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Reelin in the Years: decline in the number of reelin immunoreactive neurons in layer II of the entorhinal cortex in aged monkeys with memory impairment

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Abstract

The glycoprotein reelin has been implicated in both memory-related synaptic plasticity and Alzheimer's disease pathogenesis. Aged rats with memory impairment display decreased reelin expression in layer II of the entorhinal cortex (EC) relative to memory-intact subjects, and here we tested whether this effect extends to the primate brain. Seven young adult (8–10 years) and 14 aged (27–38 years) rhesus monkeys (*Macaca mulatta*) were examined, including 7 old animals classified as impaired based on their scores from a delayed nonmatching-to-sample recognition memory test. Histological sections spanning the rostrocaudal extent of the intermediate and caudal divisions of EC were processed by immunohistochemistry and the total number of reelin-positive neurons in layer II was estimated using design-based stereological techniques. The main finding was that the number of reelin expressing neurons in EC layer II is decreased selectively in aged monkeys with memory deficits relative to young adult and aged subjects with intact memory. The results add to evidence implicating EC-hippocampal integrity in neurocognitive aging, and they suggest that disrupted reelin signaling may be among the mechanisms that mediate the associated vulnerability of this circuitry in Alzheimer's disease.

Keywords

aging; memory; nonhuman primate; stereology

1. Introduction

The entorhinal cortex (EC), together with the hippocampus and anatomically related 'rhinal' cortical areas, comprise a medial temporal lobe system critical for normal memory

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(Buckmaster et al., 2004; Insausti et al., 1987; van Strien et al., 2009). The EC is a pivotal bidirectional gateway in this system, funneling convergent neocortical input into the hippocampus, and relaying the output of hippocampal processing to adjacent rhinal areas and many of the same neocortical targets from which it receives input (Witter, 1993). Among the distinctive features of the EC, stellate cells in layer II that originate the perforant path projection to the hippocampus are robustly immunoreactive for the extracellular matrix glycoprotein reelin (Martinez-Cerdeno and Clasca, 2002; Pesold et al., 1998; Ramos-Moreno et al., 2006). Long known to play an important role in neuronal migration and positioning during development, growing evidence indicates that reelin signaling remains critical for normal brain function throughout the lifespan (for review see Stranahan et al., 2013). In young subjects, reelin signaling through apolipoprotein E (APOE) receptors modulates long term potentiation (LTP) and other measures of synaptic plasticity (Forster et al., 2010; Herz and Chen, 2006; Rogers and Weeber, 2008). Linking these anatomical and physiological observations, recent evidence demonstrates that blocking reelin's interaction with its receptors, specifically in the entorhinal cortex, impairs spatial memory mediated by the hippocampus (Stranahan, Salas-Vega et al. 2011). These findings are consistent with the view that, in multiple regions of the adult brain, reelin expression and signaling regulate synaptic structure and function critical for normal learning and memory (Brosda et al., 2011; Rogers et al., 2011; Rogers et al., 2013; Stranahan et al., 2011b; Weeber et al., 2002).

Disrupted reelin signaling has been implicated in a variety of conditions in which cognition is prominently affected, including schizophrenia and Alzheimer's disease (AD) (Guidotti et al., 2000; Herring et al., 2012; Impagnatiello et al., 1998; Kramer et al., 2011; Seripa et al., 2008). The links to AD are illuminating and raise the possibility that the reelin-positive phenotype of layer II EC neurons might contribute to their documented vulnerability early in the course of the disorder. Changes in reelin expression localized to EC-hippocampal circuitry have also been observed in association with age-related cognitive decline, i.e., a frequent prodromal feature of disease. In rats, for example, where the effects of aging can be examined in the absence of frank neurodegeneration, aged subjects with impaired hippocampal memory display a substantial decline in EC layer II reelin expression relative to both young adults and aged rats with intact memory (Stranahan et al., 2011a). Here, taking advantage of an established model of neurocognitive aging in old world macaques, we asked whether the vulnerability of reelin-positive EC-hippocampal circuity in cognitive aging extends to the primate brain.

