně signály z vnějšího prostředí, ale že informace o vnějším světelném režimu vnímá pouze zprostředkovaně přes cirkadiánní systém matky. Po posunu světelného režimu jsou nejdříve synchronizovány cirkadiánní hodiny matky a ty pak dosud neznámým mechanismem seřizují vyvíjející se cirkadiánní hodiny mláďat. Cirkadiánní hodiny mláděte mohou být mateřským cirkadiánním systémem seřízeny během pěti dnů ať už prenatálního nebo časného postnatálního vývoje.

Otázkou zůstává, jakým mechanismem seřizují cirkadiánní hodiny matky fetální SCN. Ke konci prenatálního vývoje SCN již pravděpodobně obsahuje buňky, které fungují jako autonomní cirkadiánní oscilátory. Neurony v SCN jsou však během prenatálního období navzájem jen velmi málo propojeny synapsemi (Moore a Bernstein, 1989) a v nezralém SCN tak pravděpodobně není ještě vyvinut mechanismus, který by tyto samostatné oscilátory mohl navzájem synchronizovat. Rytmické signály přicházející od matky by mohly individuální spontánně oscilující buňky navzájem synchronizovat a pomáhat tak vyvíjejícímu se SCN vytvářet synchronní oscilace. Je však také možné, že rytmická aktivita jednotlivých buněk prenatálního SCN je přímo řízena rytmickými signály od matky. S postupující synaptogenezí a dozráváním molekulárního mechanismu v SCN se cirkadiánní hodiny mláděte stávají stále méně závislými na mateřské synchronizaci. Postupná ztráta citlivosti cirkadiánního pacemakeru mláděte k signálům od matky pravděpodobně souvisí s dozráváním autonomní rytmicity a postupným vývojem mechanismů zajišťujících synchronizaci světelnými signály.

5.2 Vývoj citlivosti cirkadiánního systému potkana ke světelným podnětům

5.2.1 Vývoj citlivosti SCN ke světelným podnětům

Související články:

Sumová A, Bendová Z, Sládek M, El-Hennamy R, Matějů K, Polidarová L, Sosniyenko S, Illnerová H. Circadian molecular clocks tick along ontogenesis. Physiological Research 2008, 57, Suppl. 3, S139-S148

Matějů K., Bendová Z., El-Hennamy R., Sládek M., Sosniyenko S., Sumová A.: Development of the light sensitivity of clock gene *Period1*, *Period2* and immediate-early gene *c-fos* within the rat suprachiasmatic nucleus. European Journal of Neuroscience. 2009, 29 (3), 490-501

Hodinové geny *Per1* a *Per2* jsou nezbytné pro synchronizaci cirkadiánních hodin světlem (Albrecht et al., 2001). Indukce jejich exprese po osvětlení zřejmě zprostředkovává molekulárnímu hodinovému mechanismu v SCN informaci o světle (Shigeyoshi et al., 1997). Exprese *Per1* a *Per2* v SCN dospělých potkanů je citlivá na světelné podněty pouze v době tzv. subjektivní noci, tedy v době, kdy je hladina jejich spontánní exprese v SCN nízká (Miyake et al., 2000; Yan a Okamura, 2002). Toto tzv. vrátkování citlivosti ke světlu je vlastností cirkadiánních hodin a umožňuje seřizování jejich fáze světlem. Dřívější práce, zabývající se ontogenetickým vývojem citlivosti SCN ke světlu, využívaly jako ukazatel fotosensitivity cirkadiánních hodin v SCN světelnou indukci exprese časného raného genu *c-fos*, která je hodinami vrátkována podobně jako fotoindukce genů *Per1* a *Per2*. Světlo indukuje *c-fos* v SCN již u 1-denních mláďat (Leard et al., 1994). U 3-denních mláďat je produkce proteinu c-FOS v SCN indukována světelnými pulsy jak během subjektivního dne, tak i během subjektivní noci a teprve u 10-denních mláďat je tato indukce omezena převážně na dobu subjektivní noci (Bendová et al., 2004).

V naší současné studii jsme zkoumali ontogenetický vývoj fotosensitivity SCN a jeho vrátkového mechanismu s použitím fotoindukce nejen exprese *c-fos*, ale také přímo hodinových genů *Per1* a *Per2*. Světelné pulsy jsme aplikovali během subjektivního dne či během první nebo druhé poloviny subjektivní noci u potkaních mláďat 1., 3., 5. a 10.

den po narození. Zaměřili jsme se také podrobněji na prostorovou lokalizaci jak spontánní, tak i světlem indukované exprese *Per1*, *Per2* a *c-fos* ve vyvíjejícím se SCN.

Exprese hodinového genu *Per1* byla v SCN 1-denních mláďat indukována světelným pulsem aplikovaným jak během subjektivního dne, tak během subjektivní noci. U 3-denních, 5-denních a 10-denních mláďat je exprese *Per1* v SCN citlivá na světlo během subjektivní noci, avšak již nikoliv během subjektivního dne. Hladina světlem indukované *Per1* mRNA v SCN narůstala zpravidla během 30 min až 1 hod po začátku světelného pulsu a pak pozvolna klesala. Množství *Per1* mRNA indukované světlem během subjektivní noci dosahovalo hodnot srovnatelných se spontánní expresí *Per1* mRNA v SCN kontrolních zvířat během subjektivního dne. S použitím emulsní autoradiografie jsme zjistili, že u 1-denních mláďat dochází ke světelné indukci exprese *Per1* v celém SCN. U 3-denních mláďat byla světlem indukovaná *Per1* mRNA již lokalizována převážně ve vlSCN. Jak SCN postupně dozrává, rozložení signálu stále více připomíná situaci u dospělých zvířat. S postupujícím vývojem byla spontánní exprese *Per1* lokalizována převážně v dmSCN, stejně jako u dospělých zvířat (Miyake et al., 2000; Yan a Silver, 2004). Mechanismus regulující citlivost hodinového genu *Per1* na světlo byl tedy v SCN přítomen již 3. postnatální den a dále se vyvíjel.

Citlivost exprese hodinového genu *Per2* v SCN na světelné podněty se vyvíjí pomaleji než *Per1*. Teprve 3. postnatální den byla pozorována indukce exprese *Per2*, a to po světelném pulsu aplikovaném během druhé poloviny subjektivní noci. V SCN 5-denních mláďat byla fotoindukce exprese *Per2* omezena především na dobu subjektivní noci, i když k mírnému zvýšení exprese došlo také po pulsu během subjektivního dne. Teprve u 10-denních mláďat byla fotoindukce exprese *Per2* omezena výhradně na dobu subjektivní noci. Hladina *Per2* mRNA narůstala v SCN po světelném pulsu pomaleji než hladina *Per1* mRNA. K významnému zvýšení docházelo až 1 až 2 hod po začátku světelného pulsu. Hladina světlem indukované *Per2* mRNA během první poloviny subjektivní noci obvykle nepřesahovala hladinu vysoké spontánní exprese v SCN kontrolních zvířat během subjektivního dne. V druhé polovině subjektivní noci bylo množství světlem indukované exprese *Per2* zpravidla nižší a dosahovalo zhruba úrovně exprese v SCN kontrolních zvířat v první polovině subjektivní noci. Emulsní autoradiografie ukázala, že po světelném pulsu aplikovaném v první polovině subjektivní noci dochází k indukci exprese *Per2* v celém SCN, zatímco slabá indukce *Per2* po pulsu během druhé poloviny subjektivní noci je lokalizována spíše ve vlSCN. Spontánní exprese *Per2* mRNA v SCN kontrolních zvířat byla lokalizována převážně

v dmSCN. Zdá se, že mechanismus vrátkující citlivost *Per2* ke světlu se začíná objevovat až 5. postnatální den a dále se postupně vyvíjí.

Exprese časného raného genu *c-fos* v SCN 1-denních mláďat byla zvýšená po světelném pulsu aplikovaném během subjektivní noci i během subjektivního dne. U 3-denních mláďat byla exprese *c-fos* v SCN indukována světlem během subjektivní noci a nikoliv během subjektivního dne. Avšak ještě 5. postnatální den byla pozorována mírná indukce exprese *c-fos* po světelném pulsu aplikovaném během subjektivního dne. Teprve u 10-denních mláďat byla fotoindukce *c-fos* omezena výhradně na dobu subjektivní noci. Exprese *c-fos* v SCN po světelném pulsu narůstala velmi rychle a 2 hod po pulsu již množství světlem indukované *c-fos* mRNA výrazně klesalo. Množství *c-fos* mRNA indukované světelným pulsem během subjektivní noci obvykle významně přesahovalo hladinu spontánní exprese *c-fos* v SCN kontrolních zvířat během subjektivního dne. Emulsní autoradiografie ukázala, že 1. postnatální den je exprese *c-fos* indukovaná světlem v celém SCN, avšak nejintenzivnější je v úzkém pásu na jeho ventrálním okraji těsně přiléhajícímu k RHT. U 3-denních a starších mláďat byla světlem indukovaná exprese *c-fos* během subjektivní noci již lokalizována ve vlSCN. Podrobná analýza prostorového uspořádání exprese odhalila, že během subjektivního dne byla v SCN 3-denních a 5-denních mláďat po světelném pulsu mírně zvýšena exprese *c-fos* ve vlSCN. Tato indukce již 10. postnatální den nebyla patrná. V souladu s předchozími výsledky (Leard et al., 1994; Bendová et al., 2004) jsme prokázali, že mechanismus, kterým cirkadiánní hodiny vymezují citlivost exprese *c-fos* ke světlu, se začíná postupně vyvíjet od 3. dne po narození, je však plně vyvinut až 10. den po narození.

Ze srovnání výsledků fotosensitivity genů *Per1*, *Per2* a *c-fos* vyplývá, že citlivost exprese *Per1* a *c-fos* v SCN ke světelným podnětům se během časné postnatální ontogeneze vyvíjejí víceméně paralelně. Mechanismy vrátkující fotosensitivitu těchto genů jsou přítomny částečně či zcela již 3. postnatální den a dále se vyvíjejí. Citlivost exprese hodinového genu *Per2* v SCN ke světelným podnětům se vyvíjí pomaleji a indukce exprese *Per2* po světelném pulsu má také poněkud odlišnou dynamiku. Zdá se tedy, že cirkadiánní hodiny regulují citlivost exprese *Per2* na světlo jiným mechanismem než citlivost *Per1* a *c-fos*.

Z výše uvedeného vyplývá, že cirkadiánní hodiny v SCN potkana začínají reagovat na světlo již 1. postnatální den, a to nejen na úrovni neuronální aktivity (jejímž markerem je exprese *c-fos*), ale také na úrovni exprese hodinového genu *Per1*. Díky

tomu může světlo ovlivňovat dosud nevyzrálý molekulární mechanismus cirkadiánního pacemakeru. Cirkadiánní hodiny jsou tedy v tomto období schopny přijímat signály z vyvíjející se sítnice (viz kapitola 5.2.2.) prostřednictvím postupně se vyvíjejícího RHT (Moore a Bernstein. 1989). Signální dráha světelné indukce *Per1* a *c-fos* v SCN je 1. postnatální den patrně již dostatečně vyzrálá, ale mechanismus vrátkující citlivost cirkadiánních hodin na světelné podněty není dosud vyvinut. Stojí přitom za povšimnutí, že vývoj mechanismu vrátkování citlivosti exprese genů *Per1*, *Per2* a *c-fos* v SCN na světlo přichází společně s vývojem cirkadiánních rytmů ve spontánní expresi hodinových genů mezi 3. a 10. postnatálním dnem (Sládek et al., 2004; **Sumová et al., 2006**). Zdá se tedy, že schopnost cirkadiánních hodin v SCN vrátkovat citlivost na světlo by mohla záviset na celkové vyzrálosti jeho vlastního molekulárního mechanismu, projevující se přítomností významných rytmů v expresi hodinových genů. Důležitou úlohu při vývoji tohoto mechanismu by také mohla hrát postupná funkční diferenciace buněk SCN na ty, které generují cirkadiánní oscilace v dmSCN a na buňky, které přijímají signály z RHT a podílí se na synchronizaci cirkadiánních rytmů světlem ve vlSCN (Hamada et al., 2001; Antle a Silver, 2005). Naše výsledky naznačují, že vývoj mechanismu regulujícího citlivost SCN ke světelným podnětům je zároveň doprovázen prostorovou redistribucí fotosensitivních buněk, a to z celého SCN do vlSCN (**Matějů et al., 2009**). Souběžně s funkční diferenciací SCN na dmSCN a vlSCN narůstá v SCN počet synapsí. Po narození roste nejvýrazněji mezi 4. a 10. postnatálním dnem (Moore a Bernstein, 1989), tedy v době kdy mechanismus vrátkující citlivost ke světlu postupně dozrává. Přítomnost mechanismu vrátkujícího citlivost na světlo je tedy pravděpodobně také podmíněna dostatečnou funkční vyzrálostí intercelulárních spojení mezi buňkami SCN.

5.2.2 Vliv světelných podnětů na sítnici potkana během vývoje

Související články:

Matějů K., Bendová Z., Sumová A. Light sensitivity of expression of clock genes *Period1, Period2* and immediate-early gene *c-Fos* within retina of early postnatal rat. *manuscript v přípravě;*

Oko jako fotoreceptivní orgán hraje důležitou úlohu při světelné synchronizaci cirkadiánních rytmů. Část gangliových buněk sítnice produkuje fotopigment melanopsin a slouží jako tzv. cirkadiánní fotoreceptory předávající informaci o světle do SCN

(Hattar et al., 2002; Berson et al., 2002). Navíc bylo prokázáno, že sítnice obsahuje periferní cirkadiánní oscilátor, který ovlivňuje její fyziologii a může tak ovlivňovat světelnou synchronizaci centrálního pacemakeru v SCN (přehled viz Tosini a Fukuhara, 2002; Tosini et al., 2008). Zdá se, že podobně jako cirkadiánní hodiny v SCN jsou i hodiny v sítnici citlivé na světlo. V sítnici dospělých potkanů byla pozorována světelná indukce exprese hodinových genů *Per1* a *Per2* lokalizovaná především ve vnitřní nukleární vrstvě (Namihira et al., 2001). V sítnici je rovněž exprimován časný raný gen *c-fos* a byla popsána jeho světlem indukovaná exprese během subjektivního dne i během subjektivní noci (Huerta et al., 1997, 1999).

Sítnice reaguje na světlo již dlouho před otevřením očí. V den porodu morfologie potkaní sítnice zhruba odpovídá sítnici čtyřměsíčního lidského plodu. Je možno odlišit diferencující se gangliovou vrstvu a vnitřní plexiformní vrstvu, zbytek vyvíjející se *pars nervosa retinae* tvoří neuroblastové buňky. Vrstva pigmentového epitelu (RPE, retinal pigmented epithelium) je v té době již dobře vyvinutá. Ostatní typy buněk sítnice se teprve postupně diferencují z neuroblastových buněk během časné postnatální ontogeneze (Weidman a Kuwabara, 1969). Gangliové buňky produkující melanopsin jsou citlivé na světlo již při narození a zároveň již v té době existují první funkční spojení mezi sítnicí a SCN (Tu et al., 2005, Sekaran et al., 2005). Tyčinky a čípky se začínají morfologicky diferencovat okolo 5. dne po narození a dozrávají až v třetím postnatálním týdnu. Během prvního týdne po narození se tak původně jednolitá silná vrstva neuroblastových buněk rozdělí na dvě a dále se morfologicky diferencuje. Gangliové buňky začínají reagovat na signály z tyčinek a čípků až mezi 10. a 12. postnatálním dnem, když začíná fungovat propojení s fotoreceptory přes bipolární buňky (Weidman a Kuwabara, 1969; Sernagor et al., 2001). Fotoreceptivní gangliové buňky jsou tedy vyvinuty dříve než klasické fotoreceptory a tzv. „cirkadiánní vidění“ je vyvinuto dříve než obrazové vidění.

V naší studii jsme se zaměřili na vliv 30-minutových světelných pulsů aplikovaných během subjektivního dne (v CT7; CT=circadian time, CT0 = doba očekávaného rozsvícení, resp. doba kdy jsou pokusná zvířata vypuštěna do konstantní tmy) nebo během první (CT15) či druhé poloviny subjektivní noci (CT21) na expresi hodinových genů *Per1*, *Per2* a časného raného genu *c-fos* v sítnici 1-denních, 3-denních, 5-denních a 10-denních potkaních mláďat. Vliv světelného pulsu byl sledován 30min, 1h, 2h a 4h po začátku pulsu. Protože byly sítnice kontrolních zvířat odebrány v časových bodech pokrývajících větší část dne, získali jsme také určitou informaci o

spontánní expresi hodinových genů *Per1* a *Per2* a časného raného genu *c-fos* v sítnici během časné postnatální ontogeneze. Již první den po narození dochází v sítnici potkaních mláďat ke spontánní expresi hodinových genů *Per1* a *Per2*. V souladu s předchozími studiemi, které uvádějí spontánní expresi časného raného genu *c-fos* v sítnici až mezi 11. a 15. postnatálním dnem (Ohki et al., 1996), nebyla až do 10. dne po narození v sítnici kontrolních zvířat pozorována výrazná exprese *c-fos*. U žádného ze sledovaných genů jsme až do 10. postnatálního dne nepozorovali rozdíly ve spontánní expresi mezi subjektivním dnem a subjektivní nocí.

Spontánní exprese *Per1* nevykazovala v sítnici 1-denních, 3-denních a 5-denních kontrolních mláďat přesně ohraničenou lokalizaci. Až 10. postnatální den byla pozorována zvýšená exprese *Per1* ve vnější části sítnice, pravděpodobně ve fotoreceptorové vrstvě. V sítnici dospělých zvířat však byla exprese hodinových genů *Per1* a *Per2* i produkce PER1 proteinu popsána zejména ve vnitřní nukleární vrstvě a částečně i v gangliových buňkách (Namihira et al., 2001; Witkovsky et al., 2003; García-Fernández et al., 2007; Dinet et al., 2007; Ruan et al., 2008). Další práce ukázaly expresi *Per1* i ve fotoreceptorové vrstvě (Tosini et al., 2007). Zdá se tedy, že lokalizace exprese *Per1* se v průběhu vývoje mění. Navíc naše dosud nepublikovaná pozorování ukázala, že hybridizační signál *Per1* mRNA je lokalizován ve vnitřní nukleární vrstvě již 15. postnatální den, tedy v období otevírání očí.

Hybridizační signál *Per2* mRNA byl u kontrolních zvířat 1. postnatální den lokalizován na vnitřní straně vrstvy neuroblastových buněk. S postupující diferenciací sítnice byla exprese *Per2* lokalizována v její vnitřní nukleární vrstvě. Při narození není ještě sítnice potkaního mláděte morfologicky zralá, ale na vnitřní straně vrstvy neuroblastových buněk již byly pozorovány jednotlivé buňky charakteristické pro vnitřní nukleární vrstvu, která funkčně dozrává koncem prvního postnatálního týdne (Weidman and Kuwabara, 1969; Sernagor et al., 2001). Spontánní exprese hodinového genu *Per2* tedy svou lokalizací pravděpodobně „kopíruje“ postupně se vyvíjející vnitřní nukleární vrstvu sítnice a zdá se, že na rozdíl od spontánní exprese *Per1*, by exprese *Per2* mohla být spojena spíše s morfologickou než s funkční zralostí vnitřní nukleární vrstvy.

Světelný puls aplikovaný 1-, 3-, 5- a 10-denním mláďatům během subjektivního dne i subjektivní noci způsobil indukci exprese časného raného genu *c-fos* v gangliových buňkách sítnice. Exprese hodinových genů *Per1* a *Per2* v těchto buňkách ovlivněna nebyla. Gangliové buňky vykazující světlem indukovanou expresi *c-fos* se 1.,

3. a 5. postnatální den nacházely zejména v dorsální části sítnice a 10. postnatální den byly již na ploše sítnice rozloženy víceméně rovnoměrně. Počet *c-fos* mRNA pozitivních buněk byl zvýšen 30min a 1hod po začátku světelného pulsu a pak postupně klesal. S postupujícím věkem celkový počet gangliových buněk vykazujících citlivost exprese *c-fos* na světlo v sítnici rostl, zejména mezi 5. a 10. postnatálním dnem.

U dospělých zvířat světlo indukuje expresi časného raného genu *c-fos* v gangliových buňkách sítnice obsahujících melanopsin a také ve vnitřní nukleární vrstvě sítnice (Sagar a Sharp, 1990, Yoshida et al., 1993, 1995, Koistinaho a Sagar, 1995, Semo et al., 2003). Krom toho byla pozorována v 10-18% gangliových buněk v sítnici dospělých myší spontánní exprese *c-fos*, která je patrně způsobena signály z fotoreceptorové vrstvy (Semo et al., 2003). Vzhledem k tomu, že v naší studii bylo několik *c-fos* mRNA-pozitivních gangliových buněk pozorováno v sítnici kontrolních mláďat až 10. postnatální den, je pravděpodobné, že v prvních dnech po narození nejsou ještě gangliové buňky propojeny se zbytkem sítnice. Exprese časného raného genu *c-fos* po osvětlení je tedy výsledkem spíše vlastní fotosensitivity gangliových buněk sítnice než mezibuněčné signalizace. Výsledky světelné indukce exprese *c-fos* mRNA v gangliových buňkách vyvíjející se sítnice jsou tak v souladu s předchozími pracemi uvádějícími, že gangliové buňky sítnice jsou již od narození citlivé na světlo (Tu et al., 2005, Sekaran et al., 2005, Hannibal a Fahrenkrug, 2004).

Světlem indukovaná exprese *c-fos* byla 1. až 5. postnatální den lokalizována v gangliových buňkách převážně v dorsální části oka. V oku 10-denních mláďat již byly gangliové buňky reagující na světelný puls expresí *c-fos* rozloženy rovnoměrně. Podobná změna lokalizace během vývoje byla pozorována u M-opsinu ve fotoreceptorové vrstvě sítnice křečka (Glösmann a Ahnelt, 2002). Polarizace sítnice podle dorso-ventrální a naso-temporální osy oka je jevem doprovázejícím vznik retinotopické projekce gangliových buněk do mozku. Tato polarizace se v ontogenezi vyvíjí poměrně záhy (Peters 2002, McLaughlin et al., 2003, Harada et al., 2007), ačkoliv např. čtyři podtypy gangliových buněk, které jsou součástí dráhy řídící reflexní pohyby očí, začínají být funkční až v období otevírání očí (Elstrott et al., 2008). Zdá se tedy, že v průběhu vývoje sítnice se vlastnosti jednotlivých jejích oblastí mohou měnit.

Analýza emulsních autoradiogramů také ukázala, že po světelném pulsu aplikovaném během subjektivního dne i během subjektivní noci dochází v sítnici 1-denních a 3-denních mláďat k indukci exprese hodinového genu *Per1* a časného raného genu *c-fos* v ostře ohraničené vrstvě na vnějším okraji sítnice. Vzhledem k tomu, že

pars nervosa retinae je ještě 3. postnatální den jen málo diferencovaná, je onou vrstvou s největší pravděpodobností RPE. Zvýšené množství světlem indukované *Per1* i *c-fos* mRNA v RPE bylo u 1-denních mláďat pozorováno 1-2 hod po zahájení světelného pulsu a pak klesalo. U 3-denních mláďat citlivost exprese *Per1* a *c-fos* v RPE ke světlu pomalu odeznívá a u 5-denních mláďat již nebyla pozorována.

RPE je struktura důležitá pro zajištění optimální funkce fotoreceptorové vrstvy sítnice. U dospělých zvířat dochází k cyklické obnově vnějších segmentů fotoreceptorů v procesu označovaném jako „disc shedding“. Nejdistálněji položené terčíky vnějších segmentů fotoreceptorů jsou při něm fagocytovány buňkami RPE a nahrazovány novými terčíky odštěpenými z vnitřního segmentu (přehledně viz Strauss, 2005). Fagocytóza vnějších segmentů fotoreceptorů byla v *in vitro* podmínkách mj. provázena indukcí exprese časného raného genu *c-fos* v buňkách RPE (Ershov et al., 1996). Proces „disc shedding“ vykazuje rytmus s maximem během světlé části dne (La Vail, 1980), který zůstává zachován i u zvířat s lézí SCN a není tedy pod přímou kontrolou centrálního pacemakeru (Terman et al., 1993). Mimo to zajišťuje RPE obousměrný transport živin, vody a iontů mezi fotoreceptorovou vrstvou sítnice a kapilárami cévnatky. Buňky RPE také během vývoje oka produkují neurotrofické faktory důležité pro diferenciaci fotoreceptorové vrstvy sítnice a vývoj kontaktů mezi RPE a kapilárami (souhrn viz Strauss, 2005).

Světelná indukce exprese časného raného genu *c-fos* byla pozorována v podmínkách *in vitro* u buněčné linie odvozené z lidského RPE (Yam et al., 2003), což dokládá, že citlivost ke světlu je vlastností RPE a není např. důsledkem činnosti fotoreceptorů. Peirson et al. (2004) popsali v RPE myší expresi melanopsinu a je tedy pravděpodobné, že mechanismus světelné indukce *c-fos* v RPE by mohl být podobný jako v gangliových buňkách sítnice. U dospělých potkanů zvýšená koncentrace NO vede k indukci exprese *c-fos* v RPE a nikoliv v *pars nervosa retinae*. Tento mechanismus indukce exprese *c-fos* zahrnuje i fosforylaci CREB (Ohki et al., 1995) Taktéž byla na buněčné linii pocházející z lidského RPE v podmínkách in vitro pozorována rytmická exprese hodinových genů *Per1* a *Per2* po tzv. sérovém šoku (Pavan et al., 2006).

Citlivost exprese *Per1* a *c-fos* v RPE na světlo je vývojově ohraničená na dobu prvních dnů po narození a do 5. postnatálního dne mizí. K postupné ztrátě citlivosti exprese *Per1* a *c-fos* na světlo dochází přibližně v době, kdy se začínají morfologicky diferencovat fotoreceptorové buňky (Weidman and Kuwabara, 1969). Vrstva

fotoreceptorových buněk je během svého vývoje v těsném kontaktu s RPE (Weidman and Kuwabara, 1969), je tedy teoreticky možné, že dočasná fotosensitivita RPE hraje svou roli při vývoji fotoreceptorové vrstvy.

Ve stejné době, kdy v RPE mizí světlem indukovaná exprese *Per1* a *c-fos*, v SCN potkaních mláďat přestává být exprese *c-fos* indukována dopaminem (Weaver et al., 1992; Weaver a Reppert, 1995). Dopamin hraje v sítnici důležitou roli v mechanismu cirkadiánních hodin (Ruan et al., 2008) a také se účastní regulace exprese melanopsinu v gangliových buňkách sítnice (Sakamoto et al., 2005). Dopaminergní signalizace je součástí mechanismu indukce exprese *Per1* v sítnici po osvětlení (Yujnovsky et al., 2006). Aktivace tyrosinhydroxylasy – enzymu důležitého pro syntézu dopaminu - byla po osvětlení pozorována v amakrinních buňkách sítnice (Iuvone et al., 1978) a také v RPE ryb (McCormack a Burnside, 1993). Vzhledem k tomu, že dopaminové D1 receptory jsou funkční již během vývoje sítnice (Reis et al., 2007), je možné, že se dopamin podílí i na námi pozorované světelné indukci exprese *Per1* a *c-fos* v RPE.

Je třeba zdůraznit, že účinek světla na RPE bylo možno pozorovat jen díky tomu, že byla k experimentům použita mláďata albinotického kmene Wistar. U pigmentovaných zvířat je v RPE přítomen pigment melanin, který pohlcuje světelné záření a chrání sítnici, resp. cévnatku před toxickými účinky světla (Peters et al., 2006), ale také na emulsním autoradiogramu překrývá případný hybridizační signál. Pro albinismus je charakteristická mutace v genu pro tyrosinasu – enzym klíčový pro syntézu melaninu (přehled viz Jeffery, 1997). Snížená funkce tyrosinasy se projeví nejen nedostatkem melaninu v RPE, ale také sníženou koncentrací jeho prekursoru L-DOPA, což vede k atypickému vývoji sítnice (Tibber et el., 2006). Námi pozorované jevy mohou být u albinotických potkanů také výsledkem nedostatečné ochrany vyvíjející se sítnice před toxickým působením světla a zvýšená exprese *c-fos* je součástí stresové odpovědi buněk RPE na světlo. Zda je vývojově ohraničená fotosensitivita exprese *Per1* a *c-fos* v RPE přítomna i u pigmentovaných zvířat bude možné rozhodnout až s použitím metod které nebudou interferovat s melaninem a zároveň nebudou ovlivněny vysokou aktivitou enzymů v buňkách RPE.

Během časné postnatální ontogeneze je sítnice potkana citlivá na světelné podněty ještě před otevřením očí. Již od 1. postnatálního dne působí světlo indukci exprese časného raného genu *c-fos* v gangliových buňkách sítnice. Počet gangliových buněk vykazujících světlem indukovanou expresi *c-fos* se v průběhu vývoje mění a mezi

5. a 10. postnatálním dnem se jejich lokalizace postupně rozšiřuje z dorsální oblasti oka na celou plochu sítnice. Ke spontánní expresi hodinových genů *Per1* a *Per2* dochází v sítnici potkaních mláďat již 1. postnatální den. Exprese *Per1* během časné postnatální ontogeneze postupně mění svou lokalizaci a v *pars nervosa retinae* nevykazuje citlivost ke světelným podnětům. Vývojově ohraničená citlivost exprese *Per1* a *c-fos* ke světlu byla pozorována ve vnější vrstvě sítnice, která je s největší pravděpodobností totožná s RPE. Exprese *Per2* během časné postnatální ontogeneze kopíruje diferencující se vnitřní nukleární vrstvu sítnice a až do 10. postnatálního dne není nijak ovlivněna světlem. Citlivost exprese *c-fos* v gangliové vrstvě sítnice na světlo a přechodná fotosensitivita exprese *Per1* a *c-fos* v RPE není časově regulována (vrátkována) jako je tomu v sítnici dospělých zvířat (Namihira et al., 2001), nebo v SCN potkaních mláďat v časné postnatální ontogenezi (**Matějů et al., *přijato do tisku***). Ačkoliv tedy sítnice potkana v prvních dnech po narození již obsahuje některé komponenty molekulárního mechanismu cirkadiánních hodin, jsou cirkadiánní hodiny v sítnici během časné postnatální ontogeneze ještě nezralé.

5.2.3 Vliv fotoperiody na expresi hodinových genů v SCN potkana během postnatálního vývoje

Související články:

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Sumová A, Bendová Z, Sládek M, Kováčiková Z, El-Hennamy R, Laurinová K, Illnerová H.: The rat circadian clockwork and its photoperiodic entrainment during development. Chronobiology International 2006, 23 (1-2), p.237-43.

Cirkadiánní rytmicita většiny obratlovců je ovlivněna délkou dne, tzv. fotoperiodou. Fotoperiodě se přizpůsobuje např. rytmus v pohybové aktivitě či v produkci melatoninu (Illnerová a Vaněček, 1980). Fotoperioda ovlivňuje přímo funkční stav cirkadiánních hodin v SCN. Dřívější studie ukázaly, že fotoperioda má vliv jak na dobu kdy je exprese časného ranného genu *c-fos* v SCN indukovatelná světlem (Sumová

et al., 1995), tak ovlivňuje i rytmus ve spontánní expresi *c-fos* v SCN (Sumová et al., 2000). U dospělých potkanů dochází ke komplexnímu ovlivnění molekulárního mechanismu cirkadiánních hodin. Ovlivněn je jak profil exprese fotosensitivního hodinového genu *Per1*, tak také profily exprese dalších hodinových genů *Cry1* a *Bmal1* jejichž exprese není přímo indukovatelná světlem. U potkanů chovaných na dlouhé letní fotoperiodě (LD16:8; 16 hodin světla a 8 hodin tmy) dochází k rannímu nárůstu exprese *Per1* v SCN dříve než u zvířat chovaných na krátké zimní fotoperiodě (LD8:16) a tak je doba po kterou je hladina *Per1* mRNA v SCN vysoká delší u zvířat chovaných na dlouhé fotoperiodě než u zvířat chovaných na krátké fotoperiodě. Ranní nárůst exprese hodinového genu *Cry1* v SCN i její večerní pokles nastává dříve u zvířat chovaných na dlouhé fotoperiodě než u zvířat chovaných na krátké fotoperiodě. Délka doby, po kterou je hladina *Cry1* mRNA v SCN vysoká, tedy není fotoperiodou ovlivněna, ale rytmus v expresi *Cry1* v SCN zvířat chovaných na dlouhé fotoperiodě je oproti zvířatům na krátké fotoperiodě fázově posunutý. Ranní pokles exprese hodinového genu *Bmal1* v SCN nastává později u zvířat chovaných na krátké fotoperiodě a doba kdy je hladina *Bmal1* mRNA v SCN zvýšená je tedy delší u zvířat chovaných na krátké fotoperiodě než u zvířat chovaných na dlouhé fotoperiodě. Navíc rytmus v expresi *Per1*, *Cry1* i *Bmal1* v SCN vykazuje větší amplitudu u zvířat chovaných na krátké fotoperiodě než u zvířat chovaných na dlouhé fotoperiodě (Sumová et al., 2003).

Ve studii zaměřené na vývoj reakce cirkadiánního pacemakeru v SCN na délku dne (**Kováčiková at al., 2005**) jsme zkoumali vliv dlouhé letní a krátké zimní fotoperiody na denní profily v expresi hodinových genů *Per1*, *Per2*, *Cry1* a *Bmal1* v SCN 3-denních, 10-denních a 20-denních potkaních mláďat. 3. den po narození nebyly profily exprese ani jednoho ze sledovaných genů modulovány fotoperiodou. U 10-denních mláďat docházelo pouze k ovlivnění profilů exprese *Per1* a *Per2*, tedy genů, jejichž exprese je citlivá na světlo. Ranní nárůst exprese *Per1* a *Per2* v SCN 10-denních mláďat chovaných na dlouhé fotoperiodě nastal – podobně jako u dospělých potkanů - významně dříve u než u mláďat chovaných na krátké fotoperiodě. U mláďat chovaných na dlouhé fotoperiodě byla navíc posunuta i doba večerního poklesu *Per2* mRNA v SCN. Díky tomu byl interval nízké exprese *Per1* a *Per2* delší u mláďat chovaných na krátké fotoperiodě než u mláďat chovaných na dlouhé fotoperiodě. Na profily genů, jejichž exprese není fotosensitivní, měla fotoperioda vliv až v pozdějších vývojových stádiích. 20. den po narození byla fotoperiodou ovlivněna exprese *Cry1* mRNA. Podobně jako u dospělých zvířat exprese *Cry1* narůstala i klesala o 4-6 hod

dříve u mláďat chovaných na dlouhé než na krátké fotoperiodě. Vliv fotoperiody na expresi hodinového genu *Bmal1* v SCN se neprojevil ještě ani u 20-denních mláďat (**Kováčiková et al., 2005**; **Sumová et al., 2006**). Synchronizace cirkadiánního systému fotoperiodou se tedy u potkana začíná vyvíjet okolo 10. dne po narození, během postnatální ontogeneze postupně dozrává a dospělé úrovně dosahuje pravděpodobně až v období odstavu.

Ačkoliv je 3. postnatální den exprese hodinových genů *Per1, Per2* a časného raného genu *c-fos* indukovatelná světelným pulsem a citlivost *Per1* a *c-fos* ke světlu již začíná být vrátkována (**Matějů et al., *přijato do tisku***), profily spontánní exprese *Per1* a *Per2* v SCN 3-denních mláďat ještě zatím nereagují na délku dne. Fototoperioda začíná modulovat denní profily ve spontánní expresi *Per1* a *Per2* v SCN až u 10-denních mláďat a na expresi hodinových genů *Cry1* a *Bmal1* má vliv až 20. postnatální den, resp. ještě později (**Kováčiková et al., 2005**). Zdá se tedy, že ukončení vývoje mechanismu vrátkujícího citlivost cirkadiánních hodin ke světlu by mohlo být nezbytným předpokladem pro modulaci denních profilů spontánní exprese hodinových genů fotoperiodou. Vzhledem k tomu, že 10. postnatální den dosahuje vývoj RHT a počet synapsí v SCN dospělé úrovně (Speh a Moore, 1993; Moore a Bernstein, 1989) a zároveň je již dostatečně zralý mechanismus vrátkující citlivost vlSCN ke světlu, může být informace o světle předávána z vlSCN do dmSCN a následně tak mohou být ovlivněny spontánní rytmy v genové expresi (**Kováčiková et al., 2005; Sumová et al., 2006**). Jelikož naše výsledky jednoznačně prokázaly dřívější ovlivnění profilů v expresi *Per1* a *Per2* a teprve pozdější vliv fotoperiody na expresi *Cry1* a *Bmal1*, je pravděpodobné, že vývoj reakce cirkadiánních hodin v SCN na fotoperiodu je závislý jak na zralosti mechanismu, kterým je informace o světle předávána z vlSCN do dmSCN, tak na celkové maturaci molekulárního mechanismu cirkadiánních hodin v dmSCN.

6. Shrnutí

Výsledky předložených prací pomáhají vytvořit obraz ontogenetického vývoje synchronizace cirkadiánního systému potkana. Během prenatálního vývoje se informace o světelném režimu dostávají k fétům zprostředkovaně přes cirkadiánní systém matky. Cirkadiánní hodiny *fétu* nejsou při změně světelného režimu synchronizovány zároveň s matkou, ale jsou seřizovány mateřskými signály až poté, co se cirkadiánní hodiny matky

přizpůsobí změně (**El-Hennamy et al., 2008**). Mateřská synchronizace hraje nejvýznamnější roli v prenatálním období vývoje a v prvních dnech po narození. S postupující maturací se cirkadiánní hodiny mláděte „osamostatňují" od synchronizačního vlivu matky a postupně převládá synchronizace světlem (**Sumová et al., 2006**). Pro synchronizaci cirkadiánních rytmů světlem je důležitá fotosensitivní funkce sítnice a funkční propojení sítnice s centrálním pacemakerem v SCN. SCN samo pak musí být dostatečně zralé, aby mohl být cirkadiánní pacemaker synchronizován světelnými podněty.

K prvnímu propojení sítnice s SCN dochází již první den po narození a již první postnatální den je sítnice potkaních mláďat citlivá na světlo. Fotosensitivita gangliových buněk sítnice se již 1. postnatální den projevuje expresí časného raného genu *c-fos* po osvětlení a během časné postnatální ontogeneze se dále vyvíjí (**Matějů et al., manuskript v přípravě**). Již od prvního dne po narození dochází také ke spontánní expresi hodinových genů *Per1* a *Per2* v sítnici. Zdá se, že exprese *Per1* a *Per2* není v *pars nervosa retinae* citlivá na světlo. V prvních dnech po narození však byla přechodně pozorována indukce exprese hodinového genu *Per1* a také časného raného genu *c-fos* v RPE. Vzhledem k tomu, že nebyly pozorovány rozdíly ve spontánní expresi hodinových genů mezi subjektivním dnem a subjektivní nocí se zdá, že mechanismus cirkadiánních hodin v sítnici potkaních mláďat je během časné postnatální ontogeneze ještě nezralý.

Již první den po narození reagují cirkadiánní hodiny v SCN na světelné podněty, a to jak na úrovni aktivace neuronů SCN reprezentované indukcí exprese časného raného genu *c-fos*, tak také indukcí exprese hodinového genu *Per1* v SCN. Mechanismus kterým cirkadiánní hodiny v SCN vrátkují citlivost exprese *Per1* a *c-fos* ke světelným podnětům se začíná uplatňovat od 3. postnatálního dne. Citlivost exprese hodinového genu *Per2* a její vrátkování se vyvíjí pomaleji, což naznačuje, že by mohlo být řízeno jiným mechanismem než u *Per1* a *c-fos*. Jak postupně dozrává molekulární mechanismus cirkadiánních hodin (**Sumová et al., 2008**), vrátkovací mechanismus se dále vyvíjí a citlivost exprese *Per1*, *Per2* a *c-fos* je 10. postnatální den již omezena pouze na dobu subjektivní noci. Vývoj vrátkovacího mechanismu je doprovázen funkčním rozdělením SCN na dmSCN, kde dochází pouze ke spontánní rytmické expresi hodinových genů a vlSCN, kde je exprese *Per1* a *c-fos* indukována světelným pulsem během subjektivní noci (**Matějů et al., 2009**).

Mechanismus, kterým jsou cirkadiánní hodiny v SCN synchronizovány světlem, má více součástí. Kromě okamžité reakce na světelné podněty dochází i ke komplexnímu ovlivnění denních profilů ve spontánní expresi hodinových genů v SCN. Zdá se, že přítomnost mechanismu vrátkujícího citlivost vlSCN ke světelným podnětům je předpokladem pro to, aby spontánní cirkadiánní rytmicita v dmSCN byla ovlivněna fotoperiodou. Spontánní exprese hodinových genů *Per1* a *Per2* v SCN začíná být modulována délkou dne 10. postnatální den a reakce na fotoperiodu se dále vyvíjí. Profil v expresi hodinového genu *Cry1* je ovlivněn fotoperiodou až 20. postnatální den a exprese *Bmal1* je ovlivněna délkou dne ještě později (**Kováčiková et al., 2005**).

