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Melatonin receptor type 1A gene linked to Alzheimer's disease in old age

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Abstract

Disruption of the circadian rhythms is a frequent preclinical and clinical manifestation of Alzheimer's disease. Furthermore, it has been suggested that shift work is a risk factor for Alzheimer's disease. Previously, we have reported association of intolerance to shift work (job-related exhaustion in shift workers) with a variant rs12506228A, which is situated close to melatonin receptor type 1A gene (*MTNR1A*) and linked to *MTNR1A* brain expression levels. Here, we studied association of that variant with clinical and neuropathological Alzheimer's disease in a Finnish whole-population cohort Vantaa 85+ ($n = 512$, participants over 85 years) and two follow-up cohorts. Rs12506228A was associated with clinical Alzheimer's disease ($p = 0.000073$). Analysis of post-mortem brain tissues showed association with higher amount of neurofibrillary tangles ($p = 0.0039$) and amyloid beta plaques ($p = 0.0041$). We then followed up the associations in two independent replication samples. Replication for the association with clinical Alzheimer's disease was detected in Kuopio 75+ ($p = 0.012$, $n = 574$), but not in the younger case-control sample ($n = 651 + 669$). While melatonin has been established in regulation of circadian rhythms, an independent role has been also shown for neuroprotection and specifically for anti-amyloidogenic effects. Indeed, in vitro, RNAi mediated silencing of *MTNR1A* increased the amyloidogenic processing of amyloid precursor protein (APP) in neurons, whereas overexpression decreased it. Our findings suggest variation close to *MTNR1A* as a shared genetic risk factor for intolerance to shift work and Alzheimer's disease in old age. The genetic associations are likely to be mediated by differences in *MTNR1A* expression, which, in turn, modulate APP metabolism.

Alzheimer's disease, melatonin receptor, genetic association study, gene expression, amyloid beta, neurofibrillary tangles

Statement of Significance

The previous systematic search for molecular genetic risk factors for intolerance to shift work identified a risk variant close to melatonin receptor 1a (*MTNR1A*). Here, we showed association of the same risk variant with clinical and pathological Alzheimer's disease in the very elderly. In line, modulation of the *MTNR1A* expression level in cell culture regulated amyloid beta production. Thus, we revealed a novel molecular genetic connection between circadian phenotype in the working age and neurodegeneration in old age, which supports the view that circadian regulation, possibly through weaker physiological melatonin signaling, contributes to the pathological cascade of Alzheimer's disease. Future studies are needed to clarify whether the poor tolerance to shift work per se indicates increased risk of Alzheimer's disease.

Introduction

Alzheimer's disease is the most common cause of dementia. It is clinically characterized by progressive cognitive decline and neuropathological changes, including amyloid beta (Aβ) plaques, neurofibrillary tangles, and neuronal loss. The cognitive symptoms in people with Alzheimer's disease are usually accompanied by dysfunction of the circadian system, which manifests for example as a fragmented sleep-wake cycle, decreased rhythmicity of melatonin secretion, and evening restlessness and agitation [1–3]. This dysfunction has already been reported in the preclinical phase [4–6]. In addition, disruption of circadian rhythms and sleep deprivation can interfere with the pathological cascades of Alzheimer's disease at least two ways: by increasing the generation of the Aβ peptide [7] and by disturbing the normal clearance of Aβ and other metabolites from the brain during sleep [8].

Shift work represents a common environmental source for disruption of circadian rhythms and sleep deprivation. There is evidence of transient cognitive decline in shift workers [9, 10], as well as association with the risk of Alzheimer's disease [11] and dementia [12]. No association with the risk of dementia or accelerated cognitive decline at later life was visible in the recent longitudinal studies [13, 14]. However, cardiovascular death as a competing risk is likely to mask potential associations with dementia, because shift workers have increased risk for cardiovascular mortality [11] and, thus, less likely to become old enough to have dementia, like demonstrated for smoking and dementia [15]. To our knowledge, no studies have examined the risk of dementia of those workers with poor tolerance to shift work.

In our previous genome-wide study, we identified a genetic risk variant for intolerance to shift work by studying shift workers from the general Finnish population and specific occupational cohorts [16]. The risk variant, rs12506228, which is situated downstream of melatonin receptor type 1A gene (*MTNR1A*), was associated with reduced *MTNR1A* expression levels in cerebellar brain tissue in the eGWAS Mayo data. The allele-specific differences in gene expression were likely mediated by changes in DNA methylation in the 5′ regulatory region of *MTNR1A* [16].

Melatonin has been reported to have neuroprotective and anti-amyloidogenic effects: it reduces Aβ production in multiple neuronal cell lines [17, 18]. It also decreases amyloid pathology and memory deficits in animal models of Alzheimer's disease [19, 20], and reverses the age-related changes in normal aged mice [21]. However, it has been unclear if these effects are mediated by the two high-affinity melatonin receptors (coded by *MTNR1A* and *MTNR1B*) [22] or by other pathways [23]. Melatonin secretion decreases with age, and this is especially pronounced in people with Alzheimer's disease [24]. A similar trend of age-related decrease has been reported for the expression of *MTNR1A* in the suprachiasmatic nucleus (SCN), the master circadian clock of the body [25].

