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Sex- and age-related changes in GABA signaling components in the human cortex

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Abstract

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the nervous system. Previous studies have shown fluctuations in expression levels of GABA signaling components—glutamic acid decarboxylase (GAD), GABA receptor (GABAR) subunit, and GABA transporter (GAT)—with increasing age and between sexes; however, this limited knowledge is highly based on animal models that produce inconsistent findings. This study is the first analysis of the age- and sex-specific changes of the GAD, GABA_{A/B}R subunits, and GAT expression in the human primary sensory and motor cortices; superior (STG), middle (MTG), and inferior temporal gyrus (ITG); and cerebellum. Utilizing Western blotting, we found that the GABAergic system is relatively robust against sex and age-related differences in all brain regions examined. However, we observed several sex-dependent differences in GABA_AR subunit expression in STG along with age-dependent GABA_AR subunit and GAD level alteration. No significant age-related differences were found in $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 3$, and $\gamma 2$ subunit expression in the STG. However, we found significantly higher GABA_AR $\alpha 3$ subunit expression in the STG in young males compared to old males. We observed a significant sex-dependent difference in $\alpha 1$ subunit expression: males presenting significantly higher levels compared to women across all stages of life in STG. Older females showed significantly lower $\alpha 2$, $\alpha 5$, and $\beta 3$ subunit expression compared to old males in the STG. These changes found in the STG might significantly influence GABAergic neurotransmission and lead to sex- and age-specific disease susceptibility and progression.

Keywords: Sex difference, Aging, GAD, GABA_A receptor, GABA_B receptor, GABA transporter, Human brain

Introduction

GABAergic interneurons account for approximately 20% of cortical neurons in the human brain that modulate neuronal activity via GABA based neuronal inhibition [1]. The balance between excitatory and inhibitory circuits is fundamental for all aspects of brain function. Existing data suggest that age and sex are significant contributors of altered neurotransmission between individuals and these differences might contribute to aging-related impairments and sex-specific vulnerability to disease conditions, for instance, depression, schizophrenia, presbycusis, and Alzheimer's disease [2–12].

GABA is synthesized by glutamic acid decarboxylase (GAD) and is then recruited into synaptic vesicles.

Following membrane depolarization, GABA is released into the synapse and binds to either ionotropic GABA_A receptors (GABA_ARs) or metabotropic GABA_B receptors (GABA_BRs). Released GABA is cleared from the synapse by membrane-bound GABA transporters, localized to neurons and astrocytes. Previous studies have reported aging-related alterations in the levels of both GAD isoforms, GAD65 and GAD67, in different species and brain areas. Using magnetic resonance spectroscopy several studies have found a reduction in concentration of GABA levels with age in animal models, nonhuman primates, and humans [13–15]. However, evidence suggesting loss of grey matter tissue fraction that causes an overall reduction in GABA concentrations confounds the correlation of age-related loss of GABA [16, 17]. In addition, alterations of GAD expression at the mRNA level and the protein level do not always follow the same trend and can be followed by changes in GABA level [18]. Furthermore, the literature also shows controversial

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results and species differences in GAD expression, some studies demonstrating an increase rather than a decrease in GAD levels in prefrontal cortical areas [2, 18, 19].

GABA_ARs are ligand-dependent Cl⁻ channel pores assembled from five subunits [20]. Over 20 GABA_AR subunits have been identified; six alpha subunits (α 1/2/3/4/5/6), three beta subunits (β 1/2/3), three gamma subunits (γ 1/2/3), delta (δ), theta (θ), epsilon (ϵ), pi (π), and rho (ρ 1/2/3), forming many possible combinations of pentameric GABA_ARs [21–23]. Literature implies that the expression pattern of subunits is brain region specific and is involved in region-specific function [24, 25]. Therefore, previous studies hypothesized regional brain function loss of hearing impairment, learning, and memory deficit, as an implication of regional GABA_AR subunit expression changes in aging [7, 8, 26, 27].

GABA_BRs are metabotropic, heterodimers formed by two subunits, GABA_BR1 and GABA_BR2, of which R1 binds GABA and R2 is associated with G proteins [28–30]. Evidence from animal studies demonstrates a loss of R1 subunit expression in the prefrontal cortex (PFC) and hippocampus in aged mice, leading to an overall downregulation of GABA_BRs, reduced inhibitory currents, and associated functional implications such as learning deficits and reduced memory formation [3, 31–33]. On the contrary, administration of a GABA_BR antagonist demonstrated improvements in working memory in aged rats [19] and olfactory discrimination learning in mice [34]. Both GABA_BR subunits show reduced expression in the rat PFC [19] and reduced GABA_BR binding in the inferior colliculus and cortex with age [35–37]. These data indicate there is complex regulation of age-related GABA_BR function.

The GABA transporters (GATs), GAT1/2/3 and beta-ine transporter 1 (BGT1), are present on interneurons and surrounding glial cells and regulate removal of GABA from the synaptic cleft [9, 38, 39]. Only few studies examined the age-related GAT changes; one study reported a decreased GAT1 expression in the rat medial PFC [19] and another in the human frontal cortex [40]. Age-related reduction in expression of GAT1 and GAT2 were also observed in the rhesus macaque visual cortex [41] corresponding with age-related visual retrogression in these primates [42].

The confounding issue of sex-based variability in the brain at the molecular and cellular level is well established. Gene and hormonal differences are the leading cause of behavioral and physiological changes observed between sexes with few studies suggesting a magnitude of asymmetrical sex-led differences in the GABAergic system [41, 43]. The changes in hormonal levels (estradiol and progesterone) during the menstrual cycle have been suggested as causative of fluctuation of brain GABA levels in healthy females [44, 45]. Furthermore,

the GABA level fluctuations coincide with behavioral changes such as mood, cognitive function, and physical symptoms [44–46]. Gonadal steroidal hormones, such as estrogens, are known manipulators of synaptic transmission through genomic mechanisms as well as rapid alteration in cell to cell communication [47–53]. Estrogens also regulate the release of GABA and induce bursts in GABA_AR-dependent inhibitory postsynaptic currents in gonadotropin-releasing hormone neurons [49, 54, 55]. Another ovarian hormone progesterone and its metabolite allopregnanolone are also regulators of inhibitory neurotransmission through their influence on GABA_AR [5, 56, 57]. Studies show that short-term exposure to allopregnanolone leads to upregulation of the α 4 subunit [58]. The ability of ovarian hormones to regulate GABA_AR subunit composition and alter their function, pharmacology, and GABA-gated current is another mechanism that could lead to changed neurotransmission. Predominantly, these hormonal changes are drastic in females during puberty, through the menstrual cycle, pregnancy, and post-menopausal period, and therefore, hypothetically, females are more susceptible towards hormonal driven changes in the GABAergic system. These findings suggest a differential mechanism and response in males and females towards changing hormonal levels throughout stages of life and aging.

Sex and age bias has been observed in many neurological disorders such as Alzheimer's disease and depression disorder [5, 59–63] and has also been linked to the GABAergic system [6, 27, 60, 64, 65]. Therefore, a thorough investigation is required to identify the link between sex and age and the GABAergic changes observed in these and other neurological conditions.

This study is the first comprehensive analysis of the sex- and age-specific expression of GABA signaling components in the human neocortical areas; primary, secondary, and association areas from each lobe; and cerebellar cortex. In the present study, we observed only a few alterations in the expression of GAD, GABA_AR, GABA_BR subunits, and transporters GAT-1/3 in the primary sensory and motor cortices, middle (MTG) and inferior temporal gyrus (ITG), and cerebellum, except the superior temporal gyrus (STG) that displayed numerous sex- and age-related expression changes, mainly affecting GAD65 and the GABA_ARs.