Získané výsledky naznačují, že funkčně nejprve dozrává vlSCN, ve kterém dochází k okamžitému ovlivnění exprese hodinových genů světlem. Když je dostatečně vyvinutý mechanismus vrátkující citlivost vlSCN ke světlu a synaptické propojení vlSCN a dmSCN dosáhne dospělé úrovně, může být informace o světle zpracovaná ve vlSCN předána do dmSCN, kde dojde k modulaci exprese hodinových genů. Jak komplexní bude ovlivnění spontánní exprese hodinových genů v dmSCN délkou dne závisí i na celkové zralosti molekulárního mechanismu cirkadiánních hodin.

Závěr

V předložených publikacích, které tvoří základ disertační práce, jsme mapovali vývoj světelné synchronizace cirkadiánního systému potkana. Pozornost byla věnována zejména tomu, jak během prenatálního a postnatálního období působí světelný režim na vyvíjející se cirkadiánní hodiny mláděte. V prenatálním období se informace o světle dostává k mláďatům zprostředkovaně přes cirkadiánní systém matky a během postnatálního období se postupně vyvíjí jak okamžitá reakce cirkadiánního pacemakeru v SCN mláďat na světelné podněty, tak i modulace cirkadiánní rytmicity mláďat délkou světlé části dne. Rovněž jsme se věnovali reakci vyvíjející se sítnice na světelné podněty během časného postnatálního vývoje.

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Minireview

Setting the biological time in central and peripheral clocks during ontogenesis

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Abstract In mammals, the principal circadian clock within the suprachiasmatic nucleus (SCN) entrains the phase of clocks in numerous peripheral tissues and controls the rhythmicity in various body functions. During ontogenesis, the molecular mechanism responsible for generating circadian rhythmicity develops gradually from the prenatal to the postnatal period. In the beginning, the maternal signals set the phase of the newly developing fetal and early postnatal clocks, whereas the external light–dark cycle starts to entrain the clocks only later. This minireview discusses the complexity of signaling pathways from mothers and the outside world to the fetal and newborn animals' circadian clocks.

Keywords: Circadian system; Suprachiasmatic nucleus; Peripheral circadian clocks; Ontogenesis

1. Introduction

Organisms are exposed to environmental changes that recur mostly in 24-h cycles as a consequence of the Earth's rotation. The most prominent changes are cycles in light and darkness. In response to such changes, organisms evolved an endogenous clock, i.e., a mechanism that enables them to anticipate rhythmically occurring events. Even under constant environmental conditions, the clock generates rhythmic signals in about 24-h cycles and is, therefore, called circadian (from Latin circa diem). Under natural conditions, the circadian clock is entrained to the 24-h day by external cyclically occurring events, mainly by the light period of the day. Due to the entrainment, periods of rest and activity and of many other physiological functions are restricted to a certain time of the day to ensure the best strategy for obtaining food, exposure to optimal outside temperature, protection against predators and excess of sun light, etc.

In mammals, the principal circadian clock resides in cells grouped in two suprachiasmatic nuclei (SCN) of the hypothalamus [1]. In rodents, the paired nuclei are composed of about 20 000 neurons. These neurons are themselves circadian oscillators and are mutually synchronized [2]. Morphologically and functionally, the rodent SCN is divided into at least two parts, namely the ventrolateral (VL) part called the core and the dorsomedial (DM) part called the shell. The VL part receives the photic information from the retina (see below) and expresses mostly light dependent rhythms, e.g., in photoinduction of the immediate early genes (IEGs) *c-fos* and *junB* [3]. The DM part exhibits spontaneous oscillations of many rhythmic variables, like expression of the *arginine vasopressin* and *c-fos* genes [4,5]. Apart from the SCN, nearly every tissue of the body, e.g., liver, kidneys, heart, muscle, spleen, etc., contains a peripheral clock driving local rhythms specific for the tissue function [for review see 6]. Under entrained conditions, the phase of the peripheral clocks is set by the SCN program. However, the peripheral clocks rhythmicity persists even in tissue culture and may not depend on the SCN [7].

The basic molecular core clock mechanism responsible for generation of the rhythmicity within the SCN and peripheral rhythmic cells is formed by interactive transcriptional–translational feedback loops between the clock genes, namely two *Per* (*Per1,2*), two *Cry* (*Cry1,2*), *Clock*, *Bmal1*, *Rev-erbα* and *casein kinase 1 epsilon* (*CK1ε*), and their protein products PER1,2, CRY1,2, CLOCK, BMAL1, REV-ERBα, CK1ε [for review see 8]. Briefly, CLOCK and BMAL1 as a heterodimer positively activates the rhythmic expression of *Per*, *Cry* and *Rev-erbα* genes. In the cytoplasm, the PER and CRY proteins form a complex important for nuclear translocation of both proteins. After shuttling into the nucleus, the PER:CRY complex directly interacts with the CLOCK:BMAL1 heterodimer and inhibits CLOCK:BMAL1 mediated transcription. Regulation of *Bmal1* transcription is mediated mostly by REV-ERBα. The SCN and peripheral clocks operate with similar components and share a similar molecular core clock mechanism. However, some tissue-dependent differences may exist [9]. Also, phasing of clock gene expression differs between the SCN and various peripheral tissues. Peripheral clocks may be phase delayed relative to the SCN by 3–9 h. Although the molecular basis of the circadian clock has been partially defined, the molecular clock outputs that ultimately control circadian rhythms at cellular, organ and system-level are still poorly understood. Components of the core clock mechanism within the SCN and peripheral tissues may serve as down-

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stream transcription factors. At a certain time of day, they switch on transcription of a great array of tissue specific clock controlled genes that are relevant to distinct functions of these organs [10,11]. For example, about 10% of the liver transcriptome is under circadian control [12,11]. In the SCN, the arginine vasopressin (AVP) gene is one of the best-recognized clock controlled genes: it is expressed in a circadian manner and appears to augment SCN excitability [13].

2. Setting the biological clocks

2.1. Entrainment of the central clock

The phase of the central clock is set mostly by photic stimuli: exposure of animals to light in the first part of the subjective night phase-delays and in the second half phase-advances the clock [14]. The photic information is transferred from the retina to the SCN via the monosynaptic retinohypothalamic (RHT) and polysynaptic geniculohypothalamic (GHT) tracts. Besides the classical retinal photoreceptors cones and rods, a small subset of retinal ganglion cells containing the opsin-like protein melanopsin is also photosensitive and projects to the SCN [for review see 15]. The RHT and GHT terminate on a subset of retino-recipient cells in the VL SCN. The spontaneously rhythmic cells in the DM SCN receive photic information only through the VL part. In the late day, the signal of darkness may be neuropeptide Y, the main neurotransmitter of the GHT [16]. During the night, release of the RHT neurotransmitter glutamate signals "light" to the clock [for review see 17]. Light-induced clock resetting may involve sequential activation of glutamatergic NMDA and non-NMDA receptors. Depending on the time when light impinges on the retina at night, the SCN signals downstream of glutamate may diverge. In the early night, signal transduction leads to activation of ryanodine receptors and release of Ca^{2+}. In the late night, the activated cGMP-dependent pathway downstream of glutamate involves Ca^{2+} influx, nitric oxide synthetase and intracellular movement of nitric oxide. Nitric oxide can activate soluble guanylylcyclase, which increases cGMP and activates cGMP-dependent protein kinase (PKG) [18]. Activation of the second messenger pathways is followed by activation of transcription factors. The Ca_i^{2+}/cAMP response element binding protein (CREB) is phosphorylated [19] and IEGs, namely *c-fos* and *jun-B* [3] and clock genes *Per1* and *Per2* [20] are transcriptionally activated, mostly in the VL SCN. Light induced P-CREB may directly regulate transcription of *Per* genes via a CRE element in the 5′-flanking regions of their promoters [21]. Importantly, light may induce CREB phosphorylation and transcription of IEGs and clock genes only during the interval when light entrains the clock, i.e., during the subjective night [19,3]. While the role of IEGs in photic entrainment has not yet been solved, induction of *Per1* and *Per2* genes is believed to be involved in resetting the core clock molecular mechanism. Via the above-mentioned pathways, the clock may attain a new phase in response to a photic stimulus experienced at night. Also, a long day length, i.e., a long photoperiod, such as during summer days, may modulate the SCN rhythmicity as well as its molecular clockwork [for review see 17,22].

Non-photic stimuli, like enforced locomotor activity, arousal, serotonergic drugs, melatonin, dark pulses, etc., are also supposed to reset the central clock when administered at a critical time of the day, e.g., in the late day [for review see 23]. Due to the complexity of the stimuli, their resetting pathways may vary. These may, however, converge at the same endpoint since it has been demonstrated that several non-photic cues acutely downregulate the *Per1* and *Per2* genes, i.e., act opposite to light stimuli. Hence, the *Per* genes may represent the molecular target for the modulating effect of non-photic stimuli on light signaling to the clock.

2.2. Entrainment of peripheral clocks

Peripheral clocks are indirectly entrained by light via setting their phase by the light entrainable SCN clock. However, they may also be directly entrained by changes in their local environment. Under normal conditions, the indirect and direct pathways act in concert. The SCN-controlled rhythm in spontaneous feeding represents one of the strongest entraining cues for many peripheral clocks. In nocturnal animals, the feeding rhythm is related to another SCN-controlled rhythm, i.e., to the rhythm in locomotor activity. Both locomotor activity and feeding mostly occur during night. However, under certain circumstances, the local entraining cue might be in conflict with the SCN signaling. This may happen in the case when access to food is restricted to an unusual time of the day, i.e., to the daytime rest period. Under such restricted feeding, the rhythmic gene expression in liver, kidneys, heart, and other tissues is phase-shifted relative to that in animals fed ad libitum, whereas the phase of gene expression within the SCN does not change [24]. Under such conditions, entrainment of the peripheral clock mediated via the nutrition supply may uncouple from the SCN entrainment. Besides the feeding rhythm, the SCN may control peripheral clocks by humoral as well as neural pathways. In the liver, glucocorticoids have been proposed to play a role in setting the phase, as administration of dexamethasone acutely shifts rhythmic gene expression in the liver and induces rhythmic *Per* expression in cell cultures [25]. Neural pathways may involve the autonomic nervous system since adrenaline may control gene expression in the liver [26].

3. Ontogenesis of the biological clocks

3.1. Ontogenesis of the SCN clock

Development of the SCN clock proceeds in more stages from fetal to postnatal periods. In the rat, the SCN is formed as a component of periventricular cell groups during embryonic days (E)14 through E17. Neurogenesis is complete at E18 although morphological maturation proceeds until postnatal day (P)10. During prenatal period, the SCN neurons only form a few synapses [27]. In this respect, the fetal SCN might resemble an in vitro culture of dissociated SCN cells where connections between the individual cells are sparse or do not exist. Synaptogenesis progresses slowly around birth and then markedly increases from P4 to P10 [27].

It seems that appearance of the first significant rhythms in clock genes expression within a population of the rat SCN neurons proceeds in parallel with the SCN development. At E19, no rhythms of clock genes expression and no clock proteins PER1, PER2 and CRY1 are detectable [28]. At E20, formation of a rhythm in *Per1* expression is indicated and rhythms of *Per2*, *Cry1* and *Bmal1* only appear during the first postnatal days [29]. In another study, rhythms in *Per1* and *Per2* expression in the rat SCN have been reported at E20 [30,31]. Impor-

tantly, amplitude of the rhythms in *Per1* and *Per2* expression in rhythmic SCN cells increases with age until P10 [28] as the synaptogenesis progresses. The parallelism points to the importance of mutual communication between individual clock cells for generating a marked rhythmic signal. Interestingly, *Bmal1* is strongly expressed in the fetal SCN of rats [28] as well as of hamsters [32], while *Per1* and *Cry1* are expressed only weakly.

The rat SCN clock starts to drive output rhythms only around birth as the rhythm in *AVP* heteronuclear RNA is undetectable in the rat SCN at E20, i.e., 1–2 days before birth, but is clearly present at P1 [29]. The rhythm in *AVP* mRNA is detectable at E21 [33], whereas the rhythm in firing rate only at E22 [34]. Altogether, these data are in favor of the hypothesis that the rat is born with a rather immature SCN clock that develops further postnatally. It remains to be ascertained whether the day–night difference in the SCN metabolic activity, monitored by a 2-deoxyglucose uptake and detected as soon as at E19 [35], i.e., well before the first appearance of the rhythm in clock genes expression, represents an intrinsic SCN rhythmicity or a maternal cue driven change. Also, it is of utmost importance to reveal whether the lack of rhythmicity in clock genes expression within the fetal SCN is due to a lack of synchronization between single oscillating SCN cells. However, the undetectable levels of clock proteins throughout the circadian cycle at E19 [28] suggest rather a not yet fully developed core clockwork in the fetal SCN.

3.2. Ontogenesis of peripheral clocks

Development of peripheral clocks depends on maturation of the organ housing the clock as well as on maturation of the molecular clockwork. The first appearance of molecular oscillations might be thus highly organ- and species-specific. In the rat heart, rhythmic expression of *Per1* and *Bmal1* genes begins between P2 and P5 whereas that of *Per2* begins at P14 [36]. In the rat liver, rhythms in clock gene expression may start from P2 and develop further through P10 until P20 [28]. In the murine cerebral cortex, daily rhythms of *Per1* and *Per2* mRNA are detected from P14 [37].

4. Entrainment of developing clocks

4.1. Maternal signaling to fetal clocks

The fetal SCN clock is supposed to be entrained exclusively by cues delivered periodically by the mother. Though light under certain circumstances may reach the fetus even in the uterus [38], the photic pathways to the fetal SCN in altricial rodents are not fully developed. Therefore, non-photic maternal entrainment appears to be dominant. There is extensive evidence that primarily the maternal SCN sets the phase of the developing fetal clock. First, the rhythm in the fetal SCN metabolic activity is synchronized by the maternal SCN [39]. Second, the newly forming and appearing rhythms in clock genes expression in the very late fetal and early neonatal stages are, from the beginning, in phase with the maternal clock [29]. Moreover, although the maternal SCN does not generate fetal rhythms per se, it ensures the postnatal within litter synchrony [for review see 40]. In hamsters, the postnatal within litter synchrony is established very early during the fetal development as a maternal SCN lesion at E10 but no more at E12 abolishes

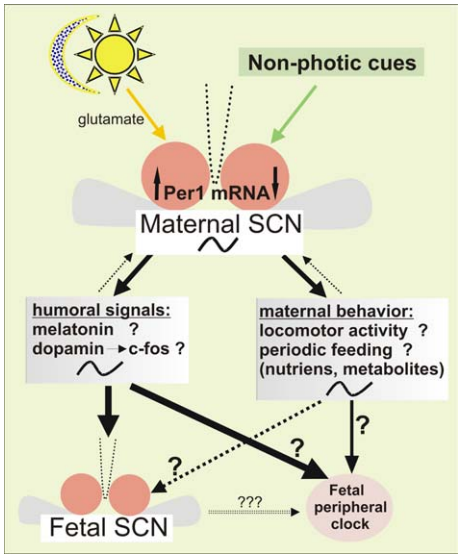

Fig. 1. Signaling to developing fetal clocks. The maternal circadian clock within the suprachiasmatic nucleus (SCN) is entrained mostly by photic and also by non-photic cues with time of the day. The underlying molecular mechanism is symbolized by Per1 mRNA that is upregulated by photic and downregulated by non-photic entraining stimuli. The entrained maternal SCN controls overt humoral and behavioral rhythms that may feedback to the maternal SCN. At the same time, the fetal SCN and perhaps peripheral clocks are entrained via as yet only partially recognized rhythmically delivered maternal stimuli. Although the fetal clocks begin to exhibit intrinsic rhythmicity of the molecular clockwork only around birth and early postnatally, the phase of the newly forming and appearing rhythms in the fetal SCN is set by the maternal SCN early prenatally. Pathways from the maternal to fetal clocks may involve signaling by dopamine via induction of c-fos and/or by melatonin (thick arrow). Also, behavioral maternal rhythms, e.g., locomotor activity and feeding may, hypothetically, entrain the fetal clocks (thin arrows). For more detail see Section 4.1.

the synchrony [41]. In rats, it is suggested that the maternal synchronization of fetal clocks occurs even before the SCN is formed [42]. If this is the case, what is the fetal anatomical substrate that is synchronized by the mother's clock? And as the molecular clockwork in the rat SCN develops mostly postnatally, what is the fetal molecular mechanism that is synchronized by maternal signals? There is also confusion concerning the photoperiodic entrainment of fetuses and newborn rodents. Djungarian hamsters maintain memory of the photoperiod experienced during their fetal stage even postnatally, i.e., the photoperiodic entrainment should be set by their mothers. However, rhythms in clock genes expression or in *c-fos* photoinduction in the neonatal rat SCN are not modulated by the photoperiod experienced by mothers during pregnancy [43,44], though photoperiod modulates the rhythms in the adult rat SCN [for review see 22]. The above-mentioned rhythms, as well as the overt rhythm in the pineal melatonin production, start to be photoperiod dependent only around P10 [for review see 40]. A question arises as to where the memory of the photoperiod experienced during the fetal stage is stored, if not in the neonatal rodent SCN?

Also the entraining signal from mother to fetus is still not completely understood. A designed candidate must exhibit a circadian variation, penetrate the placenta and act at a functional receptor or affect neuronal activity of the fetal SCN. It is difficult to imagine how the fetal clock might become en-

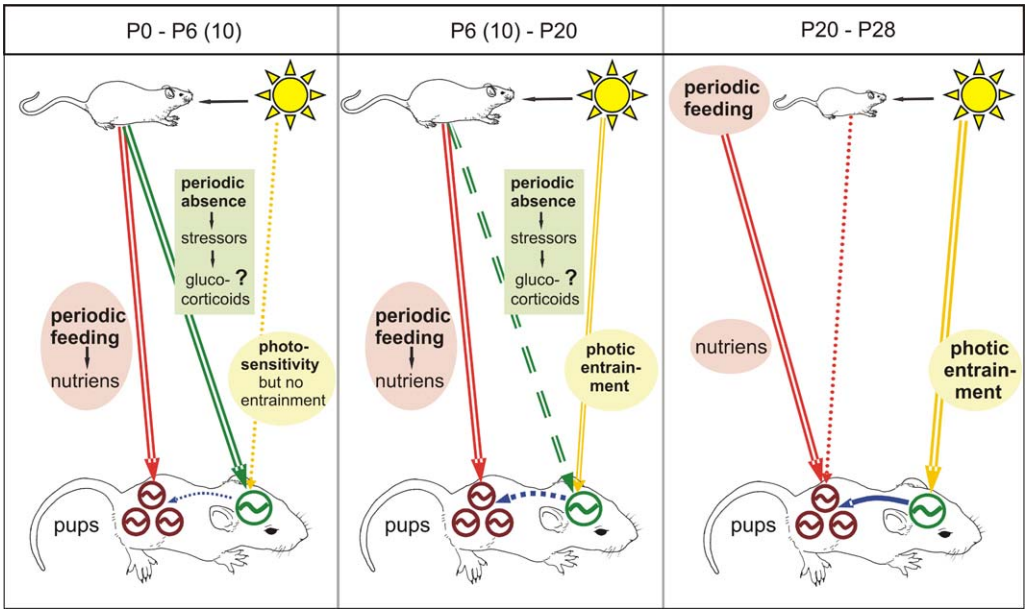

Fig. 2. Signals entraining clocks after birth. Three developmental periods in rodents are depicted: (i) about first week of life, i.e., between the postnatal day 0 and 6–10 (P0–P6(10)); (ii) since the end of the first week until P20 involving the start of weaning (P6(0)–P20); and (iii) between P20 and the end of weaning at P28 (P20–P28). P0–P6(10): During this period, pups are fully dependent on maternal care and maternal entrainment prevails. Periodic absence of the mother might entrain molecular oscillations within the pup's SCN clock via glucocorticoids. Periodic breast feeding and maternal care entrain molecular oscillations in the peripheral clocks. Although the newborn pup's SCN clock is already photosensitive, the photic entrainment does not yet occur. P6(10)–P20: during this period, pups open their eyes and start to be partially independent of their mothers. Significance of maternal absence as an entraining cue of the pup's SCN is losing and the pup's SCN clock begins to be entrained by photic stimuli. At the same time, the pup's SCN may start to control peripheral clocks. Moreover, apart from the maternal day-time feeding, pups begin gradually to forage themselves during the night-time and molecular oscillations of peripheral clocks shift accordingly. P20–P28: during this period, pups become completely independent of their mothers and maternal entrainment is lost. Similarly as in adults, the SCN clock is entrained dominantly by photic cues and peripheral clocks by nocturnal feeding regime. The SCN clock may entrain molecular oscillations in peripheral clocks either directly or rather indirectly via entraining the feeding regime with the external daytime. For more details see Sections 4.2 and 4.3.

trained before it becomes rhythmic itself. Hypothetically, the mechanism might be similar to induction of oscillations in peripheral clock cells in vitro following the addition of serum [45]. At a certain time of day, an entraining maternal cue may trigger a signaling pathway that might impinge onto the yet non-rhythmic clock cells and induce expression of certain genes. Consequently, an imprinting of time awareness might be initiated. Alternatively, a maternal signal might synchronize already existing oscillations in individual cells. Maternal melatonin may fulfill all the criteria of a functional entraining cue and was considered as a first class candidate at least for the photoperiodic entrainment. However, it appears that hormones are not exclusive entraining cues for the fetal clock. Activation of dopaminergic pathways through D1 receptors entrains rodent fetuses as well [46]. Dopamine receptors, as well as melatonin receptors, are present in fetal SCN cells [47]. While melatonin might be considered as the signal of night, dopamine might be the signal of day. The dopaminergic signaling includes activation of the IEG *c-fos* within the fetal SCN [48]. In the adult rat SCN, *c-fos* expression is spontaneously high during the daytime and low during the nighttime [5]. *c-fos* expression is likely related to neuronal activation which is also high during the day and low during the night. Importantly, a marked rhythm in cFos protein immunoreactivity in the neonatal rat SCN is present at P3, i.e., at the earliest time tested [44]. Preliminary data show that the rhythm might be present even at earlier developmental stages (El-Hennamy et al., unpublished results). It is therefore possible that maternal cue-induced *c-fos* expression may provide the fetal clock with a daytime signal and elevation of neuronal activity. It is not yet clear, however, how the suggested signals and pathways may induce rhythmic expression of clock genes. The signaling pathways activating *c-fos* and *Per* genes share a common element, i.e., phosphorylation of CREB. This step might represent the crucial point triggering rhythmic clock gene expression. As more cues share the ability to induce phosphorylation of CREB and expression of *c-fos*, the induction might, hypothetically, represent a common step setting the daytime in the fetal clock (see Fig. 1).

4.2. Signals entraining the central clock postnatally

In rodents, such as rats, mice and hamsters, the newborn neonates are fully dependent on their mothers. In the laboratory, the exquisite maternal entrainment of their rhythmicity becomes less important after the first week of their life when the photic entrainment starts to override the maternal entrainment. In nature, the switch from maternal to photic entrainment may correlate with the ability of pups to leave their underground burrows and get exposed to the environmental light. The mechanism underlying the change in sensitivity of the clock to entraining signals is not fully understood. The phase of the newborn rat SCN clock is set prenatally, synchronously with the mother's clock. Rat pups are born, however, with a low-amplitude oscillation in clock genes expression and the amplitude increases only gradually [28,29]. At the early developmental stage, pups may be partly entrained to the different circadian phase of a foster mother [for review see 40]. This maternal entrainment may be facilitated or even enabled by the low amplitude of pups' clock

oscillations. As the clock rhythmicity strengthens, maternal cues may lose the ability to entrain it and a stronger entraining agent, i.e., light, may take their place.

Maternal cues entraining the pups' clock postnatally may not be the same as those entraining the fetal clock. Many potentially entraining substrates, such as, e.g., melatonin, may be delivered in milk. Recent studies however indicate that this pathway may contribute only little to resetting the pups' clock. When blinded newborn rats are reared by a foster mother on an inverted light–dark regime, the phase of rhythms in *Per1* and *Per2* mRNAs within the pups' SCN is shifted only marginally by about 2 h [30]. Maternal behavior, namely absence of the mother, may, however, strongly entrain the neonatal clock. When newborn pups are deprived of their mothers during the light phase, i.e., at the time when they usually suckle milk, the rhythmic SCN expression of *Per1* and *Per2* genes is completely phase-reversed within six days [31]. Likely, the feeding regime and the periodic partial maternal absence are not the crucial resetting cues for the pups' SCN clock, as they are also reversed under the fostering experiment [30]. The complete absence of the mother at the time when pups are usually fed may be, however, a strong stressor for pups altering expression of stress related genes, such as *corticotropine releasing hormone*, *glucocorticoid receptor* and *AVP* [31]. Hypothetically, the signaling pathway involved in the maternal postnatal entrainment might employ glucocorticoids similarly as with entrainment of peripheral clocks in adults. Sensitivity of the SCN clock to stress diminishes with postnatal age [49].

As innervation of the VL SCN via RHT and GHT develops mostly during the first days after birth, pups become more sensitive to light and gradually the photic entrainment of the SCN clock prevails. The signaling cascade responding to light is functional at least partly immediately after birth: light pulses induce *c-fos* expression in the rat SCN on the day of birth [48] or at P1 [50]. The light induced gene expression is, however, not the only pre-requisite for photic resetting the circadian clock. The photic entrainment may be accomplished mostly due to the fact that light induces the signaling cascade only during a restricted time window that corresponds to the duration of subjective night. During the subjective night, the SCN clock is sensitive to light and photic stimuli may phase delay or phase advance the clock depending upon the time of their administration. The mechanism of how the molecular clockwork gates the response to light is still not understood. The gate for insensitivity to light is not yet developed at P3 since light pulses administered at any time within a 24-h cycle induce high cFos immunoreactivity in the SCN no matter whether it is day or night [44]. In another study [48] a slight gate was indicated at P2. However, the gate for insensitivity to light becomes present only at P10 [44]. This day corresponds well with the developmental stage when photic entrainment begins to override maternal entrainment [for review see 40]. Moreover, at P10 the rat SCN clock starts to be entrained by the photoperiod [43]. In comparison with adult rats [51], the photoperiodic control of the molecular clockwork is only partial and even at P20 it is not yet complete [43]. The data suggest that at least in rodents, the postnatal photic and photoperiodic entrainment develops in dependence on advancement of the mechanism that gates the clockwork insensitivity to light. The development proceeds gradually and may be accomplished at the end of the weaning time (see Fig. 2).

4.3. Signals entraining peripheral clocks postnatally

During postnatal ontogenesis, the circadian expression of clock genes in the rat peripheral clocks might be entrained not only by signals from the developing SCN clock, but also by maternal behavior, namely by the rhythm in breast feeding and care of the newborns. The latter possibility seems to be the case in the first weeks of life. The mother feeds her pups and thus keeps them active mostly during the day. Adult rats, however, are active and consume food mostly at night. During the weaning period, between P14 and P28, the pup's feeding and activity regimes apparently change. In parallel with the changes, the phases of rhythms in genes expression in the heart change as well [36]. First, the phases shift by several hours between P14 and P20. The shifts, though smaller, continue, together with a drastic change of the rhythm's amplitude between P20 and P30, when the matured circadian system seems to have been established. Similarly, during development of the molecular clockwork in the rat liver, rhythms of clock genes expression appear to phase shift during the first weeks of life [28]. Apparently, at this developmental stage, setting peripheral clocks by the feeding regime may prevail upon entrainment by the SCN (see Fig. 2).

5. Concluding comments

This minireview cannot encompass all known data on biological clocks and their entrainment during development. From the data summarized it is, however, obvious how little is known about biochemical signals setting the time in the clocks. Many questions still remain to be answered. What may be the pathways setting the phase of the SCN clock prenatally by the mother at the time when the fetal SCN is not yet formed or at the time when the molecular clockwork is not yet functioning? And what pathways mediate maternal entrainment of the central and peripheral clocks during the first weeks after delivery?

It is of great importance to recognize principles of maternal and photic entrainment of the circadian system during development. This system plays a significant role in controlling many physiological processes and understanding the mechanisms of its entrainment during ontogenesis might facilitate optimization of conditions necessary for its healthy development in animals, as well as human beings.

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Maternal Control of the Fetal and Neonatal Rat Suprachiasmatic Nucleus

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Maternal Control of the Fetal and Neonatal Rat Suprachiasmatic Nucleus

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Abstract The molecular clockwork underlying the generation of circadian rhythmicity within the suprachiasmatic nucleus (SCN) develops gradually during ontogenesis. The authors' previous work has shown that rhythms in clock gene expression in the rat SCN are not detectable at embryonic day (E) 19, start to form at E20 and develop further via increasing amplitude until postnatal day (P) 10. The aim of the present work was to elucidate whether and how swiftly the immature fetal and neonatal molecular SCN clocks can be reset by maternal cues. Pregnant rats maintained under a light-dark (LD) regimen with 12 h of light and 12 h of darkness were exposed to a 6-h delay of the dark period and released into constant darkness at different stages of the fetal SCN development. Adult rats maintained under the same LD regimen were exposed to an identical shifting procedure. Daily rhythms in spontaneous *c-fos*, *Avp*, *Per1*, and *Per2* expression were examined within the adult and newborn SCN by *in situ* hybridization. Exposure of adult rats to the shifting procedure induced a significant phase delay of locomotor activity within 3 days after the phase shift as well as a delay in the rhythms of *c-fos* and *Avp* expression within 3 days and *Per1* and *Per2* expression within 5 days. Exposure of pregnant rats to the shifting procedure at E18, but not at E20, delayed the rhythm in *c-fos* and *Avp* expression in the SCN of newborn pups at P0-1. The shifting procedure at E20 did, however, induce a phase delay of *Per1* and *Per2* expression rhythms at P3 and P6. Hence, 5 days were necessary for phase-shifting the pups' SCN clock by maternal cues, be it the interval between E18 and P0-1 or the interval between E20 and P3, while only 3 days were necessary for phase-shifting the maternal SCN by photic cues. These results demonstrate that the SCN clock is capable of significant phase shifts at fetal developmental stages when no or very faint molecular oscillations can be detected.

Key words circadian system, suprachiasmatic nucleus, ontogenesis, maternal entrainment, rat

The intrauterine period is important for postnatal development of mammals. During this period, a developing organism is mostly protected from the influence of the external environment, but it is continuously exposed to the internal maternal milieu. The majority of maternal signals that are delivered to the fetus transplacentally exhibit circadian rhythms. Maternal circadian rhythms are driven by an endogenous clock located within the suprachiasmatic nuclei (SCN) of the hypothalamus (Klein et al., 1991). The

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clock is dominantly entrained by the light-dark regimen of the solar day, mainly by its light period (Pittendrigh, 1981). Therefore, timing of maternal cues may provide fetuses with information about the external time. However, it is still not known whether and how maternal cues impinge on the fetal SCN clock.

In the rat, the prenatal period lasts for about 22 days. Neurogenesis of the SCN begins on embryonic day (E) 14 and is completed at E18, but the morphological maturation of the SCN neurons proceeds gradually until postnatal day (P) 10 (Moore, 1991). Synaptogenesis within the SCN appears to be a slower process: at E19, only sparse synapses can be observed. The process begins to progress only in the late prenatal and early postnatal periods, and then increases noticeably between P4 and P10 (Weinert, 2005). Thus, during the prenatal period, although SCN neurons are present, the multilevel intercellular coupling may not yet be functional. The coupling strengthens during the 1st postnatal week, and the rat SCN is developed to its full complexity only at P10. Therefore, morphological development of the SCN extends well into the postnatal period. The development is genetically determined and occurs without input from the external environment (Jud and Albrecht, 2006). A question remains as to whether the morphologically immature SCN clock serves as a self-sustaining clock or whether it functions first as an hourglass oscillator and only later becomes an autonomous clock (Sumová et al., 2008). During the late prenatal period, the SCN exhibits day-night variation in metabolic activity (Reppert and Schwartz, 1984), in *Avp* mRNA levels (Reppert and Uhl, 1987), and in the firing rate of its neurons (Shibata and Moore, 1987). In adults, all of these rhythms are supposed to be driven by the SCN clock. However, within the fetal SCN, the rhythms might arise also from cyclically appearing maternal cues that impinge on fetal SCN neurons. Such maternal "zeitgebers" might trigger rhythms in neuronal and metabolic activity as well as in *Avp* mRNA levels. Indeed, it has been demonstrated that transcription of *Avp* might be regulated by a non-clock-related mechanism (Iwasaki et al., 1997; Burbach et al., 2001). Recent data using a more reliable marker of transcription rate than detection of mRNA, that is, detection of heteronuclear RNA as a nascent transcript, revealed circadian rhythmicity in transcription of the *Avp* gene in the rat SCN only at P1, but not at E20 (Kováčiková et al., 2006).

A self-sustained clock generates circadian rhythmicity through molecular clockwork composed of interactive transcriptional-translational feedback loops. A contemporary model of the molecular core clockwork presumes that rhythmic expression of clock genes, namely, *Per1*, *Per2*, *Cry1*, *Cry2*, *Rev-erba*, *Bmal1*, as well as their proteins, drives the circadian clock in a cell-autonomous fashion (for review, see Fu and Lee, 2003; Ko and Takahashi, 2006; Reppert and Weaver, 2001). The molecular core clockwork develops gradually during ontogenesis. Ohta et al. reported clear daily rhythms of *Per1* and *Per2* mRNA in the rat SCN at E20 (Ohta et al., 2002, 2003). However, other authors did not detect significant rhythms in *Per1*, *Per2*, *Cry1*, and *Bmal1* mRNA in the rat SCN at E19 (Sládek et al., 2004), when the fetal rat SCN is already formed (Moore, 1991) and the rhythm in metabolic activity has become apparent (Reppert and Schwartz, 1984). Moreover, at that stage, clock gene proteins PER1, PER2, and CRY1 not only did not exhibit any circadian variation, but were in fact undetectable (Sládek et al., 2004). At E20, the rhythm in *Per1* expression began to form, but the amplitude was very low (Kováčiková et al., 2006). Rhythms in clock gene expression developed gradually during the postnatal period, and adult-level amplitudes were achieved only at P10 (Kováčiková et al., 2006). Similarly, molecular oscillations equivalent to those observed in adults were not detected in the fetal hamster SCN (Li and Davis, 2005). In mice, Shimomura et al. reported a significant oscillation in *Per1* but not in *Per2* mRNA in the SCN at E17, and that the amplitude of the oscillations increased progressively with postnatal age (Shimomura et al., 2001). The aforementioned data suggest that during fetal development, the SCN circadian clock is not able to generate high-amplitude synchronized oscillations in clock gene expression and, therefore, may not be able to function as a self-sustained clock.

In the present study, we aimed to determine whether and how swiftly the immature fetal and neonatal molecular SCN clock can be reset by maternal cues. The maternal SCN clock was shifted by a 6-h delay of the dark period at different stages of fetal SCN development. The impact of the shift on the prenatal SCN clock was assessed by determining profiles of spontaneous oscillations in *c-fos* and *Avp* expression on the 1st postnatal day. *c-fos* and *Avp* expression profiles were chosen as phase markers because clock gene expression exhibits only very low amplitude oscillations in the rat SCN at this developmental stage (Kováčiková et al., 2006). To investigate an impact of the shift on the molecular core clockwork in the SCN on the postnatal day 3 and 6, *Per1*

and *Per2* mRNA profiles were monitored because at these developmental stages amplitudes of both rhythms already allow determination of phase shifts. We assumed that a circadian clock that is not capable of generating synchronized molecular oscillations, as is the case for the fetal rat SCN, would not be entrained by maternal cues. However, we found a significant phase shift of the SCN clock at fetal developmental stages when no or only faint molecular oscillations were detected. This finding might suggest that maternal cues drive rather than entrain the immature fetal rat SCN clock.

MATERIALS AND METHODS

Animals

Male and female Wistar rats (Bio Test s.r.o., Konárovice, Czech Republic) were maintained for at least 4 weeks at a temperature of 23 ± 2 °C and under a light-dark cycle with 12 h of light and 12 h of darkness (LD 12:12) per day, with lights on from 0700 to 1900 h. The rats had free access to food and water throughout the whole experiment. Light was provided by overhead 40-W fluorescent tubes, and illumination was between 50 and 200 l× depending on cage position in the animal room. Vaginal smears were taken from females to determine the day of estrus; on the same day, females were mated with males. The day when female rats were found to be sperm-positive was designated embryonic day 0 (E0); the day of delivery, which occurred at about E22, was designated postnatal day 0 (P0).

All experiments were conducted under license No. A5228-01 with the U.S. National Institutes of Health and in accordance with Animal Protection Law of the Czech Republic (license No. 42084/2003-1020).

Experimental Protocol

On gestational days 18 and 20, pregnant rats were divided into 2 groups. A control group remained untreated under the previous LD regimen. The other group was exposed to a 6-h delay of 1 dark period, so that the light was switched off and on by 6 h later than before, that is, at 0100 and 1300 h, respectively (Fig. 1). At the next cycle, rats of both groups were released into constant darkness (DD) at 1900 h and were kept in darkness until pups were born and sampled at 2-h intervals at P0-1, P3, and P6. The time of the original light onset experienced by pregnant rats was designated as circadian time (CT) 0 and the time of the original light offset was designated as CT12. Simultaneously, adult male rats were subjected to treatment identical with that experienced by control and phase-shifted groups of pregnant rats, to provide evidence for the efficiency of the procedure for phase-delaying circadian rhythmicity within the adult SCN. Adult rats were monitored for locomotor activity and/or killed under anesthesia at 2-h intervals throughout the 24-h cycle, 3 or 5 days after the 6-h delay of the dark period. Pups were killed by rapid decapitation at 2-h intervals of the 24-h cycle at P0-1, P3, and P6. Brains were removed, immediately frozen on dry ice, and stored at –80 °C. They were sectioned into 5 series of 12-µm-thick slices in alternating order throughout the whole rostrocaudal extent of the SCN. Sections were further processed for *in situ* hybridization to determine profiles of *c-fos* mRNA and *Avp* hnRNA at P0-1 and in adults, and of *Per1* and *Per2* mRNA at P3, P6, and in adults.

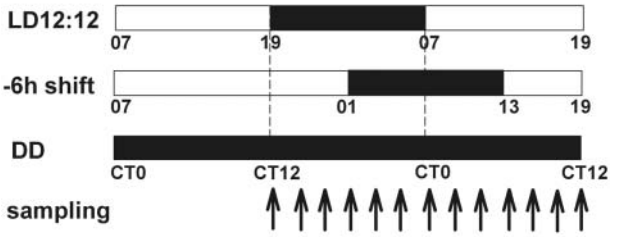

Figure 1. Experimental protocol for the shifting procedure. Pregnant rats were maintained in a light-dark cycle with 12 h of light and 12 h of darkness per day (LD 12:12) with lights on at 0700 h and lights off at 1900 h. On gestational day 18 or 20, rats were divided into 2 groups. For the group exposed to the shifting procedure, the light period was extended so that lights-off and lights-on were delayed by 6 h and occurred at 0100 h and 1300 h, respectively. Rats in the control group remained under the previous LD regimen. Thereafter, the lights were turned off at 1900 h. The next morning, the lights were not turned on and rats of both groups were maintained in constant darkness (DD) until sampling of their pups. Time of the original light onset was designated as CT0 and the time of the original offset as CT12. Pups were sampled at 2-h intervals at postnatal day P0-1, P3, and P6 starting at CT12. Simultaneously, adult male rats were subjected to treatments identical to those of the control and phase-shifted groups of pregnant rats and sampled 3 and 5 days after the treatment.

Locomotor Activity Monitoring

Adult male and female rats were maintained individually in cages equipped with infrared movement detectors attached above the center of the cage top, enabling detection of locomotor activity across the

whole cage. Activity was measured every minute using a circadian activity monitoring system (Dr. H.M. Cooper, INSERM, France) and was analyzed by Actiview Biological Rhythms Analysis software (Mini Mitter, Bend, OR). Double-plotted actograms were generated for visualization of data. The activity onset and offset was determined by 2 independent observers by fitting lines connecting at least 5 successive activity onsets or offsets by eye before and after the shift of the light-dark cycle.

