Od člověka k buňce: Nové poznatky a metody ve studiu cirkadiánních rytmů u pacientů s afektivními poruchami a schizofrenií

From human to cell: New approaches and methods in studies of circadian rhythms in patients with affective disorders and schizophrenia

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Abstrakt:

V posledních letech je stále více zdůrazňována role centrálních cirkadiánních hodin ve vzniku řady psychiatrických onemocnění. Bývá dávána do souvislosti především s onemocněními vykazujícími změny nálad a spánkových rytmů, které souvisí se změněným funkčním stavem cirkadiánního systému a jsou nejčastěji sledované u pacientů s některými typy afektivních poruch nebo u schizofrenních pacientů. U většiny těchto onemocnění dochází k narušení pravidelného nástupu spánku, jeho nestabilitě nebo změně spánkové architektury a snížené schopnosti synchronizace rytmu spánku a bdění s vnějšími podmínkami. Propojení těchto

onemocnění s funkčním stavem cirkadiánních hodin je od objevu molekulárního hodinového mechanismu potvrzováno i řadou studií sledujících molekulární markery hodin. Díky pokročilým molekulárním metodám dokážeme získat přesné informace o endogenním stavu cirkadiánního systému, který velmi dynamicky reaguje na změny vnějšího prostředí. Je citlivý zejména na změny světelných podmínek, reaguje však také na stres nebo příjem potravy. Zdá se, že tyto faktory mohou i dlouhodobě ovlivnit a měnit chod hodin. V poslední době se uvažuje nad tím, že tyto dlouhodobé změny mohou být způsobeny epigenetickými modifikacemi DNA a histonů v oblastech kódujících proteiny podmiňující chod hodin. V současné době tak kromě genetických studií nabývají na významu i studie epigenetických modifikací molekulárního mechanismu cirkadiánního systému. Rozvoj psychiatrických onemocnění často nebývá determinován pouze geneticky, ale mnohdy souvisí právě se stresem nebo výživou. Některé nemoci se rozvíjejí až v určitém věku, což může poukazovat na pozvolný vliv faktorů vnějšího prostředí na molekulární prostředí mozku prostřednictvím epigenetických modifikací. Neuroanatomické studie ukázaly, že cirkadiánní hodiny v suprachiasmatických jádrech hypotalamu přímo ovlivňují mnoho oblastí mozku, včetně amygdaly, prefrontální kůry a oblastí hypotalamu regulujících spánek. Přesto role cirkadiánního systému ve vzniku a průběhu psychiatrických onemocnění není dosud zcela objasněna. Behaviorální výstupní rytmy jeho roli sice naznačují, ovšem chybí jednoznačné důkazy na úrovni molekulárních markerů, genetické variability a epigenetických modifikací.

Klíčová slova: cirkadiánní rytmy, hodinové geny, afektivní poruchy, schizofrenie, lithium, ketamin, spánková deprivace

Abstract:

Recently, the role of the central circadian clock has been widely discussed in connection with the pathology of various psychiatric disorders. It has been observed to be primarily associated with diseases that include changes in mood and sleep rhythms, which are linked to changes in the functional state of the circadian system. These changes are typically observed in patients with affective disorders or schizophrenia. In most of these diseases, regular sleep onset is compromised or unstable; changes in sleep architecture occur and the ability to synchronize the rhythm of sleep and wakefulness with external conditions is reduced. Since the discovery of the circadian molecular mechanism, a series of studies dealing with molecular markers have affirmed the association of these conditions with the functional state of the circadian clock. With advanced molecular techniques, we can obtain accurate information about the

state of the endogenous circadian system that responds dynamically to changes in the external environment. It is particularly sensitive to changes in lighting conditions but also responds to stress or food intake. It appears that these factors may also affect the long-term changes and operation of the biological clock. Taking these facts into account, it can be deduced that these long-term changes can be caused by epigenetic modifications of DNA and histones in the protein-coding region that conditions the clockwork mechanism. Apart from the genetic studies, we are witnessing an increase in the importance of epigenetic modification studies of the molecular mechanism of the circadian system. The development of psychiatric disorders is not necessarily determined by genetic factors only – it is often connected with aspects such as stress or nutrition. Some diseases emerge at a certain ages, which may indicate a gradual influence of environmental factors on the molecular environment of the brain through epigenetic modifications. Neuroanatomical studies have demonstrated that circadian clock in the suprachiasmatic nucleus of the hypothalamus directly affects many areas of the brain, including the amygdala, the prefrontal cortex and the hypothalamus regions, regulating sleep. The role of the circadian system in the aetiology and progress of psychiatric disorders is not yet fully understood. Behavioural output rhythms indicate that it does play a role, but lacks evidence on the level of molecular markers, genetic variability and epigenetic modifications.

Key words: circadian rhythms, clock genes, affective disorder, schizophrenia, ketamine, sleep deprivation

1. Úvod:

Cirkadiánní rytmy jsou endogenní biologické cykly opakující se s přibližně 24h periodou, a to i v neperiodickém prostředí, např. v podmínkách konstantní tmy. Existence cirkadiánních rytmů byla popsána u většiny organismů, od bakterií až po savce. Cirkadiánní systém (CS) je evolučně velmi starý a umožňuje organismům předvídat rytmicky se opakující děje, které mají přibližně denní periodu. Mezi nejzřetelnější cirkadiánní rytmy savců patří rytmus spánku a bdění, pravidelný příjem potravy nebo rytmus pohybové aktivity. Hůře sledovatelné, ale velmi důležité pro správnou funkci každého organismu, jsou rytmické změny na úrovni metabolických nebo hormonálních procesů změn během dne (Hastings et al., 2003). Za synchronizaci jednotlivých tělesných rytmů je zodpovědný centrální oscilátor, který se u savců, a tedy i u člověka, nachází v suprachiasmatickém jádře v hypotalamu (SCN). Funkci CS lze modifikovat pomocí tzv. zeitgeberů neboli časovačů. Jako časovače mohou

sloužit zejména pravidelné změny ve střídání světla a tmy, které tvoří dominantní časový signál vnějšího prostředí. CS je také citlivý na změny v teplotě (Buhr et al., 2010), v dostupnosti potravy (Sherman et al., 2012), v přítomnosti odorantů (Abraham et al., 2013) nebo ve fyzické aktivitě (Hughes and Piggins, 2012).

Celá řada studií poukazuje na to, že CS hraje roli v patofyziologii některých neuropsychiatrických onemocnění, především spánkových poruch, afektivních onemocnění, nebo u schizofrenie. U těchto onemocnění jsou typicky popisovány změny rytmu spánku a bdění, v tělesné teplotě, rytmu některých hormonů, jako např. v hladině kortizolu nebo melatoninu (Bromundt et al., 2011; Buckley and Schatzberg, 2010; Kennedy et al., 1996; Novakova et al., 2015), ale i změny na úrovni samotného regulačního mechanismu tzv. vnitřních biologických hodin. Hlavní charakteristikou vnitřních hodin je jejich schopnost generovat endogenní cirkadiánní rytmus, který probíhá i v podmínkách konstantní tmy. Endogenní rytmus hodin je charakterizován vlastní periodou tau (τ), která se mírně liší od přesné 24 hodinové periody vnějšího dne. Tento rytmus je generován molekulárním hodinovým mechanismem probíhajícím v jednotlivých buňkách (Golombek and Rosenstein, 2010) . K jakým změnám chodu endogenních hodin vlivem psychiatrických nemocí dochází, není dosud dostatečně prostudováno. Zdá se, že za změnami τ, fáze nebo amplitudy rytmu mohou stát změny exprese hodinových genů, nebo změny jejich posttranslačních modifikací, histonových modifikací, nebo změny na úrovni nekódujících molekul RNA (Akashi et al., 2002; Sahar and Sassone-Corsi, 2013).

Sledování exprese hodinových genů v periferních buňkách je relativně moderní metodika studia stavu cirkadiánního systému člověka. Každá jaderná buňka v těle obsahuje kompletní sadu hodinových genů a je schopna vykazovat cirkadiánní oscilace. Ve zdravém a harmonizovaném organismu jsou tyto oscilace synchronizované se signály z SCN. První práce popisující komplexní funkci molekulárního hodinového mechanismu tvořeného zpětnovazebnými transkripčně-translačními smyčkami hodinových genů, jejich funkci a signalizační dráhy řídící jejich expresi, se objevily kolem roku 2000 (Jin et al., 1999; Ko and Takahashi, 2006; Reppert and Weaver, 2002; Zylka et al., 1998). Od této doby jsou změny molekulárního mechanismu intenzivně studovány v mnoha směrech medicíny a velmi intenzivně i na poli psychiatrie. Již dříve bylo pozorováno, že u některých afektivních poruch (bipolární deprese, unipolární deprese, sezonní afektivní porucha), nebo i schizofrenie, dochází ke změnám cirkadiánních rytmů nejen na úrovni výstupních rytmů, ale také na úrovni endogenních markerů cirkadiánního systému (McClung, 2007b; Mendlewicz, 2009a; Novakova et al., 2015).

Jisté úspěšnosti v léčbě především afektivních poruch dosáhlo využití chronoterapeutických postupů (např. léčba jasným světlem), a zároveň bylo prokázáno, že řada současných léků využívaných k léčbě těchto onemocnění vede ke změnám cirkadiánních rytmů. Zdá se tedy, že chronobiologické aspekty mohou významně obohatit oblast psychiatrie (Benedetti et al., 2005a; Echizenya, 2012; Wirz-Justice et al., 2005). Následující kapitoly detailněji objasní fungování molekulárního mechanismu generování cirkadiánních rytmů a představí jednotlivé nálezy, které vedly k hypotézám poukazujícím na propojení cirkadiánního systému s některými psychiatrickými chorobami.

2. Organizace a molekulární mechanismus biologických hodin

Centrálním oscilátorem cirkadiánního systému člověka a dalších savců jsou SCN nacházející se v hypotalamu. Jedná se o malou párovou strukturu čítající asi 20 tisíc neuronů. Téměř každý neuron SCN je schopný generovat endogenní cirkadiánní rytmus nezávisle na okolních buňkách (Hofman et al., 1988), avšak jednotný výstupní rytmický signál celého SCN vzniká až na úrovni neurálních sítí buněčných subpopulací SCN (Kalsbeek et al., 2010; Kalsbeek et al., 2006a; Vujovic et al., 2008).

SCN není jedinou strukturou generující cirkadiánní rytmus, další cirkadiánní oscilátory se nacházejí jak v mnoha strukturách CNS, tak i ve většině periferních orgánů (srdce, plíce, střevo, játra, ledviny, kosterní sval). SCN je ostatním oscilátorům hierarchicky nadřazeno, je odpovědné za udržování jejich vzájemné synchronizace, i za synchronizaci se světlenými podmínkami vnějšího prostředí (Yoo et al., 2004). Tyto synchronizační signály z centrálního do periferních oscilátorů mají neuronální i humorální povahu (Abe et al., 2002; Buijs et al., 2003; Yoo et al., 2004).

Za potenciální endogenní synchronizátor periferních hodin je považován melatonin, jehož tvorba je přímo řízena z SCN. K jeho syntéze dochází v epifýze, odkud je uvolňován do mozkomíšního moku a následně do celé oběhové soustavy. Melatonin působí na melatoninové receptory (MT1, MT2), skrze které zpětně ovlivňuje elektrickou a metabolickou aktivitu SCN. Melatoninové receptory byly současně popsány i v řadě periferních tkání (Kalsbeek et al., 2006b).

2.1 Molekulární hodinový mechanismus

Za generování autonomního rytmu probíhajícího v jednotlivých buňkách, a to i v *in vitro* podmínkách, odpovídají negativní a pozitivní transkripčně-translační zpětnovazebné smyčky

hodinových genů. Mezi pozitivní komponenty zpětnovazebné smyčky patří proteiny CLOCK a BMAL1. CLOCK a BMAL1 mají stejnou strukturní proteinovou bHLH PAS doménu, která jim umožňuje vzájemnou interakci. Utváří spolu heterodimer, který reguluje rytmickou expresi hodinových genů *Period (Per1-3)* a *Cryptochrom (Cry1* a *Cry2)*. Heterodimer CLOCK-BMAL1 se váže přímo na specifické sekvence E-box v promotorech těchto hodinových genů a indukuje jejich transkripci. Produkty transkripce, jednotlivé mRNA, jsou translokovány do cytoplazmy, kde vznikají PER a CRY proteiny. Po dosažení kritické koncentrace spolu tvoří, za pomoci proteinové PAS domény, heterodimer PER-CRY, který je zpět translokován do jádra, kde negativně reguluje svoji vlastní transkripci tím, že inhibuje pozitivní transkripční faktory CLOCK-BMAL1 (Reppert and Weaver, 2002). Heterodimer CLOCK-BMAL1 aktivuje, kromě genové exprese *Per* a *Cry*, také expresi transkripčních faktorů *Nr1D1* (Nuclear receptor subfamily 1, group D, member 1; jehož proteinový produkt je označován jako REV-ERBα) a *Rorα*, které následně zpětně regulují transkripci *Bmal1*. Proteiny RORα působí jako aktivátory exprese a REV-ERBα jako represor vazbou na RORE, element v promotoru *Bmal1* genu (Preitner et al., 2002). Přídavnou zpětnovazebnou smyčku dále tvoří CLOCK/BMAL1 komplexem aktivovaný hodinový gen albumin D-box vazebný protein (DBP), který pozitivně ovlivňuje expresi *Per* (Yamaguchi et al., 2000). Exprese genů *Per* a *Cry* probíhá v opačné fázi než exprese genu *Bmal1*. *Per* a *Cry* dosahují maxima během dne a *Bmal1* dosahuje maxima naopak během noci (Mohawk et al., 2012).

Významnou roli v molekulárním hodinovém mechanismu také hrají posttranslační modifikace, jako jsou fosforylace/defosforylace, ubiquitinizace (Hirano et al., 2013; Yoo et al., 2013), acetylace/deacetylace nebo sumoylace jednotlivých proteinových produktů hodinových genů (Asher et al., 2008; Bellet and Sassone-Corsi, 2010). Za důležité posttranslační úpravy odpovídají především aktivované kinázy, kasein kináza δ/ε (CKIδ, CKIε) a glykogen syntáza kináza 3β (GSK3β). Fosforylací proteinů dochází především ke změně jejich stability, k iniciaci tvorby komplexů vedoucích k jejich degradaci v proteosomálním aparátu nebo ke změně rychlosti jejich translokace do jádra (Akashi et al., 2002; Lee et al., 2009; Sahar et al., 2010).

Tento molekulární mechanismus indukuje cirkadiánní rytmické změny v jednotlivých neuronech a v buňkách periferních tkání, ale fáze individuálních rytmů jednotlivých neuronů v SCN jsou desynchronizované a každý neuron vykazuje maximum a minimum svého rytmu v jinou denní dobu (Brown and Piggins, 2009; Evans et al., 2011; Green and Gillette, 1982; Meijer et al., 2010; Welsh et al., 1995). Pro vyjádření jednotného cirkadiánního rytmu

pacemakeru jako populace neuronů je nezbytná synaptická komunikace mezi buňkami SCN (Honma et al., 2000; Shirakawa et al., 2000; Yamaguchi et al., 2003). Experimenty z poslední doby prokázaly, že elektrická aktivita zablokovaná tetrodotoxinem může zcela zrušit cirkadiánní změny v hladině intracelulárního vápníku a rytmus v transkripci závislé na vazebném místě CRE (z angl. cAMP responsive element) na promotorech genů *Per1* a *Per2* (Brancaccio et al., 2013; O'Neill and Hastings, 2008). Tento pokus ukázal, že k udržení jednotné fáze molekulárních zpětnovazebných smyček v jednotlivých buňkách zásadně přispívá i aktivita buněčné membrány a ovlivňuje tak i transkripci hodinových genů.

Výsadou centrálního oscilátoru je jeho unikátní schopnost synchronizace s vnějšími větelnými podmínkami. Střídání světla a tmy během dne a noci je vnímáno neuronální sítí SCN, která následně informuje periferní oscilátory a indukuje kompresi či dekompresi cirkadiánních rytmů tak, aby odpovídaly měnícímu se poměru délky světlé a tmavé části dne v průběhu roku. Informaci o světle zprostředkovávají fotosenzitivní gangliové buňky sítnice obsahující melanopsin, které projikují glutamátergní retinohypotalamickou dráhou do SCN. Výlevem glutamátu na synapsi dojde k aktivaci NMDA receptorů, která způsobí vstup Ca^{2+} do buňky a tvorbu cyklického adenosin monofosfátu (cAMP). Nárůst Ca^{2+} v buňce a cAMP aktivují mitogen-aktivované protein kinázy (MAPK). MAPK fosforyluje cAMP response-element binding protein (CREB), který se váže na CRE response element v promotoru *Per1* a *Per2* a indukuje tak jejich expresi podobně jako v případě intercelulární synchronizace mezi neurony SCN (Antle and Silver, 2005).

2.2 Vliv epigenetických modifikací hodinový mechanismus

Epigenetické změny, způsobené vlivem vnějšího prostředí buňky, se projevují na úrovni modifikací DNA nebo histonů. Mohou iniciovat DNA metylace nebo histonové modifikace, jako jsou fosforylace, metylace, acetylace, ubiquitinace, citrulinace, biotinylace, ribosylace, palmitoylace, které následně dokáží zesílit, zeslabit nebo zcela umlčet expresi cílových genů (Bannister and Kouzarides, 2011; Feil and Fraga, 2011; Lim et al., 2014; Portela and Esteller, 2010; Sahar and Sassone-Corsi, 2013). Epigenetické změny mohou také působit různé typy nekódujících RNA (Peschansky and Wahlestedt, 2014). Mezi tyto RNA patří i v poslední době hojně studované mikro-RNA (miRNA), o kterých se uvažuje také jako o významných modulátorech funkce molekulárního hodinového mechanismu (Han et al., 2015). MiRNA v buňkách obecně fungují především jako tzv. silencery genové exprese, které se komplemen-

tárně váží do cílové oblasti mRNA. Jejich vazbou dochází k inhibici translace, nebo až k destabilizaci mRNA a k její následné degradaci.

MiRNA vznikají v jádře ve formě prekurzorů jako krátké RNA úseky o přibližně 70 nukleotidech. Pre-miRNA zaujímají vlásenkovou konformaci a jsou translokovány do cytoplazmy, kde dochází k jejich maturaci hydrolytickým štěpením pomocí diceru, enzymu s endonuklézovou aktivitou (He and Hannon, 2004).

Experimenty na myších s genetickou delecí Dicer (tedy bez funkčních miRNA), i práce s buněčnými kulturami těchto myší ukázaly, že miRNA i Dicer jsou nezbytné pro udržení cirkadiánní periody hodinového mechanismu. Tyto myši i jejich buňky vykazovaly zkrácenou cirkadiánní periodu díky zrychlené translaci PER1 a PER2 proteinů, které hrají významnou roli jako negativní regulátory zpětnovazebné smyčky (viz kap. 2.1). Bylo tak prokázáno, že miR-24, miR-29a a miR-30a se specificky váží na mRNA Per1 a Per2 a tím spoluurčují délku cirkadiánního cyklu (Chen et al., 2013). Dalšími miRNA, které se mohou podílet na regulaci CS, jsou miR-219 a miR-132. Experimenty na buněčných kulturách ukazují, že tyto miRNA fungují jako pozitivní modulátory transkripce *Per1*. Exprese MiR-219 je aktivovaná komplexem CLOCK/BMAL1 a vykazuje robustní denní rytmus s maximem v první fázi dne společně s PER1 a PER2. Exprese miR-132 je indukována světlem, stejnými dráhami, kterými dochází k indukci exprese hodinových genů *Per1*, *Per2* a k následné synchronizaci centrálních hodin (Cheng et al., 2007). Zdá se, že právě tyto miRNA by mohly hrát důležitou roli v modifikaci funkce CS ovlivněného nepřirozenými světelnými změnami (práce na směny, přelety časových pásem…atd.) a mohly by se podílet na rozvoji onemocnění souvisejících se změnami CS (Liu and Wang, 2012). Zatím však o jejich funkci a významu nemáme dostatečné množství informací.

3. Možnosti studia cirkadiánního systému člověka

Při studiu funkčního stavu hlavního cirkadiálního pacemakeru v SCN člověka jsme odkázáni pouze na jeho nepřímé ukazatele. Funkci SCN je možné sledovat pouze na míře synchronizace a amplitudě jeho výstupních rytmů. Ty můžeme rozdělit na tzv. endogenní markery CS, mezi které patří např. rytmická produkce některých hormonů, teplotní rytmus, nebo exprese hodinových genů v periferních tkáních, a výstupní behaviorální markery CS, mezi které řadíme např. rytmus pohybové aktivity, nebo rytmus spánku a bdění. Jelikož SCN udržuje rytmy v periferních oscilátorech (včetně jednotlivých buněk) neustále synchronizované, lze využívat periferních tkání jako nejpřesnějších dostupných ukazatelů

funkčního stavu vnitřních hodin. (O'Neill and Reddy, 2011). V závislosti na typu hypotézy je nutné dodržovat podmínky specifické pro experimenty sledující cirkadiánní rytmy. Lze provádět cirkadiánní experimenty sledující jak reálné životní podmínky subjektu, tak i experimenty v konstantních sledovaných podmínkách (např. protokol konstantní rutiny nebo nucené desynchronizace), které je nutné provádět ve světelně izolovaných spánkových laboratořích s uměle řízeným osvětlením (Novakova and Sumova, 2014).