Methods

Human brain tissue preparation and neuropathological analysis

This study was conducted at the University of Auckland, Centre for Brain Research. The tissue was acquired through a donor program to the Neurological Foundation of New Zealand Human Brain Bank, and the procedures were approved by the University of

Auckland Human Participant's Ethics Committee (Approval number: 011654). Seven control younger females (YF, 51.7 years \pm 5.1 years), six younger males (YM, 47.5 years \pm 1.5 years), eight older females (OF, 76 years \pm 1.3 years), and seven older males cases (OM, 80 years \pm 1.5 years) with a maximum post-mortem time of 26 h (Table 1) were chosen. Processing of tissue followed the procedure described previously [66]. Firstly, the brain was dissected in half separating the hemispheres; the left hemisphere of the brain was cut into anatomical blocks, freshly frozen, and stored at -80°C . All cases included in this study had no history of any primary neurodegenerative, psychiatric disorder, neurological disease abnormalities, or excessive alcohol consumption. Standard pathological sections from all cases, including the middle frontal, middle

temporal, and cingulate gyrus; hippocampus; caudate nucleus; substantia nigra; locus coeruleus; and cerebellum, were examined and confirmed as pathologically normal by a neuropathologist.

Western blotting

The fresh human cortical tissue samples were collected from the regions of interest (sensory and motor cortex; cerebellum; superior, middle, and inferior temporal gyrus) using a cryostat (CM3050, Leica Microsystems, Germany) at 60- μm thickness on glass slides. The grey matter tissue was collected with a blade, homogenized in a buffer containing 0.5 M Tris, 100 mM EDTA, 4% SDS, pH 6.8, and protein extracts prepared using 0.5-mm glass beads (Mo BIO, USA) and a Mini Bullet

Table 1 Human brain case details for all experimental groups. YF, younger female, OF, older female, YM, younger male, OM, older male, PM, post-mortem

Case	Age	Sex	PM delay	Cause of death	Weight (g)	Classification
110	83	F	14	Aortic aneurysm	1200	OF
111	46	M	10	Coronary artery disease	1424	YM
112	79	M	8	Bleeding stomach ulcer	1190	OM
121	64	F	6.5	Pulmonary embolism	1205	YF
122	72	F	9	Emphysema	1230	OF
123	78	M	7.5	Abdominal aortic aneurysm	1260	OM
124	49	M	13	Ischemic heart disease	1495	YM
126	36	F	11	Asphyxia	1320	YF
127	59	F	21	Pulmonary embolism	1310	YF
128	34	F	18.5	Myocardial infarction	1140	YF
129	48	M	12	Pulmonary embolism	1318	YM
131	73	F	13	Ischemic heart disease	1210	OF
132	63	F	12	Rupture aorta	1280	YF
137	77	F	21	Coronary atherosclerosis	1227	OF
152	79	M	18	Congestive heart failure	1425	OM
156	89	M	19	Atherosclerosis	1430	OM
159	53	M	16.5	Ischemic heart disease	1215	YM
165	43	F	26	Nitrogen poisoning	1318	YF
169	81	M	24	Asphyxia	1225	OM
181	78	F	20	Aortic aneurysm	1292	OF
189	41	M	16	Asphyxia	1412	YM
190	72	F	19	Myocardial infarction	1264	OF
202	83	M	14	Abdominal aortic aneurysm	1245	OM
209	48	M	23	Ischemic heart disease	1470	YM
238	63	F	16	Aortic aneurysm	1324	YF
241	76	F	12	Metastatic cancer	1094	OF
243	77	F	13	Ischemic heart disease—coronary atherosclerosis	1184	OF
244	76	M	16	Ischemic heart disease—coronary atherosclerosis	1508	OM

Blender Tissue Homogenizer (Next Advance, Inc., New York, USA) at speed 8 for 8 min. The homogenates were incubated for 1 h on ice, then centrifuged at 10,000 rpm for 10 min; the supernatant collected and stored at -20°C . The protein concentration of the samples was measured using detergent-compatible protein assay (DC Protein assay, 500-0116, Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. Protein samples from each case were randomized, by a person not involved in the study, and numbered from 1 to 24. Twenty micrograms of each protein extract was run on a gradient SDS PAGE gel (NU PAGE 4–12% BT 1.5, NP0336BOX, Life technologies, California, USA) and then blotted. Proteins were separated in XCell SureLock Mini-Cell system (Invitrogen, Victoria, Australia) and transferred onto nitrocellulose membranes using XCell Blot Module (Invitrogen, Victoria, Australia). Three molecular weight ladders, Molecular weight, SeeBlue or Magic mark (Life technologies, California, USA), were also loaded in gels as verification of labeled band size. Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, USA) at room temperature for 30 min, followed by incubation with the primary antibodies (Table 2), at 4°C overnight. The following day membranes were washed 3×10 min in Tris-buffered saline pH 7.6, 0.1% Tween (TBST) and incubated with an appropriate IRDye (1:10,000, goat anti-rabbit IRDye[®]680RD, 926-68071, RRID:AB_10956166; goat anti-mouse IRDye[®]800CW, 926-32210, RRID:AB_621842; donkey anti-goat IRDye[®]800CW, 926-32214, RRID:AB_621846; LI-COR Biosciences, Germany) secondary antibody for 1 h at room temperature. Membranes were washed and scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences, USA).

Nissl staining

Nissl staining was performed for identification of the sensory and motor cortex regions on each block. Fresh frozen section were stained with a cresyl violet solution (2% Cresyl violet in 0.1 M glacial acetic acid and 0.0136 M sodium acetate solution) for a period of 45 min, mounted onto glass slides, dried, dehydrated through a graded series of ethanol, and cleared in xylene. Sections were examined using a Leica (Wetzlar, Germany) DMRB light microscope. Tissue sections were examined for features such as cortical thickness and the presence of large motor neurons.

Imaging and analysis

Odyssey Infrared Imaging System (LI-COR Biosciences, USA)-based detection of immunofluorescence signal was carried out at 680-nm and 800-nm spectrum. The analyses were conducted using the Image Studio Lite software (version 5.2, LI-COR Biosciences, USA) to measure signal intensities of each sample and were normalized to β -actin. To examine the averaged signal intensity differences between groups (younger females ($n = 6$) vs younger males ($n = 6$); younger females ($n = 6$) vs older females ($n = 6$); younger males ($n = 6$) vs older males ($n = 6$); older females ($n = 6$) vs older males ($n = 6$)), a non-parametric Kruskal-Wallis test was used. Data in all experiments was expressed as mean \pm SEM. All statistical analyses were conducted using Prism (version 6; GraphPad Software) with a value of $p < 0.05$ considered significant.

Results

The expression levels of GABA signaling components, GABA_AR subunits ($\alpha 1/2/3/5$, $\beta 3$ and $\gamma 2$), GABA_BR

Table 2 Primary antibodies used in this study

Antigen	Host species, source, catalogue, number	Concentration	Immunogen
Anti-GABA _A R $\alpha 1$	Rabbit, Alomone, AGA-001	1:1000	Peptide QPSQDELKDNTTVFTR
Anti-GABA _A R $\alpha 2$	Rabbit, Alomone, AGA-002	1:200	Peptide (C)TPEPNKKPENKPA
Anti-GABA _A R $\alpha 3$	Rabbit, Alomone, AGA-003	1:200	Peptide QGESRRQEPGFVKQ
Anti-GABA _A R $\alpha 5$	Rabbit, Thermo Fischer, PA5-31163	1:200	Recombinant fragment corresponding to amino acids 142 and 379 of human GABA _A R $\alpha 5$
Anti-GABA _A R $\gamma 2$	Goat, Santa Cruz, SC-131935	1:100	Extracellular domain of human GABA _A R $\gamma 2$
Anti-GABA _A R $\beta 3$	Mouse, Novus, NB-1-47,613	1:500	Peptide corresponding to amino acids 370-433 of mouse GABA _A R $\beta 3$
Anti-GAD65	Mouse, Millipore, MAB351	1:1000	Purified rat brain glutamic acid decarboxylase
Anti-GAD67	Mouse, Millipore, MAB5406	1:200	Recombinant GAD67 protein
Anti-GABA _B R R2	Mouse, NeuroMab, 75-124	1:400	Fusion protein amino acids 861-912 of rat GABA _B R R2
Anti-GAT1	Rabbit, Alomone, AGT-001	1:100	Peptide (C)ERNMHQMTDGLDK
Anti-GAT3	Rabbit, Alomone, AGT-003	1:100	Peptide (C)REARDKAVHERGH
Anti- β -actin	Rabbit, Abcam, ab8227	1:1000	Human β -actin amino acids 1-100
Anti- β -actin	Mouse, Abcam, ab6276	1:1000	Peptide DDDIAALVDNNGSGK

subunit R2, GAT1, GAT3, GAD65, and GAD67, were examined by Western blotting in the sensory and motor cortices, cerebellum, and human inferior (ITG), middle (MTG), and superior (STG) temporal gyrus (Figs. 1, 2, 3, 4, 5, 6, and 7).

