Age-related aggregation of amyloid-β (Aβ) is an upstream pathological event in Alzheimer’s disease (AD) pathogenesis, and it disrupts the sleep–wake cycle. The amount of sleep declines with aging and to a greater extent in AD. Poor sleep quality and insufficient amounts of sleep have been noted in humans with preclinical evidence of AD. However, how the amount and quality of sleep affects Aβ aggregation is not yet well understood. Orexins (hypocretins) initiate and maintain wakefulness, and loss of orexin-producing neurons causes narcolepsy. We tried to determine whether orexin release or secondary changes in sleep via orexin modulation affect Aβ pathology. Amyloid precursor protein (APP)/ Presenilin 1 (PS1) transgenic mice, in which the orexin gene is knocked out, showed a marked decrease in the amount of Aβ pathology in the brain with an increase in sleep time. Focal overexpression of orexin in the hippocampus in APP/PS1 mice did not alter the total amount of sleep/wakefulness and the amount of Aβ pathology. In contrast, sleep deprivation or increasing wakefulness by rescue of orexinergic neurons in APP/PS1 mice lacking orexin increased the amount of Aβ pathology in the brain. Collectively, modulation of orexin and its effects on sleep appear to modulate Aβ pathology in the brain.
Figure 1. Marked reduction of Aβ pathology in the APP/PS1/OR−/− mice compared with APP/PS1 mice. (A–I) The amount of Aβ pathology was noted at 3.5 (A–C) and 8.5 mo (D–F) in APP/PS1-21 mouse line and at 6 mo in APP/PS1E9 mouse line (G–I). (J and K) Amount of wakefulness at 3 mo of age before the onset of Aβ pathology in the brain was compared between APP/PS1E9 and APP/PS1E9/OR−/− mice. Each mouse was investigated independently one time. Data are presented as mean ± SEM (n = 4–12 in each group, two-tailed Student’s t test in C, F, and I and Mann-Whitney test in J). *, P < 0.05; **, P < 0.01; and ***, P < 0.001. Bar, 500 µm.
and loss of orexin-producing neurons causes narcolepsy (de Lecea et al., 1998; Chemelli et al., 1999).

Levels of soluble Aβ in the extracellular interstitial fluid (ISF) of the hippocampus in mice are dynamically and positively associated with minutes awake per hour and negatively associated with time asleep (Kang et al., 2009). In addition, the sleep–wake cycle also affects the Aβ pathology in the brain. A study in mice showed that intracerebral administration of orexin can acutely increase both wakefulness and Aβ levels and systemic treatment with an orexin receptor antagonist decreased Aβ deposition in amyloid precursor protein (APP) transgenic mouse models (Kang et al., 2009). Pharmacological experiments suggest that orexin and the sleep–wake cycle appear to be related to regulation of Aβ levels. We sought to determine for the first time whether genetic manipulation of orexin has similar effects as pharmacological manipulation and, importantly, whether orexin is influencing Aβ levels and Aβ pathology directly via orexin signaling or indirectly via its effects on the sleep–wake cycle.

