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Research report

# Decreased proportion of GABA neurons accompanies age-related degradation of neuronal function in cat striate cortex

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**Abstract**

Electrophysiological studies indicate that a decline of GABAergic inhibition in the visual cortex may underlie age-related degradation of visual function [A.G. Leventhal, Y. Wang, M. Pu, Y. Zhou, Y. Ma, GABA and its agonists improved visual cortical function in senescent monkeys, Science 300 (2003) 812–815; M.T. Schmolesky, Y. Wang, M. Pu, A.G. Leventhal, Degradation of stimulus selectivity of visual cortical cells in senescent rhesus monkeys, Nat. Neurosci. 3 (2000) 384–390]. To date, there is little direct evidence to support this hypothesis. Using Nissl staining and immunohistochemical techniques, we quantitatively compared the density of total neurons (Nissl-stained neurons) and GABA-immunoreactive neurons as well as the proportion of GABA-immunoreactive neurons to total neurons in the primary visual cortex between 4 young adult (1–3 year old) cats and 4 old (12 year old) cats, which had been previously examined in a single-unit recording study [T. Hua, X. Li, L. He, Y. Zhou, Y. Wang, A.G. Leventhal, Functional degradation of visual cortical cells in old cats, Neurobiol. Aging 27 (2006) 155–162]. In that study, we found the function of $V_1$ (area 17) neurons in the old cats was significantly degraded relative to young adult cats. Our present results indicate that the density of total neurons in each cortical layer of $V_1$ exhibit no significant difference in the two age groups of cats. However, the density of GABA-immunoreactive neurons in old cats is significantly lower than in young adults. Further, the ratio of GABA-immunoreactive neurons to total neurons in each layer of $V_1$ in old cats is also significantly decreased when compared to young adult cats. These results provide direct morphological evidence of decreased GABAergic inhibition in the striate visual cortex of old animals, which accompany the functional degradation of visual cortical neurons.


*Keywords:* Morphology; GABA-immunoreactive neurons; Primary visual cortex; GABAergic inhibition; Young adult cats; Old cats

## 1. Introduction

Human visual function degrades with age. As shown in previous psychological experiments, aged humans exhibit decreased visual acuity, binocular summation, contrast sensitivity and wavelength sensitivity [11,35,28,29,9,17,26,33,15,49] as well as poor or slowed performance at tasks requiring orientation discrimination and/or motion direction detection [51,2,27,44,45]. Most of the age-dependent visual function deterioration cannot be interpreted by changes in the eye [46,43,30] and subcortical visual components [41]. Therefore, it is reasonable to assume that a degradation of cortical function in old animals may contribute to those visual capacity declines.

Actually, age-related changes in the function of visual cortical cells have been observed in several species [47,23,36,52,14,48]. However, the mechanism underlying these changes is not yet clear. Mendelson and Wells [23] observed a temporal process-

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ing decline in the visual cortex of aged rats. They argued that this could be related to cell loss or morphological changes of neurons in the visual cortex. Nevertheless, these general morphological changes cannot appropriately interpret various declines of visual function that accompany old age. Schmolesky et al. [36] suggested that aging results in a decrease in GABAergic inhibition. This could account for the decreased stimulus selectivity of visual cortical neurons they observed in senescent macaque monkeys. Leventhal et al. [19] showed that both GABA and agonists of GABAa receptors significantly improved the function of $V_1$ neurons in old monkeys, while antagonists of GABAa receptors exerted stronger effects in young than in old monkey cortex. Although these results strongly suggest that a weakness of intracortical GABAergic inhibition may underlie visual cortical function degradation during aging, no direct evidence is now available to validate the conclusion.

In a previous study, we comparatively examined the stimulus selectivity and responsiveness of $V_1$ neurons in 4 old (12 years old) and 4 young adult (1–3 years old) cats using extracellular single-unit recording techniques. $V_1$ neurons of old cats exhibited degraded functions as indicated by a lower signal-to-noise ratio, higher spontaneous activity, less selectivity to stimulus orientations and motion directions than do neurons of young adults [14]. Using the same subjects in current study, we compared the density of total neurons and GABA-immunoreactive neurons as well as the ratio of GABA-immunoreactive neurons to total neurons in the primary visual cortex ($V_1$) of old cats with that of young adults, attempting to evaluate whether intracortical GABAergic inhibition in the old brain is reduced or not.

