ORIGINAL SCIENTIFIC ARTICLE

INHIBITORY, BUT NOT EXCITATORY SYNAPSES ARE REDUCED IN THE HIPPOCAMPUS OF THE SIX-MONTH-OLD ALZHEIMER'S DISEASE MODEL MOUSE

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ABSTRACT

Alzheimer's disease (AD) is a devastating neurodegenerative disorder and the most common cause of elderly dementia. One of the main features of AD diseased brain are amyloid plaques, pathological depositions made of β-amyloid peptide, derived from β-amyloid precursor protein (APP). To assess how AD pathology affects synapses in the hippocampus, brain region to be one of the earliest with obvious pathological changes, we examined APPPS1 mice, transgeneticaly expressing human APP mutation ("Swedish mutation") and human presenilin-1 mutation under the neuron-specific promoter, which develop AD symptoms early in life. We analyzed inhibitory and excitatory synapses using immunoflourescent staining and laser scanning confocal microscopy. In APPPS1 mice, inhibitory synaptic terminals labeled with vesicular inhibitory transmitter transporter (VGAT) were reduced in CA1 and CA3 regions of the hippocampus in APPPS1 mice compared to controls. This was true for both parvalbumin-positive and parvalbumin-negative terminals. On the other hand, excitatory synapses, coming from either hippocampal or entorhinal projections were similar between the genotypes. We conclude that first changes in the hippocampus caused by amyloid pathology affect inhibitory, but not excitatory synapses.

Keywords: *Alzheimer's disease, hippocampus, GABAergic interneurons, synapses, vesicular transmitter transporters.*

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder affecting mostly, but not only, elderly individuals causing progressive dementia and loss of cognitive functions. The disease is characterized by massive neurodegeneration in brain regions involved in learning, memory and emotions including neocortex, hippocampus and amygdala, basal forebrain cholinergic system and brainstem monoaminergic system, which are reduced in size due to neuronal death, astrogliosis, microgliosis and synaptic degeneration (1-4). All these changes are thought to be caused by accumulations of insoluble protein depositions: intracellular neurofibrillary tangles, composed of microtubule-associated tau protein and extracellular amyloid plaques, composed of β-amyloid peptide (Aβ), derived from amyloid precursor protein (APP). Two hypotheses were proposed to explain the etiology of AD, based on these two main pathological features of the disease. The "amyloid cascade hypothesis" states that aggregation of Aβ is the initial event which triggers pathological cascade leading to development of AD (5). This hypothesis currently dominates the field of AD research. On the other side, the "neuronal cytoskeletal degeneration hypothesis" proposes cytoskeletal changes related to tau aggregation as the basis of neurodegeneration in AD (6). Synapse loss is the strongest correlate of cognitive decline in AD, and synapses are attractive as a potential therapeutic target in AD, due to their natural plasticity which gives hopes for recovery (3, 7).

In this study we used the mouse transgenic model of parenchymal amyloidosis, with an early onset of amyloid deposition, further referred to as APPPS1 mice (8). These mice co-express human APP double Swedish mutation and human PS1 mutation (8). Both the PS1 and the APP constructs are under control of the Thy1 promoter, which provides high levels of neuron-specific transgene expression in the postnatal brain. Human APP expression in APPPS1 mice is confined mainly to neurons in the cortex, hippocampus and brain stem and to a lesser extent in the thalamus and striatum. The first amyloid plaques appear in the cortex already at the age of 6 weeks. Global neuron loss is not present, but it is observed locally in the dentate gyrus (DG) (8). The advantages that make this mouse model valuable for investigation of mechanisms of cerebral amyloidosis are the early onset of amyloid pathology, C57BL/6J background known to reduce the variability in Aβ metabolism and deposition (9) and no gender differences in Aβ level. We have previously demonstrated, using these mice, that they have a high load of amyloid beta at 7 months of age, coupled with heavy gliosis (10). The aim of this study was to determine the density and ratio of inhibitory and excitatory synaptic terminals in the hippocampus of 6-month-old APPPPS1 mice using the presynaptic markers (vesicular transmitter transporters) and quantitative confocal microscopic analysis (11, 12).