**RESULTS AND DISCUSSION**

**Marked reduction of Aβ pathology in the APP/Presenilin 1 (PS1)/Orexin knockout (OR−/−) mice**

To more specifically address the role of orexin in regulating Aβ levels and pathology, we used APP/PS1-21 transgenic mice (Radde et al., 2006), which begin to develop Aβ deposition at ~2 mo of age, and bred them to OR−/− mice (de Lecea et al., 1998; Chemelli et al., 1999). When orexin was knocked out in APP/PS1-21 mice (APP/PS1-21/OR−/− mice), there was a marked reduction of Aβ plaque pathology at 3.5 mo as well as at 8.5 mo of age compared with APP/PS1-21 mice (Fig. 1, A–F). Similar patterns of reduction in Aβ deposition were also seen when orexin was knocked out in another mouse model that develops Aβ deposition, APP/PS1E9 mice (Jankowsky et al., 2004), which begin to develop Aβ deposition at 4 mo of age. APP/PS1E9/Orx−/− mice had a marked reduction in Aβ deposition at 6 mo of age compared with APP/PS1E9 mice expressing orexin (Fig. 1, G–I). APP/PS1E9/Orx−/− mice demonstrated significantly decreased wakefulness.
by 11.5% during the 12-h dark phase at 3 mo of age compared with mice expressing orexin, before the onset of Aβ pathology in the brain (Fig. 1, J and K). The difference in wakefulness was more prominent during the early phases of the dark period (6 p.m. to 12 a.m. with an 18.3% difference). Fibrillar Aβ deposition assessed by X-34 staining was also markedly reduced in the brains of APP/PS1-21/OR−/− mice and APP/PS16E9/OR−/− mice compared with mice expressing orexin (Fig. 2). We also assessed Aβ levels biochemically. The total amount of both Aβ40 and Aβ42 solubilized by 5M guanidine was also significantly reduced in the hippocampus as well as in the cortex in APP/PS1-21/OR−/− mice compared with APP/PS1-21 mice. Similar patterns of pathological Aβ reduction were also noted in APP/PS16E9/OR−/− mice compared with APP/PS16E9 mice (Fig. 3).

Reversal of Aβ pathology by modulation of sleep rather than focal overexpression of orexin

Orexin receptors are present on hippocampal neurons, so we focally overexpressed orexin by stereotaxic injection of a lentiviral vector expressing orexin into the hippocampus of APP/PS16E9 mice to determine whether local orexin signaling was directly leading to the effects observed on Aβ deposition. This treatment resulted in no changes in levels of Aβ deposition in the brain nor changes in sleep time despite focal increases in the level of orexin in the hippocampus (Fig. 4). To further investigate whether the reduction in Aβ pathology in orexin knockout mice is mainly caused by the effects of orexin signaling on sleep, expression of orexin specifically in orexinergic neurons was instituted in 1.5-mo-old APP/PS1-21/OR−/− mice. We used an orexin lentiviral vector in which orexin is driven by the hypocretin/orexin promoter. After injection of this vector bilaterally in the hypothalamus, expression of orexin in orexinergic neurons was restored (Fig. 5 F). 6 wk after injection with the hypocretin promoter–driven orexin lentiviral vector, APP/PS1-21/OR−/− mice had a significantly increased amount of wakefulness as well as increased amount of Aβ deposition in the hippocampus and cortex compared with mice injected with a control vector (Figs. 5 E and 6). Collectively, results from focal and generalized orexin expression in different brain regions using different promoters strongly support the idea that the reduction in Aβ pathology noted in APP/PS1/OR−/− mouse models is caused by secondary changes in sleep time induced by hypothalamic expression of orexin rather than by alteration in orexin signaling in hippocampus and other brain regions.

We also analyzed the functional effect of APP/PS1/OR−/− mice in which orexin expression was rescued by hypocretin/orexin promoter. After injection of this vector bilaterally in the hypothalamus, expression of orexin in orexinergic neurons was restored (Fig. 5 F). 6 wk after injection with the hypocretin promoter–driven orexin lentiviral vector, APP/PS1-21/OR−/− mice had a significantly increased amount of wakefulness as well as increased amount of Aβ deposition in the hippocampus and cortex compared with mice injected with a control vector (Figs. 5 E and 6). Collectively, results from focal and generalized orexin expression in different brain regions using different promoters strongly support the idea that the reduction in Aβ pathology noted in APP/PS1/OR−/− mouse models is caused by secondary changes in sleep time induced by hypothalamic expression of orexin rather than by alteration in orexin signaling in hippocampus and other brain regions.

