Premature Aging Phenotype in Mice Lacking High-Affinity Nicotinic Receptors: Region-Specific Changes in Layer V Pyramidal Cell Morphology

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The mechanisms by which aging leads to alterations in brain structure and cognitive deficits are unclear. A deficient cholinergic system has been implicated as one of the main factors that could confer a heightened vulnerability to the aging process, and mice lacking high-affinity nicotinic receptors (β2<sup>−/−</sup>) have been proposed as an animal model of accelerated cognitive aging. To date, however, age-related changes in neuronal microanatomy have not been studied in these mice. In the present study, we examine the neuronal structure of yellow fluorescent protein (YFP<sup>+</sup>) layer V neurons in 2 cytologically distinct cortical regions in wild-type (WT) and β2<sup>−/−</sup> animals. We find that (1) substantial morphological differences exist between YFP<sup>+</sup> cells of the anterior cingulate cortex (ACC) and primary visual cortex (V1), in both genotypes; (2) in WT animals, ACC cells are more susceptible to aging compared with cells in V1; and (3) β2 deletion is associated with a regionally and temporally specific increase in vulnerability to aging. ACC cells exhibit a prematurely aged phenotype already at 4–6 months, whereas V1 cells are spared in adulthood but strongly affected in old animals. Collectively, our data reveal region-specific synergistic effects of aging and genotype and suggest distinct vulnerabilities in V1 and ACC neurons.

Keywords: aging, anterior cingulate cortex, β2 nAChR, primary visual cortex, selective vulnerability

Introduction

Cognitive abilities usually become impaired during aging (Lai et al. 1995; Burke et al. 2012; Engle and Barnes 2012); however, the underlying neurobiological factors remain unclear. Studies examining structural and morphological parameters have produced conflicting results. Neuron numbers may decline with age (Zoli et al. 1999; Yates et al. 2008; Stranahan et al. 2012) or remain stable (Morrison and Hof 1997; Hof et al. 2000), and so does the size of specific brain regions (O’Donnell et al. 1999; Long et al. 2012). Analysis of neuronal microanatomy has also generated the entire spectrum of possible results, as dendrites and spines have been found to decrease (von Bohlen und et al. 2006; Pannese 2011), remain stable (McNeill et al. 1990; Jacobs et al. 1997), or even increase with aging (Hinds and McNelly 1977; Buell and Coleman 1979). This spectrum partly reflects a true biological variability, indicative of a selective age-related vulnerability of different neuronal compartments, types of neurons, or specific brain regions (Wong et al. 1998; Lemaitre et al. 2012; Jagust 2013). However, it also reflects an inconsistency in experimental methodologies, including the range of ages examined, cutting and staining procedures, and heterogeneity among neuronal populations tested (Coleman and Flood 1987; Giannakopoulos et al. 2009). Thus, it is important to examine the effect of aging on identified and distinct neuronal subpopulations, and under identical experimental conditions.

A central yet presently unresolved issue in aging research concerns the distinction between normal/successful aging, consisting of a moderate decline in cognitive performance, and pathological aging, manifested as mild cognitive impairment or full-blown neurodegeneration and dementia. In particular, it has been proposed that the age-related decline in cognitive abilities may be an age-related escalation of early-life cognitive limitations, rather than an abruptly emerging neuropsychological process that occurs in old age (Elias et al. 2000; Small et al. 2000; Sarter and Bruno 2004; Amieva et al. 2005; Tyas et al. 2007). In this scenario, early abnormalities or incompletely matured neural systems would interact with age-related processes to explain the cognitive decline in later ages. However, this proposal remains controversial (Nilsson et al. 2009; Salthouse 2009) and, to our knowledge, has not been explored at the morphological/structural level. Hence, it is important to identify factors that may confer a predisposition to pathological aging and to examine how they interact with the process of aging per se.

One such factor is the integrity of the cholinergic system: Cholinergic basal forebrain neurons and their projections to the cortex show increased vulnerability to aging (Fischer et al. 1987; Altavista et al. 1990; Casu et al. 2002), and cognitive decline is associated with selective loss of neuronal nicotinic acetylcholine receptor (nAChR) function (Hellstrom-Lindahl and Court 2000; Schliebs and Arendt 2011). In this respect, animals with specific cholinergic deficits are important tools for understanding the neurobiology of successful aging. One such animal model is the β2<sup>−/−</sup> mouse, in which the gene encoding the β2 subunit of the nAChR is genetically deleted (Picciotto et al. 1995). Aged β2<sup>−/−</sup> mice have been proposed as a model of accelerated cognitive aging, based on structural alterations and spatial learning deficits only evident in old animals (Zoli et al. 1999; Picciotto and Zoli 2002). However, a systematic comparison of neuronal microanatomy in adult and aged animals has not been done to date.