Za jeden z nejpřesnějších nepřímých ukazatelů vnitřního času je dlouhodobě považována a využívána rytmická změna produkce melatoninu v epifýze. Transkripce enzym N-acetyltransferázy (AANAT), který řídí přeměnu serotoninu na melatonin, je řízena multisynaptickou drahou z SCN. Noční syntéza melatoninu může být také okamžitě zablokována osvitem a jeho hladina pak ihned klesá (Illnerova et al., 1979; Lewy et al., 1980). Sledování hladin melatoninu tak poskytuje informaci o světelných podmínkách, kterým je organismus vystavován, i o jeho vnitřní synchronizaci s vnějšími podmínkami (Illnerova, 1991).

Dostupným lidským materiálem pro *in vivo* studium CS jsou nejčastěji využívané opakované odběry vzorků lymfocytů, epiteliálních buněk bukální sliznice, nebo vlasové folikuly. Metoda využívající ke stanovení exprese hodinových genů stěry bukální sliznice byla v řadě prací ověřena jako spolehlivá jak pro určení fáze, tak i pro měření amplitudy exprese jednotlivých komponent CS. Současně tento ukazatel koreluje s fází rytmu v hladinách melatoninu a spánkového rytmu subjektu. Velkou výhodou této metody je možnost neinvazivního odběru vzorků, který je subjekt schopný provádět i samostatně (Novakova et al., 2012; Novakova et al., 2015; Novakova et al., 2013). Nevýhodou těchto studií je jejich časová náročnost vyžadující od sledovaného subjektu minimálně 24 hodinovou účast v experimentu. Proto je v posledních letech snaha vyvinout metodu sledující lidské cirkadiánní hodiny *in vitro (ex vitro)*. Nejvhodnější metodou je odebrání kožní biopsie a následná kultivace primárních lidských fibroblastů. Buňky kožních fibroblastů mohou být synchronizované řadou vnějších signálů, jsou schopné autonomně generovat a dlouhodobě udržet cirkadiánní rytmus, který přetrvává i po buněčném dělení (Nagoshi et al., 2004). Buněčné kultury kožních fibroblastů nám umožňují sledovat expresi jednotlivých hodinových komponent pomocí kvantitativní real-time PCR nebo westernblotu. S využitím luminiscenčních reportérů zapojených za promotorové oblasti sledovaného genu a retrovirálně transfekovaných do buněk je možné sledovat expresi cílových genů v reálném čase (Brown et al., 2005; Brown et al., 2008).

Tento systém by mohl sloužit jako odraz vnitřních cirkadiánních rytmů a vnitřní perioda hodin fibroblastů by měla odpovídat periodě centrálních hodin v SCN. Na základě srovnání fyziologické periody učené měřením denního profilu tvorby melatoninu od zdravých subjektů *in vivo* a jejich srovnáním s periodou naměřenou z jejich kožní biopsie v *in vitro* podmínkách byl tento předpoklad potvrzen ve studii Pagani et al. (2010). Perioda naměřená *in vivo* korelovala s periodou naměřenou *in vitro* (Pagani et al., 2010). Pozdější podobná studie tuto korelaci ovšem nepotvrdila a ukázala, že v *in vivo* podmínkách byla perioda kratší než v *in vitro* podmínkách (Hasan et al., 2012). Do jaké míry mohou tedy buněčné kultury fibroblastů sloužit jako *in vitro* model korelující s funkčním stavem centrálních hodin svého dárcovského subjektu, bude možné určit až na základě dalších studií. Podobně jako fibroblasty mohou sloužit i kultivované lymfocyty z krve. Lymfocyty je možné imortalizovat a transformovat na lymfoblastoidní buněčné linie. Takto získané buněčné linie je možné využívat podobně jako kultury fibroblastů pro studování patologických změn molekulárního hodinového mechanismu (Kittel-Schneider et al., 2015; McCarthy et al., 2011). Některé studie však ukazují, že proces imortalizace může výrazně zkreslovat expresi hodinových genů (Price and Luftig, 2014). Přes značné nepohodlí, které představují 24 hodinové experimenty, jsou tedy v současnosti pro přesnou diagnostiku cirkadiánních změn pacientů nadále vhodnější opakované odběry v přesných časových intervalech než kultivace buněk.

Existují ovšem i studie sledující stav centrálních hodin člověka přímo v SCN. Tyto studie ke své analýze využívají lidské vzorky získané post-mortem. Sledované subjekty bývají rozděleny do 3-4 skupin podle času úmrtí a hodnoty sledovaných parametrů jsou následně vyhodnoceny napříč skupinami v průběhu dne (Cermakian et al., 2011; Li et al., 2013). Interpretace takto získaných výsledků je ovšem velmi omezená přinejmenším proto, že neznáme světelnou historii těchto subjektů. Závěry o stavu cirkadiánního systému z takto získaných vzorků bývají proto často velmi diskutabilní.

4. Psychiatrická onemocnění nejvýrazněji propojená s poruchou CS

SCN jsou zapojeny do řady neurálních okruhů v CNS, projikují do přibližně 15 oblastí mozku, především pak do hypotalamických oblastí. Přímé projekce do amygdaly, laterálního septa, habenuly nebo nepřímé projekce přes paraventrikulární talamická jádra do mediálního prefrontálního kortexu (mPFC) jsou zodpovědné za zapojení CS do komplexních procesů CNS (Abrahamson and Moore, 2001; Peng and Bentivoglio, 2004; Sofroniew and Weindl, 1978). Projekce SCN do dorsomediálních jader hypotalamu je zodpovědná za cirkadiánní

rytmus spánku a bdění a projekce do mPFC ovlivňují rytmické oscilace nálady a kognitivních funkcí během dne (Morin, 2013). Cirkadiánní systém tedy ovlivňuje řadu kognitivních a mentálních funkcí člověka, a z tohoto důvodu je úzce provázán s etiologií řady psychiatrických onemocnění. U zdravého člověka kolísá pravidelně v průběhu dne mentální výkon, lze sledovat i pravidelné oscilace nálady. Nepravidelné, neočekávané změny nálad a narušený rytmus spánku a bdění jsou chronobiologické markery, které mohou poukazovat na rozvoj depresivních onemocnění, jako jsou unipolární deprese (UD), bipolární afektivní porucha (BAP), nebo také schizofrenie (McClung, 2007a; Wirz-Justice, 2008).

3.1 Bipolární afektivní porucha

 BAP je chronické onemocnění, pro které je typické střídání manické a depresivní fáze doprovázené změnami a nestabilitou spánkového rytmu (Jones et al., 2005). Podle výskytu depresivních a manických epizod rozlišujeme u BAP několik typů onemocnění. Nejčastěji jsou v populaci zastoupeny BAP typu I, BAP typu II (podle DSM-IV). U BAP typu I dochází ke klasickému střídání epizod deprese a mánie, BAP typu II je periodická depresivní porucha s hypomanickými epizodami. Ve většině studií cirkadiánního systému jsou pacienti s různými typy BAP zahrnuti do jedné experimentální skupiny, což může být jednou z příčin variability získávaných výsledků.

Porucha spánku je jedním ze základních diagnostických kritérií BAP a jeho rytmus a délka se v závislosti na její epizodě mění. V průběhu manických epizod dochází ke snížené potřebě spánku, až k insomnii, naopak během depresivní fáze se potřeba spánku výrazně prodlužuje a dochází k hypersomnii (Harvey, 2008; Ohayon and Roth, 2003). Změny behaviorálních markerů cirkadiánních rytmů byly u pacientů s BAP popsány již před lety. Bylo sledováno fázové předbíhání rytmu jejich pohybové aktivity oproti zdravým subjektům, což poukázalo na zkrácenou délku periody vnitřních hodin pacientů s BAP (Wehr et al., 1979).

Na změny cirkadiánní rytmicity u BAP poukazují i endogenní markery. U depresivních pacientů bylo sledováno snížení noční hladiny melatoninu oproti zdravým kontrolám a u manických pacientů došlo k jejímu zvýšení zcela netypicky také v průběhu dne (Kennedy et al., 1996; Novakova et al., 2015; Robillard et al., 2013). Kromě rytmických hormonálních markerů byly popsány změny i na úrovni exprese hodinových genů v periferních tkáních. Nováková et al. (2015) ve své práci popsali fázové předběhnutí rytmů v expresi hodinových genů v buňkách získaných z bukálních stěrů pacientů a zdravých kontrolních subjektů. Srovnávali rozdíly exprese genů *Per1* a *Nr1d1* v průběhu manické fáze ve srovnání s depresivní fází a se zdravými kontrolami. U pacientů v manické fázi došlo ve srovnání s kontrolními subjekty

k fázovému předběhnutí exprese hodinových genů, což poukazuje na zkrácení endogenní periody hodinového mechanismu. Současně také popsali zvýšení amplitudy exprese *Nr1d1* i *Per1* během manické epizody oproti epizodě depresivní (Novakova et al., 2015). V primárních buněčných kulturách fibroblastů od pacientů s BAP, které byly odebírány bez ohledu na epizodu, ve které se pacient nacházel, došlo ke snížení amplitudy u *Bmal1* a *Nr1D1* (Yang et al., 2009). Rozdílné nálezy v expresi *Nr1D1* ve dvou výše popsaných studiích lze obtížně srovnávat, design obou experimentů je odlišný, první experiment používá *in vivo* model na rozdíl od druhého, který pracuje s *in vitro* buněčnou kulturou. Dále došlo v kultuře fibroblastů k signifikantnímu snížení hladiny fosforylované formy GSK3β, tedy zvýšení její aktivity (Yang et al., 2009). GSK3β hraje důležitou roli v hodinovém molekulárním mechanismu. Za běžných podmínek aktivovaná GSK3β fosforyluje REV-ERBα (Yin et al., 2006), BMAL1 (Sahar et al., 2010), PER2 (Iitaka et al., 2005), CRY2 (Kurabayashi et al., 2010) i CLOCK (Spengler et al., 2009). Fosforylace jednotlivých komponent vede ke stabilizaci proteinových produktů hodinových genů, a tím se podílí na dynamice transkripčně-translační zpětnovazebné smyčky. Fosforylace na Serinu9 GSK3β vede k inhibici její aktivity a defosforylace tohoto místa naopak k její zvýšené aktivaci. Snížení hladiny fosforylované GSK3β může tedy zásadním způsobem ovlivňovat funkční stav zpětnovazebné transkripčně-translační smyčky molekulárního hodinového systému (Yang et al., 2009). Rozvoj bipolární poruchy typu I by mohl souviset i s SNP (z angl. single nucleotide polymorphism) v promotorové oblasti genu pro GSK3β. Dvě nezávisle běžící studie informovaly, že SNP (-50T/C) může ovlivnit klinickou odpověď na terapeutické podávání lithia (Adli et al., 2007; Benedetti et al., 2005b). Současně některé studie naznačují, že SNP GSK3β v homozygotní T/T formě by mohl souviset s ontogeneticky časnějším rozvojem tohoto onemocnění (Benedetti et al., 2004).

Nedávné studie využívající lymfoblastoidní buněčné linie získané od pacientů s BAP, potvrdily prodloužení délky periody exprese DBP ve srovnání s její délkou u kontrolních vzorků (Kittel-Schneider et al., 2015).

Souvislost cirkadiánního systému s BAP potvrzují i genetické studie popisující přímou spojitost polymorfismů hodinových genů s výskytem BAP. U hodinového genu *Per3* se ukázal vliv variability v počtu tandemových repetitivních sekvencí (VNTR) na rozvoj bipolární poruchy typu I. U pacientů, kteří nesou kratší, čtyřikrát se opakující repetitivní motiv homozygotně na obou alelách genu *Per3* (*Per3*[4/4]), se onemocnění projevuje v nižším věku než u pacientů nesoucí delší variantu v obou alelách genu *Per3* (*Per3*[5/5]) (Benedetti et al., 2008). Dále byl sledován výskyt SNP hodinového genu Clock u pacientů s BAP, kde došlo k záměně thyminu za cytosin v poloze 3111 genu (3111C/T). Ukázalo se, že u nositelů homozygotní C

alely byla zvýšena incidence BAP (Lee et al., 2010), nositelé vykazovali dvojnásobně častější recidivu manické či depresivní epizody (Benedetti et al., 2003), a byla pozorována také častější recidiva insomnických potíží (Serretti et al., 2003).

3.2 Unipolární deprese

Řada klinických studií potvrdila, že během depresivní epizody dochází u pacientů s UD ke změnám cirkadiánní rytmicity spánku a bdění (Mendlewicz, 2009b), ke změnám rytmické oscilace nálady (Wirz-Justice, 2008) a ke změně rytmické sekrece některých hormonů, např. melatoninu (Buckley and Schatzberg, 2010). Během depresivní epizody popisuje 80% pacientů insomnické příznaky, jako jsou problémy s usnutím nebo se setrváním ve spánku, dále velmi časné ranní probouzení a zkrácenou dobu nástupu REM fáze (Mendlewicz and Kerkhofs, 1991). Studie prováděná u pacientů s UD post-mortem prokázala oproti zdravým kontrolám vnitřní desynchronizovaný rytmus exprese hodinových genů v pěti sledovaných mozkových strukturách, kterými byly amygdala, prefrontální kortex, hipokampus, cingulární kortex a nucleus accumbens (Li et al., 2013). Dosud žádné studie neprokázaly souvislost mezi polymorfismy hodinových genů a unipolární depresí (Desan et al., 2000; Serretti et al., 2010). Exprese hodinových genů v periferních buňkách pacientů s UD nebyly doposud sledovány.

3.3 Schizofrenie

Narušené spánkové i další cirkadiánní rytmy jsou popisovány také u 80 % pacientů se schizofrenií (Monti et al., 2013; Wulff et al., 2012). Řada studií zjistila, že tito pacienti mají sníženou noční hladinu melatoninu (Bromundt et al., 2011; Monteleone et al., 1992; Wulff et al., 2006). Pravděpodobnou příčinu ukázala nedávná studie porovnávající velikosti epifýz magnetickou rezonancí. Tato studie poukázala na to, že u pacientů se schizofrenií dochází k signifikantnímu zmenšení epifýzy ve srovnání se zdravými kontrolními subjekty (Findikli et al., 2015). Genetické studie zabývající se výskytem specifických genových polymorfismů potvrdily spojitost schizofrenie se zvýšeným výskytem specifických SNP u hodinových genů *Clock* (T3111C), *Per1* a *Per3* (Mansour et al., 2006; Takao et al., 2007; Zhang et al., 2011), avšak případnými změnami amplitudy nebo fáze rytmu exprese hodinových genů se u pacientů se schizofrenií dosud nikdo nezabýval. Bylo ovšem prokázáno, že u těchto pacientů je signifikantně zvýšený výskyt duplikace VIPR2 genu v chromozómové oblasti 7q36 (Vacic et al., 2011). VIPR2 je gen kódující receptor pro vazoaktivní intestinální peptid, který hraje vý-

znamnou roli v komunikaci mezi populacemi neuronů v SCN a je stěžejní pro vznik integrovaných cirkadiánních oscilací celého SCN (Hastings et al., 2014).

5. Ovlivnění molekulárního mechanismu cirkadiánního systému léčbou psychiatrických onemocnění

K léčbě depresivních onemocnění se v některých případech používají léky nebo terapeutické postupy, u kterých bylo prokázáno, že přímo interagují s hodinovým molekulárním mechanismem. Některé používané látky ovlivňují funkci kináz, způsobují fázové posuny rytmů nebo interferují se světelnou synchronizací (Duncan et al., 1998; Li et al., 2012). Mezi tyto látky patří lithium, v poslední době se ukazuje, že takto působí i ketamin známý pro svůj rychlý antidepresivní účinek nebo dlouhodobě osvědčená léčba pomocí spánkové deprivace nebo terapie jasným světlem (Prasko, 2008). Ukazuje se, že cirkadiánní systém také ovlivňuje řada antidepresiv, především pak antidepresiva ze skupiny SSRI (z angl. selective serotonin reuptake inhibitors) interferující s modulačním serotoninovým systémem (Carvalho et al., 2009; Sprouse et al., 2006; Tan et al., 2007).

5.1 Lithium

Lithium je malý alkalický ion, který volně prochází buněčnou membránou. Standardně je ve formě chloridu lithného (LiCl) používán jako lék ke stabilizaci nálady a bývá často thymostabilizérem první volby pro pacienty s BAP. Podávání lithia prodlužuje cirkadiánní periodu jak na úrovni rytmu tělesné teploty, rytmu pohybové aktivity, rytmu spánku a bdění (Abreu and Braganca, 2015). V *in vitro* kulturách SCN byl prokázán jeho přímý účinek na periodu jednotlivých neuronů SCN. Měření rytmu jejich elektrické aktivity ukázalo, že neurony SCN prodlužují po aplikaci lithia svojí endogenní periodu (Abe et al., 2000; Johnsson et al., 1983).

Na molekulární úrovni působí lithium jako inhibitor GSK3β kinázy kompeticí o vazné místo pro hořčík. Aktivace GSK3β je závislá na přítomnosti ATP, zvýšená hladina Mg^{2+} vede naopak k inhibici aktivity GSK3β (Klein and Melton, 1996). Předpokládá se, že inhibice aktivity GSK3β způsobená podáním lithia hraje klíčovou úlohu v modulaci cirkadiánních rytmů. Přítomnost lithia v mediu s buněčnou kulturou lidských fibroblastů zvyšuje amplitudu exprese *Cry1* a *Per2*, a snižuje expresi *Per3*, *Cry2*, *Bmal1* a *Nr1D1* (Li et al., 2012; McCarthy et al., 2013; Osland et al., 2011). Změny hladin proteinových produktů

hodinových genů pravděpodobně přímo souvisí s fosforylační kinázovou aktivitou GSK3β. Inhibicí GSK3β způsobené lithiem nedochází ke stabilizující fosforylaci Nr1D1, což vede k její okamžité proteozomální degradaci (Yin et al., 2006). Prokazatelná změna funkce jedné komponenty molekulárního hodinového mechanismu může výrazně ovlivnit expresi ostatních hodinových genů a mohla by tak vysvětlovat jednotlivé změny sledované v předchozích experimentech. Nedávná studie pracující s lymfoblastoidními buněčnými liniemi prokázala, že chronické podávání lithia vede ke snížení exprese DBP (Kittel-Schneider et al., 2015). Předchozí studie s DBP knockoutovanými myšmi prokázaly, že absence DBP vede ke změnám jejich pohybové aktivity, rytmus spánku a bdění a zkrácení endogenní periody (Lopez-Molina et al., 1997). Řada pacientů s BAP na léčbu lithiem neodpovídá. Zdá se, že odpověď na léčbu lithiem může souviset i s výskytem konkrétního polymorfismu některých hodinových genů. V europoidní populaci byl prokázán signifikantně zvýšený výskyt polymorfismu *Cry1* varianty rs8192440 a *Nr1D1* varianty rs2071427 u pacientů odpovídajících na léčbu lithiem (McCarthy et al., 2011).

5.2 Ketamin

Ketamin byl využíván jako disociativní anestetikum v dětské chirurgii. V posledních letech je experimentálně ve formě infuze podáván pacientům s unipolární depresí a pacientům s bipolární depresí. Výsledky tohoto zásahu prokázaly výrazný okamžitý antidepresivní efekt ketaminu u obou typů onemocnění. Zlepšení nálady vykazovali pacienti už po 40 minutách od podání ketaminu, ovšem délka antidepresivního účinku u většiny pacientů nepřesáhla 1-2 týdny (Berman et al 2000). Mechanismus tohoto rychlého, avšak přechodného antidepresivního účinku ketaminu není dosud zcela objasněn. Ketamin je farmakologicky definován jako nekompetitivní antagonista NMDA glutamátových receptorů, váže se do fencyklidinového vazebného místa a blokuje tak funkci NMDA receptorů. Světelná synchronizace SCN je závislá na glutamátergní signalizaci z RHT, účinek ketaminu tedy logicky snižuje odpověď hodin na světelný stimul a snižuje tak i glutamátergní signalizací aktivovaný nárůst hladin mRNA hodinových genů *Per1* a *Per2* (Abe et al 1992, Paul et al 2003). V neuronálních buněčných kulturách (NG108-15) bylo prokázáno, že ketamin inhibuje cirkadiánní remodelaci chromatinu v oblasti E-box motivu nacházejícího se v promotorové oblasti řady hodinových genů, a tím snižuje schopnost vazby aktivačního CLOCK/BMAL1 komplexu (Bellet et al., 2011). Vlivem ketaminu může tedy docházet v důsledku remodelace heterochromatinu k modulaci exprese hodinových genů indukované jak heterodimerem CLOCK-BMAL1, tak ke změnám světlem, respektive glutamátem indukované exprese

hodinových genů *Per* vazbou CREB na CRE element jejich promotorové oblasti (Bellet et al., 2011; Ripperger and Schibler, 2006). Zdá se, že v mechanismu účinku ketaminu na cirkadiánní systém je zapojena i GSK3β. Specifickým zablokováním její aktivity dojde ke snížení účinku ketaminu na expresi hodinových genů (Bellet et al., 2011). Vliv ketaminu na molekulární hodinový mechanismus v buněčných kulturách ukazuje na jeho potenciál v regulaci CS. Správné načasování jeho podání by mohlo napomoci resynchronizaci narušeného CS u depresivních pacientů. Je možné, že harmonizace CS by mohla prodloužit dobu jeho působení v organismu a zesílit jeho antidepresivním účinek.