2. Material and Methods

2.1. Animals

Seven young adult (8–10 years at the time of perfusion, mean = 8.9 years; 5 females, 2 males) and 14 aged (27–38 years, mean = 31.7 years old; 7 females, 7 males) rhesus monkeys (*Macaca mulatta*) were used in this study. Subjects were maintained and behaviorally tested as previously described (Rapp and Amaral, 1991) at the California National Primate Research Center, Davis, California. All experiments were carried out in accordance with The National Research Council's Guide for the Care and Use of Laboratory

Animals and approved by the Animal Care and Use Committee at the University of California, Davis.

2.2. Behavioral testing

Delayed nonmatching-to-sample (DNMS) testing was conducted in a Wisconsin General Test Apparatus (WGTA) as previously described (Rapp and Amaral, 1991). Briefly, trials (20/day) consisted of a sample object presentation followed by a recognition test. In the sample phase, an object was presented for a response over the baited center well of the WGTA stimulus tray. After a predetermined delay, during which the test tray was hidden from view, the sample was presented together with a novel object that covered a food reward. During task acquisition, the retention interval between the sample presentation and recognition test was 10 sec, and animals were tested (nominally 5 days/wk) until they reached a criterion of 90% correct over 100 trials. Training subsequently continued with successively longer delays of 15, 30, 60, and 120 seconds (100 trials each, over 5 days). At the longest delay of 600 sec, a total of 50 trials were administered (5 trials/day).

2.3. Perfusion and tissue preparation

After completing an extensive neuropsychological test battery, animals were deeply anesthetized, transcardially perfused with aldehyde fixatives, and the brains prepared for histological processing (see Supplemental Material for details). Serial sections were cut on a freezing sliding microtome in the coronal plane at a nominal thickness of 40μm. Sections were stored individually in 1-in-10 series (400μm spacing) in cryoprotectant at −80°C until processing.

2.4. Immunohistochemistry

An evenly spaced series of histological sections from each brain was processed for the immunocytochemical visualization of reelin using a monoclonal antibody with validated cross-reactivity in primates, directed against an N-terminus epitope of mouse reelin, (Anti-Reelin, clone 142, Millipore, Corp., Billerica, MA; catalog # MAB5366). Standard immunocytochemical methods using 3, 3′ diaminobenzidine for the chromogenic reaction were employed (Supplemental Material).

2.5. Anatomical boundaries

A Nissl stained series from each subject was used to determine the rostrocaudal extent of the intermediate and caudal subdivisions of the entorhinal cortex (Amaral et al 1987). The rostral border of the intermediate EC was defined as the first section containing cell islands in layer II and a clearly defined, relatively cell free layer IV “lamina dessicans”. In the coronal plane, the caudal subdivision of entorhinal cortex borders the intermediate subdivision rostrally. The posterior border of the caudal division of EC was defined as the last section containing layer II neurons. Layer II cells were distinguished from the other laminae by their immunostaining intensity and relatively large size. The boundary delineating the lateral and medial EC divisions was placed at the medial lip of the rhinal sulcus, with the lateral EC comprising the medial bank of the rhinal sulcus (Figure 2A).

2.6. Stereological Analysis

All stereological counting was carried out by one experimenter (JML), blind to the age and cognitive status of subjects. The total number of reelin-immunoreactive (IR) layer II neurons in the intermediate and caudal divisions of the EC (Amaral et al., 1987) was estimated using the optical fractionator technique (West, 1993), implemented on a computer-aided system (StereoInvestigator, MBF Bioscience, Williston, VT). Sampling parameters were selected on the basis of pilot analyses and were sufficiently stringent to ensure that observed variability in the estimated total cell counts reflected biological variability between individuals and not insufficient sampling within subjects (see Supplemental Material).

2.7. Statistical Analysis

The number of trials to reach criterion in the DNMS task was analyzed by t-test, and performance across delays was tested by repeated measures ANOVA with subsequent group contrasts tested by t-tests. The number of reelin positive cells was analyzed by one-way ANOVA, t-tests and a repeated measures ANOVA to explore potential differences along the rostrocaudal EC axis. All statistical analyses were conducted using Prism 8 (GraphPad Software, San Diego, CA).