## 2. Materials and methods

### 2.1. Subjects

The data were acquired from 4 young adult (1–3 years old, male) cats and 4 old (12 years old, male) cats. Each subject was a healthy domestic cat with the history of age and healthcare filed by veterinarians in the Animal hospital and was examined ophthalmoscopically before the experiment to confirm that no optical or retinal problem that would impair their visual function. All animals were treated strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.2. Tissue preparation

All the subjects in this experiment had been physiologically studied previously [14]. In that study, single-unit recording result showed a significant functional degradation of visual cortical neurons in the old cats relative to the young adult cats. After single-unit recording, cats were deeply anesthetized with ketamine HCl (80 mg/kg) and xylazine (6 mg/kg) and then perfused through the heart with 1000 ml of saline solution, followed by 500 ml of fixative solution containing 1% paraformaldehyde, 2% glutaraldehyde and 0.2% picric acid dissolved in 0.1 M phosphate buffer solution (PBS, pH 7.2–7.4). Brains were removed from the skull. Blocks of tissue containing V1 were dissected out, post-fixed overnight in cold fixative solution (same as perfusion solution) and then embedded with wax. Serial coronal sections (8 μm thick) were cut perpendicularly to the pial surface of the lateral gyrus in $V_1$ and then dried onto APES-coated glass slides for Nissl staining and immunohistochemical reaction. From each animal, 20 sections (10 sections in each hemisphere at an interval of about 400 μm apart) were sampled for Nissl staining. Two adjacent sections were used for immunohistochemical labeling of GABA neurons. Another adjacent section was treated as an immunoreaction control sample.

### 2.3. Immunohistochemistry and staining procedure

Antiserum to GABA (rabbit polyclonal; 1:1500; Lab Visio Corporation), which had been well characterized previously [32,24,5,12], was applied to visualize GABA-immunoreactive neurons in the visual cortex. After deparaffinizing and rehydrating, sections were first rinsed in 0.1 M PBS (pH 7.4) for 10 min, then incubated with 0.3% $H_2O_2$ in PBS for 15 min to quench endogenous peroxidase activity. Following washing in PBS (3 × 10 min), the sections were incubated with 5% normal goat serum in PBS for 10 min at room temperature to block non-specific reactions. Subsequently, the sections were incubated with primary antibody against GABA for 24 h at 4 °C, washed in PBS (3 × 10 min) and then incubated with biotinylated goat anti-rabbit IgG for 10 min at room temperature. After further rinsing in PBS (3 × 10 min), the sections were incubated at room temperature with an ABC solution (including 10 min of treatment with streptavidin peroxidase, 10 min of rinsing in PBS and then 10 min of incubation with a mixture of DAB chromogen and DAB substrate). After rinsing in PBS, dehydrating in gradient alcohol and clearing in xylene, the sections were finally coverslipped with permont. Control sections were stained simultaneously following the same procedure as described above with the exception that the primary antibody was omitted. In order to optimally characterize the GABAergic neurons in $V_1$ of both old and young adult cats, we varied the concentration of anti-GABA serum for GABA neurons labeling in some sections (excluded for statistical analysis). At a low dilution concentration (1:2500 or 1:3000) of anti-GABA serum, the GABA-immunoreactivity in cells of young cats was nearly as strong as that at the higher dilution concentration of 1:1000, 1:1500 and 1:2000, while much less GABA-positive neurons could be detectable in the $V_1$ of old cats. Increasing the dilution concentration had little effect on displaying GABAergic neurons but produced enhancing background staining. We used the optimal dilution (1:1500) of anti-GABA serum for GABA cells visualization and quantitative study, which either yielded an appropriate background or stained GABA neurons equally well in both age groups of cats. Nissl staining (0.5% thionine 37 °C, 40 min) was used for identification of cortical layers and statistical evaluation of the density of total cortical neurons (thionine-stained neurons) in each layer of the primary visual cortex.