5.3 Spánková deprivace

Celá řada studií opakovaně potvrdila úspěšnost léčby depresivních symptomů pomocí spánkové deprivace. K léčbě se využívá buď totální deprivace, při které je pacient 48h udržován bdělý, nebo částečná, kdy je pacientovi zkrácena doba spánku a v závislosti na typu studie či léčby je tak udržována po několik dní až týdnů (Wirz-Justice and Van den Hoofdakker, 1999). U 40-60% pacientů bylo při této léčbě potvrzeno signifikantní zlepšení nálady (Benedetti et al., 2007; Wu and Bunney, 1990). Ovšem depresivní symptomy se po vysazení spánkové deprivace a dostatečném následném spánku mohou rychle vrátit. Současně může také spánková deprivace fungovat jako spouštěč depresivní nebo manické epizody u BAP. Jak působí akutní spánkové deprivace na cirkadiánní systém, nebylo dosud zcela objasněno. Bylo prokázáno, že účinkem spánkové restrikce dojde k 1,9% redukci všech cirkadiánních transkriptů v krvi, a pokud je spánek přesunut z noci do denní fáze, 97% rytmicky exprimovaných genů ztrácí svojí rytmicitu a třetina veškerých genů vykazuje fázové posuny v jejich rytmické expresi (Archer and Oster, 2015). Ackermann et al. (2013) ve své studii detailně popsali expresi 12 hodinových genů v krvi u zdravých subjektů. U subjektů nebyly zaznamenány žádné výrazné změny rytmu v jejich expresi, u většiny z nich ovšem došlo po spánkové deprivaci k mírnénu snížení amplitudy. Pouze u hodinového genu *Bmal1* došlo k jejímu signifikantnímu snížení ve srovnání se spánkově nedeprivovanými kontrolami (Ackermann et al., 2013). Studie Kavcic et al. (2011) popsala po spánkové deprivaci ztrátu rytmické exprese hodinového genu *Per2* (Kavcic et al., 2011). Na změně transkriptomu způsobeného spánkovou deprivací se pravděpodobně budou podílet i některé epigenetické mechanismy. Bylo prokázáno, že akutní spánková deprivace signifikantně zvyšuje metylaci v promotorové oblasti *Per1* a *Cry1* v tukové tkáni člověka, což vede ke snížení exprese těchto genů (Cedernaes et al., 2015). Na základě těchto studií se usuzuje, že spánková deprivace, podobně jako lithium či ketamin, ovlivňuje chod molekulárního hodinového systému, který

následně mění nastavení celého CS organismu. Tyto změny mohou následně hrát důležitou roli v terapii a při vhodném časování mohou přispívat k antidepresivnímu účinku terapeutik, při nesprávně načasované aplikaci by mohly zvýšit desynchronizaci a zpomalit působení terapeutika.

Závěr:

Celá řada studií se snaží definovat vztah mezi cirkadiánními rytmy a afektivními poruchami nebo schizofrenií. Experimenty sledující rytmy na behaviorální úrovni změny rytmického chování pacientů nezpochybnitelně prokázaly, ovšem komplexní porozumění tomuto vztahu nám dosud stále uniká. Hodinové geny hrají stěžejní úlohu v udržení vnitřní cirkadiánní rytmicity, změna jejich rytmické exprese a následná desynchronizace periferních a centrálních hodin vede k patologickým metabolickým procesům, které následně mohou ústit v rozvoj depresí. Ovšem důkazy potvrzující změny cirkadiánních rytmů u depresivních pacientů na molekulární úrovni jsou stále spekulativní a celá řada otázek zůstává stále nezodpovězena. Odpovědi na některé z nich by mohly přinést rozsáhlejší genetické studie a v poslední době významně se rozvíjející pole epigenetiky. Problém, se kterým se řada studií zabývající se pacienty s afektivními poruchami potýká, je obtížná diagnostika onemocnění. Afektivní poruchy nemají jednoznačnou etiologii a stejná diagnóza často zahrnuje pacienty se škálou různých projevů onemocnění. Skupiny pacientů v experimentech tak většinou nereprezentují endofenotypově uniformní skupinu. Toto je pravděpodobné vysvětlení, proč různé výzkumné skupiny prezentují výrazně variabilní výstupy svých studií.

Sledování cirkadiánní rytmicity pacientů může mít významné klinické využití. Cirkadiánní změny v pohybové aktivitě nebo ve spánkovém rytmu mohou sloužit jako prediktory relapsu u BAP (Ohayon and Roth, 2003) nebo u i schizofrenie (Spaniel et al., 2008). Současné metody umožňují přímý vhled do molekulárního aparátu cirkadiánních hodin. Řada vědeckých týmů se věnuje genetickým a epigenetickým analýzám variability hodinového mechanismu u afektivních poruch či schizofrenie. Výsledky jejich práce by mohly usnadnit léčbu těchto poruch zapojením chronoterapeutických přístupů. Hojně využívaný stabilizátor nálady lithium je po dlouhou řadu let využíván k experimentálním manipulacím s CS u animálních modelů. Stejně tak účinek ketaminu a spánkové deprivace zasahuje významně cirkadiánní systém. Výzvou pro budoucí studie je odhalit přesný mechanismus účinku těchto léčiv a především najít časové „okno" v průběhu cirkadiánní periody, kdy je cirkadiánní

systém připraven správně reagovat a vyvolat žádoucí terapeutický zásah do nastavení vnitřních hodin.

Velkým tématem mnoha studií je otázka, do jaké míry může časté narušování přirozené rytmicity, např. prací na směny, přelety přes časová pásma, silným nočním osvětlením, působit jako spouštěč duševních onemocnění u citlivých osob. Pracoviště zabývající se výzkumem příčin vzniku řady somatických poruch, jako je diabetes typu II, obezita, hypertenze či různých druhů karcinomů nalezla v posledních letech zcela zásadní vazby mezi cirkadiánními poruchami a těmito závažnými onemocněními. Cirkadiánní systém tak hraje důležitou roli v celé řadě onemocnění a cílení na stabilizaci cirkadiánních rytmů nebo resynchronizaci CS by mohlo vést ke zlepšení celkové fyzické i psychické kondice pacienta.

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RESEARCH ARTICLE

Moderate Changes in the Circadian System of Alzheimer's Disease Patients Detected in Their Home Environment

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease often accompanied with disruption of sleep-wake cycle. The sleep-wake cycle is controlled by mechanisms involving internal timekeeping (circadian) regulation. The aim of our present pilot study was to assess the circadian system in patients with mild form of AD in their home environment. In the study, 13 elderly AD patients and 13 age-matched healthy control subjects (the patient's spouses) were enrolled. Sleep was recorded for 21 days by sleep diaries in all participants and checked by actigraphy in 4 of the AD patient/control couples. The samples of saliva and buccal mucosa were collected every 4 hours during the same 24 h-interval to detect melatonin and clock gene (*PER1* and *BMAL1*) mRNA levels, respectively. The AD patients exhibited significantly longer inactivity interval during the 24 h and significantly higher number of daytime naps than controls. Daily profiles of melatonin levels exhibited circadian rhythms in both groups. Compared with controls, decline in amplitude of the melatonin rhythm in AD patients was not significant, however, in AD patients more melatonin profiles were dampened or had atypical waveforms. The clock genes *PER1* and *BMAL1* were expressed rhythmically with high amplitudes in both groups and no significant differences in phases between both groups were detected. Our results suggest moderate differences in functional state of the circadian system in patients with mild form of AD compared with healthy controls which are present in conditions of their home dwelling.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder causing a variety of irreversible cognitive impairments leading to dementia. Apart from memory deficits [1], AD pathological symptoms involve impairments in regulation of various physiological processes, including circadian regulations of behavior, sleep patterns and hormonal secretion [2]. These physiological functions are temporally controlled by a circadian system which consists of the central clock in the suprachiasmatic nuclei (SCN) and peripheral clocks in neuronal and non-neuronal cells

and tissues [3–5]. The central SCN clock drives systemic rhythms, mainly sleep/wake cycle and rhythm in pineal hormone melatonin levels [6], and synchronizes the peripheral clocks which drive rhythmically the tissue specific physiological programs [3]. The circadian signal is generated at the cellular level via autonomous molecular mechanism which drives rhythmically expression of clock genes, namely *PER1,2*, *CRY1,2*, *REV-ERBα*, and *BMAL1*. As a result of the molecular clock mechanism, circadian expression of *PER1,2*, *CRY1,2*, and *REV-ERBα* is in anti-phase to that of *BMAL1* (reviewed in [7]).

Among people over 65 years old, more than 80% suffer from abnormalities in sleep/wake rhythmicity [8–10]. The function of the circadian system changes with age with an individually variable progression speed in elderly people even without AD pathology [11]. Therefore, it might be difficult to distinguish between the age- and AD-related modifications in circadian regulation These age-related changes of the circadian system involve alterations in amplitudes and phases of circadian rhythms [12], as well as changes in timing of the sleep/wake cycle with respect to the circadian cycle, i.e., shortening the phase angle of entrainment [13, 14]. In AD patients, incidence of sleep/wake cycle disturbances was found to be higher compared to age-matched controls. They mostly exhibit exacerbated disruption of sleep, such as fragmented nighttime sleep and a higher frequency and duration of nighttime awakenings and daytime sleep episodes (naps) [9, 15, 16]. Importantly, disruption of the sleep/wake cycle was diagnosed in AD patients already in mild and moderate stages of the disease [17].

Due to the SCN control, production of hormone melatonin exhibits pronounced circadian rhythms so that its levels are high during the subjective night and very low during the subjective day [18]. In healthy elderly people, the circadian rhythm in melatonin levels was dampened because their nocturnal melatonin secretion was decreased [19]. However, this issue is rather controversial because other studies did not confirm that reduction of plasma melatonin concentration is a general characteristic of healthy aging [20, 21]. In AD patients, more pronounced decrease in melatonin secretion was detected at early stages of the disease when their cognitive functions were still intact [22] and the rhythm dampening correlated with the AD neuropathology progression [22, 23]. These results suggest that in AD patients, the circadian function of the central SCN clock which drives rhythms in the aforementioned functions, might deteriorate further beyond that of what happens in elderly without the AD pathology [24].

It is still not known whether the worsening of the circadian regulation in AD is due to changes of the SCN morphology and function or due to changes of functions downstream the central clock. The age-dependent changes in the SCN were detected in healthy elderly but they occurred earlier and were more pronounced in AD patients [25–32]. However, whether the morphologic pathology causally accounts for the circadian SCN dysfunction is not clear. It is possible that the SCN clock mechanism itself is not affected in AD patients but the nuclei are disconnected from the rest of the brain. Such disconnection would result in aberrant circadian regulation of the downstream brain areas which contain subordinate circadian clocks. For example, in healthy subjects, clock gene expression was found to be rhythmic in the bed nucleus of stria terminalis (BNST), the cingulate cortex and the pineal gland [33, 34]. In the brains of AD patients, most of these clocks also exhibited well pronounced 24-hour rhythmicity, however, their mutual synchronization was altered compared to controls [35]. In contrast, the rhythms in clock gene expression in pineal glands were completely lost in both clinical and preclinical AD patients [33].

As mentioned above, the aberrant circadian regulation was shown to precede clinical onset of memory deficits in AD (reviewed in [36]) and might be associated with AD development [37], Therefore, the present study was aimed to find out whether disruption of the circadian system, more pronounced than that caused by physiological aging, is present in patients with

the mild form of AD. Specifically, the study was designed to reveal whether the circadian disruption is present during the everyday life of patients who do not require hospitalization and live in their home environment. To achieve this, the couples of age-matched patients and their healthy spouses (who lived together and took care of them) were examined. The functional state of the circadian system was assessed based on the analysis of daily profiles of behavioral patterns, melatonin levels in saliva and clock gene expression in the peripheral cells of buccal mucosa.

Materials and Methods

Participants

In the study, 13 healthy subjects (6 females and 7 males) and 13 AD patients (7 females and 6 males) were enrolled. The subjects of both groups were matched for age (mean ± S.E.M.; controls: 78.1 ± 2.0 years, AD patients: 78.9 ± 1.9 years) and education level (mean ± S.E.M. of years of schooling; controls: 14.5 ± 2.0, AD patients: 14.0 ± 3.0). The exclusion criteria for participation in the study were traveling across the time zones or working night shifts one month or less before the beginning of the study. For inclusion of patients in the study, the willingness of their spouses living in the same home environment to cooperate in the study was conditional.

The AD patients were recruited from the outpatients of the Memory Clinic, AD Center, Charles University in Prague, Czech Republic. The diagnosis was based on diagnostic guidelines using clinical evaluation in combination with results of brain magnetic resonance imaging (hippocampal atrophy), single photon emission computed tomography (SPECT, temporo-parietal hypoperfusion) or analysis of cerebrospinal fluid concentrations (abnormal for amyloid-beta, total tau protein or phosphorylated tau protein) ([38]). They had dementia due to the AD; their cognition, examined with the MMSE, was significantly impaired when compared to the controls (21 ± 3 and 29 ± 1 points, respectively). All AD patients were on stable treatment of antidementia drugs (acetylcholinesterase inhibitors, memantine). Prior to and during the sampling period, the AD patients were free of sleep medication; only one subject reported previous using of sleep medication which was withdrawn during the testing period. The clinical and biomarker characteristics of 13 patients with Alzheimer disease involved in the study are summarized in S1 Table.

The controls included the elderly patient's spouses of similar age and gender proportion as the AD patients group. They were exposed to the same photic and non-photic environmental cues of their home dwellings long before and throughout the study. The spouses had to be without diagnosis of AD or other form of dementia according to clinical judgment based on long-term observations during visits with their patients prior to this study. They had normal cognitive functions assessed with the MMSE (Mini-Mental State Examination) [39] and the clock drawing test. They reported good health, were free of medication before and throughout the study and had no sleep problems; only one control subject reported previous use of a sleep drug which was withdrawn during the testing period. The controls helped patients with sampling and checked that it was performed correctly (especially important for collection of buccal samples) and at proper time intervals.

Most subjects of both groups had age-adequate deterioration in eye function, they wore glasses (10 controls and 13 AD patients) and some of them reported cataract (8 controls and 7 AD patients).

Ethical statement

The patients and control subjects were informed in detail about the purpose and procedures of the study and signed an informed consent form. The protocol and the consent form were in agreement with the Declaration of Helsinki and were approved by the Ethics Committees of the Institute of Physiology, the Czech Academy of Sciences and of the University Hospital Kralovske Vinohrady Prague Psychiatric Centre, Alzheimer Disease Centre, Czech Republic.

Protocol of the study and sample collection

The protocol of the study is summarized in Fig 1. Before beginning of the study, the participants were informed about the basic scientific background and the experimental design of the study at an instructional meeting. Especially, importance of light intensity control on the sampling day was explained. To check whether the instructions were understood and feasible to follow, the authors (KW) visited the subjects' homes to help and/or advice them with adjustments in order to ensure that the light intensity during the night when the sampling was performed did not exceed the limit (see below). The controls and AD patients were asked to maintain their normal sleep schedule throughout the entire study. All subjects were examined during the same period of April—May 2013.

The study started with sleep/wake cycle recording using individual sleep diaries in all study participants (the diaries of AD patients were recorded by their spouses) and Acti-watches in 4 randomly selected couples for 21 days prior to sampling. Thereafter, collection of samples from all study participants was accomplished within one day (the same day for all participants) in the patient/control couple's homes. This arrangement ensured exactly the same outside seasonal and lighting conditions during the study for all subjects. On the day of sampling, the sunset occurred at 21:02 and the sunrise at 4:57. The ceiling lights were turned off during the habitual sleeping time and turned on during the habitual waking time. The light exposure during the night was controlled and was the same for the controls and patients; dim light was provided by the bedside lamp covered with a fabric and was controlled not to exceed 20 lux (measured by luxmeter at the time of the researcher visit). Light of this intensity did not to affect melatonin levels in our previous studies [40−43]. During the entire 24-h interval of sampling, the subjects were not allowed to drink alcoholic and caffeinated beverages, use chewing gum and brush their teeth. Moreover, for 1 h before each sampling, they did not consume food or drink. The subjects provided saliva samples directly into test tubes that were stored in the fridge overnight. Thereafter, in the morning the samples were transferred to the laboratory and stored at −20°C until assay. Immediately after providing the saliva, buccal mucosa samples were collected by gently scratching the inner cheek on both sides using a cytological brush. The oral mucosa samples were immediately placed into RNA-later reagent (Sigma-Aldrich, St. Louis, USA) and maintained at room temperature overnight. In the morning following the sampling, they were transferred to the laboratory together with the saliva samples and stored at −20°C until analysis. The sampling of saliva and buccal swabs began at 07:00 and continued every 4 h throughout the 24 h until 07:00 on the following day; the last sampling at 07:00 was performed in darkness. Additional time points when only saliva but not buccal scrubs were collected occurred at 21:00 and 09:00 h. This protocol provided more frequent sampling of saliva around the time of expected melatonin rise and decline and, at the same time, prevented damaging the oral mucosa by too frequent brushing. The sampling procedure was tolerated by all participants without any complaints.
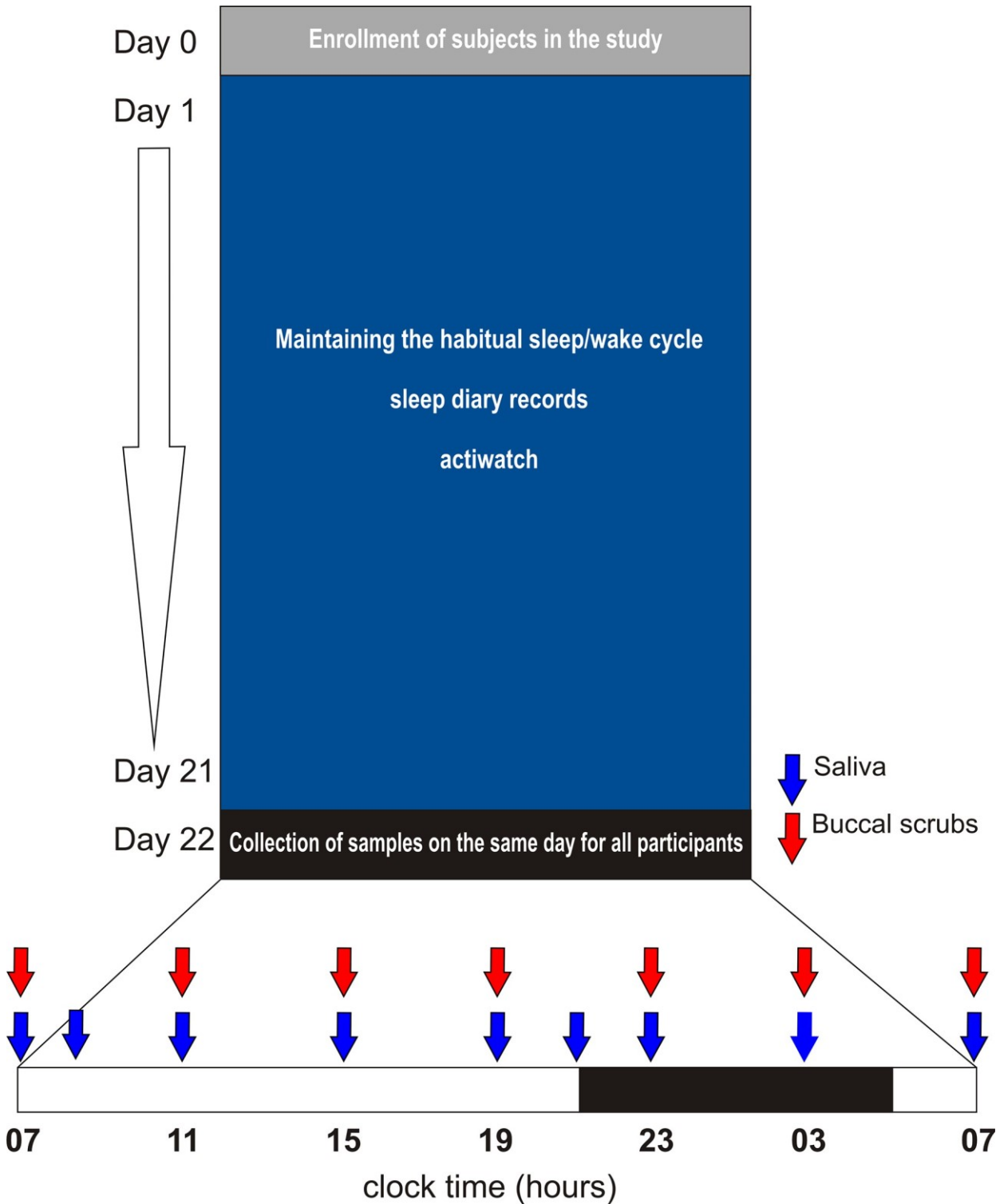
Fig 1. Protocol of the study. The study began (day 1) with recording of sleep/wake schedule in diaries (all participants) together with Actiwatch monitoring (4 couples) for 21 days. During the monitored period, the subjects maintained their habitual sleep/wake regime. Thereafter, the samples were collected throughout the 24 h on the same day from all subjects. Time when saliva (blue arrows) and buccal mucosa samples (red arrows) were collected is depicted. The dark bar on the time scale corresponds to hours between the sunset and the sunrise on the sampling day. For more details, see Materials and Methods.

doi:10.1371/journal.pone.0146200.g001

Activity and sleep/wake recording

Sleep/wake schedules were recorded for all participants in sleep diaries during 21 days preceding the sampling (one couple was able to keep the record only for 17 days). The subjects were asked to log the time intervals (30 min resolution) when they had been asleep during 24 h. The records were analyzed manually to determine timing of the sleep onset and offset in order to count the sleep duration as well as to identify daytime sleep episodes, i.e., naps (see below).

