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ORIGINAL ARTICLE

Reversal of Age-Related Neuronal Atrophy by α5-GABAA Receptor Positive Allosteric Modulation

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Abstract

Aging is associated with reduced brain volume, altered neural activity, and neuronal atrophy in cortical-like structures, comprising the frontal cortex and hippocampus, together contributing to cognitive impairments. Therapeutic efforts aimed at reversing these deficits have focused on excitatory or neurotrophic mechanisms, although recent findings show that reduced dendritic inhibition mediated by α_5-subunit containing GABA-A receptors (α_5-GABAA-Rs) occurs during aging and contributes to cognitive impairment. Here, we aimed to confirm the beneficial effect on working memory of augmenting α_5-GABAA-R activity in old mice and tested its potential at reversing age-related neuronal atrophy. We show that GL-II-73, a novel ligand with positive allosteric modulatory activity at α_5-GABAA-R (α_5-PAM), increases dendritic branching complexity and spine numbers of cortical neurons in vitro. Using old mice, we confirm that α_5-PAM reverses age-related working memory deficits and show that chronic treatment (3 months) significantly reverses age-related dendritic shrinkage and spine loss in frontal cortex and hippocampus. A subsequent 1-week treatment cessation (separate cohort) resulted in loss of efficacy on working memory but maintained morphological neurotrophic effects. Together, the results demonstrate the beneficial effect on working memory and neurotrophic efficacy of augmenting α_5-GABAA-R function in old mice, suggesting symptomatic and disease-modifying potential in age-related brain disorders.

Key words: aging, cognition, GABA, neurotrophic effect, positive allosteric modulator

Introduction

With progress in medicine and health care, the human population tends to live longer. Since 2018, persons aged 65 or above have outnumbered children below 5 years of age globally (United Nations 2019a, 2019b). By 2050, it is estimated that 16% of the global population will be over 65 years of age, compared with 9% in 2019 (United Nations 2019a). Extended lifespan is accompanied by increased prevalence of cognitive decline and age-related brain disorders.

Despite its critical importance to a population growing older, "normal" brain aging and its association with late-life diseases are an understudied area of research, compared with neurodegenerative disorders. Various neurocognitive, structural, functional, and cellular changes are identified as hallmarks of aging (Harada et al. 2013). Cognitive changes with aging are not necessarily associated with poorer functions. For example, vocabulary use or general knowledge and expert skills remain intact or can even improve with aging (Teubner-Rhodes et al. 2016; Moscoso Del Prado Martin 2017). On the other hand, functions such as

problem-solving (Zahr et al. 2009), memory (Salthouse 2003; Nyberg et al. 2012), processing speed (Salthouse 2000), and psychomotor abilities (Shanmugaratnam et al. 2010) (also known as executive functions) suffer gradual impairment with increasing age. More specifically, episodic memory (Souchay et al. 2000) (memory of personal experienced events) and working memory (Salthouse 1994) (temporary maintenance, storage, and update of information while performing a task) are altered during aging (Nyberg et al. 2012). Similar cognitive functions can be assessed in animal models of aging that recapitulate the decline observed in human populations (Mitchell et al. 2015).

Cognitive decline is linked to brain changes on multiple levels, defined as "morphomolecular senescence" (Hof and Morrison 2004). Aging causes a gray matter volume decline, particularly in the frontal cortex (Terribilli et al. 2011) and hippocampus (Apostolova et al. 2012), 2 brain regions mediating various cognitive processes. Decrease in gray matter appears to be caused by neuronal shrinkage and to a lesser extent by neuronal death (Terry et al. 1987; Cykowski et al. 2017). Pyramidal neurons are particularly vulnerable during aging (De Brabander et al. 1998; Luebke et al. 2013; Shukla et al. 2019), exhibiting reduced spine density and dendritic shrinkage. These cellular morphometric changes have been reported in humans (Jacobs et al. 1997), rodents (Wallace et al. 2007), and monkeys (Dickstein et al. 2013). Causal factors include reduced neurotrophic support (Oh et al. 2016), increased neuroinflammation (Ownby 2010), accumulation of β-amyloid (Nishimura et al. 1998; Rodrigue et al. 2009, 2012; Ten Kate et al. 2017), or dysregulation of the neuronal excitation–inhibition balance (Shukla et al. 2019), together leading to reduced cell-to-cell structure and function (i.e., communication).

Neuronal activity is impaired during normal and pathological aging, often affecting components of the excitation/inhibition balance. Studies have suggested decreased neuronal excitability toward a hyperinhibition state (Wong et al. 2006; Legon et al. 2016) or decrease in both excitation and inhibition (Hua et al. 2008; Stanley et al. 2012; Sibille 2013). GABAergic neurons represent the main cells providing inhibition and control excitability of excitatory cells. Using magnetic resonance spectroscopy, γ-aminobutyric acid (GABA) levels are downregulated during normal and pathological aging and correlate with cognitive decline (Cuypers et al. 2018). Among the various GABAergic interneurons, the subtype expressing somatostatin (SST) is particularly sensitive to stress (Fee et al. 2017) and aging (Burgos-Ramos et al. 2008). Studies showed a decrease in markers of SST functions due to normal (McKinney et al. 2015) or pathological (Burgos-Ramos et al. 2008) aging that contributes to cognitive deficits (Epelbaum et al. 2009), either directly through SST regulating pathways or indirectly through GABAergic signaling (Sandoval et al. 2011; Martel et al. 2012).

In cortical-like layers, SST cells mainly inhibit the distal dendrites of pyramidal neurons (Tremblay et al. 2016), through both GABA-B (Urban-Ciecko et al. 2015) and GABA-A receptors, specifically the α_5-subunit-containing GABA-A receptor (α_5-GABAA-R) (Schulz et al. 2018). The α_5-GABAA-Rs are located synaptically and extrasynaptically in distal dendrites of pyramidal neurons (Ali and Thomson 2008; Brickley and Mody 2012) where they regulate tonic inhibition via chloride ion influx into the postsynaptic cell (Bormann 2000; Möhler 2006). α_5-GABAA-Rs are located almost exclusively in brain regions highly involved in cognitive processes, such as the hippocampus and the prefrontal cortex (Hörtnagl et al. 2013). The expression of *GABRA5*, the gene coding for the α_5-subunit, is significantly downregulated in the aging human brain and correlates with reduced expression of SST (Erraji-Benchekroun et al. 2005; Oh et al. 2016), together suggesting a downregulation of the SST-neuron/α_5-GABAA-R signaling pathway with increasing age. Additional preclinical and clinical data showed that the α_5-subunit is downregulated in aging (Howell et al. 2000; Rissman et al. 2003, 2004), contributes to neurobiological processes (Jacob 2019), and is critical for cognitive functions in old rats (Koh et al. 2020). A recent study also showed that the synaptic localization of α_5-GABAA-Rs contributes to dendritic outgrowth and spine maturation (Brady and Jacob 2015). Altogether, the data suggest a critical role for α_5-GABAA-R in the regulation of cognitive function mediated by the GABAergic system during aging, directly through receptor activity and indirectly through cell-to-cell communication and dendritic growth. This suggests that augmenting α_5-GABAA-R function may have therapeutic effects at both symptomatic and cellular pathological levels.

Drugs acting on α_5-GABAA-Rs already exist, such as diazepam, the most commonly used benzodiazepine (BZ). However, BZs are not selective, bind at the interface of 4 α-subunits ($\alpha_{1,2,3,\text{ or }5}$-subunit) and the γ_2-subunit of the GABAA-R (Sigel and Ernst 2018), and act as nonselective positive allosteric modulators (PAMs). BZs are the most commonly prescribed medication for the treatment of anxiety and are widely prescribed to elderly despite issues and complications linked with age (Markota et al. 2016). The wide range of activity at different GABAA-R subunits is responsible for the considerable side effects of this drug class, which can also interact with GABAergic dysregulation occurring during normal or pathological aging (Ruano et al. 2000). Efforts aimed at targeting age-related pathologies while reducing the side effects of BZs have focused on increasing the ligand selectivity to the α_5-subunit and reducing selectivity to the α_1-subunit (Prevot et al. 2019). Previous data from our group showed that 2 α_5-GABAA-R PAMs, namely GL-II-73 and GL-II-75, improve working memory in mouse models of chronic stress and aging and also exhibit predictive anxiolytic and antidepressant properties (Prevot et al. 2019). Both ligands are imidazodiazepine derivatives with activity at the α_5-GABAA-R. GL-II-73 displays preferential efficacy at α_5-GABAA-R and minor activity at the $\alpha_{1/2/3}$-GABAA-Rs, whereas GL-II-75 has broad efficacy at all subunits ($\alpha_{1/2/3/5}$). Although both compounds showed beneficial effect on working memory acutely, only GL-II-73 did when administered subchronically for up to 10 days (Prevot et al. 2019). Other groups have demonstrated improved cognitive functions in old rats using different α_5-PAMs, namely Compound 44 and Compound 6 (Koh et al. 2013).

