Altered temporal patterns of anxiety in aged and amyloid precursor protein (APP) transgenic mice

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Both normal aging and dementia are associated with dysregulation of the biological clock, which contributes to disrupted circadian organization of physiology and behavior. Diminished circadian organization in conjunction with the loss of cholinergic input to the cortex likely contributes to impaired cognition and behavior. One especially notable and relatively common circadian disturbance among the aged is "sundowning syndrome," which is characterized by exacerbated anxiety, agitation, locomotor activity, and delirium during the hours before bedtime. Sundowning has been reported in both dementia patients and cognitively intact elderly individuals living in institutions; however, little is known about temporal patterns in anxiety and agitation, and the neurobiological basis of these rhythms remains unspecified. In the present study, we explored the diurnal pattern of anxiety-like behavior in aged and amyloid precursor protein (APP) transgenic mice. We then attempted to treat the observed behavioral disturbances in the aged mice using chronic nightly melatonin treatment. Finally, we tested the hypothesis that time-of-day differences in acetylcholinesterase and choline acetyltransferase expression and general neuronal activation (i.e., c-Fos expression) coincide with the behavioral symptoms. Our results show a temporal pattern of anxiety-like behavior that emerges in elderly mice. This behavioral pattern coincides with elevated locomotor activity relative to adult mice near the end of the dark phase, and with time-dependent changes in basal forebrain acetylcholinesterase expression. Transgenic APP mice show a similar behavioral phenomenon that is not observed among age-matched wild-type mice. These results may have useful applications to the study and treatment of age- and dementia-related circadian behavioral disturbances, namely, sundowning syndrome.

Alzheimer's disease | cholinesterase | nucleus basalis of Meynert | behavioral and psychological signs and symptoms of dementia

Both normal aging and dementia are associated with disturbances in the biological clock that contribute to dramatic circadian disorganization, including the sleep-wake cycle, body temperature rhythm, and daily patterns of hormone release (1). Generally, aging is associated with reduced amplitudes among these daily rhythms; the most severe changes are associated with Alzheimer's disease (AD) (2). Circadian disorders affect more than 80% of individuals over age 65 (3) because of several converging factors; loss of neurons in the suprachiasmatic nucleus, decreased melatonin and melatonin receptor sensitivity, and minimal *zeitgebers* because of lifestyle all contribute to circadian dysfunction (1, 4).

Normal aging and dementia are also associated with impairments in cognition and behavior, in part caused by loss of cholinergic input to the cortex (5). In particular, parts of the basal forebrain, most notably the nucleus basalis of Meynert (NBM), undergo degenerative changes, such as down-regulation of choline acetyltransferase (ChAT) activity (the rate-limiting enzyme in the production of acetylcholine) and dysregulation of acetylcholinesterase (AchE), the enzyme responsible for degrading Ach (6). In AD the loss of cholinergic markers in the basal forebrain is more marked than in normal aging and is linked to cognitive dysfunction. Taken together, circadian and cholinergic abnormalities may interact to provoke severe behavioral disturbances.

Recently, interest has arisen in the behavioral and psychological signs and symptoms of dementia (BPSD). BPSD are a tremendous burden to caregivers, often cited as the primary reason for institutionalization of elderly individuals, and are detrimental to the quality of life for both the caretaker and the person experiencing them (7, 8). In some cases, the caretaker may develop clinical depression as a result (9). One especially notable circadian disturbance is the so-called "sundowning syndrome," which is characterized by exacerbated anxiety, agitation, locomotor activity, and delirium occurring in the hours before bedtime (10). Although it is most commonly associated with dementia, cognitively intact, elderly institutionalized individuals also experience sundowning (11, 12). Prevalence rates vary, depending on environment and severity of illness (i.e., in the case of dementia patients), and estimates as high as 66% have been reported for dementia patients living at home (13). Sundowning syndrome represents an understudied area in terms of BPSD. Little is known about temporal patterns in anxiety and agitation, and the neurobiological basis of these rhythms remains unspecified. A few studies using 3xTg-AD and amyloid precursor protein-23 (APP23) transgenic AD mouse models described hyperactivity and reproduced certain aspects of sundowning syndrome, namely afternoon increases in locomotor activity (14, 15); however, these studies failed to consider the anxiety symptoms central to the disorder and have not proposed mechanisms underlying this phenomenon.

In the present study, we explored the diurnal pattern of anxiety-like behavior in adult and aged wild-type mice, as well as in transgenic amyloid precursor protein (APPSWE; Taconic 1349) mice, which serve as a model for AD. In our first experiment, we compared locomotor activity and anxiety-like behavior at different time points using aged wild-type mice versus middle-aged adults. Given the clinical interest in melatonin as a treatment for sundowning symptoms and its positive effects on sleep and affect (16), we attempted to ameliorate the observed behavioral disturbances in the aged mice using chronic nightly melatonin treatment. Finally, we tested the hypothesis that time-of-day differences in AchE and ChAT expression and general neuronal activation (i.e., c-Fos expression) would coincide with the behavioral symptoms. In a second experiment, we investigated whether a similar phenomenon of temporal changes in anxiety-like behavior exists in transgenic APP mice.