In the sensory cortex, most GABA signaling components showed similar expression across the four age and gender groups; however, a significant sex-related GAD65 expression difference was observed (Fig. 2). GAD65 expression in young males was significantly higher compared to young females ($p = 0.0189$). The motor cortex did not show significant changes in the expression level of any of the GABA signaling components examined between the four groups (Fig. 3).

In the cerebellum, most GABA signaling components were well preserved during aging. One significant sex-related GAT1 expression difference observed

was that older females showed significantly higher expression of GAT1 compared to the older male group ($p = 0.0249$) (Fig. 4).

In the ITG, most GABA signaling components displayed similar expression level across all the groups (Fig. 5). However, significant age-related alteration was observed in GABA_AR $\beta 3$ subunit expression as older males show significantly higher $\beta 3$ subunit levels compared to young males ($p = 0.035$). Also, GAT1 expression was significantly higher in younger males compared to younger females ($p = 0.024$) (Fig. 5).

In the MTG, all GABA signaling components were well preserved across the examined four groups as no significant changes were observed (Fig. 6).

In the STG, significant sex-related changes were observed in expression of the GABA_AR $\alpha 1$ subunit, as males show much higher expression of this subunit

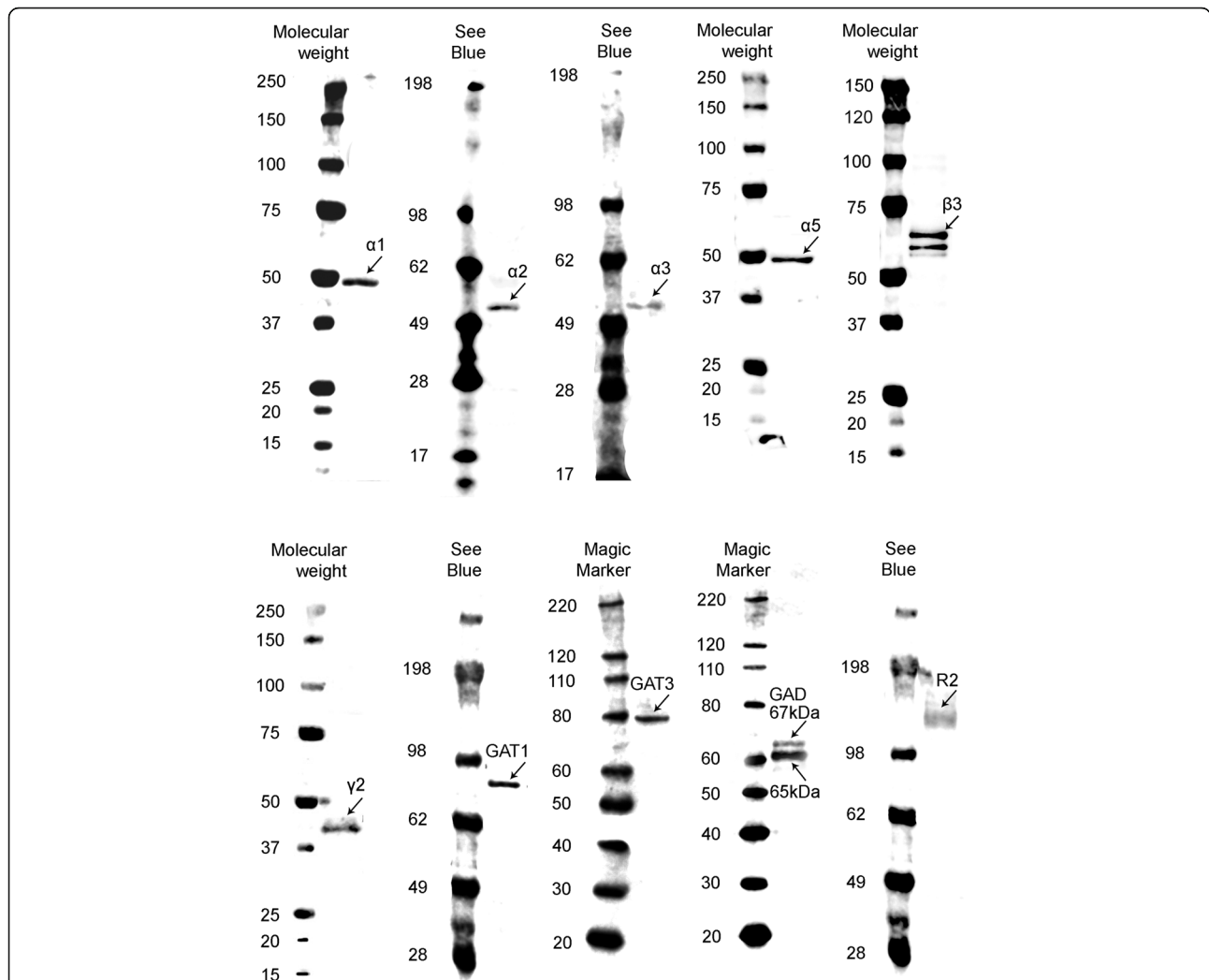
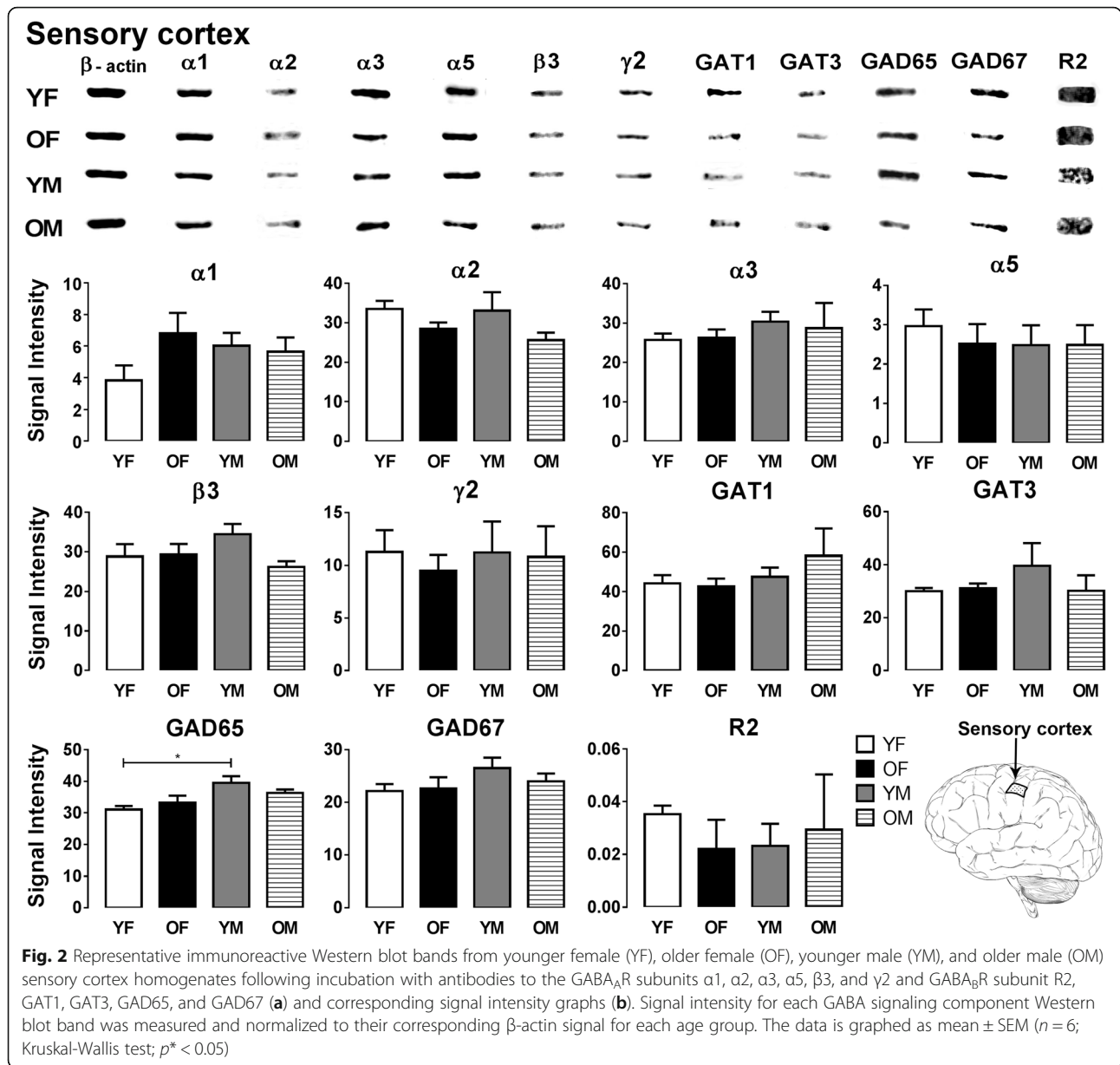