In the present study, adult (4–6 months) and old (22–24 months) wild-type (WT) and β2<sup>−/−</sup> animals were used to examine the respective contributions of age and genotype on neuronal structure. We focus on layer V pyramidal cells because: (1) they constitute the main cortical output (DeFelice and Farinas 1992; Romand et al. 2011); (2) they are often reported to exhibit increased sensitivity to aging (Nakamura et al. 1985; Baskys et al. 1990; de Brabander et al. 1998; Turner et al. 2005); (3) they possess a high density of cholinergic terminals (Houser et al. 1985) and, in contrast to layer III cells, they exhibit strong presynaptic modulation by β2-containing...
nAChRs and are activated by nAChR stimulation (Poorthuis et al. 2013); hence they would be a sensitive readout for the lack of high-affinity nicotinic receptors. Furthermore, to examine the degree of age-related vulnerability across distinct cortical areas, we used yellow fluorescent protein (YFP-H) mice that express YFP in specific populations of thick-tufted layer V pyramidal neurons across the cortical mantle (Feng et al. 2000; Sugino et al. 2006). Mutants were crossed with YFP+ mice in order to have the same labeled populations in both genotypes and we examined cells in primary visual cortex (V1) and anterior cingulate cortex (ACC), 2 cortical regions that receive similar cholinergic inputs (Jacobowitz and Creed 1983; McKinney et al. 1983; Everitt and Robbins 1997; Laplante et al. 2005), but have a distinct cytoarchitecture and functional role (Elston et al. 2005). We ask whether neurons in old β2−/− mice exhibit greater structural deficits than age-matched controls, and whether deficits appear in old age or are already present earlier.

Indeed, we find that animals lacking the β2 subunit exhibit alterations in layer V YFP+ cells that are spatially and temporally regulated: in ACC, they are evident already in adult-creamed animals, whereas in V1 they appear in old age. Our data indicate that the chronic lack of β2 nAChRs is linked to severe alterations in ACC morphogenesis and may confer an increased vulnerability to cognitive aging. To our knowledge, this is the first study that examines the combined effects of aging and genetic predisposition on neuronal subpopulations with distinct areal identities and connectivity patterns but same layer identity and comparable intrinsic properties, thereby allowing an examination of their respective contributions to the aging process.

Materials and Methods

Animals

Brains from 21 adult (4–6 months) and old (22–24 months) C57Bl/6j WT and β2−/− mice selectively expressing YFP in layer V pyramidal cells were used in this study (WT adult n = 5; WT old n = 5; β2−/− adult n = 5; β2−/− old n = 6). Mice were bred in the facility of the Center for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens (BRFAA) from animals that were obtained from the University of California at Davis. The BRFAA facility is registered as a breeding and experimental facility according to the Presidential Decree of the Greek Democracy 160/91, which harmonizes the Greek national legislation with the European Council Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. C57Bl/6j mice selectively expressing YFP in specific neuronal populations including layer V pyramidal cells (YFP+ mice; H-line) were initially obtained from Jackson Labs (Feng et al. 2000; Lichtman and Sanes 2008) and bred with C57Bl6j animals lacking the β2 subunit (β2−/− mice, Fl-line) were initially obtained from Jackson Labs (Feng et al. 2000; Lichtman and Sanes 2008) and bred with C57Bl6j animals lacking the β2 subunit (β2−/− mice, Fl-line). Live animals were examined under a dissecting microscope. Argon laser (488 nm) was used for YFP excitation, and Z-series of images were captured by the Leica Confocal Software (LAS AF). The step size between consecutive optical sections was set at 2 μm. For the cell body volume measure, a different Z-series of images were taken with lower gain to avoid fluorescence saturation (due to the large volume of the cell body compared with its processes) and to ensure correct volume estimates (Supplementary Video 1).

We examined the morphology of neurons from V1 and ACC. Complete neurons were defined as YFP+ pyramidal cells whose soma, basal dendrites, apical dendrite with its branches, and apical tuft could be clearly distinguished under the fluorescence microscope. Argon laser (488 nm) was used for YFP excitation, and Z-series of images were captured by the Leica Confocal Software (LAS AF). The step size between consecutive optical sections was set at 2 μm. For the cell body volume measure, a different Z-series of images were taken with lower gain to avoid fluorescence saturation (due to the large volume of the cell body compared with its processes) and to ensure correct volume estimates (Supplementary Video 1).

Measurements were restricted to parameters that could be fully visualized and quantified within the section thickness in order to avoid errors associated with distal dendrites that would be absent due to the cutting procedure, thereby biasing our sample in favor of smaller dendritic trees. Hence, measurements were taken for the following 12 parameters, with respect to the 3 neuronal compartments, as illustrated in Supplementary Figure 1: (1) soma: volume (in 3 dimensions, for optimal estimation of absolute size; Supplementary Video 1), horizontal, and vertical Feret diameter (dotted white lines) which gives additional information on the shape of the cell body; (2) apical dendrite: length of dendrite from soma to the apical tuft bifurcation, number of apical branches, diameter (measured at a distance of 60 μm from the soma), vertical, and horizontal coverage of the apical tuft (dotted black lines); (3) basal dendrites: the number of primary dendrites, diameter of all primary dendrites (measurements taken at 2 μm from the soma), length of all primary dendrites (up to first bifurcation point), and number of all secondary dendrites. More distal dendritic segments could not be measured reliably in all cells and were excluded from analysis. Several additional parameters were calculated from these measurements: “Tuft area” was estimated by multiplying the vertical and the horizontal dimensions of the apical tuft to indicate the extent of its areal coverage; “cell body elongation” was calculated as the horizontal divided by the vertical diameter of the cell body. “Tortuosity” of apical and primary basal dendrites was calculated as the total length of the dendrite divided by its radial length (i.e., the shortest distance between the cell body and the beginning of tuft, or the first bifurcation of basal dendrites, respectively). Cortical thickness was measured as the distance between the pia and ventral border of layer 6 (Supplementary Fig. 1) in all sections from which cells were imaged. In total, we measured 127 pyramidal neurons (WT adult n = 26; WT old n = 26; β2−/− adult n = 36; β2−/− old n = 39) from 92 sections (WT adult n = 22; WT old n = 20; β2−/− adult n = 26; β2−/− old n = 24) for V1 and 134 pyramidal neurons (WT adult n = 27; WT old n = 33; β2−/− adult n = 41; β2−/− old n = 33) from 75 sections (WT adult n = 13; WT old n = 18; β2−/− adult n = 23; β2−/− old n = 21) for ACC.