The subjective recordings of sleep/wake cycle by diaries obtained from all study participants were checked in 4 randomly selected patient/control couples who wore Actiwatch devices (AW4 model; Cambridge Neurotechnology Ltd, UK) on their non-dominant hands during the 21 days. Unfortunately, the number of Actiwatches which could be used in the study, was limited to 8, and therefore, it was not possible to monitor all participants of the study. The device recorded movement in 1-min bins and the data were analyzed by Actiwatch Activity & Sleep Analysis V 5.42 software (Cambridge Neurotechnology Ltd, UK). The activity patterns were graphically presented as daily records (actigraphs) throughout the 21 days. For better clarity, the records were double plotted, i.e., each line of the actigraphs represented two consecutive days and the next line started with the record of the previous day. The data of Actiwatch analysis provided information on the overall daily activity profiles, sleep parameters and daytime naps. For the overall daily activity profiles, the results were expressed as sums of 1 min activity epochs recorded within 30-min intervals throughout the 24 h. The data for each interval were cumulated for the 21-day recording period separately for controls and AD patients and were expressed as a mean activity ± S.E.M. of each of the 30-min values. For sleep duration and sleep quality analysis, following parameters were analyzed: i) *assumed sleep duration*—i.e., the time interval between falling asleep and waking, based on the data from diary; ii) *number/duration of wake bouts*—i.e., the number and duration of episodes which the algorithm recognized as a state of wakefulness; iii) *percentage of real sleep duration* —i.e., percentage of the real sleep (without waking episodes) of the entire sleep duration; iv) *sleep efficiency*—i.e., the percentage of sleep duration of the entire time spent in the bed; v) *fragmentation index*—i.e., the percentage of 1-min intervals of immobility vs. number of all 1-min intervals during the sleep period. For the nap analysis, the software setting determined a nap as an interval of inactivity longer than 15 min during the time of day that the subject designed as "active" daytime when being awake (based on diary data). To distinguish between immobility in vigilance state, the interval marked as naps by the software were cross-checked with the records in the diaries.

Melatonin assay

A direct double-antibody radioimmunoassay was used for the melatonin assay (Bühlmann Laboratories, Allschwil, Switzerland) as previously described (26). The kit was used according to the manufacturer's instructions. The analytical sensitivity was 0.2 pg/ml. The intra-assay coefficient of variation was 3% for samples of 18.5 ± 1.0 pg/ml and 4% for samples of 2.4 ± 0.2 pg/ml. The inter-assay coefficient of variation was 12% for samples of 18.5 ± 1.0 pg/ml and 14% for samples of 2.4 ± 0.2 pg/ml. The melatonin levels at each time point were expressed in pg/ml as mean ± S.E.M.

Determination of clock gene expression by quantitative real-time polymerase chain reaction (RT-qPCR)

As described previously [41], mRNA was isolated using the Dynabeads mRNA Direct Micro Kit (Invitrogen, Carlsbad, California, USA) and the whole mRNA sample was reverse-

transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) in 10-µl reactions incubated at 42°C for 1 h. The cDNA was then diluted 1:2 with RNase-free water and 2 µl was used to determine gene expression in 16 µl qPCR reaction. Each reaction also contained 10.3 µl of PCR-grade water, 3.1 µl of HOT FIREPol Probe qPCR Mix Plus (Solis BioDyne, Estonia) and 0.6 µl of TaqMan Gene Expression Human FAM-MGB assay (Life Technologies, CA, USA) specific for the following genes: *Period 1* (*PER1*, NM 002616, cat. no. Hs01092603_m1), *Aryl hydrocarbon receptor nuclear translocator-like* (*ARNTL*, syn. *BMAL1*, NM 001178, cat. no. Hs00154147_m1), *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, NM 002046, cat. no. Hs99999905_m1) and *Ribosomal protein large P0* (*RPLP0*, NM 001002, cat. no. Hs99999902_m1). The reaction were amplified in a sealed 96-well microplate using ViiA 7 Real-Time PCR system (Life Technologies, CA, USA) with default cycling conditions for TaqMan assay (50°C—2 min; 95°C—10 min; 50x 95°C—15 sec, 60°C —1 min). Resulting amplification curves were analyzed using ViiA7 Software 1.1 (Life Technologies, CA, USA). Negative control without the cDNA showed no amplification. To generate calibration curves and compare differences between samples, a serially diluted cDNA from total RNA isolated from cultured human primary fibroblasts was used. The levels of expression of *PER1* and *BMAL1* were normalized to the mean expression of both reference genes (*GAPDH* and *RPLP0*).

Statistical Analysis

The data of activity levels for AD patients and healthy controls were plotted as the mean ± SEM for each 30-min bin of the 24 h interval during the 21 days of recording. The profiles in both groups were compared using the repeated measures 2-way ANOVA. The parameters from the sleep quality and nap analyses were averaged for each group and compared between controls and AD patients by the Student's t-test corrected for multiple comparisons.

The data for melatonin levels at each time point were plotted as mean ± SEM for each group and compared by repeated measures 2-way ANOVA. The individual melatonin profiles were analyzed by cosinor analysis (see below) to assess the presence/absence of daily rhythms in each subject. Also, area under the curve was calculated for each individual melatonin profile, averaged for the group of healthy subjects and AD patients and the values were compared between the groups by t-test.

The data for clock gene expression were expressed as mean ± SEM for each group and analyzed by cosinor analysis (see below). The acrophases of the profiles were compared by t-test. The expression profiles of controls and AD patients were compared by repeated measures 2-way ANOVA.

Cosinor analysis: The data were fitted with two alternative regression models: either a horizontal straight line (null hypothesis) or a single cosine curve (alternative hypothesis) as defined by the equation $Y = mesor + [amplitude \times cos(2 \times \pi \times (X-acrophase)/period)]$ with a constant period of 24 h. The extra sum-of-squares F test was used for comparison, and cosine curve parameters, such as amplitude (*i.e.*, the difference between the peak or trough and the mean value of a cosine curve), acrophase (*i.e.*, the phase angle of the peak of a cosine curve) and the coefficient of determination R^2 (*i.e.*, goodness of fit) were calculated unless the P value exceeded 0.05.

The statistical, information theory and least-squares regression methods implemented in Prism 6 software (GraphPad, La Jolla, USA) were applied. Where appropriate, data were checked for normal distribution.

Results

Total inactivity period during 24 h and number of daytime naps are increased in AD patients

For analysis of the overall rest/activity and sleep/wake daily patterns and of the nighttime and daytime sleep parameters, data from sleep diaries (13 controls and 13 AD patients) and/or from Actiwatch record analyses (4 controls and 4 AD patients) were used.

Sleep time during the 24-h period was analyzed in all participants of the study based on records in their sleep diaries. The total time of sleep duration during the 24-h period (Fig 2A), expressed as mean ± SEM of the 21 day recording period, was 7.3 ± 0.4 h in controls (n = 13) and 10.4 ± 0.8 h in AD patients (n = 13). In AD patients, the mean total sleep duration was significantly longer compared to controls (Student's t-test, P = 0.002). Additionally, daily activity/rest profiles cumulated for the 21 days of the recording period obtained from the Actiwatch records of 4 patient/control couples are depicted in Fig 2B. The repeated measures two-way ANOVA revealed significant effect of time (F = 17.020; P < 0.0001) confirming the presence of daily variation in activity for both groups; however, these activity/rest profiles did not differ significantly (F = 0.394; P = 0.553) between both groups.
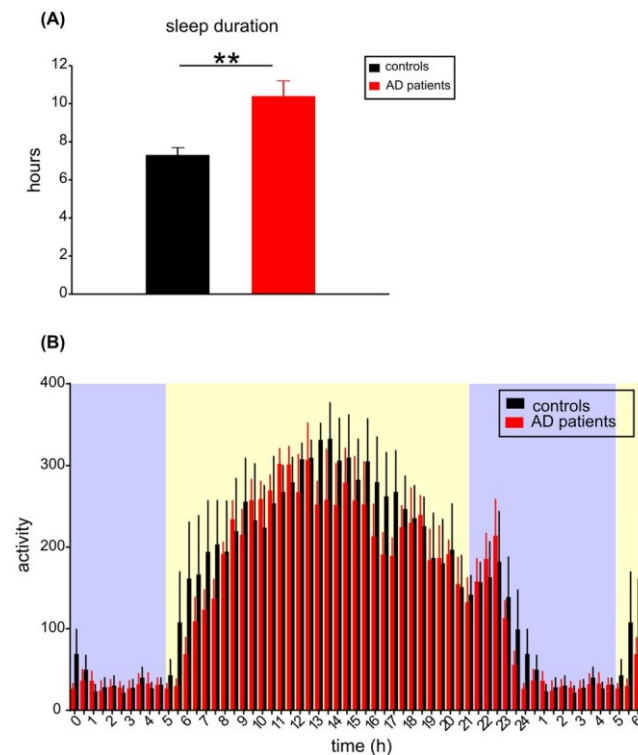

Fig 2. Analysis of sleep and activity rhythms of controls and AD patients. (A) Total sleep duration of the 24-h period (in hours) was assessed by the diary data (mean ± SEM) collected during the 21 day recording period from 13 controls (black column) and 13 AD patients (red column). In AD patients, the mean total sleep duration was significantly longer compared to controls, ** P = 0.002; (B) The mean daily activity profiles of AD patients and controls. The activity was recorded by Actiwatch during 21 days and the cumulated activity levels (mean ± SEM) in 30 min bins throughout the day and night are depicted; for clarity, part of the day (00:00 to 06:00 h) was re-plotted. The activity levels in 4 controls (black columns) and 4 AD patients (red columns) are depicted. The blue and yellow areas on the graph background correspond with time of the environmental darkness and daylight, respectively, as occurred during the recording period. X axis represents clock time (hours). For more details, see Material and Methods.

doi:10.1371/journal.pone.0146200.g002

The analysis of sleep parameters was performed by the Actiwatch software, and/or using records of the sleep diaries, separately for the night sleep and the daytime naps. The interval designated in the diaries by the subjects as time of falling asleep at night and waking up in the morning was considered the nighttime sleep and the intervals designated by the subjects as short sleep during the day were considered as naps. In one AD patient (AD#103; data not shown), the sleep was fragmented, occurring in regular intervals throughout the day and night and, therefore, the nighttime sleep and daytime naps could not be distinguished. The couple of the AD#103 patient/control was thus excluded from this part of analysis. For all other subjects, the nighttime sleep was more or less consistent and clearly distinguishable from the sporadic daytime napping. The actigraphs of 4 control/AD patient couples recorded throughout the 21-day period are depicted in Fig 3; the intervals designated in the sleep diaries as the nighttime sleep and the daytime naps are marked. One of the 4 couples reported not having naps (see below).

The mean assumed nighttime sleep duration determined from the sleep diaries was 7.3 ± 0.3 h (n = 12) in controls and 9.1 ± 0.7 h (n = 12) in AD patients. For the AD patient/control couples, whose sleep parameters were analyzed by the Actiwatch software, it was 7.8 ± 0.6 h (n = 4) in controls and 9.4 ± 0.4 h (n = 4) in AD patients. Although both measurements showed longer assumed sleep duration in AD patients than in controls, the differences between both groups were not statistically significant, be they detected by the diary records or Actiwatch software (Student's t-test; P = 0.056 and P = 0.071, respectively). The analysis of the sleep parameters by the Actiwatch software detected the number and duration of wake bouts, percentage of real sleep duration, sleep efficiency and fragmentation index (see Methods for definition). As demonstrated in Fig 4A, none of the sleep parameters were significantly different between both groups and there was only a trend in AD patients towards slightly longer assumed sleep duration (P = 0.071). The sleep efficiency and sleep fragmentation also did not differ significantly between both groups (P = 0.550 and P = 0.569).

The mean number of daytime naps during the 21-day recording period was counted for each subject based on the data from the sleep diaries. Most subjects of both groups reported napping; frequency of the naps was variable within and among all subjects and only one of the 13 AD patients and 5 of the 13 controls reported not having daytime naps throughout the 21- day period of recording. The mean number of daytime sleep bouts in AD patients was significantly higher (0.81 ± 0.23, n = 12) than in controls (0.25 ± 0.13. n = 12) (Student's t-test; P = 0.046). The same borderline significance was reached also for data acquired from Actiwatch analysis (Student's t-test; P = 0.041) where the mean number of daytime naps was significantly higher in AD patients (1.76 ± 0.61, n = 4) than in controls (0.17 ± 0.05, n = 4). Moreover, the Actiwatch analysis suggested that duration of naps might be longer in AD patients (0.62 ± 0.32 h, n = 4) than in controls (0.12 ± 0.02 h, n = 4), but the difference was not statistically significant (Student's t-test; P = 0.170).

The distribution of individual data for sleep parameters and daytime naps in each of the 4 control/AD patient couples is depicted in Fig 4B and 4C. The data revealed that in 3 out of the 4 couples, the patients had lower or the same sleep efficiency and higher fragmentation index compared with their corresponding control spouses. Nevertheless, even in controls the individual values of both markers demonstrated high variability. Interestingly, in one couple, the AD patient had a better nighttime sleep quality than the control (his sleep efficiency was higher and fragmentation index was lower); however, this one AD patient exhibited also the highest mean number of daytime naps compared with other AD patients who exhibited poorer sleep quality based on their sleep efficiency and fragmentation index (Fig 4C). Therefore, the better night sleep quality in this patient was not in parallel with less sleep during the daytime, it rather reflected higher total sleep time during the 24 h. In summary, from comparison of the
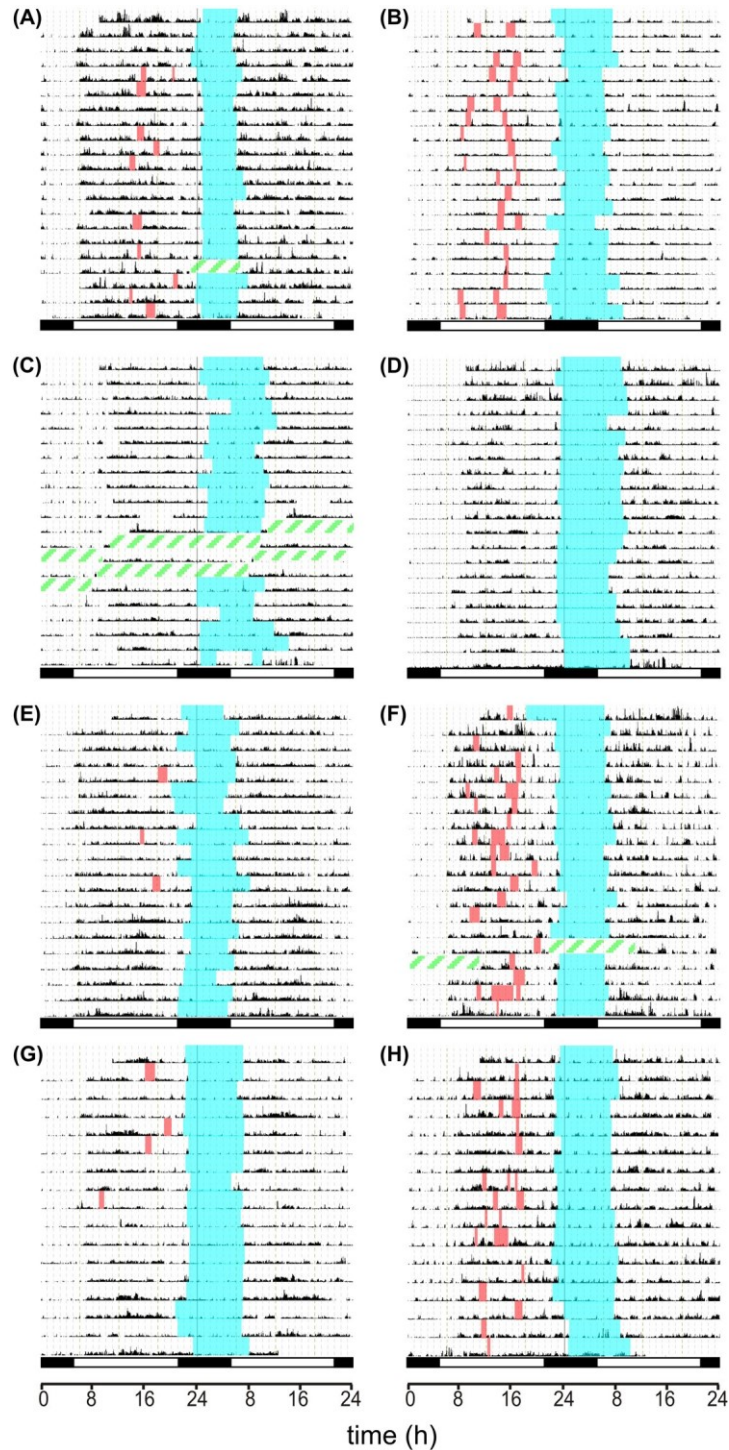
Fig 3. Individual actigraphs of the control/AD patient couples. Actigraphs from controls (A,C,E,G) and AD patients (B,D,F,H) are depicted, representing the AB, CD, EF and GH couples. The activity was recorded during 21 days (A-F) or 17 days (G,H). The record of activity (black area) from the Actiwatch analysis was completed with manual marking of nighttime sleep periods (blue area) and daytime naps (orange area) according to data from the individual sleep diaries. The green dashed areas represent intervals when the device was notoperating.