Fig. 1 Western blot against human brain protein homogenates probed with GABA_A receptor $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 3$, and $\gamma 2$ subunit, GAT1, GAT3, GAD65 and GAD67 and GABA_BR R2 subunit antibodies. Each lane has 20 μ g of protein loaded. Observed band sizes: $\alpha 1$, $\alpha 2$: ~ 52 kDa; $\alpha 3$, $\alpha 5$: ~ 55 kDa; $\beta 3$: ~ 63 kDa; $\gamma 2$: ~ 44 kDa; GAT1: ~ 85 kDa, GAT3: ~ 80 kDa; GABA_BR R2: ~ 120 kDa



compared to females in both age groups ($p = 0.049$ YF vs YM; $p = 0.018$ OF vs OM) (Fig. 7). Similarly, expression of GABA_AR α 2, α 5 and β 3 subunits is significantly higher in older males compared to the older female group ($p = 0.040$, α 2; $p = 0.010$, α 5; $p = 0.004$, β 3) (Fig. 7). The GABA_AR α 3 subunit and GAD65 showed age-specific expression changes in the STG. The α 3 subunit shows significantly lower expression in older males compared to younger males ($p = 0.035$) (Fig. 7). GAD65 expression was significantly higher in younger females compared to older females ($p = 0.019$) (Fig. 7). In the STG, all other GABA signaling components displayed similar expression level across all the groups (Fig. 7).

Discussion

In this study, we report that the GABAergic system in the human primary sensory and motor cortices, cerebellum, and ITG and MTG is generally well protected against sex- and age-related alterations. GABA_BRs are especially robust, with no expression level differences found between groups in any of the brain regions examined. The major finding of our study is the presence of strong sex-related differences in the STG, as well as a few minor differences in the other cortical areas examined, supporting the importance of accounting for sex differences between groups in future studies and the development or prescription of treatment therapies. The STG also displayed age-specific GABA_AR

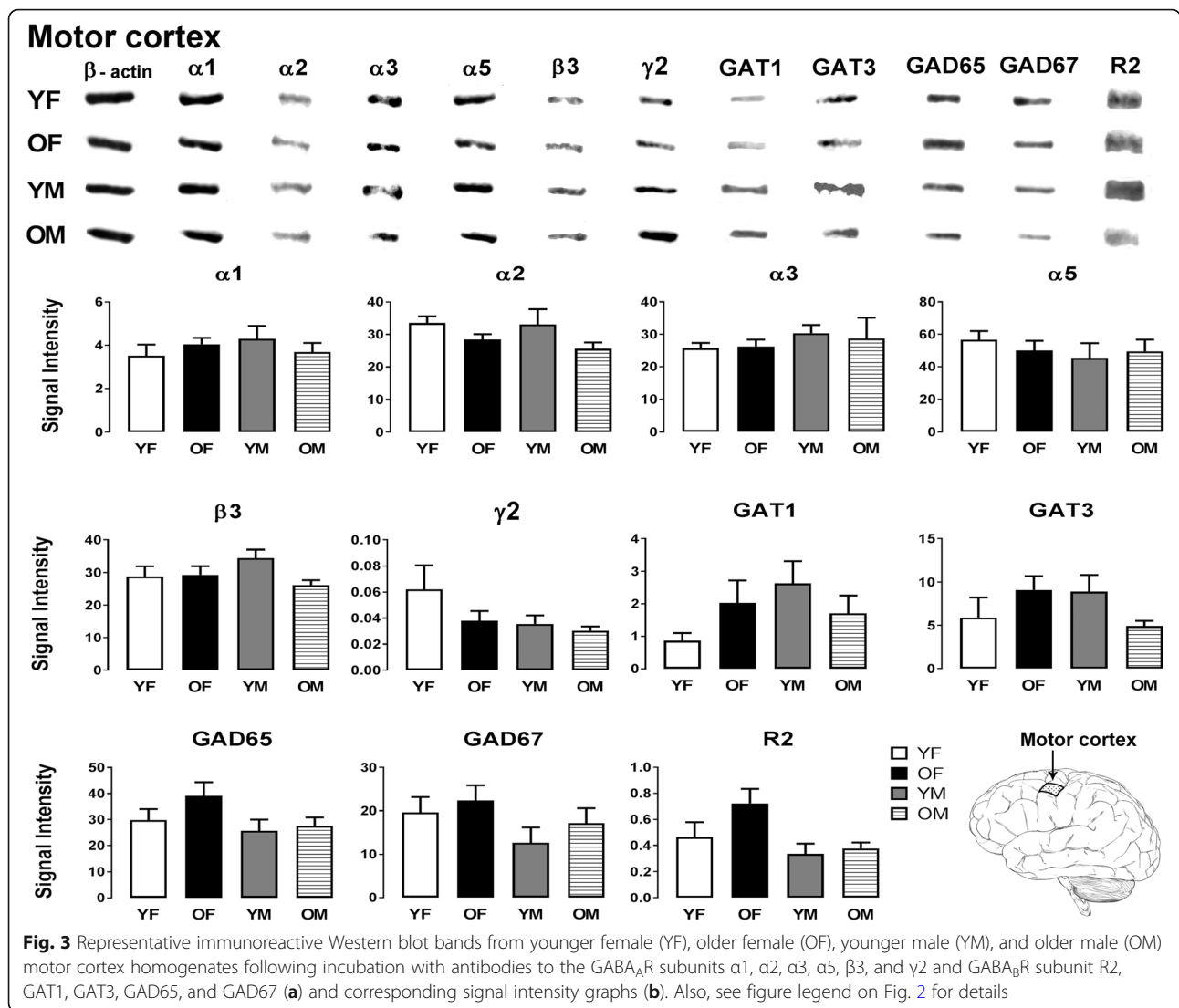
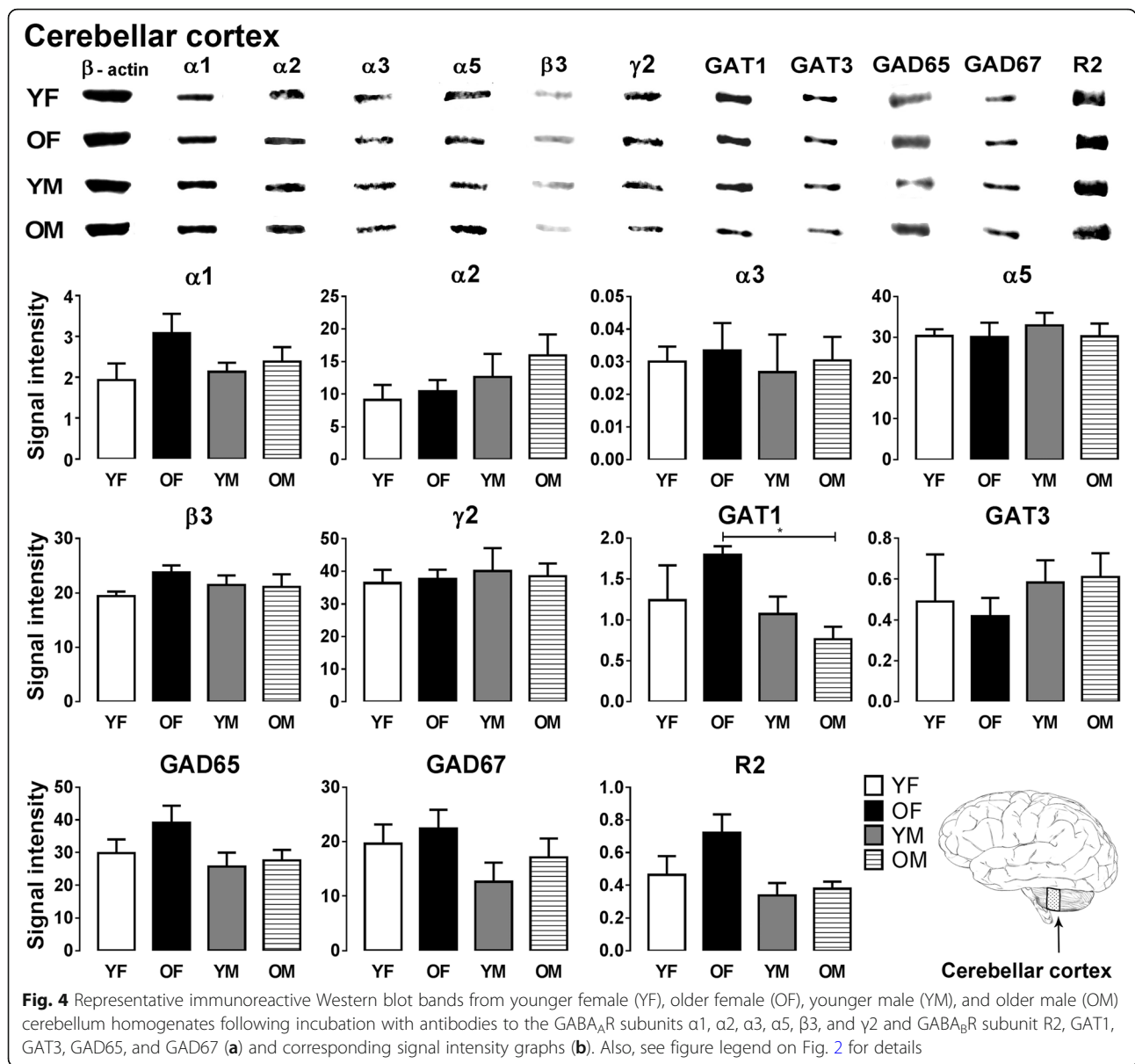