To represent the morphological changes for individual parameters associated with either aging or the lack of β2 subunit, we calculated the mean % change in old and β2−/− animals relative to adult and WT animals, respectively (Supplementary Table 3). For example, a value of 10 in the third column (% decrease with aging, β2−/−, V1) indicates that old β2−/− mice exhibit a 10% reduction compared with adult values in this genotype. Significant changes are indicated by shaded areas and bold characters.

Furthermore, in order to represent the overall extent of age-dependent and genotyp-dependent changes, we devised 2 qualitative indices that are conceptually straightforward: (1) “Total Change Index,” or “TCI,” represents the sum total of the % statistically significant changes for all parameters in any given comparison and thus provides an indication of the extent of age- or genotype-related deficits; (2) “Mean Change Index,” or “MCI,” represents the average value of the % statistically significant changes in a given comparison and thus indicates the magnitude of change as a function of age or genotype. To ensure optimal representation of the differences, only statistically significant changes between ages and/or genotypes were included for the estimation of MCI and TCI (indicated as bold in Supplementary Table 3).

Blood Vessel Staining

Fresh-frozen 20-μm coronal sections were cut with a cryostat (Leica) and post-fixed in 4% PFA for 10 min at room temperature (RT). The sections were treated with 5% normal donkey serum (NDS) diluted with phosphate-buffered saline (PBS) for 1 h at RT and immunostained...
with rat anti-mouse PECAM-1 (BD) for 12 h at 4°C (dilution 1:200 in PBS supplemented with 2% NDS). The following day sections were reacted with Alexa™-594 goat anti-rat IgG (Molecular Probes, Inc., USA) (dilution 1:200 in PBS supplemented with 2% NDS). After reaction, the sections were washed 3 × 10 min in PBS, mounted in Vectashield (Vector Laboratories), and examined under fluorescence microscopy. Vessels in the ACC were analyzed semimanually in 2D using the image analysis software Image J (NIH, USA). Measurements were taken for area and total length of all blood vessels within the ACC, and values are expressed with respect to the ACC surface area.

**Statistical Analysis**

The morphological data are hierarchically structured and may therefore contain within subjects effects, as neural parameters are derived from a number of sections of a mouse. Therefore, we used mixed models containing 2 fixed effects (age and genotype) and 2 random effects (animal and brain section) to verify the independence of neuron samples. For each dependent measurement, we took into account the P-values of the best-fit model (smallest Akaike Information Criterion) among all possible models with different combinations of fixed and random effects. We found that the models with interaction and without random effects provided the best-fit models for our measurements, leading to P-values that are identical with the two-way analysis of variance (ANOVA) analyses using the same statistical hypothesis testing. For the comparison between ACC and V1, we performed t-tests for within each of the 4 groups, since our data were obtained from independent samples.

**Results**

**Sampling Issues and Measurements**

For this study, we used YFP-H mice to monitor the morphology of genetically labeled layer V cells as this option offers important advantages. First, the label is stable and uniquely inheritable (Feng et al. 2000; Zuo et al. 2005), hence ensuring we monitor the same neuronal population(s) in all animals; this reduces sampling variability and maximizes the chance to observe true age and/or genotype differences. Secondly, layer V YFP+ neurons across spatially distinct brain regions have different developmental origins and areal identities (Rakic 1988; Bishop et al. 2000; Joshi et al. 2008; Elsen et al. 2013) as well as distinct connectivity patterns, but similar electrophysiological properties and gene expression profiles (Sugino et al. 2006; Yu et al. 2008); they also receive similar cholinergic input from the basal forebrain (Jacobowitz and Creed 1983; McKinney et al. 1983; Everitt and Robbins 1997). For these reasons, we are presented with the unique opportunity of examining the consequences of age and genotype on their respective morphology, thereby providing important clues on the possible factors that confer heightened age-related vulnerability. Cells were imaged from the entire extent of V1 and ACC, as illustrated in Supplementary Figure 1A–D. Measurements were restricted to cellular parameters that could be fully and reliably visualized in all cells and therefore excluded distal parts of the dendritic tree. In total, we quantified 19 features, with respect to the 3 neuronal compartments as outlined in the Materials and Methods (Supplementary Fig. 1E–G, Supplementary Video 1, and Supplementary Tables 1 and 2).

