Age-related Changes of Glu/GABA Expression in the Primary Visual Cortex of Cat

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Abstract: Recent studies show that a reduced effect of inhibitory transmitter system in the visual cortex may underlie aged visual function degradation. Whether excitatory transmitter system changes with age and hence affects intracortical excitation-inhibition balance is not clear. To explore this issue, we used Nissl staining and immunohistochemical methods as well as Image-Pro Express software to examine the density of Nissl-stained neurons, Glutamic acid-immunoreactive (Glu-IR) neurons and γ-Aminobutyric acid-immunoreactive (GABA-IR) neurons in the primary visual cortex of young adult and aged cats. The results showed that there was no significant difference in the density of Nissl-stained neurons between young and old cats ($P>0.05$). However, the density of Glu-IR neurons and GABA-IR neurons in the primary visual cortex of aged cats was significantly lower than that of young ones ($P<0.01$). The ratio between Glu-IR neurons and GABA-IR neurons was significantly increased in old cats compared to that in young adult ones ($P<0.01$). These results indicated that the effect of excitatory transmitter system in the old visual cortex was increased relative to the inhibitory transmitter system, which might cause an imbalance between cortical excitation and inhibition and might be an important factor mediating the visual function decline during aging.

Key words: Glutamic acid (Glu); γ-Aminobutyric acid (GABA); Balance of excitation and inhibition; Aging; Primary visual cortex; Cat

猫初级视皮层内 Glu/GABA 表达比率的衰老性变化

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摘要：最近的一些研究结果显示，视皮层内抑制性递质系统作用减弱可能是导致老年性视觉功能衰退的重要因素。是否皮层内兴奋性递质系统亦伴随衰老而发生改变并影响皮层内神经兴奋与抑制的平衡尚不清楚。为此，利用 Nissl 染色和免疫组织化学染色方法以及 Image-Pro Express 图像分析软件对青、老年猫初级视皮层(17 区)内各层神经元密度、兴奋性递质谷氨酸免疫反应阳性(Glu-immunoreactive, Glu-IR）神经元密度以及抑制性递质 γ-氨基丁酸免疫反应阳性(γ-aminobutyric acid-immunoreactive, GABA-IR）神经元密度进行了统计分析。结果显示，青、老年猫初级视皮层各层神经元密度均没有明显的年龄性差异（$P>0.05$）；与青年猫相比，老年猫初级视皮层 Glu-IR、GABA-IR 神经元密度均显著减少（$P<0.01$），而 Glu-IR/GABA-IR 神经元密度比率却显著增大（$P<0.01$）。结果提示，老年猫初级视皮层内兴奋性递质系统作用相对增强，而抑制性递质系统的作用相对减弱，导致皮层内兴奋−抑制平衡关系失调，这可能是引起老年个体视觉功能衰退的重要原因之一。

关键词：谷氨酸；γ-氨基丁酸；兴奋−抑制平衡；衰老；初级视皮层；猫

Many visual functions of human beings as well as animals degade with age, such as reduction of visual acuity (Rubin et al, 1997; Hu et al, 2000), decrease of contrast sensitivity to visual signal (Elliott et al, 1990;

Nomura et al, 2003), decline of sensitivity to wavelength (Johnson et al, 1988; Gray et al, 1995) as well as the selectivity to orientation and/or motion direction of visual stimulus (Willis & Anderson, 2000; Kline et al, 2001). The primary visual cortex receives signal input directly from lateral geneculate nucleus (LGN) and plays a quite important role in the preliminary processing of visual information and relaying processed visual information to higher cortical loci for visual perception (Shou, 2006). Similar to other cerebral cortex, the gray matter of primary visual cortex can be divided into six layers from the pial surface to white matter, including molecular lamina, exeternal granular lamina, external pyamidal lamina, internal granular lamina, internal pyramidal lamina and multiform lamina. The layer Ⅳ, which is thicker than other layers, is the main input region of visual information, while the layer Ⅱ and Ⅲ are the main region of visual information output to the higher visual cortices, and the layer Ⅴ and Ⅵ export feedback signals to subcortical nucleus, including superior colliculus (SC) and lateral geneculate nucleus (LGN) (Shou, 2006). Therefore, age-related morphological or functional changes in the primary visual cortex may exert a great impact on various visual functions.