Fig. 3 Representative immunoreactive Western blot bands from younger female (YF), older female (OF), younger male (YM), and older male (OM) motor cortex homogenates following incubation with antibodies to the GABA_AR subunits α1, α2, α3, α5, β3, and γ2 and GABA_BR subunit R2, GAT1, GAT3, GAD65, and GAD67 (a) and corresponding signal intensity graphs (b). Also, see figure legend on Fig. 2 for details

subunit expression decrease in older males and GAD65 level decrease in older females. Previous literature suggests that GABA_AR and GAD level alterations may subsequently lead to compensatory changes in order to maintain homeostasis, and this may affect regional network functionality [2–9, 67–69]. This is the first study to explore the sex- and age-specific expression of GABA signaling components in the aforementioned brain regions and to predict the potentially resulting functional alterations.

Importantly, of all the cortical regions examined, the temporal lobe is the only one that displays an age-related decrease in GABA signaling component expression besides the observed sex-specific GABA_AR subunit alterations within the STG. The temporal lobes have a unique architecture and functional characteristics that make them particularly vulnerable to certain disease processes. They are interconnected through the anterior commissure, the

corpus callosum, and the hippocampal commissure, and these connections are among the underlying mechanisms that contribute to disease processes. The optic tract and radiation may also spread pathology from the optic chiasm to both temporal lobes via Meyer’s loop that passes through the STG [70, 71]. The STG is also the most proximal gyrus of the temporal lobe to the anterior commissure and has direct connections with the corpus callosum [71, 72]. Some disease processes have selective affinity to specific areas of the temporal lobe due to selective limbic system vulnerabilities that might be immune-mediated, related to sensitivity to hypoxia and aging [70, 73, 74]. The temporal lobe is the location of the primary auditory cortex, which is important for the processing and interpretation of sounds and language. This lobe integrates auditory, sensory, visual, and limbic function, including memory processing and formation.



Our current knowledge of GABAergic sex- and age-related alterations across different regions of the human brain, including the temporal lobe, is limited. Previous studies have reported conflicting findings and species differences in GAD and GABA level changes in pre-frontal cortical areas [2, 18, 19]. However, developmental GABAergic changes in the human visual cortex and across the lifespan are relatively well documented [75]. Our study showed significant reductions in GAD65 expression in the STG with age in females. This is in agreement with the previously reported slight decline in GAD65 expression in the visual cortex in older adults (> 55 years) [75] and rhesus macaque (19–20 years) [41]. In the STG, we observed a small non-significant increase in GAD67 expression in the older female group

compared with the younger group. A similar trend towards an increase in GAD67 levels has been reported in the humans [75] and a significant increase in GAD67 has also been observed in the rhesus macaque visual cortex [41]. While previous GAD65 knock-out mouse studies have demonstrated no GAD67 or GABA level deficits [76, 77], several studies suggest that GAD65 loss may result in region-specific hyperexcitability and functional implications, such as susceptible to seizures [76–80]. GAD65 is involved in rapid GABA release and provides most of the GABA for neurotransmitter release, and under pathological conditions, GAD67 could play a similar role. Previous studies imply a consequential increase in vesicular GABA transporter (VGAT) expression in response to GAD65