We hypothesized that intolerance to shift work and late-onset Alzheimer's disease would share genetic risk factors. To test that hypothesis, we examined whether *MTNR1A* variant rs12506228 would confer an increased risk of Alzheimer's disease in an elderly Finnish population cohort with clinical and neuropathological characterization of Alzheimer's disease. The associations with clinical traits were studied further in another Finnish population-based sample and in a case-control sample of Alzheimer's disease. Because of the previously reported association of rs12506228 with decreased *MTNR1A* expression, we also studied the effects of gene silencing by RNAi, as well as overexpression of *MTNR1A* on amyloidogenic processing of β-amyloid precursor protein (APP) in N2A cells.

Materials and Methods

Participants

Alzheimer's disease was studied primarily in the Vantaa 85+ study [26], a Finnish population-based cohort comprising all participants aged 85 years or older from Vantaa, Finland as of April 1, 1991 ($n = 601$). The participants were studied at the baseline and survivors were re-examined in 1994, 1996, 1999, and 2001. A clinical examination was carried out on 553 participants, 534 with DNA samples available. Dementia status was tested in the baseline and during follow-up visits according to the Diagnostic and Statistical Manual of Mental Disorders (third ed., rev., DSM-III-R) [27], while clinical Alzheimer-type dementia was diagnosed in accordance with criteria from the National Institute of Neurologic and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) [28] as presented in detail previously [26]. In addition, all non-demented individuals were followed-up until death for symptoms of dementia through a review of their health-care records. Mixed cases of vascular and Alzheimer-type dementia were classified as vascular dementia and not Alzheimer's disease. Thus, participants with Alzheimer-type dementia were "pure" cases of Alzheimer's disease without known vascular events. Consequently, the frequency of Alzheimer-type dementia was relatively low in Vantaa 85+ (Table 1). Cognition was evaluated using Mini-Mental State Examination (MMSE) [29] and Short Portable Mental Status Questionnaire (SPMSQ) [30] cognitive tests, and performance in everyday life was evaluated by a nurse using a scale from 1 (independent) to 6 (fully dependent). Age of onset for dementia was assessed according to the estimated age of onset for symptoms.

Table 1.

Characteristics of the study samples

	Vantaa 85+	Kuopio 75+	Kuopio case-control study, cases	Kuopio case-control study, controls
Type of sample	**Whole-population**	**Population-based**	**Case-control**	**Case-control**
*n**	535	590	673	686
Age (range)†	92.4 (85–106)	84.0 (75–99)	71.4 (43–89)	69.1 (37–87)
Males	21%	26%	33%	40%
Neuropathological Alzheimer's disease	36.5%			
Dementia, Alzheimer-type	25.8%‡	17.6%	100%	0%
Dementia, all types	56.8%‡	32.2%	100%	0%
MMSE at baseline, mean (*SD*)	17.8 (9.2)	22.2 (8.12)	19.4 (4.6)	NA

*DNA samples available, before quality control.

†At the latest exam or age of death when followed-up until death (Vantaa 85+).

‡Diagnosis ever (followed-up until death).

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Neuropathological autopsies were completed in 304 participants (51%) and carried out as described earlier [26]. The neurofibrillary tangles were assessed using Braak staging [31], and the Aβ plaques using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) protocol to evaluate the maximum density of the plaques in the cortical sections. As an additional measure for neocortical amyloid plaque load, we performed morphometric analysis of methenamine-silver stained sections [32, 33]. That trait was transformed with natural logarithm to increase normality of the distribution. Descriptive statistics for the neuropathological measures are shown in Supplementary Table S1. Neuropathological Alzheimer's disease diagnosis was assessed using the modified neuropathological National Institute on Aging and Reagan Institute (NIA-RI) criteria [34] in order to create a group of individuals with no Alzheimer's disease or a low probability for Alzheimer's disease and another group of individuals with a high or intermediate probability for Alzheimer's disease (controls: CERAD scores of no or sparse neuritic plaques and Braak stages 0–II, cases: CERAD scores of moderate or frequent neuritic plaques and Braak stages IV–VI). We only included cases with diagnosed clinical dementia. Participants who failed to fulfill the criteria for either of the groups were excluded from that analysis.

The Kuopio 75+ cohort was collected using a protocol resembling that used in the Vantaa 85+, and it is described in detail elsewhere [35, 36]. The baseline phase, carried out in 1998, consisted of a representative sample (n = 700, 5.3%) of inhabitants over 75 years of age of Kuopio, Finland, 601 of which were examined. A follow-up examination was performed 5 years later for 339 persons. Information for diagnostics was gathered from questionnaires, from structured clinical examination and interview by geriatrician and trained nurse, as well as from interviews of relatives and caregivers, the clinical records of local hospitals, Kuopio University hospital, health care centers and home nursing if needed. Dementia diagnoses were established in consensus meetings by a neurogeriatrician (RS) using DSM-III-R criteria for dementia, and DSM-IV criteria [37] for clinical Alzheimer-type dementia (probable Alzheimer's disease). Similarly to Vantaa 85+, mixed cases of vascular and Alzheimer-type dementia were classified as vascular dementia. Age of onset for dementia was assessed according to reported age of onset for symptoms. If dementia was diagnosed in the follow-up but not in the baseline, the age of onset was estimated to the midpoint of the 5 years follow-up. DNA samples were given by 590 individuals. All phenotypes were determined using the latest measure available. At that point, most individuals were over 80 years old (86.4%), and 36.7% were also over 85 years of age.