**Increased Susceptibility to Aging in ACC Neurons in WT Animals**

Representative examples of the neuronal microanatomy are shown in Figure 1 and Supplementary Figure 2. Aged (22–24 months) WT animals exhibited morphological degeneration in both cortical areas and in most parameters, pertaining to both apical and basal neuronal compartments. However, aging effects were unevenly distributed between the 2 regions: overall, age-related changes in V1 were relatively modest, ranging between 6% and 33%, with an MCI of 16 (Supplementary Table 3). These included reductions in: (1) the length of primary basal dendrites (reduction was in the order of 14–24%, and applied to all measures: average, maximum, and total length; Fig. 2A,B; Supplementary Table 3); (2) diameter of primary basal dendrites (10–12%, for average and maximum diameter; Fig. 2C,D); (3) number of primary and secondary basal dendrites (6–9%; Supplementary Table 3); (4) diameter of apical dendrite (16%; Fig. 2G); (5) number of apical branches (33%; Fig. 2H), and (6) horizontal extent of the apical tuft (20%; Supplementary Table 3). There was no effect of aging on cortical thickness (Supplementary Table 3).

ACC neurons showed more extensive age-related reductions, as indicated by the larger values of both total and mean change indices (TCI = 225 vs. 160, and MCI = 28.12 vs. 16.0; Supplementary Table 3). This regional selectivity was due to a comparatively more severe age effect on the apical tuft and length of primary basal dendrites of ACC neurons (Supplementary Table 3). Specifically, parameters affected by aging included: (1) the length of primary basal dendrites (average, maximum, and total measures: 16%, 33%, and 36%, respectively; Fig. 2J,K, Supplementary Table 3); (2) maximum diameter of primary basal dendrites (16%; Fig. 2D); (3) apical tuft area (>50%; Fig. 2R), with significant reductions in both horizontal and vertical dimensions (Supplementary Table 3); and (4) number of apical branches (14%; Fig. 2Q). As in V1, we found no effect of age on cortical thickness (Supplementary Table 3). These data revealed an increased susceptibility to aging in layer V YFP+ cells located in the ACC, a region associated with higher cognitive functions, and prompted us to examine the corresponding parameters in the same neuronal populations in mice lacking high-affinity nicotinic receptors, a pathway that has previously been related to accelerated cognitive aging.

**The Absence of the β2 Subunit Is Associated with a Premature Aging Phenotype Selectively Expressed in ACC Pyramidal Cells**

We first examined neuronal microanatomy in adult (4–6 months) WT and β2−/− animals and documented genotype differences that were strongly region-specific. In V1, genotype effects were minimal and pertained to only 2 parameters: (1) diameter of primary basal dendrites, which showed a moderate (9–11%) reduction in average and maximum measures (Fig. 2C,D) and (2) number of apical branches, which was reduced by 26% (Fig. 2H). In contrast, genotype effects in the ACC were quite substantial and involved most aspects of neuronal structure, including (1) a 24% and 31% reduction in the average and maximum diameter of primary basal dendrites (Figs 1 and 2L, M); (2) a shortening of primary basal dendrites (12–37%, Fig. 2K); (3) a 34% reduction in soma volume (Figs 1M and 2V); (4) a large restriction in the tuft area (53.5%, Fig. 2R) mainly due to a shortening in the horizontal extent of the tuft (Supplementary Table 3); and (5) a 36% reduction in apical diameter (Fig. 2P and Supplementary Fig. 2).

This regionally specific effect of β2 deletion in adult animals is aptly reflected in the mean and total indices for genotype-dependent morphological changes (Supplementary Table 3),
whereas MCI and TCI values for V1 were 15.3 and 46, the corresponding values for ACC cells were 30.5 (MCI) and 305.5 (TCI). It may well be significant that the neuronal parameters that exhibit the largest age-dependent decrease in WT animals were the ones, where mutants showed an “aged” phenotype already in adulthood (e.g., apical tuft and length of basal dendrites in the ACC; number of apical branches in V1, see Fig. 2H,K,R).

These results indicate that the lack of the β2 subunit is associated with an appearance of premature aging in layer V pyramidal cells, which is preferentially expressed in the ACC. In morphological terms, ACC neurons already look “old” at 4–6 months, whereas V1 cells are minimally affected. So, we next examined the morphology of these neurons in aged animals, in order to evaluate the temporal progression of the effect of β2 deletion.

The Absence of the β2 Subunit Is Associated with Enhanced Age-Related Morphological Deficits in V1 Neurons

The regionally specific pattern of age-related deficits observed in WT animals was reversed in animals lacking β2-nAChRs, where V1 cells were more severely affected by age than ACC cells (Supplementary Table 3). In particular, V1 neurons in mutant mice exhibited more pronounced age-related changes than in the WT group, both in the number of parameters affected and in the magnitude of changes. Specifically, all 10 measurements that decreased with aging in WT mice were also reduced in β2−/− animals, in many cases to a greater extent.