MATERIALS AND METHODS

Animals

Six-month-old transgenic male APPPS1 mice were obtained from a breeding colony at the University of Tübingen, Germany. These mice co-express transgenes human APP containing KM670/671NL mutation (Swedish double mutation) and human PS-1 with L166P mutation under the neuron-specific murine Thy-1 promoter on C57BL/6J genetic background (8). The wild-type C57BL/6J control mice were obtained from the breeding colony at the central animal facility of the Universitätsklinikum Hamburg-Eppendorf. All experiments were conducted in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985), as well as with German and European Community laws on the protection of experimental animals. The procedures used were approved by the responsible committee of the State of Hamburg (permit number 098/09). Data acquisition and analyses were performed by an examiner blinded to genotypes.

Tissue preparation

Mice were anaesthetized with a lethal dose of 16% solution of sodium pentobarbital (Narcoren, Merial, Hallbergmoos, Germany, 5 µl per g body weight, intraperitoneally). The animals were transcardially perfused with fixative containing 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) for 15 min at room temperature (RT). Following perfusion, the brains were removed and postfixed in the same fixative overnight and immersed in 20% cryoprotective sucrose solution for 2 days, at 4°C. Brains were then frozen by immersion for 2 min in 2-methyl-butane precooled to -30°C and stored at -80°C until sectioned.

Immunohistochemistry

Spaced serial brain sections containing dorsal hippocampus were first dried at RT for 1 h. To retrieve antigens in fixed tissue, water-bath antigen de-masking was performed by incubating sections in 0.01M sodium citrate solution (pH 9.0) for 30 min at 70°C. Sections were then washed twice briefly in PBS and non-specific binding was blocked in blocking solution containing 5% normal goat serum and 0.2% triton X100 in 0.1M PBS for 1 h at RT. The sections were then incubated for 2 days at 4°C in primary antibodies dissolved in blocking solution. Primary antibodies that we used were monoclonal anti-mouse parvalbumin (PV, 1:500, Sigma, Deisenhofen, Germany) and polyclonal anti-rabbit vesicular inhibitory transmitter transporter (VGAT, 1:1000, Synaptic Systems, Wiesbaden, Germany) for double immunofluorescence and polyclonal anti-rabbit vesicular glutamate transporter 1 (VGLUT1, 1:1500, Synaptic Systems) and polyclonal anti-rabbit vesicular glutamate transporter 2 (VGLUT2, 1:500, Synaptic Systems) for single immunofluorescence staining were used at previously established optimal concentrations (13). After washing in PBS (3 x 15 min at RT), the appropriate fluorescently-labeled secondary antibody, diluted 1:200 in PBS-lambda-carrageenan solution, was

applied for 2 h at RT in the dark. After a subsequent wash in PBS, cell nuclei were counterstained for 10 min at RT with bis-benzimide solution (Hoechst 33258 dye, 5 µg/ml in PBS, Sigma). Finally, the sections were washed again, mounted in anti-quenching medium Fluoromount G (Southern Biotechnology Associates) and stored in the dark at 4°C. As a negative control instead of primary antibody, sections were incubated with a normal serum from the animal in which the primary antibody has been produced with the resulting lack of fluorescent signal.

Light-microscopic analysis of perisomatic terminals

Estimation of inhibitory perisomatic puncta and area of principal cell bodies was performed as described (11). Stacks of images of 1 µm thickness were obtained from different hippocampal subfields in PV/VGAT doubly immunostained sections using an LSM 510 confocal microscope (Zeiss, 63x oil immersion objective, 1024×1024 pixel resolution). One image per cell at the level of the largest cell-body cross-sectional area was used to measure soma area and count individually discernible perisomatic puncta. Numbers of VGAT-positive puncta were normalized to the perimeter of the cell profile. At least 25 cells per hippocampus region were counted, 4 animals per genotype. All measurements were performed using Image Tool 2.0 freeware (University of Texas Health Science Center, San Antonio, TX).