Results

Activity and Corticosterone. Home-cage locomotor activity was continuously recorded over several days in middle-aged adult (7 mo) and aged (29 mo) mice. Adult mice displayed a distinct pattern of activity, with three peaks occurring throughout the dark phase (Fig. 1*A*). In contrast, aged mice had a fairly flat increase in activity throughout the duration of the dark phase (Fig. 1*B*). Repeated-measures ANOVA revealed a main effect of time of day ($F_{1,23}$ = 41.787, $P < 0.0001$) and an age-by-time interaction ($F_{1,23}$ = 3.368,

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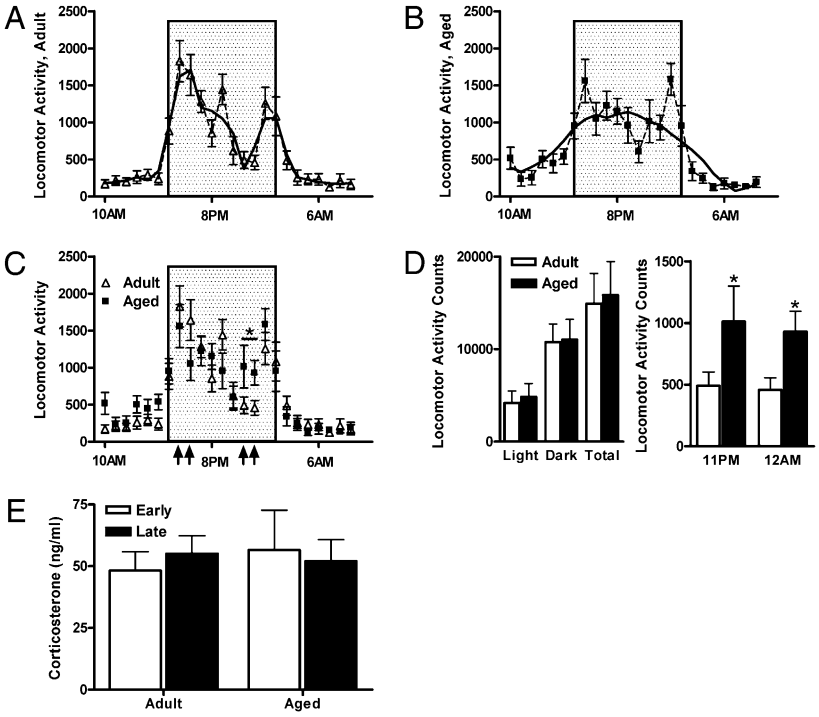

Fig. 1. Home-cage locomotor activity (mean number of beam breaks) and baseline corticosterone concentrations in adult versus aged mice. Adult mice exhibit three distinct peaks in activity (*A*), whereas aged mice have a flattened rhythm (*B*). During the 2 to 3 h before lights on, the time which would correspond with late afternoon sundowning symptoms in diurnal humans, aged mice are significantly more active than adult mice (*C*). Arrows in *C* indicate the time anxiety tests and blood/brain collections were performed. Total activity counts were equivalent for adult and aged mice (*D*). Adult and aged mice had equivalent baseline plasma corticosterone concentrations regardless of time point (*E*). n = 10–13 mice per group for activity; n = 4–6 mice per group for hormone assay. Activity graphs depict mean ± SD and bar graph depicts mean ± SEM. In *A* and *B*, dotted lines connect datapoints and solid lines represent smooth curves added for purposes of visualizing the general pattern of activity. *$P < 0.05$.

$P < 0.0001$). Aged mice were significantly more active than adults during the 2 to 3 h before the lights were illuminated (post hoc, $P < 0.05$) (Fig. 1*C*), which is reminiscent of human sundowning behavior. In nocturnal mice, this period corresponds with the late afternoon/early evening hours for a human because it is approaching the termination of daily activity. Total daily locomotor activity counts, total light phase, and total dark phase counts were equivalent in aged versus adult mice ($P > 0.05$ for all comparisons) (Fig. 1*D*) and aged mice were more active than adults at 2300 hours and 0000 hours ($P < 0.05$) (Fig. 1*D*). Baseline corticosterone concentrations were equivalent between age groups and by time of day (Fig. 1*E*).

