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Alterations in the transcriptional profile of genes related to glutamatergic signalling in animal models of Alzheimer's disease. The effect of fingolimod

Dedicated in memory of our colleague dr Henryk Jęśko (1975-2021)

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Abstract

Alzheimer's disease (AD) is a multi-factorial illness that leads to progressive cognitive impairment. A glutamatergic system dysfunction has been reported to be implicated in the pathomechanism of AD. Therefore, in the current study we characterized the transcriptional profile of glutamate-related genes in transgenic AβPP V717I (TgAD) and sporadic (SAD, streptozotocin-induced) models of AD. Genes encoding glutamate membrane-bound (GLAST, GLT1, EAAC1) and vesicular (VGLUT1-3) transporters as well as ionotropic (AMPA, NMDA) and metabotropic (mGluR3, mGluR5) receptors were analysed. Based on qPCR analysis, we observed a discrepancy between TgAD and SAD mice in the profile of targeted genes. We noticed age-dependent upregulation of genes encoding VGLUT1, NMDAR1 and mGluR3 in 12-month-old TgAD mice. In the SAD model upregulation of genes encoding AMPAR1 and NMDAR1 as well as downregulation of GLAST, VGLUT3 and mGluR5 were found. Next, the effect of fingolimod (FTY720) was indicated. In the TgAD model, the drug reversed altered transcription of the mGluR3 glutamate receptor to the control level, whereas in the SAD model it downregulated the genes encoding VGLUT1, AMPAR2 and mGluR3. Interestingly, FTY720 influenced mGluR3 mRNA in both examined models. Observed alterations of gene transcription and the effects of FTY720 may potentially constitute an interesting target for further pharmacological studies.

Key words: *Alzheimer's disease, sphingosine-1-phosphate, streptozotocin, glutamate transporters, glutamate receptors, FTY720.*

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory impairment and cognitive failure, which lead to the most severe dementia in aged populations worldwide. According to the amyloid hypothesis of AD, senile plaques from Aβ (amyloid β) oligomers as well as the formation of neurofibrillary tangles are characteristic neuropathological features of this disease and the main cause of synaptic loss and neuronal degeneration [52]. In parallel, progressive impairment of neuro-

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transmission, particularly cholinergic, glutamatergic and aminergic transmission is also observed [41]. An array of experimental data indicates that the glutamate excitotoxicity, i.e. glutamate-mediated neurotoxicity, is one of pathomechanisms responsible for neuronal cell death during the course of AD. The loss of glutamatergic neurons in layers III and IV of the neocortex, as well as damage of glutamatergically-innervated cortical and hippocampal neurons has also been observed [1]. Evidence also exists that ionotropic glutamate receptors (iGluRs), particularly N-methyl-D-aspartate (NMDA) receptors, appear to be targeted by Aβ oligomers [12] leading to the suppression of cognitive processes.

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS), whose extracellular level is tightly regulated in order to shape excitatory postsynaptic currents, limit glutamate signalling and prevent overstimulation of glutamate receptors, which uncontrolled, may lead to excitotoxicity. Glutamate present in axonal cytoplasm of glutamatergic neurons is packed by the vesicular glutamate transporters (VGLUTs), driven by a proton electrochemical gradient generated by the vesicular H^+-ATPase, into the synaptic vesicles where it is stored until release. Following the release from the glutamatergic nerve endings, glutamate activates functionally distinct iGluRs and metabotropic glutamate receptors (mGluRs) located in different parts of the synapse. After release by Ca^{2+}-dependent exocytosis and transduction of signals through an array of its receptors, glutamate is cleared from the synaptic cleft by the specific high affinity sodium-dependent transporter (GluT) systems in the glutamate-glutamine cycle based on neuron-glia interactions. In the tripartite synapse, glutamate is predominantly taken up by the GLT-1 transporter which is highly expressed on astroglial cells. Other types of GluTs such as GLAST, EAAC1 and EAAT4 contribute to this effect to a lesser extent. In astrocytes, glutamate is metabolized to glutamine, which is transported into neurons and converted back into glutamate therein. Dysfunctional transport systems lead to overflow of extracellular glutamate and produce an agonist-evoked response of its receptors. Excessive stimulation of NMDA receptors and subsequent influx of calcium are the primary events associated with excitotoxic neuron injury [10].

Despite the extensive efforts of scientists and a large number of tests with new agents, until today there has been no successful therapy for AD. One of the drugs used with moderate positive effect in the treatment of AD is memantine, a non-competitive antagonist of glutamatergic NMDA receptors. Under conditions of excitotoxicity-mediated prolonged activation, memantine effectively blocks the activity of glutamate receptors [19].

Since glutamate is tightly connected with pathomechanisms operating during AD, it is reasonable to search for agents which target this pathway.

Fingolimod (FTY720) is a drug whose action relies on modulation of the sphingolipid signalling pathway, which is ubiquitous in the CNS. After being phosphorylated by sphingosine kinase, FTY720 binds to G protein-coupled sphingosine-1-phosphate receptors (S1P1-S1P5 with exception of S1P2) and promotes activation of the pro-survival signalling pathway mainly PI3K/Akt through S1P1 and S1P3 receptors [5,11,23,35,46,50]. Activation of those receptors has been shown to exert neuroprotective functions, including a positive influence on cellular levels and secretion of amyloid-β protein precursor (AβPP) in a murine model of AD [54].

Current therapeutic application of this substance is based on its immunomodulatory activity and, it has been recently approved by the Food and Drug Administration for the treatment of multiple sclerosis (MS) [7]. Epigenetic mechanisms of FTY720-induced regulation of histone acetylation has been also reported. These mechanisms, independent of drug immunosuppressive action, were found to be associated with regulation of several transcription factors and gene expression, improvement of learning and memory, as well as reduction of neuronal damage in an experimental model of AD [2,35,36]. Moreover, FTY720 has been shown to be equally effective as the memantine in partially reversing changes in expression of several genes such as mitogen activated protein kinases (MAPKs) after injection of Aβ [32].

Hence, the protective activity of FTY720 should be further explored in the context of AD therapy, particularly since it has been reported to exert a positive effect against excitotoxic cell death, which is among the pathomechanisms operating during the course of this disease [16].