### 2.4. Measurement and statistical analysis

Under an Olympus BX51 microscope, statistical studies were done by persons who were blind to information regarding which age group and cat they were counting from. Quantitative measurement was performed at the medial bank of the lateral gyrus ($V_1$), a region where the cortical surface runs approximately parallel to the white matter. In each Nissl-stained section, the vertical depth from the pial surface to the upper border of each cortical layer (layer I, II–III, IV, V, VI) (Fig. 1) was measured randomly for 10 visual fields by using an optical calibrator at a low magnification (×4 or ×10 objective lens with ×10 eyepieces). The mean depth and thickness of each cortical layer was attained and used for laminar localization in cell counting. At high magnification (×40 objective lens with ×10 eyepieces), the number of total cortical neurons and GABA-immunoreactive neurons within each cortical layer (layer I, II–III, IV, V and VI) were randomly counted for 20 visual fields by using a grid of counting frame (50 μm × 50 μm) that was positioned at a low magnification. For an unbiased counting, neurons whose nuclear profile intersected the right and upper border of the frame were included for analysis while those crossing the left and lower border were excluded. The density of neurons (cells/mm$^2$) was calculated in two dimensions. The mean density of total neurons in each cortical layer was obtained in each Nissl-stained section. To increase the sample size of GABA neurons, the mean density of GABA-immunoreactive neurons was calculated every two sections that are adjacent to the Nissl-stained section. The ratio of GABA neurons to total neurons in different cortical layers were acquired in each adjoining sections group and defined as the mean density of GABA neurons divided by the mean density of Nissl-stained neurons (total neurons).

The criteria for acceptance as a neuron in Nissl staining sections were a clear differentiation from background staining of a soma and the presence of a nucleus. A cell with a nucleus and a brown or dark brown-labeled soma that clearly distinguished from yellow background staining was designated as a GABA-positive neuron. All data were expressed as mean ± standard deviation. The significance of the differences between cortical layers, individuals and age groups was eval-

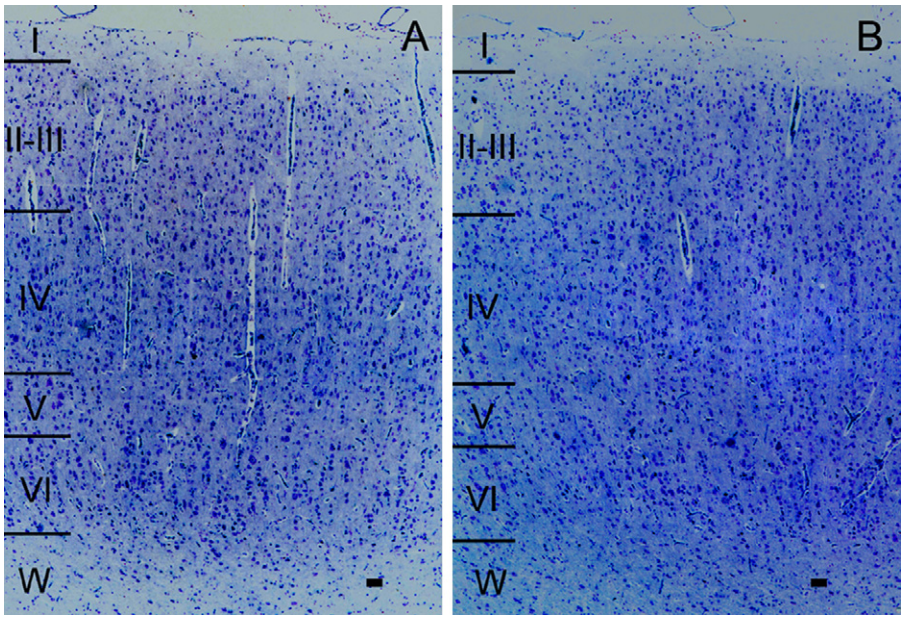


Fig. 1. Nissl staining shows laminar identification in $V_1$ of young adult (A) and old (B) cat. I, II–III, IV, V and VI indicates cortical layer 1, 2–3, 4, 5 and 6, respectively. W represents white matter. Scale bar = 50 μm.