doi:10.1371/journal.pone.0146200.g003
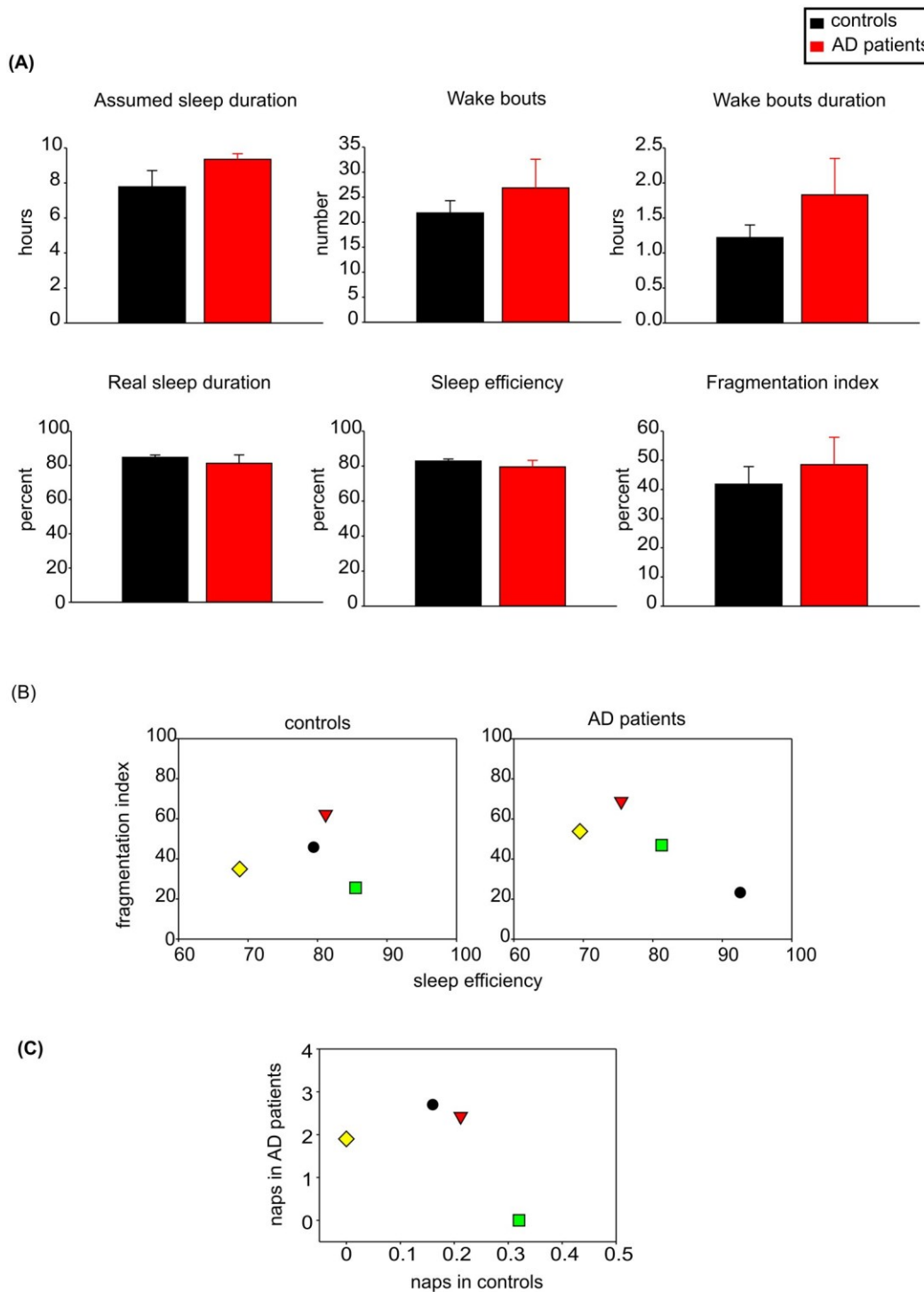
Fig 4. Nighttime sleep parameters and naps of controls and AD patients. (A) The nighttime sleep parameters (mean ± SEM), i.e., the length of the assumed sleep duration (hours), the number of awake episodes, the duration of awake episodes (hours), the real sleep duration (percent), the sleep efficiency (percent) and the fragmentation index (percent) were detected by Actiwatch analysis and compared between the group of 4 controls (black columns) and 4 AD patients (red columns); (B) The values of the sleep efficiencies and fragmentation indexes of the individual controls (left side) and AD patients (right side). For identification of the control/AD patients couples, the subjects of each couple are depicted with the same symbol (couple 1: yellow diamond, couple 2: black dot, couple 3: red triangle, couple 4: green square); (C) Mean number of daytime naps as detected by Actiwatch analysis (and confirmed by the sleep diaries records) for the control/AD patients couples. The symbols identifying the couples correspond to those depicting the nighttime sleep parameters in B). For more detail, see Material and Methods.

doi:10.1371/journal.pone.0146200.g004

individual sleep and nap parameters it appeared that in the control subject, the poor night sleep quality (sleep efficiency lower than 75%) was not compensated by higher daytime napping and that AD patients had more frequent daytime naps even in case of good night sleep quality.

Mean daily profiles of melatonin levels are not significantly different but more individual profiles are aberrant in AD patients than in controls

In each subject, the melatonin levels were determined at 9 time points during the 24 h. The daily profiles of the mean melatonin levels for the groups of controls and AD patients (mean ± SEM, n = 13 for each group) are depicted in Fig 5. The repeated measures 2-way ANOVA revealed significant effect of time (F = 18.470; P < 0.0001); however, the difference between the mean melatonin profiles in AD patients and controls was not significant (F = 0.026; P = 0.874). The result was confirmed by calculation of area under the curve (AUC) for each profile. These values were averaged for each group and then compared between AD patients and controls by Student's t-test (P = 0.585).

Examination of individual melatonin profiles revealed a high variability within both groups (data summarized in Table 1). From the 13 control subjects, 8 subjects exhibited the typical rhythmic profiles (in 7 of them the amplitudes were between 20 and 40 pg/ml and in 1 of them it was 60 pg/ml). In 4 control subjects, the melatonin levels did not rise during the night (the nighttime levels were lower than 5 pg/ml) and the profile did not exhibit circadian rhythm. One control subject had an advanced low-amplitude rhythm (maximal levels of approximately 20 pg/ml at 21:00 and 23:00). From the group of 13 AD patients, 6 subjects had melatonin profiles that did not exhibit circadian rhythms (the levels were suppressed throughout the 24-h interval to less than 5 pg/ml), or the rhythm was shallow and amplitudes were very low (10 pg/
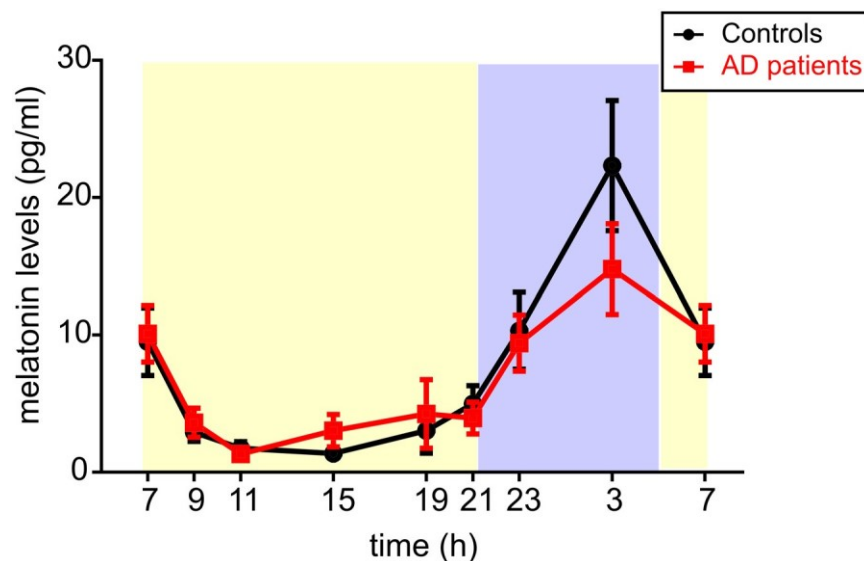

Fig 5. Daily profiles of melatonin levels in the saliva of controls and AD patients. Melatonin levels were detected during the 24 h and expressed in pg/ml. (A) The daily melatonin profiles expressed as the means ± S.E.M. in controls (black circles and black lines, n = 13) and AD patients (red squares and red lines, n = 13); (B) The individual melatonin levels in controls (black circles) and AD patients (red squares) are depicted. Moreover, the mean cosine fits (black curve for controls and red curve for AD patients) were obtained by the cosinor analysis. X-axes represent the time of day in hours, the blue and yellow areas on the graphs backgrounds correspond with time of the outside darkness and daylight, respectively, as occurred during the recording period.

doi:10.1371/journal.pone.0146200.g005

Table 1. Summary of the individual melatonin profile characteristics in controls and AD patients.

	Daily melatonin profile			
group	Total number	Circadian rhythms	Shallow rhythms	Atypical phase
Controls	13	8	4	1
AD	13	4	6	3

Number of daily melatonin profiles of controls and AD patients which exhibited circadian rhythm (nocturnal peak above 10 pg/ml), shallow rhythm (nocturnal peak below 10 pg/ml) or a rhythm with atypical phase. For detailed explanation, see Results.

doi:10.1371/journal.pone.0146200.t001

ml or lower). In 4 AD patients, the melatonin profiles exhibited typical circadian rhythms with peaks at 03:00 and the amplitudes between 20−40 pg/ml. In 3 AD patients, the peak was advanced; in 1 case to 19:00 (20−30 pg/ml) and in 2 cases to 23:00 (10−20 pg/ml). From the analysis of individual profiles it appeared that the typical circadian rhythm with the peak during the nighttime was present more often in controls than in AD patients. Therefore, based on the analysis of the individual melatonin profiles, more aberrancies and lower amplitudes of the melatonin rhythms were present in AD patients compared to controls.

PER1 and *BMAL1* daily expression profiles are not significantly affected in AD patients

The *PER1* and *BMAL1* expression profiles in buccal mucosa of controls and AD patients are depicted in Fig 6. The cosinor analysis of the daily profiles revealed significant circadian rhythms for both genes and experimental groups (Table 2). In controls and AD patients, *PER1* expression rhythms (Fig 6A) exhibited maximal levels (acrophases) during the day and the minimal levels during the night. Notably, *BMAL1* expression rhythm (Fig 6B) was in opposite phase to *PER1* in both groups. However, a closer inspection of phases of the clock gene expression rhythms suggested that whereas the *PER1* rhythm was expressed in the same phase in controls and AD patients, the *BMAL1* rhythm seemed phase-delayed in AD patients. The comparison of the expression rhythms between controls and AD patients using the repeated measures 2-way ANOVA revealed significant effect of time (*PER1*: $F = 9.435$, $P < 0.0001$; *BMAL1*: $F = 4.637$, $P < 0.0003$) but not significant difference between the clock gene expression profiles in AD patients and controls (*PER1*: $F = 0.537$, $P = 0.471$; *BMAL1*: $F = 1.655$, $P = 0.211$). Moreover, comparison of acrophases between both groups by unpaired Student's t-test did not reveal significant differences (*PER1*: $P = 0.953$; *BMAL1*: $P = 0.278$).

Discussion

In this pilot field study, we examined actual state of the circadian system in patients with mild form of AD and healthy age-matched controls who lived together in their home dwellings and were thus exposed to the same environmental cues of their everyday life. Our results suggest that under real-life conditions, circadian regulation of behavior, melatonin levels and peripheral clock gene expression in the AD patients may modestly differ compared with the controls. The limitation of this study is a low number of enrollees (13 patients and 13 healthy controls); this was mainly caused by the special arrangement of the study. Due to the relatively high inter-individual variability of the data even in control elderly subjects, the low number of participants likely accounts for the fact that our results demonstrate trends rather than significant differences.

In AD patients, the circadian activity/rest rhythm was maintained, however, their inactivity interval during the 24 h assessed by sleep diaries was significantly longer compared with that in
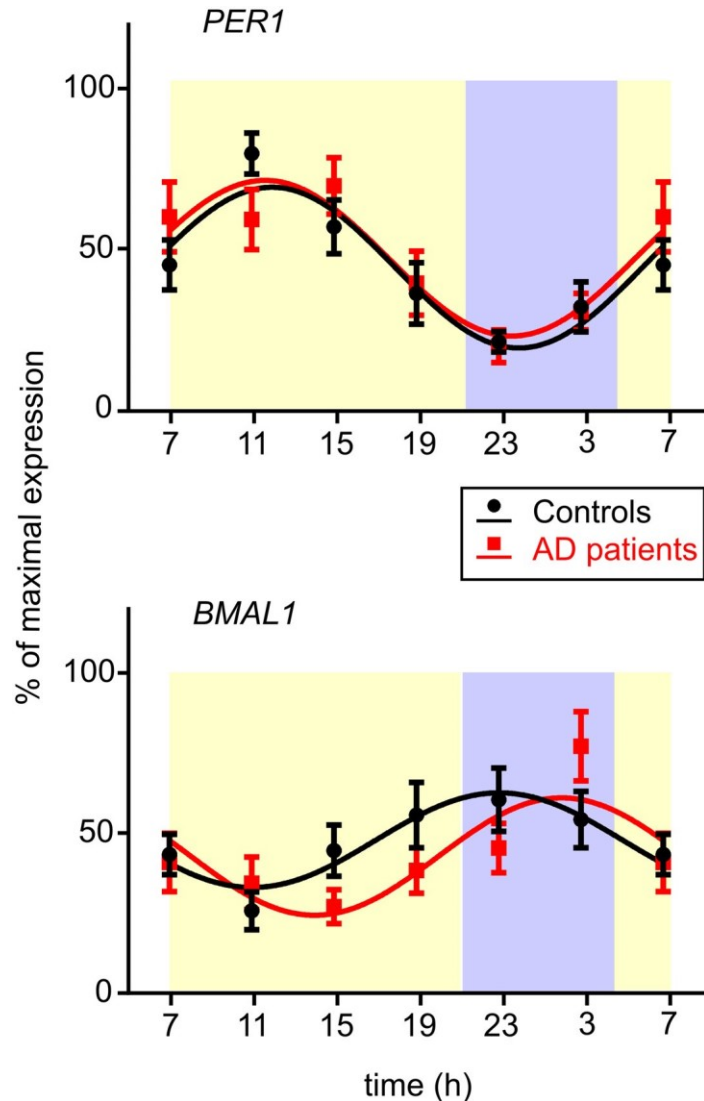
Fig 6. Daily profiles of clock gene expression in buccal cells of controls and AD patients. *PER1* (upper graph) and *BMAL1* (lower graph) expression profiles were compared in controls (black circles, n = 13) and AD patients (red squares, n = 13). Data are expressed as % of maximal expression levels (means ± S.E.M.) and fitted with cosine curves (controls: black curves, AD patients: red curves). The X-axes represent the time of day in hours.

doi:10.1371/journal.pone.0146200.g006

controls. The analysis of individual sleep/wake cycles revealed that the AD patients and controls exhibited more or less consistent rhythm in nighttime sleep and daytime wakefulness; only in 1 out of 13 patients the rhythm was completely abolished and the sleep occurred in bouts equally distributed over the 24 h period. Subsequent analysis of sleep parameters in 4 AD patient/control couples by Actiwach revealed that whereas the AD patients spent more time inactive in bed, their sleep was likely of poorer quality compared with controls. However, the worse sleep quality in AD patients was only suggested because differences in sleep efficiency and fragmentation index between both groups did not reach statistical significance. This might be related with the low number of subjects which we could include in the actigraphic study due to limited number of the devices. Additionally, the data revealed that the AD patients slept more often during the day as their number of daytime naps was significantly higher than that

Table 2. Results of cosinor analysis of the clock gene expression profiles.

Cosinor analysis	PER1		BMAL1	
	Controls	AD patients	Controls	AD patients
P	< 0.001	< 0.001	0.0058	0.0015
R^2	0.2831	0.2213	0.1105	0.1490
Acrophase (mean ±SEM)	12.0 ± 0.6	11.6 ± 0.7	23.0 ± 1.1	2.1 ± 0.9
Amplitude (mean ± SEM)	25.1 ± 4.3	24.3 ± 5.1	14.8 ± 4.5	18.3 ± 4.9
Mesor (mean ± SEM)	44.1 ± 2.9	47.1 ± 3.4	47.8 ± 3.1	42.7 ± 3.3

P (statistical significance); R^2 (coefficient of determination); Acrophase (the phase angle of the peak of a cosine curve in hours); Amplitude (the difference between the peak or trough and the mean value of a cosine curve); Mesor (the average value around which the variable oscillates)

doi:10.1371/journal.pone.0146200.t002

in controls, be it assessed by sleep diaries in all subjects or by Actiwatch in 4 couples. Analysis of individual data revealed that the napping was present in nearly all AD patients (in 12 out of 13 patients) but less often in controls (in 8 out of 13 controls). Interestingly, one AD patient exhibited a better nighttime sleep quality than all examined controls (sleep efficiency higher than 90% and fragmentation index only about 20%) but the same patient also had the highest count of daytime naps. This case result suggests that the higher number of naps in AD patients might be a consequence of sleep/wake cycle disruption rather than an aftereffect to compensate the poor nighttime sleep quality.

Our data demonstrating the longer inactivity during the 24 h in AD patients compared to controls in their home-dwellings are in agreement with previously published findings in hospitalized patients [44, 45]. With age, the percentage of slow wave sleep and REM (rapid eye movement) sleep decreased [46]. Previous studies also revealed that in AD patients the incidence of the sleep/wake cycle aberrancies increased [15, 47]. During the nocturnal sleep, they exhibited significant increase in number and duration of wake up bouts and more pronounced decrease of SWS [15]. Additionally, during the day, they exhibited longer duration and higher frequency of daytime naps and the effect was associated with more profound functional impairments [47]. Unfortunately, our field study did not allow us to follow sleep parameters by polysomnography. However, our analysis of the less accurate actigraphy, which cannot distinguish between a state of wakefulness and a movement without awaking during the sleep, also suggested that sleep quality was likely poorer in AD patients than in their healthy spouses serving as their caregivers and living together with them in the same home-dwelling. The similarities in aberrancies of the rest/activity and sleep/wake cycles found in hospitalized AD patients and our home-living patients provides confirmation that they were not due to a worse adaptation of the AD patients to the hospital conditions. The factor of home environment has also been considered in several previous studies in AD patients. Accordingly, disturbances in the activity/rest rhythm were found in moderately demented home-dwelling AD patients [48]. However, in another study, differences between the AD patients living at home and healthy controls were only minimal and more pronounced disturbances of activity/rest rhythms were only demonstrated in hospitalized versus at home living AD patients [45].

The mean melatonin profiles exhibited circadian rhythms both in AD patients and controls. Decrease in amplitude of the melatonin rhythm in AD patients compared to controls was not significant, however, inspection of the individual profiles suggested a higher incidence of the rhythm disruptions (absence, abnormal shift of the peak) in AD patients than in control subjects. Generally high inter-individual variability was found in both groups and, therefore, we cannot be sure that the indicated differences were not due to relatively small sample size

employed in the study. Nevertheless, another study confirmed decrease in melatonin rhythm amplitude already in preclinical cognitively intact patients [49]. The studies of melatonin levels in cerebrospinal fluid in postmortem samples collected from AD patients in advanced stage of the disease demonstrated more distinct suppression of the nocturnal levels [23], however, their light exposure history was unclear.

In our study, we cannot exclude a possible masking effect of environmental cues, mainly the light, on the endogenous melatonin production, because light was controlled only during the sleep hours on the night when samples were collected. Therefore, although we could not confirm a statistically significant effect of the disease on melatonin levels under real-life conditions, we cannot completely rule out this effect on the endogenous mechanism producing melatonin rhythm. On the other hand, we can exclude the possibility that the differences in melatonin profiles demonstrated in this study in AD patients were due to their different light exposure because the protocol of the study was arranged to ensure that the AD patient/control couples were together throughout the study and were thus exposed to the same environmental cues. In accordance with our results, another hormonal marker controlled by the circadian clock, cortisol, exhibited a significant circadian rhythm in moderately demented home-dwelling AD patients whose activity/rest cycle was disrupted [48]. This result, together with our data, supports the concept that in the AD patients in mild stage of the disease, the endogenous timekeeping mechanism in the central clock is likely preserved but its outputs regulating sleep, behavior and rhythmic hormonal production might become affected. The concept is further supported by our finding of robust circadian oscillation in clock gene expression in oral mucosa cells, providing final evidence that the cellular molecular clock mechanism in our AD patients was intact. Similar conclusion was drawn based on the results of a post-mortem study of circadian clock gene expression profiles in various brain areas in AD patients [35].

In summary, our results suggest that compared with their caregivers of the same age and exposed to the same environmental cues, the AD patients exhibit differences in activity/rest cycles which are manifested as significantly higher duration of the inactivity during 24 h and more daytime naps. In AD patients, in spite of the significant disruption in activity/rest and sleep/wake cycles, only marginal disruption, if any, in regulation of melatonin and clock gene expression profiles were detected. Therefore, the endogenous mechanism generating the circadian rhythmic signal is likely preserved during the mild stage of the disease. However, it is necessary to mention that the healthy controls were the patients' spouses who took care of them likely around the clock which might contribute to undervaluation of the results. Nevertheless, we believe that the experimental design of this pilot field study, testing the subjects in real-life situation, has its obvious relevance because it may reveal the effect of AD on adaptability of the circadian system to environmental conditions.

Supporting Information

S1 Table. Clinical and biomarker characteristics of 13 patients with Alzheimer disease involved in the study. Clinical status of the patients was characterized using Mini-Mental State Examination (MMSE) and Functional Activities Questionnaire (FAQ) [39, 50]. Changes on brain magnetic resonance imaging (MRI) were evaluated using medial temporal lobe atrophy (MTA) score [51]. The evaluation of MRI scans was done by scoring the extent of periventricular hyperintensities (PVH) and deep white matter lesions (DWML) according to the Fazekas scale [52]. Absence of such features is classified „0", caps or pencil-thin lining of PVH– 1, smooth halos– 2 and irregular PVH extending into the deep white matter– 3. DWML constituting only punctate foci score 1, beginning confluent foci are 2 and large confluent areas of DWML were evaluated as 3. Each side of the mediotemporal region on Neurogam—

processed SPECT 3D images was assessed by simple in-house unpublished semiquantitative scale (0 −negative, 1 −borderline, 2 −positive). A combination of scores from both sides resulted in total brain score: 0 −negative, 1 −borderline, 2 −positive on one side, 3 −positive on both sides. Cut-off cerebrospinal fluid concentrations were established on well characterized samples measured in our lab, i.e., 334 pg/ml for total tau protein, 57 pg/ml for phospho-tau protein p181-tau and 448 pg/ml for beta-amyloid.
(DOCX)

Author Contributions

Conceived and designed the experiments: AS. Performed the experiments: KW AB MN. Analyzed the data: AS KW MS. Wrote the paper: AS.