The "Kuopio case-control study" consisted of 673 Alzheimer's disease cases and 686 controls [38]. All patients were drawn from Eastern Finland and were examined in the Kuopio University Hospital's memory clinic, and probable Alzheimer's disease was diagnosed according to the NINCDS–

ADRDA criteria [39]. The community-based voluntary unrelated control participants were age-matched with the cases and derived from the same restricted area of eight communities in the province of Northern Savo [40]. The mean age of onset of dementia was 71.4 (*SD* = 7.4) for cases, and the mean age at examination was 69.1 (*SD* = 6.2) for controls. Controls had no symptoms of cognitive impairment based on a clinical interview and neuropsychological examination. The individuals were nonoverlapping with the Kuopio 75+ study.

Ethical permissions

All participants, and their relatives in case of dementia, gave their informed consent including for the use of genetic data. Local ethical committees approved the study protocols. The Ministry of Social Affairs and Health granted permission to use the health and social care notes for the Vantaa 85+ study participants. The Center for Medico-Legal Affairs granted permission to collect and use autopsy tissue for the Vantaa 85+ study participants.

Genotyping

In the Vantaa 85+ cohort, genotypes were extracted from Illumina 370 K platform and underwent quality control as described previously [41]. Call rates for individuals and markers in genome-wide genotyping were >95%. In Kuopio 75+, genotyping of rs12506228 was performed with capillary sequencing using sequencing primer 5-GGGGCATATCTTTGTCTATGG-3. The genotypes were read by hand by two persons. Samples were excluded due to missing phenotype data for dementia ($n = 1$), disrupted sample ($n = 10$) or failed or low-quality sequencing ($n = 4/579$, 0.7%). Genotyping of rs12506228 in the Kuopio case-control study (Hardy-Weinberg equilibrium $p > 0.001$) was performed by MassARRAY genotyping using iPLEX Gold chemistry (Sequenom, San Diego, CA) (an average call rate of ≥90%).

Whole-genome sequencing

Samples for whole-genome sequencing from the Vantaa 85+ Study were selected based on DNA quality and quantity (more than 4 ug left). Three hundred nine samples underwent genome sequencing, and, of these, 300 were successfully sequenced. Genome sequencing was performed using the Illumina TruSeq DNA PCR Free library preparation kit and the HiSeq X10 sequencing system to produce 150-base pair paired-end reads according to the manufacturer's protocol. Raw sequence data was processed according to Broad Best Practices API (application program interface).

Downstream analysis was performed with PLINK v1.9, https://www.cog-genomics.org/plink2 [42]. Additional quality control criteria were applied: related individuals (IBD > 0.185), individuals with discordant sex information, elevated missing data rate (>3%) or outlying heterozygosity rate (±2 *SD*) were excluded. Variants with missing per person rate >10%, missing rate >5% or significantly different genotype call rates between cases and controls ($p < 0.00001$) were excluded. Variants not in Hardy-Weinberg equilibrium ($p < 0.00001$) were also excluded.

Statistical analysis

SNP association analyses were performed using PLINK, version 1.07, http://pngu.mgh.harvard.edu/purcell/plink/ [43] for all samples, with the exception of the Kuopio case-control sample, where SPSS (IBM SPSS Statistics for Windows, version 21.0, IBM Corp. Armonk, NY) was used. Quantitative traits were analyzed using linear regression model and dichotomous traits with logistic regression model using additive model adjusting for sex. Fixed-effect meta-analyses were performed using GWAMA [44]. The statistical tests were two-tailed and the alpha-level of $p < 0.05$ was used for this candidate gene study. No correction for multiple testing was performed.

In vitro analysis of the modulation of APP metabolism by *MTNR1A*

A stable mouse neuroblastoma N2A cell line (N2A/APP) overexpressing the human APP_{751} isoform was selected and maintained using G418. Human *MTNR1A* cDNA (BC116900) was purchased from OpenBiosystems/ThermoFisher and subcloned to the pcDNA6/V5-His vector. For the characterization of the effects of *MTNR1A* on APP metabolism, N2A/APP cells were transiently transfected with either pcDNA6-*MTNR1A* overexpression plasmid or *MTNR1A*/B3 shRNA plasmid using the JetPEI reagent (Polyplus) according to the manufacturer's instructions (Supplementary Methods). The media was changed at 24-hour post-transfection and melatonin (1 or 10 μM; Sigma) or a vehicle was added to the cells for another 24 hours. Conditioned media was collected at 48-hour post-transfection and cleared of debris by centrifugation at 3000× g. Cells were washed twice with ice-cold PBS and harvested, with the total protein content determined using a BCA protein assay kit (Pierce/Thermo), and an equal amount of protein per sample was analyzed in Western blots as described previously [45]. For soluble amyloid precursor protein-β (sAPPβ) detection, equal volumes of conditioned media were analyzed on Western blots. The following antibodies were used: APP C-terminal (A8717, Sigma),

sAPPβ (Covance), APP N-terminal ($sAPP_{total}$, 22C11, Millipore), *MTNR1A* (Santa Cruz), BACE1 (Santa Cruz), GAPDH (Millipore), and β-tubulin (isoforms I+II; Sigma). Western blot images were quantitated using the Quantity One software package (Bio-Rad).