Therefore, in the present study, we focused on the effect of fingolimod (FTY720) on the transcription of genes involved in the homeostasis of the glutamatergic system in brains of AD mice. The expression

of genes encoding cellular (GLT-1, GLAST, EAAC1) and vesicular (VGLUT1-3) glutamate transporter systems, as well as selected types of ionotropic glutamate receptors (AMPA1, AMPA2, NMDA1) and metabotropic glutamate receptors (mGluR3, mGluR5) was examined in animal models of AD (genetic and sporadic form).

Mice expressing V717I AβPP under the control of a neuron-specific promoter were used as a model of the genetic form of AD. The familial/early-onset AD-linked V717I "London" AβPP mutation results in AβPP cleavage into Aβ thereby increasing the highly neurotoxic isoform Aβ42 [47], which correlates with early onset of the disease. The V717I "London" mutation of APP has been reported in numerous cases of familial AD [65]. This model recapitulates a relatively broad set of AD-specific histochemical, behavioural, electrophysiological, and biochemical features appearing in an age-dependent sequence [44]. Furthermore, age-dependent progressive increase of both soluble and insoluble Aβ40 and Aβ42 levels are observed in brain extracts of London APP mice, where soluble Aβ42/40 ratios increased up with age, whereas insoluble Aβ42/40 ratios were 5-10 times higher [14,55]. The gradual appearance of Aβ42-enriched deposits formed near acetylcholinesterase-immunoreactive structures was confirmed. Moreover, V717I mice reproduce morphological patterns, ultrastructural aspects, and biochemical composition of the vascular amyloid deposition described in human cerebral amyloid angiopathy [6,60]. Impaired NMDA-dependent long-term potentiation and decreased NMDA-receptor activation in hippocampal CA1 region have been demonstrated at a pre-plaque age stadium of London transgenic mice [55].

As a model of sporadic AD, we used the widely accepted streptozotocin-induced model. Streptozotocin (STZ) is a glucose-amine derivative of nitrosourea which has been found to cause prolonged impairment of brain glucose and energy metabolism accompanied by impairment of learning and memory, and decreased choline acetyltransferase levels in the hippocampus [24,33]. When administered into a rodent's brain by intracerebroventricular injection, it induces cerebral aggregation of Aβ and increased levels of total tau protein. These changes are accompanied by neuroinflammation, oxidative stress, biochemical alterations, death of neuronal cells and spatial learning deficit [3,13], reflecting sporadic AD-like pathology.

Material and methods

Animal models of Alzheimer's disease

The animals were housed under specific pathogen-free (SPF) conditions with controlled temperature and humidity and a 12-h light/dark cycle in the Animal House of the Mossakowski Medical Research Centre PAS, Warsaw, Poland. Efforts were made to reduce the number of animals and their distress during the experiment. All procedures were carried out in accordance with the EC Council Directive (86/609/EEC) following the ARRIVE guidelines and were approved by the IV Local Ethics Committee in Warsaw (approval no. 67/2015). The procedures using genetically modified organisms (GMO) were approved by the Ministry of Environment (approval no. 139 of 22/8/2016).

Two animal models were used:

Transgenic model of AD: Female FVB-Tg(Thy1; APP LD2/B6) mice, 3 months old (mature) and 12 months old (old), were used throughout the study. In the transgenic model of AD, the mice overexpress human AβPP with the "London" V717I mutation under control of a fragment of Thy1 promoter with specificity towards brain and spinal cord neurons (APP^+). Mice without the transgene were used as controls (APP^-).

Sporadic model of AD: The streptozotocin (STZ)-induced experimental model in rodents mimics the human age-related AD pathology of the sporadic type. STZ (10 µl, 2.5 mg/kg b.w.), dissolved in artificial cerebrospinal fluid (ACSF), was injected bilaterally, slowly (1 µl/min) into the *ventriculus lateralis cerebri* of 3-month-old mice (females, C57BL/6J) under anaesthesia (ketamine 70 mg/kg b.w. + xylazine 6 mg/kg b.w.). Then injection of STZ was repeated 48 h later. Control animals were identically treated with the same volume of ACSF.

Experimental design

Both 3-month-old mice and 12-month-old mice were treated daily with fingolimod (FTY720) (Cayman Chemical, Ann Arbor, Michigan, USA), a sphingosine analog and an S1P receptor modulator for 2 weeks. Before injection, the animals were weighed to assess the correct doses. FTY720 dissolved in 0.9% NaCl, was administered intraperitoneally in a dose of

1 mg/kg b.w., which was selected based on our previous studies [36]. Controls (ACSF + STZ, APP^{-} and APP^{+}) received the appropriate vehicle. In case of STZ-mice, the administration of the drug started on the same day as injection of STZ. One day after the last treatment, animals were sacrificed by decapitation to isolate brain tissue.

Analysis of gene expression by qPCR

Brain cerebral cortices were isolated from animals and put on ice and flash-frozen in liquid nitrogen. Total RNA was extracted using TRI-reagent (Sigma-Aldrich, St. Louis, MO, USA) and its purity was assessed spectrophotometrically. Reverse transcription of 2 µg of total RNA was performed using Applied Biosystems' High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems, Forest City, CA, USA). The qPCR reaction was run on a Light Cycler® 96 System (Roche Diagnostics GmbH, Mannheim, Germany) using 5 µl of RT product and the TaqMan Gene Expression Assay kit (the total volume 20 µl) and the following cycling conditions: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Each sample was analysed in triplicate. The relative level of mRNA was calculated against Actin beta (*Actb*) as a reference gene on the basis of the $^{\Delta\Delta}$Ct method. The specific mouse primers obtained from Applied Biosystems (Foster City, CA, USA) are listed in Table I.

Statistical analysis

The results of mRNA analysis are presented as means ± SEM from the experiments performed using the number of animals indicated under the figure. Differences between groups were assessed by one- (STZ-mice) or two-way (APP-mice) analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *P* values < 0.05 were considered significant. Analysis was performed using GraphPad Prism Software, version 6.0 (San Diego, CA, USA).

Results

Transcriptional changes in glutamate transporter systems in brain cortex of SAD and TgAD mice. The effect of FTY720

The mRNA levels of cellular excitatory amino acid (EAATs) and vesicular (VGLUTs) transporters were analysed in the mice model of genetic and sporadic AD. A number of genes were included in the analysis whose expression is important for maintaining the proper extra- and intracellular concentration of glutamate. Genes from the *Slc* family such as *Slc1a2*, *Slc1a3* and *Slc1a1* which encode transporters operating in the cellular membrane, i.e. GLT-1, GLAST and EAAC1 were examined. Among *Slc* genes encoding transporters operating in the vesicular compartment, *Slc17a7* (VGLUT1), *Slc17a6* (VGLUT2), *Slc17a8* (VGLUT3) were investigated.