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Original Article

Circadian rhythms of melatonin and peripheral clock gene expression in idiopathic REM sleep behavior disorder

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a r t i c l e i n f o

a b s t r a c t

Objective: To evaluate changes in the expression of clock genes and melatonin levels in patients with idiopathic REM sleep behavior disorder (RBD) as a potential early stage of synucleinopathies.
Methods: We assessed the rhythmicity of circadian clock genes using real time-quantitative polymerase chain reaction and 24-h blood melatonin profiles using radio-immunoassay in 10 RBD patients and nine age-matched controls.
Results: The RBD patients did not show circadian rhythmicity for clock genes *Per2*, *Bmal1*, and *Nr1d1* but the rhythmicity of *Per 1* remained, and the amplitude of *Per3* was diminished. The 24-h melatonin rhythm did not differ between RBD patients and healthy control subjects. Melatonin profile in RBD patients was delayed by 2 h compared to controls, the habitual sleep phases were phase delayed by about 1 h, however no phase shift occurred in any of the clock genes studied. The control group had stable acrophases of melatonin rhythms of approximately 5 h whereas the RBD patients had a more dispersed range over 11 h.
Conclusions: Our results suggest that RBD could be associated with altered expression of clock genes and delayed melatonin secretion. Thus, we argue that circadian system dysregulation could play a role in RBD.

1. Introduction

Idiopathic rapid eye movement (REM) sleep behavior disorder (RBD) is a parasomnia characterized by dream-related vocalizations and/or complex motor behaviors due to loss of physiological muscle atonia during REM sleep [1]. The available evidence suggests that RBD might be a precursor of synucleinopathies such as Parkinson's disease (PD) and other neurodegenerative disorders [2e6]. Sleep disturbances are common non-motor symptoms in Parkinson's disease [7,8] and it has been suggested that they may reflect a more fundamental pathology at the molecular level underlying circadian rhythms in PD patients [9,10].

Regulation of sleep/wake patterns is complex and the circadian system is an important part of this regulatory process. One of these mechanisms involves regulation of melatonin synthesis in the pineal gland. Melatonin synthesis is directly regulated by the major circadian pacemaker, which is located in the suprachiasmatic nucleus (SCN). Melatonin levels increase at night and decline in the morning. Melatonin not only promotes sleep and affects sleep quality, it is also involved in the immune system, reproduction, and metabolism regulation [11e13]. Abnormal changes in melatonin blood levels may negatively influence sleep regulation. These abnormalities may also disrupt the circadian machinery that is directly upstream from its synthesis.

Circadian clock genes such as *Period* (*Per*), *Cryptochromes* (*Cry*), *Clock*, and *Bmal1* are group of genes that compose the autoregulatory positive and negative transcriptional/translational feedback loops underlying the circadian rhythmicity of most cells [14,15]. In the morning, CLOCK and BMAL1 heterodimers promote

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transcription of *Per* and *Cry* genes by binding to E-boxes in their promoters. In the early evening, PER and CRY proteins have accumulated in the cytosol; and after dimerization, they enter the nucleus and interact with CLOCK/BMAL1 heterodimers to inhibit their own transcription. A second regulatory feedback loop includes *Rev-erb*a (*Nr1d1*) and *Ror*a, which compete for Rev-erb/Ror elements in the *Bmal1* promoter and regulate its expression [16]. Circadian clock genes are transcribed by most mammalian cells as well as many non-mammalian organisms. Transcriptional deregulation and genetic mutations have been intensively studied in the context of several diseases. In addition, they have been associated with sleep and neurodegenerative disorders; an example being the discovery of disrupted diurnal rhythmicity of clock genes expression in fibroblasts of idiopathic hypersomnia patients [17]. Similarly, relative BMAL1 levels in leukocytes correlate positively with the severity of Parkinson's disease (PD). Additionally, specific polymorphisms in *Bmal1* and *Per1* genes were also identified in a wide range of PD patients [18,19]. These and other data suggest that expression of peripheral clock genes mirror individual period length, phase, and amplitude of the circadian clock [20,21] and can thus serve as a suitable and easily available marker to assess molecular-based circadian disturbances in the body.

We hypothesized that changes in blood melatonin concentrations and expression of clock genes detected in Parkinson's disease are also present before motor symptoms occur, and may manifest as a stage of RBD, which might be detectable many years before the onset of PD. To investigate this, we examined the rhythmicity of circadian clock genes and assessed 24-h melatonin serum profiles in idiopathic RBD patients. Alterations of these circadian rhythms may serve as early markers for the development of Parkinson's disease.

2. Methods

2.1. Participants

We included 10 male patients, at our university hospital, who had been diagnosed with idiopathic RBD. RBD was defined using the ICSD-3 criteria [1]. Patients underwent a complete clinical interview and a complex neurological and cognitive examination by an experienced neurologist, which was then followed by a full-scale polysomnography. The onset of RBD symptoms was determined by patients and their spouse at the time of the RBD diagnosis. None of the patients were taking melatonin, clonazepam, or other psychoactive medications at the time of the polysomnography recording. We selected nine age-matched male controls from our database of healthy elderly individuals. All participants were carefully examined for symptoms of parkinsonism or other neurodegenerative disorders. All participants completed the Montreal cognitive assessment [22], RBD-Screening Questionnaire [23], Beck Depression Inventory II [24], and MorningnesseEveningness Questionnaire [25]. All the participants were asked to keep a sleep diary for the three week period preceding the examination in our lab to confirm that they kept a regular sleep regime. This was meant to exclude extreme circadian preferences and detect potential differences between RBD patients and the control group. Exclusion criteria for the control group were the presence of any clinically significant somatic (including acute pain) or psychiatric disorder, any sleep complaints, or use of chronic medication that could influence sleep. All participants gave written informed consent and the protocol was approved by the hospital Ethics Committee.

2.2. Video-polysomnography

All idiopathic RBD patients underwent overnight video-polysomnography which included electro-encephalography (EEG), electro-oculography (EOG), electromyography (EMG, according to SINBAR montage) [26], electrocardiography (ECG), and measurement of nasal pressure, oropharyngeal sounds (via a tracheal microphone), chest and abdominal efforts (via belts), pulse oximetry, as well as synchronized video and audio monitoring. The sleep stages, arousals, periodic leg movements, respiratory events, and EMG activities were scored according to international criteria [27] by trained evaluators. Video recordings were carefully examined to detect any movement that might have occurred during REM sleep.

2.3. Circadian rhythm analysis

The experiment was conducted under standard clinical settings and under a semi-constant routine protocol. Participants entered the laboratory 2 h before the first blood sampling at 10 am. Subjects were seated in single rooms with a light (<200 lux) until habitual sleep onset or until 22:00 when the lights were turned off. Daytime naps were not allowed. The room temperature was constant at approximately 21 °C, and meal times were adjusted to individual needs. During sleep, window shades were used to prevent external light from entering the room. Blood samples were collected every 3 h during the 24-h period; this was done through a peripheral venous cannula. Red lighting (via a red headlamp) was used for illumination to prevent disruption of melatonin production at night. This approach allowed patients to sleep through the night undisturbed, even when blood samples were being taken. While there was a long connector line that extended into an adjacent room, it was not used.

2.3.1. Melatonin assay

Serum melatonin concentrations were evaluated using RIA kits (Demeditec Diagnostics GmbH, Germany) according to the manufacturer's protocol. All samples, both patients and controls, were always processed using the same RIA kit; the reported intra- and inter-assay coefficients of variation for the kits were 9.8% (patients) and 8.0% (controls) and the analytical sensitivity was 2.3 pg/ml. Two assays were run for each serum sample.

2.3.2. Determination of clock genes expression by quantitative real-time polymerase chain reaction

2.3.2.1. *(RT-qPCR).* Peripheral blood mononuclear cells were isolated from approximately 7 ml of blood using gradient centrifugation through Histopaque 1077 medium (Sigma-Aldrich, St. Louis, MI, USA), washed, lysed using 700 ml of RNA isolation reagent (TRI Reagent; Zymo Research Corporation, CA, USA), and subsequently frozen at 80 °C. The mRNA was isolated using a Direct-zol RNA MiniPrep Plus kit (Zymo Research Corporation), and the whole mRNA sample was reverse-transcribed using a SuperScript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA) in 20-ml reactions incubated at 42 °C for 1 h. The cDNA was then diluted 1:2 with RNase-free water and 2 ml was used to determine gene expression in 16 ml qPCR reactions. Each reaction also contained 10.3 ml of PCR-grade water, 3.1 ml of HOT FIREPol Probe qPCR Mix Plus (Solis BioDyne, Estonia), and 0.6 ml of TaqMan Gene Expression Human FAM-MGB assay (Life Technologies, Carlsbad, CA, USA), which was specific for the following genes: Period circadian clock 1 (PER1, NM 002616, cat. no. Hs01092603_m1), Period circadian clock 2 (PER2, NM 022817, cat. no. Hs00256143_m1), Period circadian clock 3 (PER3, NM016831, cat. no. Hs00213466_m1), Aryl hydrocarbon receptor nuclear translocator-like (ARNTL, syn. BMAL1, NM 001178, cat. no. Hs00154147_m1), Nuclear receptor subfamily 1 group D member 1 (NR1D1, NM 021724, cat. no. Hs00253876_m1) and Beta-2-microglobulin (B2M, NM004048, cat. no. Hs00187842_m1). All qPCRs were performed in duplicate on a LightCycler® 480 Instrument (Roche Life Science, Indianapolis, IN, USA) using the following temperature profile: initial denaturation at 95 °C for 15 min, followed by

50 cycles consisting of denaturation at 95 °C for 20 s and annealing/elongation at 60 °C for 60 s. The mean of the crossing point (Cp) obtained from qPCR was normalized to the level of the B2M housekeeping gene and then used for analysis of relative gene expression using the DDCT method [28].

2.4. Statistical analysis

To compare basic group data, we first tested distribution normality using the ShapiroeWilk test, then the non-parametric Mann Whitney U test was used to compare samples.

Circadian profiles of clock genes mRNA and melatonin levels were expressed as mean ± SEM for each group. The rhythmicity was evaluated using a one-way ANOVA for the effect of time, and further analyzed using cosinor analysis defined by the equation: [Y ¼ mesor þ(amplitude × cos (2p (X — acrophase))/wavelength)], with a constant wavelength of 24 h [29] and with a horizontal line fit model (H0) and cosinor analysis as the alternative hypothesis. The area under the curve (AUC) was calculated as a measure of the amount of melatonin produced during a 24-h period (trapezoidal method). Circadian phases were assessed using acrophase and dim-light melatonin onset (DLMO), which was calculated as 2 SDs above the mean baseline of samples (ie, mean melatonin levels at 10:00, 13:00, and 16:00). The difference in acrophase, DMLO, AUC, habitual sleep time, and amplitude between data sets was tested using the two-way ANOVA.

3. Results

Table 1 summarizes the clinical characteristics of patients and Table 2 shows the main polysomnography features of our RBD patients and controls. Scores on the Movement Disorders Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) part III were slightly higher in RBD patients than in controls, but the difference was not statistically significance. Two RBD patients and two controls had periodic limb movements during sleep with a periodic limb movement index (PLMI) greater than 15 per hour, while another patient who had obstructive sleep apnea, had an ApneaeHypopnea Index (AHI) index greater than 15 events per hour.

On average, RBD patients went to bed at 11:45 pm (±60 min). Healthy individuals went to bed in about 1 h earlier at 10:55 pm (±40 min), which was a statistically significant difference (P ¼ 0.0345). Similarly, sleep offset in RBD patients was shifted to later morning. RBD patients woke up around 7:45 am (±60 min) whereas healthy individuals woke up at 6:40 am (±45 min; P ¼ 0.0277).

3.1. Melatonin rhythms

The daily profiles of mean melatonin levels for controls and RBD patients are shown in Fig. 1A. The one-way ANOVA revealed a significant time effect (controls: F (8, 48) ¼ 2.761; P 0.0136, RBD: F (8, 72) ¼ 4.437; P ¼ 0.0002); however, the difference between mean melatonin profiles in RBD patients and controls was not significant (repeated-measures two-way ANOVA interaction: P ¼ 0.3479). When acrophases of melatonin profiles were compared, we found a statistically significant difference between RBD patients and controls (P ¼ 0.0337; Fig. 1B). Although we found a phase delay in the melatonin profile of RBD patients compared to controls (assessed using the DMLO), this difference was not statistically significant (Fig. 1C). Similarly, the amplitude of circadian rhythms, as assessed using 24-h AUC, did not differ significantly between patients and controls (RBD, 389.54 ± 25.1; controls, 355.75 ± 43.2).

3.2. Peripheral clock genes expression

We measured the rhythmic expression of several clock genes in mononuclear blood cells from RBD patients and control subjects. Selected clock genes cover major attributes of the molecular clockwork; hPer1 and hPer2 genes, convey exogenous signals to clockwork also represent an important auto-regulatory loop. Stabilizing loop controls Bmal1 expression and is represented by Nr1d1 gene, which also functions as an orphan receptor that links the metabolic and immune systems with the circadian clock [30]. The Per3 gene, which has a human circadian phenotype, is an example of a clock output gene that has been widely studied. In controls, the daily profiles of all tested clock genes exhibited significant circadian variation (hPer1: P ¼ 0.0076; hPer2: P < 0.0001; hPer3: P ¼ 0.0454; hBmal1: P 0.0148; hNr1d1: P < 0.0001). However, in RBD patients, only hPer3 expression profiles exhibited significant variation as a function of time, as revealed by ANOVA (P ¼ 0.0497); although, time variation as it related to the other genes was not significant (hPer1: P ¼ 0.1107; hPer2: P ¼ 0.3818; hBmal1: P ¼ 0.7434; hNr1d1: P ¼ 0.2077). ANOVA results were supported by cosinor analysis, which also failed to confirm rhythmicity for hPer2, hBmal1, and hNr1d1 in RBD patients, yet did reveal rhythm in hPer1 gene expression (P ¼ 0.0002). Furthermore, the amplitude of hPer3 was diminished, which was statistically significant (P ¼ 0.0322). A two-way ANOVA revealed significant differences between the clock genes profiles of controls and RBD patients for hPer1 (P ¼ 0.0324), hPer2 (P ¼ 0.0039), hBmal1 (P ¼ 0.0160), and hNr1d1 (P ¼ 0.0046). (See Fig. 2).

4. Discussion

Our findings suggested a potential alteration in the circadian system in RBD patients. To our knowledge, there are no data published on clock genes expression and melatonin rhythmicity in patients with idiopathic RBD in the literature. Longitudinal studies strongly suggest that RBD is an important prodromal marker for

Table 1
Clinical characteristics of idiopathic RBD patients and controls.

Clinical characteristics	RBD patients mean (SD)	Controls mean (SD)
Number of participants	10 males	9 males
Age, years	76 (3.4)	73 (4.2)
Age when RBD was diagnosed	69.3 (5.2)	e
Time since the diagnosis was made (years)	4.1 (3.4)	e
RBD-SQ score	11.2 (1.5)	4.0 (0.9)
MDS-UPDRS part III score	3.4 (2.8)	0.7 (0.2)
MoCA score	22.6 (6.1)	27.2 (0.8)
BDI score	6.8 (2.3)	5.2 (4.0)
MEQ score	55.00 (7.7)	52.1 (6.3)

RBD-SQ, REM sleep behavior disorder-screening questionnaire; MDS-UPDRS, Movement Disorders Society-Unified Parkinson's Disease Rating Scale; MoCA, Montreal Cognitive Assessment; BDI, Beck depression inventory; MorningnesseEveningness Questionnaire.

Table 2
Main polysomnographic features of idiopathic RBD patients and controls.

	RBD patients median (IQR)	Controls median (IQR)	Z score	P value
Total sleep time, min	322 (54.5)	355 (80.0)	—0.064	0.949
WASO, min	22.4 (7.0)	22.6 (16.6)	—0.576	0.620
Sleep efficiency, %	77.6 (7.0)	77.0 (16.6)	—0.192	0.902
Stage N1, %	7.8 (2.3)	5.5 (5.1)	—1.215	0.259
Stage N2, %	33.9 (23.4)	40.8 (17.3)	—0.192	0.902
Stage N3, %	8.4 (12.85)	16.7 (8.0)	—1.215	0.259
Stage REM %	20.4 (4.7)	16.0 (3.5)	—1.471	0.165
PLMS index, /h	48.5 (66.0)	0 (29.1)	—1.995	0.053
AHI, /h	10.0 (13.5)	9.0 (26.5)	—0.256	0.805

AHI, apneaehypopnea index; PLMS, periodic leg movements during sleep; WASO, wake after sleep onset; REM, rapid eye movement stage; IQR, interquartile range.
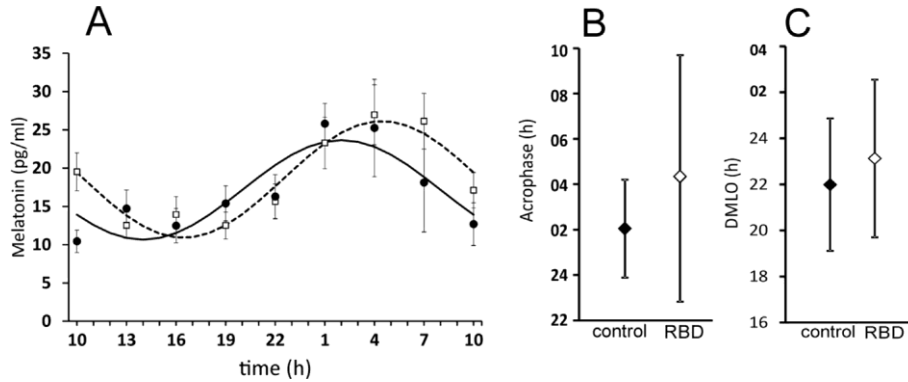
Fig. 1. Changes in serum melatonin levels in RBD patients (white squares) and control subjects (black dots). Blood was sampled at 3-h intervals starting at 10 am over a 24-h period. (A) Rhythmic profiles of melatonin level of control subjects and RBD patients. The data are expressed as the mean ± SEM. (B) The range of time points when maximum melatonin level was revealed by cosinor analysis of individual melatonin profiles. Diamonds demonstrate the mean acrophase time for control subjects (at 2.005 h), and for RBD patients (at 4.451 h). (C) The range of time points when DMLO of individual melatonin profiles was revealed. Diamonds demonstrate the mean DMLO for control subjects (at 21.99 h), and for RBD patients (at 23.13 h).

Parkinson's disease (PD) and may precede a PD diagnosis by over a decade [2,3,31,32]. So far, only two studies have analyzed peripheral clock genes expression in patients with an early form of Parkinson's disease. Cai et al., [18] quantified *Per1* and *Bmal1* expression in leukocytes of 17 patients with PD and 16 controls at four overnight time points. Breen et al., [9] published a study looking at the expression of clock genes, levels of melatonin, cortisol, and actigraphy measurements. Similar to our findings,

they discovered altered rhythmicity of *Bmal1, Per2,* and *Rev-Erb*a genes expression compared to healthy controls. In contrast, they found reduced melatonin production in PD compare to controls and no phase shift.