$A\beta_{1–40}$ and $A\beta_{1–42}$ peptides from the conditioned media were analyzed using commercially available sandwich ELISA kits according to the manufacturer's protocols (Human Amyloid-β (1–40) Assay Kit 27718 and Human Amyloid-β (1–42) Assay Kit 27719, IBL International GmbH, Germany). Three replicate wells per sample were analyzed from three independent experiments. Statistical analyses were based on ANOVA with Bonferroni's correction.

Results

Association of rs12506228 near *MTNR1A* with clinical and pathological Alzheimer's disease

To test the hypothesis of shared genetic background for intolerance to shift work and Alzheimer's disease, we examined association of rs12506228 with clinical dementia and pathological hallmarks of Alzheimer's disease in the Vantaa 85+ cohort. We first analyzed associations with clinical variables (overall dementia vs. no dementia, age-of-onset of dementia, Alzheimer-type of dementia according to NINCDS–ADRDA criteria vs. no dementia, baseline cognition and general performance). The A allele of rs12506228 was significantly associated with dementia ($p = 0.0012$, odds ratio [OR] = 1.62), earlier age-of-onset for dementia ($p = 0.0070$, $\beta = -1.15$), Alzheimer-type of dementia ($p = 7.3 \times 10^{-5}$, OR = 2.06), worse baseline cognitive tests (MMSE: $p = 2.1 \times 10^{-4}$, $\beta = -0.26$; SPMSQ: $p = 1.1 \times 10^{-5}$, $\beta = 1.15$), and worse general performance evaluated by nurse ($p = 0.0085$, $\beta = 0.29$) (Figure 1A, Supplementary Figures S1–S3).

Figure 1.

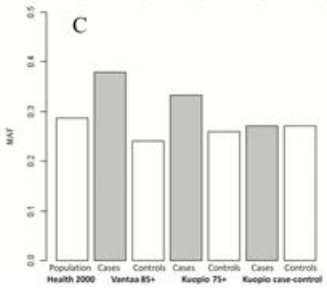
A

Trait	Vantaa 85+ P	Vantaa 85+ BETA (SE)	Vantaa 85+ OR (CI 95%)	Vantaa 85+ n (cases)	Kuopio 75+ P	Kuopio 75+ BETA (SE)	Kuopio 75+ OR (CI 95%)	Kuopio 75+ n (cases)	Meta-analysis P	Meta-analysis BETA (SE)	Meta-analysis OR (CI 95%)	Meta-analysis n (cases)
Clinical dementia AD type	**0.000073**		2.06 (1.44-2.95)	357 (136)	**0.012**		1.53 (1.10-2.12	490 (102)	**0.0000059**		1.75 (1.38-2.23)	847 (238)
Clinical dementia all types	**0.0012**		1.62 (1.21-2.17)	512 (291)	0.14		1.23 (0.94-1.60)	574 (186)	**0.0011**		1.39 (1.14-1.70)	1086 (477)
MMSE	**0.00021**	-2.41 (0.65)		498	**0.040**	-1.07 (0.52)		574	**0.000081**	-1.59 (0.41)		1017
SPMSQ	**0.000011**	1.15 (0.26)		504								
Age of onset for dementia, years	**0.0070**	-1.15 (0.42)		251	0.90	0.078 (0.65)		181	**0.027**	-0.79 (0.35)		432
Age of onset for AD, years	0.62	-0.33 (0.67)		117	0.77	-0.24 (0.84)		101	0.57	-0.30 (0.52)		218
Performance	**0.0085**	0.29 (0.11)		512								

B

Trait	P	Beta (SE)	OR (CI 95 %)	n (cases)
Neuropathological AD	**0.0028**		2.57 (1.38-4.78)	137 (89)
CERAD score	**0.0041**	0.27 (0.093)		274
Amyloid load	**0.0016**	0.26 (0.082)		274
BRAAK stage	**0.0039**	0.40 (0.14)		274

C



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Association of rs12506228 with clinical and neuropathological measures of Alzheimer's disease. (A) Association of rs12506228 with clinical measures of Alzheimer's disease (AD) using separate analyses of linear or logistic regression in the Vantaa 85+ and Kuopio 75+ cohorts and meta-analysis. (B) Association of rs12506228 with neuropathological diagnosis of Alzheimer's disease and parameters for neocortical amyloid pathology (CERAD score, amyloid load) and neurofibrillary pathology (Braak stage) in Vantaa 85+. (C) Minor allele frequency (MAF) for rs12506228 in the Finnish general population sample Health 2000 and in the cases and controls for Alzheimer's disease in the study samples.