3. Results

3.1. Cognitive characterization

DNMS results for the young and aged monkeys tested here were similar to earlier reports (Moss et al., 1988; Presty et al., 1987; Rapp and Amaral, 1991; Shamy et al., 2006). Specifically, aged monkeys required many times the number of trials as young adults to acquire the non-matching rule of the task with a short, 10 sec delay (Figure 1A; $t_{(1,19)}$=5.17, p=0.001). With sufficient training, however, all subjects performed at or above the 90% correct learning criterion. The memory demands of the task were subsequently increased by imposing successively longer retention intervals of 15 sec to 10 min. Recognition accuracy declined in both groups across longer delays, as expected, (Figure 1B; repeated measures ANOVA, main effect of delay: $F_{(4,76)}$ = 45.4, p=0.001), and the aged group scored more poorly than young adults (main effect of age; $F_{(1,19)}$ = 11.2, p=0.003). Although the magnitude of age-related impairment increased across retention intervals up to 120 sec, the interaction between age and delay was not statistically significant ($F_{(4,76)}$ = 1.04, p=0.390), and recognition accuracy in the aged group was reliably worse than for young at all delays longer than 15 sec (30s, $t_{(19)}$=2.2, p=0.042; 60s, $t_{(19)}$=3.0, p=0.007; 120s, $t_{(19)}$=3.2, p=0.004; 600s, $t_{(19)}$=2.9, p=0.008). Consistent with earlier reports (Calhoun et al., 2004) there was considerable individual variability in memory among the aged monkeys, and half scored within the range of young subjects (Fig. 1C). There was no indication of a sex difference in recognition memory in either the young or aged groups (p-values > 0.05) although sample size for subgroup analysis was small.

3.2. Reelin-positive neurons in monkey EC layer II: qualitative observations

The organization and cytoarchitecture of reelin-immunoreactive neurons in layer II of the monkey EC is illustrated in Figure 2. The low power photomicrograph in Figure 2A

illustrates the medial and lateral divisions of the EC (dotted line), highlighting the reelin positive layer II (black arrows), and characteristic layer II cell islands (white arrow). The vast majority of layer II EC neurons were intensely immunoreactive, permitting the unambiguous identification of laminar and regional boundaries. The magnification used for quantification and a counting frame are shown in Figure 2B, where individual labeled cells are prominent. Figure 2, C-H illustrate that the overall staining characteristics of the histological material was qualitatively similar in young and aged brains. In an effort to ensure that the material in panels C-H is representative, the brains chosen for illustration were selected because they contained the median total number of reelin positive neurons in their respective groups. It is notable in this context that the density of reelin positive cells in the AI EC at high power (Fig. 2H) appears subjectively lower than in the Young (Fig. 2D) or AU monkey (Figure 2, C–F), in agreement with the quantitative stereological assessment. At least a few histological sections from each case included the rostral hippocampus proper, and in a non-systematic survey, we failed to detect instances of the reelin-positive plaques that reportedly develop during aging in laboratory rodents and marmosets (Knuesel et al., 2009).

3.3. Reelin-positive neuron number in monkey EC layer II: quantitative observations in relation to age and memory status

Independent of age, across all monkeys, the estimated total unilateral number of reelin-IR neurons in layer II of the intermediate and caudal EC averaged 205,590±8,881. This value is comparable to previous stereological estimates from non-selective Nissl stained material (Gazzaley et al., 1997), consistent with the conclusion that the vast majority of layer II neurons in the rhesus monkey EC express reelin. Although group comparisons based on chronological age revealed nearly 11% fewer reelin-IR neurons in the aged EC (mean ± SE; young = 221,714±12,608; aged = 197,528±11,417), this numerical difference was not statistically significant ($F_{(1,19)} = 1.7$, p=0.207). Within each age group, reelin-IR cell number estimates were also statistically equivalent in males and females (young $T_{(5)} = 0.62$, p = 0.56; aged $T_{(12)} = 1.16$, p = 0.27).