Knowing the distribution of the α_5-subunit in the brain, its downregulation during aging, its potential role in dendritic growth, and the known beneficial effect on working memory of GL-II-73 in old mice, when administered acutely or subchronically, we hypothesized that facilitating the action of GABA at the α_5-GABAA-Rs with GL-II-73 will improve working memory in old mice, directly through activity at the receptor and indirectly through reversal of neuronal atrophy due to normal aging. To validate our hypothesis, we first used primary neuronal culture from mouse frontal cortex to test the effect of GL-II-73 at promoting dendritic growth in vitro. We then tested the effect of GL-II-73 in vivo, in old mice, to investigate if chronic treatment, for 30 days, can improve age-related cognitive deficits and reverse neuronal morphometric changes at the dendrite and spine levels and if such effects are maintained when the treatment stops.

Materials and Methods

Animals

For the cell culture experiment, heterozygous Camk2a-Cre mice (The Jackson Laboratory, stock no. 005359) were crossed with homozygous Rosa26-flox-stop-GFP mice (stock no. 007906) to express the green fluorescent protein (GFP) exclusively in pyramidal cells. The pregnancy of the females was timed in order to collect embryos at E14 (6–8 pups per female). Pups were quickly decapitated, and the frontal cortex was harvested for primary neuronal culture. For the behavioral experiment, 2 separate cohorts of 30 male C57BL6 mice were purchased as retired breeders from Charles River Laboratories at the age of 9–10 months and kept in the animal facility until they reached the age of 22 months. Seven retired breeders from the first cohort and 12 from the second cohort died of normal aging before the initiation of the behavioral tests. Two cohorts of young male C57BL6 mice (N1 = 11; N2 = 8) were purchased separately as control groups for each cohort of old mice. Animals were handled following in-house procedure to limit their reactivity to the experimenter, critical for proper assessment of working memory function in the Y-Maze. Handling was performed prior to Baseline assessment for 3 days (5 min per mouse per day) and was then conducted once a week for 3 min to maintain proper habituation level throughout the entire duration of the study. Maintenance and use of animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at CAMH.

Primary Cortical Neuron Culture and Morphometric Analysis

To visualize dendritic and spine morphology of major output neurons in culture, we prepared primary cortical neurons (Kaech and Banker 2006; Tomoda et al. 2019) from E14 CAMKII-GFP mouse embryos. Neurons were maintained in culture for 10–12 days in vitro (DIV) by replacing half the media (Neurobasal media supplemented with B-27 [GibcoBRL]) with fresh media every other day. The neurons were then treated with drugs for 24 h and fixed at 11–13 DIV with 4% paraformaldehyde in PBS and imaged using a fluorescence microscope (IX81 equipped with Disk Spinning Unit and a 60× objective lens [NA = 1.3], Olympus). To evaluate dendritic branching complexity, ImageJ (NIH) with the Sholl analysis plug-in (http://fiji.sc/Sholl_Analysis) was used to score the number of intersections of dendritic arbors with a series of concentric circles drawn from the soma with 10-µm interval. Numbers of spines along all apical dendritic branches (except for the main shaft of apical dendrite) per cell were scored by ImageJ with the Bio-Formats plug-in (https://imagej.net/Bio-Formats), and average spine numbers per 100 µm length were calculated.

Drug Preparation

The PAM with preferential activity at the α_5-GABAA-R (α_5-PAM, code name GL-II-73) was synthesized in collaboration with Dr Cook's group (University of Wisconsin-Milwaukee) as described (Li et al. 2018). For the neuronal culture, α_5-PAM was infused in the media for 24 h at the final concentration of 1 µM, in 0.01% DMSO. For the behavioral studies, it was administered through the drinking water at a dose of 30 mg/kg/day for 30 days. This dose is based on the 30% bioavailability of the drug, corresponding to a similar free fraction as when given acutely with intraperitoneal administration at 10 mg/kg. Previous studies from our group using oral administration at the same dose have demonstrated preferential activity at the α_5-GABAA-R and procognitive efficacy in chronic stress- and age-related models (Prevot et al. 2019).

Experimental Design

In the first experiment (i.e., Study #1), 11 young and 22 old mice were included. Among the 22 old mice, 8 received α_5-PAM in drinking water at the dose of 30 mg/kg, while the other 14 received only water. This group design is due to the limited availability of the drug that did not allow more animals to be included in the treatment group. Similarly, in the second study (i.e., Study #2), 8 young and 18 old mice were included. Among the old mice, 6 received water, 6 received α_5-PAM, and 6 received α_5-PAM with a 1-week washout. All young mice from both behavioral experiments received water for the entire duration of the study. In each group, 4 mice were used for the Golgi staining analyses. Selection of the animals for this study was randomized and checked for proper representation of average performances of the groups at large.

Open Field

Mice were habituated to the room lit at 75 lux for 30 min prior to testing. The apparatus was a gray PVC arena (43 × 43 cm) with walls 43 cm high. A digital camera was mounted to record the animals' activity in the arena for 10 min. Post-acquisition videos were analyzed using the software ANYmaze (Stoelting). Distance travelled in meters (m) was measured as a proxy for locomotor activity.

Y-Maze Alternation Task

The apparatus is made of black PVC, shaped like a Y, with a sliding door at the entry of each arm. Mice were habituated to the maze, by letting them explore the maze freely for 10 min per day, for 2 days. On the third day, mice were trained to alternate in the maze. Each animal was placed in the starting box for 30 s, before opening the door. Once the door was opened, the animal could decide to visit the right or left arm of the maze. Once chosen, the animal was confined into that arm for 30 s. Then, the animal was gently transferred back to the starting box, with 30-s inter-trial interval (ITI), prior to the next trial, identical to the previous one. If the animal did not alternate over 3 consecutive trials, the animal was then forced to alternate in order to prevent any reinforcement of one arm. This sequence was followed for 7 trials. Finally, on the fourth day, animals were subjected to the same sequence of testing but with a 60-s ITI. Also, an eighth trial with a 5-s ITI is implemented to assess potential loss of motivation. In the event of a lack of alternation at the eighth trial, mice were removed from the statistical analysis. Mice were tested twice in the Y-Maze: once before treatment initiation to control for performances before group affiliation, and after the completion of the treatment regimen.

Brain Collection and Staining

Twenty-four (24) hours after completion of the last behavioral test, mice were euthanized using cervical dislocation, and their brains were harvested for downstream analysis. Four brains per group were used for the Golgi-Cox staining. The remaining brains were kept as backups or for follow-up analyses. The brain

extraction and initial immersion of Golgi staining procedure were conducted at CAMH, with Golgi-Cox staining solutions provided by NeuroDigiTech LLC. The brain samples were then shipped to NeuroDigiTech for sectioning and blinded morphological analysis in layers II/III of the prefrontal cortex (PFC; anterior cingulate cortex) and CA1 region of the dorsal hippocampus (CA1).