The profile of changes in the expression of analysed genes was found to be different depending on the model. In the SAD model we revealed changes in the expression of *Slc1a3* (GLAST) and *Slc17a8* (VGLUT3) whose mRNAs significantly decreased relative to control (ACSF-treated) mice (Figs. 1 and 2). The expression of other *Slc* genes (*Slc1a2* (GLT1), *Slc1a1* (EAAC1), *Slc17a7* (VGLUT1), *Slc17a6* (VGLUT2)) were unaltered in STZ-injected mice compared to control animals treated with ACSF (Figs. 1 and 2). Administration of FTY720 produced significant effects in the SAD model by reduction of *Slc17a7* (VGLUT1) mRNA levels compared to controls (Fig. 2).

The presence of the AβPP transgene did not significantly change the mRNA levels of EAATs genes either in the cortex of the mature (3-month-old) or aged (12-month-old) animals (Fig. 1) compared with APP^{-} mice. Significant upregulation of *Slc17a7* (VGLUT1) was the only change observed in the brain cortex of 12-month-old APP^{+} mice (Fig. 2). Administration of FTY720 was found to increase the levels of *Slc1a2* (GLT1) and *Slc17a7* (VGLUT1) mRNA in 12-month-old APP^{-} mice but did not exert a similar effect in the APP^{+} mice (Figs. 1 and 2).

Table I. Specific primers used for qPCR analysis

Transporter/receptor	Gene	Primer
GLAST (EAAT1)	*Slc1a3*	Mm 00600697_m1
GLT1 (EAAT2)	*Slc1a2*	Mm 01275814_m1
EAAC1 (EAAT3)	*Slc1a1*	Mm 00436590_m1
VGLUT 1	*Slc17a7*	Mm 00812886_m1
VGLUT 2	*Slc17a6*	Mm 00499876_ m1
VGLUT 3	*Slc17a8*	Mm 00805413_m1
NMDA1	*Grin1*	Mm 00433790_m1
GluR1 (AMPA1)	*Gria1*	Mm 00433753_m1
GluR2 (AMPA2)	*Gria2*	Mm 00442822_m1
mGluR3	*Grm3*	Mm 00725298_m1
mGluR5	*Grm5*	Mm 00690332_m1

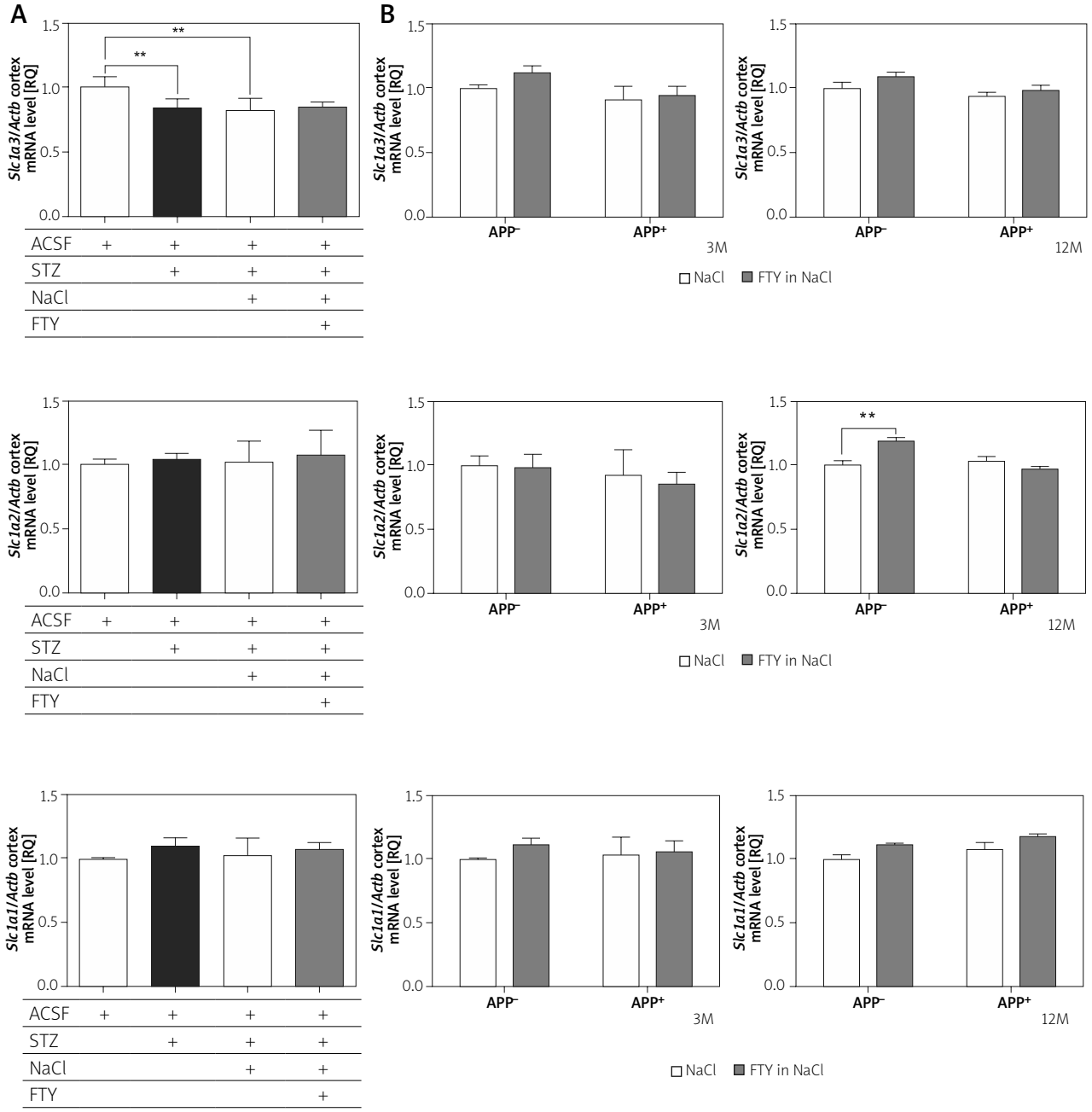

Fig. 1. Transcriptional changes of excitatory amino acid transporters: *Slc1a3* (GLAST), *Slc1a2* (GLT-1), *Slc1a1* (EAAC1) in the brain cortex of SAD and TgAD mice: **A**) 3-month-old STZ mice and **B**) 3- and 12-month-old *AβPP* (V717I)-transgenic mice. The effect of fingolimod. Expression of *Slc1a3*, *Slc1a2*, *Slc1a1* mRNA was measured with real-time PCR in the brain cortex of 3-month-old STZ mice and *AβPP*-transgenic mice at the age of 3 and 12 months (3 M and 12 M) as well as in appropriate controls. *Slc1a3*: $^{**}p < 0.01$ ($n_a = 6$, $n_b = 3$-5), *Slc1a2*: $^{**}p < 0.01$ ($n_a = 3$-4, $n_b = 3$-5), *Slc1a1*: ($n_a = 3$-4, $n_b = 3$-5) as compared to the corresponding controls (ANOVA with post-hoc Tukey's test). The relative level of mRNA was calculated against Actin beta (*Actb*) as a reference gene (RQ – relative quantification = $2^{-\Delta\Delta Ct}$).