In order to evaluate EC reelin results in relation to individual differences in the cognitive outcome of aging, we adopted an approach from earlier work and operationally classified aged monkeys as either impaired or unimpaired, depending on whether they scored within the range for young subjects when the memory demands of DNMS task were increased (i.e., using mean percent correct scores across delays of 15 to 600 sec; Fig. 1C; Calhoun et al., 2004). As illustrated in Fig. 3A, this analysis revealed that the modest numerical difference observed between the young and aged groups was almost entirely attributable to decreased reelin-IR cell number in aged monkeys that exhibited impaired memory. Indeed aged-impaired subjects displayed over 17% fewer reelin-positive EC neurons than animals with normal memory (young and aged unimpaired combined; $T_{(19)} = 2.24$, p = 0.038). This effect was predominantly driven by aged females with recognition memory deficits (aged impaired females vs. aged impaired males, $T_{(5)} = 3.2$, p = 0.024), although studies in larger numbers of monkeys are needed to confirm potential sex-linked differences in EC reelin-positive cell number and cognitive aging.

Related research in memory-impaired aged rats has reported that decreased reelin expression predominantly targets the lateral entorhinal cortex, targeting a zone of the EC homologous to that affected early in the course of AD (Stranahan et al., 2011a). Here we found a qualitatively similar pattern in the monkey when the cell count data were considered separately for the most lateral portion of the EC, comprising the medial bank of the rhinal sulcus (Fig. 2A). Reelin-IR cell number in this area averaged 26% less than in memory-intact young and aged animals, and this effect was statistically significant ($T_{(19)} = 2.09$, $p=0.05$; Fig 3B). By comparison, parallel analysis of the data for the remaining, medial portion of the EC revealed a non-significant, trend-level numerical difference between groups of 17% ($T_{(19)} = 1.80$, $p=0.09$, Fig. 3C).

The topography of EC projections to the hippocampus differs in rats and monkeys, and the prominent medial/lateral organization observed in rodents instead reportedly follows a coarsely organized anterior/posterior gradient in the primate brain (Burwell and Agster, 2008; Witter, 2007). Accordingly, in a final analysis we examined potential regional selectivity in the effects of aging along the rostrocaudal axis, evaluating the estimated total number of reelin-IR cells at 11 contiguous anterior-posterior (A-P) levels of the EC (Fig. 3D). This analysis revealed significant main effects of cognitive status (young and aged unimpaired vs. aged impaired; $F_{(1,19)} = 4.49$, $p<0.05$) and A-P level ($F_{(10,190)} = 21.06$, $p=0.001$), reflecting the overall decrease in reelin expressing neurons in aged impaired monkeys, and the gradually increasing size of the EC at posterior levels, respectively. The magnitude of the difference between groups, however, was relatively constant along the rostrocaudal axis of the EC (group by A-P level interaction; $F_{(10,190)} = 1.07$, $p=0.39$).

4. Discussion

The EC is anatomically positioned to influence memory capacities known to be vulnerable to aging and AD (Buckmaster et al., 2004). Reelin expression is enriched in superficial layers of the entorhinal cortex, and previous studies have reported that the number of these neurons is decreased in aged mice (Knuesel et al., 2009) and rats (Stranahan et al., 2011a) with memory impairments. This effect appears particularly pronounced in lateral portions of the EC, a cortical area arguably homologous to the transentorhinal region affected early in the course of AD (Braak and Braak, 1991, 1995, 1997). The current experiments extend these findings to the nonhuman primate brain, demonstrating a significant decrease in reelin-IR cell number in layer II of the EC in aged monkeys with recognition memory deficits relative to age-matched and younger adults with normal memory. Previous studies using non-selective Nissl staining indicate that layer II neuron number is relatively preserved in the aged rhesus monkey EC (Gazzaley et al., 1997; Merrill et al., 2000). A lack of EC neuron death has also been noted in aged rats (Merrill et al., 2001; Rapp et al., 2002). Thus, together the findings suggest that aged monkeys with memory impairment display decreased EC reelin expression rather neuronal dropout, and that EC vulnerability is an evolutionarily conserved feature of cognitive aging. Whether or not the disproportionate loss we noted in aged female monkeys reflects a reliable sex difference in age-related circuit vulnerability merits consideration in human studies with much larger sample sizes.