Recent studies show that a reduction of inhibitory transmitter system effect in the visual cortex may mediate aged visual function degradation (Schmolesky et al, 2000; Leventhal et al, 2003; Hua et al, 2006, 2008). Whether excitatory transmitter system modifies with age and thus impacts intracortical excitation-inhibition balance remains unknown. To probe into this issue, we used Nissl staining and immunohistochemical methods as well as Image-Pro Express software to examine the density of Nissl-stained neurons, Glu-IR and GABA-IR neurons in the primary visual cortex of young adult and aged cats, attempting to explore if the excitatory transmitter system changes during senescence and provide morphological evidence for further researches on mechanisms of visual function decline during aging.

1 Material and methods

1.1 Preparation of subjects and tissue

Four young adult cats (1−3 years old) and 4 old cats (11−13 years old), all of which were healthy cats and examined ophthalmoscopically before the experiment to confirm that there is no optical or retinal problem that would impair their visual function, were studied in this experiment. All the animals were treated strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The animals were deeply anaesthetized with ketamine hydrochloride (40 mg/kg) and then perfused with 0.9% NaCl solution through the ascending aorta till the liver became pale, followed immediately by 500 mL of a fixative solution containing 4% Paraformaldehyde and 2.5% Glutaraldehyde in 0.1 mol/L phosphate buffer solution (PBS, pH7.2−7.4). After perfusion, the skull was opened and the blocks of tissue containing V_1 were dissected out and cut into about 1 cm in length. After further fixation for 20−24 h in the perfusing fixative solution, the samples were washed in PBS (3×5 min), dehydrated in ethanol, transparentized in xylene and embedded in paraffin. Consecutive coronal sections of 7 μm thick were cut perpendicularly to the pial surface of the lateral gyrus in V1 and then dried onto APES-coated glass slides at 37℃ for 2−3 days. Every other 10 slices, 4 adjacent ones were choosed and used respectively for Nissl staining, immunohistochemical labeling of Glu and GABA neurons as well as negative control experiment.

1.2 Procedure of immunohistochemical and Nissl staining

Paraffin slices used for immunostaining were first washed in 0.1 mol/L PBS (pH7.2−7.4) for 10 min after deparaffinizing in xylene and rehydrating in gradient ethanol, then incubated with 0.3% H_2O_2 in PBS (3×5 min) to quench endogenous peroxidase activity. Following washed in PBS (3×5 min), the slices were incubated with 5% normal goat serum in PBS for 10 min at room temperature to block non-specific reactions. Subsequently, the slices were incubated respectively with primary antibody against Glu and GABA for 24h at 4℃, washed in PBS (3×5 min) and then incubated with biotinylated rabbit anti-cat IgG for 15−20 min at 37℃. After further rinsing in PBS (3×5 min), the slices were incubated with an ABC solution for 15−20 min. After rinsing in PBS (3×5 min), dehydrating in gradient alcohol and clearing in xylene, the slices were finally coverslipped with permont. Slice used for control experiment was stained simultaneously following the same procedure as described above with the exception that the primary antibody was omitted. The paraffin slice used for Nissl staining was put into 0.25% cresyl violet solution for 1−2 h at room temperature after deparaffinizing and rehydrating, then dehydrated, cleared and finally coverslipped. Nissl staining was used for

identification of cortical layers and statistical evaluation of the density of total cortical neurons in each layer of the primary visual cortex.