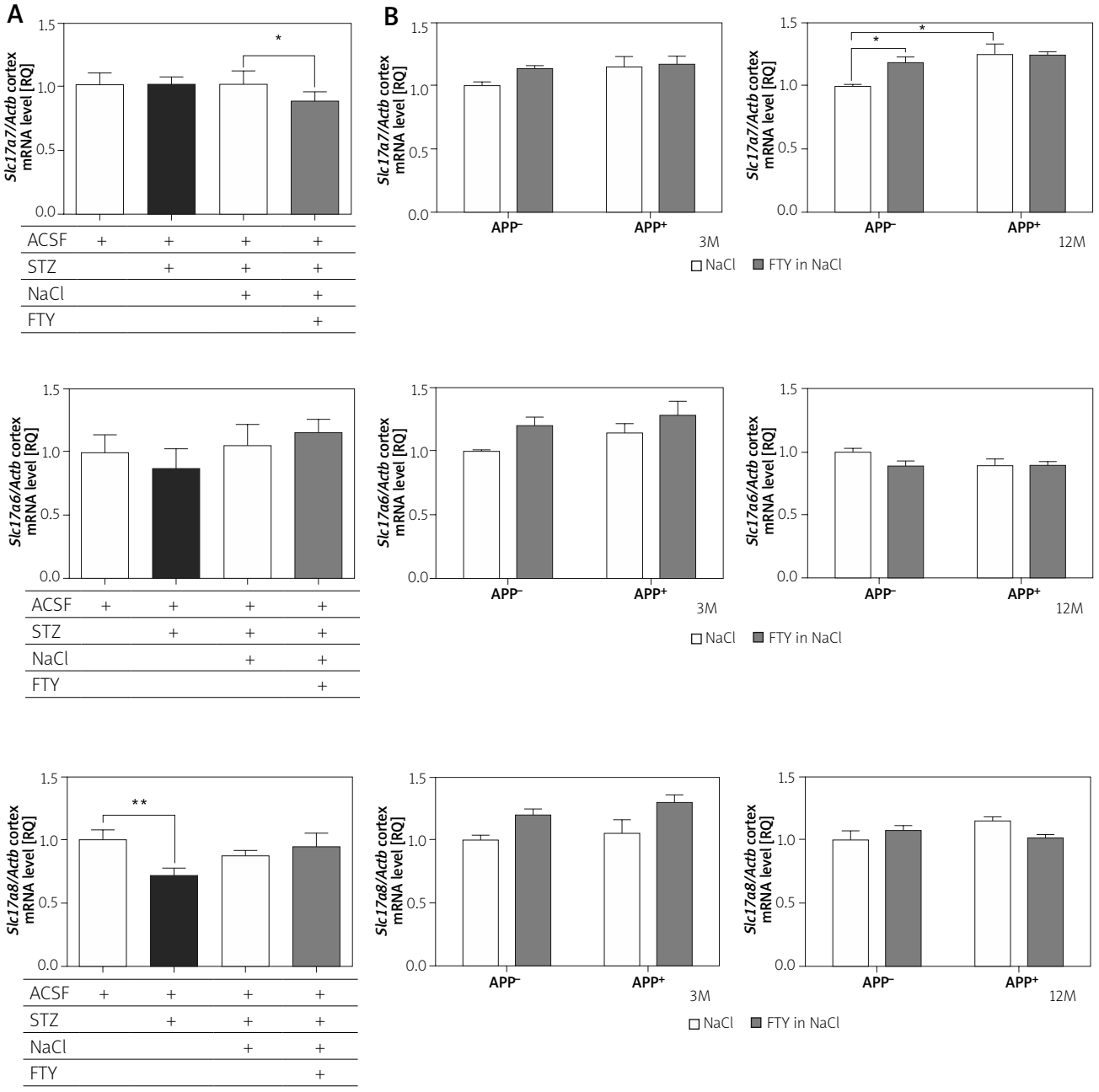

Fig. 2. Transcriptional changes of vesicular glutamate transporters: *Slc17a7* (VGLUT1), *Slc17a6* (VGLUT2), *Slc17a8* (VGLUT3) in the brain cortex of SAD and TgAD mice: **A**) 3-month-old STZ mice and **B**) 3- and 12-month-old AβPP (V717I)-transgenic mice. The effect of fingolimod. Expression of *Slc17a7*, *Slc17a6*, *Slc17a8* mRNA was measured with real-time PCR in the brain cortex of 3-months STZ mice and AβPP-transgenic mice at the age of 3 and 12 months (3 M and 12 M) as well as in appropriate controls. *Slc17a7*: $^{*}p < 0.05$ (n_a = 6-8, n_b = 3-5), *Slc17a6*: (n_a = 3-4, n_b = 3-5), *Slc17a8*: $^{**}p < 0.01$ (n_a = 3-4, n_b = 3-4) as compared to the corresponding controls (ANOVA with post-hoc Tukey's test). The relative level of mRNA was calculated against Actin beta (Actb) as a reference gene (RQ – relative quantification = $2^{-\Delta\Delta Ct}$).

Transcriptional changes in ionotropic and metabotropic glutamate receptors in brain cortex of SAD and TgAD mice. The effect of FTY720

The changes in expression of ionotropic glutamate receptors mRNAs were observed in the SAD model, where *Gria1* (AMPAR1) and *Grin1* (NMDAR1) were upregulated compared to ACSF control mice (Fig. 3) whereas the mRNA level of *Gria2* (AMPAR2) remained unaltered (Fig. 3). Moreover, FTY720 significantly reduced mRNA expression of *Gria2* (AMPAR2) compared to the appropriate control (Fig. 3). In the SAD mice, the mRNA expression of mGluR3 was found to be unaffected whereas mGluR5 mRNA decreased significantly compared to controls (Fig. 4). FTY720 did not influence this change but downregulated the expression of *Grm3*, which encodes mGluR3 receptor (Fig. 4).