Next, we looked at association of rs12506228 with neuropathological findings of Alzheimer's disease. The rs12506228 variant associated both with amyloid plaques (CERAD score: $p = 0.0041$, $\beta = 0.27$) and neurofibrillary tangles (Braak stage: $p = 0.0039$, $\beta = 0.40$).

Finally, we studied association with the combined clinical and histopathological measures (modified NIA-RI criteria), which represent

neuropathological diagnosis of Alzheimer's disease. The rs12506228 was associated with neuropathologically defined Alzheimer's disease (p = 0.0021, OR = 2.69) (Figure 1B, Supplementary Figure S4).

Replication of rs12506228 with clinical Alzheimer's disease in another population-based but not in a younger case-control sample

We performed replication analysis in Kuopio 75+. A significant association of the A allele of rs12506228 was found with clinical Alzheimer-type dementia (p = 0.012, OR = 1.53), as well as with cognitive test (MMSE: p = 0.040, β = −1.07). The association was driven by Alzheimer-type dementia, as the analysis for all types of dementia (combined) was nonsignificant (p = 0.14, OR = 1.23).

We then performed a fixed-effect meta-analysis for the old cohorts Vantaa 85+ and Kuopio 75+. The association signals became stronger (more significant) for clinical Alzheimer-type dementia ($p = 5.9 \times 10^{-6}$, OR = 1.75), all types of dementia (p = 0.0011, OR = 1.39), and MMSE ($p = 8.1 \times 10^{-5}$, β = −1.59) (Figure 1A).

Finally, in order to test the association in the third sample with participants at relatively younger age (mean 71 years for cases and 69 years for controls), we studied a clinical case-control sample from the Kuopio region. In contrast to the two population-cohorts comprising the oldest old, no association with clinical Alzheimer-type dementia was detected (p = 0.90, OR = 1.01, Figure 1C, Table 2). When restricting the analysis to cases and controls over 75 years, the number of controls was limited to 100, and the same direction of effect with Vantaa 85+ and Kuopio 75+ was nonsignificant (p = 0.53, OR = 1.13, Table 2).

Table 2.

Association of rs12506228 with clinical Alzheimer-type dementia in the Kuopio case-control sample

Age group	Minor allele frequency		*N*		*P*	OR (95% CI)
	Cases	Controls	Cases	Controls		
All	0.27	0.27	651	669	0.903	1.01 (0.85–1.20)
≤75	0.27	0.27	458	569	0.953	0.99 (0.82–1.21)
>75	0.27	0.25	193	100	0.531	1.13 (0.77–1.66)

CI, confidence interval. The genetic association was examined using binary logistic regression with an additive model in multivariate analysis corrected for sex.

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Fine mapping of the association signal

To search for potentially causative variants linked to rs12506228, we studied whole-genome sequencing data for 278 Vantaa 85+ participants and then subjected additional variants to association analysis. Variants in the 500 kb region around rs12506228 were investigated. Supplementary Table S2 shows those 130 SNPs which have $r^2 > 0.2$ with rs12506228. Out of these variants, the strongest associations with clinical Alzheimer's disease were detected with three SNPs, rs11723770 (p = 9.05E-07), rs7684546 (p = 1.66E-06) and rs78783920 (p = 2.83E-06). The fourth strongest association was found with the original SNP rs12506228 (p = 2.89E-05, Supplementary Table S3). The strongest association with CERAD was found with rs4862701 (p = 0.02) and the second strongest with rs12506228 (Supplementary Table S4). With Braak, no associations stronger than that of rs12506228 were detected (Supplementary Table S5). Rs11723770 was genotyped also as part of the genome-wide genotyping in the whole Vantaa 85+ cohort [41]. The association of that variant with clinical Alzheimer's disease (OR = 2.2, $p = 1.17 \times 10^{-5}$) as well as all types of clinical dementia (OR = 1.65, p = 0.00059) remained slightly stronger than that of rs12506228, but the association with age of onset for dementia (β = −0.51, p = 0.23), and neuropathological markers, Braak (β = 0.30, p = 0.027) and CERAD (β = 0.23, p = 0.012) were weaker to that of rs12506228. All these associating SNPs were in high linkage disequilibrium ($r^2 > 0.8$) with rs12506228 and they are located, as rs12506228, downstream of *MTNR1A* in the intron of the RNA gene F11-AS1.

In addition, rs78783920 (known also as rs2023257), the SNP in the highest LD ($r^2 = 0.91$) with rs12506228, is located inside a microsatellite length polymorphism at bp 187380340-187380372 (GRCh37/hg19). The intermediate-sized 13m allele of the microsatellite was tightly linked to both the T

allele of rs78783920 and the risk allele A of rs12506228 [16]. Here, we found an association of the 13m allele of the microsatellite, assessed by fragment analysis, with clinical Alzheimer-type dementia in a joint analysis of the Vantaa 85+ and Kuopio 75+ cohorts (Supplementary Table S6), but the association was statistically weaker as compared to that of rs12506228. Thus, there are several plausible variants downstream of *MNTR1A,* which may contribute to the signal of association obtained with rs12506228.