Table 1
Statistical evaluation showing the mean density of total neurons, the mean density of GABA-immunoreactive neurons and the mean ratio of GABA neurons to total neurons in each cortical layer of $V_1$ for young adult and old cats

| Subject | Layer | | | | |
|---|---|---|---|---|---|
| | I | II–III | IV | V | VI |
| Density of total neurons (cells/mm$^2$) | | | | | |
| $YC_1$ | 302 ± 50.3 | 1229 ± 71.9 | 1250 ± 96.0 | 1222 ± 82.8 | 1226 ± 135 |
| $YC_2$ | 299 ± 41.9 | 1249 ± 38.3 | 1264 ± 40.3 | 1210 ± 85.4 | 1258 ± 116 |
| $YC_3$ | 304 ± 57.5 | 1231 ± 103 | 1231 ± 113 | 1246 ± 119 | 1225 ± 153 |
| $YC_4$ | 297 ± 50.6 | 1250 ± 129 | 1245 ± 96.0 | 1221 ± 105 | 1251 ± 105 |
| $OC_1$ | 297 ± 36.6 | 1231 ± 82.7 | 1258 ± 108 | 1222 ± 192 | 1213 ± 232 |
| $OC_2$ | 297 ± 30.1 | 1243 ± 25.2 | 1246 ± 83.2 | 1216 ± 144 | 1222 ± 175 |
| $OC_3$ | 296 ± 36.3 | 1243 ± 110 | 1261 ± 138 | 1232 ± 152 | 1270 ± 199 |
| $OC_4$ | 300 ± 33.3 | 1244 ± 54.7 | 1255 ± 123 | 1209 ± 133 | 1219 ± 184 |
| Density of GABA neurons (cells/mm$^2$) | | | | | |
| $YC_1$ | 118 ± 45.0 | 246 ± 88.5 | 303 ± 119 | 257 ± 109 | 210 ± 83 |
| $YC_2$ | 130 ± 51.3 | 285 ± 44.4 | 249 ± 70.8 | 231 ± 75.3 | 242 ± 77.4 |
| $YC_3$ | 147 ± 41.4 | 296 ± 40.2 | 291 ± 88.4 | 280 ± 83.9 | 266 ± 71.3 |
| $YC_4$ | 141 ± 21.2 | 272 ± 52.4 | 272 ± 79.7 | 249 ± 46.4 | 255 ± 62.4 |
| $OC_1$ | 71.7 ± 33.6 | 105 ± 28.3 | 139 ± 44.4 | 96.9 ± 24.1 | 117 ± 37.5 |
| $OC_2$ | 74.4 ± 34.3 | 129 ± 30.5 | 124 ± 29.2 | 103 ± 32.1 | 102 ± 44.5 |
| $OC_3$ | 78.2 ± 30.7 | 125 ± 33.9 | 121 ± 31.1 | 103 ± 41.4 | 132 ± 54.7 |
| $OC_4$ | 73.3 ± 26.8 | 117 ± 24.4 | 121 ± 28.9 | 96.3 ± 38.5 | 126 ± 57.0 |
| Ratio of GABA neurons to total neurons | | | | | |
| $YC_1$ | 0.40 ± 0.14 | 0.20 ± 0.07 | 0.24 ± 0.08 | 0.21 ± 0.09 | 0.17 ± 0.06 |
| $YC_2$ | 0.43 ± 0.14 | 0.22 ± 0.03 | 0.19 ± 0.06 | 0.19 ± 0.05 | 0.20 ± 0.08 |
| $YC_3$ | 0.49 ± 0.11 | 0.24 ± 0.03 | 0.24 ± 0.07 | 0.22 ± 0.06 | 0.22 ± 0.05 |
| $YC_4$ | 0.48 ± 0.09 | 0.22 ± 0.04 | 0.22 ± 0.07 | 0.20 ± 0.04 | 0.21 ± 0.06 |
| $OC_1$ | 0.24 ± 0.10 | 0.09 ± 0.02 | 0.11 ± 0.04 | 0.08 ± 0.03 | 0.10 ± 0.03 |
| $OC_2$ | 0.25 ± 0.12 | 0.10 ± 0.02 | 0.10 ± 0.02 | 0.09 ± 0.03 | 0.08 ± 0.03 |
| $OC_3$ | 0.27 ± 0.11 | 0.10 ± 0.03 | 0.10 ± 0.03 | 0.08 ± 0.03 | 0.11 ± 0.04 |
| $OC_4$ | 0.25 ± 0.11 | 0.09 ± 0.02 | 0.10 ± 0.02 | 0.08 ± 0.03 | 0.11 ± 0.05 |