Selection of the Regions of Interest and Samplings

Brains were sectioned on a cryostat (coronal section, 100 µm thickness), and slices were mounted on glass slides. The slides included serial coronal sections that covered the anterior-to-posterior axis of the brain. The sampling of the regions of interest (ROIs) included the basal and apical dendrites of pyramidal cells in layers II/III of PFC and the CA1 of the hippocampus. The ROIs were then chosen and analyzed using the stereology-based software NeuroLucida v10 (Microbrightfield, VT), installed on a Dell PC workstation that controlled Zeiss Axioplan 2 image microscope with Optronics MicroFire CCD (1600 × 1200) digital camera, motorized X, Y, and Z focus for high-resolution image acquisition and digital quantitation. The sampling process was conducted as follows: The investigators first previewed the entire rostrocaudal axis of ROIs, under low-magnification Zeiss objectives (10× and 20×), compared and located those with the least truncations of distal dendrites as possible under high-magnification Zeiss objectives (40× and 63×), and then used a Zeiss 100× objective with immersion oil to perform 3D dendritic reconstruction, followed by counting of the spines throughout the entire dendritic trees. The selection of candidate neurons for analysis (n = 6 per animal) was based on the following criteria: visualization of a completely filled soma with no overlap of neighboring soma and completely filled dendrites, the tapering of most distal dendrites, and the visualization of the complete 3D profile of dendritic trees using the 3D display of imaging software. Neurons with incomplete impregnation and/or neurons with truncations due to the plane of sectioning were not collected. Moreover, cells with dendrites labeled retrogradely by impregnation in the surrounding neuropil were excluded (derived from Wu et al. 2004).

For spine sampling, only spines orthogonal to the dendritic shaft were resolved and included in this analysis, whereas spines protruding above or beneath the dendritic shaft were not sampled. After completion, the digital profile of neuron morphology was extrapolated and transported to a multipanel computer workstation for the quantitative analysis, including the dendrograms, spine counts, and Sholl analyses.

Statistical Analysis

In the cell culture experiment, the average number of intersections depending on the distance from the soma and the treatment group was analyzed using 2-way analysis of variance (ANOVA) with repeated measures. Statistical differences between conditions were then assessed using Bonferroni's test. The average spine counts per group were analyzed using a t-test (two-tailed). Behavioral performances were analyzed using 1-way ANOVA followed by post hoc analyses when significance was reached (Scheffe's test). To quantify the dendritic length, spine count, and spine density of the samples, 6 cells per animal and per brain region were used. The total of each feature was quantified, as well as the detail of each feature on the apical or basal segment of the dendrite. Repeated measures ANOVA, taking into consideration each cell quantified for its dendritic length, spine count, and spine density in each segment (apical or basal), was performed with "group" as the independent variable. If a significant difference was found, Tukey's multiple comparison test was performed to identify the differences between groups. Correlation analyses were also performed, using Spearman's rank correlation between morphological features and behavioral scores in the Y-Maze.

Results

Infusion of the α_5-PAM in Neuronal Culture Increases Pyramidal Neuron Dendritic Complexity and Spine Number

α_5-GABAA-R is predominantly expressed in CaMKII-positive pyramidal neurons in cortical areas of the brain (Hu et al. 2019). As a first step to study the effects of α_5-PAM on dendritic morphology, we prepared primary cortical neurons from E14 CAMKII-GFP mouse embryos (resulting from Rosa26-lox-stop-lox-GFP X Camk2a-cre cross), which specifically express GFP in CaMKII-positive neurons. GFP epifluorescence started to be expressed after approximately 8 days in culture. On 10–12 DIV, neurons were treated with α_5-PAM for 24 h, fixed, and imaged to evaluate their morphology (Fig. 1A). Sholl analysis revealed a significant effect on dendritic branching complexity, as measured by the number of intersections between dendrites and concentric circles 10 µm apart from each other (Fig. 1B). Repeated measures ANOVA of number of intersections at each radius step for 10 neurons per experimental conditions showed a significant effect of α_5-PAM ($F_{(15,135)} = 10.87$, $P < 0.001$). Post hoc analysis identified an increase in number of intersections of the neurons treated with α_5-PAM between 30 and 50 µm from the cell body ($P < 0.001$ at 30 and 40 µm, $P < 0.05$ at 50 µm). Higher magnification view of the secondary dendritic branches showed increased spine-like protrusions from the shaft after α_5-PAM treatment (Fig. 1A, bottom). Analysis of the spine number showed a significant increase in neurons treated with α_5-PAM compared with neurons treated with vehicle ($P < 0.001$; Fig. 1C).

Chronic Treatment with α_5-PAM Increases Spatial Working Memory Performances in Old Mice

Twenty-two-month-old mice were first tested in the Y-Maze prior to drug treatment to assess baseline working memory performances (Supplementary Fig. 1). Spontaneous alternation rates were low (~55%; 50% = random alternation) as expected for old mice (Prevot et al. 2019) and not different between old mice and old mice about to receive the drug treatment in the drinking water ($F_{(1,8)} = 0.43$; $P = 0.84$). Young mice were not included at that stage, as they were not in the facility at that time. Instead, their baseline testing, assessed separately from the old mice a week after arrival (on day 27 on Fig. 2A), was high (~80%) as expected from young mice (Prevot et al. 2019).

A group of old mice was then exposed to 30 mg/kg of α_5-PAM for 30 days ("old + α_5-PAM" group), provided in their drinking water, in parallel to 2 groups of young or old mice receiving only water ("young" and "old," respectively). "young," "old," and "old + α_5-PAM" mice were tested in the open field to assess locomotor activity and in the Y-Maze to assess spatial working memory (Fig. 2A). Old mice appeared to travel less (Fig. 2B), although the difference did not reach statistical significance ($F_{(2,31)} = 2.4$; $P = 0.11$). ANOVA performed on the percentage of alternation

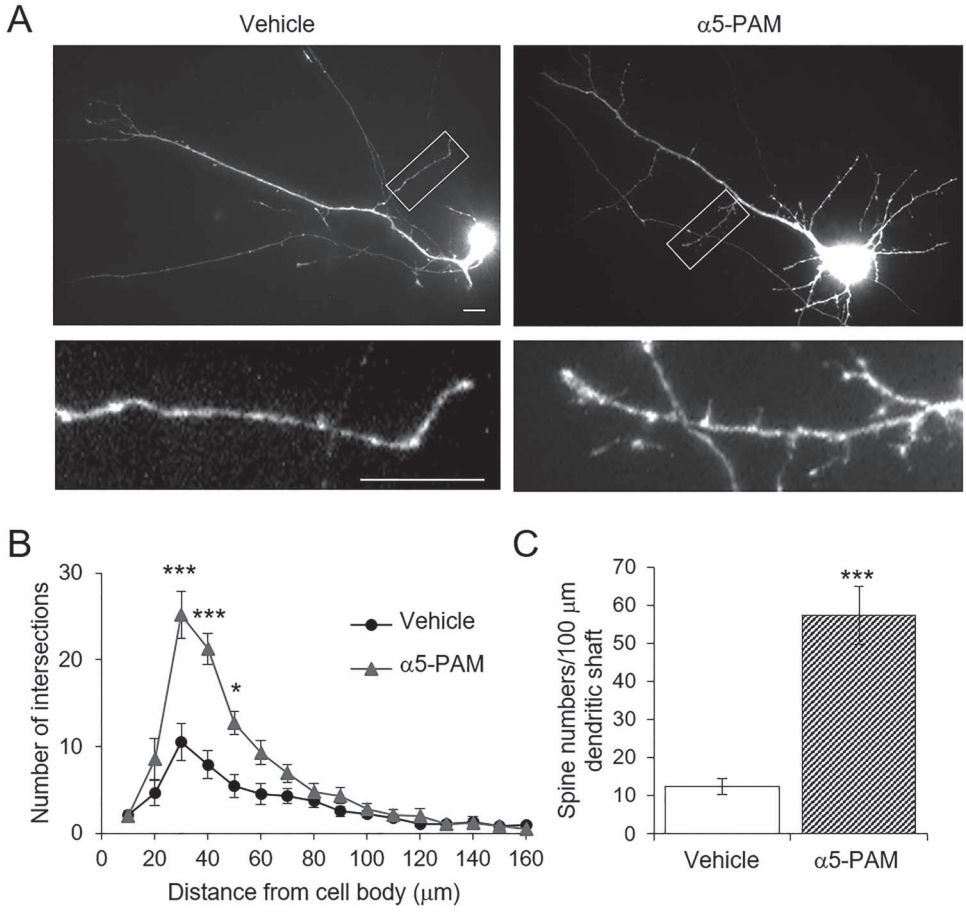

Figure 1. Effect of α_5-PAM on dendrite morphology and spine numbers in primary cortical neurons in vitro. Cortical neurons, prepared from E14 mouse embryos expressing GFP in CaMKII-positive pyramidal neurons, were cultured for 10–12 days and then incubated for 24 h with vehicle (0.01% DMSO in culture media) or α_5-PAM (GL-II-73: 1 μM). (*A*) Representative images of isolated GFP-positive neurons after 24 h of incubation with α_5-PAM or vehicle. The dotted box areas in top panels were magnified underneath to render a view of spines and protrusions from a secondary dendritic shaft. Scale bars: 10 μm. (*B*) Dendritic branching complexity evaluated by Sholl analysis. The number of intersections every 10 μm from the soma was analyzed. A significant increase in the number of intersections was identified in neurons incubated with α_5-PAM between 30 and 50 μm of distance from the soma. Vehicle ($N = 10$), α_5-PAM ($N = 10$). (*C*) Spine density was also increased after incubation with α_5-PAM. $^*P < 0.05$; $^{***}P < 0.001$.

in the Y-Maze (Fig. 2C) showed significant differences between groups ($F_{(2,25)} = 11.34$; $P < 0.001$). Post hoc analysis identified a significant decrease of alternation with age ("old" vs. "young"; $P < 0.001$) that was reversed by chronic treatment with α_5-PAM ("old + α_5-PAM" vs. "old"; $P < 0.05$). The "old + α_5-PAM" group was not significantly different from the "young" group.