Figure 1. Single optical sections of soma and primary basal dendrites of layer V YFP+ pyramidal neurons in V1 (A–H) and ACC (I–P) from the 4 experimental groups (WT adult, WT old, β2−/− adult, and β2−/− old). Scale bars: 7 μm (left column) and 2 μm (right column) in V1 and ACC.
Cortical Pyramidal Morphological Changes in $\beta_2^{-/-}$ Mice with Aging

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V1 Basal dendrites

Figure 2. Comparison of morphological parameters (indicated at the middle) in V1 (A–I) and ACC (J–R) in the 4 experimental groups (x axis). Bars indicate mean values and SEMs (V1: WT adult neurons, n = 26; WT old, n = 26; $\beta_2^{-/-}$ adult, n = 36; $\beta_2^{-/-}$ old, n = 39; ACC: WT adult neurons, n = 27; WT old, n = 33; $\beta_2^{-/-}$ adult, n = 41; $\beta_2^{-/-}$ old, n = 33). Symbols represent significant age effect in the ACC of $\beta_2^{-/-}$ mice, there was a pronounced genotype effect in adult animals (Supplementary Table 3, asterisks), suggestive of a "ceiling effect" on morphological degeneration established by the mutation already in adulthood.

Taken together, our data indicate that, compared with V1, ACC pyramidal neurons in layer V exhibit greater vulnerability both to the lack of high-affinity nicotinic receptors and to the process of aging. In $\beta_2^{-/-}$ mice, adult ACC neurons already manifest an aged phenotype, which only mildly deteriorates in the following 16–20 months. The same parameters affected by the mutation are also the ones most prominently affected by aging in normal animals, suggesting possible common underlying mechanisms. In contrast, V1 cells are less affected by aging in WT animals, and the impact of the mutation is only apparent in aged individuals.

The Absence of $\beta_2$ Subunit Does not Affect Cerebral Vasculature in ACC

We also assessed the specificity of these findings by examining whether the genotype effects on microanatomy may have been secondary to an impact of the mutation on cortical vasculature, as blood vessels in the cortex are known to receive cholinergic innervation (Kalaria et al. 1994; Vaucher and Hamel 1995) and vascular processes are known to contribute to the decline in cognitive status (Wang et al. 2012; Kling et al. 2013). For this, we examined cortical vasculature in the ACC region where the impact of $\beta_2$ deletion on morphology was most pronounced. Although both size and diameter of cortical vessels were affected by age equally in WT and $\beta_2^{-/-}$ mice, there was no effect of genotype on either parameter (Supplementary Fig. 3).

Region-Specific Differences in Neuronal Microanatomy in WT and $\beta_2^{-/-}$ Animals

A direct comparison between YFP$^+$ cells in the 2 regions (Supplementary Tables 1 and 2) revealed significant structural differences. The most obvious discriminating features were cell body volume, apical dendrite and tuft. In contrast, elements of the basal compartment were highly similar, with a small difference in the maximum diameter of the primary basal dendrites. In Table 1, measurements for each parameter are normalized to the values obtained in V1 neurons, in order to facilitate comparison among features with disparate absolute sizes. Adult WT cells in the ACC had substantially larger cell bodies (37%), tuft areas (137%), and thicker primary basal dendrites (18%), whereas their apical dendrite was shorter (27%) and contained fewer branches (56%), compared with V1.
significantly larger values in ACC neurons relative to V1. Bold/shaded values indicate statistically significant differences.

| Table 1 | Comparison of morphological parameters between ACC and V1 neurons in the 4 experimental groups |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Apical Soma volume | WT adult | WT old | β2−/− adult | β2−/− old |
| Apical length | 27 | 25 | 31 | 24 |
| Apical diameter | −11 | −14 | 26 | 10 |
| # apical branches | 56 | 42 | 39 | 42 |
| Tuft area | −137 | −34 | −25 | −39 |
| Basal | # primary basal dendrites | 6 | 4 | 16 | 6 |
| Max length of primary basal dendrites | −9 | −4 | 22 | −8 |
| Total length of primary basal dendrites | −4 | 20 | 36 | 15 |
| # secondary basal dendrites | 9 | 5 | 17 | −2 |
| Average diameter of primary basal dendrites | −8 | −26 | 8 | 11 |
| Max diameter of primary basal dendrites | −18 | −10 | 11 | 10 |

Note: Numbers reflect mean ± SD differences in ACC neurons relative to V1 cells. Negative numbers indicate larger values in ACC neurons relative to V1. Bold/shaded values indicate statistically significant differences.

(Table 1; WT adult column). These differences (also illustrated graphically in Fig. 3) suggest that YFP+ cells in ACC and V1 are morphologically distinct types. It is noteworthy that, despite significant age-related changes in individual parameters in cells of both areas, region-specific morphological identity is not altered with aging (Table 1; WT old column).

