universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Quantification of synapse loss in human brains with mixed Alzheimer's and Lewy body disease"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (M.Sc.)

Wien, 2016 / Vienna 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 863
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Biologische Chemie
Betreut von / Supervisor:	Assoz. Prof. Mag. Dr. Wolfgang Wadsak, Privatdozent

This Master Thesis was carried out from 15. 01. 2016 till 09. 09. 16 at DZNE in Munich under leadership from Prof. Dr. Jochen Herms, supervised by Dr. Dr. Mario Dorostkar.

Acknowledgment

I want to thank Dr. Dr. Mario Dorostkar for his supervision of my master thesis, the great support and assistance I received from him as well as the possibilities and freedom of research he was offering. Open doors and ears were promoting my ideas for the project.

I thank Prof. Dr. Jochen Herms for the immediate acceptance of my master thesis project in form of an Erasmus internship at the DZNE in Munich.

Just as much I want to thank all the members of the group of Prof. Dr. Herms for the help and support they gave me, whenever I asked for it.

I appreciate the tips and equipment I got from Michael Schmidt.

Finaly I might thank Prof. Dr. Wolfgang Wadsak, who took the supervision of my Erasmus internship from the University of Vienna.

Self-reliance

I hereby confirm my Master Thesis was written independently and just with the specified resources. Other works that were used are cited and labeled with the source.

Abstract

Synapses mediate the communication between neurons and their function alters during physiological processes such as learning and memory. Cognitive decline in neurodegenerative disease has been shown to result from loss of synapses. However, previous studies only analyzed specific types of synapses in one or few specific brain regions.
The aim of this study was to map the loss of excitatory and inhibitory synapses distinct brain regions in the brains of patients suffering from Alzheimer's disease and Lewy body disease.
I analyzed 67 brains from donors at the Newcastle Brain Tissue Resource, which had been neuropathologically classified as Alzheimer's disease, Lewy body disease or controls. Tissue microarrays from several brain regions were immunofluorescence labelled with antibodies against DLG4 (also known as PSD-95 scaffold protein) to stain excitatory synapses and GABRA2 (GABAA Receptor subunit α2) to stain inhibitory synapses. Images were recorded on a confocal microscope and synapse densities were quantified automatically using custom-written image analysis algorithms.
I found different patterns of synapse loss, depending on the disease. Particularly in Alzheimer's disease brains, there was a marked loss of inhibitory synapses in several brain regions, including the entorhinal cortex, temporal and parietal cortex, cingulate, visual cortex, putamen and pallidum.
This loss of inhibitory synapses may cause disruption of long-range neuronal networks, which has been demonstrated in several animal studies.

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1. Introduction

Anatomy of the brain

The human brain can be divided anatomically into many different regions. The classification into Brodmann's areas is one of the main systems used, which is based on the microscopic appearance of the different areas. Furthermore, the functions of the areas are well correlated with this classification. The Brodmann's area functions can be summarized in executive functions (Brodmann's area 9, 10, 44, 45, 46), motor functions (Brodmann's area 4, 6, 8), somatosensory functions (Brodmann's area 1, 2, 3, 5, 40), attention (Brodmann's area 7 and 39), visual functions (Brodmann's area 17, 18, 19), memory (Brodmann's area 20, 21, 37), emotional regulation (Brodmann's area 11, 38, 47) and sound recognition and handling (Brodmann's area 22, 41, 42).