In the cerebral cortex of TgAD mice, we did not identify changes in the expression of *Gria1* (AMPAR1) and *Gria2* (AMPAR2) genes (Fig. 3). However, in 12-month-old TgAD mice the increased mRNA expression of NMDAR1 (compared to APP^{-} mice) was observed (Fig. 3). Administration of FTY720 was found to induce significant upregulation of *Gria1* (AMPAR1) in 3-month-old APP^{+} mice as well as *Grin1* (NMDAR1) in 12-month-old APP^{-} mice (Fig. 3).

The mRNA level of gene encoding metabotropic receptor mGluR3 was unaffected in TgAD mature mice whereas increased significantly in TgAD aged mice. Administration of FTY720 reversed this change to the control level (Fig. 4). No differences were observed in the expression of mGluR5 in both age groups of TgAD mice and the effect of FTY720 was not present (Fig. 4). All changes that were observed in sporadic (streptozotocin) and 12-month-old transgenic mice were compiled and presented in Table II.

Discussion

Different patterns of transcriptional changes within the two models of AD

A series of previous studies on AD brain tissue have identified alterations of the glutamatergic system such as reduced concentration of neurotransmitter [34] and diminished uptake of glutamate into synaptosomes [30]. These effects indicate a loss of glutamatergic terminals, which has been confirmed further in histological studies [56]. The deficiency of cortical neurons utilizing glutamate for neurotransmission correlates closely with cognitive impairment and progression of AD [20].

Although it is well recognized that the glutamatergic system is involved in the pathophysiology of AD, the mechanisms of its contribution to the course of disease are not well understood. In our study, we attempted to assess the differences in transcription of genes related to glutamate transport and glutamate receptors in genetic and sporadic models of AD and to evaluate the possible neuroprotective effect of fingolimod.

While analysing the expression of several genes encoding glutamate transporters and receptors in the two models of AD, we observed alterations in the expression of selected genes involved in the glutamatergic transmission which markedly differ between TgAD and SAD mice models. In the genetic model of AD, an interesting observation was that VGLUT1, NMDAR1 and mGluR3 genes are upregulated in aged animals but not in mature animals. On the other hand, in the SAD model genes that encode GLAST, VGLUT3 and mGluR5 are downregulated whereas the gene encoding AMPAR1 is upregulated. Of note, the expression of NMDAR1 mRNA increases in both models (TgAD and SAD).

Among glutamate transporter systems, the changes in the expression of genes encoding vesicular glutamate transporters (VGLUTs) responsible for the uploading of glutamate into synaptic vesicles are markedly visible. VGLUTs have an essential role in regulation of quantal glutamate release [63]. This regulation mainly depends on the expression level of the two predominant isoforms, VGLUT1 and VGLUT2, among which VGLUT1 is functionally more significant, being specifically associated with glutamatergic synapses involved in long-term potentiation (LTP) and memory formation [62]. The pattern of VGLUT1 and VGLUT2 expression was shown to correlate positively with the learning and memory [9]. Moreover, cortical loss of VGLUT1 and VGLUT2 proteins in AD patients was found to be highly correlated with the degree of cognitive impairment [37]. Based on the "glutamatergic hypothesis" of AD and the evidence obtained from AD samples, one might expect diminished expression of vesicular transporters. Our qPCR results revealed downregulation of *Slc17a8* gene that encodes VGLUT3 in SAD mice and upregulation of gene *Slc17a7* encoding VGLUT1 in APP^{+} mice. Since overexpression of VGLUTs is connected with increased