Anxiety. Adult and aged mice were tested for anxiety-like behavior in the elevated-plus-maze either early or late in the dark phase based on the observed patterns of locomotor activity (see arrows in Fig. 1*C*). Age and time interacted to affect the duration of time mice spent in the open arms of the maze ($F_{1,17} = 7.139$, $P < 0.05$). Aged mice tested late in the dark phase spent less time in the open arms than aged mice tested early (post hoc, $P < 0.01$) (Fig. 2*A*). There was an effect of time of day on number of open-arm entries ($F_{1,17} = 6.229$, $P < 0.05$), as aged mice made fewer entries late in the dark phase compared with all other groups (post hoc, $P < 0.05$ in all cases) (Fig. 2*B*). The number of closed-arm entries, however, was not affected by time of day ($P > 0.05$), suggesting that general activity in the elevated-plus-maze is unlikely to have affected anxiety responses. Latency to enter the open arms was affected by age ($F_{1,18} = 7.629$, $P < 0.05$), with an interaction of age and time of day ($F_{1,18} = 6.219$, $P < 0.05$). Aged mice tested late had a significantly greater latency to enter the open arms compared with all other groups (post hoc, $P \leq 0.01$ in all cases) (Fig. 2*C*). In the open field, there was an interaction of age and time on rearing behavior ($F_{1,18} = 4.186$, $P = 0.05$). Aged mice engaged in less exploration in the form of rearing during the late dark phase relative to early (post hoc, $P = 0.05$) (Fig. 2*D*).

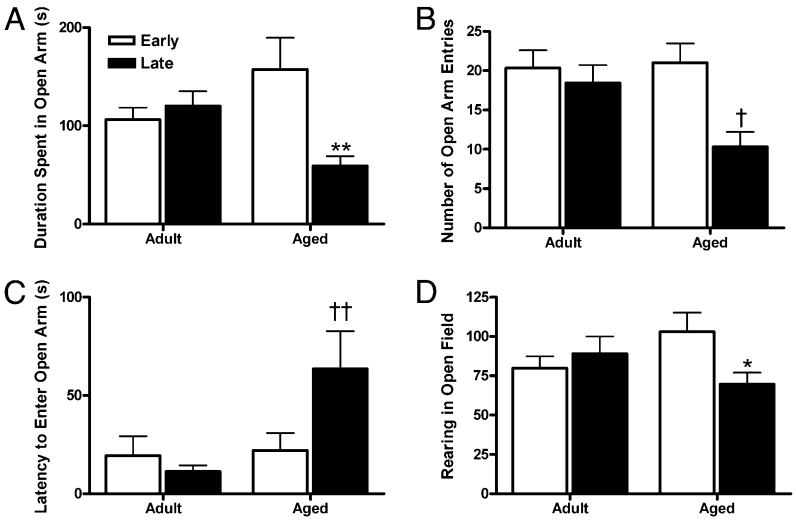

Fig. 2. Anxiety-like behaviors in the elevated-plus-maze and open field. Late in the dark phase compared with early, aged mice spend less time exploring the open arms (*A*), make less entries into the open arms (*B*), and have a greater latency to first enter the open arms (*C*). In the open-field test, aged mice exhibit less exploratory rearing behavior late in the dark phase compared with early (*D*). n = 3–7 mice per group. Mean ± SEM *$P < 0.05$ aged early vs. aged late; **$P <$ 0.01 aged early vs. aged late; $^{\dagger}P < 0.05$ aged late vs. all other groups; $^{\dagger\dagger}P < 0.01$ aged late vs. all other groups.

Melatonin Treatment. Four weeks of nightly melatonin treatment did not ameliorate sundowning symptoms in the elevated-plus-maze (Fig. S1). Age ($F_{1,17} = 16.654$, $P < 0.001$) and time of day ($F_{1,17} = 5.244$, $P < 0.05$) affected duration spent in the open arms of the maze; there was also an interaction of age and time ($F_{1,17} = 6.481$, $P < 0.05$). Aged mice tested late in the dark phase spent significantly less time exploring the open arms compared with those tested early (post hoc, $P < 0.01$). There was no effect of age on locomotor activity ($P > 0.05$), indicating no differences between adult and aged mice following melatonin treatment.

AchE, ChAT, and c-Fos Expression. Time of day affected the relative optical density of AchE staining in the NBM ($F_{1,15} = 5.116$, $P < 0.05$). There was significantly greater expression of AchE in aged mice late in the dark phase compared with early (post hoc, $P < 0.05$) (Fig. 3 and Fig. S2). There was a main effect of age on ChAT-positive cells in the NBM ($F_{1,12} = 5.466$, $P < 0.05$) (Fig. 4), as aged mice had fewer ChAT-positive cells than middle-aged adults. There was also an effect of age on Fos-positive cells in the pedunculopontine tegmentum (PPTg), which sends projections to the NBM ($F_{1,14} = 17.160$, $P < 0.01$). Aged mice had fewer Fos-positive cells, regardless of time of day (post hocs, $P < 0.05$ for all comparisons) (Fig. 5). There was no effect of age or time of day on Fos-immunoreactivity in any of the other brain regions analyzed (Table 1).