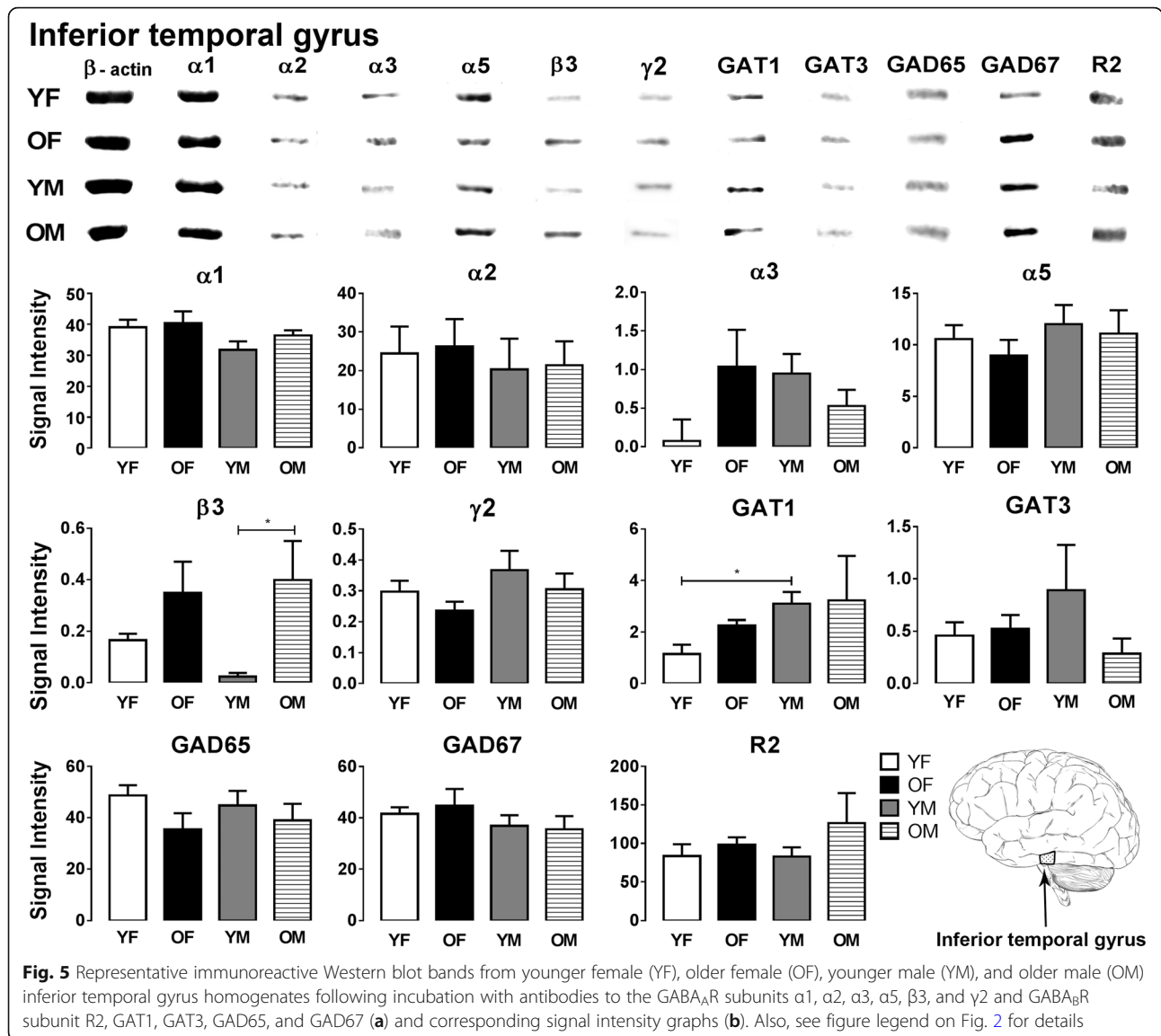
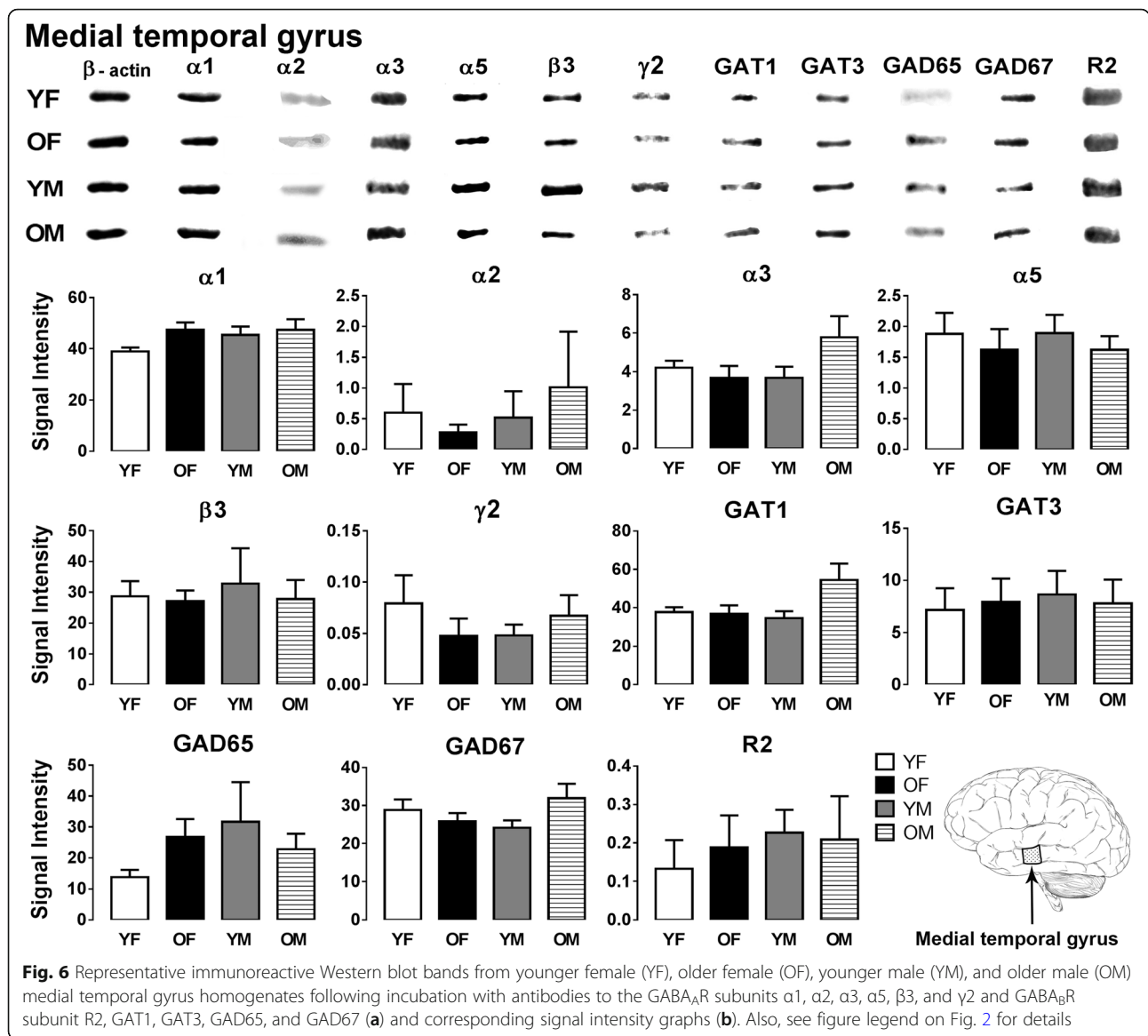


Fig. 5 Representative immunoreactive Western blot bands from younger female (YF), older female (OF), younger male (YM), and older male (OM) inferior temporal gyrus homogenates following incubation with antibodies to the GABA_AR subunits α 1, α 2, α 3, α 5, β 3, and γ 2 and GABA_BR subunit R2, GAT1, GAT3, GAD65, and GAD67 (a) and corresponding signal intensity graphs (b). Also, see figure legend on Fig. 2 for details