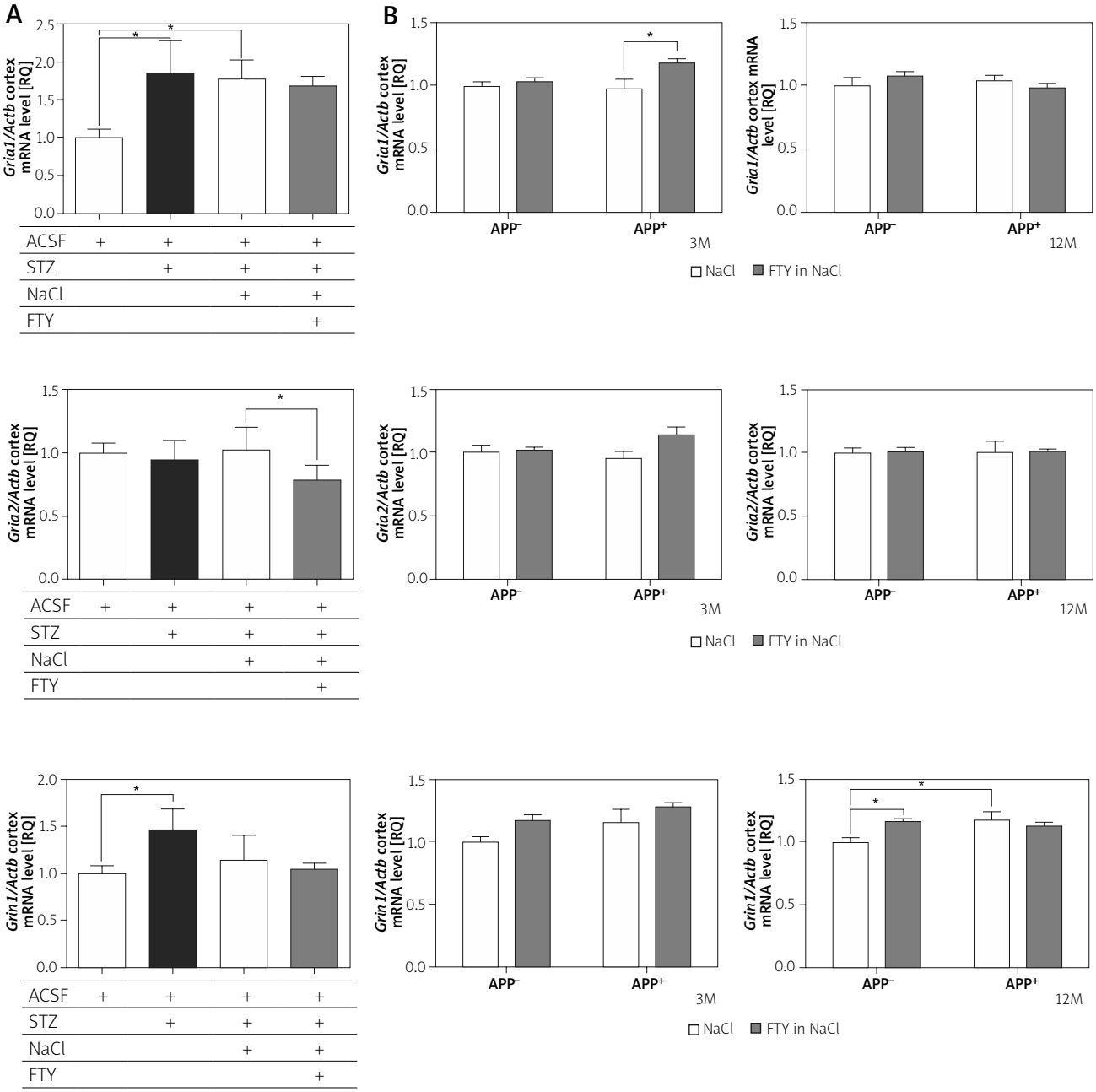

Fig. 3. The mRNA level of ionotropic glutamate receptors (AMPAR1, AMPAR2 and NMDAR1) in the brain cortex of SAD and TgAD mice: **A**) 3-month-old STZ mice and **B**) 3- and 12-month-old AβPP (V717I)-transgenic mice. The effect of fingolimod. Expression of *Gria1*, *Gria2* and *Grin1* mRNA was measured with real-time PCR in the brain cortex of 3-month-old STZ mice and AβPP-transgenic mice at the age of 3 and 12 months (3 M and 12 M) as well as in appropriate controls. *Gria1*: *$p < 0.05$ (n_a = 3-4, n_b = 3-5), *Gria2*: *$p < 0.05$ (n_a = 5-7, n_b = 3-5), *Grin1* *$p < 0.05$ (n_a = 3-4, n_b = 3-4) as compared to the corresponding controls (ANOVA with post-hoc Tukey's test). The relative level of mRNA was calculated against Actin beta (Actb) as a reference gene (RQ – relative quantification = $2^{-\Delta\Delta Ct}$).

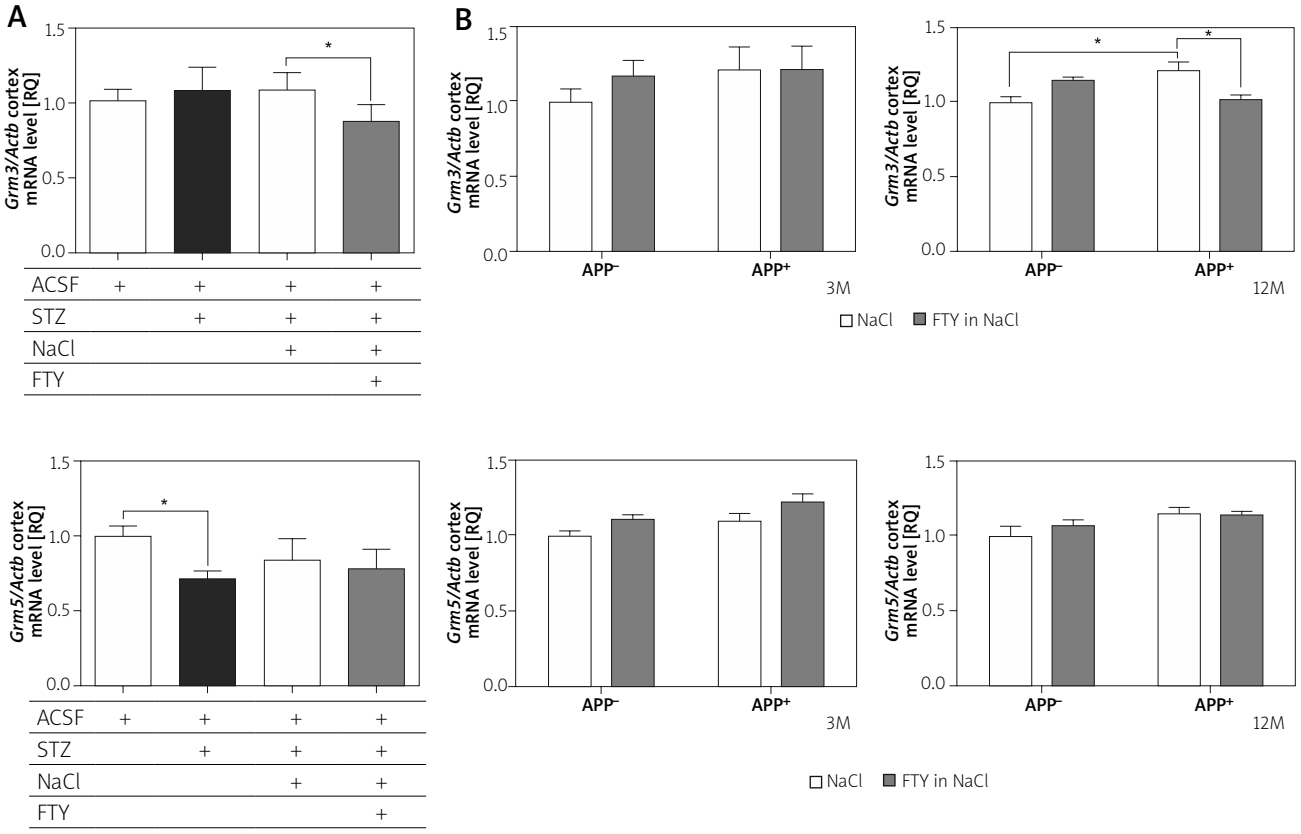

Fig. 4. The mRNA level of group II (mGluR3) and group I (mGluR5) metabotropic glutamate receptor in the brain cortex of SAD and TgAD mice: **A**) 3-month-old STZ mice and **B**) 3- and 12-month-old *AβPP* (V717I)-transgenic mice. The effect of fingolimod. Expression of *Grm3* and *Grm5* mRNA was measured with real-time PCR in the brain cortex of 3-month-old STZ mice and *AβPP*-transgenic mice at the age of 3 and 12 months (3 M and 12 M) as well as in appropriate controls. *Grm3*: $*p < 0.05$ (n_a = 6-7, n_b = 3-4), *Grm5*: $*p < 0.05$ (n_a = 3-4, n_b = 3-5) as compared to the corresponding controls (ANOVA with post-hoc Tukey's test). The relative level of mRNA was calculated against Actin beta (*Actb*) as a reference gene (RQ – relative quantification = $2^{-\Delta\Delta Ct}$).