APP Mice. The AD model used in these experiments develops behavioral impairments and Aβ accumulation at ~9 mo of age (17), and thus we investigated behavior at this age. At 9 mo of age, APP mice had increased dark phase activity compared with wild-type mice (Fig. 6*A*). APP mice tested in the elevated-plus-maze late in the dark phase spent less time exploring the open arms of the maze compared with those tested early ($t_{11} = 2.543$, $P < 0.05$) (Fig. 6*B*). Wild-type mice spent equivalent time exploring the open arms regardless of time of day. At 5 mo of age, APP mice had increased dark phase activity in the home-cage (Fig. 6*C*). In the elevated-plus-maze, there were no effects of time and APP mice were indistinguishable from wild type mice in terms of time spent exploring the open arms (Fig. 6*D*).

Discussion

In the present study, we describe a temporal pattern of anxiety-like behavior that emerges in elderly mice. This behavioral pattern appears in conjunction with elevated locomotor activity relative to adult mice near the end of the dark phase, and coincides with time-dependent changes in basal forebrain AchE expression. Transgenic APP mice show a similar behavioral phenomenon that is not observed among age-matched wild-type mice, despite also showing nonspecific hyperactivity across the dark phase. These results may have useful applications to the study of age- and dementia-related circadian behavioral disturbances, namely sundowning syndrome, as well as provide insights into therapeutic interventions.

Sundowning syndrome has been described in the geriatric clinical literature for over 70 y as a diurnal pattern of behavioral disturbances that worsen in the late afternoon or early evening (18). No efficacious treatment for sundowning exists, largely because of the paucity of information regarding its neurobiological mechanisms and the lack of an animal model to study the disorder. Transgenic AD mouse models have been used to reproduce certain aspects of sundowning syndrome, namely afternoon increases in locomotor activity (14, 15); however, these few studies failed to model the anxiety symptoms central to the disorder. Development of an animal model should facilitate understanding the etiology of sundowning syndrome. The phenomenon we describe with our present results may be useful for this purpose.

In the clinical literature, one criticism of the notion of sundowning has been that it occurs near the end of the work day, at

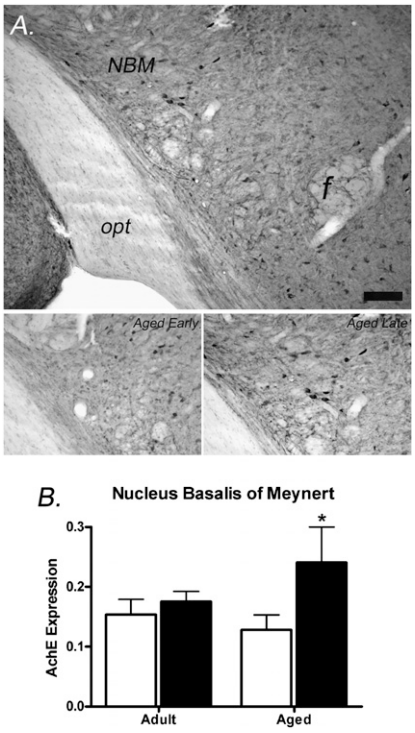

Fig. 3. AchE staining in the NBM. Relative optical density was quantified in sections containing the NBM (*A*). Aged mice had greater AchE expression late in the dark phase compared with other groups (*B*). n = 3–6 mice per group. Images captured at 10×. Open bars, early dark phase; filled bars, late dark phase. (Scale bar, 100 μm.) Graph depicts mean relative optical density ± SEM *$P < 0.05$. NBM: nucleus basalis of Meynert; opt: optic tract.