Considerable debate centers on the idea that the neurobiological effects of aging and neurodegenerative disease are neuroanatomically specific and differentially target distinct brain regions or circuitry. A specific conceptualization of this sort is that compromised EC integrity signals an AD-related neurodegenerative process, whereas aging preferentially impacts the dentate gyrus of hippocampus (Small et al., 2011). Studies challenging this view have demonstrated by high resolution diffusion tensor imaging that the perforant path projection from the EC to the hippocampus is degraded in healthy, dementia-free older humans (Yassa et al., 2011) and that this disruption is coupled with deficits in memory processes dependent on the hippocampus (Yassa et al., 2011). Functional MRI results additionally point to regional specificity, demonstrating hypoactivity in anterolateral portions of the EC (alEC) in non-demented older adults relative to young controls, coupled with impairment in pattern separation abilities mediated by the hippocampal projection targets of the EC (Reagh et al., 2018). A similar association with cognitive status has also been reported for alEC volume in older adults (Olsen et al., 2017). Although older participants in these studies were cognitively healthy, defining the potential contribution of preclinical neuropathological processes to neuroimaging observations is an endemic challenge in human research. Wild-type rats fail to spontaneously develop AD, and the decreased EC reelin expression seen in aged subjects with memory impairment (Stranahan et al., 2011a) therefore counts against the possibility that this effect is a consequence of disease. Our findings extend these observations to the primate brain and suggest the conclusion that EC vulnerability may be an important element of the neurobiological condition that renders aging a major risk for AD.

Other experiments are needed to identify the specific mechanisms that link decreased reelin expression to regional EC vulnerability and cognitive outcomes in aging. Nonetheless, there is substantial evidence that reelin signaling powerfully modulates synaptic plasticity critical for memory mediated by the hippocampus. Reelin modulates NMDA receptor activity via phosphorylation of intracellular tyrosine residues of the NR2 subunit of the NMDA receptor (Chen et al., 2005), enhancing glutamate-stimulated calcium influx necessary for induction and maintenance of LTP (Herz and Chen, 2006; Malenka, 2003). Other findings document that LTP is enhanced after reelin administration and impaired in genetically modified mice lacking reelin receptors (Weeber et al., 2002). Plasticity-related structural connectivity also appears sensitive to reelin. For example, hippocampal and neocortical dendritic spine density are decreased in reelin deficient mice (Pappas et al., 2001; Qiu et al., 2006) whereas relative spine density is increased after reelin supplementation (Niu et al., 2004; Niu et al., 2008; Rogers et al., 2012).

As might be predicted on the basis of the available anatomical and physiological data, considerable behavioral evidence indicates that reelin potently regulates learning and memory (Brosda et al., 2011; Stranahan et al., 2011b; Weeber et al., 2002). In one study, for example, interference with reelin receptor binding in the lateral EC profoundly disrupted hippocampal memory in rats tested in the water maze (Stranahan et al., 2011b). In addition to demonstrating that suppressed reelin signaling can negatively impact memory, these findings directly implicate the involvement of lateral entorhinal cortical circuitry known to be vulnerable early in the course AD, and that features decreased reelin expression in both

cognitively impaired aged rats (Stranahan et al., 2011a) and, as we demonstrate here, aged monkeys with memory deficits.

Memory in the reelin-deficient reeler mouse is improved after intracerebroventricular injection of recombinant reelin, together with positive effects on dendritic spine density and synaptic plasticity (Rogers et al., 2011; Rogers et al., 2013). These findings point to reelin as a potential target for therapeutic intervention. In the context of AD-related pathogenesis, Pujadas et al. (2014) have demonstrated that reelin directly interacts with amyloid-ß fibrils in vitro, whereas reelin overexpression in AD mice slows plaque formation and rescues recognition memory. The possibility that increasing reelin might blunt the effects of age-related precursors of AD vulnerability, including the disrupted excitatory/inhibitory balance observed in aged rats and monkeys with memory impairment (Haberman et al., 2017b; Thome et al., 2016; Wilson et al., 2006), merits testing. Initial results are encouraging, demonstrating that low-dose levetiracetam administration – i.e., a treatment that reduces neuronal hyperactivity in the aged hippocampus and improves memory – rescues reelin expression in the lateral EC in aged rats (Haberman et al., 2017a). The findings reported here support the translational potential of this approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Long-Reelin Verification statements

The data contained in the manuscript being submitted has not been previously published, has not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging. All required verification information is included in the manuscript or cover letter.