Interestingly, the pattern of region-specific differences was partly reversed in β2−/− mutants, as all neuronal parameters were smaller in ACC, compared with V1, cells (positive numbers in Table 1; β2−/− adult column; Fig. 3C,G). This reversal in comparative microanatomy is largely a consequence of the asymmetrical impact of β2 deletion, which selectively affects ACC neurons in adult mice. In old mutant animals, the region-specific differences in microanatomy were severely reduced, as the majority of the morphological parameters did not differ between ACC and V1 (Table 1; β2−/− old column). While this partly reflects the delayed impact of β2 deletion on V1 cells, it also indicates that the combined effect of age and genotype is a pronounced “flattening” of regional differences in neuronal and dendritic morphology.

Collectively, these results indicate that layer V YFP+ cells in V1 and ACC have a distinct neuronal structure, which is differentially affected both by aging and by the chronic absence of high-affinity nicotinic receptors.

Discussion

In this work, we address the issues of selective age-related vulnerability and the distinction between normal and pathological aging. We compare the morphology of identified populations of layer V pyramidal cells from 2 distinct cortical regions and examine the consequences of the chronic absence of high-affinity nicotinic receptors in adult (4–6 months) and old (22–24 months) animals. We describe a region-specific and interactive effect of age and genotype on the neuronal structure of YFP+ cells in ACC and V1.

Region-Specific Effect of Aging in Layer V YFP+ Pyramidal Cells in WT Animals

The issue of selective age-related vulnerability of different neuronal components is still debated. While there is a consensus that some cells are more affected by aging than others, the mechanisms for this heightened sensitivity are not clear. The 2 neuronal populations examined in the present study (ACC and V1) share several defining characteristics including laminar location and pyramidal identity, similarities in intrinsic excitability and genome-wide expression profiles (Sugino et al. 2006; Yu et al. 2008), as well as common cholinergic input from the same area of the basal forebrain, the horizontal limb of the diagonal band of Broca (Jacobowitz and Creed 1983; McKinney et al. 1983; Everitt and Robbins 1997; Laplante et al. 2005). On the other hand, they also have important differences, including distinct developmental origin and areal identities along the anteroposterior axis (Rakic 1988; Bishop et al. 2000; Joshi et al. 2008), as well as diverse input-output connectivity and functional role (Elston et al. 2005). Hence, investigating how their respective morphology is affected with age, or particular mutations, could be valuable in the search for mechanisms that differentiate successful from pathological aging.

The more extensive age effects we observed in ACC pyramidal neurons are consistent with previous studies in humans documenting increased age-related vulnerability in prefrontal...
compared with sensory cortical areas (Waldemar 1995; Raz et al. 1997). However, other studies have either reached the opposite conclusion (Yates et al. 2008) or revealed similar effects of age (Raz et al. 2004). While this could partly reflect true variation among species, differences in parameters quantified as well as technical limitations particular to each study make it difficult to generalize. Instead, available data suggest that even within a given cortical region, neuronal populations in different layers are unevenly affected by aging, although no particular layer appears consistently more vulnerable than others (de Brabander et al. 1998; Wong et al. 1998; Grill and Riddle 2002; Majdi et al. 2007; Yates et al. 2008; Allard et al. 2012).

It should be noted that the age-related deficits reported here do not constitute the full effects of aging since, due to technical limitations, not all dendritic parameters were quantified. Hence, it is likely that either or both of the 2 subpopulations would exhibit additional effects in more distal dendritic segments or spine numbers (Wong et al. 2000; Allard et al. 2012; Lozada et al. 2012). Nevertheless, this does not alter the main conclusion that YFP+ layer V pyramidal cells in ACC and V1 are differently affected by aging. Combined with findings from previous studies, this suggests that the impact of age is determined at the cellular level and is not a consequence of any single feature such as layer or areal identity, or global gene expression profiles.

The mechanisms leading to targeted dendritic atrophy are not clear. It is noteworthy that the increased susceptibility to aging in ACC microanatomy documented in the present study is largely a consequence of a comparatively more severe age effect in the apical tuft (Fig. 1 and Supplementary Table 3). A study using the same YFP-H mouse line has shown that the apical tuft is particularly vulnerable to ischemia (Enright et al. 2007). Previous studies (Sonntag et al. 1997; del Zoppo 2012) and present results (Supplementary Fig. 3) indicate that cortical vasculature is reduced with aging. Hence, it is possible that the pronounced decrease in the size of the tuft is a reflection of reduced blood supply with aging. Why this should be especially prominent in ACC is presently unclear. Alternatively, the enhanced tuft vulnerability could reflect selective loss of presynaptic elements to this region as a result of aging. Earlier studies have shown that cholinergic innervation is substantially reduced with aging, and that this reduction is more pronounced in distal compared with proximal dendritic segments (Casu et al. 2002). Provided that the nonuniform loss of presynaptic elements also extends to the tuft region, this could lead to loss of postsynaptic dendritic elements in this area.

**Region-Specific Effect of β2 Deletion on Pyramidal Cells in Prefrontal Cortex**

In addition to having greater vulnerability to aging, YFP+ cells in ACC were also much more susceptible to the lack of high-affinity nicotinic receptors. This increased impact of β2 deletion in prefrontal areas is consistent with previous findings on (non-YFP) layer II/III pyramidal cells in β2−/− mice, in which the density of dendritic spines was reduced in prelimbic cortex but not in V1 (Ballesteros-Yanez et al. 2010).