The dilution concentration of GABA antiserum is 1:1500. All values are expressed as mean ± standard deviation. *Notes:* $YC_{1–4}$ and $OC_{1–4}$ represent four young adult cats and four old cats, respectively. Layer I, II–III, IV, V, VI represents cortical layer 1, 2–3, 4, 5 and 6.

uated by a two-way analysis of variance (ANOVA), and a $P$-value of <0.05 was considered significant.

## 3. Results

The density of total neurons and GABA-immunoreactive neurons as well as the ratio of GABA neurons to total neurons in each cortical layer of $V_1$ were quantitatively studied in four old cats and four young adult cats. For convenience, subjects studied were named as $OC_{1–4}$ for old cats 1–4 and $YC_{1–4}$ for young cats 1–4 (Table 1).

### 3.1. Density of total cortical neurons

The density of total neurons in each cortical layer was compared within and between the young adult and old cat groups. Two-way ANOVA analysis indicated that the density of total neurons is highly dependent on cortical layers in both cat groups (main effect of layer: $F(4,380) = 1544.256$, $p < 0.0001$ in young adult cats; $F(4,380) = 840.765$, $p < 0.0001$ in old cats), with lower density in layer I and higher density in layers II–III, IV, V and VI (Table 1). The density of total neurons in each cortical layer between individual cats showed no significant difference in either old ($F(3,380) = 0.357$, $p = 0.784$) or young adult cat ($F(3,380) = 0.235$, $p = 0.872$) group. Between-group analysis neither revealed age difference in the density of total neurons in each cortical layer (main effect of age: $F(1,760) = 0.058$, $p = 0.809$; interaction of age and cat: $F(3,760) = 0.429$, $p = 0.732$; interaction of age and layer: $F(4,760) = 0.118$, $p = 0.976$) (Fig. 2).

### 3.2. Density of GABA-immunoreactive neurons

GABA-immunoreactive neurons were found throughout all layers of $V_1$ in young adult and old cats as indicated by the presence of brown or dark brown-stained cell bodies (Fig. 3A–H). Most of GABA-positive neurons in young cats exhibited a clear labeling of the proximal dendrites and/or an initial segment of the axons but no visible immunoreaction in the distal dendritic processes (Fig. 3A, C, E and G). Very few of GABA neurons in old cats showed any clear GABA-immunoreactive dendrites or axons (Fig. 3B, D, F and H).

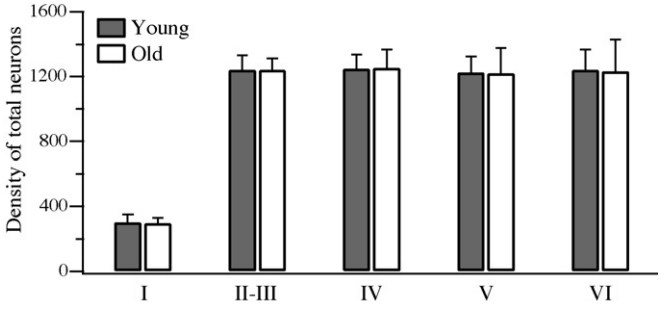


Fig. 2. Mean density (neurons/mm$^2$) of total neurons (thionine-stained neurons) in each cortical layer of young adult and old cat group. I, II–III, IV, V and VI represents cortical layer 1, 2–3, 4, 5 and 6. The mean density of total neurons of each cortical layer in both age groups showed no significant difference ($F(1,790) = 0.06$, $p = 0.807$).