Chronic Treatment with α_5-PAM Reverses Cellular Morphological Changes Related to Aging

From each group, 4 mice were euthanized and brains were collected for Golgi staining and quantification of dendritic length, spine count, and spine density in the PFC and CA1 of the dorsal hippocampus. Representative images of pyramidal neurons from the PFC are presented for each group in Figure 3A, with a close-up visualization of the spines at the apical segment of the pyramidal neuron dendrite. Total dendritic length was significantly different between groups (ANOVA, $F_{(2,46)} = 5.5$; $P < 0.001$; Supplementary Fig. 2A). Post hoc analyses showed that "old" mice had shorter dendritic length compared with "young" mice, while "old + α_5-PAM" mice were neither significantly different from the "old" group nor the "young" group ($P > 0.05$). When analyzed per dendritic segment (apical vs. basal), data showed no difference between groups in the basal segment (ANOVA, $F_{(2,46)} = 1.07$; $P > 0.05$; Fig. 3B), while a group difference was observed in the apical segment (ANOVA, $F_{(2,46)} = 5.3$; $P < 0.05$; Fig. 3C). Post hoc analyses identified a significant decrease in dendritic length due to normal aging ("old" vs. "young"; $P < 0.01$) that was reversed by chronic treatment with α_5-PAM ("old + α_5-PAM" vs. "old"; $P < 0.05$).

Similarly, repeated measures ANOVA of the total spine counts showed a significant difference between groups ($F_{(2,46)} = 16.29$; $P < 0.001$; Supplementary Fig. 2B), explained by a significant decrease of spine counts with age ("young" vs. "old"; $P < 0.001$), reversed by chronic treatment with α_5-PAM ("old + α_5-PAM" vs. "old"; $P < 0.05$). However, mice receiving the drug still had less spines than young mice ($P < 0.05$). At the dendritic segment level, repeated measures ANOVA of the spine counts from the basal segment showed significant differences ($F_{(2,46)} = 6.09$; $P < 0.001$; Fig. 3D). Post hoc analysis identified a significant decrease with age ("old" vs. "young"; $P < 0.01$) but no further significant differences. In the apical segment (Fig. 3E), spine counts were also significantly different between groups (repeated measures ANOVA, $F_{(2,46)} = 10.54$; $P < 0.001$), explained

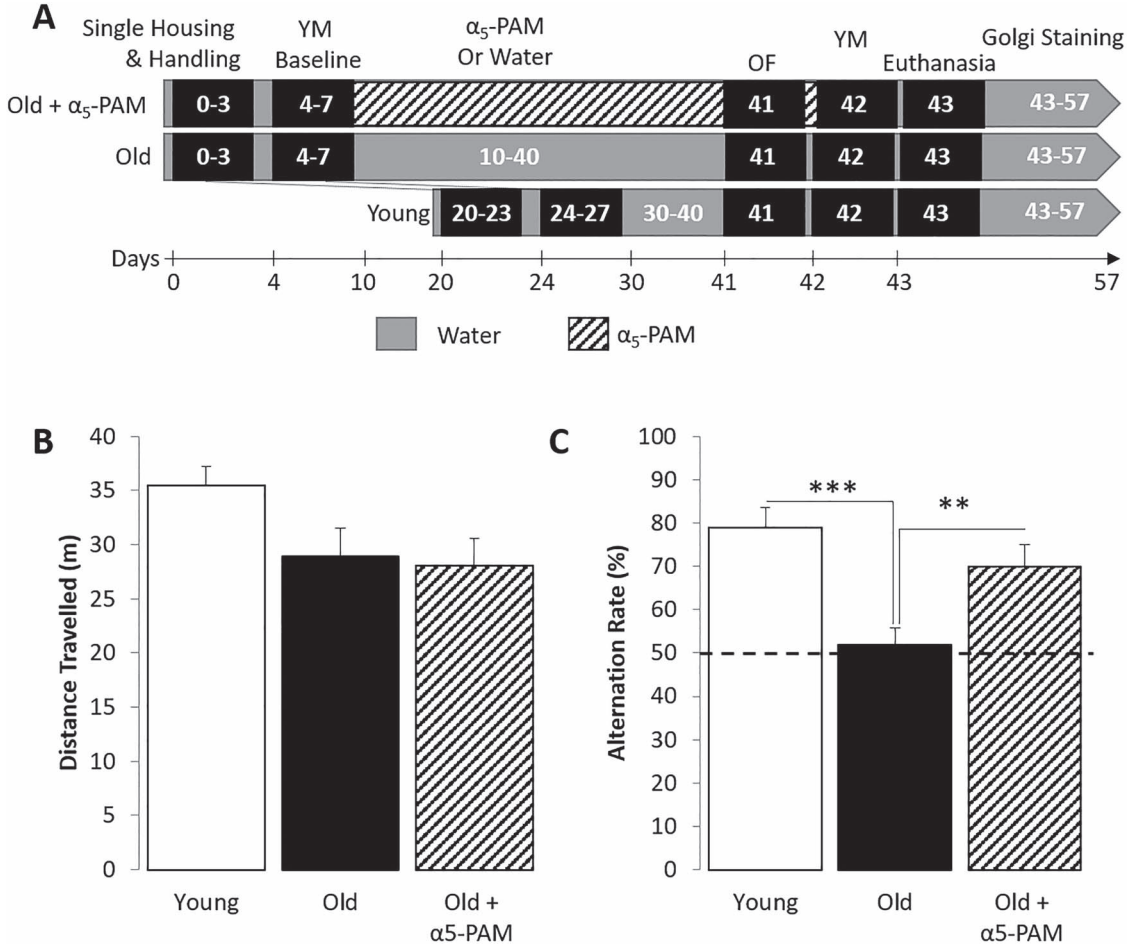

Figure 2. Chronic treatment with the α_5-PAM reverses age-related deficit in working memory. C57BL/6 male mice were used: young (2 months old) and old (22 months old). (A) Experimental overview. Numbers in boxes indicate days in experiment. Old mice received either α_5-PAM in drinking water ($N = 8$) or water ($N = 14$). Young mice received water only ($N = 11$). After 30 days of treatment, mice were tested in the open field to assess locomotor activity. (B) No significant differences in the total distance travelled were observed between "young," "old," and "old + α_5-PAM" mice. (C) Mice were then tested in the alternation task in the Y-Maze (YM). ANOVA revealed a significant difference in alternation between groups, explained by a decreased alternation in old mice, compared with young, that is reversed by chronic treatment with the α_5-PAM. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with "old." Dash line represents chance level.

by a significant decrease with age ("old" vs. "young"; $P < 0.001$), reversed by chronic treatment ("old + α_5-PAM" vs. "old"; $P < 0.05$). No statistical difference was observed between "young" and "old + α_5-PAM" ($P > 0.05$).