In Situ Hybridization

The cDNA fragments of rat *c-fos* (1160 bp; corresponds to nucleotides 141-1300 of the sequence in GenBank accession number X06769), *Avp* (506 bp; identical to nucleotides 796-1302 of the intronic sequence in GenBank accession number X01637), *Per1* (980 bp; corresponds to nucleotides 581-1561 of the sequence in GenBank accession number AB002108), and *Per2* (1512 bp; corresponds to nucleotides 369-1881 of the sequence in GenBank with accession number NM031678) were used as templates for in vitro transcription of complementary RNA probes. The *Per1* and *Per2* fragment-containing vectors were generously donated by Professor H. Okamura (Kobe University School of Medicine, Kobe, Japan) and the *c-fos* fragment-containing vector was generously donated by Dr. Tom Curran (Children's Hospital of Philadelphia, Philadelphia, PA). The *Avp* cDNA was cloned in our laboratory (Kováčiková et al., 2006). Probes were labeled using ^{35}S-UTP, and the in situ hybridizations were performed as described previously (Kováčiková et al., 2006; Shearman et al., 2000; Sládek et al., 2004). The sections were hybridized for 20 h at 60 °C. Following a posthybridization wash, the sections were dehydrated in ethanol and dried. Finally, the slides were exposed to the film BIOMAX MR (Kodak) for 10 to 14 days and developed using the ADEFO-MIX-S developer and ADEFOFIX fixer (Adefo-Chemie Gmbh, Dietzenbach, Germany). Brain sections from control and phase-shifted animals were processed simultaneously under identical conditions.

Autoradiographs of sections were analyzed using an image analysis system (Image Pro, Olympus, New Hyde Park, NY) to detect relative optical density (OD) of the specific hybridization signal. In each animal, mRNA or hnRNA was quantified bilaterally, always at the midcaudal SCN section containing the strongest hybridization signal. Each measurement was corrected for nonspecific background by subtracting OD values from the same adjacent area in the hypothalamus. The background signal of that area served as an internal standard and it was consistently low and did not exhibit marked changes with time of the day. Finally, slides were counterstained with cresyl violet to check the presence and the midcaudal position of the SCN in each section. For each time point, 3 or 4 rats were killed. The OD for each animal was calculated as a mean of values for the left and right SCN.

Data Analysis

Data were analyzed by 2-way analysis of variance (ANOVA) for the group and time differences. Subsequently, the Student-Newman-Keuls multiple range test was used, with a significance level of $p < 0.05$. Cross-correlation analysis was used to test phase differences between the profiles of gene expression.

RESULTS

Effect of a 6-h Phase Delay of the Dark Period on the Locomotor Activity and Profiles of *c-fos*, *Avp*, *Per1*, and *Per2* Expression in the SCN of Adult Rats

We first aimed to determine how the shifting procedure affects the maternal SCN. Dynamics of the SCN entrainment in adult rats were assessed from the locomotor activity recordings before and after the 6-h shift of the dark period. A representative locomotor activity actogram is depicted in Figure 2. The phase delay of locomotor activity was apparent by the 3rd day after the shift of the dark phase.

To provide further evidence that the adult SCN had phase shifted, we determined daily profiles of *c-fos* and *Avp* expression on the 3rd day and of *Per1* and *Per2* expression on the 5th day after the delay of the dark period in the SCN of control rats and those exposed to the shifting procedure (shifted group).

For *c-fos* mRNA levels (Fig. 3A), the 2-way ANOVA revealed a significant effect of time ($F = 35.9$, $p < 0.001$). Although the effect of group was not significant, the highly significant interaction effect ($F = 10.2$, $p < 0.001$) suggested the presence of differences at specific time points between the *c-fos* mRNA profiles for the control and shifted groups. *c-fos* mRNA levels at CT24 and CT2 were significantly higher ($p < 0.001$) and levels at CT12 and CT6 were significantly lower ($p < 0.001$) in controls than in

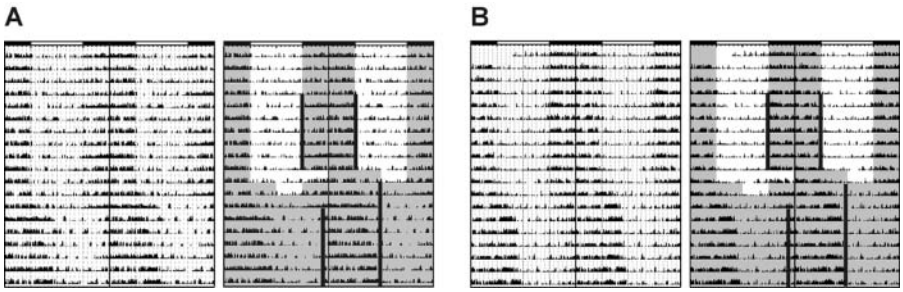

Figure 2. Representative double-plotted actogram of locomotor activity of 1 female (A) and 1 male (B) rat subjected to the experimental procedure described in Figure 1. The white and black bars on the top of the actogram represent the light and dark periods of the LD cycle prior to exposure to the shifting procedure. On the left side, the actogram shows raw data without labeling. On the right side, the actogram is depicted with a shaded area that marks time when the rat was maintained in darkness. Activity onset and offset before and after the phase shift was determined by 2 independent observers by fitting lines connecting at least 5 successive activity onsets or offsets by eye (the vertical lines on the right side actogram).

the shifted group. The entire daily profile of *c-fos* expression was phase delayed by about 2 h in animals exposed to the shifting procedure compared with the profile of control animals (correlation coefficient $R = 0.967$, $p < 0.001$).

For *Avp* hnRNA levels (Fig. 3B), the 2-way ANOVA revealed a significant effect of time ($F = 189.5$, $p < 0.001$) and of group ($F = 30.8$, $p < 0.001$) as well as a significant interaction effect ($F = 14.2$, $p < 0.001$). *Avp* hnRNA levels were significantly higher at CT22, CT24, and CT2 ($p < 0.001$) and significantly lower at CT12 ($p < 0.05$) in controls compared with the shifted group. Apparently, the rise but not the decline in *Avp* expression was phase delayed by about 2 h in animals exposed to the shifting procedure compared with the control animals (correlation coefficient $R = 0.977$, $p < 0.001$).

For *Per1* mRNA levels (Fig. 3C), the 2-way ANOVA revealed significant effects of time ($F = 167.4$, $p < 0.001$), group ($F = 6.2$, $p < 0.05$), and interaction ($F = 33.3$, $p < 0.001$). The levels were significantly higher at CT2 and CT4 ($p < 0.001$) and lower at CT12, CT14 ($p < 0.001$), and CT16 ($p < 0.05$) in the control than in the shifted group. The entire daily profile of *Per1* expression was phase delayed by about 2 h in animals exposed to the shifting procedure compared with the controls (correlation coefficient $R = 0.978$, $p < 0.001$).

For *Per2* mRNA levels (Fig. 3D), the 2-way ANOVA revealed a significant effect of time ($F = 58.7$, $p < 0.01$). Although the effect of group was not significant, the highly significant interaction effect ($F = 7.7$, $p < 0.001$) suggested the presence of differences at specific time points between the *Per2* expression profiles for the control and the shifted groups. *Per2* mRNA levels were significantly higher at CT2 and CT4 ($p < 0.001$) and significantly lower at CT18 ($p < 0.001$) in controls than in the shifted group. The entire daily profile of *Per2* expression was phase delayed by about 2 h in animals exposed to the shifting procedure compared with that of the control animals (correlation coefficient $R = 0.947$, $p < 0.001$).

Effect of the Maternal Phase Shift at E20 on Profiles of *c-fos* and *Avp* Expression in the SCN at P0-1 and of *Per1* and *Per2* Expression at P3 and P6

Our second aim was to investigate whether exposure of pregnant rats to the shifting procedure on gestational day 20 (E20) entrains the fetal SCN. To determine whether the shift could be accomplished during prenatal period within 3 days, the profiles of *c-fos* and *Avp* expression were assessed in newborn pups at P0-1. To determine whether the shift is detectable during the postnatal period, the profiles of *Per1* and *Per2* expression were determined at P3 and P6.

For levels of *c-fos* mRNA at P0-1 (Fig. 4A), the 2-way ANOVA revealed a significant effect of time ($F = 28.2$, $p < 0.001$) and of group ($F = 10.6$, $p < 0.01$) as well as a significant interaction effect ($F = 3.6$, $p < 0.001$). Although there were significant differences in *c-fos* mRNA levels between the control and the shifted group at specific time points, a significant rise in *c-fos* mRNA levels occurred at the same time, that is, at CT2, in controls and the shifted group. The elevated levels declined at CT16 in the control and at CT18 in the shifted group. Altogether, at P0-1, the profile of *c-fos* expression was not phase shifted in pups of mothers exposed to the shifting procedure at E20 compared with the profile of pups born to control mothers. The same hold true for *Avp* hnRNA profiles. At P0-1 (Fig. 4B), the 2-way ANOVA revealed a significant effect of time ($F = 22.1$, $p < 0.001$) and group ($F = 5.3$, $p < 0.05$), but not for the interaction. The daily profile of *Avp* expression in pups of mothers exposed to the shifting procedure at E20 was not phase shifted

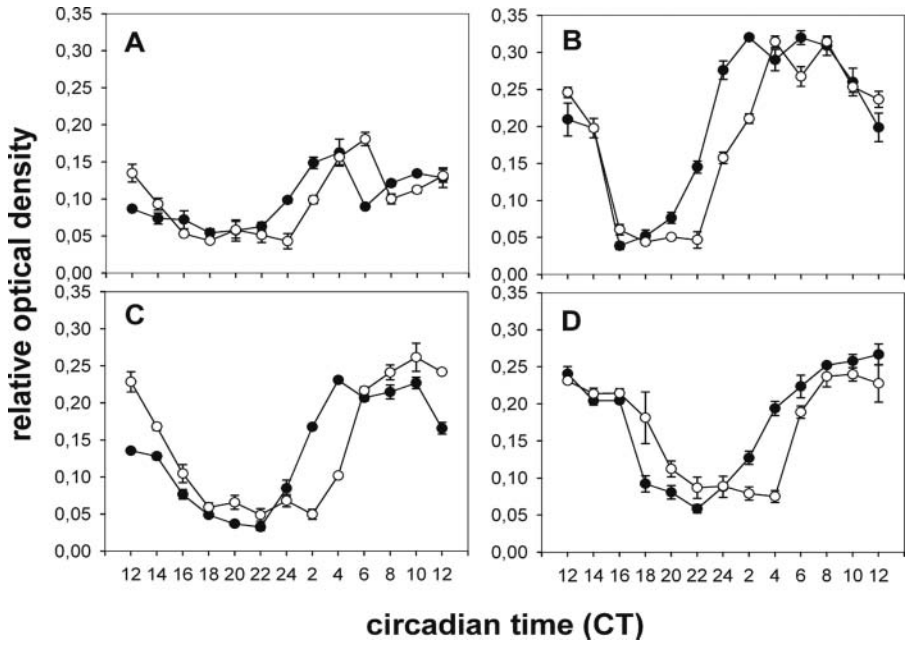

Figure 3. Effect of the shifting procedure on gene expression within the adult suprachiasmatic nucleus (SCN) clock. Daily profiles of *c-fos* (A), *Avp* (B), *Per1* (C), and *Per2* (D) mRNA were determined within the SCN of adult male rats. Control rats (full circles) were released into constant darkness and sampled 3 (A, B) and 5 (C, D) days later throughout the 24-h cycle starting at CT12. Group of rats exposed to the shifting procedure and released into darkness (open circles) were sampled 3 (A, B) and 5 (C, D) days after the treatment starting at CT12. For further details of experimental protocol, see Materials and Methods. Data represent mean values of 4 animals per time point and SEM.

relative to the profile of pups born to control mothers.

Regarding levels of *Per1* mRNA at P3 (Fig. 5A), the 2-way ANOVA revealed a significant effect of time ($F = 39.7$, $p < 0.001$). Although the effect of group was not significant, the highly significant interaction effect ($F = 6.3$, $p < 0.001$) suggested the presence of differences at specific time points between both groups. *Per1* mRNA levels at CT24, CT2, and CT4 were significantly higher ($p < 0.001$) and at CT8, CT10, and CT12 significantly lower ($p < 0.001$, 0.05, and 0.001, respectively) in controls compared with the shifted group. Similarly, at P6 (Fig. 5C), the 2-way ANOVA revealed a significant effect of time ($F = 24.1$, $p < 0.001$) though not of group for levels of *Per1* mRNA. However, the highly significant interaction effect ($F = 6.2$, $p < 0.001$) suggested that there may indeed be differences between the control and the shifted group. *Per1* mRNA levels were significantly higher at CT24 ($p < 0.05$), CT2, and CT4 ($p < 0.001$) and lower at CT8, CT10 ($p < 0.01$), and CT14 ($p < 0.001$) in the control group compared with the shifted group. Altogether, at P3 as well as at P6, the entire daily profile of *Per1* expression was phase delayed by about 2 h in pups born to mothers exposed to the shifting procedure at E20 relative to the profile of pups born to control mothers (correlation coefficient $R = 0.988$ and 0.920, respectively, $p < 0.001$).

For levels of *Per2* mRNA at P3 (Fig. 5B), the 2-way ANOVA revealed a significant effect of time ($F = 50.9$, $p < 0.001$) and of group ($F = 12.9$, $p < 0.001$) as well as a significant interaction effect ($F = 11.2$, $p < 0.001$). *Per2* mRNA levels at CT14 ($p < 0.001$), CT16, and CT18 ($p < 0.01$) were significantly lower and at CT24 and CT8 ($p < 0.001$) were significantly higher in controls than in the shifted group. Similarly for levels of *Per2* mRNA at P6 (Fig. 5D), the 2-way ANOVA revealed a significant effect of time ($F = 89.1$, $p < 0.001$). Although the effect of group was not significant, a highly significant interaction effect ($F = 8.5$, $p < 0.001$) again suggested the presence of differences at specific time points in the expression profiles for the control and the shifted group. *Per2* mRNA levels were significantly higher at CT2 and CT4 ($p < 0.001$) and lower at CT18 ($p < 0.001$) in controls compared with the shifted group. In summary, at P3 as well as at P6, the entire daily profile of *Per2* expression was phase delayed by about 2 h in pups born to mothers exposed to the shifting procedure at E20 relative to the profile of pups born to control mothers (correlation coefficient $R = 0.934$ and 0.950, respectively, $p < 0.001$).

The data indicate that the fetal SCN did not entrain in parallel with the maternal SCN, that is, within 3 days after the shifting procedure, but rather later during the postnatal period at P3, that is, within 5 days after the shift. A similar phase delay as that observed at P3 was also confirmed at P6.

Effect of the Maternal Shift at E18 on Profiles of *c-fos* and *Avp* Expression in the SCN at P0-1

To elucidate whether the prenatal SCN can be entrained by maternal cues, pregnant rats were

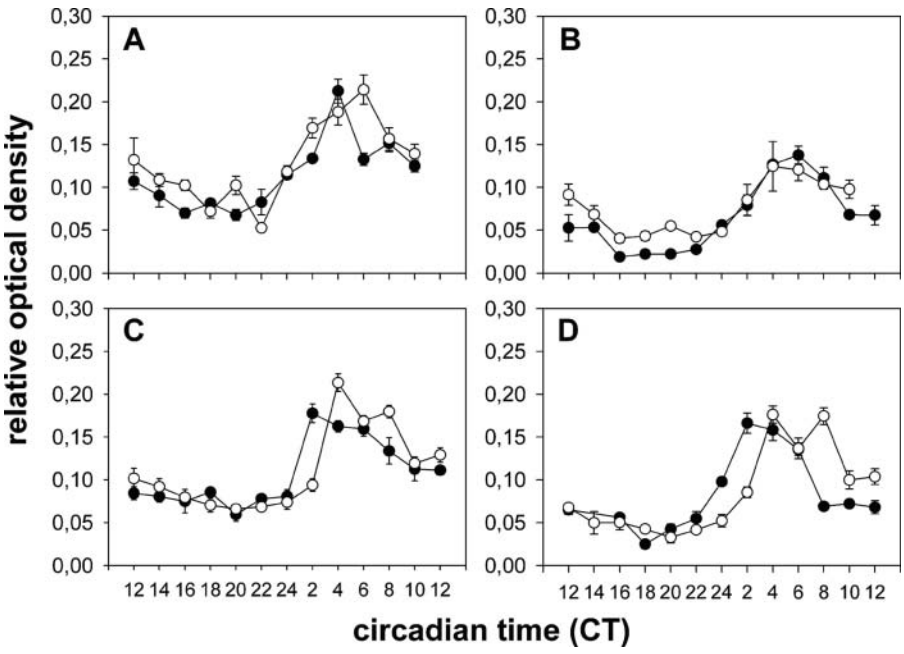

Figure 4. Effect of the shifting procedure on gene expression within the prenatal suprachiasmatic nucleus (SCN) clock. Daily profiles of *c-fos* mRNA (A, C) and *Avp* hnRNA (B, D) were determined within the SCN of pups at P0-1 born either to control mothers (full circles) or to those exposed to the shifting procedure (open circles) at gestational day 20 (A, B) or 18 (C, D). For further details of the experimental protocol, see Material and Methods. Data represent mean values of 4 (occasionally 3) animals per time point and SEM.

exposed to the shifting procedure at E18 rather than at E20, and profiles of *c-fos* and *Avp* gene expression were determined in the newborn pups at P0-1. This experimental design allowed the fetal SCN to be exposed to the shifting maternal entraining cues for 5 days instead of 3 days, that is, for the same time interval as the period between a shift at E20 and sampling of pups at P3 (see above). This arrangement permitted exclusively prenatal maternal cues to impinge on the pup's SCN.

For levels of *c-fos* mRNA at P0-1 (Fig. 4C), the 2-way ANOVA revealed a significant effect of time ($F = 47.6$, $p < 0.001$). The effect of group was not significant, but a highly significant interaction effect ($F = 8.0$, $p < 0.001$) implied differences at specific time points between the control and the shifted group. *c-fos* mRNA level at CT2 was significantly higher ($p < 0.001$) and at CT4 and CT8 lower ($p < 0.001$) in the control compared with the shifted group. The daily profile of *c-fos* expression in pups born to mothers exposed to the shifting procedure at E18 appeared to be phase delayed by about 2 h relative to the profile of pups born to control mothers (correlation coefficient $R = 0.963$, $p < 0.001$).

For levels of *Avp* hnRNA at P0-1 (Fig. 4D), the 2-way ANOVA revealed a significant effect of time ($F = 39.7$, $p < 0.001$). The effect of group was not significant, but the highly significant interaction effect ($F = 9.2$, $p < 0.001$) indicated the presence of differences at specific time points between the control and the shifted group. *Avp* hnRNA levels at CT24 and CT2 were significantly higher ($p < 0.01$ and $p < 0.001$, respectively) and at CT8, CT10, and CT12 lower ($p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively) in the control compared with the shifted group. At P0-1, the entire daily profile in *Avp* expression in pups born to mothers exposed to the shifting procedure at E18 was phase delayed by about 2 h relative to the profile of pups born to control mothers (correlation coefficient $R = 0.920$, $p < 0.001$).

These data indicate that the fetal SCN did entrain within 5 days after exposure of pregnant rats to the shifting procedure at E18.

DISCUSSION

Our data demonstrate that exposure of pregnant rats to a 6-h delay in the dark period induced a significant phase delay in the profiles of *c-fos* and *Avp* expression within the newborn pup's SCN at P0-1 if the shifting procedure was performed at E18, but not at E20. The shifting procedure at E20 was, however, able to phase-shift the profile of *Per1* and *Per2* expression in pups at P3 and P6.

The efficiency of the procedure for phase-shifting the maternal SCN clock was proven by a noted phase delay of locomotor activity as well as of profiles of *c-fos*, *Avp*, *Per1*, and *Per2* gene expression within the SCN of adult rats. Locomotor activity recordings revealed that exposure of adult rats to the shifting procedure induced a significant phase delay within 3 days after shift. On the 3rd and 5th day after the shifting procedure, profiles of *c-fos* and *Avp*, and *Per1* and

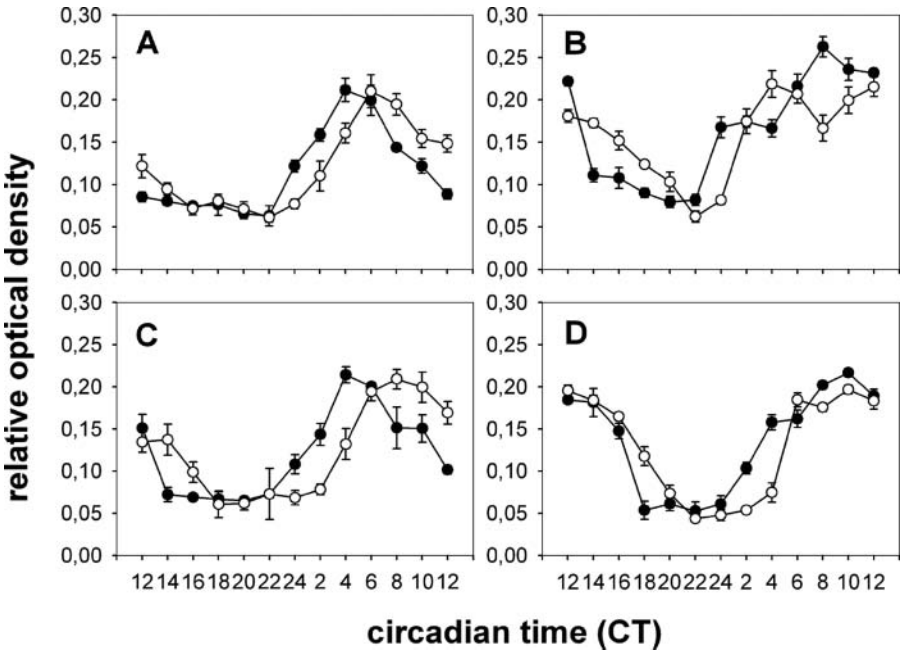

Figure 5. Effect of the shifting procedure on gene expression within the postnatal suprachiasmatic nucleus (SCN) clock. Daily profiles of *Per1* (A, C) and *Per2* (B, D) mRNA were determined within the SCN of rat pups at P3 (A, B) and P6 (C, D) born to control mothers (full circles) and those exposed to the shifting procedure at gestational day 20 (open circles). For further details of the experimental protocol, see Materials and Methods. Data represent mean values of 4 animals per time point and SEM.

Per2, gene expression, respectively, within the SCN of adult rats released into DD were significantly phase delayed compared with the profiles of control rats. Similarly, another study showed that profiles of *Per1*, *Per2*, and *Cry1* expression in the mouse SCN were phase delayed within 2 cycles after the mice were exposed to a 6-h delay of the LD cycle and kept in a new LD regimen (Reddy et al., 2002). The magnitude of phase delays was larger, however, in mice held in LD than in rats kept under DD conditions. Nevertheless, our data clearly demonstrate that after the shifting procedure and subsequent release of rats into DD, the adult and hence also the most likely maternal SCN was significantly phase delayed. The delay was clearly detectable on the 3rd day after the shift both at the locomotor activity as well as the gene expression profile levels.

In newborn rat pups at P0-1, significant circadian rhythms in *c-fos* and *Avp* expression were detected (this study; Kováčiková et al., 2006; Leard et al., 1994). Therefore, we used profiles of *c-fos* mRNA and *Avp* hnRNA as phase markers of circadian rhythmicity within the newborn SCN. At P3 and P6, we used *Per1* and *Per2* mRNA profiles as phase markers of the rhythmicity, as our recent studies have shown that those rhythms exhibit already significant amplitude at these developmental stages. In previous studies, maternal entrainment during fetal stages was studied by monitoring various overt rhythms postnatally, for example, the pineal arylalkylamine N-acetyltranferase in rats after P10 (Reppert and Schwartz, 1986b; Duncan et al., 1986), the running wheel activity and drinking behavior rhythm in rats and hamsters following weaning (Davis and Gorski, 1985a,1985b; Viswanathan et al., 1994; Weaver and Reppert, 1989; Bellavia et al., 2006), or the corticosterone rhythm after P28 (Honma et al., 1984). The general outcome of the studies was that the fetal SCN was entrained by maternal cues. However, in these reports, a relatively long time elapsed between manipulation of pregnant rats and the recording period, and, therefore, postnatal entraining cues might have also interfered. In the fetal rat SCN, a rhythm in metabolic activity detected by 2-deoxyglucose uptake was used to study maternal entrainment (Reppert and Schwartz, 1984, 1986a). Usually, 1 day time point and 1 night time point were determined and significance of difference between the 2 time points increased between E19 and E21 (Reppert and Schwartz, 1984). After complete surgical removal of the maternal SCN, the day-night difference was abolished (Reppert and Schwartz, 1986b). It was concluded that this resulted from a desynchronization of the rhythms between individual fetuses rather than to loss of the fetal SCN rhythmicity. The maternal SCN was thus recognized as an important component of maternal entrainment during fetal development.

In the current study, we examined the impact of a phase shift of an intact maternal SCN on the phase of the newborn rat SCN. Exposure of mothers to a 6-h delay of the dark period at E18 induced a significant phase delay in the rhythms of *c-fos* and *Avp* expression

in the SCN of newborn pups at P0-1. As pregnant rats were released into DD immediately after the manipulation and pups were born in darkness, the observed phase shift in both gene expression profiles was accomplished solely by nonphotic maternal cues during the fetal development. This phase delay was similar to the phase shift observed in adult rats. However, when the same shifting procedure was applied to pregnant rats at E20, profiles of *c-fos* and *Avp* expression at P0-1 were not phase shifted. This result could not be attributed to the possibility that the phase-shifting of the maternal SCN had not completed within 3 days (see above), nor to the possibility that the fetal SCN was not sensitive to the shift, because a significant delay in the *Per1* and *Per2* expression profiles was detected at P3 and at P6. Rather, it seems that the interval elapsing between the maternal manipulation and detection of the phase shift within the pup's SCN is important: 5 days were necessary for phase-shifting the pup's SCN clock by maternal cues, whether the interval between E18 and P0-1 or the interval between E20 and P3. In contrast, only 3 days were needed to significantly phase-shift the adult and hence also maternal SCN by photic cues. Our data thus support the hypothesis that the maternal SCN is necessary for entrainment of the fetal SCN clock. If entrainment of the fetal SCN were mediated independently of the maternal SCN, for example, via a direct effect of a cue upon the fetal SCN, the maternal and fetal SCN would shift at the same rate.

Our results show for the first time a maternal entrainment of rhythms in clock gene expression at such early developmental stages. In previous studies, only about 2-h phase shifts of *Per1* and *Per2* rhythms were demonstrated at P6 in the SCN of blinded pups, induced by nursing of the pups by foster mothers synchronized to a reverse LD cycle. Between P6 and P13, maternal cues were ineffective (Ohta et al., 2002). The ability of maternal cues to entrain the pup's molecular clockwork thus decreases during postnatal development. Maternal entrainment is gradually replaced by developing photic entrainment, which may be capable of overriding the maternal cues after P6 (Duncan et al., 1986).

The most intriguing question that still remains is what is the substrate that mediates the maternal entrainment of the fetal SCN clock. In the rat SCN, synchronized spontaneous rhythms in the SCN clock gene expression had only begun to develop and the rhythm in clock-controlled *Avp* gene expression was not detectable by late fetal stages (Kováčiková et al., 2006; Sládek et al., 2004). This might result from a lack of intercellular communication between individual SCN neurons at fetal stages when the rate of synaptogenesis was still very low. Indeed, the increase in amplitude of clock gene expression rhythms nicely paralleled synaptogenesis; the amplitude achieved the adult-like magnitude only at P10 when synaptogenesis was completed. The data suggest that only a small population of the SCN cells may be spontaneously rhythmic during late fetal stage. As synapses gradually maturate during early postnatal development, more and more SCN cells may become synchronized and rhythmic. Alternatively, a minority of the SCN cells may become rhythmic because they are selectively sensitive to the cyclically appearing maternal SCN-derived cues. These cues might, in turn, directly drive the activity of these cells during fetal stages. Later on, as synaptogenesis progresses, previously insensitive cells may also become rhythmic, allowing development of the autonomous SCN clock to proceed spontaneously. With the gradual development of the autonomous clock, the original mechanism that drove the rhythmicity might become redundant. The developmental decline in sensitivity of the SCN clock to maternal cues may favor the latter hypothesis. To achieve more insight into the underlying mechanisms, future studies should investigate the nature of the maternal entraining cues and the phenotype of the rhythmic fetal SCN cells.

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MINIREVIEW

Circadian Molecular Clocks Tick along Ontogenesis

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Summary

The circadian system controls the timing of behavioral and physiological functions in most organisms studied. The review addresses the question of when and how the molecular clockwork underlying circadian oscillations within the central circadian clock in the suprachiasmatic nuclei of the hypothalamus (SCN) and the peripheral circadian clocks develops during ontogenesis. The current model of the molecular clockwork is summarized. The central SCN clock is viewed as a complex structure composed of a web of mutually synchronized individual oscillators. The importance of development of both the intracellular molecular clockwork as well as intercellular coupling for development of the formal properties of the circadian SCN clock is also highlighted. Recently, data has accumulated to demonstrate that synchronized molecular oscillations in the central and peripheral clocks develop gradually during ontogenesis and development extends into postnatal period. Synchronized molecular oscillations develop earlier in the SCN than in the peripheral clocks. A hypothesis is suggested that the immature clocks might be first driven by external entraining cues, and therefore, serve as "slave" oscillators. During ontogenesis, the clocks may gradually develop a complete set of molecular interlocked oscillations, i.e., the molecular clockwork, and become self-sustained clocks.

Key words

Circadian clock • Ontogenesis • Suprachiasmatic nucleus • Peripheral clock • Clock gene

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Introduction

The circadian system has evolved as an adaptation to cyclic changes in light and darkness due to the Earth's rotation that occurs within a period of solar day, i.e., 24 hours. It ensures the proper timing of vital processes in most organisms studied thus far. In mammals, the circadian system consists of a central clock in the brain and numerous peripheral clocks that are subordinate to the central clock. Via the molecular clockwork in cells, the clocks generate circadian rhythmicity, which controls bodily functions through rhythmic regulation of gene transcription. The rhythmicity is thus manifested at the behavioral as well as at the physiological levels. The mammalian central clock is strategically located in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus just above the optic chiasm. It receives photic input from the retina, which synchronizes it with the 24-h day. The SCN is a paired organ, and each of the two nuclei is composed of about 10 000 mutually interconnected cells (for review see Moore *et al.* 2002, Lee *et al.* 2003). The peripheral clocks in mammals have lost their photosensitivity during evolution. They are synchronized with the 24-h day mostly via outputs from the central SCN clock, as well as by their local environment (Hastings *et al.* 2003, Yoo *et al.* 2004). The ontogenetic development of the mammalian circadian system has already been the subject of an extensive review (Weinert 2005). This mini-review mainly summarizes recent data that addresses the crucial question of when and how the mammalian circadian molecular clockwork develops during ontogenesis. Are the central and peripheral clocks functional at the time of

birth? Do they develop even earlier, i.e., during the fetal stage? Data has recently accumulated to suggest that, although mammalian circadian clocks are genetically equipped to generate rhythmicity well before birth, they undergo a gradual postnatal development in order to function adequately.

The molecular clock within a single cell

Cells in the mammalian body are equipped with a set of genes that are indispensable for circadian clock function. The principles of circadian rhythmicity generation have been partly ascertained at the molecular level and are similar for the central and peripheral clocks. The basic components of the clockwork are the clock genes that encode clock proteins. Malfunction or absence of the clock components render severe abnormalities in circadian rhythmicity (Bae *et al.* 2001, van der Horst *et al.* 1999, Zheng *et al.* 2001). The abnormalities may range from lengthening or shortening of the circadian period to complete arrhythmicity. A contemporary model of the molecular core clockwork presumes that rhythmical expression of clock genes and their proteins drives the circadian clock in a cell-autonomic fashion (for review see Fu and Lee 2003, Ko and Takahashi 2006, Reppert and Weaver 2001). In principle, clock proteins CLOCK and BMAL1 serve as transcriptional activators that switch on the transcription of genes that contain E-box response elements (CACGTG) in their promoters. Both of these proteins contain a basic helix-loop-helix DNA-binding domain and two PAS (Per-Arnt-Sim) protein interaction domains. E-boxes are present in the promoters of the clock genes *Per1,2* and *Cry1,2* and two orphan nuclear receptors *Rev-erbα* and *Rora*, as well as in the promoters of the clock-controlled genes, i.e., the genes that are not part of the core clockwork, but are controlled by it and thus transmit the rhythmical signal outside of the clock. After the CLOCK:BMAL1 heterodimer switches on transcription of *Per1,2*, *Cry1,2* clock genes, the proteins corresponding to these genes are formed with a clock protein-specific delay. The PER1,2 and CRY1,2 proteins accumulate in the cytoplasm and form homo- and heterodimers via their PAS domains. The dynamics of this checkpoint are controlled by post-translational modifications of the clock proteins, mainly by phosphorylation and subsequent proteasomal degradation. PER protein phosphorylation by CASEIN KINASE 1ε (CKIε) and CKIδ facilitates PER1 and PER2 ubiquitinylation and degradation and masks their nuclear localization signals. Consequently, the entry of the PER:CRY heterodimer into the nucleus is delayed (Lee *et al.* 2001, Lowrey *et al.* 2000, Akashi *et al.* 2002). This may be a crucial step for maintaining the circadian period of the molecular clock. After entering the nucleus, the PER:CRY heterodimers inhibit CLOCK:BMAL1 mediated transcription, most likely by mechanisms involving directed histone deacetylation and other chromatin modification (Etchegaray *et al.* 2003). Later on, PER:CRY repression is relieved by degradation of PERs and CRYs. The rhythmical transcription of the CLOCK:BMAL1 transcription activator complex is controlled via circadian oscillations in the transcription of clock gene *Bmal1*. REV-ERBα and RORA compete to bind to ROR-response elements in the *Bmal1* promoter, and repress or activate its transcription, respectively (Shearman *et al.* 2000, Preitner *et al.* 2002, Sato *et al.* 2004). These interlocked positive and negative transcriptional-translational feedback loops repeat with a circadian period and thus form the basis for a self-sustained circadian clock.

However, recent findings suggest that the core clockwork mechanism might be much more complex and the current model may represent only a part of the complete system. It is plausible that not only more genes, but even additonal mechanisms not considered in the current feedback loop model, may be involved. For example, miRNA, namely miR-219, has recently been implicated in the regulation of the central circadian clock (Cheng *et al.* 2007). miRNAs are small molecules that act as potent silencers of gene expression *via* translational repression of mRNA degradation. miR-219 is a target of the CLOCK:BMAL1 complex and exhibits robust circadian rhythms of expression as a clock-regulated gene. Moreover, *in vivo* knock-down of miR-219 lengthens the circadian period in a manner similar to a knock-out of the clock gene *Clock*. Translation control via miRNAs may, therefore, represent a novel regulatory level of the circadian clock. Another novel regulatory mechanism might be based on the finding that bZIP transcription factor *E4BP4* is a key negative component of the circadian clock (Ohno *et al.* 2007). Moreover, three recent reports confirmed the importance of targeted protein degradation as a key feature of the circadian clock (Siepka *et al.* 2007, Busino *et al.* 2007, Godinho *et al.* 2007). They revealed that the clock protein CRY is targeted for degradation by a member of F box family of ubiquitin E3 ligases, FBXL3. *Overtime* (Siepka *et al.* 2007) and *after hours* (Godinho *et al.* 2007) mutations

both lie in the region of FBXL3 that binds to CRY. Due to these mutations, binding of FBXL3 to CRY is disrupted, CRY degradation is prevented and the duration of its repressive function on clock gene activation is prolonged. Therefore, mice with a targeted mutation of FBXL3 have a longer circadian period than wild-type mice. Although the mechanisms of the degradation pathways have not been fully ascertained, targeted degradation is likely to control not only the rate at which clock protein complexes accumulate in the cytoplasm, but also the rate of their degradation.

Although the basic principles of the core clockwork seem to be conserved across the central and peripheral clocks, they might not be absolutely identical. In contrast to peripheral clocks, the central clock within the SCN is formed of a web of inter-connected cell-autonomous oscillators (see below). Recent data have demonstrated that the inter-cellular clock mechanisms may significantly contribute to the robustness of the clock system (Liu *et al.* 2007). The oscillator network interactions in the SCN can partly compensate for *Per1* or *Cry1* deficiency and preserve sustained rhythmicity in behavior and *in vitro* in the SCN slices of *Per1* or *Cry1* mutant mice. In contrast, *Per1* and *Cry1* genes are implicitly required for sustained rhythms in peripheral tissues, cells and dissociated SCN neurons (Liu *et al.* 2007). Therefore, a new model that is specific for the central SCN clock is needed to incorporate the ability of inter-cellular coupling among the SCN neurons to confer the robustness of molecular oscillations.

SCN clock: a single cell oscillator or a web of coupled oscillators?

To function properly as a master clock, the SCN must not only be able to generate circadian oscillations, but must also entrain the oscillations at single cell level to cyclically occurring cues and transmit the synchronized rhythmic information to the rest of the body. These tasks are highly dependent upon inter-neuronal coupling within the SCN. In the adult SCN, information regarding photic entraining cues is first processed by a set of neurons located in the retinorecipient zone of the SCN. In rodents, this zone is called the ventrolateral (VL) part or the core of the SCN. Thereafter, the information is sent *via* intra-SCN coupling pathways to the non-photosensitive cells located mostly within the dorsomedial (DM) part or the shell of the SCN (Yamaguchi *et al.* 2003, Yan and Okamura 2002, Yan and Silver 2004, Yan *et al.* 1999). Inter-cellular communication between clusters of SCN cells has recently been considered to be important for entrainment of the central clock to a change in day length, i.e., in the photoperiod (Inagaki *et al.* 2007, VanderLeest *et al.* 2007). The mechanism by which the coupling is accomplished is not well understood. Several mechanisms underlying the intercellular synchrony have been considered, namely electrical coupling (Aton and Herzog 2005) and coupling by neurotransmitters, such as vasoactive intestinal polypeptide (VIP) (Harmar *et al.* 2002, Aton *et al.* 2005, Maywood *et al.* 2006) and gamma aminobutyric acid (GABA) (Albus *et al.* 2005, Aton *et al.* 2006).

Recent findings support the idea that inter-cellular coupling is crucial not only for entrainment and transmission of the synchronized output signals out of the clock, but also for the time-keeping mechanism *per se*. In particular, the VIP signaling through the VIPR2 receptor has been shown not only to contribute to synchrony between cells, but also to help maintain a robust rhythmicity in individual SCN neurons. In $Vip^{-/-}$ and $Vipr2^{-/-}$ mice, the circadian firing rhythm was abolished in about half of all SCN neurons (Aton *et al.* 2005). Similarly, the rhythmicity was disrupted in cells within the SCN organotypic slices from $Vipr2^{-/-}$ mice carrying *Per1::luciferase* and *Per1::GFP* reporter transgenes as reporters of activity within the core circadian feedback loop (Maywood *et al.* 2006). Recently, it has been shown that coupling between single cell SCN oscillators may amplify and stabilize unstable component oscillators, and, therefore, establish a more reliable rhythmicity at the SCN and behavioral level (Liu *et al.* 2007, To *et al.* 2007). Thus, the coupling between individual rhythmic cells is likely to contribute the autonomous time-keeping mechanism and ensure stability of the central clock.

Development of the central SCN clock

From the data summarized above, it is obvious that the adult central clock is not only a simple sum of self-oscillating neurons, but is rather a well organized entity. The multi-level organization includes coupling between individual neurons as well as coupling between the defined subdivisions of the nucleus. Therefore, development of the central clock within the SCN obviously does not depend only on the presence of individual components of the molecular core clockwork and the ability of single cells to oscillate, but also on development of a hierarchical organization of the

nucleus. Only maturation of the complex clock enables the development of synchronized oscillatory signaling from the central clock to the rest of the body. In this context, data regarding the morphological development of the mammalian SCN might be highly relevant.

Morphologically, the rodent SCN develops gradually (Moore 1991). Gestational periods among different rodent species differ and therefore, for simplification, most of the further discussion relates to development of the rat SCN. In the rat, the prenatal period lasts about 22 days. Neurogenesis of the SCN begins on embryonic day (E) 14 and continues through E17 from a specialized zone of the ventral diencephalic germinal epithelium as a component of periventricular cell groups. Neurons of the VL SCN are generated at E15 - E16 and those of the DM SCN at E16 - E17. The neurogenesis is completed at E18, but the morphological maturation of the SCN neurons gradually proceeds until postnatal day (P) 10. Synaptogenesis in the SCN is a slower process; at E19, only very sparse synapses may be observed. It begins to progress only in the late prenatal and early postnatal periods, and then increases noticeably from P4 to P10 (Weinert 2005). Therefore, during the prenatal period, the SCN neurons are present but the multi-level inter-cellular coupling may not yet be functional. The coupling strengthens during the first postnatal week and, the rat SCN is fully developed to its full complexity only at P10.