The dyestuff of cresyl violet used in this experiment was bought from the corporation of Sigma. The primary antibodies against Glu and GABA, the reagent box for immunohistochemistry and the DAB colored fluid were all bought from the company of Lab Vision Corporation.

1.3 Statistical analysis

Under an Olympus BX51 microscope, statistical studies were performed at the medial bank of the lateral gyrus (V1) where the cortical surface runs approximately parallel to the white matter. In each Nissl-stained section, the mean depth of each cortical layer was measured with an optical calibrator at a low magnification ($\times 4$ or $\times 10$ objective lens with $\times 10$ eyepieces) and used for laminar localization in cell counting. At a high magnification ($\times 40$ objective lens with $\times 10$ eyepieces), the number of total cortical neurons, Glu-IR neurons and GABA-IR neurons within varied cortical layers (layer Ⅰ, Ⅱ-Ⅲ, Ⅳ and Ⅴ-Ⅵ) were randomly counted for 6 visual fields in each section using a grid of counting frame (50 $\mu m \times 50 \mu m$). 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 Nissl-stained neurons, Glu-IR neurons and GABA-IR neurons in different cortical layer was obtained in each

Nissl-stained and immuno-stained section, and the ratios of Glu-IR neurons to GABA-IR neurons in different cortical layers were acquired for each adjoining section group. The criteria for acceptance as a neuron in Nissl-stained section 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 background staining was designated as a Glutamate- or GABA-positive neuron in the immunostaining sections.

All the data were expressed as mean $\pm$ standard deviation. The significance of the differences between cortical layers, individuals and age groups was evaluated by one- or two-way analysis of variance (ANOVA), and a difference with *P*-value of <0.05 was accepted as statistical significant.

2 Results

2.1 Result of Nissl staining

In Nissl-stained sections, the gray matter of primary visual cortex of cat can be devided into 6 layers from the outside to the inside (Fig. 1), including molecular lamina (Ⅰ), exetermal granular-pyamidal lamina(Ⅱ−Ⅲ), internal granular lamina (Ⅳ), internal pyramidal lamina(Ⅴ) and multiform (Ⅵ). Our statistical analysis indicated that the density of Nissl-stained neurons in each cortical layer showed no significant difference between individuals of either young adult cat group
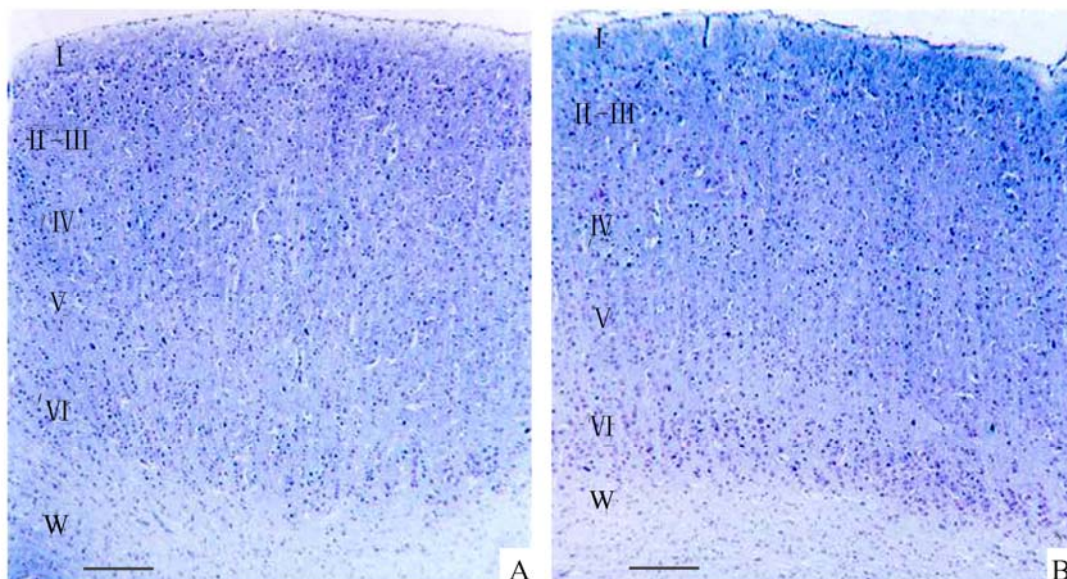