Spine density was also calculated. Repeated measures ANOVA of total spine densities (Supplementary Fig. 2C) showed significant differences between groups ($F_{(2,46)} = 17.93$; $P < 0.001$), explained by a decrease in spine density with age ("old" vs. "young"; $P < 0.001$), reversed by chronic treatment with α_5-PAM ("old + α_5-PAM" vs. "old"; $P < 0.05$). A significant difference was observed between "old + α_5-PAM" and "young" ($P < 0.01$). When focusing on the basal segment (Fig. 3F), a significant difference was identified (repeated measures ANOVA, $F_{(2,46)} = 9.24$; $P < 0.001$), characterized by decreased spine density with age ("old" vs. "young"; $P < 0.001$). In the apical segment (Fig. 3G), repeated measures ANOVA showed significant group differences ($F_{(2,46)} = 10.1$; $P < 0.001$), characterized by reduced spine density due to aging, regardless of the drug treatment ("old" vs. "young" and "old + α_5-PAM" vs. "young"; $P < 0.05$).

The same analyses were carried out in the CA1 region of the dorsal hippocampus (Fig. 4 and Supplementary Fig. 3). Similarly, total dendritic length, total spine counts, and spine density (Supplementary Fig. 3A–C) were significantly decreased by age (repeated measures ANOVA, $F_{(2,46)} = 9.1$, $F_{(2,46)} = 27.8$, and $F_{(2,46)} = 18.8$, respectively, all $P < 0.01$—post hoc $P < 0.05$ for all comparisons between "old" vs. "young" groups) and reversed by chronic treatment with α_5-PAM ($P < 0.05$ for all comparison between "old" vs. "old + α_5-PAM"). Looking at dendritic length, spine counts, and spine density in the basal segment (Supplementary Fig. 3D–F), repeated measures ANOVA showed a trend toward significance ($F_{(2,46)} = 3.05$, $P = 0.057$) for dendritic length or significant differences ($F_{(2,46)} = 14.2$ and $F_{(2,46)} = 10.7$, all $P < 0.01$) for spine counts and spine density. These differences were explained by reduced spine counts ($P < 0.001$) and spine density ($P < 0.001$) due to age ("old" vs. "young") and reversal of reduced spine counts ($P < 0.01$) and spine density ($P < 0.05$) after chronic treatment with α_5-PAM ("old" vs. "old + α_5-PAM").

Dendritic length, spine count, and spine density in the CA1 of the hippocampus were also quantified in the apical segment of young, old, and old + α_5-PAM mice (Fig. 4B–D). Repeated measures ANOVA showed significant differences

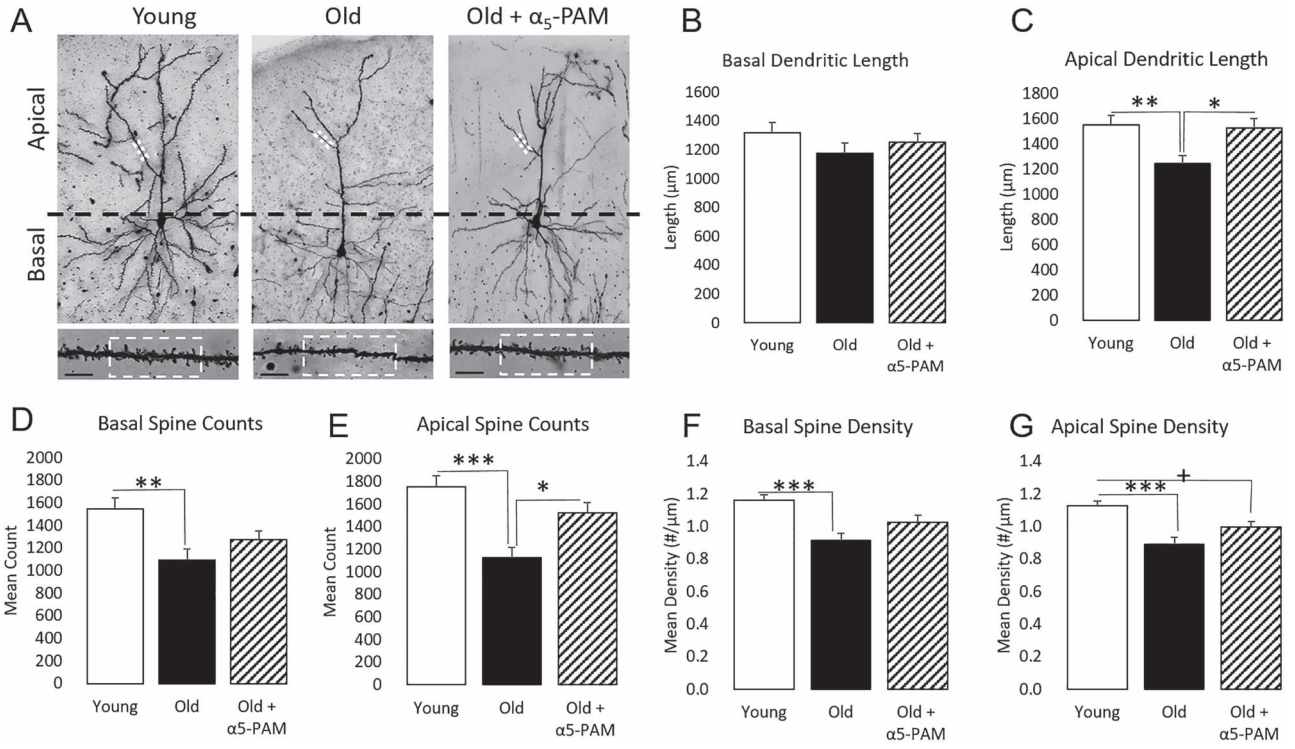

Figure 3. Chronic treatment with α_5-PAM reverses cellular morphological changes related to aging in the PFC. After completion of the behavioral screening, mice were euthanized and brains were stained with Golgi-Cox solution. (*A*) Pyramidal neurons ($N = 6$ per mouse) from 4 mice per group were analyzed for dendritic length, spine counts, and spine density. Basal (*B*) and apical (*C*) dendritic lengths were measured. No statistical differences were observed in the basal segment, but ANOVA in the apical segment revealed significant differences between groups. This difference was explained by a decrease in dendritic length in old mice, compared with young mice, that was reversed by chronic treatment with α_5-PAM. (*D*,*E*) In the same brain region, basal and apical spine counts were assessed. In the basal segment, a significant decrease was observed in old mice. In the apical segment, a similar decrease was observed with age that was reversed by chronic treatment with α_5-PAM. Similarly, basal (*F*) and apical (*G*) spine density were measured. With age, a significant decrease in spine density was observed in the basal segment and in the apical segment. However, chronic treatment with α_5-PAM only reversed this decrease in the apical segment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with "old"; $^{+}P < 0.05$ compared with "young." Scale bar in (*A*) represents 50 µm.

between groups in all 3 parameters ($F_{(2,46)} = 6.9$, $F_{(2,46)} = 15.25$, and $F_{(2,46)} = 7.9$, respectively; all $P < 0.01$). Post hoc analyses identified these differences to be due to a significant decrease of dendritic length ($P < 0.01$), spine counts ($P < 0.001$), and spine density ($P < 0.001$) with aging ("old" vs. "young"). Additionally, reduced dendritic length and spine count in old mice were reversed with chronic α_5-PAM treatment ($P < 0.05$ for all). In both brain regions, dendritic complexity was assessed using Scholl analyses, confirming the effect of age and treatment described above (Supplementary Results and Supplementary Fig. 4). Also, using Spearman's rank correlation, apical dendritic length in the CA1 and the PFC was correlated with behavioral scores in the Y-Maze and showed significant positive correlation (Supplementary Fig. 5 and Supplementary Table 1).

One-Week Treatment Cessation after Chronic α_5-PAM Treatment Results in Loss of Procognitive Efficacy, but Sustained Reversal of Age-Related Cellular Morphological Changes

A separate cohort of old mice was tested in the Y-Maze, first at baseline to ensure lack of random group differences prior to treatment (Supplementary Fig. 6). ANOVA of alternation rates measured for the 3 arbitrary groups of old mice ("old," "old + α_5-PAM," and "old + washout") did not show significant differences between groups ($F_{(2,13)} = 0.038$; $P = 0.96$). Mice were then tested again in the Y-Maze after 30 days of treatment with α_5-PAM, or after 30 days of treatment and a subsequent washout period of 1 week ("washout" group; Fig. 5A), in parallel to a group of young mice receiving only water. ANOVA of the alternation rate showed significant differences between groups ($F_{(3,17)} = 9.48$; $P = 0.007$), explained by significant decrease of alternation with aging ("old" vs. "young"; $P < 0.01$), reversed by chronic treatment ("old + α_5-PAM" vs. "old"; $P < 0.01$). However, mice in the "washout" group had decreased alternation rate compared with "young" and "old + α_5-PAM" ($P < 0.01$; Fig. 5B).