To estimate the density of glutamatergic synaptic terminals labeled with anti-VGLUT1 and anti-VGLUT2, antibodies, we selected 3–5 sections containing the LDCN of each animal in all experimental groups and captured 2–3 confocal optical slices (1-µm-thick) per section using a 63x objective. Analysis was performed using the ‘‘Measurement’’ function of the ImageJ software. Briefly, after subtraction of background brightness and defining the size and circularity of presynaptic terminals, the average immunostained area per image expressed as percent was evaluated. Finally, the average of synaptic densities was calculated per tissue section, and these values were used for statistical analysis.

Photographic documentation and statistical analysis

Images were documented on an an Axiophot 2 microscope equipped with a digital camera AxioCam HRC and AxioVision software (Carl Zeiss, Oberkochen, Germany) and processed for brightness and contrast using Adobe Photoshop CS5 software (Adobe Systems Inc., San Jose, CA).

All numerical data are presented as group mean values with standard deviations (SD). Statistical difference was tested by Student’s t test, after Shapiro-Wilk test confirmed normal distribution. The threshold value for acceptance of differences was 5%. Analyses were performed using the SYSTAT 9 software package (SPSS, Chicago, IL, USA).

RESULTS

APPPS1 mice have reduced number of inhibitory synapses around pyramidal cells in the CA1 and CA3 subregions of the hippocampus

We first investigated inhibitory synapses around the cell bodies of principal neurons in the CA1, CA3 and dentate gyrus. To that end, we stained the sections for VGAT, vesicular inhibitory transmitter transporter, which labels all inhibitory synapses, both GABA-ergic and glycin-ergic (ref.). Additionally, as the most important source of inhibition to these cells represent parvalbumin-expressing basket cells (14), we co-stained the sections with parvalbumin. Thus, we could demonstrate both parvalbumin-negative (single stained for VGAT, red on the images) and parvalbumin-positive (double stained for VGAT and PV, yellow on images) presynaptic terminals (Figure 1A). The number of both parvalbumin+ and parvalbumin- terminals around pyramidal cells in the CA1 and CA3 subregions of the hippocampus was significantly reduced in APPPS1 mice compared to controls (Figure 1B). However, the number of inhibitory terminals around dentate gyrus granule cells was similar between the genotypes (Figure 1B). We conclude that Alzheimer’s disease pathology affects inhibitory synapses in the hippocampus of APPPS1 mice in region-specific manner.

APPPS1 mice have density of excitatory synapses in the hippocampus similar to controls

Next, we investigated excitatory synapses in the hippocampus. Glutamatergic synapses in the hippocampus express mainly either VGLUT1 or VGLUT2 in a layer-specific manner. Most of the interhippocampal synapses and synapses and synapses from the commissural projections express VGLUT1, whereas most of the input from the entorhinal cortex is VGLUT2+ (12). We tested for the expression of both VGLUT1 and VGLUT2 in various, well defined layers of the hippocampus (Figure 2A,C), but found no difference between the genotypes (Figure 2B,D). We conclude that excitatory neurotransmission is not affected at the beginning stage of Alzheimer’s disease in the hippocampus.