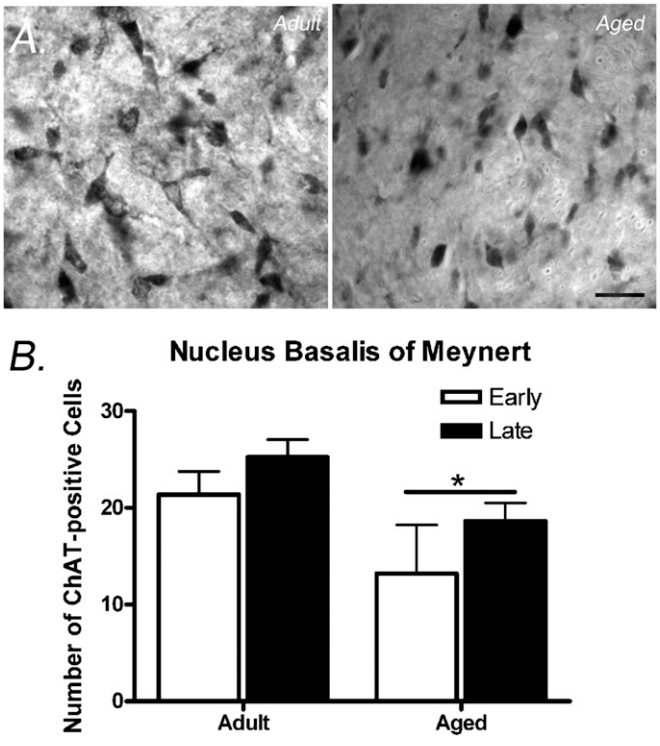

Fig. 4. Choline acetyltransferase staining in the NBM. ChAT-positive cells were quantified in sections containing the NBM (*A*). Aged mice had fewer ChAT-positive cells than adult mice regardless of timepoint (*B*). Open bars, early dark phase; filled bars, late dark phase. (Scale bar, 25 μm.) n = 3–5 mice per group. Graph depicts mean ± SEM. Representative images are from an adult at the early time point and an aged mouse at the late time point. *$P < 0.05$.

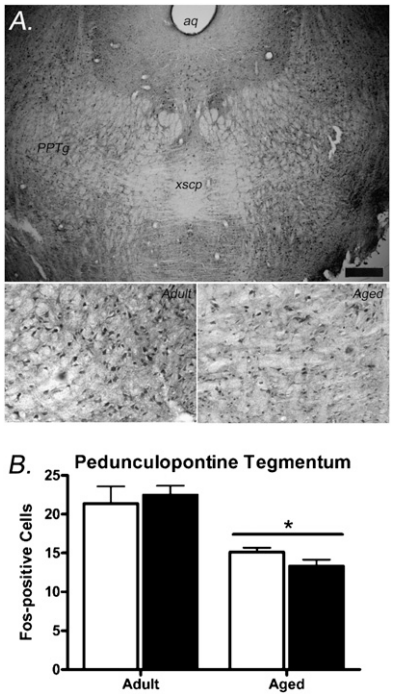

Fig. 5. c-Fos staining in the PPTg. Fos-positive cells were quantified in sections containing the PPTg (*A*). Aged mice had fewer Fos-positive cells than adult mice regardless of time point (*B*). Open bars, early dark phase; filled bars, late dark phase. (Scale bar, 100 μm.) n = 3–5 mice per group. Graph depicts mean ± SEM. Representative images are from the late time point. *$P <$ 0.05. aq, cerebral aqueduct; PPTg, pedunculopontine tegmentum; xscp, decussation of superior cerebellar peduncle.

the same time of day that nurses or caregivers change shifts or become fatigued themselves (19). Thus, critics have argued that the perception of increased afternoon/evening behavioral disruptions may well arise from the caretakers' own fatigue and not any change on the part of patients themselves (20). However, studies quantifying temporal patterns in disruptive behavior have, for the most part, ruled out this hypothesis (21–23). Moreover, our data support sundowning syndrome as a legitimate condition by demonstrating its central symptoms in a mouse model and documenting circadian and cholinergic dysregulation that occur in parallel to the behavioral changes.

Table 1. Other brain regions analyzed for AchE, ChAT, and Fos expression

Region	Adult early	Adult late	Aged early	Aged late
AchE relative optical density				
BLA	0.18 ± 0.02	0.19 ± 0.03	0.17 ± 0.02	0.22 ± 0.02
PVN	0.26 ± 0.05	0.20 ± 0.03	0.19 ± 0.02	0.30 ± 0.08
MS	0.22 ± 0.08	0.16 ± 0.04	0.11 ± 0.03	0.17 ± 0.04
LS	0.21 ± 0.05	0.29 ± 0.04	0.18 ± 0.03	0.23 ± 0.07
Cg Ctx	0.18 ± 0.02	0.22 ± 0.02	0.17 ± 0.01	0.22 ± 0.05
Fos-positive cells				
BLA	22.9 ± 2.1	23.6 ± 1.9	23.3 ± 3.6	23.2 ± 4.6
PVN	23.8 ± 3.4	26.4 ± 2.6	22.1 ± 2.5	19.4 ± 0.1
MS	23.7 ± 3.2	25.4 ± 2.1	22.7 ± 2.5	18.3 ± 2.1
Cg Ctx	19.6 ± 1.0	20.1 ± 0.6	19.8 ± 2.7	16.8 ± 1.3
PrL Ctx	33.8 ± 6.4	32.6 ± 0.9	37.0 ± 6.4	34.8 ± 3.6
PPTg	20.7 ± 1.8	19.6 ± 1.2	13.3 ± 2.1	12.3 ± 0.8
NBM	24.3 ± 2.9	25.3 ± 2.6	22.4 ± 0.6	23.0 ± 2.5
ChAT-positive cells				
PPTg	26.8 ± 2.5	31.1 ± 4.8	21.4 ± 3.2	24.6 ± 2.6

Data represent mean ± SEM. BLA, basolateral amygdala; PVN, paraventricular nucleus; MS, medial septum; LS, lateral septum; Cg, Ctx-cingulate cortex; PrL Ctx, prelimbic cortex; PPTg, pedunculopontine tegmentum; NBM, nucleus basalis of Meynert.