After completion of the behavioral testing, mice were euthanized and brains collected for Golgi staining (Supplementary Fig. 7). ANOVA performed on total dendritic length, spine counts, and spine density in the PFC showed significant differences ($F_{(3,92)} = 3.5$, $F_{(3,92)} = 17.1$ and $F_{(3,92)} = 38.1$, respectively; $P < 0.05$ for all; Supplementary Fig. 8). These differences were explained by a decrease of dendritic length in old mice, compared with young mice ($P < 0.05$). Old mice also showed decreased total spine counts compared with young ($P < 0.001$), which was reversed by chronic treatment with the α_5-PAM ("old + α_5-PAM" vs. "old"; $P < 0.001$) even after a 1-week washout ("old + washout" vs. "old"; $P < 0.001$). Similarly, the significant differences in the total spine density were explained by reduced spine density in old mice compared with young ($P < 0.001$), partially reversed by chronic

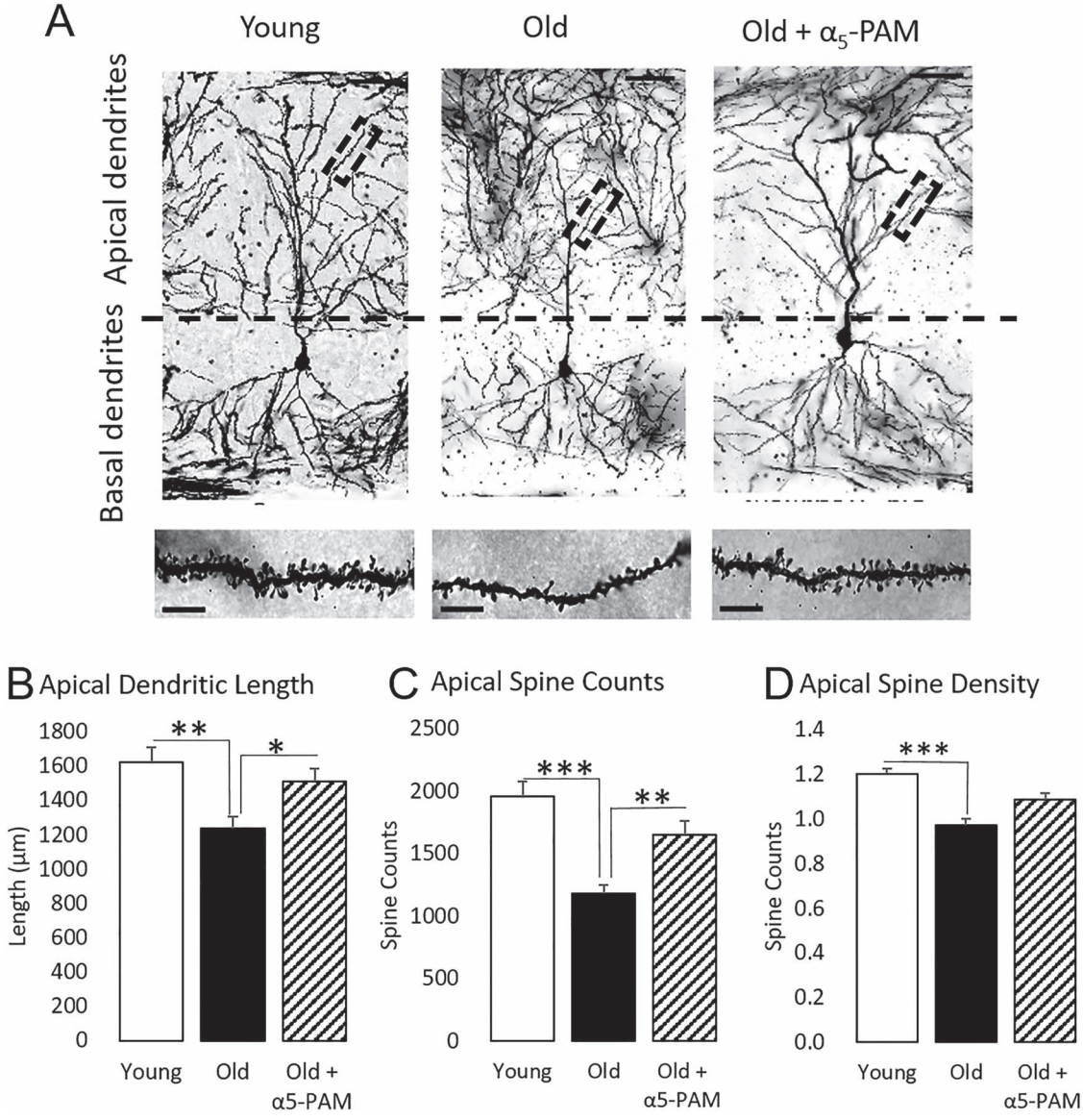

Figure 4. Chronic treatment with α_5-PAM reverses cellular morphological changes related to aging in the hippocampal CA1 region. The same brains as in Figure 3 were analyzed for dendritic length, spine count, and spine density in the CA1 of the hippocampus. (*A*) Pyramidal neurons (N = 6 per mouse) from 4 mice per group were analyzed for dendritic length, spine counts, and spine density. (*B*) Dendritic length in the apical segment was significantly decreased by aging. Chronic treatment with α_5-PAM significantly reversed the reduced dendritic length in old mice. (*C*) Spine counts in the apical segment were also reduced with age. Chronic treatment with α_5-PAM reversed the reduced spine counts in old mice. (*D*) Spine density in the apical segment of pyramidal neurons in the CA1 was also significantly decreased in old mice. Chronic treatment with α_5-PAM did not significantly reversed the spine density reduction induced by aging. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with "old." Scale bar in (*A*) represents 50 μm.

treatment, even with a 1-week washout ("old" vs. "old + α_5-PAM," "old" vs. "old + washout," "young" vs. "old + α_5-PAM," and "young" vs. "old + washout"; $P < 0.05$).

In the apical segment, ANOVA of the total dendritic length did not reach significance ($P = 0.1$; Fig. 5C). ANOVA of the apical spine counts and spine density reached significance ($F_{(3,92)} = 9.6$ and $F_{(3,92)} = 24.1$, respectively; $P < 0.001$ for both). The significant difference in the apical spine count (Fig. 5D) was explained by reduced spine count during aging ("old" vs. "young"; $P < 0.001$), reversed by chronic treatment with the α_5-PAM ("old + α_5-PAM" vs. "old"; $P < 0.05$), even after a 1-week washout ("old + washout" vs. "old"; $P < 0.001$). Finally, the significant difference in apical spine density (Fig. 5E) in the PFC was explained by a significant decrease with age ("old" vs. "young"; $P < 0.001$), partially reversed by chronic treatment with α_5-PAM ("old + α_5-PAM" vs. "old" and "young"; $P < 0.01$), and fully reversed by chronic treatment followed by a 1-week washout ("old + washout" vs. "old"; $P < 0.001$). In the basal segment, ANOVA of the spine counts and the spine density showed significant differences ($F_{(3,92)} = 9.1$ and $F_{(3,92)} = 16.5$, respectively; $P < 0.001$ for both) but not on the dendritic length ($F_{(3,92)} = 2.3$; $P = 0.07$) (Supplementary Fig. 8D–F). Spine count and density in the basal segment were decreased