Figure 3. Strong reduction in Aβ species in APP/PS1 mice lacking orexin. (A–D) The amount of PBS-soluble and guanidine-soluble forms of Aβ40 and Aβ42 were compared in APP/PS16E9/OR−/− and APP/PS16E9 mice (left two columns) and in APP/PS1-21/OR−/− and APP/PS1-21 mice (right two columns). Results were obtained from the hippocampus (A and B) and from the cortex (C and D) of each group of mice. Each mouse was investigated independently one time. All samples were measured in triplicate. Data are presented as mean ± SEM (n = 5–9 in each group, two-tailed Student’s t test). *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
decreases in sleep time and increases in amount of Aβ levels and deposition have a reciprocal relationship (Kang et al., 2009; Roh et al., 2012; Ju et al., 2014). Intriguingly, recent results from humans also indicate that the quality or amount of sleep is inversely associated with the amount of Aβ measured in the cerebrospinal fluid (CSF; Ju and Holtzman, 2013; Ooms et al., 2014) and that the amount of sleep inversely correlates with fibrillar forms of Aβ quantified by amyloid PET scan (Spira et al., 2013). Treatment with active vaccination with Aβ42 prevented development of Aβ pathology and abnormalities of the sleep–wake cycle in APP/PS1ΔE9 mice (Roh et al., 2012). This demonstrated that Aβ aggregation and accumulation caused a sleep abnormality. In this study, the opposite is also true, i.e., an increase in sleep time caused by orexin deficiency resulted in a decrease in the amount of Aβ pathology that developed in the brain. Collectively, these findings suggest that increasing certain types of sleep should be considered.

Sleep deprivation induced Aβ pathology in OR−/− mice

Finally, we wanted to determine whether sleep deprivation increased Aβ deposition in the absence of orexin, as previously observed in the presence of orexin (Kang et al., 2009). We sleep–deprived APP/PS1-21/OR−/− mice using the platform above water method as previously described (Kang et al., 2009). Interestingly, relative to mice that were placed on a large platform for 20 h per day for 3 wk, where they can maintain a normal sleep–wake cycle, mice placed on a small platform that experienced sleep deprivation had a significant increase in Aβ plaque pathology (Fig. 7). Collectively, these findings strongly support the idea that the amount of sleep, as modulated by hypothalamic but not local orexin signaling, is crucial in regulating Aβ metabolism and AD pathogenesis.

Increasing evidence suggests that neuronal activity in many brain regions is physiologically regulated by the sleep–wake cycle (Bero et al., 2011). It has been noted in animals that decreases in sleep time and increases in amount of Aβ levels and deposition have a reciprocal relationship (Kang et al., 2009; Roh et al., 2012; Ju et al., 2014). Intriguingly, recent results from humans also indicate that the quality or amount of sleep is inversely associated with the amount of Aβ measured in the cerebrospinal fluid (CSF; Ju and Holtzman, 2013; Ooms et al., 2014) and that the amount of sleep inversely correlates with fibrillar forms of Aβ quantified by amyloid PET scan (Spira et al., 2013). Treatment with active vaccination with Aβ42 prevented development of Aβ pathology and abnormalities of the sleep–wake cycle in APP/PS1ΔE9 mice (Roh et al., 2012). This demonstrated that Aβ aggregation and accumulation caused a sleep abnormality. In this study, the opposite is also true, i.e., an increase in sleep time caused by orexin deficiency resulted in a decrease in the amount of Aβ pathology that developed in the brain. Collectively, these findings suggest that increasing certain types of sleep should be considered.

Figure 4. No changes in Aβ deposition and amount of wakefulness by focal overexpression of orexin. (A–F) Amount of Aβ pathology was compared after focal injection of ubiquitin-driven orexin lentiviral vector versus ubiquitin-driven GFP lentiviral vector in the hippocampus from 3 to 6 mo (A–C) or 5 to 9 mo (D–F) in APP/PS1ΔE9 mice. (G) Levels of orexin in the hippocampus and CSF were compared after focal injection of orexin or GFP lentiviral vector driven by ubiquitin promoter. (H) The amount of wakefulness in APP/PS1ΔE9 mice was compared after focal injection of ubiquitin-driven orexin lentiviral vector versus ubiquitin-driven GFP lentiviral vector. Each mouse was investigated independently one time. Data are presented as mean ± SEM (n = 4–5 in each group, two-tailed Student’s t test in C, F, and H; one-way ANOVA, followed by Tukey’s post hoc test in G). *, P < 0.05. Bar, 500 µm.
attenuated diurnal fluctuation of ISF amyloid-β in OR⁻/⁻ mice

Attenuated diurnal fluctuation of ISF amyloid-β in OR⁻/⁻ mice

Rescue of diurnal fluctuation by orexin replacement

as a therapeutic target to decrease Aβ-associated AD pathogenesis. Possible side effects of sleep induction such as excessive daytime sleepiness or cataplexy driven by knocking out or decreasing orexin function need to be considered in the process of designing new therapeutic interventions targeting sleep.