We observed a flattened activity rhythm in our aged mice, which is consistent with other reports in aged mice and humans (24–26). Aging humans and dementia patients commonly have reduced amplitude in the diurnal activity rhythm (27). Our aged mice were more active than adults late in the dark phase because they failed to exhibit the general quieting of activity observed in adults, not because they had an abnormal peak in activity. This finding is interesting in light of some clinical studies that have quantified absolute activity and failed to find afternoon increases among elderly individuals (28, 29). In some cases, it is possible that sundowning patients show hyperactivity relative to normal adult individuals, but not in relation to their own flat activity pattern. At least two studies, however, have revealed decreased diurnal and increased nocturnal activity in this population (27, 30).

Reductions in melatonin and melatonin-receptor sensitivity have been reported in aging individuals (31, 32). Because of the importance of melatonin in regulating the sleep-wake cycle, it has been one major target of clinical studies for the treatment of sundowning. Results, however, have been mixed; some studies find substantial improvements in sundowning symptoms, whereas others do not (16, 22, 33, 34). Because melatonin receptors are substantially reduced in the suprachiasmatic nucleus of elderly and AD individuals, there may be too little substrate for melatonin supplementation to have an effect (35). We treated aged mice with melatonin nightly for 4 wk to test the hypothesis that augmenting melatonin during the night would strengthen entrainment of the diurnal activity rhythm and diminish some of the circadian dysregulation impact on anxiety-like behaviors. This treatment regimen did not affect sundowning symptoms in the aged mice, perhaps because another major factor—cholinergic dysregulation—is also at play in provoking the temporal pattern of anxiety. Future studies must address whether ameliorating cholinergic dysfunction alone or in combination with melatonin treatment will abrogate sundowning symptoms.

Alterations in cholinergic neurotransmission have been implicated in anxiety induction; muscarinic Ach receptor antagonists are anxiogenic in rodents (36, 37), whereas nicotinic receptor agonists decrease anxiety (38–40) and AchE inhibitors reduce neophobia (41). Aged mice had elevated AchE expression in the NBM that coincided with elevated anxiety-like behavior occurring late in the dark phase. Aged mice overall had reduced NBM ChAT-immunoreactivity compared with middle-aged adult mice. The NBM is part of the ascending reticular activating system, which sends projections to the neocortex and amygdala to modulate arousal and attention (42). Activation of the NBM is governed partly by connections from the PPTg, located in the brainstem (43). In addition to AchE and ChAT, we analyzed c-Fos expression in the PPTg and observed few Fos-positive cells in aged mice, regardless of time of night. This finding may suggest that aging causes a general decline in the driving regions of the arousal circuitry, which combines with altered diurnal patterns of cholinergic tone to impact the cortical and amygdalar regions important for modulating affect and behavior. Pharmacological studies to manipulate this circuitry will be necessary to determine causal relationships.

Pharmacological treatment of sundowning syndrome has taken a scattered approach because of lack of information. One survey found that physicians prefer atypical neuroleptics for sundowning, despite safety concerns and little evidence to support use of these drugs (44). Age-related changes in the dorsal raphe serotonin system have been shown important in rodent circadian aging (45–47). In other cases, AchE inhibitors have been found effective (48). Our data are consistent with other studies that implicate reduced cholinergic tone in dementia, and suggest that AchE inhibitors may warrant further examination as a treatment specific for sundowning.

APP mice showed sundowning-like behavior on the elevated-plus-maze at 9 but not at 5 mo of age. This finding is likely explained by the progressive development of behavioral impairments and Aβ accumulation reported to appear around 9 mo of age in this model (17). At both ages, APP mice were substantially