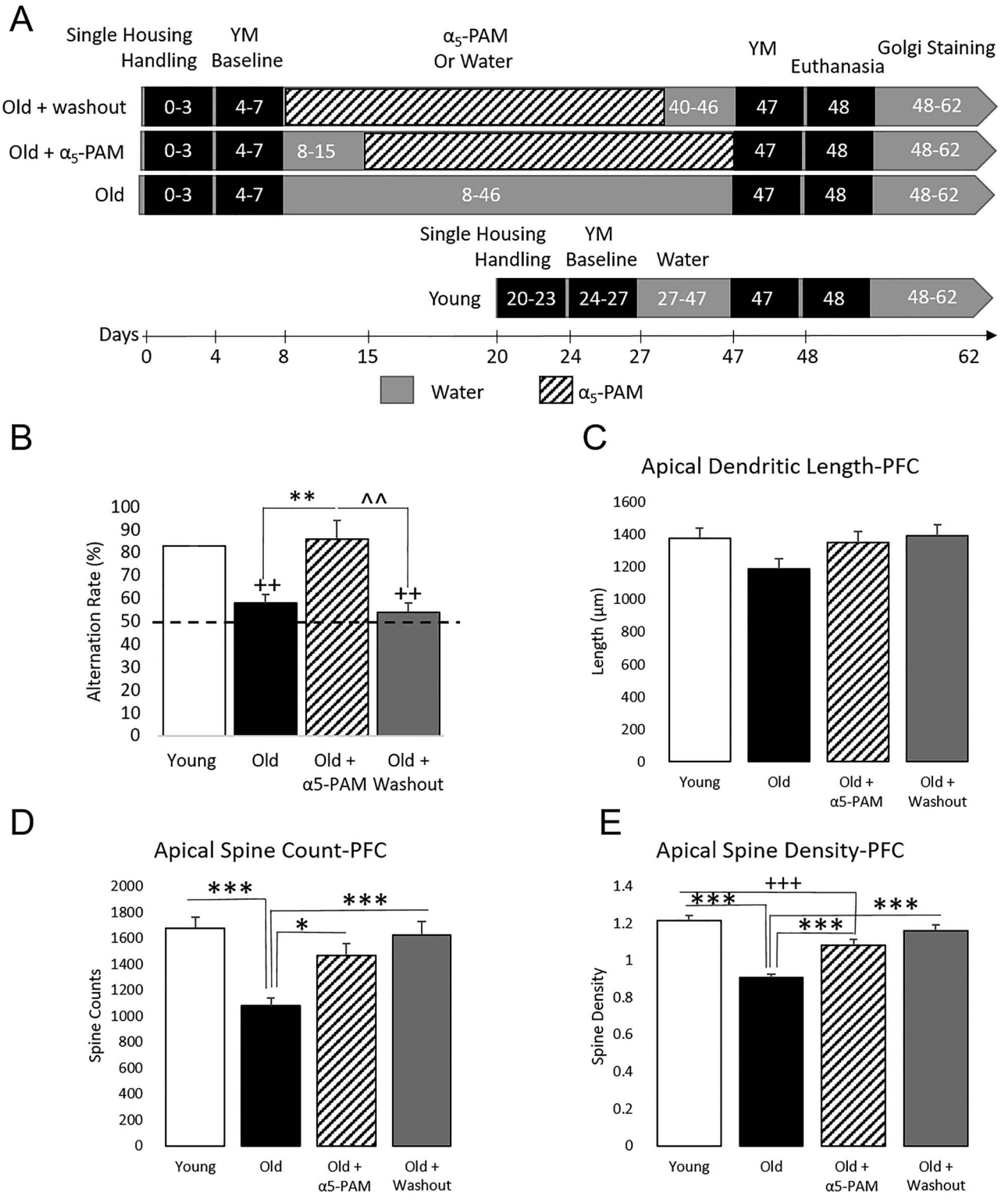

Figure 5. Cessation of chronic treatment with α_5-PAM limits the procognitive efficacy but does not reverse the neurotrophic effect on dendritic morphology. C57BL/6 male mice were used: young (2 months old) and old (22 months old). (*A*) Experimental overview. Old mice received either α_5-PAM in the drinking water ($N = 6$), water ($N = 6$), or α_5-PAM for 30 days followed by 7 days of washout ($N = 6$). Young mice received water only ($N = 8$). (*B*) After completion of the treatment schedule, mice were trained in the alternation task in the Y-Maze. ANOVA revealed a significant difference in alternation between groups, explained by a decreased alternation in old mice, compared with young, that was reversed by chronic treatment with α_5-PAM. The 7-day drug washout resulted in a loss of procognitive effect. After completion of the behavioral testing, mice were euthanized, brains were collected and stained for morphology analyses, and apical morphology features were measured. (*C*) ANOVA performed on the apical dendritic length did not reach significance ($P > 0.05$). (*D*) ANOVA performed on the apical spine count reached significance ($P < 0.05$), explained by a decrease of spine count with age that was reversed by chronic α_5-PAM treatment, including after a 7-day washout period. (*E*) Similar findings were obtained

with aging ("old" vs. "young"; $P < 0.001$) and reversed by chronic treatment ("old + α_5-PAM" vs. "old"; $P < 0.05$), even with a 1-week washout ("old + washout" vs. "old"; $P < 0.05$).

Similar analyses were performed on the dendritic length, spine count, and spine density in the CA1 of the same animals (Supplementary Fig. 9). The data obtained in the CA1 were consistent with the PFC results. There were no significant differences in total, basal, or apical dendritic length (data not shown), but a significant difference was found in total spine count ($F_{(3,92)} = 8.95$; $P < 0.001$), explained by a decrease in spine count with age ("old" vs. "young"; $P < 0.001$), that was significantly reversed only in the washout group ("old + washout" vs. "old"; $P < 0.001$; Supplementary Fig. 10A). The same effects were observed when analyzing basal and apical segments separately (Supplementary Fig. 10B,C). Total spine density also showed significant differences (ANOVA $F_{(3,92)} = 25.65$, $P < 0.001$; Supplementary Fig. 9D), explained by decreased density with aging ("old" vs. "young"; $P < 0.001$), partially reversed by chronic treatment ("old + α_5-PAM" vs. "old" and "young"; $P < 0.01$), even with a 1-week washout ("old + washout" vs. "old" and "young"; $P < 0.05$). The decrease in spine density with age was confirmed in the basal and apical segments (ANOVA $Fs_{(3,92)} = 10.5$ and $Fs_{(3,92)} = 15.7$, respectively; $P < 0.01$ for both), as well as the reversal with chronic treatment with α_5-PAM, even with a 1-week washout (Supplementary Fig. 10E and F). In both brain regions, dendritic complexity was assessed using Scholl analyses, confirming the effect of age and treatment described above (Supplementary Results and Supplementary Fig. 11). Finally, Spearman's correlation analyses showed that Y-Maze scores did not correlate with morphological features (dendritic length, spine count, and spine density) after the 1-week washout (Supplementary Table 1—Study #2).

Discussion

This study was based on the observation that normal aging induces reduced neuronal morphology that may contribute to cognitive decline and that limited number of treatment targets these 2 aspects of normal aging. To our knowledge, only riluzole, a glutamatergic modulator, showed efficacy at both symptom and cellular levels (Pereira et al. 2014, 2017). Here, we first showed that exposure of pyramidal neuron culture to an α_5-PAM fosters dendritic growth and increases the number of spines. We extended this finding to animal studies, demonstrating that chronic α_5-PAM treatment reverses age-related morphometric changes in a sustainable way. Altogether, these results demonstrate both symptomatic and "disease-modifying" efficacies of α_5-PAM treatment in old mice.

In the primary neuron culture assay, we showed that α_5-PAM treatment increases dendritic branching, morphological complexity, and spine density. This effect is observed in culture in the absence of peripheral inputs and stimuli, suggesting a direct role of α_5-GABAA-Rs in dendritic and spine growth. This observation is consistent with the previous findings that α_5-GABAA-R is involved in dendritic outgrowth and spine maturation via interaction with the synaptically localized adaptor protein gephyrin and that this interaction is maintained throughout developmental stages and into adulthood (Brady and Jacob 2015), suggesting that the observed effects of α_5-PAM on dendritic morphology are mediated through similar mechanisms throughout life.

At the behavioral level, we confirmed in 2 distinct experiments that normal aging in mice induces cognitive deficits, with old mice exhibiting impaired working memory in the alternation task, consistent with previous finding (Magnusson et al. 2003; Beracochea et al. 2016; Prevot et al. 2019). Various compounds (Vandesquille et al. 2011), drugs (Van Dam et al. 2005), natural extracts (Beracochea et al. 2016), or physical exercise (Ma et al. 2017) have demonstrated potential at reversing cognitive decline in animal models. Here, we report again through 2 independent experiments and confirm prior findings (Koh et al. 2013, 2020; Prevot et al. 2019) that augmenting the function of the α_5-GABAA-Rs is a valid approach to improve working memory in the context of aging (Koh et al. 2013, 2020; Prevot et al. 2019). Furthermore, we extended the previous finding from our group by showing that improved working memory is also observed after longer treatment duration (10 days in Prevot et al. 2019 vs. 30 days here), suggesting no desensitization of the receptors after chronic treatment. However, many of the drugs exhibiting procognitive effects in animal models have shown little-to-no success in human populations (Garner 2014; Al Dahhan et al. 2019). This lack of efficacy can be partially explained by the focus of such approaches on reversing the symptoms rather than the underlying pathology. Indeed, it is unclear whether potential treatment avenues showing procognitive efficacies in animal models may actually target and/or alleviate neuronal shrinkage and reduced cell-to-cell communication in human subjects. Companion tools like PET imaging against SV2A measuring synaptic density (Cai et al. 2019) could be used to demonstrate such morphological efficacy in human, in parallel to effects on cognitive symptoms.