Within-group analysis revealed that the density of GABA-immunoreactive neurons is highly dependent on cortical layers in both age groups of cats ($F(4,380) = 54.715$, $p < 0.0001$ in young adult cats group; $F(4,380) = 26.719$, $p < 0.0001$ in old cats group), with significantly lower density in layer I than in other cortical layers (Table 1). The density of GABA neurons of each cortical layer showed inter-subject variation in young cat group ($F(3,380) = 3.587$, $p = 0.014$) but not in the old ($F(3,380) = 0.579$, $p = 0.629$). Comparisons between the two groups indicated that the density of GABA-immunoreactive neurons showed a significant age difference (main effect of age: $F(1,760) = 1031.798$, $p < 0.0001$), which effect was independent of subjects ($F(3,760) = 1.86$, $p = 0.135$) but strongly depended on cortical layers ($F(4,760) = 20.854$, $p < 0.0001$). Compared with young adult cats, the mean density of GABA neurons in old cats decreased by 56.8, 54.7, 60.7 and 50.9% in layer II–III, IV, V and VI, respectively, while 44.5% in layer I (Fig. 4A).

### 3.3. Proportion of GABA-immunoreactive neurons to total neurons

The proportion of GABA neurons to total neurons, which directly reflects intracortical inhibition intensity, was also compared between young and old cats. The ratio of GABA-immunoreactive neurons to total neurons varied significantly with cortical layers in both old cats ($F(4,380) = 127.43$, $p < 0.0001$) and young adult cats ($F(4,380) = 149.779$, $p < 0.0001$), with higher proportion of GABA neurons in layer I than in other cortical layers (Table 1). The ratio of GABA neurons to total neurons showed interanimal difference in the young cat group ($F(3,380) = 4.951$, $p = 0.002$) but not in the old cat group ($F(3,380) = 0.341$, $p = 0.796$). However, the proportion of GABA neurons in each cortical layer of each old cat was significantly lower than of any individual young adult cat ($F(1,760) = 774.626$, $p < 0.0001$), which effect was highly dependent on cortical layers ($F(4,760) = 11.684$, $p < 0.0001$) but independent of subjects ($F(3,760) = 2.255$, $p = 0.081$). A comparison of an averaged ratio of GABA neurons to total neurons in each cortical layer between the two cat groups also exhibited a significant age difference (main effect of age: $F(1,790) = 758.86$, $p < 0.0001$), but the age effect varied considerably with cortical layers (main effect of layer: $F(4,790) = 266.923$, $p < 0.0001$; interaction of age and layer: $F(4,790) = 11.446$, $p < 0.0001$) (Table 1). Relative to young adult cats, the mean proportion of GABA-immunoreactive neurons to total neuron in old cats lowered by 56.8, 54.7, 59.9 and 50.3% in layer II–III, IV, V and VI, respectively, while 43.7% in layer I (Fig. 4B).

The above analysis indicated that both the density and proportion of GABA neurons in $V_1$ of cats were significantly reduced during aging, indicating a compromised GABAergic inhibition in the primary visual cortex of the old animals.

## 4. Discussion

Our previous single-unit recording study showed that $V_1$ neurons in the old cats exhibited lower signal-to-noise ratio