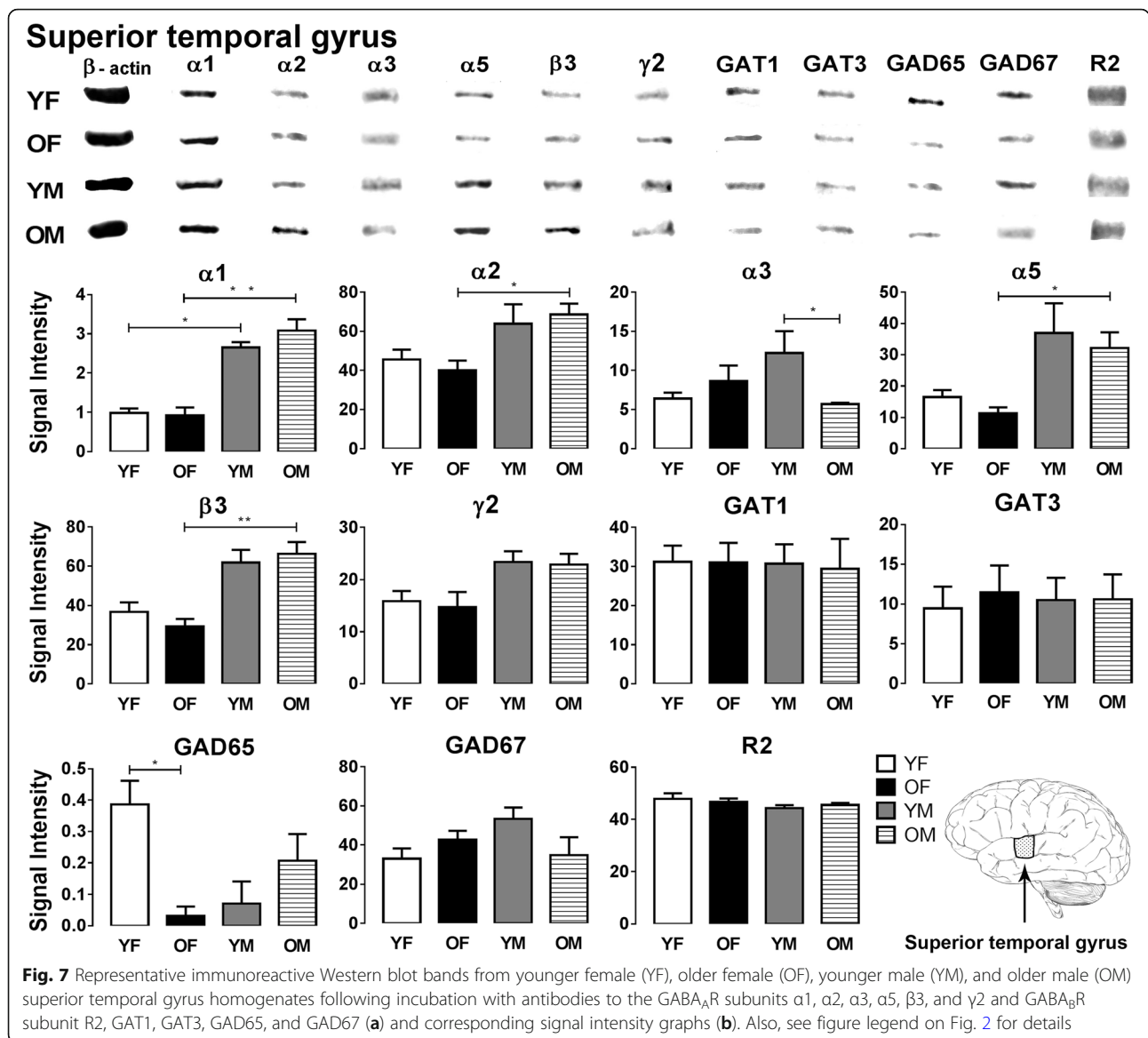
knock-out [81]. Upregulation of VGAT, however, only partially contributes to increased GABA uptake into vesicles and GAD67 might play an important compensatory role [81]. The age-related decrease in the expression of GAD65 in the STG and visual cortex and the reciprocal increase in GAD67 expression might be the result of a shift in the expression of GAD67 to compensate for the effect of GAD65 loss on GABA synthesis [82, 83]. However, the functional implications of this age-related GAD65 expression decrease in the older female group are difficult to predict, and further functional studies will be required to understand the physiological consequences of this change. However these changes might underlie age-dependent disease susceptibility and influence the progression of Alzheimer’s disease, epilepsy, or schizophrenia, conditions in which the fine balance of excitation and inhibition is impaired.

GABA_AR subunit densities exhibit varying expression profiles in different regions of the human cerebral cortex [24]. As mentioned earlier, our study is the first to examine age- and sex-related changes in the expression of GABA_AR subunits in the temporal gyri, and we demonstrate that the STG displays the greatest magnitude of age- and sex-related changes in GABA_AR subunit expression. Importantly, only a few previous studies have reported sex-specific changes in the expression of GABA_AR subunits in the primate brain [41], and the lack of human data warrants further research in this area. Examination of the STG demonstrated a sex-related difference in β 3 subunit expression; older males showed significantly higher expression compared with older females, and a similar trend has been observed between younger females and younger males as well. In the ITG, the β 3 subunit displayed age-specific



expression changes; older males had significantly higher β 3 subunit levels compared with younger males, with a similar trend between the younger and older female groups. Previous results from a human dorsolateral PFC study showed a relatively stable β 3 subunit expression with aging, but the average age of the oldest age group in the study was only 43 years of age [84]. However, we can also deduce that the differential expression pattern observed in our study might be due to regional differences in the regulation of subunit expression. The α 3 subunit also shows age-related expression changes in the STG; in younger males, the expression was significantly higher compared with older males. We observed a sex difference in α 1 subunit expression in the STG as males show significantly higher expression than females in both age groups. Furthermore, α 2, α 5, and

β 3 subunit expression is significantly higher in older males compared with older females, and a similar trend has been also observed between the younger male and female groups. The STG contains the transverse gyrus of Heschl and Wernicke's area that are involved in the processing of auditory sensory information. Based on all the expression changes found in the STG, we might suspect a sex- and age-related influence on auditory function. Evidence based on animal experiments shows that synaptic inhibitory mechanisms in the auditory cortex are particularly vulnerable to aging [26]. Results from in situ hybridization studies show an age-related reduction in the GABA_AR α 1 subunit transcript across all layers of the auditory cortex. An age-related increase in α 3 subunit expression was observed in a subset of layers of the auditory cortex (layers II and III). The



GABA_AR β 1, β 2, γ 1, γ 2s, and γ 2L subunits also showed age-related declines at the mRNA and protein levels [26]. Other changes, such as the loss of GAD65 and GAD67 in the auditory cortex and increased spontaneous neuronal activity in the inferior colliculi and auditory cortex, have also been observed, leading to a synergistic loss of GABA signaling components and impaired auditory function in aged rats [85, 86]. As mentioned before, we also observed GAD65 loss in the STG, but GAD67 levels are well preserved in the human secondary auditory cortex. The discrepancy between the rat and human data might be the result of species differences or the techniques used, as mRNA expression is not necessarily proportional to protein levels.

Data from sex-differentiated studies suggest that the etiology and progression of age-related hearing

loss (presbycusis) differs in males and females with age [12]. The magnitude of sex-related GABA_AR subunit changes observed in the STG in this study might contribute to sex-specific hearing loss with aging, besides many other possible factors. The differential expression patterns of GABA_AR subunits affects GABA binding affinity and the downstream function of the receptor, altering neuronal excitability and the activity of neuronal networks [22]. We hypothesize that the GABA signaling component expression changes in the STG might contribute to alterations in higher auditory information processing and have an effect on memory formation and processing. However, peripheral hearing impairment might also lead to central molecular and cellular changes, including the GABAergic system. As age-related hearing loss displays sex-specific

differences, these alterations might explain why the STG is the area most vulnerable to sex-specific GABAergic changes.