Intrinsic rhythms in the SCN may appear as early as the late embryonic stage. A day-night variation in metabolic activity monitored by a 2-deoxyglucose uptake was detected in the fetal rat SCN from E19 through E21 (Reppert and Schwartz 1984), in the *Avp* mRNA level at E21 (Reppert and Uhl 1987) and in the firing rate of the SCN neurons at E22 (Shibata and Moore 1987). All these rhythms are supposed to be driven by the SCN clock in adults, and the rodent fetal clock has therefore been considered to be functionally developed well before birth. However, direct evidence that the above mentioned fetal SCN rhythms are indeed driven by the molecular core clockwork is lacking. Alternatively, the observed rhythmicity might arise from cyclically appearing maternal cues, which impinge on fetal SCN neurons and driving the oscillations in a "slave" oscillator-like fashion. Such maternal "zeitgebers" might trigger the rhythm in neuronal activity, as reflected in the rhythms in firing rate and metabolic activity, as well as in gene transcription, as is the case with the observed rhythm in *Avp* mRNA levels. It is relevant to note that transcription of *Avp* might be regulated not only by the clockwork via activation of the E-box sequence in its promoter (Jin *et al.* 1999), but also via activation of CRE (Iwasaki *et al.* 1997, Burbach *et al.* 2001) and AP1 (Burbach *et al.* 2001) elements by a non-clock-related mechanism. Moreover, recent data using detection of heteronuclear RNA as a nascent transcript, which is a more reliable marker of transcriptional rate than detection of mRNA, did not reveal any circadian rhythmicity in transcription of the *Avp* gene in the rat SCN at E20. However, the expression was rhythmic at P1 (Kováčiková *et al.* 2006).

A solution to the question of whether the central clock is functional before birth might come from studies on the development of the molecular core clockwork mechanism. According to the current model described above, the circadian rhythms in the levels of the clock gene transcripts and protein products are essential for the molecular timekeeping mechanism. Therefore, several groups of researchers have measured the daily profiles of clock gene expression in the rat SCN by *in situ* hybridization, but outcome of these studies was ambiguous. Ohta *et al.* reported high amplitude rhythms of *Per1* and *Per2* mRNA in the fetal rat SCN at E20 (Ohta *et al.* 2002, 2003). Other authors studied the daily profiles of *Per1*, *Per2*, *Cry1* and *Bmal1* mRNA at E19, i.e., at the embryonic day when the fetal rat SCN is already formed (Moore 1991) and the rhythm in metabolic activity present (Reppert and Schwartz 1984). However, none of the above-mentioned clock genes were expressed rhythmically at that embryonic stage (Sládek *et al.* 2004). Moreover, levels of clock gene proteins PER1, PER2 and CRY1 not only did not exhibit any circadian variation, but were in fact undetectable at E19 (Sládek *et al.* 2004). These data suggest that at this stage of fetal development, the SCN circadian clock might not be able to generate synchronized oscillations. The same authors performed a detailed developmental study and found that at E20, some of the rhythms were just about beginning to form, but the amplitude of rhythmicity was very low or did not reach a significant level (Kováčiková *et al.* 2006). Rhythms in clock gene expression developed gradually during the postnatal period, and adult-stage-like amplitudes were achieved only at P10 (Kováčiková *et al.* 2006) (Fig 1). Similarly, molecular oscillations equivalent to those observed in adults were not detected in the fetal hamster SCN (Li and Davis 2005). In mice, Shimomura *et al.* found a significant oscillation in *Per1* but not in *Per2* mRNA in the SCN at E17, and the amplitude of the oscillations increased progressively with

postnatal age (Shimomura *et al.* 2001).

Using this approach, it was possible to study the development of synchronized rhythmicity, but not the development of single cell rhythmicity. Low amplitude rhythms in clock gene expression might already be present in individual SCN neurons, but they may not yet be mutually synchronized due to insufficient synapses in the embryonic SCN (Moore 1991). The increase in the amplitude of the rhythms in clock gene expression correlated well with synaptogenesis within the SCN. Therefore, it is plausible that mutual synchronization of the SCN neurons due to developing synapses may account for the gradual rise in the amplitude of clock gene oscillations. Theoretically, development of the synapses might also be conditional for the oscillations. Daily profiles of clock gene expression in the rat SCN at E19 seem to support the idea of undeveloped molecular oscillations in individual neurons rather than the idea of fully developed but desynchronized oscillations, since levels of constitutively expressed *Per1*, *Cry1* and *Bmal1* genes at E19 corresponded either to the minimum or maximum, but not to the mean of their P3 values. Moreover, at E19, not only rhythms in clock gene mRNA levels, but also protein products PER1, PER2 and CRY1 were undetectable. In fact, no PER1, PER2 and CRY1immunoreactive cells were detected in the fetal SCN at any circadian time (Sládek *et al.* 2004). The absence of the basic components of the molecular core clockwork is rather in favor of the hypothesis that the mechanism enabling the rhythmic expression of clock genes may not yet be mature at E19. However, the possibility cannot be ruled out that only a very small proportion of the SCN cells is rhythmic during the fetal stage, and that the number of rhythmic cells increases due to development of synaptic communication between these rhythmic cells and the non-rhythmic ones. The methodological approach used in the above-mentioned studies would not detect a very low oscillating signal, which could potentially arise from a few SCN cells.

The use of newly introduced experimental tools, such as transgenic animals, will be necessary for addressing these issues in the future. In SCN slices explanted from transgenic animals, it is possible to detect rhythms in clock gene expression with a single cell resolution. However, even in the case of detection of a significant rhythm in clock gene expression at a single cell level during the fetal stage, the question still remains as to whether these rhythms are indeed reliable markers of a functional circadian clock. If so, individual cellular oscillators without any coupling must be able to drive synchronized rhythmicity. However, such characteristics have not been observed in *in vitro* cell lines that are devoid of inter-cellular coupling like the fetal clock. Without entraining cues, these cells are desynchronized or arrhythmic. As soon as the cultured cells are subjected to a "zeitgeber", e.g., to serum shock, the cells become synchronized and exhibit synchronized rhythmicity (Balsalobre *et al.* 1998). Therefore, the question of whether the fetal SCN cells are able to maintain oscillations, or whether the oscillations would soon be dampened in the isolated SCN may still remain. The possibility that other mechanisms besides the molecular clockwork might drive the SCN rhythmicity during late embryonic development should be considered. For example, maternal cues, such as dopamine or melatonin, might directly trigger the fetal SCN rhythm in metabolic activity (Davis and Mannion 1988, Weaver *et al.* 1995). Complete lesions of the maternal SCN at E7 disrupt rhythms in SCN glucose utilization in rat fetuses (Reppert and Schwartz 1986). This disruption might be due to desynchronization among the fetuses, but also might be due to the lack of a rhythmical input to the slave fetal clock. Strikingly, a periodic feeding cue delivered to SCN-lesioned pregnant rats is sufficient to entrain the fetal SCN clock (Weaver and Reppert 1989). The fetal clock is therefore sensitive to feeding cues in a way similar to adult peripheral clocks, but not the adult SCN clock. These observations suggest that multiple and more complex pathways mediate rhythmic information to the fetal SCN clock as compared to the adult SCN clock. They also indicate that formal properties of the fetal and of the postnatal SCN clock may differ. Therefore, the possibility cannot be excluded that during ontogenesis, the SCN clock develops spontaneously from a slave oscillator at the prenatal stage to a master clock at the postnatal stage.

More strikingly, the restructuring of the slave oscillator to the master clock may occur spontaneously without entraining cues driven by the maternal SCN. Surgical ablation of the maternal SCN did not prevent development of the clock during the postnatal period (Reppert and Schwartz 1986, Davis and Gorski 1988). Moreover, genetic ablation of functional central as well as peripheral maternal clocks did not prevent spontaneous development of the clocks, since heterozygous off-spring of m$Per1^{Brdm1}/Per2^{Brdm1}$ and $mPer2^{Brdm1}/Cry1^{-/-}$ double mutant arrhythmic females crossed with wild-type males developed circadian rhythm in locomotor activity.

However, within a litter, pups were less synchronized than pups born to wild-type controls (Jud and Albrecht 2006). Also, transplantation of fetal SCN tissue to arrhythmic SCN-lesioned animals leads to a recovery in the circadian rhythm of locomotor activity (Ralph *et al.* 1990). Therefore, development of the circadian clock appears to be genetically predetermined.

Peripheral clocks during ontogenesis

Studies on the development of peripheral clocks have only recently commenced after the finding that rhythms in clock gene expression are detectable in cells of the peripheral organs, and are therefore not unique to the central SCN clock (Abe *et al.* 2001, Balsalobre 2002, Schibler and Sassone-Corsi 2002). Two methodological approaches for these studies were used. In the first approach, daily profiles of clock gene expression within a peripheral tissue sampled throughout the circadian cycle were examined. In the rat heart, circadian rhythms in the expression of clock genes *Per1*, *Per2* and *Bmal1* and a clock-controlled gene *Dbp* were not detected by Northern blot analysis on P2 (Sakamoto *et al.* 2002). Expression of *Per1*, *Bmal1* and *Dbp* began to be rhythmic between P2 and P5, but expression of *Per2* did not exhibit any rhythmicity until P14. Similarly, in the rat liver, clock gene expression as determined by RT-PCR developed gradually during postnatal ontogenesis (Sládek *et al.* 2004) (Fig.1). At E20, only *Rev-erbα* mRNA exhibited a significant, high amplitude circadian oscillation, but the expression of *Per1*, *Per2*, *Cry1*, *Bmal1* and *Clock* mRNA did not. Even at P2, *Rev-erbα* was still the only gene expressed rhythmically with high amplitude. At P10 *Per1* mRNA and at P20 *Per2* and *Bmal1* also began to be expressed in a circadian way. Only as late as at P30, all of the studied clock genes were expressed rhythmically in an adult-like pattern (Sládek *et al.* 2004). Development of the molecular oscillations in the liver was therefore similar to that in the heart. Apparently, rhythms in synchronized clock gene expression develop earlier in the central SCN clock (see above) than in peripheral oscillators. The stable detection of the high-amplitude rhythm in *Rev-erbα* expression throughout ontogenesis rules out the possibility that the lack of rhythmicity in the early development is due to desynchronization of oscillating cells in the liver. Unlike the SCN clock cells, the peripheral oscillating cells are not mutually interconnected via synapses and are likely to be synchronized by rhythmic humoral or neuronal cues

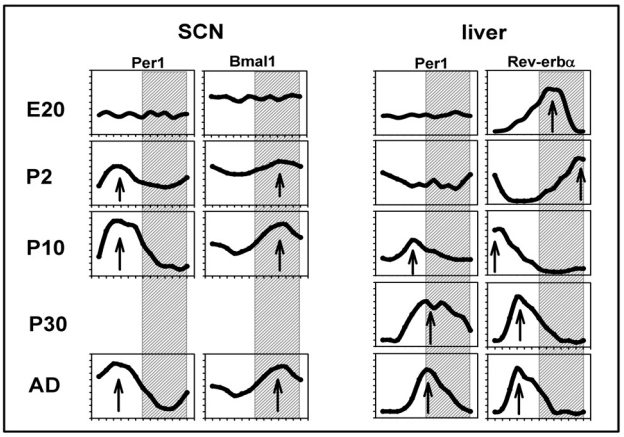

Fig. 1. Schematic drawings of development of the circadian clocks in the rat. Daily profiles of clock gene *Per1* and *Bmal1* mRNA in the SCN and *Per1* and *Rev-erbα* in the liver are depicted in 20-day-old embryos (E20), in pups at postnatal day 2 (P2), P10, P30 and in adult rats. X axis represents day time with the shaded area defining night hours. Y axis represents relative mRNA levels. Drawings are based on results published previously in Sládek *et al.* (2004), Kováčiková *et al.* (2006) and Sládek *et al.* (2007).

impinging upon individual cells. The significant rhythm in *Rev-erbα* expression in the absence of rhythms in other clock genes during ontogenesis may give us clues regarding the mechanism that underlies peripheral clock development. During an early developmental stage, rhythmic expression of *Rev-erbα* might be triggered by mechanisms other than E-box mediated induction. Apart from the E-box, the *Rev-erbα* promoter contains other response elements that may be responsible for switching on/off gene transcription, namely Rev-DR2/RORE, DBPE/D-box etc. (Adelmant *et al.* 1996, Raspe *et al.* 2002, Yamamoto *et al.* 2004). The rhythmically appearing mediators may activate transcription of *Rev-erbα* by stimulation of some of these elements independently of the core clockwork. Moreover, it is tempting to speculate that the rhythmic expression of *Rev-erbα* might trigger the newly appearing rhythms in clock gene expression, since a constant phase relationship between rhythms in the expression of *Rev-erbα* and other clock genes is maintained during different developmental stages (Sládek *et al.* 2004) (Fig.1). Therefore, theoretically, a peripheral clock may function as a slave oscillator during early ontogenesis, and may only later, with the development of clock gene oscillations, become a self-autonomous clock.

Importantly, phases of the rhythms in clock gene expression in the liver (Sládek *et al.* 2007) as well as in the heart (Sakamoto *et al.* 2002) change during development. The acrophase of these rhythms shifts in a coordinated

manner so that the expressions of individual clock genes keep stable phase relationships throughout development (Fig.1). Feeding regimes accompanied by behavioral activity may account for these phase changes. Mothers feed their pups mostly during the daytime; therefore, during the period of maternal breast feeding, pups are diurnal rather than nocturnal in their food consumption (Weinert 2005). The nocturnal feeding pattern develops during the weaning period, but it is preceded by a period when pups still suckle some maternal milk during the daytime and consume solid food during the nighttime. These changes in feeding behavior appear to be mirrored in changing phases of the rhythms in clock gene expression.

Recently, another approach was used for studying the ontogenesis of oscillations in clock gene expression (Saxena *et al.* 2007). *In vivo* rhythms in bioluminiscence were monitored *in utero* in the fetuses of transgenic rats carrying *Per1::luciferase* transgene throughout the whole gestational period (Saxena *et al.* 2007). The bioluminiscence increased dramatically at E10 and continued to increase progressively until birth. Diurnal fluctuations in *Per1* expression in the whole body were already suggested prior to birth. From this study, it is not apparent which parts of the fetal body might account for the whole-fetal bioluminiscence recorded *in vivo* or for the suggested day-night differences observed. It is possible that some peripheral clocks may start to exhibit circadian rhythms in *Per1* expression before birth. Tissue-specific differences in the development of molecular oscillations in peripheral clocks are suggested.

Conclusions

The data summarized in this mini-review cannot definitively answer the question of exactly when the central and peripheral clocks develop during the mammalian ontogenesis. Depending on the methods used, different results have been produced; therefore, more studies are still needed. However, most results support the hypothesis that synchronized oscillations in clock gene expression develop gradually during ontogenesis, and development extends well into the postnatal period. It is feasible that the ability to function as a self-sustained clock may develop gradually, and that the immature clock may function first as a "slave" oscillator. Only later, with the development of a complete set of molecular oscillations, may it become a self-sustaining clock. Such development occurs earlier in the central SCN clock than in peripheral clocks.

Conflict of Interest

There is no conflict of interest.

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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Development of the light sensitivity of the clock genes *Period1* and *Period2*, and immediate-early gene *c-fos* within the rat suprachiasmatic nucleus

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Keywords: circadian clock, ontogenesis, photic entrainment

Abstract

The molecular mechanism underlying circadian rhythmicity within the suprachiasmatic nuclei (SCN) of the hypothalamus has two light-sensitive components, namely the clock genes *Per1* and *Per2*. Besides, light induces the immediate-early gene *c-fos*. In adult rats, expression of all three genes is induced by light administered during the subjective night but not subjective day. The aim of the present study was to ascertain when and where within the SCN the photic sensitivity of *Per1*, *Per2* and *c-fos* develops during early postnatal ontogenesis. The specific aim was to find out when the circadian clock starts to gate photic sensitivity. The effect of a light pulse administered during either the subjective day or the first or second part of the subjective night on gene expression within the rat SCN was determined at postnatal days (P) 1, 3, 5 and 10. *Per1*, *Per2* and *c-fos* mRNA levels were assessed 30 min, 1 and 2 h after the start of each light pulse by *in situ* hybridization histochemistry. Expression of *Per1* and *c-fos* was light responsive from P1, and the responses began to be gated by the circadian clock at P3 and P10, respectively. Expression of *Per2* was only slightly light responsive at P3, and the response was not fully gated until P5. These data demonstrate that the light sensitivity of the circadian clock develops gradually during postnatal ontogenesis before the circadian clock starts to control the response. The photoinduction of the clock gene *Per2* develops later than that of *Per1*.

Introduction

In a non-periodic environment, many behavioural, physiological and molecular events in mammals exhibit self-sustained rhythms with a period close to 24 h. These circadian rhythms are driven by a master circadian pacemaker that resides in the suprachiasmatic nuclei (SCN) of the hypothalamus (Ralph *et al.*, 1989; Klein *et al.*, 1991; LeSauter *et al.*, 1996). The SCN circadian rhythmicity is generated by a system of interconnected transcriptional–translational feedback loops composed of clock genes and their protein products (reviewed in Ko & Takahashi, 2006). Light entrains circadian rhythms to the period of the solar day, i.e. exactly 24 h, via resetting the phase of the circadian pacemaker (Daan & Pittendridgh, 1976). Exposure to light during the first part of the subjective night delays and during the second part advances the phase of circadian rhythms. During the subjective day, light does not affect the circadian phase. This gating of light sensitivity to the specific time of day represents a formal property of the circadian clock itself that enables the entrainment of the endogenous rhythms to a light–dark cycle (Klein *et al.*, 1991). The mechanism by which light entrains the circadian clock has not yet been fully elucidated. At a molecular level, the phase-shifting effect of light correlates with the induction of expression of the clock genes *Per1* and *Per2* within the SCN (Shigeyoshi *et al.*, 1997; Albrecht *et al.*, 2001). In the adult rat SCN, *Per1* expression is sensitive to light during the subjective night, when the level of the endogenously expressed *Per1* is low, but not during the subjective day, when the endogenous *Per1* expression is high (Shearman *et al.*, 1997). Expression of *Per2* appears to be sensitive to light only during the early subjective night when endogenous *Per2* mRNA levels decline (Yan *et al.*, 1999; Miyake *et al.*, 2000). Spontaneous *Per1* and *Per2* expression exhibit circadian rhythms mostly in the dorsomedial part of the SCN (dmSCN), the site of the self-sustained circadian rhythmicity. In contrast, photoinduction of *Per1* occurs within the ventrolateral part of the SCN (vlSCN; Yan & Okamura, 2002; Yan & Silver, 2004), where the retinohypothalamic tract (RHT) that conveys photic information from the retina terminates (Johnson *et al.*, 1988). Apart from the clock genes, the expression of the immediate-early gene *c-fos* within the vlSCN is induced by phase-shifting light pulses (Aronin *et al.*, 1990; Rusak *et al.*, 1990). Though *c-fos* does not participate in the core clockwork mechanism and its role in the photic entrainment pathway has not been proven, the SCN clock precisely gates its induction in the vlSCN so that it occurs only at the time when the intrinsic clock rhythmicity is reset by photic stimuli (Jelínková *et al.*, 2000). All of these data suggest that the vlSCN is responsible for the entrainment of the circadian clock by light.

The mammalian circadian system matures gradually during ontogenesis (for a review, see Weinert, 2005). In the rat, the prenatal period lasts about 22 days, and neurogenesis within the SCN is completed roughly 5 days before birth (Moore, 1991). Synaptogenesis within the SCN develops during the late prenatal and early postnatal periods,

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increasing rapidly from postnatal day (P) 4 to P10 (Moore & Bernstein, 1989). The SCN begins to be innervated by the RHT at P1, and the connections reach their adult levels near P10 (Speh & Moore, 1993). Although the eyes open at about P15, light already induces the expression of *c-fos* within the SCN at P1 (Leard *et al.*, 1994). Light sensitivity develops further gradually between P3 and P10 (Bendová *et al.*, 2004). The spontaneous expression of the clock genes *Per1* and *Per2* is detectable within the rat SCN before birth, but the mRNA levels of these genes do not exhibit pronounced circadian rhythms (Sládek *et al.*, 2004). Low-amplitude circadian rhythms of *Per1* and *Per2* expression are detectable by P1 and P2, respectively (Kováčiková *et al.*, 2006). The rhythms then mature gradually during early postnatal ontogenesis with increases in their amplitudes (Sládek *et al.*, 2004).

While ontogenesis of the light-induced expression of *c-fos* has been studied in rats (Weaver & Reppert, 1995; Bendová *et al.*, 2004) as well as other species, it is not yet known when during ontogenesis light begins to induce *Per1* and *Per2* expression within the SCN. Therefore, the aim of this study was to elucidate when and where within the rat SCN the photic sensitivity of *Per1* and *Per2* develops during the early postnatal ontogenesis and compare it with development of *c-fos* photoinduction. The specific aim was to uncover when the circadian clock begins to gate the sensitivity to light and, therefore, when it likely begins to be entrained by photic cues.

Materials and methods

Animals

Male and female Wistar rats (BioTest s.r.o.; Konárovice, Czech Republic) were maintained under a light–dark regime with 12 h of light and 12 h of darkness per day (LD12 : 12) at a temperature of 23 ± 2°C with free access to the food and water. Light was provided by overhead 40-W fluorescent tubes, and illumination was between 50 and 200 lux, depending on the cage position. Animals were maintained at LD12 : 12 for at least 4 weeks before mating. The day of delivery was designated as P0.

All experiments were conducted under license no A5228-01 with the U.S. National Institutes of Health, and in accordance with Animal Protection Law of the Czech Republic (license no. 42084/2003-1020).

Experimental protocol

Female rats with their pups were released into constant darkness at the time of dark to light transition (designated as circadian time 0; CT0) on P1, P3, P5 or P10. During the first cycle in darkness, experimental groups of pups were exposed to a 30-min light pulse (700 lux) at either CT7 (i.e. during subjective day), CT15 (i.e. during the first part of subjective night) or CT21 (i.e. during the second part of subjective night); control groups were left untreated in darkness. Pups from both groups were subjected to maternal deprivation from the beginning of the light pulse in experimental animals until the time of decapitation and rapid tissue sampling at 30 min, 1 h and 2 h after the start of each light pulse. At each of these time points, four to eight animals from the light-pulsed group and four control animals were sampled. Whole heads (at P1, P3 and P5) or brains (at P10) were immediately frozen on dry ice and stored at −80°C.

Adult male Wistar rats were exposed to light pulses and sampled using the experimental schedule described above. Three (occasionally four) experimental and control animals at each time point were deeply anaesthetized by intraperitoneal injection of thiopental (Valeant Czech Pharma s.r.o., Praha, Czech Republic; 50 mg/kg) and decapitated. Brains were removed, immediately frozen on dry ice and stored at −80°C.

Whole heads or brains were sectioned into series of 12-μm-thick coronal slices in an alternating order throughout the rostral-caudal extent of the SCN. Levels of *rPer1*, *rPer2* and *c-fos* mRNA were assessed by *in situ* hybridization. To delineate the position of the dm-part within the developing SCN, expression of arginin–vasopressin (*rAVP*) hnRNA was detected in a few sections from control animals at P1, P3, P5 and P10 (see Dardente *et al.*, 2002; Hamada *et al.*, 2004). The position of the vl-part of the developing SCN was delineated according to the area of *c-fos* expression in sections from light-pulsed animals at P1, P3, P5 and P10.

In situ *hybridization histochemistry*

The cDNA fragments of rat *rPer1* (980 bp; corresponds to nucleotides 581–1561 of the sequence in GenBank accession no AB002108), rat *rPer2* (1512 bp; corresponds to nucleotides 369–1881 of the sequence in GenBank accession no NM031678), rat *c-fos* (1160 bp; corresponds to nucleotides 141–1300 of the sequence in GenBank accession no X06769) and rat *rAVP* (506 bp; corresponds to nucleotides 796–1302 of the intronic sequence in GenBank accession no. X01637) were used as templates for *in vitro* transcription of complementary RNA probes (T7, T3 or SP6 MAXIscript kit, Applied Biosystems/Ambion, Austin, TX, USA). The *rPer1* and *rPer2* fragment-containing vectors were generously donated by Professor H. Okamura (Kobe University School of Medicine, Japan). The rat *c-fos* fragment-containing vector (originally cloned by Dr Tom Curran from Children's Hospital of Philadelphia, PA, USA) was generously donated by Professor W. J. Schwartz (University of Massachusetts Medical School, Worcester, MA, USA). The whole cDNA was recloned into a pBluescript SK vector (Stratagene, La Jolla, CA, USA) in our laboratory. The *rAVP* fragment-containing vector was cloned in our laboratory (Kováčiková *et al.*, 2006). Probes were labelled by α-^{35}S-UTP (MP Biomedicals, Irvine, CA, USA) and purified using Chroma-Spin 100-DEPC H2O columns (Clontech Laboratories, Mountain View, USA). *In situ* hybridization was performed as described previously (Shearman *et al.*, 2000; Sládek *et al.*, 2004). Briefly, sections were hybridized for 21 h at 60°C. Following a post-hybridization wash, the sections were dehydrated in ethanol and dried. Finally, the slides were exposed to a BioMax MR film (Kodak) for 10 days (*rPer1*, *rPer2*), 12 days (*rAVP*) or 14 days (*c-fos*), and the film was developed in film processor Optimax (PROTEC GmbH, Oberstenfeld, Germany) using AdefoMix and AdefoFix solutions (ADEFO-CHEMIE GmbH, Dietzenbach, Germany). For each gene, sections of the SCN from pups of the same age were hybridized with the same probe and processed simultaneously under identical conditions.

Film autoradiographs of sections were analysed by an image analysis system ImagePro (Olympus, New Hyde Park, NY, USA) to detect the relative optical density (OD) of the specific hybridization signal in the SCN. In each animal, the mRNA level was quantified bilaterally at the mid-caudal SCN section containing the strongest hybridization signal. Each measurement was corrected for non-specific background by subtracting the OD values from neighbouring areas expected to be free of specific signal and thus serving as their own internal standard. The OD value for each animal was calculated as the mean of the left and right SCN relative OD values.

To differentiate spatial distribution of the mRNA signal within the SCN, slides from three light-pulsed and three control animals for each time point were dipped in autoradiographic emulsion LM-1 (Amersham Biosciences, Piscataway, NJ, USA). After 6 weeks of exposure, the slides were developed using the developer Fomatol LQN and fixer

FOMAFIX (FOMA, Hradec Králové, Czech Republic) and mounted for optical microscopy. Representative pictures of emulsion autoradiographs were taken using a digital Olympus DP70 camera (Olympus, New Hyde Park, NY, USA) connected to an Olympus Ax-70 microscope (Olympus). For delineating the position and shape of the SCN on brain sections from pups at P1, P3, P5 and P10, and for comparison with the signal area on the autoradiographic film and/or emulsion, parallel sections were counterstained with Cresyl violet. The intensity of the signal may depend on the thickness of the emulsion, which may vary slightly across the slides. Therefore, the results from emulsion autoradiography were used only for spatial resolution of the signal and not for quantification of the signal intensity.

Statistical analysis

Mean values of relative OD from control and experimental animals were compared at each time point using a *t*-test. Data were expressed as the percentage of the highest mean value ± SEM. For *Per1*, *Per2* and *c-fos* mRNA expression at P1, data from two independent experiments were converted into the percentage of the highest mean value, pooled and analysed with a *t*-test.

Results

Light sensitivity of Per1*,* Per2 *and* c-fos *expression within the SCN of rat pups and adult animals*

First, we investigated the effect of a 30-min light pulse delivered at CT7 (i.e. during the subjective day), CT15 or CT21 (i.e. during the first or second part of the subjective night) on the expression of the clock genes *Per1* and *Per2* and immediate-early gene *c-fos* within the SCN of both rat pups at P1, P3, P5 and P10 and adult animals. To reveal the dynamics of the photic response, the expression of each gene was detected 30 min, 1 h and 2 h after the beginning of the light

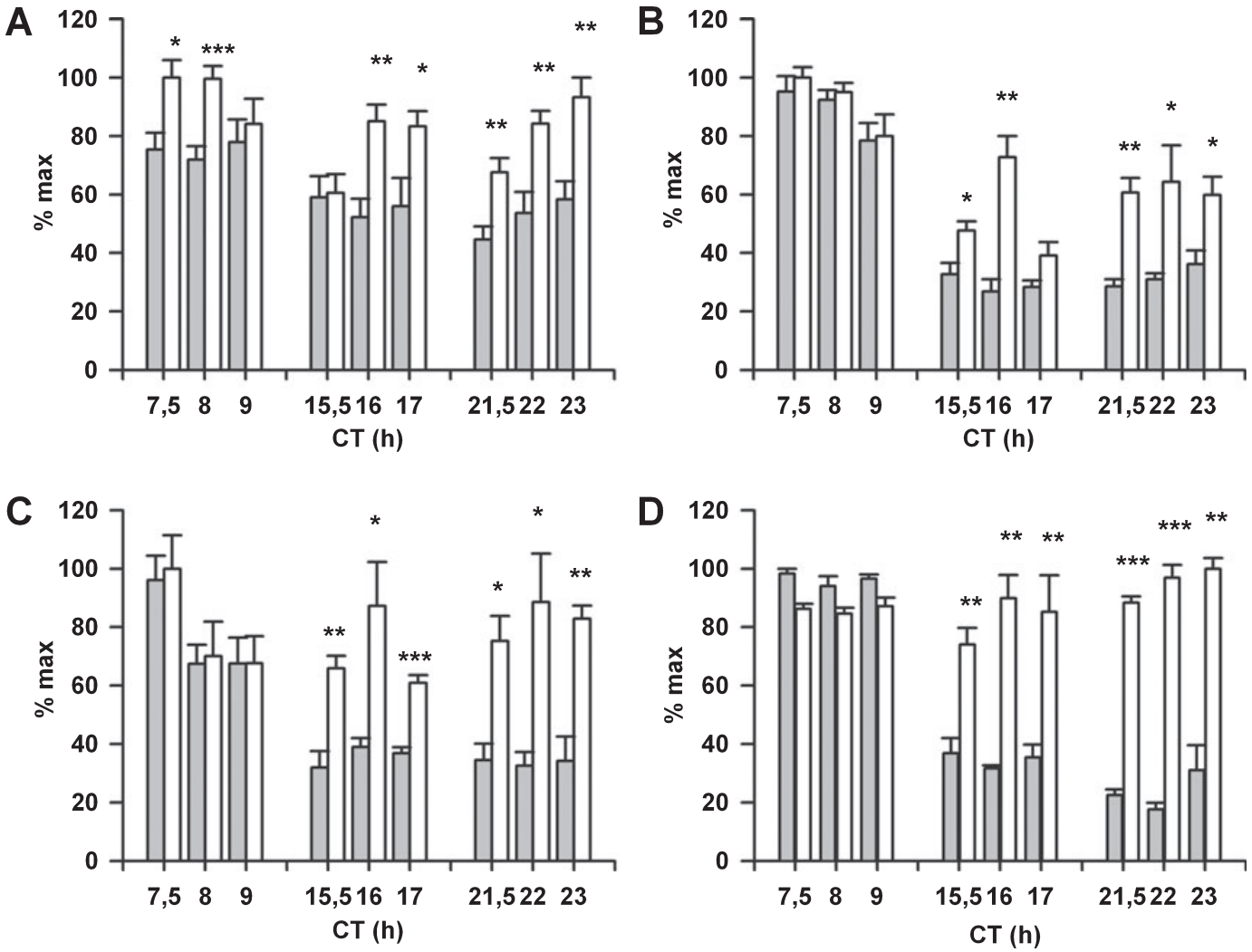

FIG. 1. Effect of light pulses on *Per1* mRNA within the SCN of rat pups at P1, 3, 5 and 10. Rat pups at (A) P1, (B) P3, (C) P5 and (D) P10 were released into constant darkness and exposed to a 30-min light pulse (700 lux) during the subjective day (CT7) or first (CT15) or second (CT21) part of the subjective night. Control pups were left in darkness. Levels of *Per1* mRNA within the SCN of control (dark columns) and light-pulsed (open columns) animals were assessed by *in situ* hybridization histochemistry. The mRNA levels were measured 30 min, 1 h and 2 h after the start of each light pulse (depicted by three couples of control-pulsed/dark-open columns for CT7, CT15 and CT21). The levels of mRNA were determined as the relative OD of the signal in the SCN region measured on autoradiographic film. Data were expressed as the percentage of the maximum OD value. Each column represents the mean of six to eight (at P1) or four (occasionally three; at P3, P5 and P10) animals ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (*t*-test). Time is expressed as circadian time (CT), where CT12 corresponds to the time of the previous lights-off and CT24 corresponds to the time of the previous lights-on.

pulse and compared with expression in controls sampled in darkness at the corresponding CT.

At P1, the levels of *Per1* mRNA (Fig. 1A) increased significantly above the corresponding control levels following light pulses delivered at CT7 (CT7.5: $t_{11} = -2.97$, $P = 0.0127$; CT8: $t_{13} = -4.29$, $P = 0.0009$), CT15 (CT16: $t_{12} = -3.82$, $P = 0.0024$; CT17: $t_{12} = -2.47$, $P = 0.0294$) and CT21 (CT21.5: $t_{13} = -3.46$, $P = 0.0042$; CT22: $t_{14} = -3.61$, $P = 0.0028$; CT23: $t_{14} = -3.83$, $P = 0.0018$). At P3 (Fig. 1B), P5 (Fig. 1C) and P10 (Fig. 1D), *Per1* mRNA levels increased significantly above control levels following light pulses administered at CT15 and CT21, but not CT7, and the response occurred as early as 30 min after the beginning of each light pulse: the levels of *Per1* mRNA increased at P3 (CT15.5: $t_5 = -3.06$, $P = 0.0282$; CT16: $t_6 = -5.49$, $P = 0.0015$; CT21.5: $t_6 = -5.79$, $P = 0.0012$; CT22: $t_6 = -2.63$, $P = 0.0388$; CT23: $t_6 = -3.04$, $P = 0.0227$), P5 (CT15.5: $t_6 = -4.84$, $P = 0.0029$; CT16: $t_6 = -3.15$, $P = 0.0199$; CT17: $t_5 = -7.37$, $P = 0.0007$; CT21.5: $t_5 = -3.68$, $P = 0.0143$; CT22: $t_6 = -3.24$, $P = 0.0177$; CT23: $t_5 = -5.54$, $P = 0.0026$) and P10 (CT15.5: $t_4 = -4.83$, $P = 0.0084$; CT16: $t_4 = -7.29$, $P = 0.0019$; CT17: $t_6 = -3.77$, $P = 0.0092$; CT21.5: $t_6 = -22.27$, $P = 0.0001$; CT22: $t_6 = -16.23$, $P = 0.0001$; CT23: $t_5 = -6.55$, $P = 0.0012$).

For *Per2* mRNA levels at P1 (Fig. 2A), no statistical differences between the control groups and pups exposed to light pulses at CT7, CT15 and CT21 were detected. At P3 (Fig. 2B), *Per2* mRNA levels increased only 1 h ($t_6 = -3.32$, $P = 0.0161$) and 2 h ($t_6 = -5.05$, $P = 0.0023$) after the beginning of a light pulse administered at CT21, whereas the administration of light pulses at CT7 and CT15 did not increase significantly *Per2* mRNA levels. At P5 (Fig. 2C), *Per2* mRNA levels increased following light pulses administered at CT15 (CT16: $t_6 = -6.68$, $P = 0.0005$; CT17: $t_5 = -10.39$, $P = 0.0001$) and CT21 (CT22: $t_5 = -2.81$, $P = 0.0374$; CT23: $t_6 = -4.46$, $P = 0.0043$), but a low induction 2 h after the light pulse at CT7 was also detected ($t_6 = -3.05$, $P = 0.0224$). At P10 (Fig. 2D), levels of *Per2* mRNA

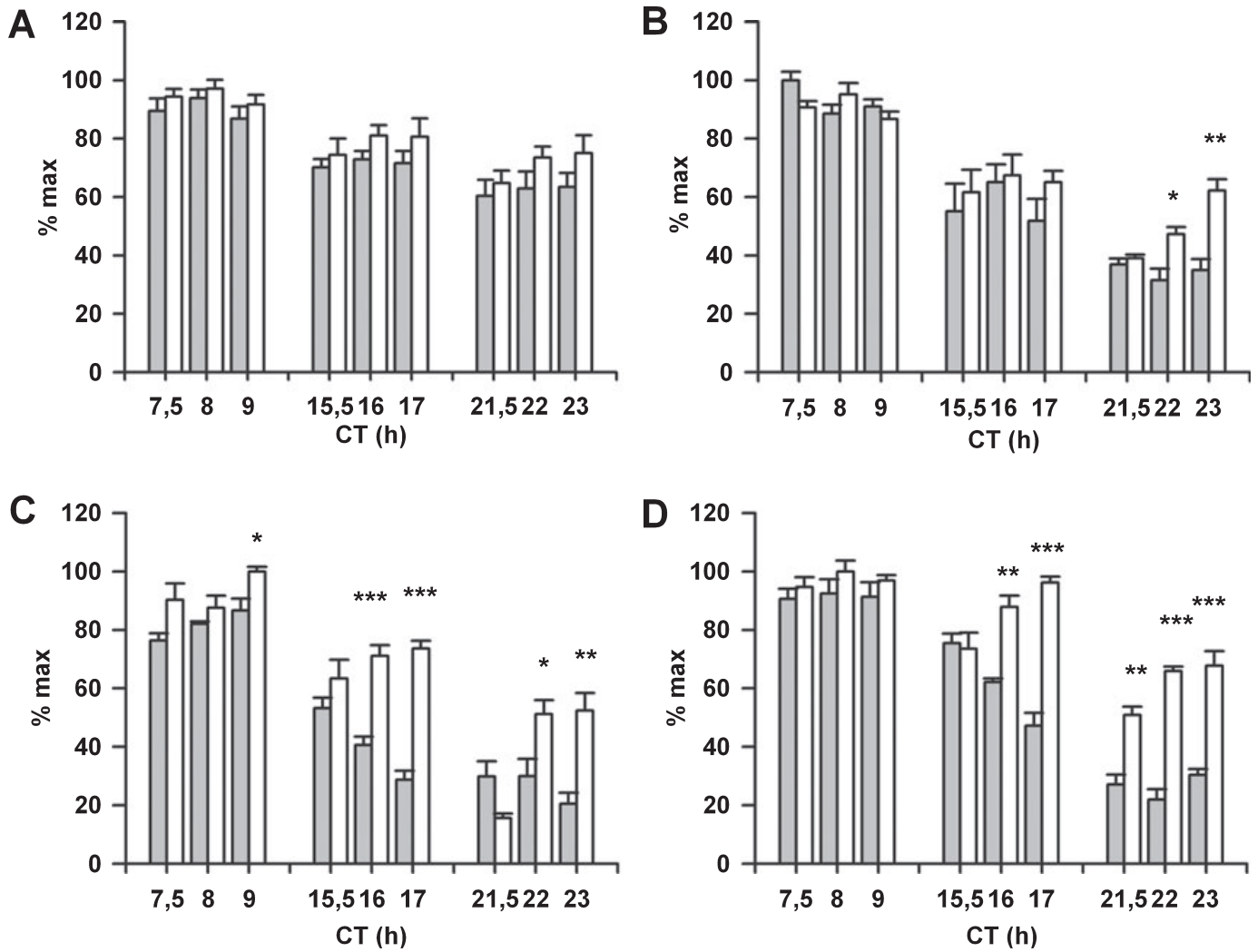

FIG. 2. Effect of light pulses on *Per2* mRNA within the SCN of rat pups at P1, 3, 5 and 10. Rat pups at (A) P1, (B) P3, (C) P5 and (D) P10 were released into constant darkness and exposed to a 30-min light pulse (700 lux) during the subjective day (CT7) or first (CT15) or second (CT21) part of the subjective night. Control pups were left in darkness. Levels of *Per2* mRNA within the SCN of control (dark columns) and light-pulsed (open columns) animals were assessed by *in situ* hybridization histochemistry. The mRNA levels were measured 30 min, 1 h and 2 h after the start of each light pulse (depicted by three couples of control-pulsed/dark-open columns for CT7, CT15 and CT21). The levels of mRNA were determined as the relative OD of the signal in the SCN region measured on autoradiographic film. Data were expressed as the percentage of the maximum OD value. Each column represents the mean of six to eight (at P1) or four (occasionally three; at P3, P5 and P10) animals ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (*t*-test). Time is expressed as circadian time (CT), where CT12 corresponds to the time of the previous lights-off and CT24 corresponds to the time of the previous lights-on.

increased significantly above control levels following light pulses administered at CT15 (CT16: $t_4 = -6.33$, $P = 0.0032$; CT17: $t_5 = -11.21$, $P = 0.0001$) and CT21 (CT21.5: $t_6 = -5.42$, $P = 0.0016$; CT22: $t_6 = -11.33$, $P = 0.0001$; CT23: $t_5 = -7.83$, $P = 0.0005$), but not CT7.