It is notable that a 12% increase in the sleep time during the dark phase was associated with >50% reduction in the development of Aβ pathology in the brain. This matches well with the previous findings that an ~15% decrease in the amount of soluble forms of Aβ in the extracellular space resulted in an up to 50% reduction of Aβ plaque growth and formation in the brain (Yan et al., 2009). It suggests that a small increase in NREM sleep or small improvement in sleep quality and the resulting decrease in Aβ release into the extracellular space could be beneficial for the delay or attenuation of emergence of Aβ-associated pathologies in AD. Although our data support a mechanism whereby decreased neuronal activity during sleep results in a decrease in ISF Aβ, we cannot yet rule out the contribution of increased Aβ clearance from the ISF as proposed by Xie et al. (2013). It will be important in future experiments to determine whether different methods of sleep or orexin modulation will trigger new methods of intervention targeting Aβ pathophysiology in the brain. Given the association between neuronal activity and release of tau in the brain, further studies to determine whether the sleep–wake cycle also influences this key molecule in the pathogenesis of AD and other neurodegenerative diseases will be critical (Yamada et al., 2014). It is important to note that other systems involved in the sleep–wake cycle are also likely to affect the pathophysiology of AD. Even though we concluded that not orexin itself but rather the secondary changes in the sleep–wake cycle affect AD pathophysiology in brains of mice, modulation of other molecules that increase or decrease wakefulness such as noradrenergic and GABAergic modulation are also likely to influence AD pathophysiology (Matsuki et al., 2009; Carter et al., 2010).

The location of Aβ deposition in the human brain overlaps very closely with a brain network called the “default mode” network, which is made up of brain regions that appear to have...
the highest measures of aerobic glycolysis when individuals are at rest (Raichle et al., 2001; Vlassenko et al., 2010). In addition to the global reduction in neuronal activity resulting from an increase in sleep time as shown here, focal alteration of brain activity, connectivity, or even focal sleep modulation through pharmacologic or other methods may also merit consideration as potential therapies in AD and other neurodegenerative disorders (Nir et al., 2011; Vyzovitskiy et al., 2011). Such manipulation may be a powerful way to modulate Aβ levels and pathology by taking advantage of normal brain physiology.

MATERIALS AND METHODS

Mice. All experiments were approved by the Animal Studies Committee at Washington University in St. Louis. Female APPwE/PS1E9 mice on a B6 background (The Jackson Laboratory) were used. A subset of OR mice was crossed with OR mice on a C57BL/6 background to produce APPwE/PS1E9 hemizygous mice. Then, APPwE/PS1E9; OR+ mice were crossed with OR mice to obtain APPwE/PS1E9 hemizygous OR+ mice (APPwE/PS1E9/OR+). APP/PS1-21/OR− mice were obtained using the same methods beginning from crossing the APP/PS1-21 mice (C57BL/6) with OR− mice (C57BL/6). APP/PS1-21 mice (C57BL/6) were provided by M. Jucker at the University of Tubingen (Tubingen, Germany).

ELISA. Microdialysis samples were analyzed for Aβ1-40, Aβ1-42, or Aβ1-x with sandwich ELISAs. In brief, Aβ1-40, Aβ1-42, and Aβ1-x were captured with monoclonal antibodies targeted against amino acids 35–40 (HJ5.1; Koenigsknecht-Talboo et al., 2008) followed by streptavidin–poly-HRP40 (Fitzgerald) was used for detection. For Aβ1-x assays, a biotinylated N-terminal domain monoclonal antibody (3D6; Koenigsknecht-Talboo et al., 2008) followed by streptavidin–poly-HRP20 (Fitzgerald) was used. The antibodies m266 and 3D6 were gifts from Eli Lilly. All assays were developed with Super Slow ELISA TMB (Sigma-Aldrich) and read on a Bio-Tek Synergy 2 plate reader at 650 nm. Hippocampal tissue lysates were analyzed for Aβ1-40 and Aβ1-42 using a denaturing, sandwich ELISA specific for human Aβ1-40 and Aβ1-42 after solubilizing the tissue in SM guanidine as previously described (Cirrito et al., 2003).