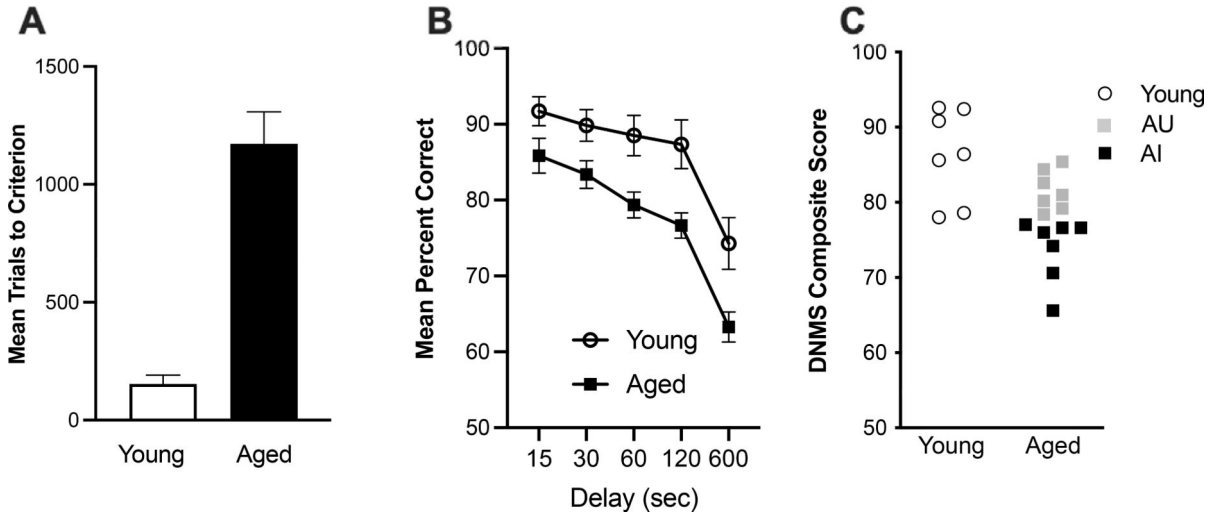

Figure 1.

Performance of young and aged monkeys on the delayed nonmatching to sample task (DNMS). A) Mean number of trials (S.E.M.) to reach a 90% correct learning criterion at a 10-second delay. B) Average percent correct of the young and aged groups across increasing delays. C) Mean percent correct for each subject across delays of 15 to 600 sec. Using this measure, aged monkeys were classified as unimpaired (AU) or impaired (AI) based on their performance relative to young (YG) monkeys.