Similarly to *Per1*, the levels of *c-fos* mRNA at P1 (Fig. 3A) increased significantly above the corresponding control levels following light pulses delivered at CT7 (CT7.5: $t_{13} = -3.09$, $P = 0.0087$; CT8: $t_{13} = -5.93$, $P = 0.0001$; CT9: $t_{13} = -2.89$, $P = 0.0126$), CT15 (CT15.5: $t_{14} = -11.87$, $P = 0.0001$; CT16: $t_{13} = -13.87$, $P = 0.0001$; CT17: $t_{12} = -5.07$, $P = 0.0003$) and CT21 (CT21.5: $t_{12} = -6.10$, $P = 0.0001$; CT22: $t_{12} = -6.91$, $P = 0.0001$; CT23: $t_{14} = -2.88$, $P = 0.0121$). At P3 (Fig. 3B), P5 (Fig. 3C) and P10 (Fig. 3D), *c-fos* mRNA levels increased significantly above control levels after the light pulses administered at CT15 and CT21, but not CT7: the levels of *c-fos* mRNA increased at P3 (CT15.5: $t_5 = -15.04$, $P = 0.0001$; CT16: $t_6 = -15.21$, $P = 0.0001$; CT17: $t_5 = -57.72$, $P = 0.0006$; CT21.5: $t_6 = -13.78$, $P = 0.00001$; CT22: $t_6 = -16.76$, $P = 0.0001$; CT23: $t_6 = -14.58$, $P = 0.0001$), P5 (CT15.5: $t_6 = -26.68$, $P = 0.0001$; CT16: $t_4 = -34.39$, $P = 0.0001$; CT17: $t_5 = -3.76$, $P = 0.01312$; CT21.5: $t_5 = -26.84$, $P = 0.0001$; CT22: $t_5 = -4.09$, $P = 0.0094$; CT23: $t_6 = -10.95$, $P = 0.0001$) and P10 (CT15.5: $t_5 = -18.48$, $P = 0.0001$; CT16: $t_5 = -10.08$, $P = 0.0002$; CT17: $t_5 = -5.99$, $P = 0.0019$; CT21.5: $t_6 = -16.61$, $P = 0.0001$; CT22: $t_6 = -24.09$, $P = 0.0001$; CT23: $t_5 = -6.33$, $P = 0.0015$). While *Per1* and *Per2* mRNA levels after the nighttime light pulses increased to levels roughly as high as endogenous daytime levels (with the exception of *Per2* photoinduction at CT21), *c-fos* mRNA levels remarkably exceeded the elevated daytime levels. From visual comparison between the developmental stages (Figs 1–3) it appears that the circadian clock starts to gate its photosensitivity gradually. The light-induced responses within the SCN at P1 were either not gated by

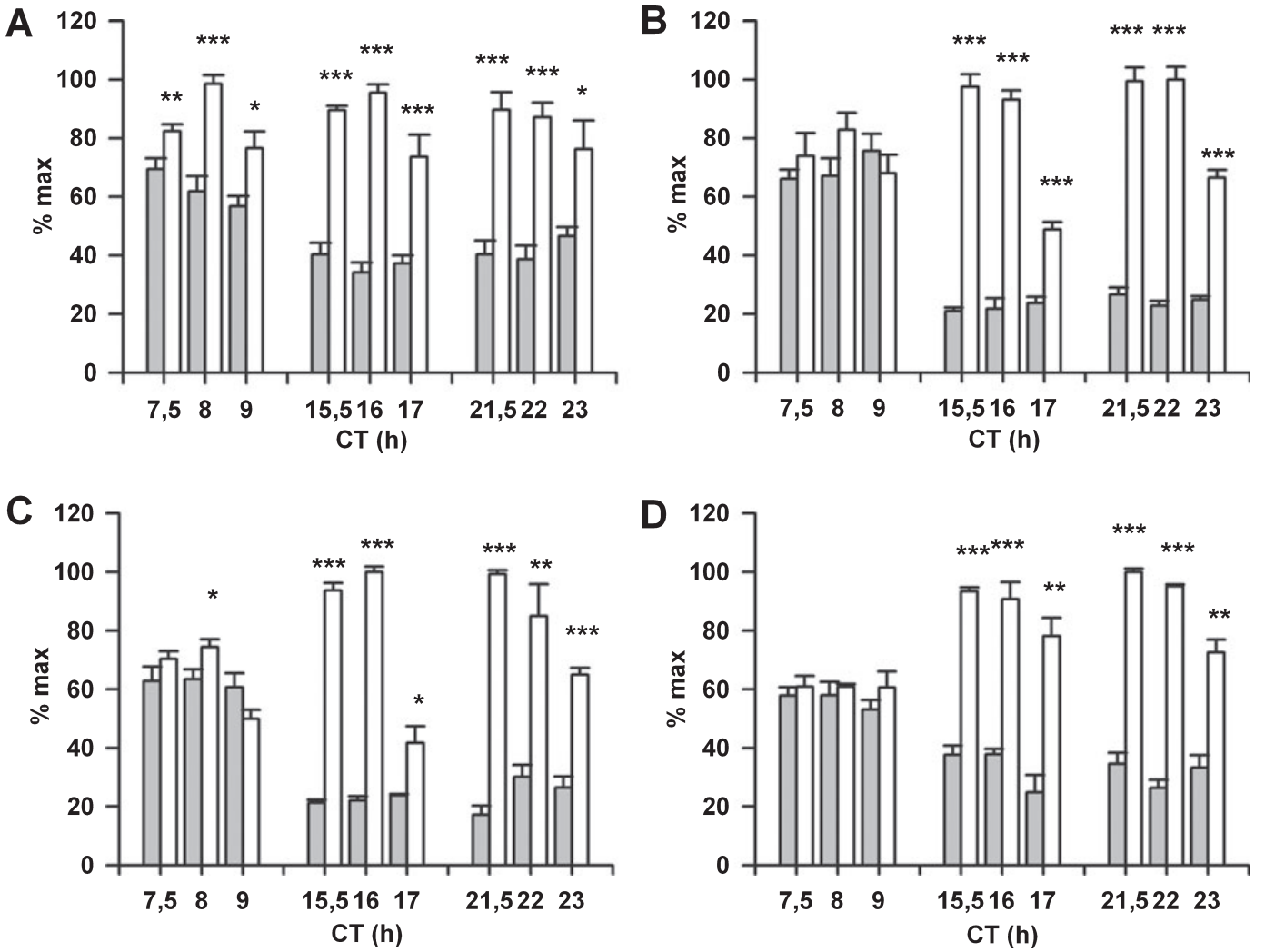

FIG. 3. Effect of light pulses on *c-fos* mRNA within the SCN of rat pups at P1, 3, 5 and 10. Rat pups at (A) P1, (B) P3, (C) P5 and (D) P10 were released into constant darkness and exposed to a 30-min light pulse (700 lux) during the subjective day (CT7) or first (CT15) or second (CT21) part of the subjective night. Control pups were left in darkness. Levels of *c-fos* mRNA within the SCN of control (dark columns) and light-pulsed (open columns) animals were assessed by *in situ* hybridization histochemistry. The mRNA levels were measured 30 min, 1 h and 2 h after the start of each light pulse (depicted by three couples of control-pulsed/dark-open columns for CT7, CT15 and CT21). The levels of mRNA were determined as the relative OD of the signal in the SCN region measured on autoradiographic film. Data were expressed as the percentage of the maximum OD value. Each column represents the mean of six to eight (at P1) or four (occasionally three; at P3, P5 and P10) animals ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (*t*-test). Time is expressed as circadian time (CT), where CT12 corresponds to the time of the previous lights-off and CT24 corresponds to the time of the previous lights-on.

the circadian clock (*Per1* and *c-fos*) or were not yet significant (*Per2*). At P3, light induced *Per1* and *c-fos* expression only during subjective night but not during subjective day. Therefore, the response to photic stimuli seems to be already gated by the circadian clock. However, the expression of *Per2* was still only marginally sensitive to light at this developmental stage. At P5, the response of all examined genes to light pulses was restricted mostly to the subjective night; following the daytime light pulse, only marginal responses were detected. Hence, the circadian clock gates the photic response in gene expression at P5, but the gating mechanism may not yet be completely precise at this developmental stage. At P10, the response of gene expression to light pulses occurred only during the subjective night but not during the subjective day. Therefore, it was completely gated by the circadian clock.

For comparison with the above-mentioned data, the sensitivity of gene expression to photic stimuli was determined within the SCN of adult rats. Levels of *Per1* (Fig. 4A) and *c-fos* (Fig. 4C) mRNA were induced by a light pulse administered at CT15 and CT21, but not at CT7. For *Per1* mRNA, the levels increased significantly above the control levels at CT15.5 ($t_6 = -3.11$, $P = 0.0208$), CT16 ($t_4 = -3.25$, $P = 0.0314$), CT17 ($t_5 = -3.13$, $P = 0.0261$), CT21.5 ($t_4 = -4.79$, $P = 0.0087$) and CT22 ($t_4 = -3.08$, $P = 0.0367$). For *c-fos* mRNA, the levels increased significantly at CT15.5 ($t_6 = -10.40$, $P = 0.0001$), CT16 ($t_4 = -2.99$, $P = 0.0404$), CT17 ($t_4 = -4.71$, $P = 0.0092$), CT21.5 ($t_4 = -4.84$, $P = 0.0084$) and CT22 ($t_4 = -10.18$, $P = 0.0005$). Light-induced *Per1* mRNA levels corresponded roughly to high endogenous daytime levels, whereas photoinduced *c-fos* mRNA levels significantly exceeded the highest daytime levels. *Per2* expression (Fig. 4B) was induced significantly 2 h after the light pulse administered at CT15 (CT17: $t_3 = -5.37$, $P = 0.0126$), but it reached only control levels 1 h earlier. Slight *Per2* photoinduction was also suggested 30 min and 1 h after the light pulse at CT21, but the rise in *Per2* mRNA levels was not significant in comparison to control levels. Light pulse administered at CT7 did not affect significantly *Per2* mRNA levels. These data demonstrate that *Per1*, *Per2* and *c-fos* gene expression in the adult SCN are induced by light pulses administered during the subjective night but not subjective day. Therefore, the response to light is fully gated by the circadian clock. In our experiments, photoinduction of *Per1* differed from that of *Per2* not only in the timing but also in the magnitude of the response.

Spatial distribution of photic induction of Per1*,* Per2 *and* c-fos *expression within the SCN of the developing rat*

Further, we aimed to differentiate the spatial distribution of endogenously expressed and photoinduced *Per1*, *Per2* and *c-fos* mRNAs within the SCN during early ontogenesis. To accomplish this task, slides exposed to films to determine relative signal intensities were

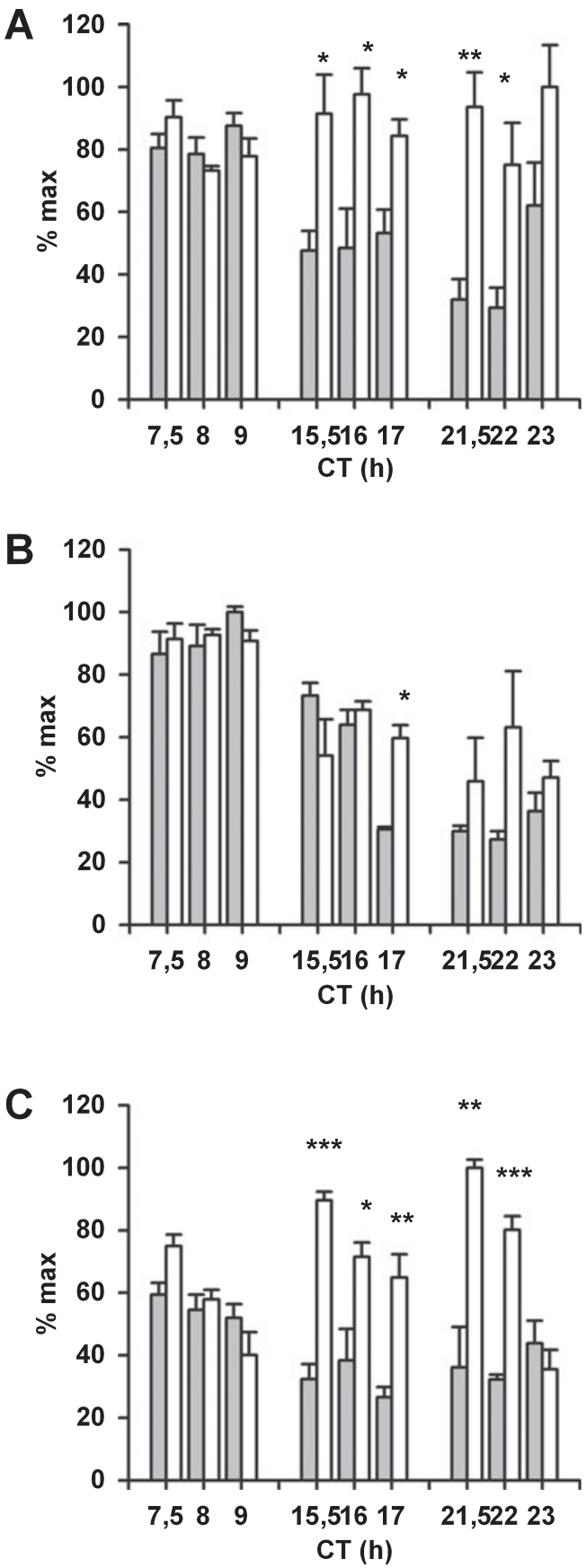

FIG. 4. Effect of light pulses on levels of *Per1*, *Per2* and *c-fos* mRNA within the SCN of adult rats. Adult male Wistar rats were released into constant darkness, and either left in darkness (controls; dark columns) or exposed to a 30-min light pulse during the subjective day (CT7) or first (CT15) or second (CT21) part of the subjective night, and sampled 30 min, 1 h and 2 h later (open columns). Levels of (A) *Per1*, (B) *Per2* and (C) *c-fos* within the SCN were assessed by *in situ* hybridization histochemistry. The levels of mRNA were determined as the relative OD of the signal in the SCN region measured on autoradiographic film. Data were converted to the percentage of the maximum OD value. Each column represents the mean of three (occasionally four or two) values ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (*t*-test). For further details, see the legend of Fig. 1. CT, circadian time.

covered by an autoradiographic emulsion and developed 6 weeks later. To delineate the dmSCN region, the endogenous expression of *Avp* hnRNA was assessed on some slices from control pups. The vlSCN region was characterized as the area of *c-fos* photoinduction in pups at the same developmental stage. Emulsion autoradiography revealed that *Avp* hnRNA was detected at P1 in only a few cells and, therefore, the dmSCN region could not be delineated using this approach at such an early age (data not shown). At P3, P5 and P10, the *Avp* hnRNA signal was localized within the dmSCN and could thus be used for characterization of the dmSCN region at these developmental stages. Figure 5 depicts representative autoradiographs with *Avp* hnRNA and *c-fos* mRNA signal within the SCN at P3 and P10.

The distribution of endogenous *Per1* mRNA (Fig. 6, control) changed during development. At P1, the signal overlapped the entire area of the SCN. At P3, P5 and P10, spontaneous *Per1* expression prevailed primarily within the dmSCN. The administration of light pulses (Fig. 6, pulse) induced *Per1* mRNA mostly within the vlSCN. An exception to this trend occurred at P1 after a light pulse at CT7 (Fig. 6A), when *Per1* expression was induced within the whole SCN 30 min and 1 h after the light pulse. Light pulses administered during the subjective night (Fig. 6B and C) induced *Per1* mRNA levels within a thin layer at the ventral border of the SCN at P1, and the area of photoinduced signal expanded gradually with age. At P10, this area already resembled the adult vlSCN pattern.

The distribution of endogenously expressed *Per2* mRNA (Fig. 7, control) at P1, P3, P5 and P10 prevailed primarily within the dmSCN. Light pulse administered at CT7 (Fig. 7A) did not affect the intensity or distribution of the autoradiographic signals in comparison to controls at any of the studied developmental stages. Similarly, administration of a light pulse at CT15 (Fig. 7B) and CT21 (Fig. 7C) at P1 did not affect the intensity and distribution of the signal. At P3, the intensity of the signal after a light pulse at CT15 (Fig. 7B) did not change; however, it increased significantly in the vlSCN 1 and 2 h after a light pulse at CT21 (Fig. 7C). At P5 and P10, a light pulse administered at CT15 (Fig. 7B) induced *Per2* expression within the entire area of the SCN, while a pulse at CT21 (Fig. 7C) induced expression mostly within the vlSCN.

At P1, P3, P5 and P10, the endogenous expression of *c-fos* (Fig. 8A, control) was located exclusively within the dmSCN. The administration of a light pulse at CT7 (Fig. 8A, pulse) induced *c-fos* expression primarily within a thin layer at the ventral border of the SCN at P1, P3 and P5 and throughout the vlSCN at P10. At P10, the intensity of the signal following the pulse did not exceed the endogenous dmSCN signal. The signal intensity of the entire SCN was thus not increased, and photoinduction was consequently not recognized by film autoradiography (see Fig. 3). Following the administration of light pulses at CT15 (Fig. 8B, pulse) and CT21 (Fig. 8C, pulse), *c-fos* expression was induced mainly at the ventral border of SCN at P1, and the ventrolateral area spread in the dorsal direction between P3 and P10. Interestingly, at P1, both these nighttime light pulses induced *c-fos* expression within the dm-part of the SCN as well. This response weakened at P3 and disappeared thereafter. At all developmental stages, the intensity of the vlSCN signal after nighttime light pulses greatly exceeded that of the dmSCN. In addition, nighttime light pulses induced *c-fos* expression very rapidly, i.e. within 30 min, and light-induced levels declined markedly 2 h after the beginning of the light pulse.

Discussion

In this study, we demonstrate the temporal and spatial development of the photosensitivity of the canonical clock genes *Per1* and *Per2* and of immediate-early gene *c-fos* within the rat SCN during the early postnatal period. *Per1* and *c-fos* expression were sensitive to light pulses on the first postnatal day, whereas expression of *Per2* was sensitive only on the third. At P1, the expression of *Per1* and *c-fos* was

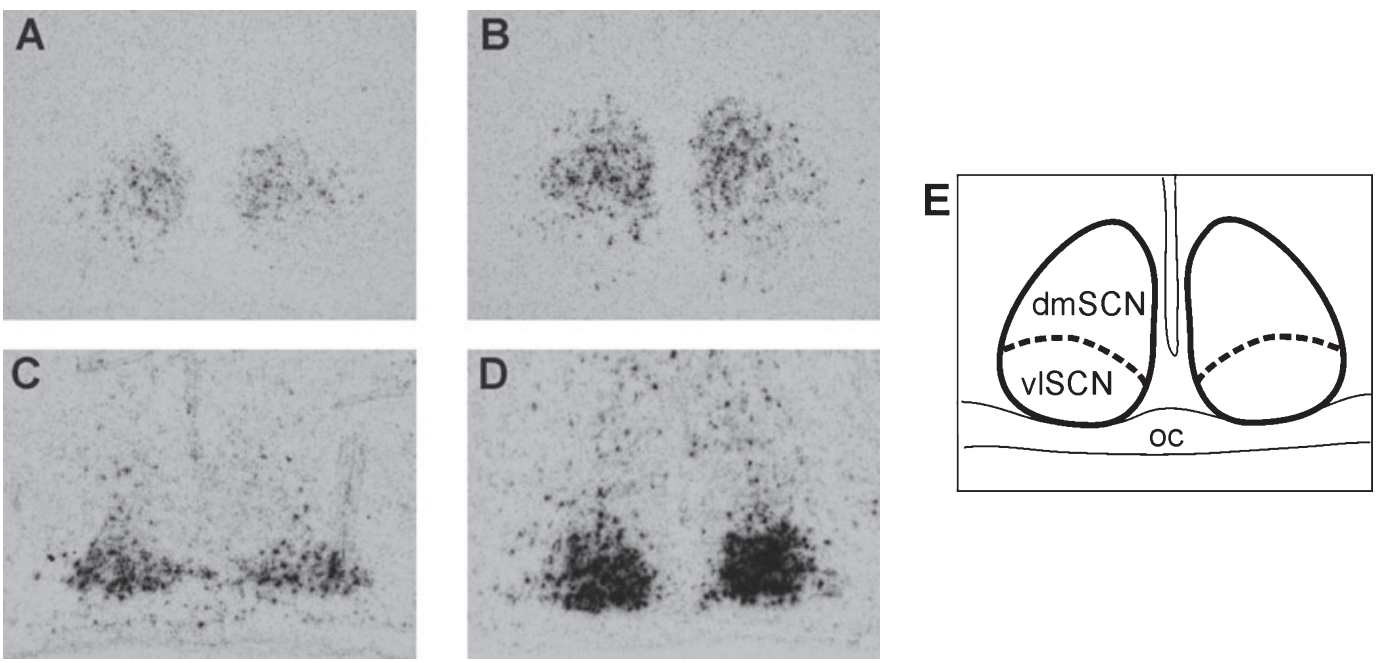

FIG. 5. Compartmentalization of the rat SCN during early postnatal ontogenesis. Representative emulsion autoradiographs delineate the distribution of cells exhibiting spontaneous *Avp* (A, B) and light-induced *c-fos* (C, D) expression within the SCN at P3 (A, C) and P10 (B, D). The images were used as templates to determine the areas of the dorsomedial (A, B) and ventrolateral (C, D) regions of the SCN at early developmental stages. The template is schematically summarized in (E). dmSCN, dorsomedial part of the suprachiasmatic nucleus; oc, optic chiasm; vlSCN, ventrolateral part of the suprachiasmatic nucleus.

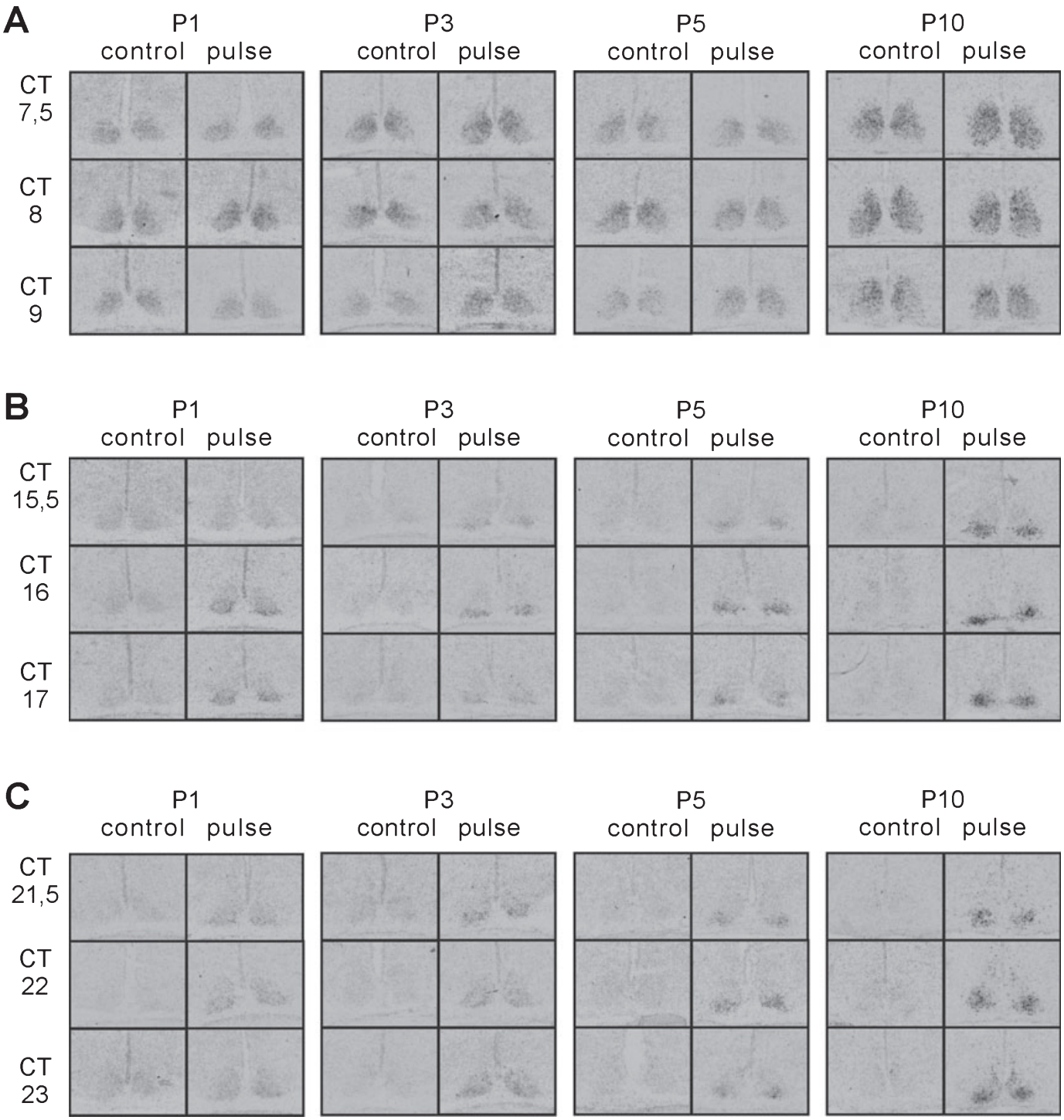

FIG. 6. Spatio-temporal distribution of *Per1* expression within the SCN of rat pups. Representative emulsion autoradiographs demonstrating the distribution of *Per1* expression in SCN sections of rat pups at P1, P3, P5 and P10. Pups were either kept in darkness (control) or exposed to a 30-min light pulse at (A) CT7, (B) CT15 or (C) CT21 (pulse), and sampled 30 min (CT7.5, CT15.5, CT21.5), 1 h (CT8, CT16, CT22) and 2 h (CT9, CT17, CT23) after the start of each light pulse. Note: the intensity of the signal on individual emulsion autoradiographs may not exactly reflect the relative mRNA levels (for details, see Materials and methods). CT, circadian time; P, postnatal day.

induced by light pulses delivered during either the nighttime or daytime. Apparently, the newborn pups lack the mechanism present in adult animals that restricts photic responses to the nighttime. The gating mechanism was present at P3, though it developed gradually further with age.

Our data, which demonstrate the photosensitivity of the rat SCN already during the first postnatal day, are in accordance with earlier reports on *c-fos* photoinduction at this developmental stage (Leard *et al.*, 1994). In the present study, light pulses at P1 affected not only *c-fos* expression as the neuronal activity marker but also the

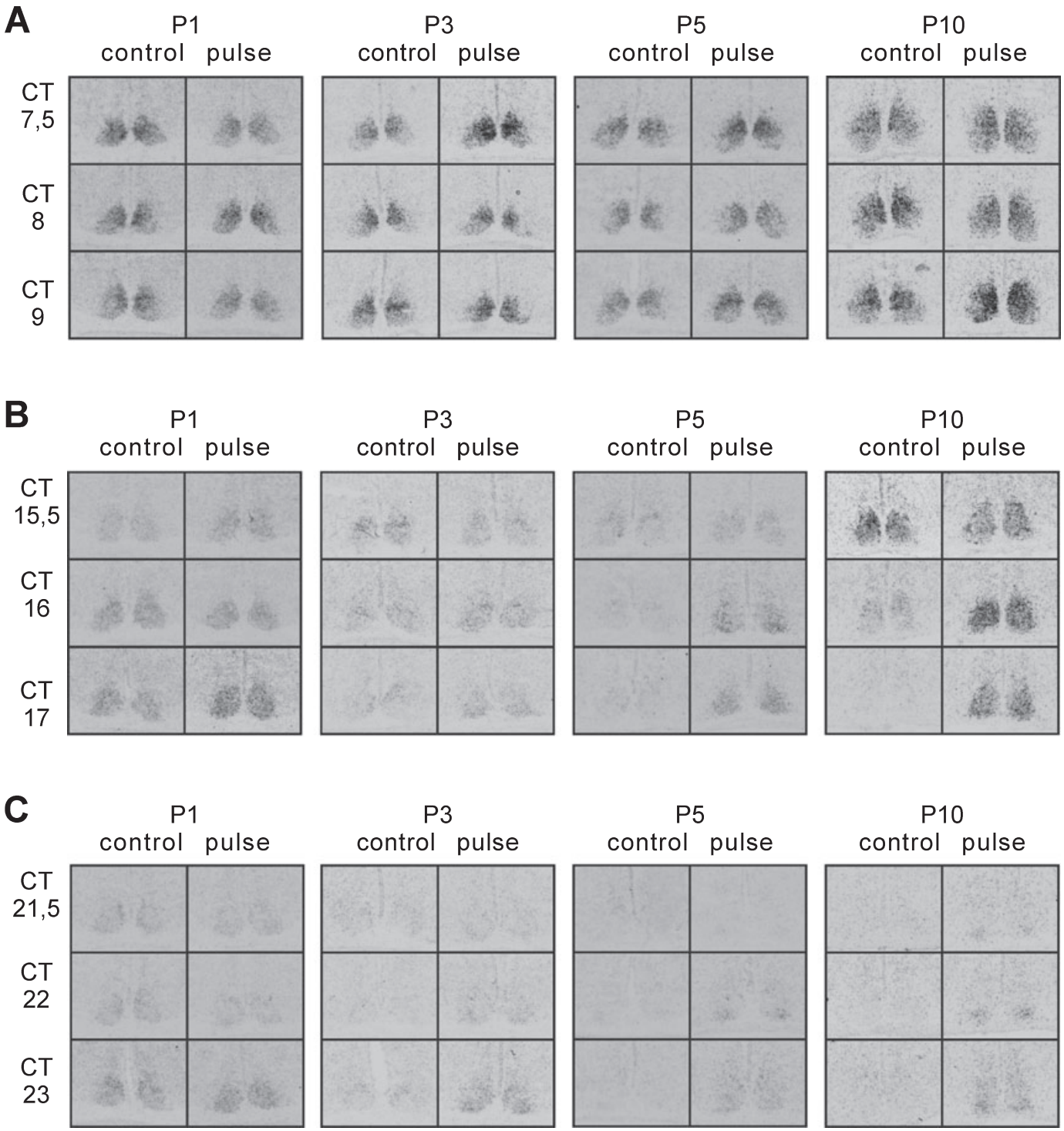

FIG. 7. Spatio-temporal distribution of *Per2* expression within the SCN of rat pups. Representative emulsion autoradiographs demonstrating the distribution of *Per2* expression in SCN sections of rat pups at P1, P3, P5 and P10. Pups were either kept in darkness (control) or exposed to a 30-min light pulse at (A) CT7, (B) CT15 or (C) CT21 (pulse), and sampled 30 min (CT7.5, CT15.5, CT21.5), 1 h (CT8, CT16, CT22) and 2 h (CT9, CT17, CT23) after the start of each light pulse. Note: the intensity of the signal on individual emulsion autoradiographs may not exactly reflect the relative mRNA levels (for details, see Materials and methods).

expression of the clock gene *Per1* mRNA. Apparently, photic information may reach the core clockwork components very early during the postnatal development. The RHT, which connects the retina with the SCN, reaches the vlSCN by P1 (Speh & Moore, 1993). The RHT contains filaments of intrinsically photoreceptive retinal ganglion cells expressing the non-visual photopigment melanopsin (Hattar *et al.*, 2002). Melanopsin-containing retinal ganglion cells are present within the retina at prenatal stages in mice and rats, and they may thus transmit information about external lighting conditions during the early postnatal period, i.e. well before the visual photopigments maturate and eyes open (Fahrenkrug *et al.*, 2004; Hannibal & Fahrenkrug, 2004; Sekaran *et al.*, 2005). These findings, together with

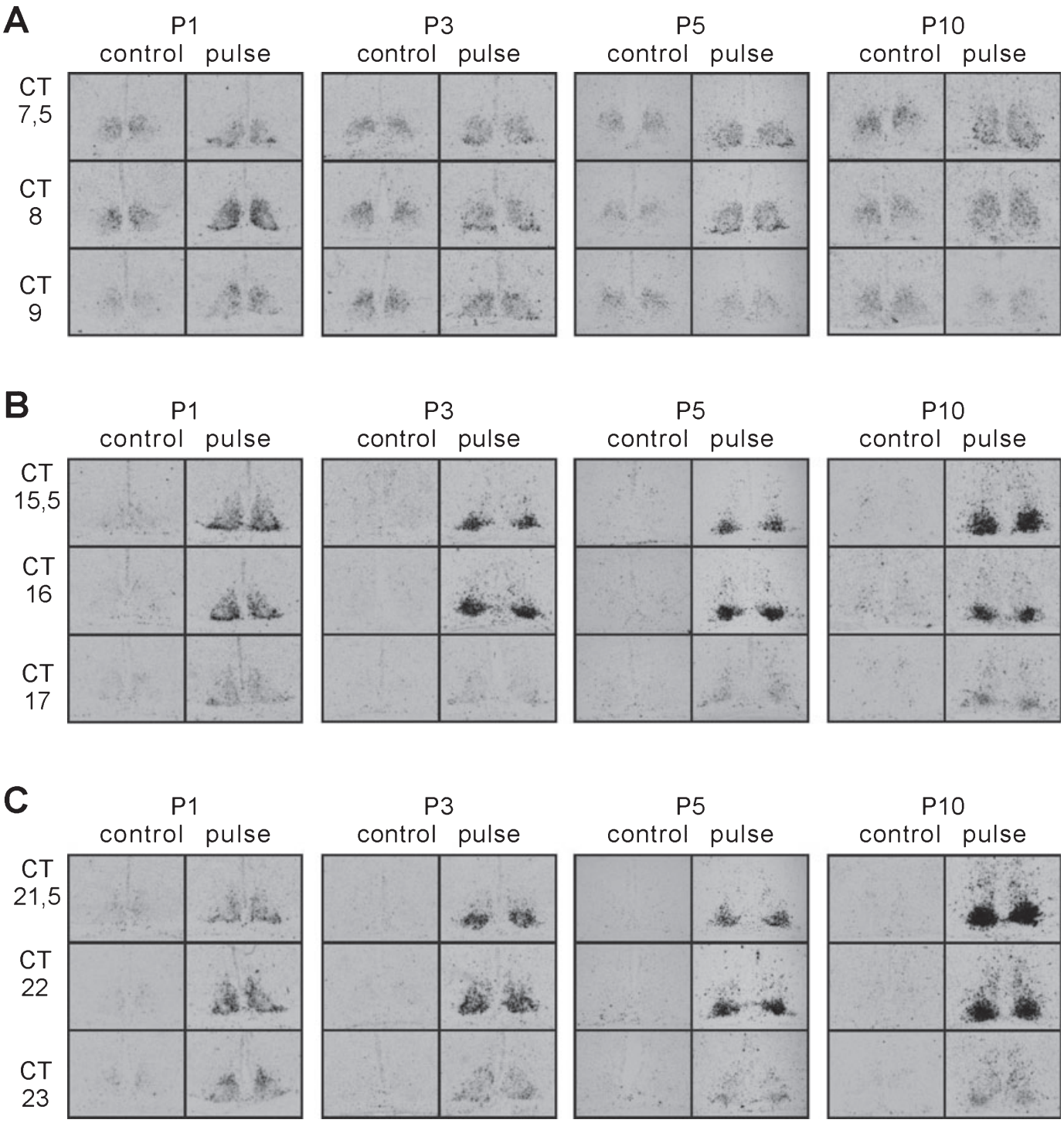

FIG. 8. Spatio-temporal distribution of *c-fos* expression within the SCN of rat pups. Representative emulsion autoradiographs demonstrating the distribution of *c-fos* expression in SCN sections of rat pups at P1, P3, P5 and P10. Pups were either kept in darkness (control) or exposed to a 30-min light pulse at (A) CT7, (B) CT15 or (C) CT21 (pulse), and sampled 30 min (CT7.5, CT15.5, CT21.5), 1 h (CT8, CT16, CT22) and 2 h (CT9, CT17, CT23) after the start of each light pulse. Note: the intensity of the signal on individual emulsion autoradiographs may not exactly reflect the relative mRNA levels (for details, see Materials and methods).

our current data, demonstrate that pathways conveying photic information to the circadian clock in the SCN are fully developed not only morphologically but also functionally at birth. Hence, the immature responses of the newborn rat SCN to photic stimulation during the daytime observed in this work are due to undeveloped functional properties of the circadian clock itself rather than non-functioning photic pathways.

In adult animals, the sensitivity of the circadian clock to light is restricted to the period of subjective night. In the rat SCN, the duration of the subjective night was correlated with either the interval in which light pulses induced high expression of *c-fos* within the vlSCN (Sumová & Illnerová, 1998) or the interval when endogenous expression of *c-fos* within the dmSCN was low (Sumová *et al.*, 1998). During this window, the SCN core clockwork permits the

photoinduction of the light-sensitive clock genes *Per1* and *Per2* from low nighttime levels. This is believed to be a crucial step in light's ability to reset the molecular core clockwork. In adult rats, *Per1* expression is light sensitive during the first and second part of subjective night, but *Per2* is supposed to be photoinduced only during the early subjective night (Yan *et al.*, 1999; Miyake *et al.*, 2000). In our study of adult rats, *Per2* expression was induced 2 h after the light pulse delivered at CT15, and also slightly (though not significantly) 1 and 2 h after the light pulse administered at CT21.

Our current data demonstrate that the gating mechanism was not present immediately after birth. At P1, light pulses induced the expression of *Per1* and *c-fos* during the daytime as well as during the nighttime. No *Per2* expression was induced at any time during this developmental stage. Interestingly, the spatial distribution of the signal induced by the daytime light pulse differed for *c-fos* and *Per1* expression. While *c-fos* expression was photoinduced primarily within a thin layer of the ventral part of the vlSCN, *Per1* expression was photoinduced throughout the entire SCN. The circadian control of *Per1* photoinduction resembled that of adult rats at P3, while that of *Per2* only at P5. At P3, P5 and P10, light pulse delivered during the second part of the night induced the expression of *Per2* within the same region as *Per1*, i.e. in the vlSCN. However, light pulse administered during the first part of the night induced the expression of *Per2* within the entire SCN region, but expression of *Per1* only in the vlSCN at P5 and P10, but not yet at P3. Therefore, it seems that the photoinduction of *Per2* differs from that of *Per1* not only in timing but also in spatial distribution, and may thus be controlled by different mechanisms since its beginning.

Interestingly, the response of the immediate-early gene *c-fos* to light pulses was more dramatic than the responses of the clock genes. Apparently, during early ontogenesis pathways mediating photic information may massively activate neuronal activity within the SCN. These pathways involve a clock-controlled step likely shared with that affecting *Per1* photoinduction. *c-fos* photoinduction was found to be gated at P3, when the signal intensity was measured on autoradiographic film from the whole SCN. A detailed examination of the spatial distribution of the *c-fos* photoinduction by emulsion autoradiography revealed that daytime light pulse induced *c-fos* expression at the ventral border of the vlSCN until P5, and a slight response in the entire vlSCN was detectable even at P10. While the photoinduction of *Per1* and *Per2* thus seemed to be restricted to the subjective night beginning at P3 and P5, respectively, the photoinduction of *c-fos* was not completely gated until P10. These data are in accordance with our previous findings on development of the circadian gate for the interval of *c-fos* protein photoinduction within the vlSCN (Bendová *et al.*, 2004). While light pulses induced *c-fos* immunoreactivity throughout the circadian cycle at P3, an interval of insensitivity to light pulses was detected at P10. Similarly, Weaver & Reppert (1995) reported the photic induction of *c-fos* expression during both day and night from P0 to P6.

The mechanism how the circadian clock in the SCN gates its response to photic stimuli has not yet been elucidated, though Ras-like G-protein Dexras1 has been recently recognized as a potential gatekeeper molecule (Cheng *et al.*, 2006). The gating is obviously property of photosensitive cells located in the vl-part of the SCN. Because the self-oscillating cells are mostly located in the dm-part, the inter-cellular rather than intra-cellular mechanisms are likely operating. The current model suggests that spontaneously oscillating cells impose their outputs upon the photosensitive cells, controlling thus the interval of their photosensitivity (Antle *et al.*, 2007). In accordance with this hypothesis, the absence of the gating mechanism during the early postnatal stage, as revealed in this study, might be due to a lack of functional synapses connecting the spontaneously oscillating cells with the photosensitive gate cells. In the rat SCN, synaptogenesis proceeds well into the postnatal stage and is completed at P10 (Weinert, 2005), i.e. just around the time when the gating mechanism develops. We further hypothesize that presence and degree of responses of *Per* genes expression to light might be conditional to the presence of a day/night variation in their endogenous expression. Although the experimental design of this study did not allow comparison of the amplitude of the rhythms, a gradual increase of the amplitude was demonstrated in our previous studies (Sládek *et al.*, 2004; Kováčiková *et al.*, 2006). The absence of the gating mechanism at P1 parallels the absence of significant circadian variations in clock gene expression within the SCN of newborn rats (Sládek *et al.*, 2004; Kováčiková *et al.*, 2006). Only very low-amplitude synchronized rhythms of *Per1* and *Per2* mRNA levels were detected at P1 and P2, respectively, and rhythms in the expression of other clock genes did not develop until P3. Apparently, the core clock mechanism may restrict the *Per1* and *Per2* photoinduction to subjective night only when synchronized circadian oscillations in their expression with low nighttime and high daytime levels have fully developed. It is plausible to speculate that these circumstances may facilitate the prevalence of the maternal influence on entrainment of the developing circadian clock over the photic influence during the first few postnatal days.