In the context of aging, the "morphomolecular" senescence has a broad impact on different systems, including the GABAergic system. Reduced GABAergic functions are frequently observed during aging, and previous studies from our group and others demonstrated that targeting α_5-GABAA-Rs to bypass such deficits could reverse age-related cognitive decline (Koh et al. 2013, 2020; Prevot et al. 2019). In the present study, we investigated the potential efficacy of α_5-PAM on both symptoms and underlying pathology, that is, working memory and neuronal shrinkage, respectively. We demonstrated and replicated that chronic treatment with α_5-PAM not only reverses working memory deficit but also reverses dendritic shrinkage and spine atrophy in the PFC and the CA1 of old mice, 2 brain regions involved in cognitive processes and expressing high levels of α_5-GABAA-Rs (Hörtnagl et al. 2013). Indeed, chronic treatment with α_5-PAM increased dendritic length, spine count, and spine density in both brain regions to levels that were mostly indistinguishable from young mice. This effect was significantly correlated between the 2 brain regions, and cognitive performances, but the effect seemed to be driven by group and age effects. To confirm a direct link between morphological features and behavioral performances, within-group correlation would need to be performed with a bigger sample size.

with apical spine density measurement, where the age-related decrease in spine density was reversed by chronic α_5-PAM treatment including after a 7-day washout period. $^{++}P < 0.01$, $^{+++}P < 0.001$ compared with "young"; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with "old"; $^{\wedge\wedge}P < 0.01$ compared with "old + α_5-PAM." Dash line represents chance level.

To our knowledge, it is the first time that a drug acting on the GABAergic system demonstrates neurotrophic effects. Non-specific GABAergic ligands such as BZs tend to decrease spine density in cortical pyramidal neurons (Curto et al. 2016). Other drugs have shown neurotrophic effects (Castrén 2004), such as ketamine, an NMDA receptor antagonist (Duman and Duman 2015; Moda-Sava et al. 2019). Moda-Sava et al. showed sustained spine formation after acute ketamine treatment as early as 12 h post administration in animal models of chronic stress, which are also characterized by decreased morphometric features. However, previous studies have shown deleterious effects of ketamine on morphometric features in cultured pyramidal neurons from the hippocampus (Jiang et al. 2018) or in developing GABAergic neurons (Vutskits et al. 2006). This discrepancy in the finding with ketamine between in vivo and in vitro studies could result from different doses applied to neurons (where only low doses seem to show beneficial effects) or from an indirect role of ketamine on morphometric features, potentially involving the GABAergic system, as recently proposed (Gerhard et al. 2020; Ghosal et al. 2020). Indeed, in vitro systems lack the complexity of in vivo microcircuits, and contribution of connections to other cell types, such as GABAergic neurons, may be missing. In comparison, our results show consistency between in vitro and in vivo studies, suggesting a direct effect through α_5-GABAA-R-mediated signaling. Further studies will need to identify the mechanisms of action through which α_5-PAM exerts neurotrophic effect, and whether it has shared pathways with ketamine. Controversies around the role of ketamine in improving or worsening cognitive functions (Zhang and Ho 2016; Chen et al. 2018; Basso et al. 2020) may relate to dosage or duration of the treatment. Neurotrophic effects of ketamine were observed at low doses, alongside with antidepressant properties (Lara et al. 2013), which were recently suggested to be mediated by the GABAergic system (Gerhard et al. 2020; Ghosal et al. 2020). This further emphasizes the need to better understand the contribution of ketamine on the GABAergic system and how both mechanisms provide antidepressant and procognitive efficacies associated with neurotrophic benefits.

Interestingly, although the neurotrophic effects of α_5-PAM were systematically observed for dendritic length, spine numbers, and spine densities, these effects were more pronounced in the apical segment compared with the basal segment of dendrites from PFC pyramidal neurons. This is consistent with α_5-GABAA-Rs located at higher levels in apical segments (Hörtnagl et al. 2013). These results further confirm that activity at the α_5-GABAA-Rs could directly mediate this neurotrophic effect of our drug treatment. A significant increase of spine counts and spine density was nonetheless observed after chronic α5-PAM treatment in the basal segments of pyramidal neurons of the hippocampal CA1 region. This effect can result from low levels of α_5-GABAA-Rs in the basal dendrites of CA1 pyramidal neurons or from a generalized neurotrophic effect occurring downstream of the apical effect. Follow-up studies will need to resolve these differences.

A critical question for potential therapeutic use is whether the beneficial effect on working memory and neurotrophic effects of augmenting α_5-GABAA-R function in old animals are long-lasting. We show here that after a week of drug cessation, the neurotrophic effect remains, but the effects on working memory did not, and this is associated with a lack of correlation between behavioral performances and morphological features. This shows that restored dendritic structure and spine numbers are not sufficient to restore working memory functions and suggests that active signaling α_5-GABAA-R is instead needed for these effects. This is consistent with the acute effects of the drugs (i.e., 30 min after injection) where in vivo neurotrophic effects are unlikely. So whereas the neurotrophic effects of α_5-PAM are a positive outcome for the brain of old animals, additional studies are needed to investigate the degree to which the pre- and postsynaptic compartments are affected by sustained increase in α_5-GABAergic signaling. The results suggest that whereas postsynaptic pyramidal neurons may be "rejuvenated," this may not be the case of presynaptic GABAergic neurons. Indeed, the absence of sustained effect on working memory despite restored pyramidal neuron morphometric features in the washout group is consistent with the fact that α_5-PAM targets mainly pyramidal neurons and not GABA interneurons per se. Aging induces a decrease in GABA function, particularly marked in SST neurons. α_5-PAM bypasses this deficit by boosting α_5-GABAA-R activity on postsynaptic pyramidal neurons. The results from this study are consistent with the hypothesis that presynaptic GABAergic deficits are not reversed by postsynaptic α_5-GABAA-R treatment, even after chronic treatment predicted to affect the homeostasis of the cortical microcircuitry. The mechanisms are unknown and may include age-related intrinsic vulnerability of SST-neurons or insufficient feedforward excitation of SST interneurons from "rejuvenated" pyramidal neurons, hence limiting the functional recovery of SST cells. Extending the duration of the treatment until recovery of the SST cell function could contribute to longer lasting effects on working memory. Investigating whether longer treatment duration with an α_5-PAM can improve the stability of cognitive recovery (and potentially restore the functions of impaired SST cells) could be tested in follow-up studies. Alternatively, the morphological improvements induced by chronic α_5-PAM administration may lead to improvements in aspects of cognitive aging not captured by the Y-Maze test.

This study has additional limitations, including the absence of female mice being tested. This was due to the lack of availability of old female mice from vendors at initiation of our study. Follow-up studies are planned to investigate the effects of the drug in old female mice. We anticipate similar beneficial effects on working memory and neurotrophic effects in females compared with males, as we previously showed no behavioral differences of this α_5-PAM exposure between sexes in adulthood (Prevot et al. 2019). Also, it is important to note that mating history between groups represent a variable in this experiment. Indeed, the old mice were obtained as retired breeders, while young counterparts were virgin males. Previous studies showed that mating history can affect behavioral outcomes (Ingram et al. 1983). In our present study, we were unable to control or ascertain any potential impact of the mating history of the retired breeders. To address this point, a follow-up study could be performed with old virgin males or middle age mice. To conclude, we demonstrated the potential usefulness of targeting α_5-GABAA-Rs for the treatment of age-related working memory deficits and underlying cellular morphometric changes, suggesting both symptomatic and "disease-modifying" clinical potential.

Supplementary Material

Supplementary material can be found at *Cerebral Cortex* online.

Notes

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