amounts of glutamate released per vesicle [61], upregulation of genes that encode this vesicular transporter (observed in transgenic mice) may suggest some type of adaptive reaction of the glutamatergic system to pathological changes (in aged APP+ animals) however, at a relatively early stage of the disease. It was previously reported that in the parietal cortex of AD patients protein expression of VGLUT1 was significantly reduced and this loss of vesicular protein may provide evidence for dysfunction of glutamatergic synapses. Unfortunately, the transcriptional level of this transporter was not estimated [38]. In light of this, one possible explanation of our observation is that the upregulation of VGLUT1 gene in APP+ mice may reflect the compensation for the persistent decrease of VGLUT1 protein levels. The results obtained in our studies of the SAD model are more in line with our expectations and previous reports. Decreased expression of gene (*Slc17a8*) that encodes VGLUT3 may indicate disturbed glutamatergic signalling and synaptic loss in AD brains [38]. However, the third isoform of vesicular transporters, VGLUT3, is less widely expressed in glutamatergic neurons but is found in cholinergic and serotoninergic neurons which may also store and release glutamate [22]. As previously reported, high-affinity choline uptake, choline acetyltransferase, and acetylcholine esterase activities in the hippocampus were reduced by STZ [64]. Therefore, downregulation of VGLUT3 in the SAD mice may

Table II. Schematic representation of changes in genes coding glutamate transporters and receptors observed in the sporadic (STZ) and transgenic (12-month-old (12 M) FVB/AβPP(V717I)) mice brain cortex

	Genetic	Models of AD	Sporadic
	 V717I "London" AβPP	Expression of genes related to glutamate homeostasis	 STZ model
AβPP⁻ vs. AβPP⁺	↑ *Slc17a7* (VGLUT1) *Grin 1* (NMDAR1) *Grm3* (mGluR3)	**Model (not treated with FTY720)**	**ACSF vs. ACSF + STZ** ↑ Gria1 (AMPAR1) Grin1 (NMDAR1) ↓ Grm5 (mGluR5) Slc17a8 (VGLUT3) Slc1a3 (GLAST)
AβPP⁺: NaCl vs. FTY720	↓ *Grm3* (mGluR3)	**FTY720 administration**	**ACSF + STZ: NaCl vs. FTY720** ↓ Slc17a7 (VGLUT1) Gria2 (AMPAR2) Grm3 (mGluR3)

potentially reflect toxic influence of STZ on cholinergic and serotoninergic neurons.

Surprisingly, except for GLAST, we did not observe changes in the expression of genes which encode other glutamate membrane transporters either in transgenic mice or in the SAD mice model. There are conflicting data concerning the expression of EAATs in human samples and mice models. In samples of the frontal cortex obtained from individuals with sporadic AD, it has been found that EAAT2-immunoreactivity is decreased whereas EAAT2 mRNA levels are increased [40]. On the contrary, Scott *et al*. reported that EAAT1 is strongly expressed in cortical pyramidal neurons expressing tau in patients showing Alzheimer-type pathology [51]. In line with our results it was found that mRNA levels of GLAST (EAAT1) and GLT-1 (EAAT2) are not affected in transgenic mice with the London mutation expressing human amyloid precursor protein [42]. Similarly, the research of Tong *et al*. on an AD model based on toxicity of $A\beta_{1-42}$ oligomers indicates that the astrocytic expression of GLT-1 and GLAST mRNAs is unaltered [57]. In turn, results presented by Twine *et al*., which were obtained by whole transcriptome sequencing of human brain regions affected by Alzheimer's disease, revealed the reduction of *SLC1A1* (EAAT3) mRNA levels in AD temporal lobe [59]. Downregulation of *SLC1A1* (EAAT3) gene expression was also observed in CA1 pyramidal neurons and regional hippocampal sections of AD patients [21].

In the SAD model we noticed a significant decrease in the expression of GLAST mRNA. A previous analysis of gene expression patterns in the SAD rat model revealed a significant increase in glutamate transporter mRNA in the cortex but a significant decrease in the striatum [25]. Unfortunately, this report did not specify which of the glutamate transporters has altered expression. It has been suggested that in SAD, expression of glutamate transporters is not altered due to disturbances at the mRNA level but rather is a result of the post-transcriptional modifications during oxidative stress [25].

Among glutamate receptors, N-methyl-D-aspartate receptors (NMDARs) attract much attention because of their involvement in both neurodegenerative mechanisms and in memory processes. Activated NMDARs play a central role in formation of LTP and long-term depression (LTD) phenomena underlying cognitive processes such as learning and memory [19]. *In vitro* studies indicate that prolonged exposure of primary cortical neurons to Aβ leads to pathological tonic activation of NMDARs and promotion of their endocytosis. In addition, neurons isolated from a genetic mouse model of AD were found to express reduced amounts of surface NMDA receptors [53]. Besides, a significant decrease in *Grin1* (NMDAR1), *Grin2a* (NMDAR2A) and *Grin2b* (NMDAR2B) mRNA levels was also observed in 5XFAD mice brain cortex [48]. These results correlate well with the reduction of *GRIN1* (NMDAR1) mRNA lev-

els observed in human AD temporal lobe as well as in hippocampal samples of elderly humans and AD patients [4,59]. Interestingly, Leuba *et al*. observed pathological reorganization of NMDA receptor subunits in AD brain cortex, which resulted in a 3-6-fold increase in protein levels of GluN1 (NMDAR1) subunit (which is encoded by GRIN1 gene), while the levels of GluN2A and GluN2B NMDA receptor subunits were decreased by 3-4 fold [39].