In conclusion, our data demonstrate that light affects the molecular core clock mechanism within the SCN immediately after birth. The photoinduction of the clock gene *Per2* develops later than that of *Per1*. The circadian clock begins to restrict *Per1* and *Per2* photoinduction to the subjective night during the first postnatal week, again earlier for *Per1* than for *Per2*. These data clearly demonstrate that the molecular circadian clock within the rodent SCN is not completely developed at the time of birth, but rather undergoes substantial postnatal maturation to function as a circadian clock in the whole complexity of the body demands. Only upon completing the development, the molecular core clockwork begins to be entrained by photic cues while maternal entrainment gradually loses importance.

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Abbreviations

AVP, arginin-vasopressin; CT, circadian time, CT0 is the time of the previous lights-on, CT12 is the time of the previous lights-off; dmSCN, dorsomedial part of the suprachiasmatic nucleus of the hypothalamus; LD, light–dark regime; OD, optical density; P, postnatal day, the day of birth is P0; *Per1,* Period1; *Per2,* Period2; r, rat; RHT, retinohypothalamic tract; SCN, suprachiasmatic nuclei of the hypothalamus; vlSCN, ventrolateral part of the suprachiasmatic nucleus of the hypothalamus.

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Expression and light sensitivity of clock genes *Period1*, *Period2* and immediate-early gene *c-fos* within retina of early postnatal rat

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ABSTRACT

Mammalian retina contains the circadian clock that is formed by the similar molecular components as the master circadian clock within the suprachiasmatic nuclei of hypothalamus (SCN). In the SCN, light-sensitive clock genes *Period1 (Per1)* and *Period2 (Per2)* and immediate-early gene *c-fos* are responsive to light already very early postnatally and may thus soon participate in the developing mechanism of circadian clock photoentrainment. The aim of the present study was to elucidate whether transcripts of *Per1*, *Per2* and *c-fos* genes are expressed also in the newborn rat retina, whether they are light sensitive and how their light sensitivity develops during early postnatal development. At postnatal day 1 (P1), P3, P5 and P10, the 30 min light pulse was administered during the subjective day or during the first or second part of subjective night and the induction of *Per1*, *Per2* and *c-fos* expression was assessed 30min, 1h, 2h and 4h from the pulse onset. In-situ hybridization followed by emulsion autoradiography revealed the endogenous expression of *Per1* and *Per2* but not of *c-fos* in the newborn retina. Endogenous expression of *Per2* was detected mainly in the inner retina and no light induction of *Per2* expression was observed. Light pulse induced the *c-fos* expression in the ganglion cells already at P1. Until P5, *c-fos* expressing cells were localized mostly in the dorsal part of the retina. At P10, they were already distributed across the entire retinal circumference. At P1 and P3, but not at P5 and P10, light pulse strongly enhanced *Per1* and *c-fos* expression in the outermost part of the retina. From the data, we hypothesize that this developmentally transient sensitivity to light occurs in retinal pigment epithelium and may play role in neural retina development. Our findings show, that the mammalian retina is light-sensitive already from birth similarly as the SCN clock, however, the light-induced genes expression developmentally change its spatial distribution and vary among the retinal layers.

Key words: development, retinal circadian clock, retinal pigment epithelium, retinal ganglion cells

INTRODUCTION

Mammalian circadian system includes master circadian clock located in the suprachiasmatic nuclei of the hypothalamus (SCN) and output pathways that translate its timing signals into daily rhythms in physiology and behavior (Hastings et al., 2007). The clockwork mechanism in the SCN is genetically determined and based on the system of interconnected transcriptional/translational feedback loops composed of several clock genes such as *Period 1,2, Clock, Bmal1, Cryptochrome 1,2, Rev-erb α* or *casein kinase 1 epsilon* and their protein products. Protein products of clock genes regulate positively or negatively its own transcription and give rise to circadian, i.e., approximately 24 h oscillations (reviewed in Reppert and Weaver, 2004). The light/dark cycle is the most potent environmental cue that entrains the endogenous circadian rhythms to the 24 h period of solar day. The photic information is conveyed to the SCN via retinohypothalamic tract (RHT) - a monosynaptic pathway consisting mostly of fibres from intrinsically photosensitive subpopulation of retinal ganglion cells (ipRGCs). IpRGCs contain non-visual photopigment melanopsin (Hattar et al., 2002) and exhibit an intraretinal network communication forming the system of circadian photoreceptors within retina (Sekaran et al., 2003; Barnard et al., 2006). In adults, the sensitivity of circadian clock in the SCN to light is temporally restricted to the period of subjective night. Light stimulus during the late night advances and during early night delays the phase of internal oscillations within SCN and output circadian rhythms. At molecular level, immediate early gene *c-fos,* a marker of neuronal activation, and clock genes *Period1 (Per1)* and *Period2 (Per2)* are light inducible during the subjective night, but not

during the subjective day and are thus the light-sensing components of clockwork mechanism within the SCN (Akiyama et al., 1999).

Beside its sensory function, vertebrate retina also contains autonomous circadian clock generating rhythmicity independently of the SCN (Ruan et al., 2006; Storch et al., 2007). It controls a broad range of retinal physiology and regulate a variety of retinal circadian rhythms as are rod outer segment disc shedding, melatonin release, dopamine synthesis, visual sensitivity, intraocular pressure and many others (for review see Tosini and Fukuhara, 2002; Green and Besharse, 2004; Tosini et al., 2008). In mammalian retina, coordinated clock gene expression was reported predominantly in dopaminergic cells of the inner nuclear layer (INL) and ganglion cell layer (GCL) (Witkovsky et al., 2003, Ruan et.al., 2006, 2008). Similarly to the SCN, circadian clock in the adult retina can be also entrained by light (Tosini and Menaker, 1996, 1998, Ruan et al., 2008) and photoinduction of *c-fos*, *Per1* and *Per2* expression in the retina was also reported (Huerta et al., 1997, 1999; Namihira et al., 2001).

During early postnatal development, circadian rhythmicity and photic entrainment of master circadian pacemaker in the SCN maturates only gradually (for review see Weinert, 2005; Sumová et al., 2006). However, expression of *c-fos* and *Per1* in the SCN of rat pups can be induced by light immediately after birth (Leard et al., 1994, Matějů et al., *in press*). Thus, the SCN is sensitive to light long before eye opening, retina maturation and image-forming vision. This is due to the non-parallel development of image forming and non-image forming vision. IpRGCs containing melanopsin are light responsive, form functional connections with the SCN neurons and are directly responsible for light-induced *c-fos* expression within SCN already at the day of birth (Tu et al., 2005, Sekaran et al., 2005). At this age, other parts of the rat retina, except well-developed retinal pigment epithelium (RPE), are still in a very premature state. The

primordial photoreceptors start to extend their outer segments at postnatal day (P) 5 and mature by the third postnatal week. The inner part of the retina consists of neuroblastic cells that begin to differentiate into two layers during the first week of postnatal life. Rod and cone ganglion cells, although morphologically differentiated, respond to photoreceptors stimulation as soon as bipolar cells become functional between P10 and P12 (Weidman and Kuwabara, 1969; Sernagor et al., 2001).

Thus, we begin to understand the sensory mechanism leading to early photoinduction of clock gene expression within the SCN that may thus be involved in the development of the photic entrainment of circadian clock. However, the expression of clock genes within immature retinal layers and its responsiveness to light stimulation remain unknown. Therefore, the aim of the present study was to elucidate whether clock genes *Per1*, *Per2* and immediate early gene *c-fos* are expressed in the retina of rat pups, how their sensitivity to light develops during early postnatal development and where within immature rat retina the photoinduction occurs.

MATERIALS AND METHODS

Animals

Male and female Wistar rats (BioTest s. r. o.; Konárovice, Czech Republic) were maintained under a light-dark regime with 12-h of light and 12-h of darkness per day (LD12:12) at a temperature of 23 ± 2°C with free access to the food and water. Light was provided by overhead 40-W fluorescent tubes and illumination was between 50 and 200lux, depending on the cage position. Animals were maintained at LD12:12 for at least four weeks before mating. The day of delivery was designated as postnatal day 0 (P0).

All experiments were conducted under license No. A5228-01 with the U. S. National Institutes of Health and in accordance with Animal Protection Law of the Czech Republic (license no. 42084/2003-1020).

Experimental protocol

Female rats with their pups were released into constant darkness at the time of dark to light transition (designated as circadian time 0; CT0) on postnatal day (P)1, P3, P5 or P10. During the first cycle in darkness, experimental groups of pups were exposed to a 30-min light pulse (700 lx) at either CT7 (i.e. during subjective day), CT15 (i.e. during the first part of subjective night), or CT21 (i.e. during the second part of subjective night); control groups were left untreated in the darkness. Pups from both groups were subjected to maternal deprivation from the beginning of the light pulse in experimental animals until the time of sampling by a rapid decapitation at 30 min, 1 h, 2 h and 4 h after the start of each light pulse. In 10-day old pups, sampling at 4 h after the light pulse was omitted. At each of these time points, four animals from the light-pulsed group and four control animals were sampled. Whole heads were immediately frozen on dry ice, stored at -80 °C and then sectioned into series of 12µm-thick coronal slices in an alternating order throughout the region containing the medial part of eye bulbs. Expression of *rPer1*, *rPer2* and *c-fos* mRNA within retina was assessed by *in-situ* hybridization.

In situ hybridization histochemistry

The cDNA fragments of rat *rPer1* (980bp; corresponds to nucleotides 581-1561 of the sequence in GenBank accession no. AB002108), rat *rPer2* (1512bp; corresponds to nucleotides 369-1881 of the sequence in GenBank accession no. NM031678) and rat *c-fos* (1160bp; corresponds to nucleotides 141-1300 of the sequence in GenBank accession no. X06769) were used as templates for *in-vitro* transcription of complementary RNA probes (T7 or T3 MAXIscript kit, Applied Biosystems/Ambion, Austin, TX, USA). The *rPer1* and *rPer2* fragment-containing vectors were generously donated by Professor H. Okamura (Kobe University School of Medicine, Japan). The rat *c-fos* fragment-containing vector (originally cloned by Dr. Tom Curran from Children`s Hospital of Philadelphia,

PA, USA) was generously donated by Professor W. J. Schwartz (University of Massechusetts Medical School, Worcester, MA, USA). The whole cDNA was recloned into a pBluescript SK vector (Stratagene, La Jolla, CA, USA) in our laboratory. Probes were labeled by α-^{35}S-UTP (MP Biomedicals, Irvine, CA, USA) and purified using Chroma-Spin 100-DEPC H2O columns (Clontech Laboratories Inc., Mountain View, USA). *In situ* hybridization was performed as described previously (Shearman et al., 2000; Sládek et al., 2004). Briefly, sections were hybridized for 21 h at 60°C. Following a post-hybridization wash, the sections were dehydrated in ethanol and dried. The slides were then dipped in autoradiographic emulsion LM-1 (Amersham Biosciences, Piscataway, NJ, USA). After six weeks of exposure, the slides were developed using the developer Fomatol LQN and fixer FOMAFIX (FOMA, Hradec Králové, Czech Republic) and mounted for optical microscopy. For each gene, sections from pups of the same age were hybridized with the same probe and processed simultaneously under identical conditions. As a control, few parallel sections were also hybridized with sense probes and no specific hybridization signal within retina was found (Fig.1). Representative pictures of emulsion autoradiographs were taken using a digital Olympus DP70 camera (Olympus, New Hyde Park, NY, USA) connected to an Olympus Ax-70 microscope (Olympus, New Hyde Park, NY, USA).

Analysis of emulsion autoradiographs

Emulsion autoradiographs were analyzed using an image analysis system ImagePro (Olympus, New Hyde Park, NY, USA). In each animal, the mRNA levels within outer and inner neuroblast cell layer (or differentiating outer and inner nuclear layer at P10), and ganglion cell layer were quantified as the relative optical density (OD) of the layer. OD of the outermost part of retina was measured only if mRNAs expression was induced; otherwise the structure was not distinguishable from surrounding tissue. OD

of subretinal area without any specific signal served as the internal standard for each section. Experiment was repeated three times and the obtained data were converted to the percentage of the highest mean value in each assay regardless of animals' age and retina layer. The first experiment included animals at P1 and P3, the second expreriment animals at P1, P3 and P5 and the third experiment animals at P10. The eye sections from each experiment were processed simultaneously in the same *in-situ* hybridization. Data from these assays were converted to the percentage of the maximum mean value from each experiment and pooled. Pooled data were then used for statistical analysis.

Concerning the *c-fos* induction within the ganglion cell layer, the emulsion autoradiography allowed us to count individual *c-fos* mRNA-positive cells. The induction of *c-fos* expression within ganglion cell layer was then plotted as a mean of a number of *c-fos* positive cells within the ganglion cell layer ± S.E.M.

Statistical analysis

In each time point, levels of *Per1* and *c-fos* mRNA expression (pooled data as described above) in neuroblast cell layer of control and light-treated animals were compared using T-test. In animals exposed to light, levels of *Per1* and *c-fos* expression (pooled data as described above) in each time point were also compared within neuroblast cell layer and the outermost part of retina using T-test. Each column in graphs represents the mean value of 4 to 9 animals ± S.E.M.

RESULTS

We have studied the effect of 30 min light pulse administered either during the subjective day (at CT7), or during the first (at CT15) or second part of subjective night (at CT21) on expression of *Per1*, *Per2*, and *c-fos* genes within the retina. The levels of *Per1*, *Per2* and *c-fos* mRNA were assessed by *in-situ* hybridization with subsequent emulsion autoradiography on the coronal sections of rat eyes at P1, P3, P5 and P10. Comparison of

slices hybridized with sense probes revealed elevated spontaneous expression of *Per1* and *Per2* (Fig.1A) in retinas of control animals as early as at P1. Until P5, *Per1* was weakly expressed throughout the retina with no specific layer location (Fig. 1B). At P10, its expression predominated in the outer part of developing retina. Clock gene *Per2* was expressed mainly in the inner part of the neuroblast cell layer already from birth and did not change its location until P10 (Fig 1B). No specific elevation of spontaneous expression of *c-fos* within retina of control animals was observed at P1 (Fig. 1A) as well as at P3 and P5. At P10, a weak endogenous expression was observed in the developing INL (Fig. 1B).

Relative OD of GCL and inner and outer part of the neuroblast cell layer was measured within retinas of light-stimulated and control rat pups. Relative OD of light-induced stripe of expression in the outermost part of the retina was measured only in animals exposed to the light pulse. In GCL, light pulse administration induced the expression of *c-fos* as early as at P1. The hybridization signal was clustered into the individual cells, thus the cells expressing the *c-fos* were numerable (Fig. 2 F, G, H). No labeled cells were observed in the GCL of control P1, P3 or P5 animals (Fig. 2 E) and only a few cells were distinguishable at P10 (Tab.1 B). Light pulse elicited the maximum *c-fos* expression within 30 min from the onset of the light pulse administered at CT7 and CT15 as well as at CT21. Two hours after the light pulse, number of cells and the intensity of their *in-situ* hybridization signal stayed still high and the effect of light disappeared between 2 and 4 h after the pulse (Tab.1 A). At P1, P3 and P5, the *c-fos* positive cells were localized mainly in the dorsal and, to a lesser extent, to the ventral part of the retina (Fig. 2 A, B, C). At P10, they were distributed along the whole retinal circumference (Fig. 2 D). No light-induced expression of *Per1* and *Per2* in ganglion cells was detected until P10.

At P1 and P3 but not at P5 or P10, light pulse administration induced the thin stripe of *Per1* and *c-fos* expression in the outermost part of the retina that has been recognized as the RPE (Fig. 4 A, C; Fig. 5 A, C). In control animals, no such expression occurred and the RPE layer was not distinguishable from the surrounding tissue (Fig. 4 B; Fig. 5 B). The OD measurement was therefore possible only when the light-induced signal defined the structure. Because OD of *Per1* and *c-fos* hybridization signal in neuroblast cell layer showed no significant differences between control and light-stimulated animals (Fig. 3), we compared the relative OD of the RPE region with the relative OD of adjacent outer part of neuroblast cell layer within retina of light-stimulated animals. At P1, light pulse administration induced *Per1* expression within RPE at CT7, CT15 as well as at CT21 (Fig 4 D). Statistical analysis revealed the significant difference between relative OD of *Per1* mRNA hybridization signal within RPE and outer neuroblast cell layer at CT 8 and CT9 ($p<0,01$ each; T-test), CT16 and CT17 ($p<0,001$ each; T-test) and also at CT22 and CT23 ($p<0,001$ each; T-test). Thus, light induces *Per1* mRNA expression within RPE of rat pups at P1 within 1 h; effect of light reaches its maximum within 2 h and between 2 and 4 h after the light pulse disappeared. At P3, *Per1* expression was induced within RPE by light pulse administration at CT7 and CT21 and slightly at CT15 (Fig. 4 E). Comparison of values of relative OD of the RPE and adjacent neuroblast cell layer revealed significant differences at CT8 ($p<0,05$; T-test), CT9 ($p<0,001$; T-test), CT17 ($p<0,01$; T-test), CT22 ($p<0,05$; T-test) and at CT23 ($p<0,01$; T-test).

Similarly, photoinduction of *c-fos* expression was observed within the RPE at P1 and P3, but not at P5 and P10. At P1 (Fig. 5, D), comparison of relative OD values of *c-fos* mRNA signal within RPE and adjacent neuroblast cell layer revealed the significant *c-fos* induction within RPE at CT8 ($p<0,001$; T-test), at CT9 ($p<0,001$; T-test), CT17 ($p<0,01$; T-test), CT22 ($p<0,05$; T-test) and at CT23 ($p<0,001$; T-test). At P3, expression

of *c-fos* was not significantly induced by light pulse administration at CT7 and only slightly by light applied at CT15 and CT21 (Fig. 5 E). Relative OD of the RPE region significantly differs from the OD of attached neuroblast cell layer at CT17 ($p<0,01$; T-test), CT22 ($p<0,05$; T-test) and CT23 ($p<0,01$; T-test). No photoinduction of *Per2* was observed at any time and age studied (data not shown).

Our data show that light pulse administration during the subjective night as well as during the subjective day induced *c-fos* expression in ganglion cell layer at P1, P3, P5 and P10. *Per1* and *c-fos* expression was also light-responsive during the subjective day and night within the outermost part of rat retina at P1 and P3 but not at P5 and P10. Our study did not reveal any diurnal oscillations in *Per1*, *Per2* and *c-fos* expression in retinas of control rat pups collected during the study as well as apparent temporal regulation of light sensitivity of studied genes in retina.

DISCUSSION

In the present study, we have monitored the mRNA expression of light-sensitive clock genes *Per1, Per2* and immediate-early gene *c-fos* within the retinas of control and light-treated newborn rats. In-situ hybridization histochemistry with subsequent emulsion autoradiography allowed us to focus the specific hybridization signal more precisely within retinal layers. We have measured the optical density from the inner and outer part of the retinal neuroblast cell layer, from light induced stripe in the outermost part of the retina and from the ganglion cell layer. Our data demonstrate the spatial and developmental difference in photosensitivity of all three genes studied as well as the differences in the distribution of their endogenous expression in the retina of neonatal rat.

Although many studies describe the expression of clock genes and immediate-early gene *c-fos* in the adult rodent retina, no data exists so far characterizing their endogenous expression in the retina during early postnatal development. Here, we show

that the clock genes *Per1* and *Per2* are expressed within rat retina early in postnatal ontogeny. At P1, P3 and P5, *Per1* is expressed throughout the retina with no specific layer location. At P10, its hybridization signal becomes stronger in the outer part of the retina, the site of developing photoreceptors. In the adult rat retina, *Per1* and *Per2* mRNA expression as well as PER1 protein was found mainly in the inner nuclear layer (INL) and small group of ganglion cells (Namihira et al., 2001; Witkovsky et al., 2003; García-Fernández et al., 2007; Dinet et al., 2007; Ruan et al., 2008). Other studies show the *Per1* presence also in photoreceptors (Tosini et al., 2007). Therefore, it seems that the spatial distribution of *Per1* gene within the retina may vary during the development. In support, our preliminary data suggest that its expression predominates in the INL at P15, i.e., immediately after the eye opening.

Interestingly, at P1, *Per2* expression was localized to the inner part of the neuroblast cell layer and during the gradual development of retinal morphology, *Per2* mRNA expression occurred predominantly within the developing INL. Several INL cell types can be distinguished already at birth, however the layer become functional at the end of the first postnatal week (Weidman and Kuwabara, 1969; Sernagor et al., 2001). It seems that, in contrast to *Per1*, *Per2* mRNA expression should be associated rather with early morphological differentiation of INL then with its functional maturation. Previous study has reported that the first spontaneous *c-fos* expression in the retina appeared between P11 and P15 (Ohki et al., 1996). Consistently with this finding, we observed no apparent spontaneous *c-fos* expression within retina of control animals.

Similarly to the adult rat SCN, also in adult rodent retina, expression of clock genes *Per1* and *Per2* exhibit circadian variation, mainly in INL (Shearman et al., 1997; Namihira et al., 2001; Witkovsky et al., 2003; Kamphuis et al., 2005; Rohleder et al., 2006; Ruan et al., 2008). Although our experiments were focused mainly on the effect of

light, intact newborn retinas were collected over the most of the 24h cycle. This allows us to refer also on circadian variation of *Per* genes in the developing retina. In the developing rat SCN, expression of *Per1* and *Per2* was detected before birth; circadian rhythm in *Per1* and *Per2* expression starts to form at P1, became apparent at P2 and develops gradually during early postnatal ontogenesis. At P10, expression of most of the clock genes in the SCN exhibit circadian rhythm (Sládek et al., 2004, Kováčiková et al., 2006). In contrast to the SCN, we have observed no circadian variation in *Per1* and *Per2* expression within retinal layers until P10. Non-visual photopigment melanopsin was previously reported as possible diurnal regulator of various retinal functions (Barnard et al., 2006). Melanopsin is in the retina present very early in ontogeny and is responsible for transduction of photic input into the SCN already from birth (Fahrenkrug et al., 2004). According to our observation, circadian rhythm in expression of *Per* genes in the retina seems to develop later during postnatal ontogenesis. Thus, melanopsin probably does not play any direct role in the generation of the rhythmic clock gene expression within retina.

As demonstrated previously, light stimulates *c-fos* expression in melanopsin-positive ipRGC and within INL (Sagar and Sharp, 1990; Yoshida et al., 1993; 1995; Koistinaho and Sagar, 1995; Semo at al., 2003). In addition, *Per1* and *Per2* expression was induced by light mainly in INL (Namihira et al., 2001). In neonatal retina, *c-fos* expression in ipRGCs can be induced by light from birth onward (Hannibal and Fahrenkrug, 2004) demonstrating thus the early photosensitivity of ipRGCs. Consistently, our study shows the *c-fos* photoinduction within ganglion cell layer as early as at P1. Interestingly, emulsion autoradiography revealed the neonatal topographic restriction of *c-fos* expressing ganglion cells mainly into the dorsal part of the eye bulb. As late as at P10, the labeled ganglion cells were distributed uniformly along the dorso-ventral extent of the retina. The nasal-temporal and dorsal-ventral topographic polarity of the retina develops

very early in ontogeny and axons of ganglion cells from a distinct part of the retina project to appropriate target fields establishing thus a specific retinotopic maps in the brain (Peters, 2002; McLaughlin et al., 2003, Harada et al., 2007). Here, we show a topographic polarity of light-induced *c-fos* expression into the dorsal part of the retina that disappeared between P5 and P10. Similar developmental change was observed for M opsin production within photoreceptor layer in hamster retina. M opsin was localized into dorsal retina only until P10 and then its distribution spread, similarly as light-induced *c-fos* in ganglion cells, across the entire circumference (Glösmann and Anhelt, 2002). On the other hand, four topographic subtypes of direction-selective ganglion cells that are responsible for reflexive eye movements are functional as late as at eye opening (Elstrott et al., 2008). Therefore, the functional properties of topographic retinal fields may change during the development. The question remains, whether these changes may influence also the appropriate retinotopic target regions in the brain.

Semo at al., (2003) reported that in the absence of light 10-18% of melanopsin ganglion cells within the adult mouse retina express *c-fos* spontaneously due to rod and cone inputs. In our study, no spontaneous *c-fos* expression in ganglion cells was observed within retina of control pups until P10, supporting the fact that ganglion cells in early postnatal retina have a low synaptic connection with other retinal layers.

No *Per1* and *Per2* photo-induction was observed in ganglion cells. In mouse retina, PER1 protein production was shown in a group of ganglion cells that do not express melanopsin (Witkovsky et al., 2003). In adult animals, these cells receive synaptic inputs from INL. Induction of c-Fos protein production may thus depend on the signalization from circadian oscillator within INL. Early after birth, this signaling pathway may not yet been developed.

We measured also optical density from inner and outer part of the neuroblast cell layer and we did not find any difference between light stimulated and control retinas for none of the genes studied even at P10. Contrary to our data, Munoz-Llamosas et al. (2000) revealed photo-induction of c-Fos protein production in the inner part of neuroblast cell layer in mice at P4. This discrepancy can be caused by the differences between species as well as by the lower sensitivity of in-situ hybridization histochemistry, which may not be able to detect sparse individual cells in the INL.

Intriguing point of this study is the light-induced strip of hybridization signal obtained with *c-fos* and *Per1* probes in the outermost part of the retina early postnatally. As mentioned previously, so early after birth neural retina and photoreceptors especially are still very immature (Weidman and Kuwabara, 1969). Thus, they are unlikely responding to photic stimulation. On the contrary, transient *c-fos* photoinduction was observed in human RPE cell line *in vitro* (Yam et al., 2003). The *c-fos* was also rapidly induced in the RPE, but not in the neural retina of adult rats by nitric oxide donor (SNP) via CREB phosphorylation (Ohki et al., 1995). Finally, rhythmic expression of *Per1* and *Per2* was induced by serum shock in human RPE cell line (Pavan et al., 2006). *In vitro* data confirm that the photosensitivity and the ability to induce gene expression upon external stimulation are the intrinsic property of the RPE cells and do not result from the retinal circuitry. Although we failed to obtain plausible proof, mainly due to biochemical properties of the RPE (Katz et al., 1978) that mask the immunohistochemical detection of specific proteins in the RPE, it seems probable that the light-induction of both genes occurs in the RPE. In support, Peirson et al. (2004) have described the presence of melanopsin in the mouse RPE that suggest that the light-induced gene expression in the RPE could be mediated via similar mechanism as in ipRGC.

The light-induced *c-fos* and *Per1* expression within the RPE is strongest at P1, still evident at P3 and disappear completely between P3 and P5. Similar developmental time course was observed for the loss of dopamine-stimulated *c-fos* expression via D1 receptor within the neonatal SCN (Weaver et al., 1992; Weaver a Reppert, 1995). In the retina, dopamine mediates acute light induction of the *Per1* gene (Yujnovsky et al., 2006), and via D1 receptors, it plays a key regulatory role of the endogenous retinal clock mechanism (Ruan et al., 2008). Light acutely increases the activity of retinal tyrosine hydroxylase, a key enzyme of dopamine synthesis in amacrine cells (Iuvone et al., 1978) and also in fish RPE (McCormack and Burnside, 1993). Light also evokes a dopamine synthesis and utilization within 30 min of light onset (Nir et al., 2000). Moreover, dopamine controls melanopsin expression in ipRGCs (Sakamoto et al., 2005). During the development, dopamine-mediated cAMP accumulation via D1 receptors was observed in the retina already before synaptogenesis (Reis et al., 2007). All these data suggest that dopaminergic system may play a role in light-induced *Per1* and *c-fos* expression within the neonatal RPE. However, here it is important to point out that in our study, the albinotic rat strain was used. The albino phenotype results from the mutation in the tyrosinase gene and consequently the insufficient release of melanin and L-DOPA, a dopamine precursor, which is considered to be responsible for the abnormal development of the neural retina (for review see Jeffery, 1997; Tibber et el., 2006). Ocular melanin protects the retina and choroid of pigmented animals against light-induced cell toxicity (Peters et al., 2006) and dopamine also protect retinal neurons from glutamate-induced excitotoxicity by reducing NO production (Yamauchi et al., 2003). Therefore, it is also possible, that our observation is limited only to albino rats with low dopamine and melanin protection against the toxic effect of light causing the stress response via activation of *c-fos* as a part of AP-1 complex. The role of *Per1* in this model would remain to be established. Noteworthy, the

level of D1 receptors expression may be even elevated in early postnatal albino compared to pigmented rat retina (Kralj-Hans et al., 2006), suggesting that the newborn albino retina is prepared to receive dopaminergic input despite its own low production. Exploration of gene photo-responsiveness within RPE of pigmented animals is complicated by the presence of melanin that cover the site of specific in situ hybridization signal. Therefore, different methodical approaches are needed for further elucidation and exploitation of the present data.

Finally, the expression of *c-fos* in GCL and *c-fos* and *Per1* in the RPE were induced by light in neonatal retina regardless of the circadian time, i.e. during the subjective night as well as during the subjective day even at P10, when the circadian clock within SCN already gates the sensitivity to light to the period of subjective night. Similarly, light sensitivity of *c-fos* expression in amacrine and ganglion cells within adult mouse retina is not temporally regulated (Huerta et al., 1999). Therefore, it seems that the rat retina lack a mechanism gating the sensitivity to light to the nighttime as it is in the SCN.

In conclusion, we show temporary spatial distribution of light-induced *c-fos* into dorsal part of ganglion cell layer of newborn Wistar rat and the developmentally transient photosensitivity of clock gene *Per1* and immediate-early gene *c-fos* in the retinal pigment epithelium. Also, we show that spontaneous *Per2* expression is localized to INL already from P1 and, contrary to the SCN (Matějů et al., *in press*), is not changed upon light stimulation until P10.

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FIGURE LEGENDS

Fig.1. **A**: Spontaneous expression of clock genes *Per1*, *Per2* and immediate-early gene *c-fos* within retina of 1-day old rat pups. Representative emulsion autoradiographs demonstrating the hybridization signal obtained with antisense and sense *Per1 Per2* and *c-fos* probes within eye sections of control animals show the specific hybridization signal for *Per1* and *Per2* within retina of rat pups at postnatal day 1. **B**: Spontaneous expression of *Per1*, *Per2* and *c-fos* within rat retina during early postnatal development. Details of representative emulsion autoradiographs demonstrating the development and localization of spontaneous expression of clock genes *Per1* and *Per2* and immediate-early gene *c-fos* within retina of control animals at postnatal day (P) 1, P3, P5 and P10. Clock gene *Per1* is expressed within rat retina without specific localization until P5 and at P10 became localized within outer part of retina. *Per2* expression is localized within the inner part of the neuroblast cell layer and as the retina gradually maturates; its expression became restricted to the developing inner nuclear layer. Immediate-early gene *c-fos* is weakly expressed within retina of control animals until P5 and at P10 only few cells were labeled within developing inner nuclear layer of control animals.

Fig.2. *c-fos* mRNA photoinduction within ganglion cells at postnatal day (P) 1 (A), P3 (B), P5 (C) and P10 (D) 1h after the start of the light pulse. The site of prevailing *c-fos* photoresponsiveness is localized in the dorsal part of the eye bulb (arrows) and as development continues, at P10 became also present within the medial and ventral part (D). Detail of representative emulsion autoradiograph of rat retina at P1 demonstrate the lack of *c-fos* mRNA hybridization signal within ganglion cells of control animals (E) and *c-fos* expression 30 min (F), 1h (G) and 2h (H) after the start of the 30min light pulse.

Fig. 3. Effect of light on *Per1* (A) and *c-fos* (B) expression within outer neuroblast layer of the retina at P1. Light pulse at any time of the circadian cycle did not increase the mRNAs levels (gray columns) above endogenous mRNA levels in control animals (black columns). Data from repeated experiments were converted to the percentage of the maximum mean value from each experiment and then merged. Merged data are plotted as mean of 7-8 values ± S.E.M.

Fig.4. Photoinduction of *Per1* expression within the outermost part of retina of rat pups. Representative emulsion autoradiographs show the localization of the *Per1* mRNA hybridization signal within the eye of 1-day old rat pup 2h after the start of 30 min light pulse (A). Details of emulsion autoradiographs demonstrate that in control animals, no *Per1* mRNA hybridization signal is distinguishable in the outermost part of retina (B), but 2 h after the light pulse onset, a strong *Per1* expression (C) occurs probably within retinal pigmented epithelium (RPE). Graphs demonstrate the effect of light on *Per1* mRNA expression within the RPE of rat pups at postnatal day (P)1 (D) and P3 (E). Expression of clock gene *Per1* was induced by a light pulse administration during the subjective day (circadian time 7, CT7) as well as during the subjective night (CT15 and CT21). Relative optical density (OD) of light induced signal in RPE (dashed columns) was compared with OD of the adjacent neuroblast cell layer (NCL) (gray columns) that did not exhibit light sensitive *Per1* expression (see Fig.3). Data from repeated experiments were converted to percentage of the maximum mean value from each experiment and then merged. Merged data are plotted as mean of 6-9 (occasionally 3) values ± S.E.M. * P<0,05; ** P<0,01; *** P<0,001 (T-test)

Fig.5. Photoinduction of *c-fos* expression within the outermost part of retina of rat pups. Representative emulsion autoradiograph show the localization of the *c-fos* mRNA hybridization signal within the eye of 1-day old rat pup 2 hours after the start of 30 min light pulse (A). Details of emulsion autoradiographs demonstrate that in control animals, *c-fos* signal is not distinguishable in the outermost part of retina (B), but 2 hours after the light pulse, strong *c-fos* expression (C) occurs probably within retinal pigmented epithelium (RPE). Graphs demonstrate the effect of light on *c-fos* mRNA expression within the RPE at postnatal day (P)1 (D) and P3 (E). Expression of clock gene *c-fos* was induced by a light pulse administration during the subjective day (circadian time 7, CT7) as well as during the subjective night (CT15 and CT21). Relative optical density (OD) of light induced signal in RPE (dashed columns) was compared with OD of the adjacent neuroblast cell layer (NCL) (gray columns) that did not exhibit light sensitive *c-fos* expression (see Fig.3). Data from repeated experiments were converted to the percentage of the maximum mean value from each experiment and then merged. Merged data are plotted as means of 4 – 8 (occasionally 3) values ± S.E.M. *P<0,05; **P<0,01; ***P<0,001 (T-test)

Table 1. Number of retinal ganglion cells exhibiting light-dsensitive *c-fos* mRNA expression. **A**: Number of ganglion cells exhibiting light-induced *c-fos* mRNA expression within retina of rat pups at postnatal day (P) 1, P3, and P5. At P1, P3 and P5 no spontaneous *c-fos* mRNA expression was observed within retina of control animals. **B**: Number of ganglion cells exhibiting light-induced *c-fos* mRNA expression within retina of rat pups at P10. At P10, slight spontaneous expression of *c-fos* mRNA was observed within ganglion cells of control animals. Within retina of 10-day old rat pups, levels of c-fos mRNA expression were not assessed 4h after the start of each light pulse. Both tables

demonstrates that expression of immediate-early gene *c-fos* is induced by a light pulse administered during the subjective day as well as during the subjective night.