Sleep–wake monitoring. Polysomnographic sleep–wake cycle analysis of mice was performed as described previously (Bero et al., 2011; Roh et al., 2012). In brief, electroencephalogram (EEG) and electromyogram (EMG) electrodes were implanted simultaneously with a microdialysis guide cannula. For EEG recording, two stainless steel screws attached to wire electrodes were placed over the right frontal bone (bregma +1.0 mm, 1.5 mm lateral to midline) and the right parietal bone (bregma +3.0 mm, 2.5 mm lateral to midline). Two wire electrodes were directly inserted into the neck musculature for EMG recording. Mice were habituated to the recording cages for 3 d. At the end of the habitation period, EEG and EMG recording began simultaneously with collection of microdialysis samples. EEG and EMG signals were displayed on a monitor and stored in a computer for analysis of sleep states. EEG and EMG recordings were assessed with a PS11K AC preamplifier (Grass-Telefactor Instruments), digitized with a DigiData 1440A Data Acquisition System (Molecular Devices), and recorded digitally with pCLAMP 10.2 (Molecular Devices). EEG and EMG signals were filtered (EEG: high pass 1 Hz, low pass 30 Hz; EMG: high pass 10 Hz, low pass 100 Hz) and used to...
Figure 7. Increased Aβ deposition with chronic sleep deprivation in APP/PS1–21/OR−/− mice. (A–F) The amount of Aβ plaques stained with HJ3.4B (A–C) and fibrillar Aβ stained with X-34 (D–F) were compared between APP/PS1–21/OR−/− mice exposed to a large platform and a small platform. Sleep deprivation experiments were performed using a small and large platform in a cage with water on the bottom, where a mouse cannot sleep on the small platform because of its size, whereas they can maintain a normal sleep–wake cycle on the larger platform. Mice exposed to small platforms (n = 3) and to large platforms (n = 3) were analyzed together within a set of experiments. The results are the sum of five repeats in different mice. Each mouse was investigated independently. Data are presented as mean ± SEM (n = 3–14 in each group, two-tailed Student’s t-test). *, P < 0.05; and **, P < 0.01.

Bars: (A) 500 µm; (D) 100 µm.

Plaque deposition analyses. After mice were perfused with PBS transcardially, brains were removed, fixed in 4% paraformaldehyde for 24 h (4°C), cryoprotected with 30% sucrose in PBS (4°C), frozen in powdered dry ice, and cut on a freezing sliding microtome. Serial coronal sections (50 µm thick) were collected from the genu of the corpus callosum to caudal hippocampus. Sections (each separated by 300 µm) were stained with biotinylated HJ3.4 (Aβ 1–13) antibody to visualize Aβ-immunopositive plaques or X-34 dye to visualize fibrillar amyloid plaques (Roh et al., 2012). Immunostained sections and X-34–stained sections were imaged with a NanoZoomer slide scanner (Hamamatsu Photonics). Quantitative analysis of percent area covered by HJ3.4– or X-34–positive staining was performed using a neurostereological method as described previously (Kim et al., 2009). In brief, images of immunostained sections were exported with NDP viewer software (Hamamatsu Photonics), converted to 8-bit grayscale with ACDSeePro 2 software (ACD Systems), thresholded to highlight Aβ–specific staining, and analyzed with ImageJ software (National Institutes of Health). Images of X-34–stained sections were converted to 16-bit grayscale, thresholded to highlight X-34–specific staining, and analyzed with ImageJ software. A mouse brain atlas was used to identify hippocampal sections relative to bregma (−1.7, −2.0, −2.3 mm) for quantitative analysis of immunostained and X-34–positive staining as described previously (Roh et al., 2012).