Fig. 1 Nissl staining shows the laminar identification in primary visual cotex of young adult and aged cats Layer identification of primary visual cortex in young cats (A) and aged cats (B)

Ⅰ, Ⅱ−Ⅲ, Ⅳ, Ⅴ and Ⅵ represents morlecular laminar, exetermal granular-pyamidal laminar, internal granular laminar, internal pyramidal laminar and multiform laminar of gray matter of primary visual cortex respectively, W represents white matter. Bar=300 μm, the dyestuff used is cresyl violet.

Tab. 1　Density of neurons of the primary visual cortex in young and old cats (Mean±*SD*)

Neurons	Young cats(cells/mm^2)	Old cats(cells/mm^2)
Neurons in layer Ⅰ	268.07±48.42	259.61±34.67
Neurons in layer Ⅱ－Ⅲ	1142.81±162.30	1127.53±145.32
Neurons in layer Ⅳ	1134.63±150.21	1115.72±129.74
Neurons in layer Ⅴ－Ⅵ	1087.29±121.56	1063.14±117.83

(main effect of cat: $F_{(3,396)}$=0.197, *P*=0.898) or old cat one [main effect of cat: $F_{(3,396)}$=0.164, *P*=0.921]. Between-group analysis neither revealed age difference in the density of Nissl-stained neurons of all cortical layers [main effect of age: $F_{(1,792)}$=2.349, *P*=0.126] (Tab. 1).

2.2　Result of Glu and GABA immunostaining

In immunostaining sections, Glu-IR neurons and GABA-IR neurons were found throughout all layers of V1 both in young adult and old cats as indicated by the presence of brown or dark brown-stained cell bodies (Fig. 2A－G). A number of Glu-IR and GABA-IR neurons in V1 of young cats exhibited a clear labeling of the proximal dendrites and/or an initial segment of the axons (Fig. 2A, C, E, G), but very few neuron in old cats showed any clear dendrites or axons (Fig. 2B, D, F, H). Compared with young adult cats, the Glu-IR neurons in V$_1$ of old cats was evidently sparser (Fig. 2A, B, C, D). Similarly, the GABA-IR neurons in V$_1$ of old cats was also less than that in young adult cats (Fig. 2E, F, G, H). There was no positive reaction in the negative control experiment.

2.2.1　Density of Glu-IR and GABA-IR neurons　The statistical result indicated that both density of Glu-IR neurons and GABA-IR neurons in each cortical layer of V$_1$ showed no significant difference within either the young adult cat group [Glu: $F_{(3,396)}$=0.198, *P*=0.897; GABA: $F_{(3,396)}$=0.604, *P*=0.613] or the old cat group

[Glu: $F_{(3,396)}$=0.174, *P*=0.914; GABA: $F_{(3,396)}$=0.686, *P*=0.561]. However, inter-group comparisons showed that both density of Glu-IR neurons and GABA-IR neurons had significant age difference [Glu: $F_{(1,792)}$= 7.343, *P*=0.007; GABA: $F_{(1,792)}$=478.213, *P*<0.0001] (Tab. 2). Compared with young adult cats, the mean density of Glu-IR neurons in old cats decreased respectively by 12.8%, 15.0%, 14.3% and 9.5% in layer Ⅰ, Ⅱ－Ⅲ, Ⅳ, and Ⅴ－Ⅵ; while the mean density of GABA-IR neurons reduced by 45.7%, 42.8%, 43.4% and 47.9% in layer Ⅰ, Ⅱ－Ⅲ, Ⅳ and Ⅴ－Ⅵ respectively.