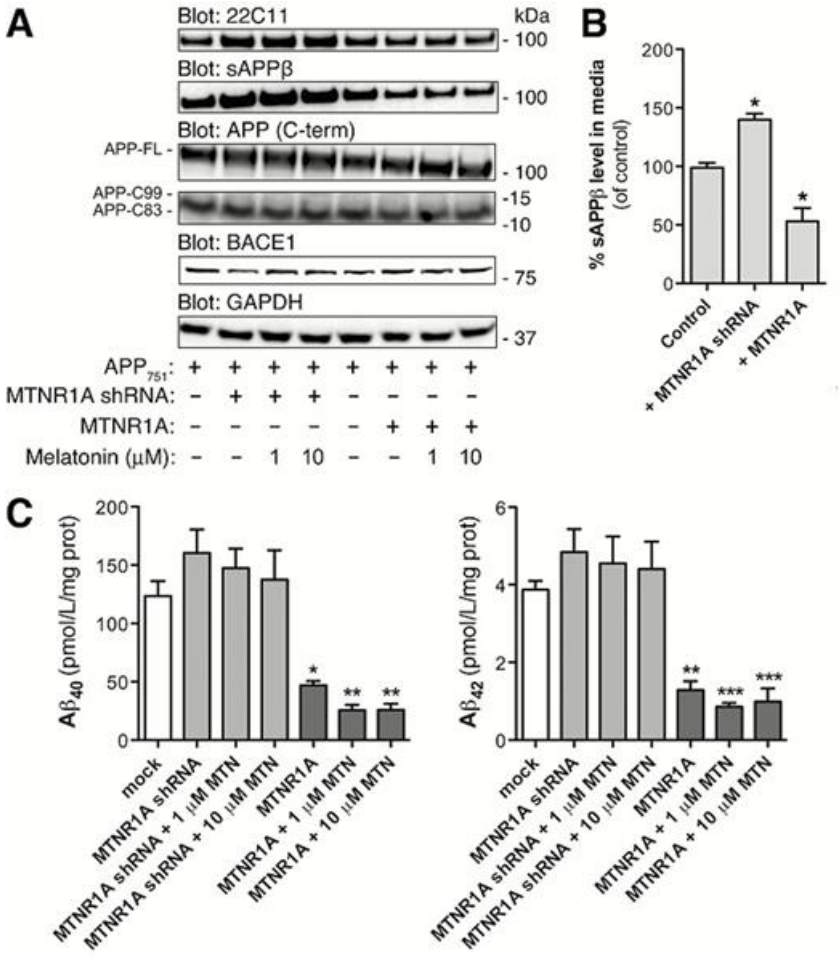
Inverse correlation of *MTNR1A* expression levels with amyloidogenic processing of APP in vitro

Next, we studied the impact of the MT1 melatonin receptors—the product of the *MTNR1A* gene—on proteolytic processing of APP by using N2A cells overexpressing the human APP_{751} isoform. MT1 melatonin receptor levels were knocked down by RNAi (transfection with *MTNR1A* shRNA plasmid), or up-regulated by transient overexpression of *MTNR1A*.

Amyloidogenic processing of APP was increased in cells where the MT1 melatonin receptor were decreased, as shown by the 40% increase in the levels of sAPPβ, the soluble APP ectodomain derived from amyloidogenic cleavage by BACE1 (Figure 2, A and B). Conversely, cells overexpressing MT1 melatonin receptors secreted 47% less sAPPβ (Figure 2, A and B). In concordance, MT1 melatonin receptor knockdown increased the levels of secreted $A\beta_{40}$ and $A\beta_{42}$ by 30% and 25%, respectively (Figure 2C), while both were decreased (by −62% and −67%, respectively; Figure 2C) in cells overexpressing MT1 melatonin receptors. Modulation of the receptor levels had no impact on the $A\beta_{42}$ / $A\beta_{40}$ ratio (which would indicate altered γ-secretase cleavage) or the levels of APP holoprotein or BACE1, the enzyme responsible for the β-cleavage of APP (Figure 2A).

Addition of melatonin (1 or 10 μM) to the media further decreased the Aβ levels in cells overexpressing the MT1 receptor (Figure 2C). In cells where MT1 receptor levels were reduced by RNAi, the addition of melatonin partially compensated for the increased Aβ secretion, likely reflecting the incomplete knockdown by RNAi.

Figure 2.



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MT1 melatonin receptor regulates the amyloidogenic processing of APP. (A) N2A cells were transfected with *APP* and *MTNR1A*

overexpression plasmids or *MTNR1A* shRNA plasmids, and treated with either a vehicle or melatonin (1 or 10 μM) for 24 hours. Soluble APP fragments were analyzed from the conditioned media with 22C11 (total sAPP) and sAPPβ-specific antibodies. APP holoprotein, C-terminal fragment, and BACE1 levels were analyzed from cell lysates. (B) sAPPβ levels were quantitated densitometrically from Western blots. (C) $A\beta_{40}$ and $A\beta_{42}$ levels in the conditioned media were determined by sandwich ELISA. *$p < 0.05$, **$p > 0.01$, ***$p < 0.001$. All the analyses were repeated three times.