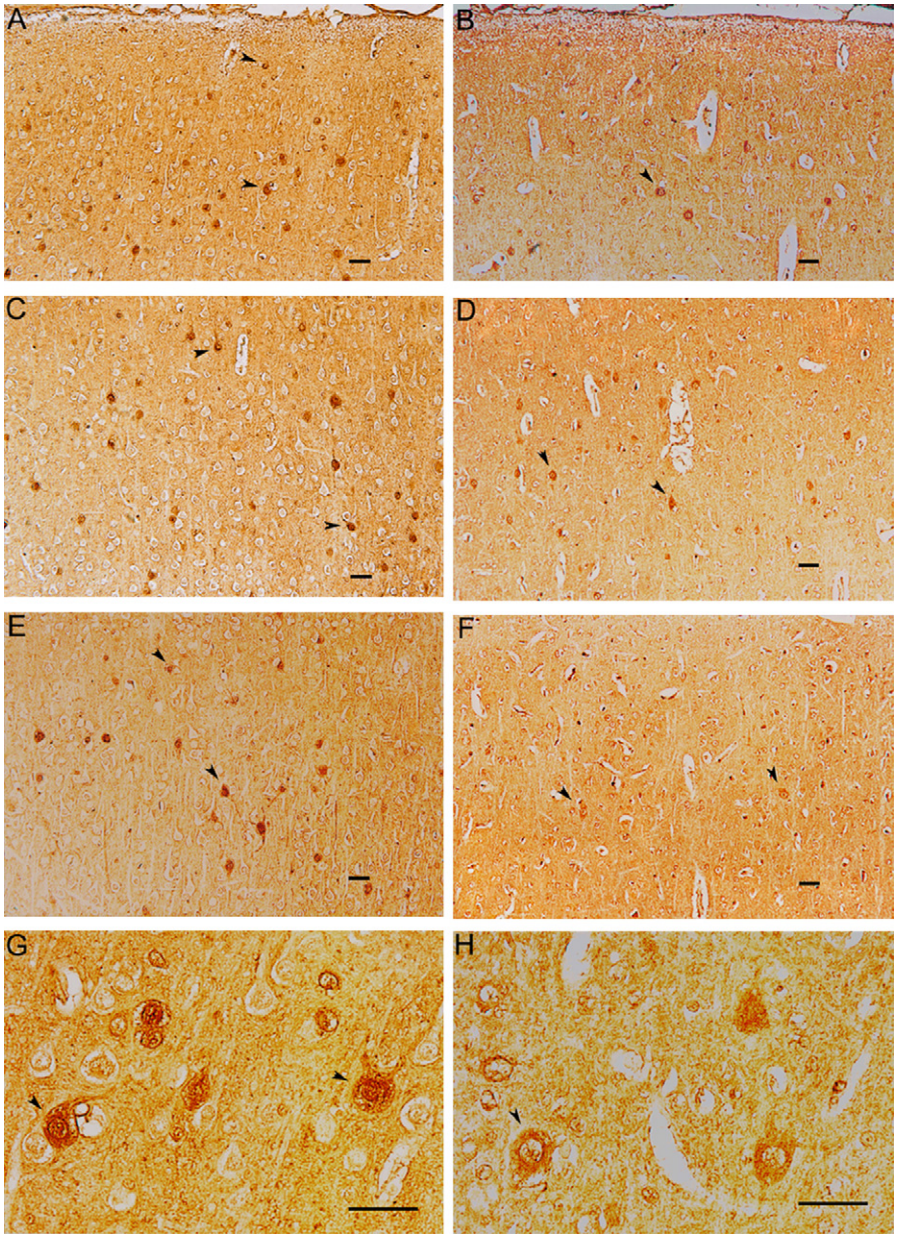


Fig. 3. GABA-immunoreactive cells in $V_1$ of young adult (A, C, E, G) and old (B, D, F, H) cats. The dark brown-stained cells (arrow head) are GABA-positive cells. (A/B), (C/D) and (E/F) show GABA-positive neurons in layer I–III, IV and V–VI, respectively (magnification ×100). (G and H) Examples of GABA-positive neurons at a higher magnification (×400) in layer II/III for young and old cats, respectively. The dilution of anti-GABA serum is 1:1500. Scale bar = 50 μm.

as well as less selectivity to visual stimulus orientations and motion directions than do cells in the young adults [14]. The functional degradation of visual cortical neurons in old animals was largely attributed to an increase of spontaneous activity and response to all visual stimulations, especially to stimuli with non-optimal orientations and motion directions [36,14]. This increased responsiveness of visual cortical neurons in the old brains could be caused by a decrease of intracortical inhibition, especially GABAergic inhibition [19,36]. The present study observed a significantly lower density and proportion of GABA neurons in $V_1$ of old cats than of young adults, and hence provided direct morphological evidence that a decreased GABAergic inhibition accompanied functional degradation of visual cortical neurons in the old brain. Taken our present result and that of previous studies together, this cortical inhibition compromise is likely attributed to a reduction of GABA transmitters. First, observations in rat cerebral cortex showed that the protein expression of the main $GABA_A$ receptors ($\alpha_1$, $\gamma_2$ and $\beta_{2/3}$) remained unchanged during aging process [13]. Second, Ling et al. [20] recently report that GABA synthetic enzyme, glutamic acid decarboxylase (GAD) in the rat primary auditory cortex is significantly declined with age. Finally, here we observed a significantly reduced density and proportion of GABA-containing neurons in the primary visual cortex of old cats relative to young adult ones (Fig. 4). Similar result was also reported in the auditory cortex of the same subjects as in this study [21]. Therefore, it is quite possible that the visual cortex in old animals contains less GABAergic neurons and thus lower level of GABA trans-