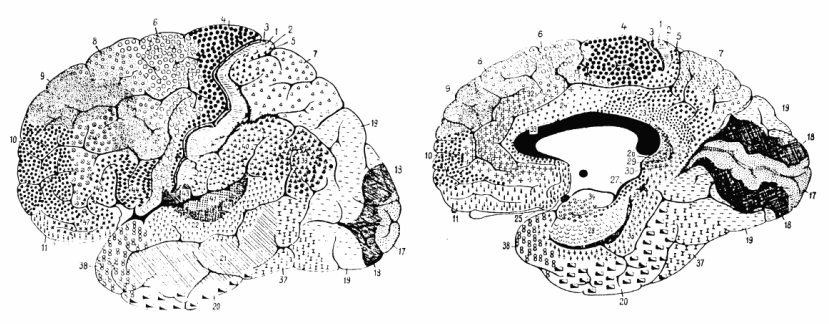


Fig. 1 Brodmann's areas, sagittal picture of a human brain

Figure from: Brodmann, K., Beiträge zur histologischen Lokalisation der Greosshirnrinde. VI. Mitteilung: Die Cortexgliederung des Menschen. J. Psychol. Neurol. (Leipzig), 1908. 10: p. 231-246 [1]

Schematic picture of the different brain regions, differentiated by brodmann. Areas 1, 2, 3 are the primary somatosensory cortex, Area 4 the primary motor cortex, Area 5 the somatosensory association cortex, Area 6: Premotor cortex, Area 7: Visuo-motor coordination, Area 8: Frontal eye fields, Area 9: Dorsolateral prefrontal cortex, Area 10: Anterior prefrontal cortex, Area 11: Orbitofrontal area, Area 12: Orbitofrontal area, Area 13, 16: Insular cortex, Area 17: Primary visual cortex, Area18: secondary visual cortex, Area 19: Associative visual cortex, Area 20: Inferior temporal gyrus, Area 21: Middle temporal gyrus, Area 22: Superior temporal gyrus, Area 23: Ventral posterior cingulate cortex, Area 24: Ventral anterior cingulate cortex, Area 25: Subgenual area, Area 26: Ectosplenial portion of the retrosplenial region of the cerebral cortex, Area 27 – Piriform cortex, Area 28: Ventral entorhinal cortex, Area 29: Retrosplenial cingulate cortex, Area 30: Part of cingulate cortex, Area 31: Dorsal Posterior cingulate cortex, Area 32: Dorsal anterior cingulate cortex, Area 33: Part of anterior cingulate cortex, Area 34: Dorsal entorhinal cortex, Area 35: Perirhinal cortex, Area 36: Ectorhinal area, now part of the perirhinal cortex, Area 37: Fusiform gyrus, Area 38: Temporopolar area, Area 39: Angular gyrus, Area 40: Supramarginal gyrus, Areas 41 and 42: Auditory cortex, Area 43: Primary gustatory cortex, Area 44: Pars opercularis, Area 45: Pars triangularis, Area 46: Dorsolateral prefrontal cortex, Area 47: Pars orbitalis, part of the inferior frontal gyrus, Area 48: Retrosubicular area, Area 49: Parasubicular area and Brodmann's area 52: Parainsular area.

The major part of the brain is the cerebrum, which is divided into two hemispheres. Figure 1 depicts a sagittal profile along the hemisphere. The cerebral cortex is divided into the frontal lobe (Brodmann's areas: 4, 6, 8, 9, 10, 11, 44, 45, 46, 47), the parietal lobe (Brodmann's areas: 1, 2, 3, 5, 7, 40), the temporal lobe (Brodmann's areas: 20, 21, 22, 37, 38, 39, 41, 42) and the occipital lobe (Brodmann's areas: 17, 18, 19). In the occipital lobe, visual processing occurs, while the temporal lobe receives input from the ears. Further language, like fluent speech, understanding and recognition of words, numbers, body and faces are processed there. The parietal lobe integrates the input of different senses, important for orientation. The frontal lobe is important in speech-language production and the activation and control of body muscles, like the eyes.

The limbic system consists of parts of the frontal, parietal and temporal lobe. Further the hippocampus and amygdala, which is part of the limbic system (Brodmann's areas: 23, 24, 25, 26, 29, 30, 31, 32, 33), as well as thalamus, basal ganglia and the cingulate gyrus, play important roles in memory and emotion. The brainstem is the connection of the spinal cord, consisting of the midbrain, pons, medulla and oblongata. Thalamus and hypothalamus send motor and sensory signals to the cortex. The cerebellum coordinates motor and autonomic functions, and is therefore important for balance and coordination. [2] All these different brain regions are responsible for many functions and disrupted regions therefore responsible for disease. Also the strong interconnection of the brain regions makes it difficult to distinguish the trigger and to inhibit the spreading of disease activators. This interconnection is due to neurons, that have their soma and most of its dendrites in one region, but their axon is reaching into another brain region. Kwon and Jang showed, that 70% of the brain is connected to all other regions, forming information processing networks [3].