On the contrary to clinical studies, our results indicate enhanced expression of *Grin1* mRNA which encodes NMDAR1 in both sporadic and transgenic AD model. This may presumably reflect reactive upregulation of this gene in an attempt to compensate some alterations in the glutamatergic system or, according to Leuba *et al*. [39], may be an effect of NMDA receptor reorganization. Recently, similar observations were also described in the TgCRND8 transgenic model of AD, where upregulation of mRNAs such as *Grin1* (NMDAR1), *Grin2a* (NMDAR2A), *Gria1* (AMPAR1), *Gria2* (AMPAR2) was present in anterior forebrain [43]. Our results also revealed that *Gria1* (AMPAR1) was upregulated, although exclusively in SAD mice. Similar results were also found in human AD hippocampal formation, particularly in the CA2-CA3 and CA4 region, where *GRIA1* (AMPAR1), *GRIA2* (AMPAR2), *GRIN2B* (NMDAR2B) were found among highly expressed genes [49]. However, the other studies showed significant downregulation of the AMPA1 receptor gene in human AD temporal lobe as well as in hippocampus [4,21,59].

Other glutamate receptors may also be targeted by Aβ oligomers, especially receptors belonging to group I mGluRs such as mGluR1 and mGluR5 [8] with the latter being closely associated with NMDARs in postsynaptic complexes of glutamatergic synapses [58]. There is strong evidence that mGluR5 mediates Aβ-induced toxicity towards synapses [28]. Selective pharmacological blockage of this receptor has been found to reduce plaque formation in mice with the Swedish mutation *via* an unknown mechanism [29]. In our genetic Tg AD model, aged mice did not exhibit changes in mRNA expression of the gene (*Grm5*) encoding mGluR5 receptor whereas they displayed an enhanced mRNA level of the *Grm3* gene which encodes mGluR3. Conversely, in SAD mice the *Grm5* (mGluR5) gene was found to be downregulated. These observations are difficult to interpret due to the small amount of data on the expression of group I mGluRs (mGluR1 and mGluR5) and group II mGluRs (mGluR2 and mGluR3) in animal models of AD, as well as in post-mortem human brain samples. Elevation of *Grm5* mRNAs was found recently in anterior forebrain of transgenic AD mice, whereas upregulation of *GRM3* gene expression was observed in the white matter of human AD brain [43,49]. Taking into account that activation of mGluR5 leads to synaptotoxicity under conditions of Aβ presence [29], downregulation of its transcript in SAD mice may be considered as a compensative reaction of a biological system to pathological changes. As suggested, activation of mGluR3 in neurons is permissive for development of mGluR5-mediated signalling leading to synaptotoxicity [15]. However, contradictory data have been also presented indicating that enhancing the activity of mGluR3 may have a beneficial effect by reducing Aβ production [17] and increasing its clearance [18].

The modulatory effect of fingolimod (FTY720) in AD mice

Fingolimod (FTY720) binds to sphingosine-1-phosphate receptors (S1PRs), which are widely distributed in the brain and in the other human organs. Activation of these receptors promotes neuronal growth and function, regulates oxidative-stress-induced cell survival, indicating a neuroprotective role of S1P-mediated signalling [5,23,46]. Of note, it is apparent that S1P levels decrease in AD [31].

Since FTY720 is primarily an S1P receptor agonist, it is expected to promote processes signalled by this receptor, and hence, to exert beneficial effects. FTY720 is also being tested in other brain pathologies such as AD, Parkinson disease (PD), ischemia and intracerebral haemorrhage [45,50]. It has also been reported to reduce the production and neurotoxicity of the Aβ peptide by modulating the S1P receptor [7]. Chronic administration of FTY720 to the rats exposed to $A\beta_{1\text{-}42}$ was found to attenuate histological changes in the hippocampus and reduce behavioural deficits of animals [32]. In addition, this drug exerts a neuroprotective effect against NMDA-mediated excitotoxicity in neuronal primary cultures isolated from mice and rats [16].

Current therapeutic administration of this drug is based on its immunomodulatory and neuroprotective properties. However, non-receptor intracellular effects of FTY720 have also been observed, including inhibition of class I histone deacetylase (HDAC) [26].

Epigenetic regulation of the gene expression underlies the effect of fingolimod on learning and memory processes [27], as well as its suppressive activity towards genes encoding nuclear factor NFκB, caspase-3 and inflammatory cytokines [32].

In light of these reports, we attempted to explore whether administration of FTY720 to the models of AD can modify the transcriptional level of glutamate homeostasis-related genes.

An interesting observation, although difficult to interpret, is that the drug has a weak effect in diseased mice, regardless of their age, in the genetic model of AD. In aged APP^+ animals FTY720 was found to restore expression of the *Grm3* gene that encodes mGluR3, whereas in mature animals it produced an increase in expression of the *Gria1* gene which encodes the AMPA1 receptor. The effect of FTY720 on mGluR3 mRNA might be regarded as protective in light of data indicating the significant role of this receptor in supporting mGluR5-mediated synaptotoxicity [15]. On the other hand, while considering the above-mentioned reports on neuroprotective potential of mGluR3, the downregulation of this receptor by FTY720 seems not to be beneficial. Concomitantly, FTY720 was found to affect gene expression in APP^- mice, particularly increasing mRNA levels of GLT1, VGLUT1 and NMDAR1. This observation indicates modulatory potential of FTY720 towards these genes that may potentially result in the enhancement of glutamatergic transmission (i.e., increased release of vesicular glutamate, elevated expression of NMDARs, acceleration of extracellular glutamate uptake).

Besides, downregulation vs. controls was observed for VGLUT1, mGluR3 and AMPAR2 whose expression profiles were unchanged in the SAD mice relative to control healthy mice. Another important observation concerns significant downregulation of the gene encoding mGluR3 by FTY720 in both models of the disease. However, this effect is difficult to interpret due to conflicting reports on the role of mGluR3.

In conclusion, the main findings of this study demonstrate differences in the expression of glutamate homeostasis-related genes in genetic and sporadic models of AD. Interestingly, observed alterations of VGLUT1, NMDAR1 and mGluR3 mRNA levels in the TgAD model were only present in aged 12-month-old animals, which may suggest that they occur in the later stages of the disease. Despite the heterogeneity of the two models applied in the current study, there is strong evidence for transcriptional changes of genes that encode cellular (GLAST) and vesicular (VGLUT1 and VGLUT3) glutamate transporters and several of glutamate receptors (NMDAR1, AMPAR1, mGluR3, mGluR5), which may have a significant implications in alteration of glutamatergic signalling.

We also observed that fingolimod downregulated gene expression of VGLUT1, AMPAR2 and mGluR3 in the sporadic AD model. Moreover, fingolimod showed efficacy in reversing the altered expression of *Grm3* encoding mGluR3 receptor in the transgenic AD model, which indicates potential impact of the sphingosine-1-phosphate receptor modulator on genes involved in glutamatergic signalling.

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Disclosure

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