2.2.2　Ratios of Glu-IR to GABA-IR neuron density

Our statistical result showed that the ratios of Glu-IR neurons to GABA-IR neurons in the primary visual cortex of old cat were significantly higher than that of young adult cat [main effect of age: $F_{(1,792)}$=960.034, *P*<0.0001]. Relative to young adult cats, the mean ratios of Glu-IR neurons to GABA-IR neurons in old ones increased by 46.1%, 64.5%, 61.7% and 60.8% in layer Ⅰ, Ⅱ－Ⅲ, Ⅳ and Ⅴ－Ⅵ respectively (Fig. 3).

In summary, the density of total neurons in primary visual cortex of aged cats showed no significant difference from that of young adult ones. Relative to young adult cats, both the density of Glu-IR neurons and GABA-IR neurons in the primary visual cortex of aged cats decreased to a different extent, but the ratios of Glu-IR neurons to GABA-IR neurons was increased remarkably.

Tab. 2　Density of Glu-IR and GABA-IR neurons in the primary visual cortex of young and old cats (Mean±*SD*)

Neurons	Young cats(cells/mm^2)	Old cats(cells/mm^2)
Glu-IR neurons in layer Ⅰ	178.63±33.55	155.76±25.28*
Glu-IR neurons in layer Ⅱ－Ⅲ	822.12±67.97	699.07±41.05*
Glu-IR neurons in layer Ⅳ	794.24±74.43	680.59±58.26*
Glu-IR neurons in layer Ⅴ－Ⅵ	717.61±58.32	649.52±46.31*
GABA-IR neurons in layer Ⅰ	69.73±8.04	37.84±5.54*
GABA-IR neurons in layer Ⅱ－Ⅲ	235.58±45.78	134.87±21.26*
GABA-IR neurons in layer Ⅳ	228.16±43.90	129.42±29.15*
GABA-IR neurons in layer Ⅴ－Ⅵ	216.88±37.59	113.63±23.07*