DISCUSSION

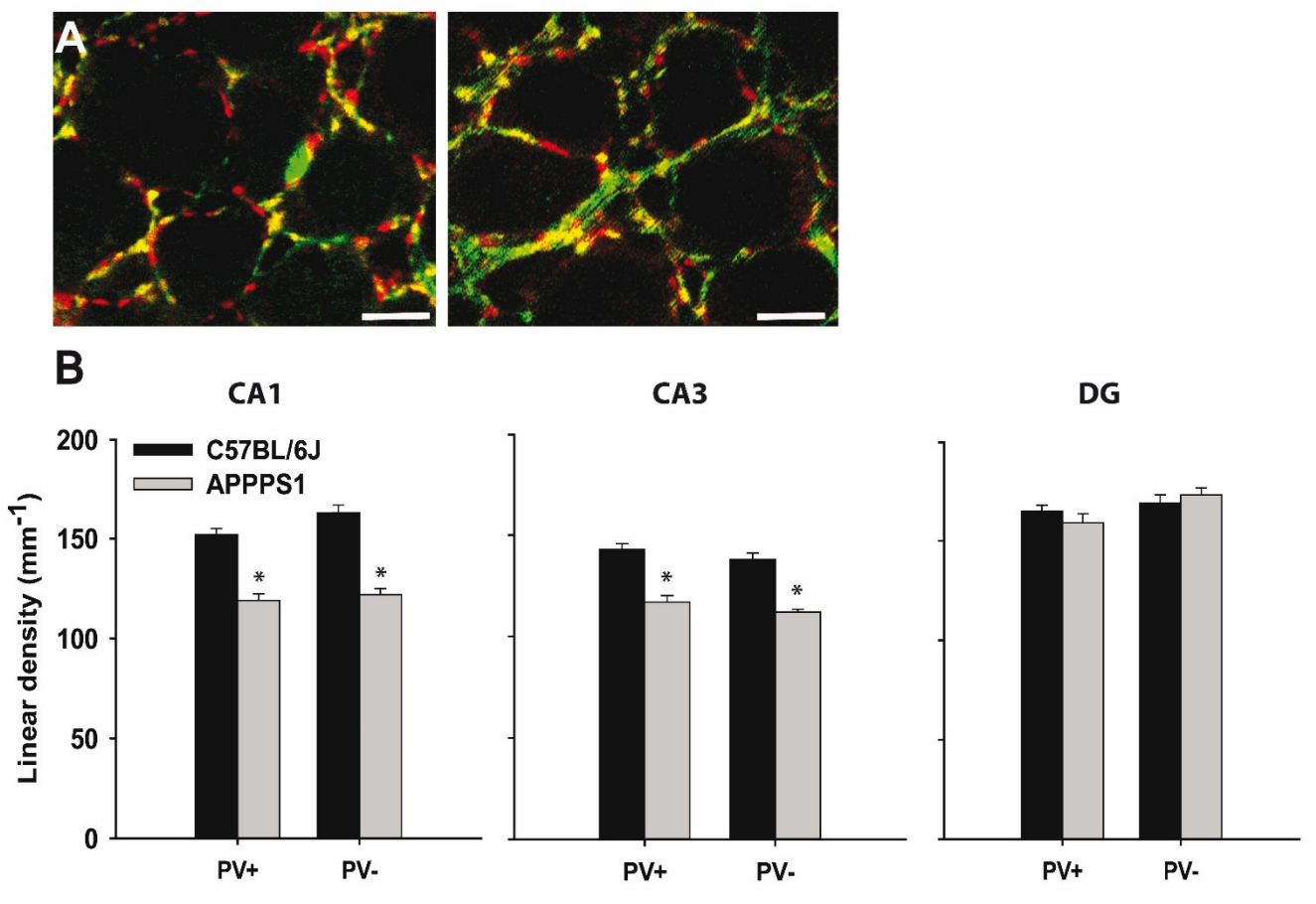
We have shown that at 6 months, which we consider an early phase of the amyloid deposition in APPPS1 mice, excitatory, glutamatergic synapses are not affected, whereas there is a significant reduction in the number of inhibitory synapses compared to control. As the age that we chose for our study was when the early amyloid plaques occur in the hippocampus (8, 10), we propose that the first changes caused by amyloid depositions adversely affect inhibitory, but not excitatory synapses in the hippocampus.

In APPPS1 mice cortical neuron loss is not apparent up to 8 months of age, but local neuron loss in the DG has observed already at 3 months of age (8). Therefore, it is not surprising that they show only subtle behavioral phenotype - impairment in working memory present at the age of 8 months,

which coincides with massive amyloidosis in the hippocampus (8). In line with this observation is electrophysiological evidence for impaired long-term potentiation in CA1 *in vivo* at the same age (15). The loss of parvalbumin-positive interneurons was reported in the olfactory cortex of a similar APP/PS1 double transgenic mouse model (16) and electrophysiological deficits in PV-positive cortical interneurons were found (17). Additionally, selective vulnerability of PV-positive interneurons has been documented under different experimental conditions, including epilepsy (18), global ischemia (19), IL6 overexpression (20) and schizophrenia (21, 22). In our study there were no indication of the loss of parvalbumin-positive interneurons in the hippocampus, based on the immunostaining pattern, thus it is rather the loss of axons and/or synaptic terminals that occurs in APPPS1 mice.