Discussion

Here, we describe an association of an *MTNR1A* genetic variant with neuropathological Alzheimer's disease as well as clinical Alzheimer-type dementia in two elderly population cohorts. Our previous study identified the same risk variant for intolerance to shift work, and also reported association of that variant with reduced *MTNR1A* expression. Here, we showed that in vitro reduction of MT1 melatonin receptors, the product of *MTNR1A*, promoted Aβ production, likely via enhanced β-cleavage of APP. These results support previous studies suggesting that the pathophysiology of Alzheimer's disease (APP metabolism) is linked to the circadian regulatory system. Our results raise the hypothesis that part of the genetic predisposition to Alzheimer's disease, especially in very old age, is mediated via differences in *MTNR1A* regulation.

The association of rs12506228 with clinical Alzheimer-type dementia was found in two cohorts of very elderly population, but not in the younger clinical case-control sample. Age is the strongest risk factor for late-onset Alzheimer's disease, and if old age is not reached, the differences in genetic risks and accumulating Alzheimer's disease pathology may remain unexpressed. In our study, higher OR was detected when neuropathological changes were taken into account in addition to the clinical dementia status, similar to that previously reported for APOE4 [46, 47]. In Vantaa 85+ none of the 23 participants with A/A genotype of rs12506228 were free of severe Aβ or neurofibrillary neuropathology (Supplementary Figure S4). In addition, previously, when using cognitively intact and old (> 85 years) "supercontrols," stronger effect size was reported in the Icelandic population for a putative protective variant, A673T substitution of *APP* [48], and for a risk variant rs75932628-T of *TREM2* [49]. Here, the very old individuals in the Vantaa 85+ and Kuopio 75+ cohorts without dementia can be considered as "supercontrols" regarding Alzheimer's disease, who may have lower than general population frequency of the risk variants for Alzheimer's disease. This consequently increases the odds to detect the associations as compared to use of younger controls which are not part of the same population-based sampling with cases. This kind of pattern of allele frequencies is apparent for rs12506228. (Figure 1C). None of the published GWASes for Alzheimer's disease, to our knowledge, have been performed in samples with a mean age of over 80 years for cases and controls (GWASes of Alzheimer's disease in Supplementary Table S7), which may explain why rs12506228 has not been detected as a risk factor for Alzheimer's disease in earlier studies.

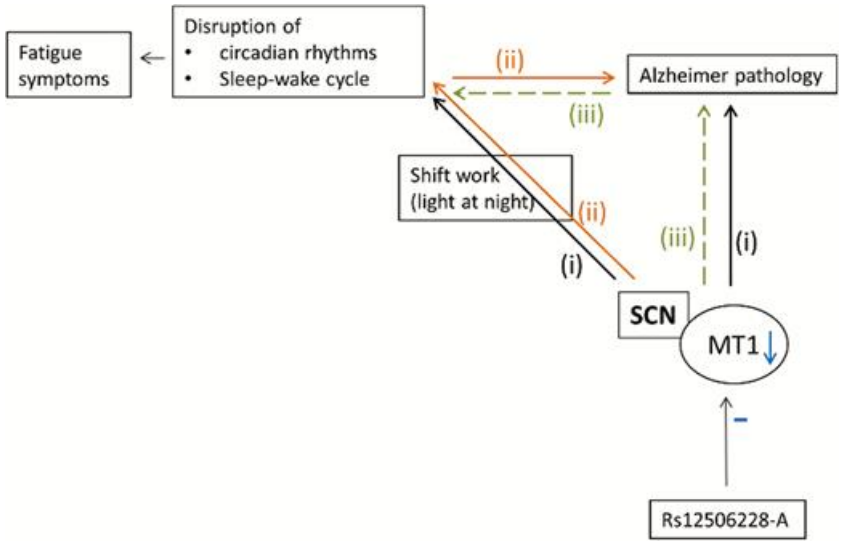
Neuroprotective effects of melatonin have become evident [50]. It has been unclear if the neuroprotective and anti-amyloidogenic effects of melatonin are mediated by the two high-affinity melatonin receptors or by other properties of melatonin. Neuroprotective effects of melatonin on mitochondrial and the Rip2/Caspase-1 cell-death pathways common to many neurodegenerative diseases, was demonstrated to be mediated by MT1 melatonin receptors [51]. One recent in vivo study in mice reported both receptor-dependent and independent effects on memory, receptor-dependent effects on antioxidant pathways but receptor-independent effects on Aβ plaques and plasma $A\beta_{1-42}$ levels [23]. In contrast, a recent in vitro study demonstrated receptor-dependent effects on the key enzymes of the amyloidogenic and non-amyloidogenic route of APP processing, BACE1, Presenilin-1, and ADAM10, as well as on the product of the non-amyloidogenic route, sAPPα [22]. This is concordant with our study, which showed that MT1 melatonin receptor levels modulate the production of Aβ and sAPPβ. In our model system, we observed no changes in BACE1 levels, which has been previously suggested as a potential mechanism for melatonin-mediated regulation of Aβ generation [22]. Although our data suggest that BACE1 activity may be directly affected by melatonin and MT1 melatonin receptor levels, it is possible that in brain tissue both the expression and activity are regulated by melatonin signaling in parallel.