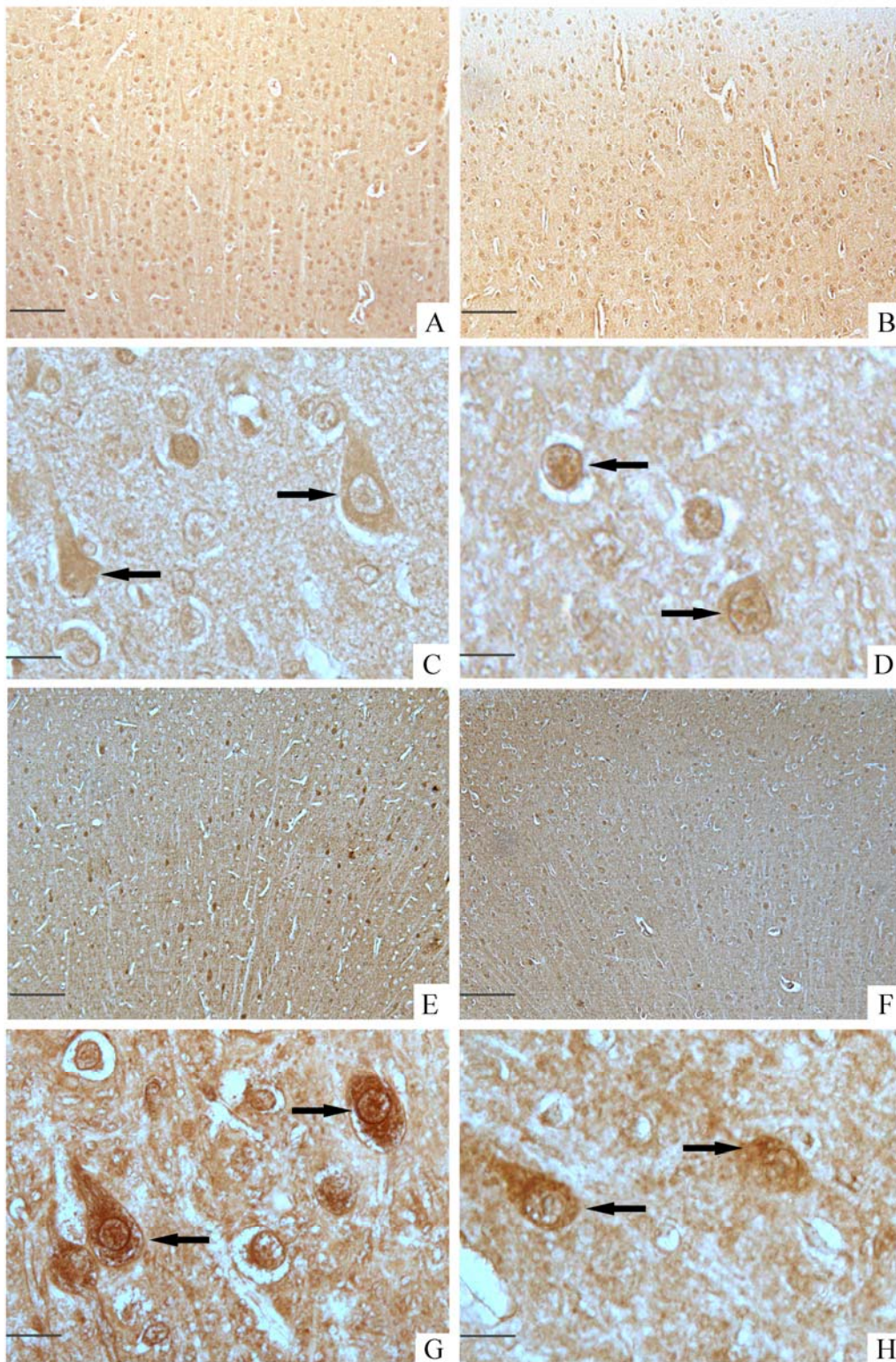*Means significant difference between young and old cats, *P*<0.01.

Fig. 2   Immunohistochemical staining shows Glu-IR and GABA-IR neurons in V1 of young adult and aged cats

Glu-IR neurons in layer Ⅱ−Ⅲ, Ⅳ of primary visual cortex in young cats (A) and aged cats (B), Bar=200 μm; Glu-IR neurons (→) in layer Ⅳ of primary visual cortex in young cats (C) and aged cats (D), Bar=25 μm; GABA-IR neurons in layer Ⅱ−Ⅲ, IV of primary visual cortex in young cats (E) and aged cats (F), Bar=200 μm; GABA-IR neurons (→) in layer Ⅳ of primary visual cortex in young cats (G) and aged cats (H), Bar=25 μm.
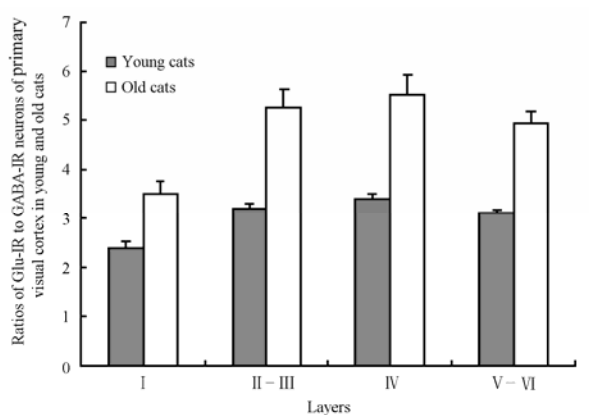
Fig. 3   Ratios of Glu-IR to GABA-IR neurons in
different layers of the primary visual cortex
in young and old cats