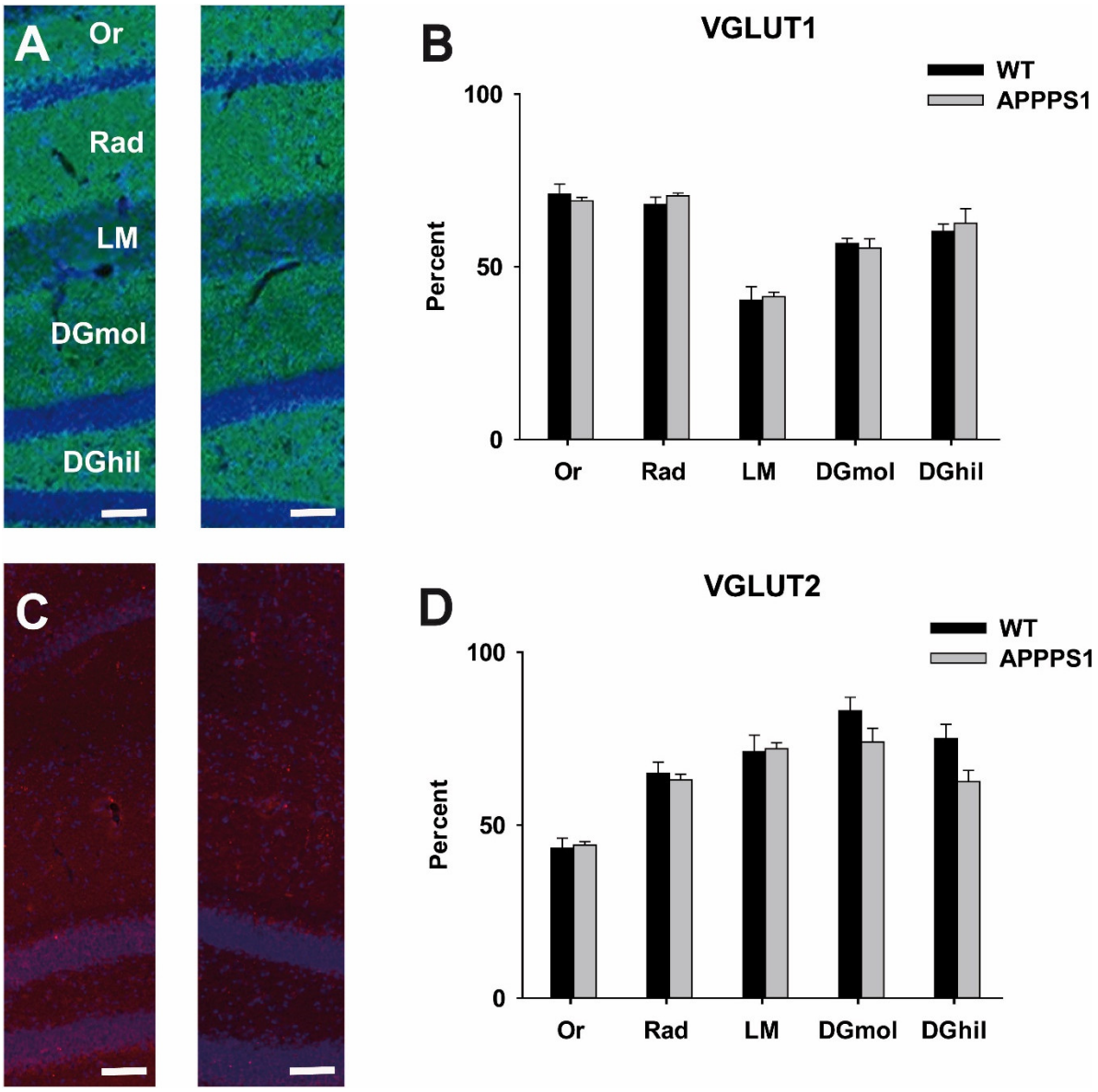
Because the early pathology of AD seems to occur at the synaptic level, the loss of dendritic spines and synaptic contacts is commonly seen in other AD mouse models (7, 23). Therefore, it was not surprising that we discovered significantly reduced numbers of inhibitory perisomatic terminals in the hippocampal CA1 and CA3 regions, compared wild-type controls of the same age. Interestingly, this loss was not observed in the DG, where the loss of granule cells occurs early (8). Furthermore, the excitatory synapses do not seem to be affected at this age, although we cannot exclude the possibility that subtle biochemical or ultrastructural changes in excitatory synapses are present as well. However, we have previously shown that our confocal analyses of synaptic terminals correlate well with the changes observed at electron microscopic level (12, 14).