Our data show that melatonin rhythmicity in RBD patients and control subjects differs only in the acrophase, which was more than 2 h phase delayed compared to controls. Similarly, the significant phase delay in habitual sleep onset and offset has been reported in
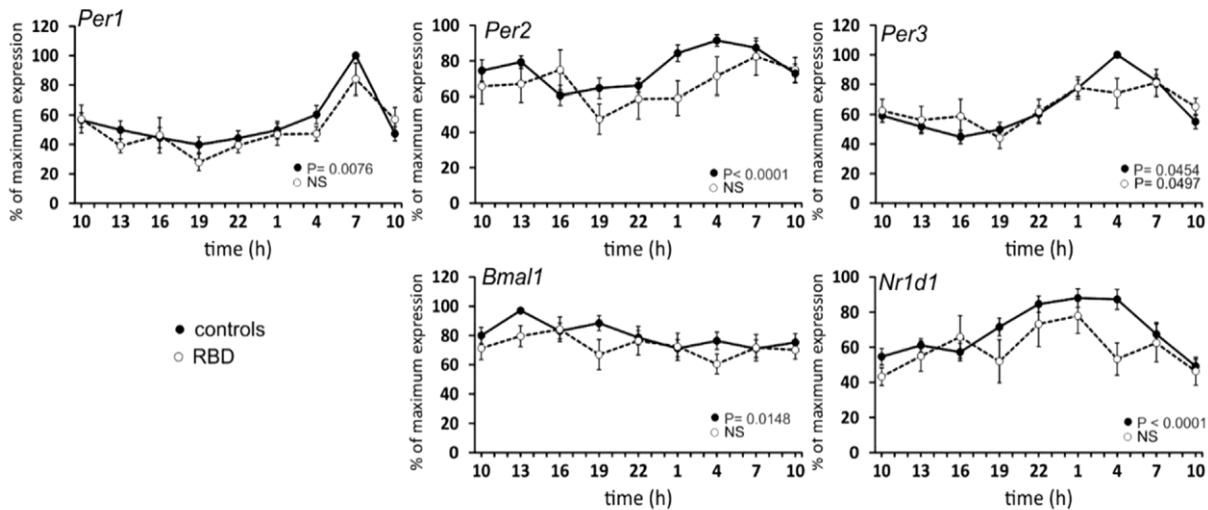

Fig. 2. Changes in clock genes expression in the RBD patients (white dots) and the control subjects (black dots). Blood was sampled at 3-h intervals starting at 10 am over a 24-h period. The data are expressed as the mean ± SEM. Statistical significance of the results was based on the results obtained from ANOVA statistical test.

our patients, which may be a consequence of the phase shift in melatonin rhythmicity. The amplitude of the rhythmicity did not differ between our groups, which is in contrast with observations done in PD patients, in which the AUCs were significantly diminished [9,10]. Thus, we concluded that melatonin regulation is not significantly compromised in RBD patients.

Bmal1 expression is regulated by nuclear receptors, positively by RORα and negatively by REV-ERBα, which are protein products of the *Nr1d1* gene. The blunted expression of the *Nr1d1* gene may cause blunted expression of *Bmal1,* since it does not balance rhythmic *Bmal1* induction by RORα. Notably, the physical interaction of PER2 with REV-ERBα allows for modulation of *Bmal1* gene regulation. Meanwhile, the binding of PER2 to the *Rev-Erbα* promoter has also been demonstrated [33]. In the striatum, the rhythmic expression of *Per2* depends on daily dopaminergic activation of D2 receptors. Treatments that deplete striatal dopamine were shown to blunt the rhythm of PER2, while daily stimulation of D2, but not D1 receptors, restored the PER2 rhythm of a DA-depleted striatum [34]. Dopamine, however, does not affect the rhythm of PER2 in the SCN and the question remains, whether low dopamine levels may affect the expression of clock genes in the blood. In general, blood cells contain circadian clock genes that express high-amplitude rhythms; and dopamine content has been shown to be reduced in peripheral blood lymphocytes in the early stages of PD [35e37]. This observation suggests that extra-clock driven regulation, namely of *Per2* expression, might be responsible for reduced *hNr1d1* and *hBmal1* rhythmicity.

The uncertain difference in rhythmicity of *Per1* expression in lymphocytes suggests that disruption of the clockwork itself would not cause the observed differences. It can be assumed that negative feedback of PER1 and CRY (which was not tested) may be sufficient to regulate the primary feedback loop and the rhythmic expression of *Per3*. The compromised stability of the clockwork caused by blunted *Bmal1* rhythmicity could be reflected in the lowered amplitude of *Per3* mRNA. The standard amplitude of rhythmic melatonin synthesis in RBD patients may indicate that the primary circadian mechanism is only partially compromised in RBD patients and the circadian clock can send oscillatory signals to the pineal gland. Based on the previous experimental work done in this field, it seems that *Per1* and *Cry1* might be sufficient to sustain oscillatory function [38,39]. Therefore, it seems that partial disruption of the circadian clock does not necessarily mean arrhythmicity in RBD patients, yet, it may increase the vulnerability of their clock to the disruptive effect of external conditions (ie, a weak light/dark Zeitgeber) [33]. From the clinical point of view, the strength of the light Zeitgeber reflects the contrast between the daytime levels of illumination and nighttime darkness. This Zeitgeber weakens with the reduction in contrast, which may open the question of whether light therapy could improve the quality of life in RBD patients.

Although it is known that the CLOCK/BMAL1 complex also regulates cerebral redox homeostasis, which may be one of the mechanisms that links an impaired circadian clock to neurodegeneration [40], the exact relationship between circadian rhythm disruption and neurodegeneration as well as the causality of these CNS diseases remains to be clarified. It is possible that circadian oscillatory deficits increase brain vulnerability to oxidative injury, neuroinflammation, or synaptic degeneration, and accelerate the development of neurodegenerative diseases [41]. Our results support the importance of clock-targeted therapeutics such as timed exposure to bright light, exercise, and melatonin supplementation in RBD patients, which was described in 1997 [42]. Subsequently, the literature and various guidelines began to support the observed positive outcomes seen when melatonin was used to treat RBD patients [43]. In addition to the results presented

in this study, it is reasonable to suggest that both melatonin and chronotherapy should be a standard part of RBD patient treatment.

We acknowledge that our study has some limitations. First, it could be argued that sample sizes were too small and that the design of the study was only a case-controlled trial and not a longitudinal follow-up. It is true that larger sample sizes could have delivered more consistent results and better reflected real-life situations. Conversely, since the data are only preliminary, we believe we can increase the sample size by continuing the experimental protocol. Additionally, longitudinally obtained follow-up data will be collected over time. Moreover, one of the main aims of this project was to follow participants for longer periods of time with a focus on both potential changes in circadian markers as well as signs of PD progression. Second, the sleep regime of our participants was only subjectively measured using a sleep dairy, MEQ questionnaire, and a structured interview, which was conducted by a clinical researcher. Actigraphy would have been better for our experiment purposes, unfortunately, due to technical limitations, this was not possible. Nevertheless, we think the monitoring methods used were able to provide all the essential information needed regarding participant routines.

Acknowledgments

Conflict of interest

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: https://doi.org/10.1016/j.sleep.2018.07.019.

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RESEARCH ARTICLE

The Effect of a Common Daily Schedule on Human Circadian Rhythms During the Polar Day in Svalbard: A Field Study

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All Arctic visitors have to deal with extreme conditions, including a constant high light intensity during the summer season or constant darkness during winter. The light/dark cycle serves as the most potent synchronizing signal for the biological clock, and any Arctic visitor attending those regions during winter or summer would struggle with the absence of those entraining signals. However, the inner clock can be synchronized by other zeitgebers such as physical activity, food intake, or social interactions. Here, we investigated the effect of the polar day on the circadian clock of 10 researchers attending the polar base station in the Svalbard region during the summer season. The data collected in Svalbard was compared with data obtained just before leaving for the expedition (in the Czech Republic 49.8175°N, 15.4730°E). To determine the circadian functions, we monitored activity/rest rhythm with wrist actigraphy followed by sleep diaries, melatonin rhythm in saliva, and clock gene expression (*Per1, Bmal1,* and *Nr1D1*) in buccal mucosa samples. Our data shows that the two-week stay in Svalbard delayed melatonin onset but did not affect its rhythmic secretion, and delayed the activity/rest rhythm. Furthermore, the clock gene expression displayed a higher amplitude in Svalbard compared to the amplitude detected in the Czech Republic. We hypothesize that the common daily schedule at the Svalbard expedition strengthens circadian rhythmicity even in conditions of compromised light/dark cycles. To our knowledge, this is the first study to demonstrate peripheral clock gene expression during a polar expedition.

Keywords: circadian system; arctic; polar day; social cues; human chronobiology

1. Introduction

The regular light/dark cycle has a crucial impact on our circadian clock. The natural daylight period varies between the seasons and its length is also specific to the latitude. In the Czech Republic (49.8175°N), the light/dark cycle during the summer is about 16 hours of light and 8 hours of darkness, and during the winter the light/dark cycle is 8 hours of light and 16 hours of darkness. In Svalbard (77.8750°N), polar days during which the sun is up for 24 h start on the 19th of April and end by the 23rd of August. The long polar night begins on the 11th of November and ends on the 30th of January. Svalbard visitors are thus exposed to extreme light conditions that affect their circadian clocks. Previous studies already reported circadian, sleep or mood disturbances due to constant illumination or constant darkness in a polar environment [1–5]. The

aim of our study was to complete this data with the evaluation of clock gene expression in peripheral tissues and with the assessment of changes in the circadian rhythm within the same subjects before departure and after a two-week adaptation period in Svalbard.

2. Materials and Methods
2.1. Participants

The participants were recruited from a group of Czech researchers attending a 14-day summer expedition in Svalbard. Ten subjects were enrolled in this study (five males and five females) of average age 34.6 ± 8.5 SD. Potential participants were excluded for: 1) having changed time zones during the study, 2) having worked night shifts up to one month before the beginning of the study, or 3) being on any medication that was known to affect their sleep.

Before the beginning of the study, the participants were informed about the underlying scientific principles and the experimental design of the study during an instructional meeting. This included information regarding proper sample storage and collection and the importance

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of avoiding artificial light exposure during the night periods. Each subject received a prepared kit with nine marked sampling tubes for saliva (opaque tubes) and 14 tubes for buccal scrubs containing RNA stabilizing solution, cytological brushes, and an actigraph device.

All participants signed an informed consent waiver that was in agreement with the Declaration of Helsinki and was approved by the Ethics Committee of the Third Faculty of Medicine of Charles University.

2.2. Protocol of the study and sample collection

The study took place over two phases, using the same protocol: The first was in the Czech Republic before departure, and the second part took place in Svalbard during the last day of the participants' two-week stay.

2.2.1. Czech Republic sampling conditions

The sleep/wake cycle was recorded using the Motion-Watch device supplemented with sleep diaries from 10–14 days before departure to the expedition. All subjects were instructed to keep their regular life/work schedules for the entire duration of the measurement period. The saliva and buccal scrub samples were collected 1–3 days before leaving for Svalbard. The sampling started at 7 AM, and all the samples were collected in 4 h intervals over a 24 h period. Two additional times, 9 AM and 9 PM, were inserted into the saliva sampling schedule for more precise estimation of melatonin rise and decline.

Saliva samples were collected directly in marked tubes and stored at −20°C until assayed. Two separate samples of oral mucosa from each side of the cheek were scrubbed by cytological brushes into sampling tubes containing an RNA stabilizing solution (RNAlater, Sigma-Aldrich, St Louis, USA) and stored at −20°C until further analysis.

2.2.2. Svalbard sampling conditions

The expedition took place at Nostoc Field Station in Petuniabukta in July. Sun at this latitude (78°41′13″N 16°31′43″E) is present for 24 hours at a time from the 7th of May to the 24th of August. The perceived light intensity during the polar day was almost stable, but actigraphic measurement revealed variations of the peak light level comparing midday points. During the night time hours, the subjects slept in containers with a small window that limited light exposure, but the light intensity measured by actigraphy was higher compared to that in the Czech Republic (**Figure 1**). On the 13th day of the expedition, the subjects were provided with the prepared kits with sample tubes and brushes and followed the same sample collection protocol as in the Czech Republic. The saliva samples were stored and transported on dry ice and the buccal scrubs were transported in cooling boxes. All samples were processed in the Czech Republic.

2.3. Activity, sleep/wake, and light intensity recording

Activity was recorded using a MotionWatch device (MotionWatch model 8; Cambridge Neurotechnology Ltd. UK). All subjects wore the MotionWatch devices on their non-dominant hands, and they were instructed to only take them off during baths or saunas. The device recorded movement and light intensity every 30 s, and the overall daily activity was expressed as a mean activity within 30-min intervals 24 h/day. The MotionWatch data was analyzed using MotionWare software (Cambridge Neurotechnology Ltd. UK). Sleep analysis was conducted with particular attention to sleep time, wake-up time, and sleep duration. Actigraphic sleep/wake data was aligned with a sleep diary, which helped to distinguish between motionless periods in a waking state and real sleep. This provided more accurate information about sleep onset and offset.
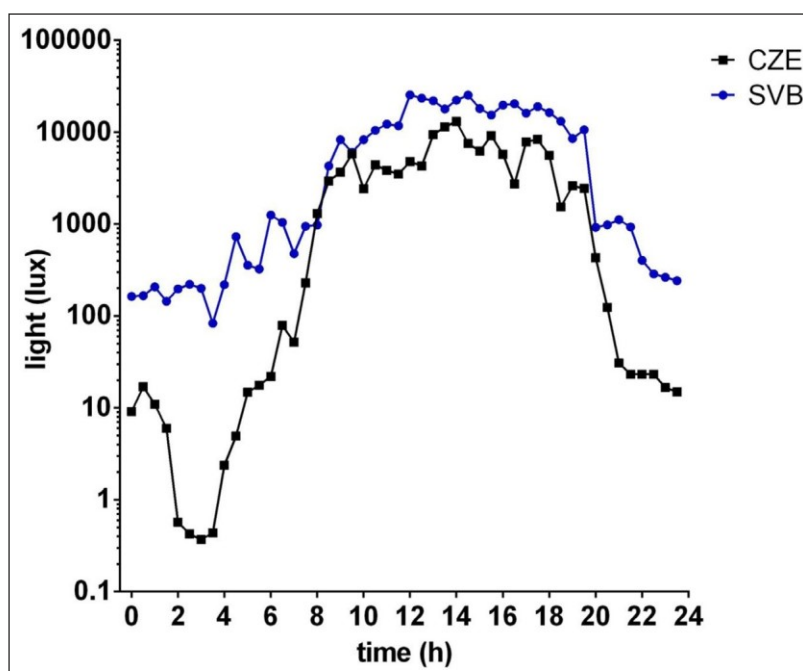

Figure 1: The average light INTENSITY in the Czech Republic (black line) and in Svalbard (blue line) during the 24 h of polar day. The light intensity is plotted on logarithmic scale.

The mean activity was analyzed with cosinor analysis from eight subjects (two subjects were excluded due to defective MotionWatch devices). We used the cosinor analysis for each of our subject separately and the results of cosinor analysis were presented as a mean of the calculated values.

2.4. Melatonin assay

Salivary melatonin concentrations were evaluated using a commercially available direct double-antibody radioimmunoassay kit (Bühlmann Laboratories. Allschvil, Switzerland) according to the manufacturer's instructions. The melatonin concentration was expressed in pg/ml as the mean ± SEM of eight subjects (two subjects did not provide a sufficient amount of saliva in every sample). Individual melatonin profiles were analyzed by cosinor analysis, as described in Chapter 2.6. Amplitude, mesor, and acrophase were assessed for individual profiles.

2.5. Determination of clock gene expression by quantitative real-time polymerase chain reaction (RT-qPCR)

The mRNA was extracted from buccal scrubs using the Direct-Zol RNA MiniPrep (Zymo Research Corporation) and each participant's full RNA sample was reverse-transcribed by High-Capacity cDNA Reverse Transcription Kit (LifeTechnologies, Applied biotechnologies) in 20 ul reaction incubated 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. The cDNA was then diluted 1:2 with RNase-free water, and 2 μl of diluents were used to determine gene expression in a 16 μl qPCR reaction. Each reaction also contained 10.3 μl of PCR-grade water, 3.1 μl of HOT FIREPol Probe qPCR Mix Plus (Solis BioDyne, Estonia), and 0.6 μl of TaqMan Gene Expression Human FAM-MGB assay (Life Technologies, CA, USA) specific for the following genes: *Period 1* (*PER1* NM 002616, cat. no. Hs01092603_m1), *aryl hydrocarbon receptor nuclear translocator-like* (*ARNTL*. syn. *BMAL1* NM 001178, cat. no. Hs00154147_m1), *nuclear receptor subfamily 1 group D member 1* (*NR1D1* NM 021724, cat. no. Hs00253876_m1), *beta-2-microglobulin* (*B2M* NM 004048, cat. no. Hs00187842_m1), and *glyceraldehyde-3-phosphate dehydrogenase* (*GADPH* NM 002046, cat. No. Hs99999905_m1). The qPCR reactions were performed in triplicate and amplified in sealed 384-well microplates on a LightCycler® 480 instrument (Roche Life Science, Indianapolis, IN, USA) using the following temperatures: initial denaturation at 95°C for 15 min, followed by 50 cycles consisting of denaturation at 95°C for 20 s and annealing/elongation at 60°C for 60 s. A negative control without the cDNA showed no amplification. As a positive control, a cDNA sample isolated from cultured human fibroblasts was used. The mean of the crossing point (Cp) was normalized to the geometrical Cp mean of *B2M* and *GADPH* housekeeping genes and then used for the analysis of relative gene expression using the ⊗⊗CT method [6].

2.6. Statistics

Activity data was plotted as a mean ± SEM in 30 min intervals bins for 24 h over the 12-day recording period. The activity profiles' Czech and Svalbard measurements were compared using repeated-measure 2-way ANOVA with Bonferroni's multiple comparison. Each of the activity profiles was analyzed with cosinor analysis, and the difference in acrophases among the groups was compared by paired Student's t-test. Sleep analysis parameters were evaluated by paired Student's t-test, while the differences between the Czech and Svalbard sleep parameters were analyzed by Wilcoxon's test.

The data for melatonin levels at each time point was plotted as mean ± SEM for each group and compared by repeated-measure 2-way ANOVA with Bonferroni's multiple comparison. The individual melatonin profiles were analyzed by cosinor analysis. The differences in acrophases and amplitude were evaluated by paired Student's t-test, while the differences between the Czech and Svalbard measurements were analyzed by Wilcoxon's test.

The data for clock gene expression was analyzed with cosinor analysis (see below) individually and in a group. The group results were expressed as mean ± SEM. The acrophase and amplitude among the groups were compared by paired Student's t-test. The expression profiles among the groups were analyzed by 2-way ANOVA for repeated measures with Bonferroni's multiple comparison.

Cosinor analysis: The data was fitted with two alternative regression models: either a horizontal line (null hypothesis) or a single cosine curve (alternative hypothesis) as defined by the equation Y = mesor + [amplitude*cos (2*π*(X-acrophase)/period)] with a constant period of 24 hours (Weissova et al. 2016). The analysis was done in Prism 8 software (GraphPad, La Jolla, USA).

3. Results

3.1. Actigraphic data

Using the actigraphic data, we performed nonparametric circadian analysis of rest/activity patterns and sleep/wake patterns for eight subjects (two subjects were excluded due to defective MotionWatch devices). We compared the data recorded in the Czech Republic before attending the Svalbard expedition with the data recorded in Svalbard.

3.1.1. Activity

The activity is presented as a mean in 30 min intervals throughout the day (**Figure 2A**). The repeated measures 2-way ANOVA using Bonferroni's multiple comparison tests revealed a significant effect of time (F = 18.25; P < 0.0001), confirming the presence of daily variation in activity in both groups; however, there was no significant difference between the groups (F = 0.9007; P = 0.6902). Cosinor analysis followed by paired t-test identified a significant difference in acrophase between Czech and Svalbard circadian activity. Activity in Svalbard had been delayed 0.97 ± 0.1 h (P = 0.0021; **Figure 2B**).

3.1.2. Sleep analysis

The analysis of sleep and nonparametric circadian parameters was performed by MotionWare software. We compared the times when participants fell asleep, the times at which they woke up, and total sleep duration.

Student's t-test revealed significantly later fall-asleep time in Svalbard (Czech Republic mean: 23.79 ± 0.98 h;

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During the Polar Day in Svalbard

Svalbard mean: 24.8 ± 1.03 h; P < 0.0005; **Figure 3A**). Similar results were observed for wake-up time means, for which the subjects' mean wake times in Svalbard were significantly delayed as well (Czech Republic mean: 7.51 ± 1.4 h; Svalbard mean: 8.51 ± 0.97 h, P = 0.0056; **Figure 3B**). We did not find any significant difference in mean sleep duration (Czech Republic mean: 6.36 ± 0.67 h; Svalbard mean: 6.65 ± 0.65 h; **Figure 3C**).

3.2. Salivary melatonin level

Melatonin levels were determined from saliva samples collected 9 times during the 24-h period. The daily melatonin is presented as a mean over the 32-h period with re-plotted values at 9*, 11*, 15*, and 19* hours. The repeated measures 2-way ANOVA using Bonferroni's multiple comparison test revealed a significant effect of time (F = 12.5; P < 0.0001; **Figure 4**), which confirmed the daily variation in melatonin secretion in both groups. However, there was no significant difference between the samples col-

lected in Svalbard and in the Czech Republic (F = 1.617; P = 0.0654). Multiple-comparison test revealed significant differences at 3 AM between the groups (P = 0.0355). Despite no significant difference between those two conditions, there was a significant difference in acrophase between melatonin rhythmicity in the Czech Republic and Svalbard (paired Student's t-test; P = 0.0030; **Figure 5A**). The melatonin rhythm in Svalbard was delayed by about 1.666 ± 1.14 h. The comparison of amplitude between the samples collected in Svalbard and in the Czech Republic did not reveal significant differences due to a large SEM in the Czech time point (**Figure 5B**).