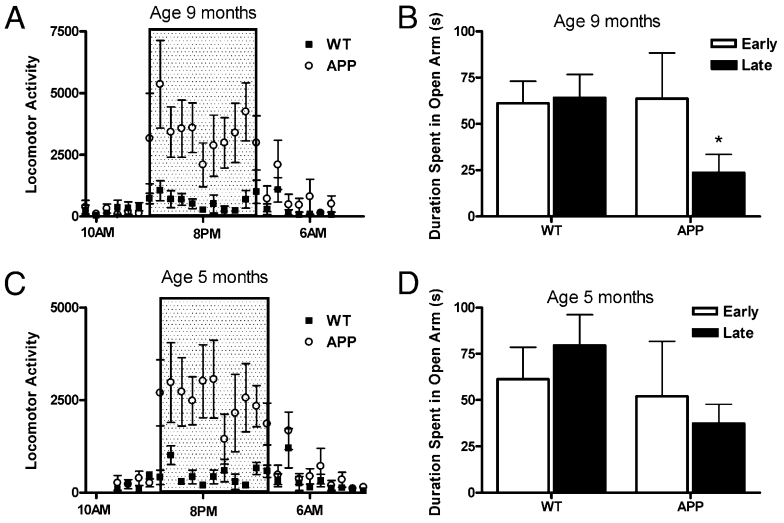

Fig. 6. Transgenic APP mice: home-cage locomotor activity (number of beam breaks) and anxiety-like behavior in the elevated-plus-maze. At 9 mo of age, APP mice were hyperactive during the dark phase compared with wild-type mice (*A*) and spent less time in the open arms of the elevated-plus-maze late in the dark phase relative to wild-type and APP mice tested early (*B*). At 5 mo of age, APP mice were hyperactive compared with wild-type (*C*), but did not display altered anxiety-like behaviors compared with wild-type mice (*D*). $n = 6$ mice per group. Activity graphs depict mean ± SD and bar graphs depict mean ± SEM, $^*P < 0.05$.

more active during the dark phase than wild-type mice. This observation is consistent with previous reports using APP models (49, 50). Future studies will investigate temporal functioning of the cholinergic system using APP mice.

Taken together, our data demonstrate temporal patterns in anxiety-like behavior in both aged and APP mice, a temporal association of changes in AchE expression with this phenomenon, and overall decreases in ChAT and c-Fos immunoreactivity in aged mice. These findings may prove useful in understanding age- and dementia-related circadian behavioral disturbances, such as sundowning syndrome. Expanding research on BPSD, and sundowning syndrome in particular, to include mouse models may yield much-needed treatments.

Materials and Methods

Experiment 1. Ten aged (29 mo) C57BL/6 male mice from the National Institute on Aging Aged Rodent Colony and 13 adult (7 mo) C57BL/6 mice from Jackson Laboratories were individually housed in polypropylene cages (30 cm × 15 cm × 14 cm) at constant temperature (22 ± 2 °C) and humidity. Food (Harlan Teklad 8640) and filtered tap water were available ad libitum. Animals were maintained on a 14:10 light/dark cycle (lights on at 0200 h). All experimental procedures were approved by the Ohio State University Institutional Animal Care and Use Committee.

Locomotor activity was assessed using a home-cage monitoring system from Columbus Instruments. Total beam breaks were continuously recorded to compare activity patterns of aged versus adult mice. Anxiety-like behavior was assessed in the elevated-plus-maze, which is a plus-shaped apparatus, elevated 50.8 cm above the floor with two dark enclosed arms and two open anxiogenic arms. Each mouse was placed in the center of the test apparatus to begin and behavior was recorded on video for 10 min. An observer unaware of experimental groups later scored videotapes for time spent in the open arms of the maze using Observer software (Noldus). Exploratory activity was also measured in the open field test using a Flex Field photobeam system (San Diego Instruments), which produced time spent in the center vs. periphery and rearing counts. Mice were tested during the dark phase under dim red light at one of two time points: during the 1 to 3 h after lights were terminated or during the 1 to 3 h before lights were illuminated.

One week after testing, mice were treated orally with melatonin as previously described (51). Briefly, crystalline melatonin (Sigma) was diluted in a minimal amount of ethanol to a concentration of 2.7 μg/μL. Stock solution was injected into small apple cubes (<5 mm^3) to yield a dose of 0.33 mg/kg. The ethanol was allowed to evaporate for 20 to 30 min in the dark before the apples were fed to the mice. Before beginning the treatment, mice were fed small pieces of untreated apple each day to habituate them to the feeding regimen. All mice quickly learned to eat the apple as soon as it was presented. Melatonin-laced apple cubes were fed every 3.5 h throughout the dark phase for 4 wk. The half-life of melatonin is 3 to 4 h (51), so the feedings were timed to maintain stable concentrations throughout the dark phase. At the end of the 4 wk, home-cage locomotor activity was measured and mice were tested in the elevated-plus-maze as described above.

Ten days after concluding treatment and behavior testing, mice were deeply anesthetized with an overdose of sodium pentobartital and transcardially perfused with 0.9% cold saline, followed by 4% paraformaldehyde in PBS (0.1 M, pH 7.4). Following perfusion, brains were collected, postfixed overnight at 4 °C, transferred to 30% sucrose solution in PBS at 4 °C for 4 d, and then frozen in cold isopentane and stored at −80 °C. Brains were sliced into 40-μm sections using a cryostat and free-floating sections were collected for AchE, ChAT, and c-Fos staining.