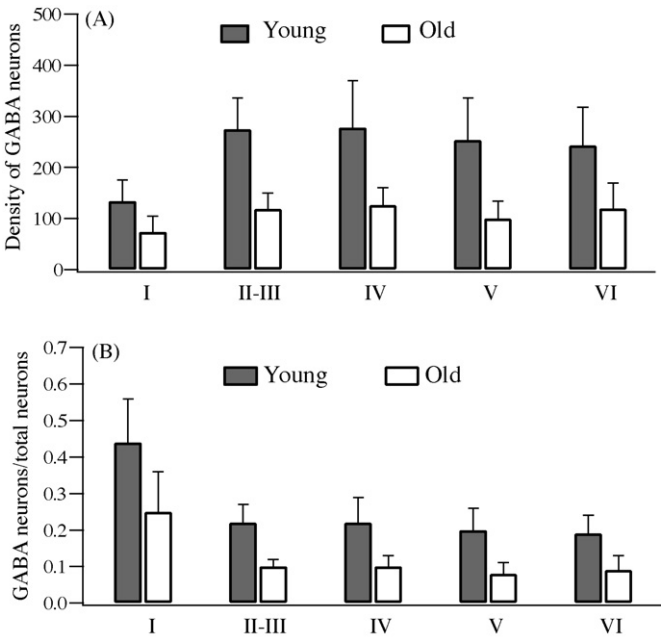


Fig. 4. Showing the density and proportion of GABA-immunoreactive neurons in $V_1$ of young adult and old cats. I, II–III, IV, V and VI represents cortical layer 1, 2–3, 4, 5 and 6. (A) The mean density (neurons/mm$^2$) of GABA neurons in each cortical layer of both age groups. The density of GABA neurons in each cortical layer of old cats is significantly lower than of young adults ($F(1,790) = 1009.889$, $p < 0.0001$). (B) The proportion of GABA neurons in each cortical layer. The mean ratio of GABA neurons to total neurons in each cortical layer of old cats is significantly smaller than of young adults ($F(1,790) = 758.86$, $p < 0.0001$).

mitters than in young adults, which may contribute to visual function declines during senescence.

Why GABAergic inhibition, a main component of intracortical inhibition, is closely related to neural processing properties has been widely probed but still remains in debate [16,40,42,3,50,18]. Specifically, blocking GABA inhibition by iontophoresis manipulation results in a reduced orientation and motion direction selectivity of visual cortical neurons [38,37,39,31,1,10]. However, visual cortical cells still demonstrate considerable orientation selectivity to visual stimulation even if the GABA-mediated intracortical inhibition is inactivated, which suggests that GABAergic inhibition is not necessary for a single cortical neuron to show orientation selectivity [25]. Further experimental evidences indicate that excitatory input connections are capable of generating the selectivity of visual cortical neurons to stimulus orientations and motion directions while intracortical inhibitory connections tend to suppress the neuronal responsiveness, especially the response to visual stimuli with non-optimal orientations and motion directions, and thus sharpen its stimulus tuning selectivity [34,6,7,8,40]. These intracortical inhibitory connections experience spatial pattern refining and clustering during postnatal development and remain spatially aligned with excitatory connections in the young and probably middle-aged adulthood so that visually evoked excitatory inputs are selectively masked and hence keep neurons responding sharply to optimal orientations and motion directions [4,22]. Therefore, a compromised GABAergic inhibition, which could happen with aging, will likely strengthen the postsynaptic efficacy of cortical neurons. As a result, the spontaneous activity of visual cortical neurons as well as the visually driven response, especially the response to non-optimal orientations and motion directions will be increased, which in turn, attenuates stimulus selectivity of neurons for orientations and motion directions [19,10].

## Conflict of interest

We declare that what we have done in this research has no conflict of interests with others published in the public journals.

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