Overexpression of orexin by lentiviral vector and measurement of orexin. Lentiviral vectors were prepared in the Hope Center Viral Vectors Core. For focal overexpression of orexin, orexin or GFP driven by an ubiquitin promoter was overexpressed via lentivirus in the hippocampus bilaterally in APPswe/PS1-21/OR mice (B6C3) starting at 5 mo of age, and the amount of sleep and Aβ pathology from both groups were compared at 9 mo. To investigate the effect of focal overexpression of orexin before Aβ plaque pathology was formed in the hippocampus, the same lentiviral injection experiments were performed in APPswe/PS1-21 mice (B6C3) with viral injection at 3 mo and pathological assessment at 6 mo. For rescue of orexinergic neurons, orexin or GFP driven by an hypocretin/orexin promoter was expressed via lentivirus in the bilateral hypothalamus in APPswe/PS1-21/Orexin knockout mice (C57BL/6) starting at 1.5 mo of age, and the amount of sleep and Aβ pathology from both groups were compared at 3 mo. The mouse hypocretin/orexin promoter was provided by L. de Lecea. Brains were sectioned on a freezing microtome at 80-µm thickness. Floating brain sections at a 1.6 series were processed for anti–orexin-A and anti–glial fibrillary acidic protein (GFAP) immunohistochemistry as follows: tissue was washed in Tris-buffered saline (TBS), then quenched in 3% hydrogen peroxide solution for 10 min, washed again in TBS, and then incubated in 0.25% Triton-X solution plus 5% normal goat serum for 30 min. Finally, the slides were incubated in primary antibody plus 5% normal goat serum, anti–orexin-A at 1:1,000 overnight (rabbit anti–mouse Orexin-A; Phoenix Pharmaceuticals, Inc.) or anti–GFAP at 1:1,000 (polyclonal chicken anti–GFAP; EMG Millipore). The next day, sections were incubated in a solution containing goat anti–rabbit IgG biotinylated secondary antibody for orexin or donkey anti–chicken secondary
antibody for GFAP at 1:1,000 dilution, and signal was then amplified using the Vectastain ABC kit at 1:1000 (Vector Laboratories) followed by visualization with DAB-nickel. Sections were carefully mounted onto glass microscope slides, air-dried, and then dehydrated in ascending ethanols and coveredlipped with Cytoseal. CSF and brain tissue lysate samples were analyzed for orexin-A (human, rat, mouse, porcine, bovine, and ovine) levels using a commercially available fluorescent enzymatic immunoassay (EIA) kit (Phoenix Pharmaceuticals). All samples were assayed in duplicate, and the mean of the two values was reported.

In vivo microdialysis. In vivo microdialysis to assess Aβ and lactate in the brain ISF of awake, freely behaving mice was performed as described previously (Cirrito et al., 2003; Roh et al., 2012). In brief, guide cannulae (BR style; Bioanalytical Systems) were stereotaxically implanted into hippocampus (bregma −3.1 mm, 2.5 mm lateral to midline, and 1.2 mm below the dura at a 12° angle). Probe placement in the regions of interest was confirmed by cresyl violet staining. Microdialysis probes (2 mm; 38-kD molecular size cutoff; BR style; Bioanalytical Systems) were connected to a syringe pump (Stoelting Co.), and artificial CSF, pH 7.35, containing 1.3 mM CaCl₂, 1.2 mM MgSO₄, 3 mM KCl, 0.4 mM KH₂PO₄, 25 mM NaHCO₃, and 122 mM NaCl was continuously perfused through the microdialysis probe. For measurement of Aβ levels, a flow rate of 0.5 µl/min was used. Guide cannulae were implanted 2 wk before the beginning of microdialysis. After insertion of the microdialysis probe, mice were habituated to a 12-h light/dark cycle for three more days. On the fourth day, samples were collected and stored for analyses.

Statistical analysis. Statistical significance was determined by two-tailed *t* test if the datasets fulfilled the normality test (Kolmogorov–Smirnov test). All statistical analyses were performed with Prism version 4.0 (GraphPad Software). *P* values were reported.

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Potential role of orexin and sleep modulation in the pathogenesis of Alzheimer’s disease
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The authors regret that Hong Jiang was omitted from the author’s list in the original version of this paper. This has been corrected in the html and pdf versions of the article.

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