Figure 1

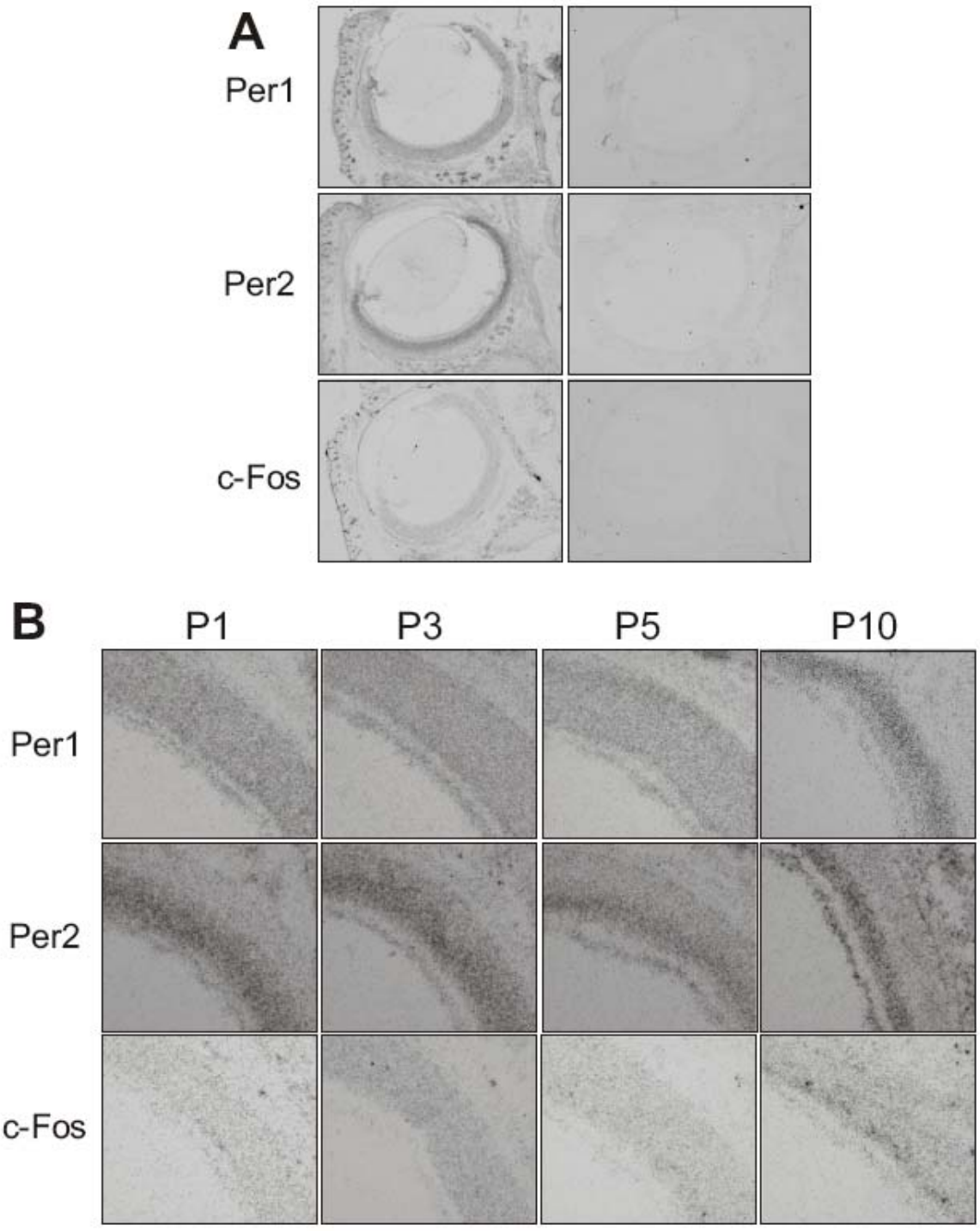

Figure 2

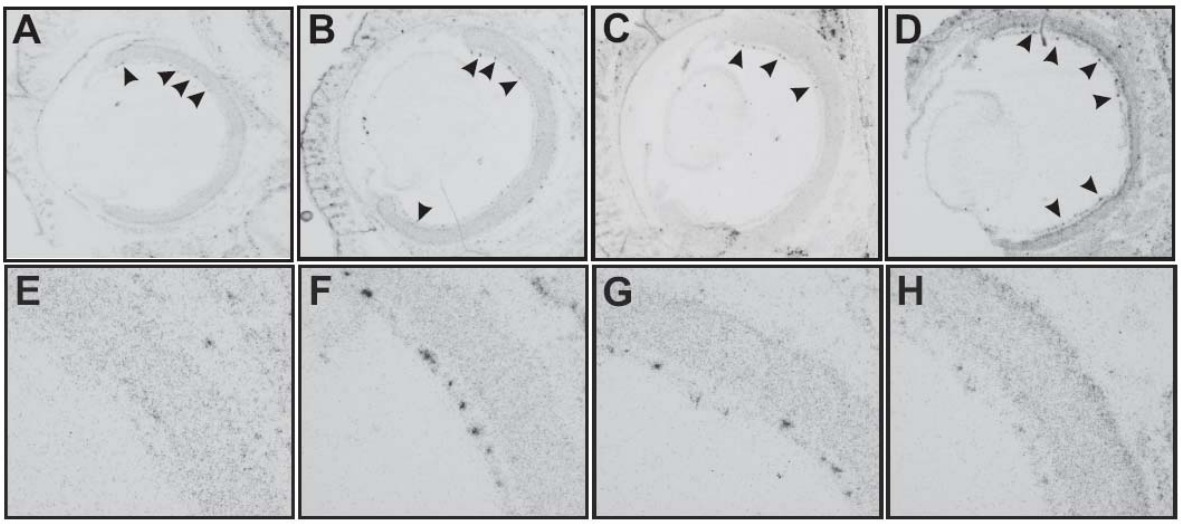

Figure 3

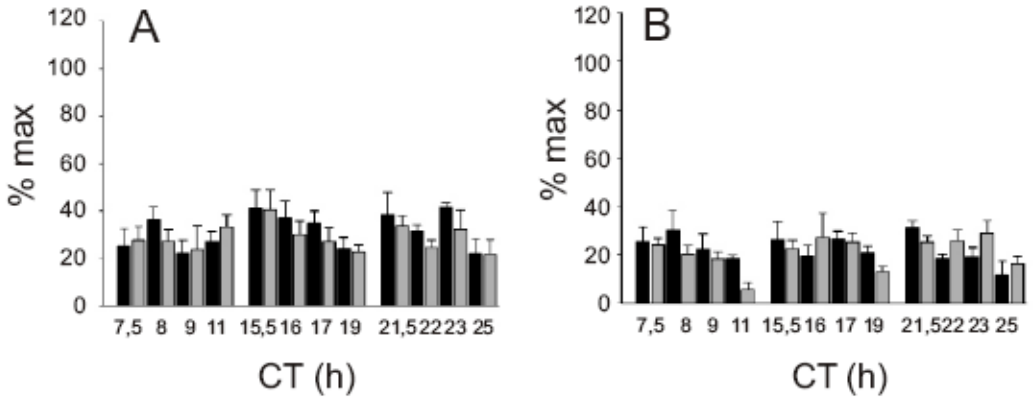

Figure 4

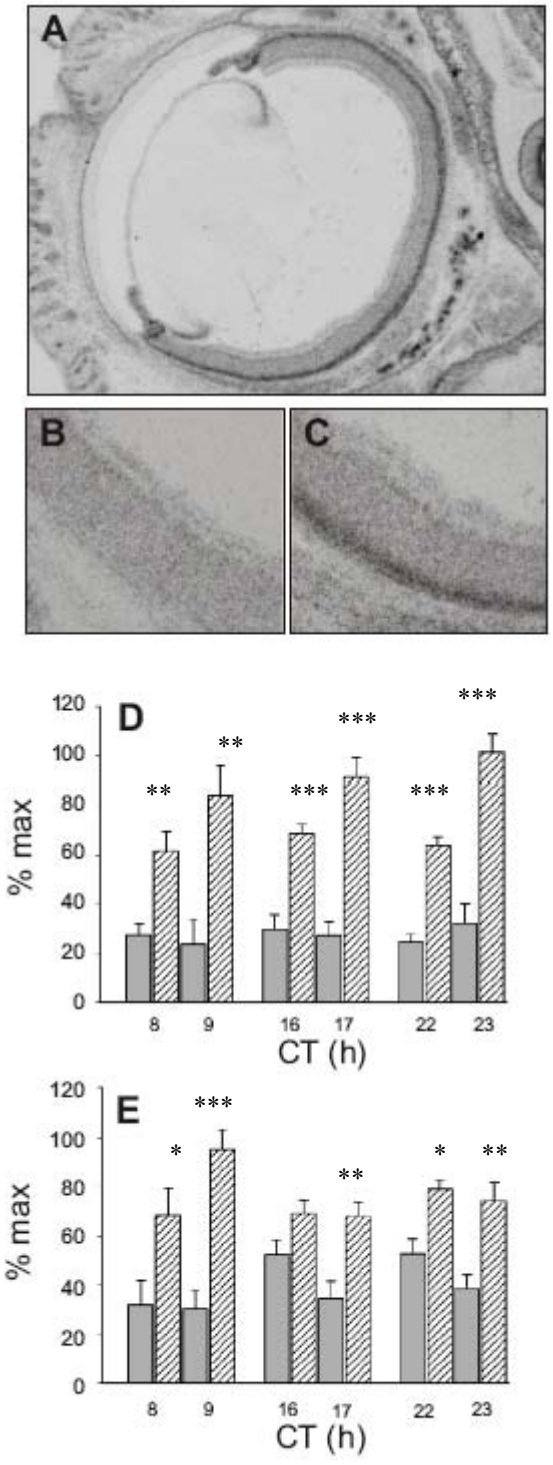
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Figure 5

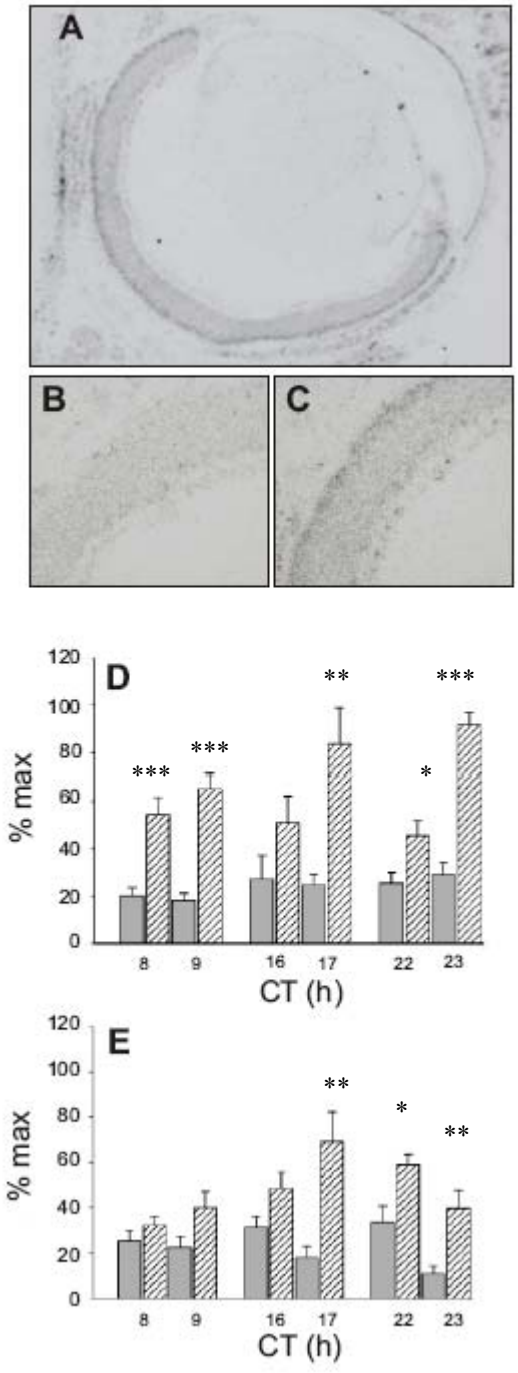

Table 1

A

time	CT7				CT15				CT21			
day	**+30 min**	**+1 h**	**+2 h**	**+4 h**	**+30 min**	**+1 h**	**+2 h**	**+4 h**	**+30 min**	**+1 h**	**+2 h**	**+4 h**
P1	10±2	12±2	7 ± 1	0	11±2	11±1	7 ± 1	1 ± 0	9 ± 1	7 ± 1	8 ± 2	1 ± 0
P3	3 ± 1	7 ± 4	5 ± 2	0	9 ± 3	8 ± 3	5 ± 1	0	7 ± 1	8 ± 1	5 ± 2	0
P5	6 ± 1	7 ± 1	4 ± 0	1 ± 1	5 ± 2	9 ± 2	3 ± 1	1 ± 2	5 ± 1	4 ± 1	1 ± 1	0

B

time	CT7			CT15			CT21		
P10	**+30 min**	**+1 h**	**+2 h**	**+30 min**	**+1 h**	**+2 h**	**+30 min**	**+1 h**	**+2 h**
pulse	21±5	14±1	17±1	23±2	16±3	19±3	25±7	21±5	14±1
control	1 ± 1	1 ± 1	2 ± 1	2 ± 1	1 ± 1	2 ± 1	3 ± 0	2 ± 1	2 ± 1

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Research Report

Ontogenesis of photoperiodic entrainment of the molecular core clockwork in the rat suprachiasmatic nucleus

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Abstract

The molecular mechanism underlying a generation of circadian rhythmicity within the suprachiasmatic nucleus (SCN) is based on interactive negative and positive feedback loops that drive the rhythmic transcription of clock genes and translation of their protein products. In adults, the molecular mechanism is affected by seasonal changes in day length, i.e., photoperiod. The photoperiod modulates phase, waveform, and amplitude of the rhythmic clock genes expression as well as the phase relationship between their profiles. To ascertain when and how the photoperiod affects the circadian core clock mechanism during ontogenesis, the rhythmic expression of clock genes, namely of *Per1*, *Per2*, *Cry1* and *Bmal1* was determined in 3-, 10- and 20-day-old rat pups maintained under either a long photoperiod with 16 h of light and 8 h of darkness per day (LD 16:8) or under a short, LD 8:16 photoperiod. The daily profiles in the level of clock genes mRNA were studied in constant darkness. The photoperiod affected the profile of *Per1* and *Per2* mRNA in 20- and 10- but not yet in 3-day-old pups. Expression of *Cry1* was affected only in 20-day-old pups, whereas expression of *Bmal1* was not yet affected even in 20-day-old rats. The results demonstrate no effect of the photoperiod on 3-day-old pups, only partial entrainment of the molecular core clockwork in 10-day-old pups and a more mature, though not yet fully complete, entrainment in 20-day-old pups as compared with adult animals. The developmental interval when the photoperiod begins to entrain the core clock mechanism completely might thus occur around the time of weaning.

Theme: Systems neuroscience
Topic: Regulatory systems

Keywords: Suprachiasmatic nucleus; Ontogenesis; Photoperiod; Clock gene; Rat

1. Introduction

All mammals exhibit daily rhythms at behavioral, physiological, hormonal, biochemical and molecular levels. The rhythms persist even in a nonperiodic environment with a period close, but not equal to, 24 h [21]. The circadian rhythms are controlled by a generator of the rhythmicity, i.e., by a clock located in two suprachiasmatic nuclei (SCN) of the hypothalamus [16]. The interactive molecular feedback loops between the expression of canonical clock genes, namely *Per1,2*, *Cry1,2*, *Bmal1*, *Clock* and *casein kinase 1ε*, and their protein products PER1,2, CRY1,2, BMAL1, CLOCK and CKIε are responsible for the SCN rhythmicity (for review see [10] and [25]. BMAL1 and CLOCK heterodimers positively activate rhythmic expression of *Per* and *Cry* genes through E-boxes on their promoters. PER and CRY proteins form heterodimers that translocate from cytoplasm to the nucleus. Beside the dimerization, the phosphorylation state of the PER monomer by CKIε also regulates its stability and dynamic of the translocation. In the nucleus, PER and CRY heterodimers inhibit the CLOCK and BMAL1 mediated transcription and chromatin remodeling may be involved [6]. Rhythmic transcription of *Bmal1* is negatively affected by REV-

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ERBα, a protein product of gene *Rev-erbα* gene regulated by CLOCK and BMAL1 heterodimer [22].

Under real-life conditions, daylight entrains the endogenous rhythmicity to the period of solar day, i.e., 24 h [21]. The entrainment proceeds through resetting the molecular core clockwork by a yet unknown mechanism. It likely starts by the instantaneous induction of transcription of the light-sensitive clock genes *Per1* and *Per2* after light intrudes into subjective night, i.e., the light sensitive zone of the circadian cycle [1,28,31,44]. The light-induced transcription is mediated through a locus on a *Per1* promoter, the CRE element, independently of the E-box stimulation [41].

In temperate zones, the duration of daylight, i.e., the photoperiod, changes with the seasons. At latitude 50°N, the duration of daylight in summer is double that in winter. Such profound changes in the photoperiod affect mammalian behavior and physiology. In rodents, the circadian clock is able to recognize the photoperiod and entrains the circadian rhythmicity accordingly to enable anticipation of the forthcoming season [34,39,43]. The molecular mechanism through which the photoperiod modulates the interactive feedback loops undergoes an intensive investigation [8,13–15,18,33,37,38,40]. In all rodents studied so far, the duration of high *Per1* and *Per2* expression and PER1 and PER2 immunoreactivity (-ir) are related to the duration of daylight, i.e., it is longer on long summer days and shorter on short winter days [14,15,18,33,37,38,40]. In the rat, in addition to changes of *Per1* and *Per2* expression profiles, the elevated *Bmal1* expression oscillating in the antiphase to *Per* genes expression persists for a longer time on short than on long days [38]. Moreover, *Cry1* expression is phased advanced on long days as compared with that on short days without any change in its duration [38]. Such changes in molecular inter-phasing might consequently affect the interval when PER and CRY proteins are available at the same time in the cytoplasm to form complexes that translocate to the nucleus and inhibit the BMAL1 and CLOCK mediated transcription. However, other mechanisms may also contribute to decoding daylight duration by the molecular clock [3,7], and the complex exact mechanism of photoperiodic entrainment still remains to be found.

Morphologically, the SCN develops through more phases [16]. In the rat, it is formed from the embryonic day (E)14 through E17 as a component of the periventricular cell groups. Synaptogenesis progresses slowly at the late prenatal and early postnatal periods and increases significantly from the postnatal day (P)4 to P10. The retinohypothalamic tract conveying information about light from the retina to the SCN forms near the time of birth, is clearly present at P4 and approaches the adult state at P10 [17].

During the embryonic stage, intrinsic rhythmicity of the SCN is already present as reflected by the day–night difference in metabolic activity monitored by the 2-deoxyglucose uptake from E19 through E21 [23], by the day–night difference in arginine vasopressin mRNA levels at E21 [24] and firing rate of the SCN neurons at E22 [30]. Data on the beginning of the rhythmic oscillation of the clock gene expression in the rat SCN are contradictory. Daily rhythms in *Per1* and *Per2* mRNA have been reported on the rat embryos at E20 [19,20]. In another study, no significant rhythms in *Per1*, *Per2*, *Cry1*, *Bmal1* and *Clock* mRNA, and moreover, no PER1, PER2 and CRY1-ir have been detected in the rat SCN at E19 [32] and E20 (Kováčiková et al., in preparation). The amplitude of the rhythmic oscillations of *Per1*, *Per2*, *Cry1* and *Bma1l* mRNA increased progressively with age from P3 to P10 [32].

The circadian clock starts to be sensitive to light soon after birth. Light induces expression of immediate-early gene c-fos and its protein cFos already in 1-day and 3-day rat pups, respectively [2,12]. Thus, light information reaches the SCN cells well before eyelid opening. However, it is likely that maternal entrainment dominates over that of the photic during the first days after birth [5]. The ability of the SCN to recognize the photoperiod may develop gradually only during postnatal ontogenesis. In the rat pups, the circadian rhythm in light-induced cFos-ir within the SCN was not affected by the photoperiod at P3 but was partially modulated at P10 while the circadian rhythm in spontaneous cFos-ir was not yet affected by the photoperiod even at P10 [2]. The aim of the present study was to determine when and how during the early postnatal ontogenesis the photoperiod starts to modulate the molecular core clock mechanism in the developing SCN and to compare the effect of the photoperiod with that in adult rats.

2. Results

Expression of *Per1*, *Per2*, *Cry1* and *Bmal1* mRNAs at P3, P10 and P20 maintained under LD16:8 at 1400, i.e., during the daytime, and at 0400, i.e., at the end of the night, is shown in Fig. 1.

To compare the effect of the long and short photoperiod on daily profiles of individual clock gene expression at P3, P10 and P20, the data were expressed as percent of the maximum daily values (Fig. 2).

For expression of *Per1* mRNA (Figs. 2A, E, I), the two-way ANOVA revealed a significant effect of time at P3, P10 as well as at P20 ($F = 15.2$, 47.4 and 67.5, respectively, $P < 0.01$). Although no significant effect of the photoperiod was revealed at any of these ages, at P10 (Fig. 2E) and P20 (Fig. 2I), but not at P3 (Fig. 2A), the significant interaction effect ($F = 4.3$ and $F = 3.4$, respectively, $P < 0.01$) suggested differences between the profile under the long and that under the short photoperiod. Indeed at P10, the morning rise under LD16:8 started already after 0400 and was highly significant at 0600 (0400 vs. 0600, $P < 0.01$), whereas under LD8:16, the rise started only after 0600 and was significant at 0800 (0400 vs. 0800, $P < 0.01$). Moreover, at 0600 *Per1* mRNA was significantly higher under the long than under

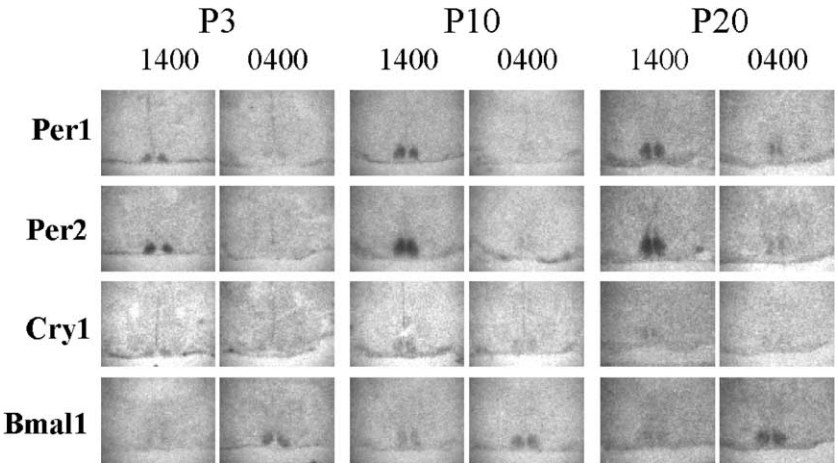

Fig. 1. Representative autoradiographs of coronal brain sections of 3- (P3), 10- (P10) and 20-day-old (P20) rats maintained under the long photoperiod LD 16:8 and sampled at external time (ExT) 1400, i.e., during the daytime, and at ExT 0400, i.e., during the end of the night time. For experimental design, see Experimental procedure. Expression of *Per1*, *Per2*, *Cry1* and *Bmal1* genes in the SCN was determined by in situ hybridization using cRNA probes.

the short photoperiod ($P < 0.01$). The decline of the *Per1* expression occurred at about the same time under the long as under the short photoperiod (1600 vs. 2000, $P < 0.01$). Therefore, at P10 the nighttime duration of low *Per1* expression lasted by about 2 h longer under the short than under the long photoperiod. At P20, the morning rise under LD16:8 started already after 0200 and was significant at 0400 (0200 vs. 0400, $P < 0.01$), whereas under LD8:16, the rise started only after 0400 and was significant at 0600 (0400 vs. 0600, $P < 0.01$). The decline of the *Per1* expression occurred at about the same time under the long as under the short photoperiod (2000 vs. 2200, $P < 0.01$). Consequently, at P20 the nighttime duration of low *Per1* expression lasted also by about 2 h longer under the short than under the long photoperiod. The difference between the *Per1* mRNA profile under long days and that under short days at P10 and P20 might be probably due to a difference in the photoperiod.

For expression of *Per2* mRNA (Figs. 2B, F, J), the two-way ANOVA revealed a significant effect of time at P3,

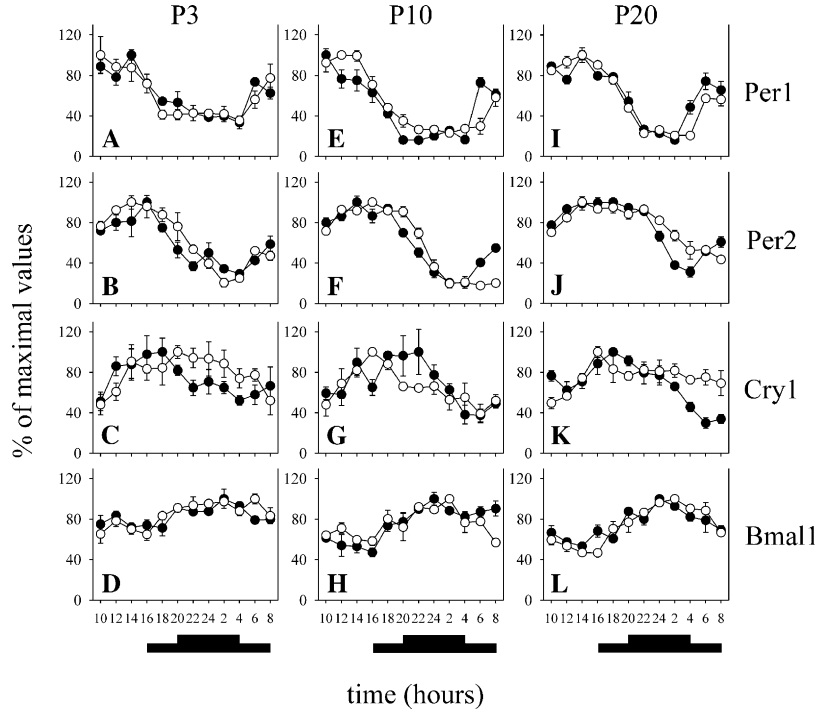

Fig. 2. Daily profiles of expression of clock genes *Per1* (A, E, I), *Per2* (B, F, J), *Cry1* (C, G, K) and *Bmal1* (D, H, L) under LD 16:8 (dark dots) and under LD 8:16 (open dots) in the SCN of early postnatal rats. Animals were sacrificed at P3 (A–D), P10 (E–H) or P20 (I–L). Time is expressed in external time (ExT) where ExT 24 (0) h is assigned to the middle of the dark period. Data are expressed as a percentage of the maximum value. Each point represents the mean ± SEM from 4 animals. Solid bars indicate dark period.

P10 as well as at P20 (F = 27.5, 104.1 and 49.0, respectively, $P < 0.01$). At P3 (Fig. 2B), neither the effect of the photoperiod nor the interaction effect was significant and, therefore, expression of *Per2* had the same profile under the long as under the short photoperiod. At P10 (Fig. 2F), though the effect of the photoperiod was not significant, the highly significant interaction effect (F = 7.1, $P < 0.01$) suggested a difference between the profile under the long and that under the short photoperiod. The rise occurred earlier under the long than under the short photoperiod: under LD16:8, it was already significant at 0600 (0400 vs. 0600, $P < 0.05$), whereas under LD8:16 only at 1000 (0400 vs. 1000, $P < 0.01$). Moreover, at 0800 *Per2* mRNA was significantly higher under long than under short days. The decline of the *Per2* expression occurred later under LD8:16 (1800 vs. 2200, $P < 0.01$) than under LD16:8 (1800 vs. 2000, $P < 0.01$). Consequently, at P10 the duration of low *Per2* RNA expression might be about by 2–4 h longer under the short than under the long photoperiod. At P20 (Fig. 2J), both the effect of the photoperiod as well as the interaction effect were significant (F = 3.9, $P < 0.05$ and F = 5.4, $P < 0.01$, respectively). The first significant rise in *Per2* expression already occurred at 0600 (0400 vs. 0600, $P < 0.05$) under the long photoperiod but only at 1000 (0800 vs. 1000, $P < 0.01$) under the short photoperiod. The decline of *Per2* expression occurred at 0200 (2400 vs. 0200, $P < 0.01$) under the long photoperiod but only at 0400 (2400 vs. 0400, $P < 0.01$) under the short one. Consequently, the duration of low *Per2* expression was by about 2 h longer under the short than under the long photoperiod.

For *Cry1* expression (Figs. 2C, G, K), the two-way ANOVA revealed a significant effect of time at P3, P10 as well as at P20 (F = 2.6, 6.7 and 11.5, respectively, $P < 0.01$). The effect of the photoperiod as well as the interaction effect was significant only at P20 (F = 3.8, $P < 0.05$ and F = 4.1, $P < 0.01$, respectively) but not at P3 and P10. Therefore, at P3 (Fig. 2C) as well as at P10 (Fig. 2G), the profile of Cry1 mRNA expression under the long photoperiod did not differ significantly from that under the short photoperiod. At P20 (Fig. 2K), the first significant rise in *Cry1* expression occurred at 1000 (0800 vs. 1000, $P < 0.05$) under the long photoperiod but only at 1600 (1000 vs. 1600, $P < 0.05$) under the short one. The decline occurred at 0600 (2400 vs. 0600, $P < 0.01$) under the long and at 1000 (2400 vs. 1000, $P < 0.05$) under the short photoperiod. Therefore, the rise as well as the decline in *Cry1* expression occurred by 4–6 h earlier under the long than under the short photoperiod.

For *Bmal1* expression (Figs. 2D, H, L), the two-way ANOVA revealed a significant effect of time at P3, P10 as well as at P20 (F = 6.3, 12.3 and 16.7, respectively, $P < 0.01$). At P3 (Fig. 2D) as well as at P20 (Fig. 2L), neither the effect of the photoperiod nor the interaction effect was significant, and therefore, no changes of the profiles due to the photoperiod might be considered. At P10 (Fig. 2H), the interaction effect (F = 2.7, $P < 0.05$) but not the effect of photoperiod was significant. The rise of the *Bmal1* expression occurred at about the same time under both photoperiods, i.e., at 2200 (1600 vs. 2200, $P < 0.01$), however, a decline was indicated at 0800 (0200 vs. 0800, $P < 0.01$) under LD8:16 but only at 1000 under LD16:8 (0200 vs. 1000, $P < 0.05$). The decline thus occurred earlier and duration of the elevated *Bmal1* mRNA level appeared to be shorter under the short than under the long photoperiod, in contrast to the photoperiodic adjustment of *Bma1* expression in the adult rat [38].

3. Discussion

The retinohypothalamic tract conveying information about light from retina to the SCN forms near the time of birth [17]. In accord with this fact, light induces the immediate-early gene c-fos and its protein product c-Fos in the rat SCN already at P1 and P3 [2,12]. Although light information reaches the SCN shortly after birth and well before eyelid opening around P15, our data suggest that the photoperiod affected the daily profile of clock genes *Per1* and *Per2* expression only at P10 and P20, but not yet at P3. Expression of *Cry1* was affected by the photoperiod only at P20, but not yet at P3 or at P10, whereas expression of *Bmal1* was not affected by the photoperiod in a manner similar to that in the adult rats [38] even at P20. These findings indicate that at P10 the molecular core clockwork starts to be partially entrained by the photoperiod while at P3 the photoperiod is without any effect. At P20, the photoperiod already affected the expression profiles of 3 out of the 4 studied genes, namely of *Per1*, *Per2* and *Cry1*, but not of *Bmal1*. However, the effect of the photoperiod on the *Per1* mRNA profile at P20 was still smaller than that in adult rats. Whereas in adult rats [38], similarly as in adult Syrian [14] and Siberian [15] hamsters, the morning rise of *Per1* expression occurred by 4 h earlier under LD16:8 than under LD8:16, in 20-day-old rats the rise occurred only by 2 h earlier under LD16:8 than under LD8:16. Therefore, adjustment to the photoperiod proceeded only gradually, and the complete entrainment was not accomplished within the first 20 postnatal days.

In adult rats, the photoperiod affects expression of *Per1*, *Cry1* and *Bmal1* mRNA [38]. Under a long photoperiod, the duration of high *Per1* expression is longer while of high *Bmal1* expression shorter, and the phase of *Cry1* expression is advanced when compared to a short photoperiod. Findings in mice and hamsters indicate that *Per2* expression is affected by the photoperiod as well [33,40]. Thus, both the light sensitive as well as the light insensitive components of the core clockwork appear to be modulated by the photoperiod.

Our results confirm a rhythmic expression of *Per1*, *Per2*, *Cry1* and *Bmal1* genes at P3 reported recently [32] and show further that the expression is not yet affected by the photoperiod. At P10, the photoperiod started to

modulate the molecular clockwork, namely, expression of the light sensitive genes *Per1* and *Per2*: the morning *Per1* and *Per2* mRNA rise occurred by about 2 and 4 h, respectively, earlier under a long than under a short photoperiod. In contrast to *Per1* and *Per2*, expression of the light insensitive gene *Cry1* was affected by the photoperiod only at P20 but not yet at P10: at P20, the *Cry1* mRNA rise and decline under the long photoperiod was phase advanced by 4–6 h relative to that under the short photoperiod. The expression of the light insensitive gene *Bmal1* was not affected by the photoperiod even at P20. The data suggest a dominant role of light sensitive genes *Per1* and *Per2* in the mechanism of photoperiodic entrainment of the SCN core clock mechanism.

In our study, we were not able to differentiate between the direct role of the photoperiod and that of the maternal entrainment which might also contribute to synchronization of the pup's SCN rhythmicity during ontogenesis. Although we cannot exclude the influence of the maternal entrainment, several data support a direct effect of the photoperiod on the pup's SCN. It is likely that the role of the maternal entrainment would rather decline than rise with the postnatal age. Previously, it was shown that a critical period for the maternal entrainment is around P5 [26]. In agreement with this finding, Ohta and colleagues did not find a significant maternal influence on phase shifts of the SCN *Per1* and *Per2* mRNA rhythms in rat pups after P6 [19]. Our results, however, show that the photoperiodic control of the molecular clockwork may start to develop only around P10. Moreover, our previous data demonstrate gradual development of the mechanism which gates the insensitivity of a rat SCN to a light stimulus during postnatal ontogenesis [2]. The gate is an important pre-requisite of photoperiodic entrainment: whereas at P3 a light pulse induces high cFos-ir within the rat SCN throughout the whole circadian cycle, at P10 the response to light is already gated only to the period roughly corresponding to subjective night. Hence, insensitivity to a light stimulus during the subjective day develops around the 10th postnatal day. Similarly as in adult rats, at P10 the interval of insensitivity is shorter under a short than under a long photoperiod. Although at P10 the photoperiod starts to modulate partially the rhythm in light sensitivity, it does not yet affect markedly the light independent rhythm in spontaneous cFos-ir within the dorsomedial SCN [2]. The latter rhythm which may serve as a marker of the SCN endogenous neuronal activity is also modulated by the photoperiod in adult rats [36].

It is tempting to speculate that the inability of the photoperiod to modulate the spontaneous rhythm in c-Fos production at P10 is due to an incomplete effect of the photoperiod on light insensitive components of the core clockwork at this age, as is demonstrated in the present study. The rhythm in the light sensitivity resides within the ventrolateral part of the rat SCN [9,11,27], whereas the spontaneous rhythms in the clock and immediate-early genes expression reside mostly within the dorsomedial part of the rat SCN [9,35,36]. The light recipient ventrolateral part appears to be modulated earlier by the photoperiod than the spontaneously rhythmic dorsomedial part [2]. The incomplete modulation of the clock gene expression by the photoperiod at P10 suggests only a partially functional photoperiodic entrainment. Indeed, at around this age, an overt pineal rhythm of arylalkylamine *N*-acetyltransferase is only partially adjusted to the photoperiod [42].

In conclusion, our data demonstrate that the molecular core clockwork within the rat SCN begins to be modulated by the photoperiod in 10-day-old pups. At this age, the modulation is still partial, matures only gradually and is not yet fully completed in 20-day-old rats. Among the core clock genes, *Per1* and *Per2* genes are the first whose rhythmic expression begins to be adjusted to the photoperiod, suggesting their leading role in the mechanism of photoperiodic entrainment.

4. Experimental procedure

4.1. Animals

Female Wistar rats (Bio Test, Konárovice, Czech Republic) were housed in a temperature of 23 ± 2 °C with free access to food and water. The animals were maintained for at least 4 weeks under either a long photoperiod with 16 h of light and 8 h of darkness per day (LD16:8) with the lights on from 0400 to 2000 or from 2000 to 1200, or under a short photoperiod with 8 h of light and 16 h of darkness per day (LD 8:16), with the lights on from 0800 to 1600 or from 2400 to 0800. For comparison between the experiments, the time scales were corrected so that for each experiment 2400 (0) h was assigned to the middle of the dark period, i.e., external time (ExT) was used [4]. Light was provided by overhead 40-W fluorescent tubes, and illumination was about 200 lx, depending on cage position in the animal room. The female rats were mated on the day of estrus, and day of delivery was designated as postnatal day 0. At P3, P10 and P20, the mothers with their pups were released into constant darkness, i.e., the morning light was not turned on, and thereafter, four pups were sampled every 2 h throughout the whole circadian cycle. For both photoperiods, the sampling was completed within 1 day. The pups were killed by rapid decapitation, their brains were removed, immediately frozen on dry ice and stored at −80 °C. Each brain was sectioned into five series of 12-μm thick slices in alternating order throughout the whole rostrocaudal extent of the SCN and processed for in situ hybridization to determine levels of *Per1*, *Per2*, *Cry1* and *Bmal1* gene mRNAs. All experiments were conducted under license No. A5228-01 with the (US) National Institutes of Health and in accordance with the Animal Protection Law of the Czech Republic (license No. 1020/491/A/00).

4.2. In situ hybridization

The cDNA fragments of rat *rPer1* (980 bp, position 736–1717 nt, GenBank No. AB002108), rat *rPer2* (1512 bp, position 369–1881, GenBank No. NM031678) rat *rBmal1* (841 bp, position 257–1098 nt, GenBank No. AB012600), and mouse *mCry1* (719 bp, position 1074–1793 nt, GenBank No. NM007771) were used as templates for in vitro transcription of cRNA probes. The *rPer1* and *mCry1* fragment-containing vectors were generously donated by Professor H. Okamura (Kobe University School of Medicine), and *rBmal1* was cloned in our laboratory. Briefly, cDNA fragments of *Bmal1* were obtained from the rat hypothalamic mRNA. After reverse transcription, cDNA was amplified by standard PCR and ligated into vector pGem-T-Easy and pBluescript, respectively. The cloned inserts were sequenced to verify the amplified products.

The probes were labeled using ^{35}S-UTP, and the in situ hybridization was performed as described previously [29]. Briefly, the sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 40 min and transferred through 0.2 N HCl for 20 min, 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min and dehydrated in 70% and 96% ethanol for 5 min. The sections were then incubated with a hybridization buffer (50% formamide, 10% dextran sulfate, 5× SSPE [0.9 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA], 2× Denhardt's solution, 500 μg/ml yeast tRNA, 500 μg/ml salmon sperm DNA, 0.1% sodium dodecyl sulfate and 5% NaPPi and 50 mM dithiothreitol in 0.1% DEPC H_2O) containing the purified radioactive labeled probes and hybridized for 20 h at 61 °C (*Per2*), 60 °C (*Per1*), 58 °C (*Bmal1*) and 55 °C (*Cry1*). Following a post-hybridization wash, the sections were dehydrated in ethanol and air dried. Finally, the slides were exposed to Hyperfilm-beta-max (Amersham) for 10–14 days and developed using the developer Fomatol LQN and fixer FOMAFIX (FOMA, Czech Republic).

As a control, in situ hybridization was performed in parallel with sense probes (apart from r*Per2*) on sections containing the SCN. All sections hybridized with the same probe were processed simultaneously under identical conditions.

Autoradiographs of sections were analyzed using an image analysis system (ImagePro, Olympus) to detect the relative optical density of the specific hybridization signal. In each animal, the mRNA level was quantified bilaterally at the mid-caudal SCN section containing the strongest hybridization signal. Each measurement was corrected for a nonspecific background by subtracting the optical density values from neighboring areas expected to be free of the specific signal, thus serving as their own internal standard. At the end, slides were counterstained with cresyl violet to check the presence and position of the SCN in each section. In no case did in situ hybridization yield any specific signal with a sense probe.

Four animals were used for each time point; occasionally one sample was missed. Data were expressed as means of optical density (OD) ± SEM; OD for each animal was calculated as a mean of the left and right SCN values.

4.3. Statistical analysis

To compare the effect of the photoperiod in the same age group, data were transformed to the percent of the maximal value and analyzed by two-way ANOVA for time and photoperiod differences and the subsequent Student–Newman–Keuls multiple range test.

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THE RAT CIRCADIAN CLOCKWORK AND ITS PHOTOPERIODIC ENTRAINMENT DURING DEVELOPMENT

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The mammalian circadian pacemaker is located in the suprachiasmatic nucleus (SCN), which is composed of dorsomedial (dm) and ventrolateral (vl) regions. The molecular clockwork responsible for the SCN rhythmicity consists of clock genes and their transcriptional-translational feedback loops. The rat SCN rhythmicity and clockwork are affected by the photoperiod. The aim of this study was to elucidate development of the rat SCN rhythmicity, namely of the rhythmicity of the dm- and vl-SCN and of expression of clock genes and to ascertain when the photoperiod starts to affect the SCN rhythmicity. Rhythmicity of the dm-SCN, measured as the rhythm in spontaneous c-FOS production, developed earlier than that of the vl-SCN, which was measured as the rhythm in c-FOS photoinduction. However, photoperiodic affection of the rhythmicity occurred earlier in the vl-SCN than in the dm-SCN. From the 4 clock genes (*Per1*, *Per2*, *Cry1* and *Bmal1*) studied, the expression of *Bmal1* and *Per1* was rhythmic already in 1-day-old rats; at this age, the *Per2* mRNA rhythm just started to form and no rhythm in *Cry1* expression was detected. After the second postnatal day, all 4 genes were expressed in a rhythmic manner. Thereafter, the rhythms matured gradually via increasing amplitude. *Per1* and *Per2* mRNA rhythms started to be affected by the photoperiod at the 10th postnatal day. The data suggest that the rhythms in clock genes expression in the rat SCN develop mostly postnatally. The molecular clockwork may start to be photoperiod-dependent around the 10th postnatal day.

Keywords Suprachiasmatic nucleus, Clock genes, Photoperiodic entrainment, Development, Circadian rhythm

INTRODUCTION

The rat, like all other mammals, exhibits circadian rhythms from the molecular to the behavioral level. The rhythms are controlled by a clock located in the two suprachiasmatic nuclei (SCN) of the hypothalamus

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(Klein et al., 1991). The SCN is composed of a ventrolateral (vl) part, also called a core, and of a dorsomedial (dm) part, called a shell (Moore et al., 2002). The SCN, itself, exhibits rhythms, *e.g.*, in spontaneous (Sumová et al., 1998) as well as light-induced (Schwartz et al., 1995) expression of immediate early genes, namely *c-fos* and in the production of arginine vasopressin (AVP) (Klein et al., 1991). Some of the rhythms are specific for the vl-SCN, *e.g.*, the rhythm in *c-fos* photoinduction (Schwartz et al., 1995), whereas others may be specific for the dm-SCN, *e.g.*, rhythms in spontaneous *c-fos* and *AVP* gene expression (Sumová et al., 1998; Jáč et al., 2000). The SCN rhythmicity is due to the SCN molecular clockwork (reviewed in Fu and Lee, 2003). The 9 mammalian clock genes cloned so far, namely 3 period genes (*Per1*, *-2*, and *-3*), 2 cryptochrome genes (*Cry1* and *-2*), *Clock*, *Bmal1*, *Rev-erbα*, and casein kinase 1ε (*CK1ε*), are thought to be involved in the clockwork by forming interacting transcriptional-translational feedback loops.

Overt circadian rhythms, namely the rhythm in the pineal melatonin production (Klein et al., 1991) as well as the SCN rhythmicity, itself, *e.g.*, the rhythm in *c-fos* photoinduction (Sumová et al., 1995) or in c-FOS (Sumová et al., 2000) and AVP (Jáč et al., 2000) spontaneous expression, are modulated by day-length, *i.e.*, by the photoperiod. The rat SCN clockwork is affected by the photoperiod as well (Sumová et al., 2003). The rat SCN is formed during the fetal stage. Synaptogenesis in the SCN progresses slowly in the late and early postnatal periods and then markedly increases from postnatal (P4) to postnatal day 10 (P10) (Klein et al., 1991). Intrinsic SCN rhythmicity may be already present in the fetal rat SCN.

The aim of the present work was to elucidate development of the SCN rhythmicity and of its photoperiodic entrainment. The following questions were addressed: 1) Does the rhythmicity of the dm-SCN and of the vl-SCN develop in parallel, and when does it start to be affected by the photoperiod (Bendová et al., 2004)? 2) When and how does the rhythmicity of clock gene expression develop (Sládek et al., 2004; Kováčiková et al., in press)? 3) At what developmental stage does the molecular clockwork respond to the photoperiod (Kováčiková et al., 2005)?

MATERIALS AND METHODS

Animals

Female Wistar rats were maintained under a light-dark (LD) cycle with 12 h of light and 12 h of darkness per day (LD 12 : 12) or under LD 16 : 8 or LD 8 : 16. The day of delivery was designated the postnatal day 0 (P0). On average, 4 pups per each time point were decapitated every 2 h. All experiments were conducted under License No. A5228-01 with the U.S.

National Institutes of Health and in accordance with the Animal Protection Law of the Czech Republic (License No. 1020/491/A/00) and the standards of the Journal (Touitou et al., 2004).