The reasons underlying this regional sensitivity are presently unclear. One possibility is that ACC cells are more responsive to nicotinic inputs, which are severely reduced in these animals. However, this appears unlikely, since β2-containing nAChRs in adult rodents are homogeneously distributed over the entire cortex and binding sites for the β2-nAChRs do not reveal significant density gradients across cortical areas (Pauly et al. 1991; Hill et al. 1993; Marks et al. 1998). It could also be hypothesized that the heightened vulnerability of ACC neurons is secondary to region-specific effects in cortical vasculature (Enright et al. 2007). However, we found no genotype effect in ACC vasculature, indicating that the lack of β2 does not lead to compromised vessel integrity in this region. Yet, another possibility relates to differences in the developmental time course of β2 mRNA expression and high-affinity nicotinic binding, both of which appear in the rodent neocortex during the third week of gestation in a caudal-to-rostral gradient (Naeff et al. 1992; Zoli et al. 1999).

The region-specific effects of cholinergic inputs during development are also documented by experimental lesions in afferent cholinergic neurons that lead to reduced dendritic branching and spine densities selectively in medial cortical areas (Robertson et al. 1998). Although this seems the most parsimonious explanation presently, concrete evidence is still lacking.

The morphological deficits in ACC cells are likely to affect their function in important ways. For instance, the smaller cell body could reflect decreased levels of perisomatic inhibition (Soltesz et al. 1995), while the smaller tuft area would imply reduced sampling and integration of inputs from distant cortical areas and thalamic locations (Spruston 2008). In addition, the thinner dendritic diameter may be associated with less favorable propagation (London et al. 1999) and/or faster calcium kinetics in spines, leading to altered regulation of synaptic plasticity (Holthoff et al. 2002). While the combined effect of these structural alterations cannot be predicted, on the basis of our findings, one would expect that functions that depend on the integrity of prefrontal cortex and in particular ACC would be affected in adult β2−/− mice, whereas functions that depend on V1 would be largely spared. Indeed, a number of studies have shown deficits in attention, behavioral flexibility, and sociability in β2−/− mice (Granon et al. 2003; Maubourget et al. 2008; Bourgeois et al. 2012) that were “rescued” after re-expression of β2-containing receptors in the prefrontal cortex (Guillem et al. 2011). In contrast, receptive fields of V1 cells in adult β2−/− mice were normal and visual cortex-dependent tasks were unaltered (Wang et al. 2009). Hence, our data confirm and extend previous findings, and suggest that prefrontal cortex is particularly sensitive to the chronic absence of high-affinity nAChRs. The delayed impact of the mutation on V1 microanatomy would further predict that V1-dependent visual functions will be compromised in old animals, a hypothesis that remains to be tested.

**Age–Genotype Interactions on Pyramidal Cell Morphology: Premature Versus Accelerated Aging Phenotypes**

The experimental design of the present study allows us to examine the interaction of age and genotype (in this case, the deletion of β2-nAChRs) and to evaluate their separate and combined contributions on neuronal structure. The data revealed a mixed picture, which was strongly dependent on cortical location. In ACC, all parameters that were affected by the absence of β2-nAChRs manifested deficits already in adults. These were usually maintained, or—in few cases—enhanced further in aged animals. In contrast, the situation was...
practically reversed in V1, where cells in adult β2−/− mice were minimally affected, but marked structural deficits appeared in aged animals. Hence, our data indicate that, when investigating patterns and mechanisms of pathological aging, it could be useful to make a distinction between premature aging, where cells in adulthood already exhibit an aged phenotype; and accelerated aging, where cells in adult animals are still normal but deficits are acquired at an accelerated pace as animals grow old. In both cases, the outcome would be similar, with more severe deficits in aged mutant animals compared with age-matched controls, but the underlying mechanisms in the 2 scenarios would be different. Our data suggest that V1 cells in β2−/− animals conform to the scenario of accelerated aging, and that the major effect of the mutation in this region may be to “prime” the cortex so that the age-related changes are exacerbated. In contrast, mutant ACC cells exhibit a prematurely aged phenotype. This could reflect the impact of the mutation on either morphogenesis and/or maintenance of neuronal microanatomy, or on a premature onset of the aging process. Although the present data cannot distinguish between these 2 alternatives, the outcome of an aged appearance in ACC neurons is clear. Further studies would be needed to examine the underlying mechanisms.