There are roughly 86,1 billion neurons in an adult male human brain, with 19% in the cerebral cortex, which makes up 82% of the total brain mass [4]. Neurons are composed of the soma, the nucleus, one axon and several dendrites, which can form synapses to other neurons in short distances 4mm [5], whereas the axon can reach other neurons at distances of up to 1m [6]. One neuron can be connected by its dendrites to 100.000 other cells [6], forming a complex network. Neurons are responsible for the reception of stimuli, processing and transfer of information in the form of electrochemical impulses. A neuron consists of the cell body, with the nucleus, many dendrites and also one axon, which can form a synaptic terminal. The axon is myelinated through oligodendrocytes, which forms myelin sheaths around the axon, to accelerate the action potential, which jumps from one ranvier knot to the next. This ranvier knot is the region between two myelin sheaths and free of contact to oligodendrocytes. On the synaptic terminal neurotransmitter can be released and can bind on postsynaptic membrane receptors, initiating a new action potential.

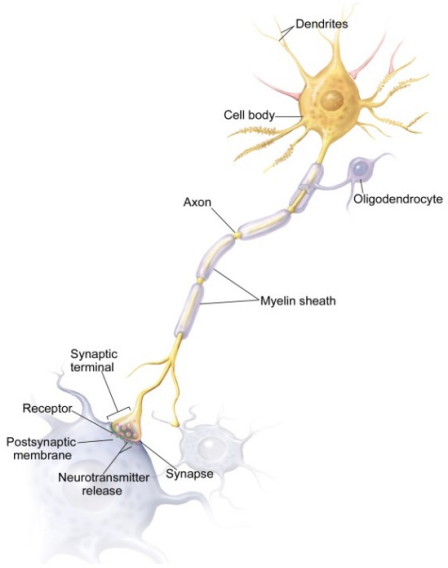


Fig. 2 Synaptic Contact between Neurons

Figure from: Winslow, T., *The neuron.* Regenerative Medicine. Departemtn of Health and HumanServices, 2006. [7]

Schematic picture of a synaptic contact. A neuron consists of a cell body with a nuleus, many dendrites and one axon. Oligodendrocytes forming myelin sheaths around axons, to facilitate rapid impulse conduction. On the synaptic terminal, neurotransmitter were released and bound on receptors on the postsynaptic membrane.

Hence neurons are responsible for the most critical and complex cellular functions in the brain. ”They undergo the greatest number of microscopic changes in response to acute and chronic cell injury and are the principal sites of damage for several of the diseases associated with the highest morbidity and mortality in our society, i.e. cerebrovascular and neurodegenerative diseases” [8]. Neurons require ion channels and pumps to hold the balance between intracellular and extracellular electrolyte concentration, especially for toxic calcium ions, but also for sodium and potassium. This balance is disturbed by micro-environmental damage to the membrane and therefore to the pumps, leading to energy deprivation. Necrosis is the consequence of that irreparable cell damage which may be caused by oxygen deficit, which affects cell energy requirements and membrane integrity. “When neurons undergo cell death and necrosis, no effective neuronal mitosis or replenishment of neurons from stem cells is present within the adult human brain” [8]. Microglia cells and macrophages remove these damaged neurons by phagocytosis. Furthermore, astrocytes proliferate in response to the injury. They seemed to have important neuroprotective functions. [8] Astrocytes outnumber neurons in the brain. They are thought to be responsible for glutamate-, ion- and water homeostasis, defense against oxidative stress, energy storage, scar formation, tissue repair and synapse formation [9].