Rhythmicity of the dm- and vl-SCN

Daily profiles of the spontaneous c-FOS immunoreactivity (ir) and of light-induced c-FOS-ir were studied as models of a dm- and a vl- SCN rhythm, respectively. At P3 and P10, in order to avoid the masking effect of the light, either the evening light onset was advanced by 4 h when the afternoon and early night-time points were followed, or the morning light was not turned on, when the late night and daytime points were studied. To analyze the spontaneous c-FOS-ir, pups were killed in darkness. To determine the photoinduced c-FOS-ir, pups were exposed to a 30 min light pulse with an intensity of 200 to 300 lx, then returned to darkness and killed 30 min later. Brains were immediately removed and processed for immunocytochemical demonstration of c-FOS in the SCN as described elsewhere (Bendová et al., 2004).

Rhythms of Clock Gene Expression

At P1, P2, P3, and P10, mothers with their pups were released into constant darkness (DD), *i.e.*, the morning light was not turned on. Pups were sampled throughout the whole circadian cycle and their brains were processed for *in situ* hybridization to determine levels of *Per1*, *Per2*, *Cry1* and *Bmal1* gene mRNAs, as described elsewhere (Sládek et al., 2004).

RESULTS

Development of the dm- and vl-Rhythmicity and its Entrainment by the Photoperiod

In 3- and 10-day-old rats, the dm-SCN rhythm in spontaneous c-FOS-ir was already well expressed, but a response to a photoperiod similar to that in adult rats was not yet been developed (Bendová et al., 2004). The vl-SCN gate for insensitivity of c-FOS production to light at certain times was detected in 10-day-old, but not yet in 3-day-old rats; in the latter, light exposure at any day time induced high c-FOS-ir. In 10-day-old pups, as in adult rats, the gate was shorter under LD 8 : 16 than under LD 16 : 8, but the difference between the short and the long photoperiod did not yet reach that of adult animals (Bendová et al., 2004).

Development of Rhythms in Clock Genes Expression and of Their Photoperiodic Control

At P1, the *Per1* expression was already rhythmic, and after P2, the rhythm was well pronounced (Figure 1A) (Kováčiková et al., in press). The rhythm amplitude continued to increase between P3 and P10 (Sládek et al., 2004). The photoperiod did not yet affect the *Per1* mRNA profile of 3-day-old rats (Figure 2A) (Kováčiková et al., 2005). However, at P10 the morning *Per1* mRNA rise occurred already by about 2 h later under LD 8 : 16 than under LD 16 : 8. The *Per2* mRNA (Figure 1B) rhythm just began to form at P1 (Kováčiková et al., in press). After P2, however, the profile was highly rhythmic (Kováčiková et al., in press), and the rhythm amplitude continued to increase between P3 and P10 (Sládek et al., 2004). The photoperiod did not affect significantly the rhythmic *Per2* expression at P3, but at P10 the duration of low *Per2* expression lasted 2 to 4 h longer under the short

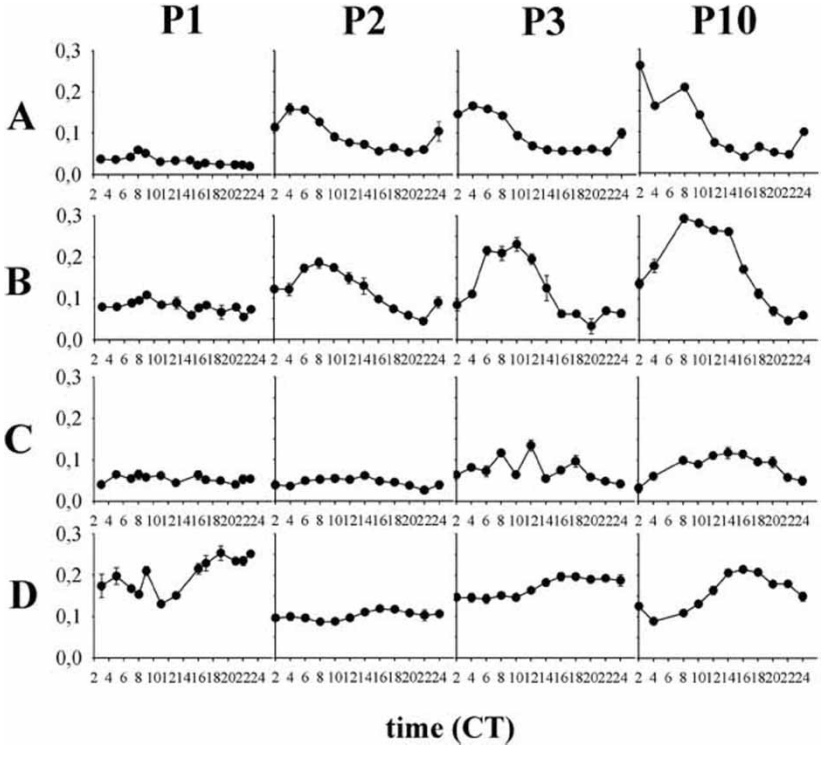

FIGURE 1 Daily profiles of *Per1* (*A*), *Per2* (*B*), *Cry1* (*C*), and *Bmal1* (*D*) mRNA levels at P1, P2, P3, and P10 in the SCN of rats maintained under LD 12 : 12 and released into darkness at the time of the expected light-dark transition. The mRNA levels are expressed as relative optical density means ± S.E.M. from 4 animals. The data are taken from Sládek and colleagues (2004) and Kováčiková and coworkers (in press).

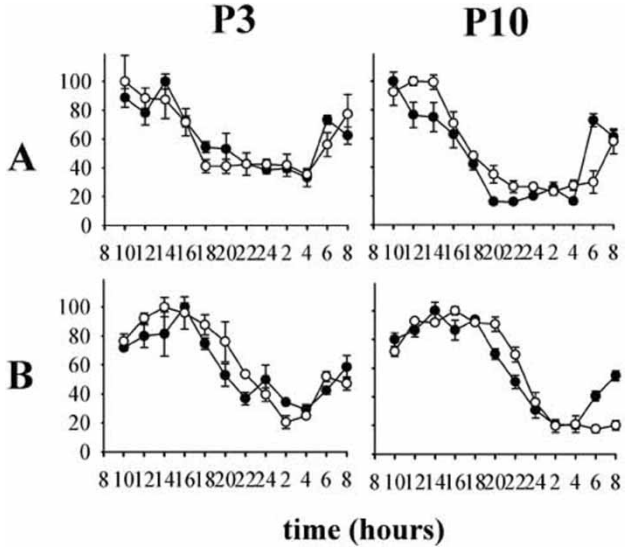

FIGURE 2 Daily profiles of *Per1* (*A*) and *Per2* (*B*) mRNA levels at P3 and P10 in the SCN of rats entrained to LD 16:8 with light on from 04:00 until 20:00 h (*dark circles*) or LD 8:16 with light on from 08:00 until 16:00 h (*open circles*) and released into darkness at the time of expected light-dark transition. The mRNA levels are expressed as percent of maximal relative optical density means ± S.E.M. from 4 animals. The data are taken from Kováčiková et al. (2005).

than under the long photoperiod (Figure 2B) (Kováčiková et al., 2005). *Cry1* mRNA rhythm (Figure 1C) started to form at P2, but there was no rhythm at P1 (Kováčiková et al., in press). Gradually, the rhythm amplitude increased (Sládek et al., 2004). At P3 as well as at P10, the profile of *Cry1* mRNA expression under LD 16 : 8 did not differ significantly from that under LD 8 : 16 (Kováčiková et al., in press). *Bmal1* mRNA (Figure 1D) exhibited a highly significant rhythm already at P1 (Kováčiková et al., in press). Initially, the phase of the rhythm was opposite to that of *Per1* expression as is the case in the later postnatal ages; however, its shape differed from that at later ages. Neither at P3 nor at P10 was the rhythmic *Bmal1* expression affected by the photoperiod in a manner similar to that in adult rats (Kováčiková et al., in press).

DISCUSSION

The data suggest that the circadian rhythmicity of the vl-SCN develops later than that of the dm-SCN. We make this suggestion with caution since only the rhythm in c-FOS photoinduction was analyzed in the vl-SCN.

The molecular clockwork develops only gradually. At P1, the rhythm in *Bmal1* and a slight rhythm in *Per1* expression were already present; whereas, the rhythm in *Per2* expression just started and no rhythm in *Cry1* expression was observed. After P2, all studied genes were expressed

in a rhythmic manner and matured mostly via increasing the amplitude. Altogether, the data suggest that rhythms in clock genes expression develop mostly postnatally and not necessarily in parallel.

The photoperiodic control of the SCN circadian rhythmicity may also develop only gradually. At P10, the rhythm of c-FOS photoinduction in the vl-SCN, *i.e.*, the rhythm in photosensitivity, but not yet the spontaneous rhythm in c-FOS production in the dm-SCN, started to be affected by the previously experienced photoperiod. From the studied clock genes, the rhythmic expression of *Per1* and *Per2*, but not that of *Cry1* and *Bmal1*, also started to be affected by the photoperiod at P10, though not yet to the same extent as in adult rats (Sumová et al., 2003). The data suggest a dominant role of light-sensitive *Per1* and *Per2* genes in the SCN photoperiodic entrainment (Hastings, 2001) during development. It was somewhat surprising that the SCN rhythmicity was not affected by the photoperiod at P3, *i.e.*, shortly after birth, as transduction of photoperiodic information from mother to fetus has been demonstrated in several species, *e.g.*, in the Djungarian hamster (Stetson et al., 1986). Thus, the photoperiodic information is not transduced via an undeveloped fetal SCN, or the SCN memory on the fetal photoperiod is only short-term. The postnatal photoperiod may start to affect the circadian system around P10.

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Ontogenetický vývoj cirkadiánního systému savců

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SOUHRN

V organizmu probíhá množství rytmických dějů. Pokud se tyto rytmické děje odehrávají i v prostředí bez periodických podnětů (např. ve stálé tmě) s periodou ± 24 hodin, označujeme je jako rytmy cirkadiánní. Jedná se např. o rytmus ve spánku a bdění, výlevech hormonů, rytmické změny v tělesné teplotě atd. Za vznik těchto rytmů a jejich synchronizaci s 24hodinovým dnem je zodpovědný tzv. cirkadiánní systém, který je u savců složen z centrálních hodin v suprachiasmatických jádrech hypothalamu (SCN), vstupních a výstupních drah umožňujících jejich komunikaci s vnějším a vnitřním prostředím a z periferních hodin v různých tkáních těla. Podkladem pro vznik cirkadiánní rytmicity je systém zpětnovazebných transkripčně-translačních smyček složených z tzv. hodinových genů a jimi kódovaných proteinů, které pozitivně či negativně ovlivňují vlastní transkripci. Výzkumy provedené na mláďatech a embryích potkanů ukazují, že jednotlivé složky cirkadiánního systému se vyvíjejí postupně během prenatální a postnatální ontogeneze. Pro vývoj vlastního molekulárního mechanizmu v SCN je důležité období okolo narození. Mechanizmus umožňující světelnou synchronizaci cirkadiánního systému se u potkanů vyvíjí až postnatálně. V době kdy ještě není plně vyvinut, hraje pro mláďata důležitou synchronizační roli matka.
Klíčová slova: cirkadiánní rytmy, suprachiasmatická jádra, vývoj, synchronizace

SUMMARY

Many behavioral, physiological and molecular processes exhibit diurnal rhythms. Endogenous rhythms with period close to 24 hours are called circa-dian rhythms. Light entrains circadian rhythms to a 24 period of solar day. Circadian system consists of pacemaker, which is in mammals located in the suprachiasmatic nuclei of hypothalamus (SCN), its input and output pathways and peripheral clocks in numerous tissues. The generation of circadian rhytmicity is based on interactive transcription-translational feedback loops in SCN. These feedback loops consist of so called clock genes and their protein products which positively or negatively regulate their own transcription. Studies in rodent embryos and neonates demonstrate that circadian system matures gradually during prenatal and postnatal period. Mechanism of light entrainment of the circadian system develops postnatally. During early postnatal period, the developing circadian system is synchronized mainly by maternal cues.
Key words: circadian rhythms, suprachiasmatic nuclei, development, entrainment

1. ÚVOD

Biologické hodiny existují v určité podobě prakticky u všech organizmů. Rytmy řízené biologickými hodinami se vyskytují na různých úrovních počínaje úrovní celého těla (jako je rytmus v pohybové aktivitě, spánku a bdění, příjmu potravy), přes fyziologické funkce (jako např. rytmy v dýchání, srdečním tepu, či změny v tělesné teplotě) až po molekulární a buněčnou úroveň (kde dochází k rytmickému přepisu některých genů a tvorbě proteinů). Základními vlastnostmi biologických rytmů je, že jsou vrozené a endogenní. Nejsou tedy pouhou reakcí na změny vnějších podmínek, ale běží i v prostředí bez periodicky se opakujících podnětů, a to s vlastní vnitřní periodou. Rytmy s periodou výrazně kratší než 24 hodin se nazývají rytmy ultradiánní a patří k nim např. rytmy v dýchání a srdečním tepu. Rytmy s periodou přibližně 24 hodin se nazývají cirkadiánní a jedná se např. o rytmus v pohybové aktivitě, spánku a bdění či tělesné tep-

lotě. A nakonec rytmy s periodou výrazně delší než 24 hodin se nazývají infradiánní; jako příklad lze uvést např. estrální cyklus. Tato práce se blíže zabývá rytmy cikadiánními.

Cirkadiánní rytmy se v evoluci vyvinuly jako mechanizmus, který umožňuje organizmu předvídat cyklické změny prostředí spjaté se střídáním dne a noci. V neperiodickém prostředí je délka periody pro jednotlivé cirkadiánní rytmy druhově specifická a zpravidla se nerovná zcela přesně 24 hodinám. Aby nedocházelo k předbíhání rytmů, jejichž perioda je kratší než 24 hodin, či naopak zpožďování rytmů s periodou delší než 24 hodin, jsou cirkadiánní hodiny pravidelně synchronizovány s 24hodinovým dnem. Při synchronizaci je fáze rytmu posunuta tak, aby byla v souladu s fází rytmu synchronizátoru, tedy s 24hodinovým dnem. Nejdůležitějším synchronizátorem je světlo, a to jak jednotlivé světelné záblesky (tzv. diskrétní synchronizace), tak i délka světlé části dne (tzv. kontinuální synchronizace). Světlo zvečera působí zpoždění rytmů s periodou delší než 24 hodin, zatímco světlo zrána předběhnutí rytmů s periodou kratší než 24 hodin (Daan a Pittendrigh, 1976). Tímto způsobem je udržována perioda rytmů přesně 24 hodin. Kromě světla působí jako synchronizátory také signály nesvětelné povahy např. změny okolní teploty nebo sociální podněty.

V laboratorních podmínkách jsou pokusná zvířata obvykle chována při světelném režimu, kde se pravidelně střídá světlá a tmavá fáze (LD, light-dark). Při zkoumání mechanizmu řídícího cirkadiánní rytmy je však nutno odstranit akutní „maskující" vliv světla a proto bývají pokusná zvířata převedena do konstantní tmy (DD, dark-dark). V DD se střídá tzv. subjektivní noc, tj. období, kdy je možno světelným pulzem vyvolat posun fáze a subjektivní den, kdy světlo fázi cirkadiánních rytmů neovlivňuje.

2. CIRKADIÁNNÍ SYSTÉM SAVCŮ

Původní představy o cirkadiánním systému vycházely z předpokladu, že cirkadiánní rytmy jsou generovány jediným tzv. centrálním oscilátorem – pacemakerem, který je u savců uložen v suprachiasmatických jádrech hypothalamu, u ptáků v epifýze, u hmyzu ve skupině buněk v centrálním nervovém gangliu, atd. Poslední studie však dokládají i u savců výskyt mnoha dalších oscilátorů v periferních orgánech (játra, srdce, plíce, ledviny, kosterní sval atd.) a ukazují, že jde ve skutečnosti o mnohem komplikovanější systém (viz obr. 1). Centrální pacemaker v něm pravděpodobně funguje jako synchronizátor fází jednotlivých periferních oscilátorů.

Centrální pacemaker savců – suprachiasmatická jádra hypothalamu (SCN, suprachiasmatic nuclei) se nachází nad chiasma opticum. Jedná se o dva shluky buněk ležící po stranách třetí mozkové komory. SCN je možno funkčně i morfologicky rozdělit na dvě části: část dorsomediální (dmSCN) a část ventrolaterální (vlSCN). DmSCN je tvořeno zejména neurony produkujícími arginin-vasopressin (AVP) a bývá funkčně spojováno se vznikem endogenní rytmicity. Neurony vlSCN jsou charakteristické produkcí vasoaktivního intestinálního polypeptidu (VIP). Ventrální část SCN těsně přiléhá k chiasma opticum a hraje důležitou roli ve světelné synchronizaci (přehled viz Ibata et al., 1999).

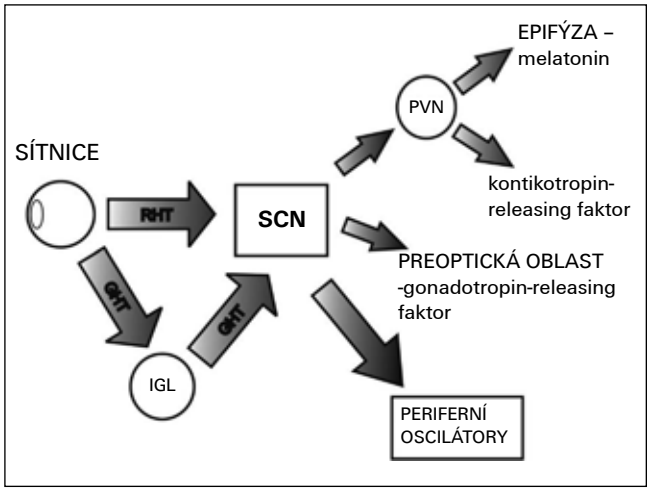
Informace o světle se do SCN dostává ze sítnice retinohypothalamickým traktem (RHT). RHT je tvořen převážně axony určité frakce gangliových buněk sítnice (RGCs, retinal ganglion cells). RGCs exprimují opsinu podobný fotopigment melanopsin, a ačkoliv se nepodílejí na tzv. obrazovém vidění, předávají do SCN informaci o světle (přehled viz Morin a Allen, 2006). Hlavním mediátorem RHT je glutamát. Další nepřímá dráha, tzv. genikulo-hypothalamický trakt (GHT), vede ze sítnice přes intergenikulátní lístek (IGL, intergeniculate leaflet) nucleus geniculatus lateralis thalamu a přivádí do SCN informace o světelných i nesvětelných podnětech. Hlavním mediátorem GHT je neuropeptid Y. SCN přijímají další podněty také serotoninergní dráhou z raphe nuclei a z dalších částí mozku.

Oscilace vznikající v buňkách SCN činností molekulárního mechanizmu (viz kap. 3.) jsou převáděny na rytmus v elektrické aktivitě neuronů a na rytmickou produkci neuropeptidů. Elektrická aktivita neuronů SCN *in vitro* je vyšší přes den a rytmus v elektrické aktivitě je přítomen i v případě že je neuronům v *in vitro* podmínkách zabráněno tvořit synapse. Za těchto okolností vykazuje každá buňka rytmus v elektrické aktivitě s fází odlišnou od okolních buněk a tento její rytmus se s nezměněnou fází obnoví i po dočasném zablokování elektrické aktivity tetrodotoxinem (Welsh et al., 1995; Pennartz et al., 2002). Z výše uvedeného vyplývá, že centrální pacemaker je patrně *in vivo* složen z více nezávislých oscilátorů, které jsou navzájem synchronizovány dosud neznámým mechanizmem.

Kromě elektrické aktivity neuronů se na přenosu rytmů z SCN do dalších částí mozku podílejí i mediátory rytmicky produkované buňkami SCN. Jedná se zejména o AVP, kyselinu γ-amminomáselnou (GABA), glutamát a další. V SCN je možno rozlišit několik subpopulací neuronů, které rytmicky produkují určitý mediátor, popř. jejich kombinaci. Tyto subpopulace hrají důležitou roli při cirkadiánní regulaci výlevu některých hormonů (souhrn viz Kalsbeek et al., 2006).

Výše popsané rytmy na úrovni SCN jsou přenášeny řadou nervových drah do dalších oblastí hypothalamu a také do dalších částí mozku (podrobnější anatomie viz Watts, 1991). Nervovými drahami z SCN je zprostředkována cirkadiánní regulace tvorby některých hormonů, např. melatoninu, kortikosteronu či gonadotropin-releasing faktoru. Melatonin (N-acetyl-5-hydroxytryptamin) je tvořen v epifýze ze serotoninu (5-hydroxytryptamin) činností N-acetyltransferázy (NAT) a hydroxyindol-O-methyltransferázy (HIOMT). Aktivita NAT je nízká přes den a vysoká v noci (Illnerová a Vaněček, 1980). Vysoká hladina melatoninu v plazmě tak působí jako významný signál, který informuje organizmus nejen o denní době, ale i o délce noci a tím o aktuálním ročním období. Mediátorem, který se podílí na aktivaci nočního výlevu melatoninu, je patrně glutamát produkovaný určitou subpopulací neuronů SCN, zatímco ranní pokles hladiny melatoninu řídí subpopulace buněk SCN produkující GABA (Kalsbeek et al., 2006). Do epifýzy se informace z SCN dostávají multisynaptickou drahou z dmSCN přes nucleus paraventricularis hypothalamu, intermediolaterální sloupec

šedé hmoty ve spinální míše a ganglion cervicale superior. Nucleus paraventricularis hypothalamu obsahuje mj. neurony produkující kortikotropin-releasing faktor a drahou z SCN je tak ovlivněna i hladina kortikosteronu v plasmě (Buijs et al., 1998). Nepřímo jsou z SCN ovlivněny i hladiny pohlavních hormonů drahou vedoucí do preoptické oblasti, kde se podílí na regulaci uvolňování gonadotropin-releasing faktoru (van der Beek et al., 1997).



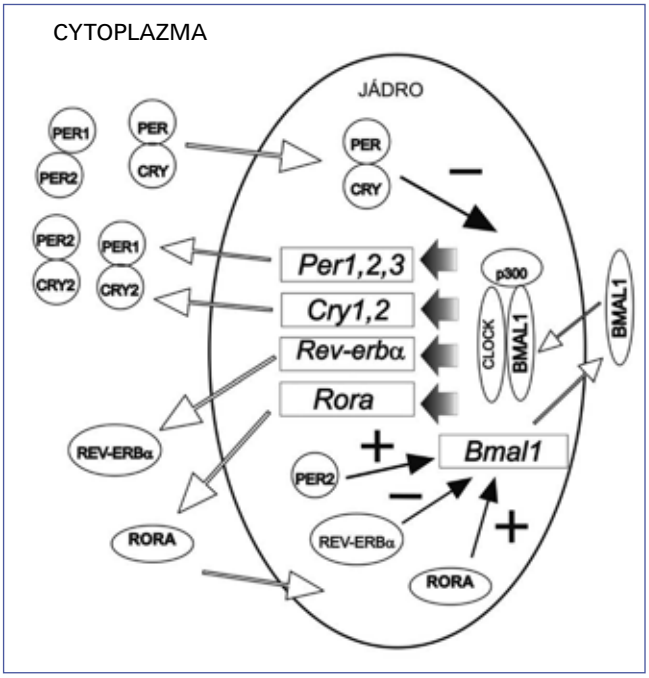
Obr. 1: *Zjednodušené schéma cirkadiánního systému savců*

RHT = retinohypothalamický trakt, GHT = genikulohypothalamický trakt, IGL = intergenikulátní lístek, PVN = nucleus paraventricularis, SCN = suprachiasmatická jádra hypothalamu; RHT a GHT představují hlavní vstupy synchronizačních podnětů do SCN. Výstupní z dráhy z SCN vedou přes PVN do epifýzy a také do preoptické oblasti i do dalších částí mozku. Dochází tak k cirkadiánnímu řízení výlevu hormonů, které dále řídí rytmické aktivity organizmu (spánek-bdění, příjem potravy atd.). Kromě oscilátoru v SCN jsou podobné oscilátory rovněž přítomny i v periferních orgánech a podílí se např. na rytmické tvorbě některých enzymů. Důležitou funkcí SCN je koordinace fází těchto rytmů.

3. MOLEKULÁRNÍ MECHANIZMUS VZNIKU CIRKADIÁNNÍCH RYTMŮ

Vlastní mechanizmus vzniku endogenních oscilací je založen na systému vzájemně propojených zpětnovazebných transkripčně-translačních smyček. Tyto smyčky jsou tvořeny tzv. hodinovými geny, tj. geny jejichž vyřazení způsobí u daného jedince narušení až ztrátu cirkadiánní rytmicity. Proteiny kódované těmito geny vstupují do jádra a regulují transkripci vlastních genů. U savců byly jako hodinové označeny geny: *Clock, Bmal1, Period1 (Per1), Period2 (Per2) a Period3 (Per3), Cryptochrome1 (Cry1) a Cryptochrome2 (Cry2), Rev-erbα, Rora a kasein-kináza1ε (CK1ε)* (přehled viz Reppert a Weaver, 2001; zjednodušené schéma na obr. 2). Proteinové produkty hodinových genů *Clock* a *Bmal1* tvoří heterodimery CLOCK:BMAL1, které se v jádře v komplexu s histonacetylázou p300 vážou na E-boxy v promotorech genů skupiny *Per a Cry*, do promotoru *Rev-erbα* a pravděpodobně také Rora a aktivují tak jejich transkripci (Etchegaray et al., 2003; Sato et al., 2004). V cytoplazmě se následně tvoří proteiny PER1, 2, 3 a CRY1 a 2, které se spojují v homo i heterodimery a jsou transportovány do jádra. Dimerizace a transport do jádra je pravděpodobně regulován fosforylací těchto proteinů CK1ε, popř. jejím homologem CK1δ. V jádře dimery PER:CRY narušují komplex CLOCK:BMAL1 – p300 a negativně tak ovlivňují vlastní transkripci. Protein REV-ERBα je rovněž transportován do jádra, kde se váže na tzv. RORE sekvence (REV-ERBα/ROR response element) a působí jako represor transkripce genu *Bmal1*. Tato represe snižuje dostupnost proteinu BMAL1, což vede k zpětnovazebné negativní regulaci vzniku REV-ERBα. Tato negativní zpětná vazba je patrně inhibována proteinem PER2, který tak působí jako pozitivní zpětnovazebný regulátor zvyšující dostupnost BMAL1 (Preitner et al., 2002). Do stejného místa jako REV-ERBα se váže také protein RORA (retionoic acid-related orphan receptor a), který naopak působí jako pozitivní regulátor exprese *Bmal1* (Ueda et al., 2002; Sato et al., 2004). Výsledkem výše popsaných vzájemně propojených zpětnovazebných smyček jsou rytmické oscilace mRNA hodinových genů a jejich proteinových produktů, přičemž rytmická exprese genů *Per, Cry* a *Rev-erbα* je v protifázi k rytmu v expresi *Bmal1*. Výjimku tvoří gen *Clock*, který v SCN nevykazuje rytmus v expresi.

Činnost molekulárního mechanizmu musí být nějakým způsobem převedena na rytmus v neuronální aktivitě a na produkci neuropeptidů v buňkách SCN. Prvkem, který zajišťuje na molekulární úrovni výstup z cirkadiánního oscilátoru, jsou tzv. geny kontrolované hodinami (CCGs, clock controlled genes), resp. jejich proteinové produkty. Jedná se o geny, které nejsou součástí zpětnovazebných smyček, ale přesto jejich exprese vykazuje cirkadiánní rytmus. Mají totiž ve svém promotoru E-boxy, na které se vážou heterodimery CLOCK:BMAL1 a působí tak rytmické zesilování jejich transkripce. Mezi CCGs patří např. AVP a některé recepto-



Obr. 2: *Schéma molekulárního mechanizmu cirkadiánních hodin. Blíže viz kapitola 3*

ry a podjednotky iontových kanálů, což se pravděpodobně odráží v rytmické aktivitě neuronů SCN.

4. SVĚTELNÁ SYNCHRONIZACE NA MOLEKULÁRNÍ ÚROVNI

Po osvětlení v noci je v SCN uvolněn glutamát z nervových zakončení RHT. Glutamát se váže na NMDA receptory neuronů SCN a spouští tak signální dráhu uvnitř buňky, která zahrnuje zvýšení intracelulární koncentrace Ca^{2+} a aktivaci NO-syntázy (Ding et al., 1994). Zvýšené koncentrace Ca^{2+} a NO vedou k aktivaci proteinkináz, které fosforylují DNA-vazebný Ca^{2+}/cAMP response element binding protein (CREB). Tento se váže na Ca^{2+}/cAMP response element (CRE) sekvenci v promotorech hodinových genů *Per1* a *Per2* a způsobují aktivaci transkripce *Per1* a *Per2* po světelném pulzu. Aktivace transkripce Per genů prostřednictvím CLOCK:BMAL1 heterodimeru popsaná v předchozí kapitole, je na CRE pravděpodobně nezávislá (Trávníčková-Bendová et al., 2002).

Zvýšení hladiny *Per1* mRNA v SCN bylo pozorováno po aplikaci světelného pulzu v první nebo druhé polovině subjektivní noci, ale nikoliv po aplikaci během subjektivního dne. Rovněž hladina *mPer2* mRNA byla zvýšená po světelném pulzu, ale pouze v první polovině subjektivní noci (Miyake et al., 2000). Má se tedy za to, že hodinové geny *Per1* a *Per2* jsou nezbytné pro světelnou synchronizaci cirkadiánních rytmů (Albrecht et al., 2001). Ke zvýšení hladiny *Per1* a *Per2* mRNA, resp. proteinů PER1 a PER2 po světelném pulzu dochází zejména ve vlSCN (Yan a Silver, 2004). Odtud je zřejmě informace předávána do dmSCN a ovlivňuje výstupy z oscilátoru.

Kromě okamžitého vlivu světelných pulzů na expresi hodinových genů *Per1* a *Per2*, dochází vlivem změny délky dne, tj. fotoperiody, ke změnám v profilech exprese hodinových genů *Per1, Cry1* a *Bmal1* (přehled viz Sumová et al., 2004).

5. VÝVOJ CIRKADIÁNNÍHO SYSTÉMU

Mláďata primátů včetně člověka a také dalších velkých savců se rodí poměrně dobře vyvinutá a některé fyziologické rytmy jsou u nich patrné ještě v prenatálním období vývoje. U novorozených dětí však ihned po porodu převažuje spíše ultradiánní rytmicita ve spánku a bdění či příjmu potravy. Cirkadiánní rytmicita začíná převládat teprve postupně během prvního měsíce života (Lohr a Sigmund, 1999). Mláďata hlodavců se rodí méně vyvinutá a různé vnější rytmy se objevují převážně až po narození. Z tohoto důvodu jsou potkani a myši v časných stadiích postnatálního vývoje vhodným objektem pro výzkum vývoje cirkadiánní rytmicity.

Březost trvá u potkanů 22-23 dnů. SCN je tvořeno mezi 13. a 16. dnem embryonálního vývoje a vlSCN vzniká dříve než dmSCN (přehled viz Weinert, 2005). Brzy po zformování SCN (19. den embryonálního vývoje) byl pozorován rytmus v metabolické aktivitě buněk SCN (Reppert a Schwarz, 1984). Od 21.dne embryonálního vývoje jsou patrné rozdíly v hladině AVP mRNA v SCN mezi dnem a nocí (Reppert a Uhl, 1987). U potkana je ode dne porodu pozorovatelná vyšší neuronální aktivita neuronů SCN během dne. Tento rytmus se postupně vyvíjí a u mláďat starých dva týdny dosahuje stejné úrovně jako u dospělých potkanů (Shibata a Moore, 1987).

Studie v posledních letech byly zaměřeny zejména na vývoj molekulárního mechanizmu cirkadiánních hodin. Exprese hodinových genů *Per1, Per2, Cry1, Bmal1* a *Clock* byla v SCN potkana detekována již 19. den embryonálního vývoje, v této době však nebyl ještě detekován cirkadiánní rytmus v jejich expresi (Sládek et al., 2004). Denní rytmy v hladině *Per1* a *Per2* mRNA v SCN začínají být patrné až 1. den po narození, exprese je prokazatelně rytmická u 2-denních mláďat a v období a mezi 3. a 10. postnatálním dnem se dále vyvíjí. První náznaky rytmu v expresi *Cry1* mRNA jsou patrné druhý den po narození (Kováčiková et al., 2006) a u 3-denních mláďat je rytmus již prokazatelný a rovněž se s věkem dále vyvíjí (Sládek et al., 2004). Hladina *Bmal1* mRNA v SCN je 19. a 20. den embryonálního vývoje vyšší než u ostatních hodinových genů a její rytmické změny jsou patrné od 1. dne po narození, přičemž u 2-denních mláďat se začíná objevovat pro *Bmal1* typický 24-hod profil, který je v protifázi k profilům *Per* a *Cry* mRNA (Kováčiková et al., 2006). Amplituda rytmu v expresi všech sledovaných hodinových genů se dále zvětšuje mezi 3. a 10. postnatálním dnem (Sládek et al., 2004). Stejně jako u dospělých potkanů není exprese hodinového genu *Clock* v SCN během prenatálního a časného postnatálního vývoje rytmická (Sládek et al., 2004; Kováčiková et al., 2006). Z výše uvedeného je patrné že jednotlivé části molekulárního mechanizmu cirkadiánních hodin se vyvíjejí postupně a převážně během časného postnatálního období (viz Tab. 1).

6. SYNCHRONIZACE SVĚTLEM VE VYVÍJEJÍCÍM SE CIRKADIÁNNÍM SYSTÉMU

Reakce vyvíjejícího se cirkadiánního systému na světelné podněty je kromě zralosti jednotlivých částí molekulárního mechanizmu podmíněna i vývojem drah, kterými se dostává informace o světle ze sítnice do SCN. Od 18. dne embryonálního vývoje začíná být ve vnitřní vrstvě neuroblastů sítnice potkana tvořen cirkadiánní fotopigment melanopsin. V období okolo porodu buňky obsahující melanopsin migrují do vrstvy gangliových buněk a v časné postnatální ontogenezi vytvářejí dendritickou síť ve vnitřní plexiformní vrstvě (Fahrenkrug et al., 2004). Fotoreceptory, které se účastní přenosu vizuálních podnětů, se u mláďat vyvíjejí později a svůj vývoj dokončují až během třetího týdne postnatálního života. Projekce RHT tvořeného z větší části vlákny RGCs do SCN a přilehlých oblastí se začíná objevovat 1. den po narození v podobě izolovaných vláken vedoucích na ventrální okraj SCN. Dospělé úrovně inervace je dosaženo přibližně 10. den postnatálního vývoje (Speh a Moore, 1993). Oči se potkanům otevírají okolo 14. a 15. dne života. I přesto však

mláďata do určité míry vnímají světlo i před tímto důležitým vývojovým mezníkem (Duncan et al., 1986). Již 1. den po porodu byl pozorován vliv světelného pulzu na zvýšení exprese časného raného genu *c-fos* v SCN (Leard et al., 1994). Teprve až od 10. postnatálního dne je však vyvinut mechanizmus, který vymezuje dobu, kdy je SCN citlivé na světelný pulz, tj. tzv. vrátkový mechanizmus. Bylo prokázáno, že teprve u 10-denních mláďat světelné pulzy indukují expresi *c-fos* převážně v době subjektivní noci (Bendová et al., 2004)

Schopnost molekulárního mechanizmu reagovat na délku dne, tzv. fotoperiodu se vyvíjí postupně. U 3-denních potkaních mláďat ještě fotoperioda neovlivňuje v SCN expresi ani jednoho z hodinových genů. U 10-denních mláďat dochází k mírnému ovlivnění profilu exprese pouze u genů citlivých na světlo, tedy *Per1* i *Per2*. V SCN mláďat chovaných na dlouhé letní fotoperiodě (LD 16:8) je 10. postnatální den patrný dřívější ranní nástup zvýšené exprese *Per1* i *Per2* mRNA než u mláďat chovaných na krátké fotoperiodě (LD 8:16). U genu *Per2* ovlivňuje fotoperioda i dobu večerního poklesu mRNA v SCN. Díky tomu je doba po kterou je hladina *Per1* a *Per2* mRNA vysoká delší u mláďat chovaných na dlouhé fotoperiodě než u mláďat chovaných na krátké fotoperiodě. Na profily genů, jejichž exprese není přímo ovlivnitelná světlem, má fotoperioda vliv až v pozdějších vývojových stadiích. Profil exprese *Cry1* mRNA je fotoperiodou ovlivněn až 20. den po narození a profil exprese *Bmal1* mRNA v SCN nebyl ovlivněn ještě ani u 20-denních mláďat (Kováčiková et al., 2005; přehledně viz Sumová et al., 2006). Vývoj synchronizace fotoperiodou je tedy u potkana ukončen až okolo období odstavu. Je pravděpodobné, že vývoj diskrétní (světelné) a kontinuální (fotoperiodické) synchronizace souvisí s vývojem „vrátkového" mechanizmu, který vymezuje dobu citlivosti ke světlu.

***Tabulka 1.**: Prenatální a postnatální vývoj cirkadiánního systému potkana*
E = den embryonálního vývoje, P = den postnatálního vývoje

E13-E16	formování SCN
E18	Začíná tvorba melanopsinu v buňkách sítnice
E19	v SCN zjištěn rytmus v metabolické aktivitě
E19-E22	v SCN detekována exprese hodinových genů:
	Per1
	Per2
	Cry1
	Bmal1
	Clock
P0	den porodu
	v SCN zjištěn náznak rytmu v neuronální aktivitě
P1	první projekce RHT do SCN
	detekován náznak rytmické exprese hodinových genů
	Per1, Per2 a Bmal1
P2	Detekována rytmická exprese Per1, Per2 a Bmal1
	náznak rytmu u Cry1
P3	detekován rytmus v expresi Cry1
	u ostatních hodinových genů se zvětšuje amplituda rytmu
P10	zjištěn vliv fotoperiody na 24hod. profil exprese Per1 a Per2
	pozorován rytmus v pohybové aktivitě
P14-P15	otevírání očí
P20	Kromě profilů exprese Per1a Per2 je fotoperiodou modulován i
	profil exprese Cry1

7. SYNCHRONIZACE NESVĚTELNÝMI PODNĚTY VE VYVÍJEJÍCÍM SE CIRKADIÁNNÍM SYSTÉMU

Mláďata savců dostávají již před narozením informace o vnějších světelných podmínkách. Děje se tak zprostředkovaně přes cirkadiánní systém matky. Pokusy s lézemi SCN u potkaních samic v různých stadiích březosti ukázaly, že mateřská synchronizace se uplatňuje zhruba od okamžiku kdy je zformováno embryonální SCN. U potomků samic kterým bylo odstraněno SCN v časných fázích březosti nedochází ke ztrátě cirkadiánní rytmicity, ale je narušena synchronizace s vnějším dnem (Shibata a Moore, 1988). SCN matky se přímo nepodílí na vývoji cirkadiánních rytmů mláďat, ale slouží jako významný synchronizátor cirkadiánních rytmů celého vrhu.

V prenatálním období přijímá embryonální cirkadiánní systém od matky řadu signálů behaviorálních i hormonálních. Důležitým signálem je noční výlev melatoninu. Melatonin přechází přes placentu a pro mláďata by tak mohl být „signálem noci". Jako „signál dne" by mohl sloužit dopamin. Melatonin a dopamin tak zřejmě působí v embryonálním cirkadiánním systému jako dva vzájemně se doplňující signály (přehledně viz Weinert, 2005).

Vliv matky na cirkadiánní systém mláděte pokračuje i po narození. Nejsilnější je v prvních dnech až týdnech postnatálního vývoje a pak postupně slábne. Mláďata hlodavců, kteří jsou aktivní v noci, jsou významně synchronizována rytmem v krmení a hlavně rytmickou přítomností, resp. nepřítomností matky v hnízdě (přehled viz Weinert, 2005). Nepřítomnost matky v době krmení, tedy přes den, je pro mláďata silným stresujícím signálem, který otáčí fázi rytmů v expresi hodinových genů *Per1* a *Per2* (Ohta et al., 2003). Obecně lze říci, že cirkadiánní systém mláděte je v prvních dnech života citlivý převážně k nesvětelným podnětům zprostředkovaným mateřskou péčí a teprve potom ke světelným signálům. Vliv matky v prenatální i postnatální ontogenezi napomáhá synchronizaci cirkadiánního systému mláděte s vnějšími podmínkami v době, kdy cirkadiánní systém mláděte není ještě plně vyvinut (Sumová et al., 2006).

8. ZÁVĚR

Během posledních let došlo k velkému pokroku ve výzkumu cirkadiánních rytmů. Zásadní podíl na tom má zejména využití metod molekulární biologie a genetiky. Do popředí zájmu se nyní dostává nejen detailní prozkoumání molekulárního mechanizmu vzniku oscilací v SCN, ale i oscilátory v periferních orgánech a jejich možný dopad na funkci těchto orgánů. Výzkum ontogeneze cirkadiánního systému napovídá mnohé o tom jak a jakými mechanizmy je tento systém schopen se adaptovat na různé světelné i nesvětelné podněty v raných fázích vývoje.

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