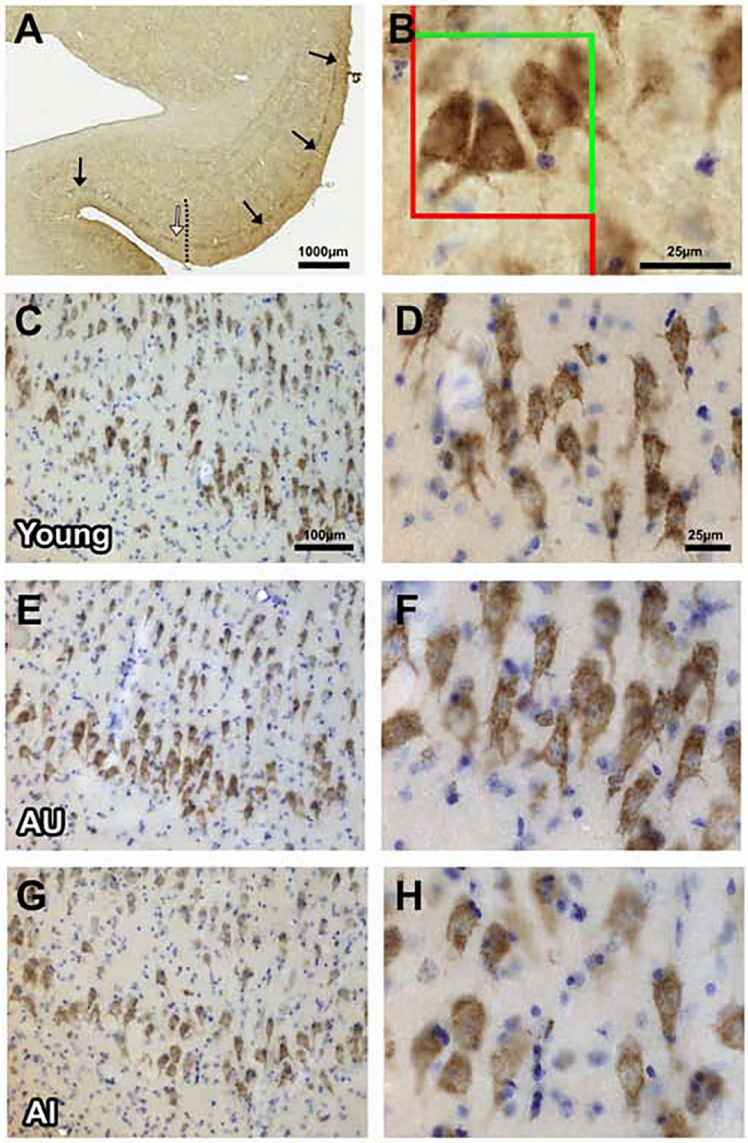

Figure 2.

Reelin immunoreactive neurons in the monkey entorhinal cortex. A) Black arrows denote EC layer II heavily populated with reelin immunopositive neurons. White arrow identifies a characteristic EC layer II cell island. Dotted line comprises the border used for the medial and lateral EC. B) High power photomicrograph of reelin immunoreactive neurons in EC layer II showing an example of an unbiased counting frame that was superimposed on live digital images for quantification. C-H) Photomicrographs of sections from Young (C&D), AU (E&F) and AI (G&H) brains showing general topographical organization, immunoreactivity and morphological characteristics of reelin positive neurons.

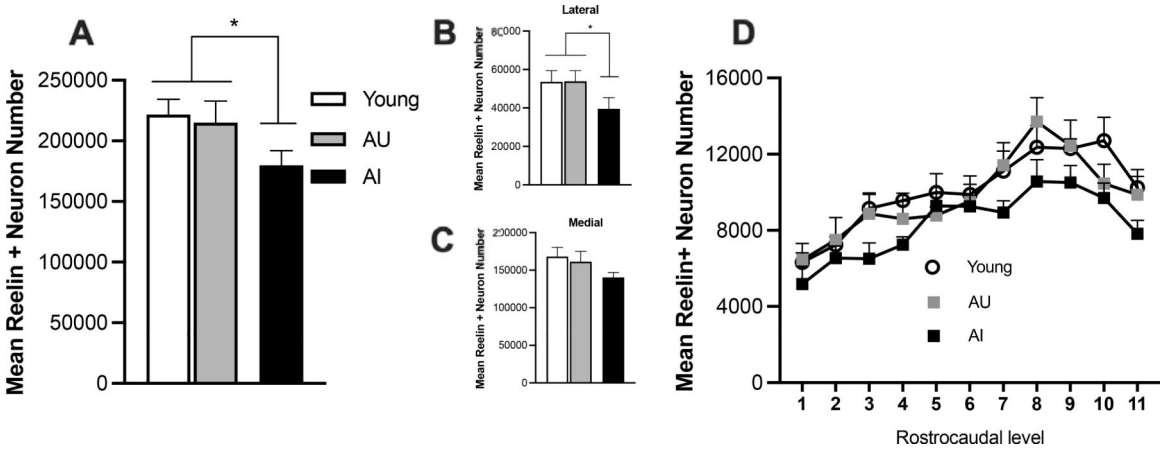

Figure 3.

Reelin immunoreactive neuron number in the monkey entorhinal cortex. A) Mean estimated total number of reelin positive cells in aged cognitively impaired monkeys relative to memory-intact monkeys (young + aged unimpaired monkeys). B) and C) Reelin positive neuron number in lateral (B) and medial (C) components of EC layer II. D) Reelin number along the rostrocaudal extent of EC layer II (bin 1 is the most rostral).