3.3. Clock gene expression in oral mucosa

The *Per1, Nr1D1* and *Bmal1* expression profiles in oral mucosa were analyzed with cosinor analysis. The *Per1* and *Nr1D1* expressions showed significant circadian rhythmicity (**Figure 6; 1a, 2a**). Neither the Svalbard samples nor the Czech samples showed significant rhyth-
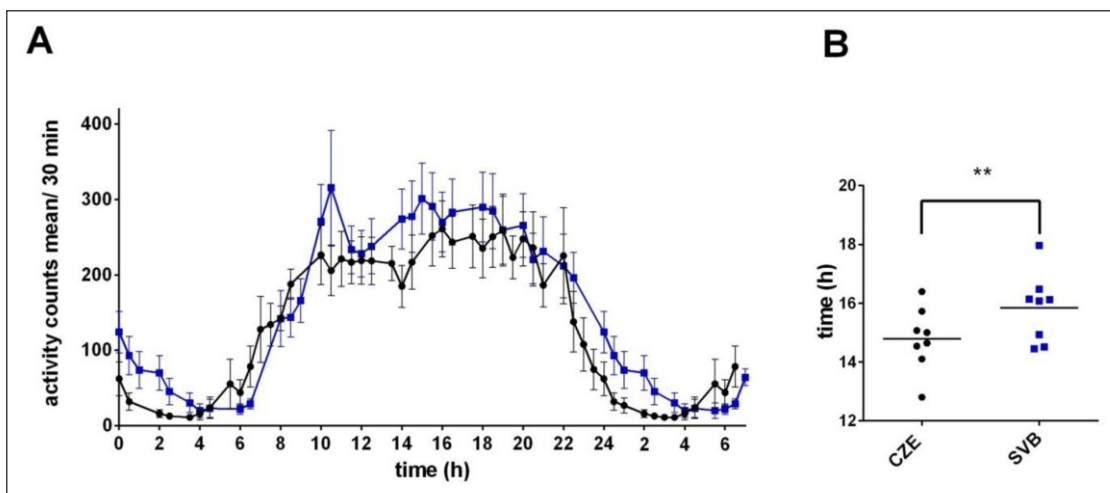

Figure 2: Activity RHYTHMS **of** SUBJECTS **in Czech Republic (CZE) and in Svalbard (SVB). A)** Mean daily activity of eight subjects in the Czech Republic and in Svalbard. The activity was recorded by MotionWatch for 12 days and displayed as a mean ± SEM in 30 min bins over 24 h. For clarity, part of the day (00:00 to 7:00) was re-plotted. In the Czech Republic, the activity counts are represented by black dots and a black line, and in Svalbard by blue boxes and a blue line. **B)** Activity acrophase plotted for each subject. Black dots represent the acrophase for each subject in the Czech Republic; blue boxes present the acrophase in Svalbard.
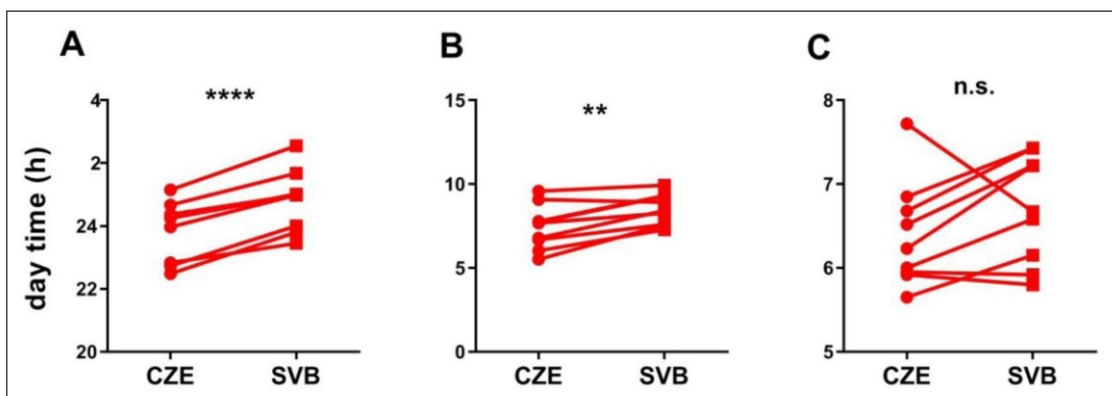

Figure 3: Sleep PARAMETERS **in the Czech Republic (CZE) and in Svalbard (SVB). A)** Fall-asleep time for each subject in the Czech Republic and in Svalbard. **B)** Wake-up time for each subject in the Czech Republic and in Svalbard. **C)** Sleep duration for each subject in the Czech Republic and Svalbard.
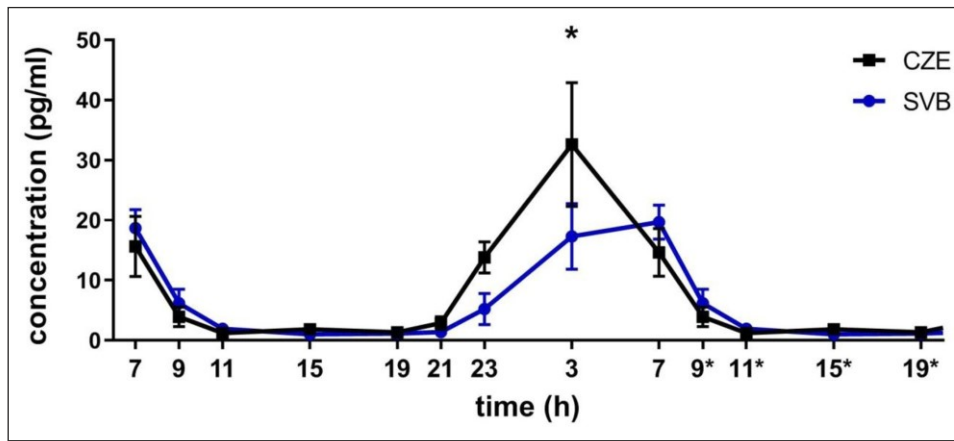
Figure 4: Daily PROFILES of melatonin LEVELS in SALIVA in the Czech Republic (CZE) and in Svalbard (SVB).
Melatonin levels were detected in saliva in 24 h profiles and expressed as mean ± SEM in pg/ml (n = 8). Black boxes and a black line represent melatonin levels in the Czech Republic and blue circles and blue lines represent melatonin level in Svalbard.
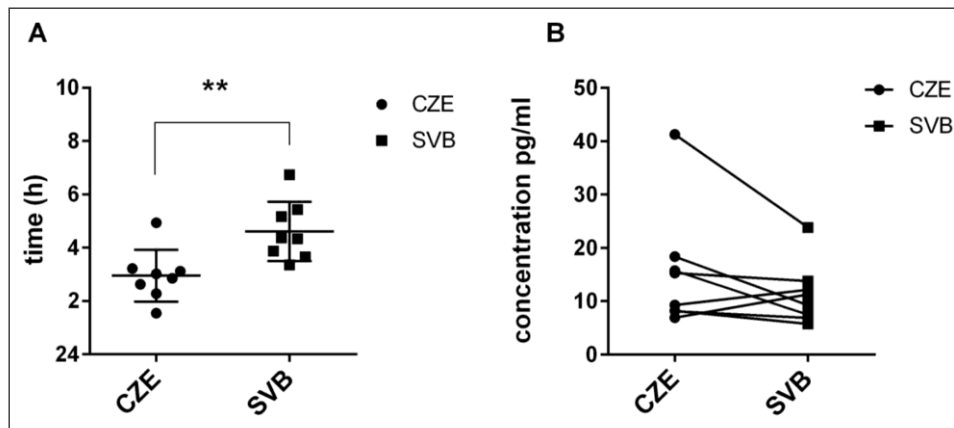

Figure 5: COSINOR ANALYSIS in melatonin daily rhythm. A) Melatonin acrophase plotted for each subject in the Czech Republic (black dots) and in Svalbard (blue boxes). **B)** Melatonin amplitude plotted for each subject in the Czech Republic (black dots) and in Svalbard (black boxes).

mic expression in *Bmal1* gene expression (**Figure 6, 3a**). The repeated measures 2-way ANOVA with Bonferroni's multiple comparison test revealed a significant effect of time (*Per1*: F = 12.5; P < 0.0001; *Nr1D1*: F = 11.08; P < 0.0001), which confirmed the presence of daily variation in gene expression in both groups. However, there was no significant difference between the groups (*Per1*: F = 0.966; P = 0.4527; *Nr1D1*: F = 1.050; P = 0.3989). The comparison of amplitude using paired Student's t-test revealed a significant difference in *Per1* and in *Nr1D1* (*Per1*: P = 0.042; *Nr1D1*: P = 0.0244; **Figure 7; 1a, 2a**). However, there was no significant difference in acrophase, although the SD range was smaller in Svalbard (*Per1*- CZE: SD ± 3.13; SVB: SD ± 1.82; *Nr1D1*-CZE: SD ± 4.36; SVB: SD ± 2.03; *Bmal1*- CZE: SD ± 6.89; SVB: SD ± 6.26; **Figure 7; 2a, 2b, 2c**).

4. Discussion

The aim of this study was to examine the actual ability of the circadian clock to adapt to the polar day in real-life conditions. Each subject enrolled in the study was exposed to both study conditions: the Czech summertime and the Svalbard polar day. To our knowledge, there was only one study reporting such results with a similar study design. That study included only three subjects and was focused on different circadian parameters (oral temperature, self-reported fatigue, grip strength, heart rate, time of waking and getting up;) [1].

Our results suggest that circadian rhythmicity in the tested group of researchers differed based on the conditions in the Czech Republic and after two weeks in Svalbard. The activity rhythm was delayed in Svalbard, similarly to the salivary melatonin rhythm. The peripheral clock gene expression displayed higher amplitude and the acrophase standard deviation was lower. Comparing the mean overall activity between Svalbard and the Czech Republic, we did not observe any significant changes in its level. Sleep analysis confirmed that the majority of subjects postponed their wake-up and fall-asleep times without any significant changes in sleep duration. The mean salivary melatonin profiles displayed circadian rhythmic production in both conditions, but the night maximum in Svalbard was decreased and its rhythm was significantly delayed in the same manner as the activity. Delayed
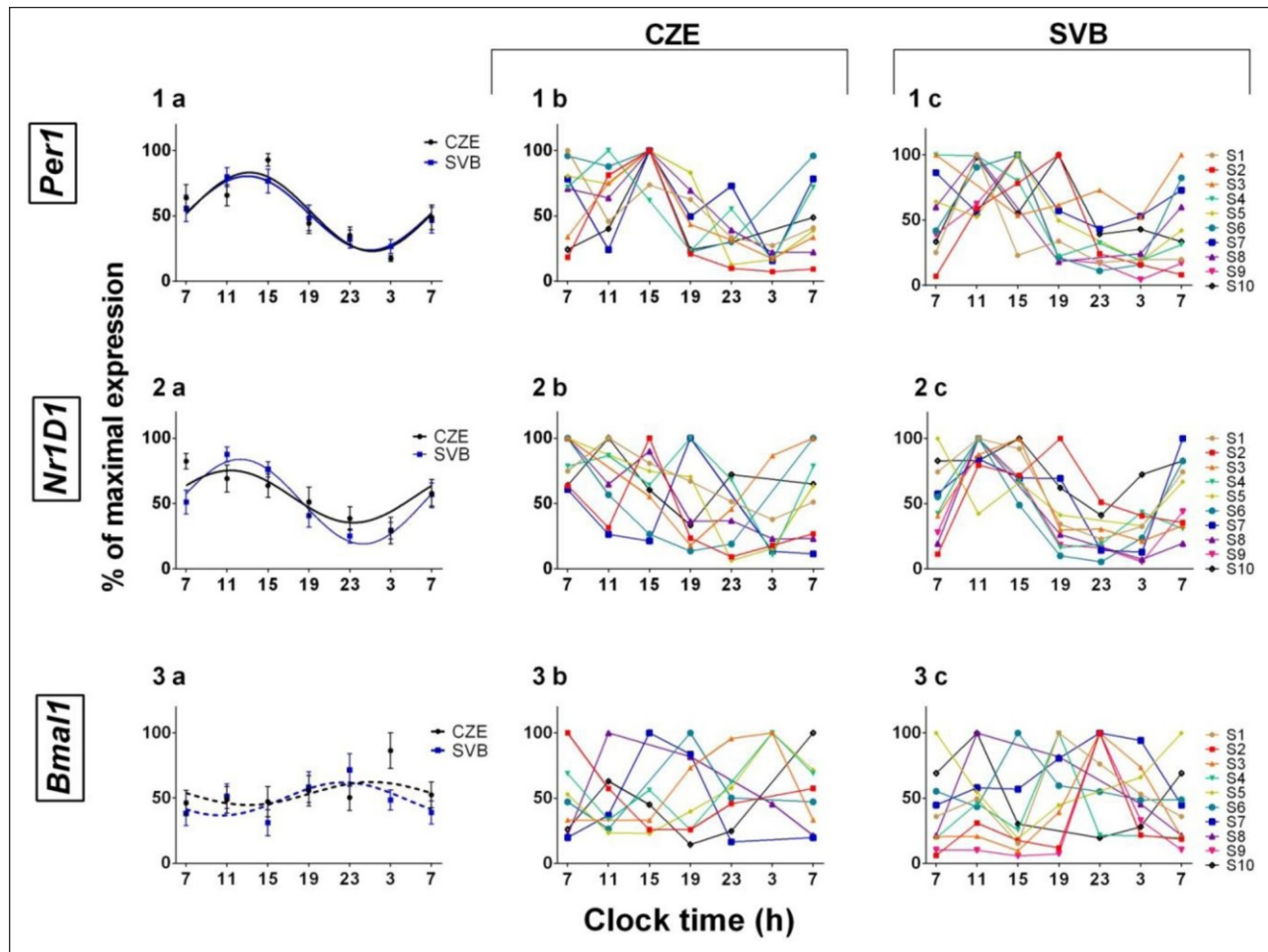
Figure 6: Individual and group clock gene EXPRESSION in oral MUCOSA. 1) *Per1*, 2) *Nr1D1*, and 3) *Bmal1*. **a)** Cosinor analysis of the group means ± SEM 24-h rhythms in *Per1, Bmal1*, and *Nr1D1* gene expression. **b)** Individually plotted clock gene expression in the Czech Republic. **c)** Individually plotted clock gene expression in Svalbard. Relative gene expression is displayed in % of maximal value of its expression. Significant rhythms (P < 0.05 for *Per1, Nr1D1*) are indicated by a solid line; non-significant rhythms are indicated by a dotted line. The group averages are means ± SEM.

sleep/activity patterns and melatonin production during the polar day correspond with previous findings by other studies [2–5] and could be the result of the exposure to bright light until bedtime (**Figure 1**). Although the subjects slept in a facility with less light, the level of light during the night was higher than in the Czech Republic, which may provide some explanation for lowered melatonin levels during the night, most significantly at 3 AM. A comparison of within-subject profiles suggests that the lowered amplitude in Svalbard may be more general, and in the group analysis, it may be masked by the distinct individual phases of the melatonin rhythms.

To assess the circadian clock gene expression, we used oral mucosa samples collected every 4 hours. Oral mucosa samples provide a sufficient amount of RNA to analyze the circadian oscillation in oral mucosa cells [7,8]. Statistical analysis did not confirm the circadian rhythmicity of *Bmal1* expression. The low amplitude of *Bmal1* in samples of peripheral tissue has been shown in many other studies [8–10]. High inter-individual variability in phase and amplitude in our samples could further enhance the masking of the grouped rhythm. We observed that *Per1* and *Nr1D1* genes display strong circadian oscillation under both conditions, but surprisingly, the amplitude

was significantly higher in the Svalbard samples and the acrophase standard deviation was lower. This might suggest a higher degree of synchronization of the circadian system in Svalbard. Increased amplitudes of *Per1* and *Nr1D1* were found even in individuals' expression profiles. The smaller acrophase variability suggests that the synchronization in Svalbard could be better even between subjects.

In humans, the absence of or weakened photic zeitgeber may be compensated for by other zeitgebers, such as physical activity, social cues, regular mealtimes or drug intake [11–13]. A field study by Reinberg et al. (1984) did not show any significant alteration in circadian parameters of activity-rest rhythm, oral temperature, or fatigue rhythm after 63, 141, and 147 days in polar day conditions. The authors concluded that social synchronization might help to maintain the rhythmicity of subjects [1]. Other studies demonstrate that the circadian rhythmicity is stronger during constant light or darkness when the subjects keep their regular daily structures and suggest that a strict daily regime could very efficiently stabilize circadian rhythms [4, 14]. On the contrary, Kennaway et al. reported free-running rhythms in melatonin and activity/rest rhythm. However, the authors discussed that the subjects in their
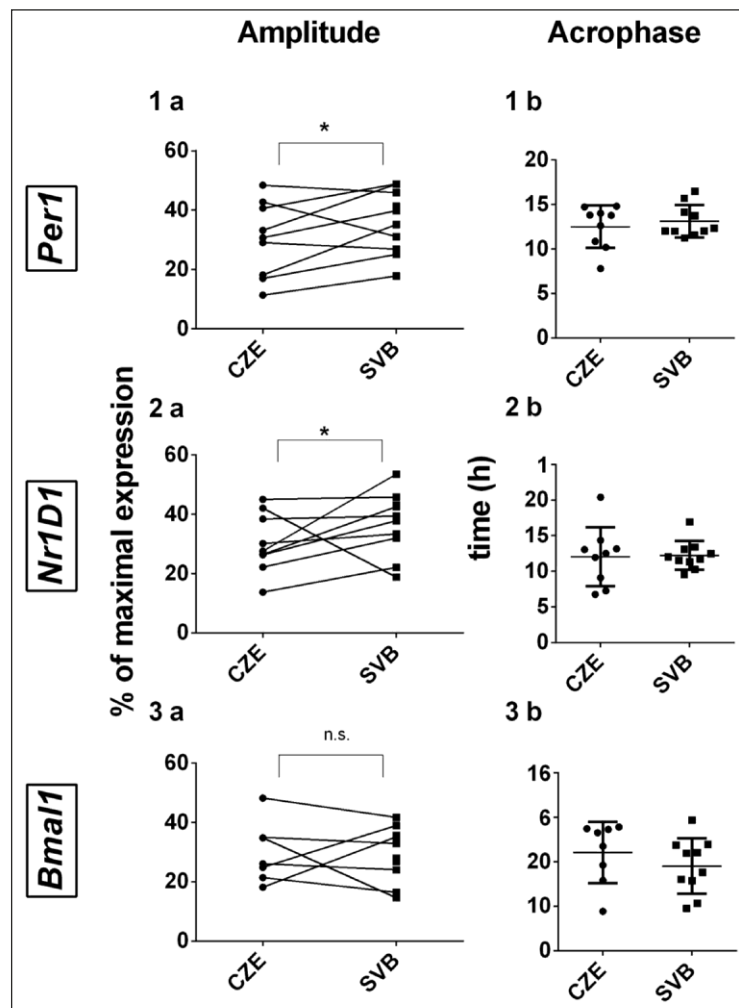
Figure 7: The DIFFERENCES in AMPLITUDES and ACROPHASES for individual clock GENES' PROFILES in the Czech Republic and in Svalbard. 1) Individually plotted amplitudes compared by paired Student's t-test. 2) Individually plotted acrophases compared by paired Student's t-test. **A)** *Per1*, **B)** *Bmal1*, and **C)** *Nr1D1*. The dots represent the values in the Czech Republic and the squares represent the values in Svalbard.

study were encouraged to follow their individual sleep needs, and there was no pressure on them to work within time constraints [15].

Concerning our data, we hypothesize that in the Czech Republic, each of the subjects followed his or her subjective daily routine and they differed in their lifestyles. This could be reflected by higher variability in clock genes' oscillation and melatonin rhythmicity as well. Regular social interaction among the subjects, shared mealtimes, and strict sleep-wake and working schedules in Svalbard could entrain the circadian rhythmicity even during extreme photoperiods such as a polar day. Alternatively, the slightly higher level of physical activity in Svalbard (see **Figure 2**) could further strengthen the synchronization of the peripheral clocks [16].

5. Conclusion

In general, circadian rhythms in activity (as a behavioral circadian marker), melatonin (as an indirect circadian marker), and peripheral clock gene expression (as a direct circadian marker) were slightly altered, but all of the subjects maintained circadian patterns in those studied parameters. Light-sensitive melatonin production was delayed by late-night light exposure; late-night illumi-

nation had the same effect on activity pattern, but the rhythm stayed consistent. A similar effect was observed in the peripheral clock genes' expression; however, the rhythm was more pronounced. The delay in circadian clock-driven parameters such as melatonin production and the sleep/wake rhythm might result from the late night light exposure and Svalbard expedition work schedule rather than reflect a delay in the inner circadian clock. We hypothesize that the clock stayed fully synchronized under the conditions of the polar day due to the shared schedule between participants, particularly the mealtimes, which would also lead to strengthened social interactions. These factors have a cumulative positive effect on circadian rhythm synchronization.

Competing Interests

The authors have no competing interests to declare.

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