3　Discussion

In the transmiting process of visual signal, the physiological function of primary visual cortex is mainly to receive the visual information (such as visual stimulus orientation, motion direction and contrast sensitivity) from LGN and relay it to higher visual cortex after preliminary information processing. A number of researches indicated that human beings and animals showed lower capacity for visual stimulus orientation and motion direction discrimination during aging. For example, aged people learned very slowly in tasks related to orientation and motion direction (Owsley et al, 1981; Tran et al, 1998), and the ability to detect moving signals also dropped down significantly with age (Willis & Anderson, 2000; Kline et al, 2001). Recent studies showed that a reduced effect of inhibitory transmitter system in the visual cortex may underlie aged visual function degradation. Hu et al (2000) found that iontophoretic application of GABA$_A$ receptors antagonists into the primary visual cortex of cat could obviously depress the selectivity of LGN neurons to visual stimuli orientations. Schmolesky et al (2000) suggested that a compromised effect of inhibitory transmitter system in aged monkeys' cortex could account for the decreased selectivity of V$_1$ neurons for visual stimulus properties, which was largely attributed to an increase of spontaneous activity and response to all visual stimulus orientations and motion directions, especially to non-optimal orientations and motion directions. Leventhal et al (2003) found that adding GABA or the agonist of GABA receptors to the visual cortex of old monkey could greatly improve the

selectivity of visual cortical neurons for stimulus orientations and motion directions. Our previous studies using extracellular single-unit recording techniques showed that the neurons in primary visual cortex of old cats exhibited degraded functions as indicated by a lower signal-to-noise ratio, higher spontaneous activity, less selectivity to orientations and motion directions of visual stimulus than did neurons of young adult cats (Hua et al, 2006), and our recent studies indicated that this declined stimulus selectivity of visual cortical neurons in the aged animals may attribute to the reduced synthesis of inhibitory neurontransmitter in the aged cortex (Hua et al, 2008). In current study, we compared the density of Glu-IR and GABA-IR neurons as well as the ratio of Glu-IR neuron density to GABA-IR neuron density in the primary visual cortex between aged and young adult cats. We found that both the density of Glu-IR neurons and GABA-IR neurons in the primary visual cortex of old cats were significantly reduced. However, the ratio of Glu-IR neurons to GABA-IR neurons in old cats was signifcantly higher than that in young adults. Therefore, the effect of excitatory transmitter system in the primary visual cortex of old cats was actually enhanced relative to the inhibitory transmitter one.

Glutamate and GABA is the most important excitatory and inhibitory neurontransmitter in the central nervous system. 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 response, especially the response to visual stimuli with non-optimal orientations and motion directions, and thus sharpen its stimulus tuning selectivity (Sato et al, 1996; Crook et al, 1996, 1997, 1998; Sohya et al, 2007), so the balance between intracotical excitation and inhibition will play a key role in keeping the normal brain function. An imbalance between cortical excitation and inhibition, which was highlighted by lots of researchers, has been deemed as an important reason for brain damnification (Lyden & Hedges, 1992; Shuaib & Kanthan, 1997; Green et al, 2000; Schwartz-Bloom & Sah, 2001). Further experimental evidences indicated that 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 (Chen et al, 2005; Marino et al, 2005). In this study, both the density of Glu-IR neurons and GABA-IR neurons in primary visual cortex of old cats reduced. However, a varied decrease rate of Glu-IR and GABA-IR neurons led to an increased ratio of Glu-IR nuerons to GABA-IR neurons in the primary visual cortex of old cats relative to young adults.

To sum up, the expression of excitatory neurontransmitter is enhanced relative to the inhibitory one in the primary visual cortex of cats during aging and hence result in a maladjustment of excitation-inhibition balance, which may be an important reason for the decline of visual function in aged individuals. Of course, expression of other neurontransmitter such as acetylcholine, noradrenalin, dopamine as well as glycin may also change with age and hence affect the intracortical excitation-inhibition balance, which need to be studied subsequently.

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