Figure 1. The loss of perisomatic inhibitory terminals on pyramidal neurons in the hippocampus of APPPS1 mice.



(A) Representative confocal micrographs of VGAT-(red) and parvalbumine (green)-immunostained perisomatic puncta around CA3 pyramidal neurons in the hippocampus of the control (C57BL/6J, left panel) and APPPS1 (right panel) mouse. Scale bars: 10 μm. (B) Numbers of parvalbumine-positive/VGAT-positive (yellow, $PV^+/VGAT^+$) and parvalbumin-negative/VGAT-positive (red, $PV^-/VGAT^+$) perisomatic terminals per mm (linear density) in the CA1, CA3 and dentate gyrus (DG) of the hippocampus of wild-type C57BL/6J control and APPPS1 mice. Data are presented as mean + standard deviation. Asterisks indicate significant difference, $p < 0.05$, t test; n = 4 mice/group).

Figure 2. Excitatory synaptic terminals in the hippocampus of APPPS1 mice.



(A) Representative confocal micrographs of VGLUT1-(green) immunostained CA1 hippocampus subfield of the control (WT, left panel) and APPPS1 (right panel) mouse. Cell nuclei are counterstained with bisbenzimide (blue). Scale bars: 100 µm. (B) Percent of VGLUT1-stained area. Data are presented as mean + standard deviation. There were no significant differences, $p > 0.05$, t test; n = 4 mice/group). (C) Representative confocal micrographs of VGLUT2-(red) immunostained CA1 hippocampus subfield of the control (WT, left panel) and APPPS1 (right panel) mouse. Cell nuclei are counterstained with bisbenzimide (blue). Scale bars: 100 µm. (D) Percent of VGLUT2-stained area. Data are presented as mean + standard deviation. There were no significant differences, $p > 0.05$, t test; n = 4 mice/group). Or – stratum oriens; Rad – stratum radiatum; LM – stratum lacunosum-moleculare; DGmol – dentate gyrus molecular layer; DGhil – dentate gyrus hilus.

CONCLUSIONS

Our study shows that early pathology in AD, before the development of symptoms, or at the very early signs of mild cognitive deficit not testable in mice, is consistent with the selective loss of inhibitory, but not excitatory synapses in the Amon's horn region of the hippocampus proper. It is tempting to speculate that, like in several other neuropsychiatric diseases, inhibition is the first to get damaged by the pathologic processes. Further studies on rodents could help elucidate behavioral consequences of this disinhibition, and studies on tissue sections from AD patients could help confirm if in humans disease follows similar pathophysiological pattern as in model animals.