The mechanisms responsible for such a distinction between different aging patterns have not been investigated. However, it is particularly noteworthy that the same dendritic features that manifested the more severe genotype effect in adult β2−/− animals (i.e., apical tuft, length of primary basal dendrites) were the ones that exhibited enhanced age effect in WT animals. In this respect, our data could indicate a possible neurobiological substrate for early abnormalities that would interact with aging processes to explain the age-related morphological deficits. Recently, 2 elegant studies have examined structural changes and cognitive performance in the same animals, and shown that changes in synaptic excitation–inhibition (E/I) ratio may underlie the degree of cognitive impairment with aging, indicating that an imbalance towards inhibitory tone is detrimental (Allard et al. 2012; Bories et al. 2013). In addition, the age-impaired animals had reduced numbers of cholinergic boutons that positively correlated with the ratio of E/I appositions on basal dendrites (Allard et al. 2012). Hence, interpreting our findings in the context of these studies we could propose the following hypothesis concerning the combined effect of age and β2-deletion: during normal aging, there is a reduction of total dendritic length, implying loss of postsynaptic elements. The degree of functional compensation (e.g., by increased presynaptic excitation, or other mechanisms) is likely to determine which animals will maintain high cognitive performance, and which will show cognitive decline (Wong et al. 2000; Bories et al. 2013). In β2−/− mice, presynaptic cholinergic modulation is constitutively severely reduced, thereby affecting pyramidal cell morphology and preventing the efficacy of compensatory changes. As a result cortical pyramidal cells manifest structural deficits, possibly leading to the observed cognitive impairments. For reasons that are presently unclear, these deficits appear earlier in ACC, and are delayed in V1. Hence, in this scenario, the impact of β2 deletion on aging would be to severely compromise the ability of cortical networks for functional compensation against age-related changes. While this hypothesis remains speculative at present, further studies combining behavioral assessment and cortical electrophysiology in the same animals should provide relevant insights to the mechanisms underlying functional compensation during aging.

Region-Specific Morphological Identity of Layer V Cells in WT Versus β2−/− Animals

A notable finding of our study is the distinct microanatomy of layer V YFP− cells in ACC and V1 (illustrated graphically in Fig. 3A,E), implying different synaptic integration properties and functional role for cells in the 2 cortical areas. Morphological differences between pyramidal cells of different brain regions have been reported previously in other types of neurons (Jacobs et al. 2001; Elston et al. 2005; Benavides-Piccione et al. 2006; Ballesteros-Yanez et al. 2010). However, while most previous studies have examined broader populations, defined largely on the basis of cortical layer and/or projection patterns (but see, Groh et al. 2010), here we have analyzed genetically labeled subpopulations with substantial similarities in global gene expression patterns and intrinsic excitability properties (Sugino et al. 2006; Yu et al. 2008). Hence, our results confirm and further extend previous evidence on the parallel between structural and functional specialization in the cortex, and suggest that neuronal morphology is strongly influenced by connectivity patterns and the particular function these cells perform.

These findings also pertain to the long-standing questions of (1) what constitutes a cell class and (2) the degree to which a single canonical circuit consisting of a set of canonical cell types can be recognized across cortical areas (Douglas and Martin 2004). Cell type identity has been defined based on several factors, both “intrinsic” to the cells (e.g., expression of chemical markers, membrane excitability and firing properties, gene expression profiles) and “extrinsic” (e.g., anatomical location and input/output connectivity). In addition, cells in different brain regions have distinct origins and developmental histories (Rakic 1988; Bishop et al. 2000; Joshi et al. 2008) that could endow them with distinct identities, even after morphogenetic gradients have ceased to be present. Our data, revealing considerable morphological heterogeneity among cells with common intrinsic features, highlight the need to reach a consensus as to the relative weight of the criteria upon which classification should be based, or indeed to revise the notion of “cell type” to reflect the full hierarchy of cellular and network properties (Nelson et al. 2006) that may also need to include the cell’s developmental history. In this respect, it would be interesting to characterize these YFP+ neurons at earlier stages to examine whether the region-specific morphology is acquired at a particular stage of development and to what extent it is controlled by input–output connectivity.

Our data demonstrating severe, temporally regulated structural alterations in pyramidal cells of β2−/− mice suggest that high-affinity nicotinic signaling contributes to the regionalization of the cerebral cortex and the acquisition of neuronal microanatomy in pyramidal cells. In addition, the pronounced “flattening” of regional differences in neuronal morphology in old mutant mice indicates that the β2 pathway continues to play a role in the maintenance of region-specific morphology across the life span. A previous study on layer II/III pyramidal cells has also reported a “uniformization” of the morphological regionalization of the cerebral cortex, caused by the absence of the β2 nAChRs, interpreting it as an alteration of the overall “associative” character of the cortex (Ballesteros-Yanez et al.
Our data are consistent with this notion and further suggest that the effect of the lack of β2-mediated signaling may be fairly widespread to different types of cells. In this respect, it would be interesting to investigate the effect of β2 deletion on the graded expression of transcription factors in cortical progenitors and to examine the possibility that β2 signaling affects regionalization of the cerebral cortex through thalamic and/or basal forebrain afferents (Lambe et al. 2003).

Concluding Remarks
We have shown that high-affinity nicotinic signaling plays a region-specific role both on morphogenesis and/or maintenance of identified layer V pyramidal neurons and on the process of aging per se, by promoting or enhancing the age-related decline in neuronal structure. Hence, the phenotype of YFP+ cells in aged β2−/− mice is the result of the interaction of aging with the absence of high-affinity nAChRs over a relatively prolonged period of life span. This, in turn, may contribute to structural degeneration especially of the circuits that participate in high-order functional connectivity of the cerebral cortex. Thus, we propose that β2−/− mice can serve as an appropriate animal model with which to study the factors that confer cell type-specific vulnerability to aging and provide a useful tool with which to examine the possible interventions that could restore successful cognitive aging.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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