The STG is strongly implicated in the pathophysiology of schizophrenia, particularly with regard to auditory hallucinations [87–90]. A significant (30%) increase in the binding of [3H] muscimol in the STG has been observed in schizophrenia patients compared with control subjects, suggesting an increase in GABA_AR density in the STG in this disease [91]. Previous studies demonstrated that working memory dysfunction in schizophrenia is mediated by altered GABAergic neurotransmission in certain dorsolateral prefrontal cortex microcircuits. Subjects with schizophrenia exhibited expression deficits in GABA signaling-related mRNA transcripts; the down-regulation of GAT1 in the presynaptic terminals of parvalbumin-containing chandelier neurons [92]; the up-regulation of the GABA_AR α 2 subunit in the postsynaptic axon initial segments of pyramidal neurons [93]; deficits in GAD67 and VGAT [94]; neuropeptides (somatostatin, neuropeptide Y and cholecystokinin); and the GABA_AR α 1, α 4, β 3, γ 2, δ [94, 95] and α 5 subunits [96]. Age- and sex-related differences are present in schizophrenia, but the mechanisms underlying these require further investigation [11, 97–102]. The sex- and age-specific differences in GABA_AR and GAD65 levels observed in the STG in this study might play a crucial role in the pathogenesis of schizophrenia and in disease susceptibility, but a direct link will have to be established.

GABA_AR subunit expression changes have been reported in other disease conditions such as epilepsy and Alzheimer's disease [27, 103]. Several studies have reported sex-specific susceptibility to the development of specific epilepsy subtypes, particularly in temporal lobe epilepsies in females [104–106]. The impairment of GABA_A receptor-mediated inhibition causes an increase in neuronal excitability and plays a critical role during epileptogenesis [107, 108]. The sex-specific reduction in GABA_AR α 1, α 2, α 5, and β 3 subunit expression observed in females in this study might be a factor underlying their higher susceptibility for temporal lobe epilepsies. Some sex hormones and neuroactive steroids are potent activators of GABA_ARs and can therefore change the expression of some GABA_AR subunits [57, 58, 108, 109]. Interestingly, sex steroids do not seem to influence the expression of the examined GABA_AR subunits during aging in females; despite the fact that some of the younger females might have been premenopausal while others post-menopausal, the hormonal levels did not lead to greater variation in GABA_AR subunit expression levels and we have not observed any subunit alterations between the younger and older female group. Animal studies have demonstrated GABA_AR subunit expression alterations during the

estrus cycle and pregnancy, although these studies have mostly implicated extrasynaptic δ subunit-containing GABA_ARs [108, 109]. Importantly, these changes have been linked to altered tonic inhibition and seizure susceptibility, anxiety, and depression [108, 109].

It is accepted that women are more likely to develop anxiety and depression than men [10, 110]. Benzodiazepines, allosteric modulators of GABA_AR function, are widely used as therapeutic agents for the treatment of anxiety [111], depression [112], and insomnia [113]. The elderly are more sensitive to the side effects of benzodiazepines, and poisoning may occur as a result of long-term use [114]. We found significant age-specific differences in the expression of GAD65 and the GABA_AR α 3 subunit, as well as sex-specific differences in GABA_AR α 1, α 2, α 5, and β 3 subunit expression levels in the STG. This suggests that the well-established α 1/2/5 β 2/3 γ 2 subunit containing benzodiazepine-sensitive receptors are upregulated in males. These findings highlight that besides differences in drug absorption, bioavailability, distribution, metabolism, and hormone balance between the sexes and between age groups [114–116], sex- and age-specific alterations in GABAergic signaling components throughout the brain should be considered in the use and prescription of benzodiazepines as they might influence the effect of these agents.

GABA_BRs did not show expression level differences between sexes and age groups in any of the brain regions examined. The reason why the GABA_BRs are spared is not known mainly due to the limited number of studies in the field. However importantly, in other brain regions, these receptors might be affected by aging or display sex-specific expression, and the robustness observed in our study might not be a general phenomenon. For example, in the macaque visual cortex, the GABA_BR R2 subunit is upregulated with age [41].

GABA transporters are essential for the maintenance of GABA levels in the synaptic cleft. We have found that younger females have significantly lower GAT1 expression compared with younger males in the ITG. GAT1 expression in the cerebellum displayed a significant sex difference, with the older female population displaying significantly higher levels of GAT1 expression than older males. GAT1 knock-out mice exhibit prolonged inhibitory post synaptic currents in cerebellar granule cells due to reduced GABA clearance from the synaptic cleft and symptoms of ataxia, disturbed thermoregulation, and circadian rhythm and tremor [117]. In comparison to GAT1 expression, we found that GAT3 showed different expression pattern in the cerebellar cortex and sensory cortex. Older females with the highest cerebellar GAT1 expression showed the lowest GAT3 levels, and older males with the lowest cerebellar GAT1 levels showed the highest GAT3

expression. High GAT1 expression is observed on interneurons whereas GAT3 is expressed mainly on astrocytes [9, 118]. These results suggest that the GAT3 upregulation in astrocytes might occur as a compensatory mechanism, but future studies using cell-type-specific markers have to be performed to test this hypothesis. Previous studies conducted in the developing mouse [119] and in perinatal hypoxia [15, 120], schizophrenia [121], and Alzheimer's disease [9] reported similar compensatory mechanisms, and these are essential for the maintenance of GABA levels in the synapse.

In neurodegenerative disorders like Alzheimer's disease, sex difference has been well documented. The mechanisms underlying AD are not well understood, but aging is considered to be the leading risk factor for the disease [60–62, 110, 122–124]. Sex- and age-specific changes in key molecular components of the major transmitter systems, as described in this study, could account for the effects of sex and age on the disease, or they might be factors that influence disease prevalence and progression. In Alzheimer's disease, the GABAergic system also undergoes significant remodeling. The STG shows downregulation of GABA_AR α 2 and α 5 subunits, and the sex-specific downregulation of these receptors in females might be implicated in disease susceptibility and the faster disease progression observed within the female population [8, 9, 27, 67]. The lack of a clear understanding of sex- and age-related disease pathology in neurodegenerative diseases, as well as in other neurological disorders, like schizophrenia, epilepsy, depression, and anxiety, suggests for the importance of the inclusion of sex and age as case selection criteria or experimental parameter in the design and interpretation of all such studies, to prevent the effect of these parameters as confounding factors and to aid in improving our knowledge of the etiology, progression, and treatment of these disorders.

Conclusions

Aging is associated with molecular, cellular, and structural changes in the brain leading to functional changes, cognitive decline, and increased vulnerability to neurological diseases, neurodegenerative conditions, sensory retrogression, and depression, just to name a few. Our study highlights that age-related GABAergic changes are brain region specific; most cortical areas are not affected. However, in the temporal lobe, we identified dramatic GABA_AR subunit and GAD65 expression changes besides several sex-specific differences. With increasing life expectancy and the dramatically growing elderly population, understanding the mechanism and consequences of aging is critically important. There is also growing evidence that GABAergic system-specific sex differences might influence disease prevalence and

progression and possibly has to be considered when designing new preventive and therapeutic options for these conditions.

Abbreviations

AD: Alzheimer's disease; EDTA: Ethylenediaminetetraacetic acid; GABA: Gamma-aminobutyric acid; GABA_AR: GABA type A receptor; GABA_BR: GABA type B receptor; GABAR: GABA receptor; GAD: Glutamic acid decarboxylase; GAT: GABA transporter; ITG: Inferior temporal gyrus; MTG: Middle temporal gyrus; OF: Older female; OM: Older male; PBS: Phosphate-buffered saline; PBST: Phosphate-buffered saline, 0.2% Triton X-100; PFC: Prefrontal cortex; SDS: Sodium dodecyl sulfate; STG: Superior temporal gyrus; TBST: Tris-buffered saline, 0.1% Tween; YF: Younger female; YM: Younger male

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Availability of data and materials

All datasets generated or analyzed during the study are included in the published article.

Authors' contributions

AK and RLF designed research; MP, THP, AK, and HJW performed research; BJLS and CT performed pathological assessment; AK, MP, and HJW wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The tissue was acquired through a donor program to the Neurological Foundation of New Zealand Human Brain Bank, and the procedures were approved by the University of Auckland Human Participant's Ethics Committee (Approval number: 011654).

Consent for publication

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Competing interests

The authors declare that they have no competing interest.

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