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REFERENCES

1. Albert MS. Changes in cognition. Neurobiol Aging 2011; 32:58-63.
2. Hardy J. A hundred years of Alzheimer's disease research. Neuron 2006; 52:3-13.
3. Jucker M, Walker LC. Pathogenic protein seeding in Alzheimer disease and other neurodegenerative disorders. Ann Neurol. 2011; 70:532–540.
4. Mattson MP. Pathways towards and away from Alzheimer's disease. Nature 2004; 430:631-9.
5. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. Science 1992; 256:184-5.
6. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 1991; 82:239-59.
7. Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol. 1991; 30:572-80.
8. Radde R, Bolmont T, Kaeser SA, Coomaraswamy J, Lindau D, Stolze L et al. Aβ42-driven cerebral amyloidosis in transgenic mice reveals early and robust pathology. EMBO reports 2006; 7:940-6.
9. Lehman EJ, Kulnane LS, Gao Y, Petriello MC, Pimpis KM, Younkin L et al. Genetic background regulates beta-amyloid precursor protein processing and beta-amyloid deposition in the mouse. Hum Mol Genet. 2003; 12(22):2949-56. doi: 10.1093/hmg/ddg322.
10. Djogo, N., Jakovcevski, I., Müller, C., Lee, H. J., Xu, J. C., Jakovcevski, M. et al. Adhesion molecule L1 binds to amyloid beta and reduces Alzheimer's disease pathology in mice. Neurobiology of disease 2013; 56:104–15. https://doi.org/10.1016/j.nbd.2013.04.014
11. Morellini F, Sivukhina E, Stoenica L, Oulianova E, Bukalo O, Jakovcevski I et al. Improved reversal learning and working memory and enhanced reactivity to novelty in mice with enhanced GABAergic innervation in the dentate gyrus. Cereb Cortex 2010; 20:2712-27.
12. Vulovic M, Divac N, Jakovcevski I. Confocal Synaptology: Synaptic Rearrangements in Neurodegenerative Disorders and upon Nervous System Injury. Front Neuroanat. 2018; 12:11. doi: 10.3389/fnana.2018.00011.
13. Schmalbach B, Lepsveridze E, Djogo N, Papashvili G, Kuang F, Leshchyns'ka I et al. Age-dependent loss of parvalbumin-expressing hippocampal interneurons in mice deficient in CHL1, a mental retardation and schizophrenia susceptibility gene. J Neurochem. 2015; 135(4):830-44. doi: 10.1111/jnc.13284.
14. Nikonenko AG, Sun M, Lepsveridze E, Apostolova I, Petrova I, Irintchev A et al. Enhanced perisomatic inhibition and impaired long-term potentiation in the CA1 region of juvenile CHL1-deficient mice. Eur J Neurosci. 2006; 23:1839-52.
15. Gengler S, Hamilton A, Hölscher C. Synaptic plasticity in the hippocampus of a APP/PS1 mouse model of Alzheimer's disease is impaired in old but not young mice. PLoS ONE 2010; 5:e9764.
16. Saiz-Sanchez D, Ubeda-Bañon I, De la Rosa-Prieto C, Martinez-Marcos A. Differential expression of interneuron populations and correlation with amyloid-β deposition in the olfactory cortex of an AβPP/PS1 transgenic mouse model of Alzheimer's disease. J Alzheimers Dis. 2012; 31:113-29.
17. Verret L, Mann EO, Hang GB, Barth AM, Cobos I, Ho K et al. Inhibitory interneuron deficit links altered network activity and cognitive dysfunction in Alzheimer model. Cell 2012; 149:708-21.
18. Schwaller B, Tetko IV, Tandon P, Silveira DC, Vreugdenhil M, Henzi T et al. Parvalbumin deficiency affects network properties resulting in increased susceptibility to epileptic seizures. Mol Cell Neurosci 2004; 25:650-63.
19. Meade CA, Figueredo-Cardenas G, Fusco F, Nowak TS, Pulsinelli WA, Reiner A. Transient global ischemia in rats yields striatal projection neuron and interneuron loss resembling that in Huntington's disease. Exp Neurol 2000; 166:307-23.
20. Samland H, Huitron-Resendiz S, Masliah E, Criado J, Henriksen SJ, Campbell IL. Profound increase in sensitivity to glutamatergic- but not cholinergic agonist-induced seizures in transgenic mice with astrocyte production of IL-6. J Neurosci Res 2003; 73:176-87.
21. Aksic M, Poleksic J, Aleksic D, Petronijevic N, Radonjic NV, Jakovcevski M et al. Maternal Deprivation in Rats Decreases the Expression of Interneuron Markers in the Neocortex and Hippocampus. Front Neuroanat. 2021; 15:670766. doi: 10.3389/fnana.2021.670766.
22. Jakovljevic A, Agatonovic G, Aleksic D, Aksic M, Reiss G, Förster E et al. The impact of early life maternal deprivation on the perineuronal nets in the prefrontal cortex and hippocampus of young adult rats. Front Cell Dev Biol. 2022; 10:982663. doi: 10.3389/fcell.2022.982663.
23. Wei W, Nguyen LN, Kessels H, Hagiwara H, Sisodia S, Malinow R. Amyloid beta from axons and dendrites reduces local spine number and plasticity. Nat Neurosci 2010; 13:190-6.