Sections spaced 240 μm apart throughout the rostrocaudal extent of the brain were stained for AchE as previously described (52). Briefly, sections were placed for 7 to 10 min in 0.1% H_2O_2, and then rinsed with 0.1 M maleate buffer (pH 6.2). Sections were then incubated 45 min in a solution of 5 mg acetylthiocholine, 0.147 g sodium citrate, 0.075 g copper sulfate, and 0.0164 g potassium ferrocyanide in 1 L of 0.1 M maleate buffer. Following incubation, sections were rinsed in 50 mM Tris buffer (pH 7.6), and then placed into a secondary incubation solution containing 0.05 g diaminobenzidine and 0.375 g nickel ammonium sulfate in 125 mL of 50 mM Tris buffer. After 10 min, 20 mL 0.1% H_2O_2 was added for an additional 2 min. Finally, sections were rinsed in 50 mM Tris buffer, mounted onto gelatin-coated slides, dried overnight, dehydrated in ethanol, and cleared with xylene before coverslipping. A Nikon Eclipse E800 bright-field microscope was used to take micropictographs of sections at 10× (N.A. 0.45) containing the basolateral amygdala, cingulate cortex, lateral septum, medial septum, paraventricular nucleus, and NBM. All settings, including microscope illumination, exposure time, and aperture, were kept constant for the collection of all images. Relative optical density of AchE staining was measured in a box of standardized size in each region using standard ImageJ techniques. Background measurements taken from white matter were subtracted for each image and then measurements from different sections and sides of the brain (i.e., for bilateral structures) were averaged to produce one value for each animal, which was used for statistical analysis. Two to six sections were analyzed per mouse, depending on the specific brain region.

Additional sets of sections spaced 240 μm apart were stained for c-Fos and ChAT, as previously described (53). Briefly, for Fos staining only, sections were preincubated in 1% sodium borohydride in 0.1 M PBS for 10 min. Sections were then rinsed in 10% normal donkey serum and 0.3% hydrogen peroxide in PBS-TX for 20 min, followed immediately by incubation in goat anti c-Fos (Chemicon; 1:10 K) or rabbit anti ChAT (Abcam; 1:2,000), respectively, in 1% normal donkey serum for 48 h at 4 °C. Sections were rinsed in PBS, incubated for 2 h with either biotinylated donkey anti-goat or biotinylated goat anti-rabbit secondary antibody (Vector Laboratories; 1:500) in PBS-TX. The sections were rinsed in PBS, incubated 30 min in avidin-biotin complex (Vector Laboratories; ABC Elite kit), rinsed again, and then developed in hydrogen peroxide and diaminobenzidine for 2 min. Sections were mounted on gelatin-coated slides, dehydrated, cleared with xylene, and coverslipped. A Nikon E800 bright-field microscope was used to capture images of Fos-ir at 10× (N.A. 0.45) through the same regions as above, as well as the pedunculopontine tegmentum and prelimbic cortex. Images of ChAT-ir were captured at 40× (N.A. 0.7) in sections containing the NBM and PPTg. Higher magnification was used here because ChAT staining was especially dense and more accurately quantified at 40×. Numbers of immunoreactive cells were counted in standard sized regions of interest by an observer unaware of treatment groups and

averaged across sections and sides of the brain (i.e., for bilateral structures) to produce one value for each animal which was used for statistical analysis.

Experiment 2. Six male transgenic APPSWE mice (Taconic Model 1349; B6/SJL mixed background) and six wild-type mice were obtained from Taconic and maintained as described above. Home-cage locomotor activity and anxiety-like behavior in the elevated-plus-maze were assessed as described above in the mice at both 5 and 9 mo of age. In this model, memory impairment and amyloid plaques have been observed beginning at 9 mo of age (17). The only difference in test procedure was that because of the small sample size, mice were tested at both time points on the elevated-plus-maze, with the two test sessions spaced 1 wk apart; the groups counterbalanced so half had their first experience in the maze during the early time point and half during the late time point, and then groups were reversed.

Statistics. Locomotor activity was analyzed using repeated-measures ANOVA, with age group as the independent variable and time as the repeated measure. Behavior and histology data were analyzed using two-way ANOVA with age group (aged vs. adult) and test time (early vs. late dark phase) as the independent variables. Main effects were followed up with Fisher's exact test for post hoc comparisons. Some aged mice died over the course of the experiments. Because APP mice were tested for anxiety-like behavior under both time points, paired *t* tests were used in this case to compare the groups by time point. Statistics were performed using Statview 5.0.1 for Windows. For all tests, results were considered statistically significant when $P \leq 0.05$.

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