In our study, most of the anti-amyloidogenic effect of *MTNR1A* overexpression was visible also without addition of melatonin (Figure 2). This is in line with previous reports of the constitutive activity of the MT1 melatonin receptors [52–54], which can cover 80%–90% of the maximal G-protein activity of the receptor [52].

Several, probably coexisting, explanations could be considered for our finding of the *MTNR1A*-linked genetic variant as a risk factor for the two traits: intolerance to shift work and Alzheimer's disease (Figure 3). First, melatonin has several targets in the body. Inadequacy of the receptors, in parallel, both increases the build-up of amyloid pathology in neurons as shown here and destabilizes the circadian rhythm in the SCN, which may lead to worse tolerance to shift work [16]. Second, the relative lack of melatonin receptors destabilizes the circadian rhythm in SCN, which leads to circadian disturbances and sleep problems, subsequently enhancing the behavioral and cellular processes associated with Alzheimer's disease pathogenesis [7, 8]. Third, decreased MT1 melatonin receptor levels in the individuals with the genetic risk variant increase the amyloidogenic processing of APP in neurons and enhance the pathological process of Alzheimer's disease. The preclinical pathological changes in the circadian regulatory areas in SCN, pineal gland, and cholinergic cells of the basal forebrain, in turn, decrease the amplitude of circadian rhythms [55] and lead to worse tolerance to

circadian challenge of shift work in middle age (mean age 43–47 years in [16]). Whether intolerance to shift work is a preclinical manifestation of Alzheimer's disease should be clarified by using amyloid imaging techniques and cerebrospinal fluid $A\beta_{1-42}$ measurements showing Aβ build-up in tolerant and intolerant shift workers.

Figure 3.



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Alternative mechanistic explanations for the association of rs12506228 both with Alzheimer's disease and intolerance to shift work. The risk allele A of rs12506228 is linked to decreased *MTNR1A* expression in brain. (i) Relative lack of the MT1 melatonin receptors both increases the build-up of the Alzheimer's disease pathology in neurons, and also destabilizes the circadian rhythm in SCN, which can be seen as worse tolerance to shift work. (ii) The relative lack of the receptors destabilizes the circadian rhythm in SCN which leads to problems of circadian rhythms and sleep which enhances the build-up of Alzheimer's disease pathology [7, 8]. (iii) Relative lack of the MT1 melatonin receptors increase the build-up of Alzheimer's disease pathology. The pathological changes in the circadian regulatory areas decrease the amplitude of circadian rhythms and lead to worse tolerance to the circadian challenge of shift work.

In addition, the effect of melatonin signaling on Alzheimer's pathology could be mediated by neuroinflammatory and oxidative stress components in the pathological cascade of Alzheimer since melatonin [56, 57] or normally functioning circadian rhythms in itself [58] has been suggested to have antioxidant and anti-inflammatory properties, partially mediated by melatonin receptors [56].

Some limitations occur which should be noted. Association of rs12506228 was studied with human neuropathology, but no with amyloidogenic processing in vitro, since the available neuronal cell lines were rodent cell lines with different genetic architecture near *MTNR1A*. We were also lacking the historical information of tolerance to shift work/circadian disruption for our elderly cohorts to evaluate the direct association of the two phenotypes. No correction for genetic structure was included in the genetic analyses because of the lack of the genome-wide data on Kuopio 75+ or Kuopio case-control samples. However, population stratification is not likely to explain the signal of association of the *MTNR1A* variant with Alzheimer's disease in the Kuopio 75+ and Vantaa 85+ samples, as both are based on population-based sampling in the area of only one city with very high participation rate (92% for Vantaa 85+ and 86% for Kuopio 75+).

Taken together, we have identified a genetic variation near *MTNR1A* that was associated with both intolerance to shift work at working age and with Alzheimer's disease at old age. We further demonstrated in vitro that levels of MT1 melatonin receptors regulate the amyloidogenic processing of APP. These results support the view that weaker physiological melatonin signaling contributes to the pathological cascade of Alzheimer's disease. Future studies are needed to clarify whether poor tolerance to shift work per se indicates increased risk of Alzheimer's disease. We also encourage clinical studies with administered melatonin to consider the availability of the melatonin receptors.

Supplementary material

Supplementary material is available at *SLEEP* online.

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Notes

Conflict of interest statement. SS has an immediate family member who is an employee and shareholder of Amialife Ltd, which is not related to this study. RS is an employee and shareholder of Amialife Ltd, which is not related to this study. HMO is consulting for Jazz Pharmaceuticals Plc on topics unrelated to the work described in this manuscript. DJS is employed by Merck and Co. HJH is an employee and shareholder of Herantis Pharma Plc, which is not related to this study. BJT holds the European patent on diagnostic testing and therapeutic interventions of the C9orf72 repeat expansion, and the US patent for the same is pending. The other authors report no conflicts of interest.

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