2. Neurotransmitters

When an action potential reaches a neuron, neurotransmitter, packed in vesicles, are transported to the membrane and released into the synaptic cleft. The synaptic cleft consists of a pre- and a post synapse with receptors on both terminals. The released neurotransmitter can bind on the receptors and initiate another action potential or inhibit it. Receptors on the pre synapse can bind neurotransmitter as a saturation signal to check the quantity of neurotransmitters in the cleft. If more neurotransmitters bind on pre synapse receptors the release of neurotransmitter may be decreased. There are many different neurotransmitters which can be summarized into different functional or chemical groups. There are small molecule neurotransmitters and peptide neurotransmitters. The first can be divided further into biogenic amines (catecholamine like dopamine or epinephrine, indole amine like serotonin and imidazole amine like histamine), amino acids (glutamate, GABA), purines (ATP) and others like acetylcholine. The second group, peptide neurotransmitter can be between 3 – 30 amino acids long, e.g. methionine encephalin. [10]

The two main types of neurotransmitters for excitatory and inhibitory synapses are glutamate and GABA, which were also analyzed for this project.

2.1. Glutamate – Excitatory neurotransmitter

Glutamate is the main excitatory neurotransmitter in the brain and the most abundant amino acid in the human body.

Signals can be excitatory or inhibitory. Excitatory postsynaptic potentials (EPSP) are caused of an influx of positive charged ions (Na^+) and results in a depolarization of the membrane, that lead to an action potential firing of the neuron, when the depolarization is high enough to overcome the gate threshold.

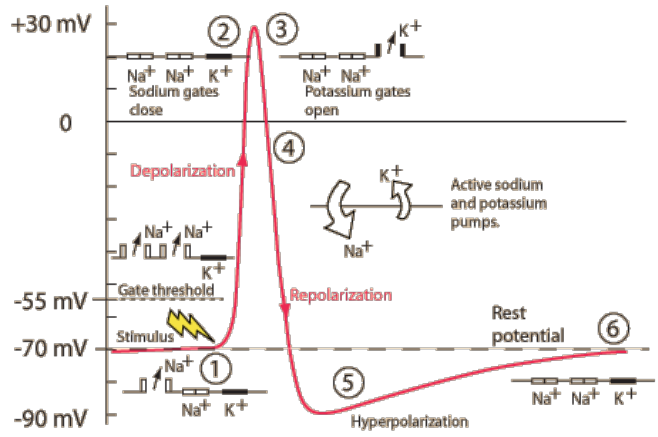


Fig. 3 Action potential

Figure from: Charand, K.X., *Action Potentials*. [11]

Demonstration of an action potential. ① The stimulus opens sodium channels and drive the potential from -70mV up to -55mV, the gate threshold, where the stimulus creates an action potential. Under this threshold no action potential is initiated. More sodium channels open and Na^+ influx into the cell ②. The membrane gets depolarized to 30mV and the sodium gates were closed, when potassium channels open, result an efflux of K^+ ③. ④ The membrane repolarize to the rest potential and undershoot it to -90mV ⑤, called "Hyperpolarization". This prevents the neuron from getting another stimulus in this time period to ensure that the signal is proceeding in one direction at the time. ⑥ Last the sodium and potassium pumps drive the potential back to -70 mV.

Glutamate is responsible for memory creation. Under normal conditions the required amount of glutamate is covered by the diet, but it can be synthesized from α-ketoglutaric acid, from the citrate cycle. Glutamate can't pass the blood-brain barrier and needs to be transported with the help of highly specific transporters. It can interact with NMDA receptors, AMPA receptors or metabotropic glutamate receptors (described later).

Furthermore, glutamate is the precursor for the inhibitory neurotransmitter GABA.

2.2. GABA

Γ-Aminobutyric acid, the major inhibitory neurotransmitter in the mammalian central nervous system, is responsible for the reduction of the neuronal excitability.

Inhibitory postsynaptic potential (IPSP), the opposite of an EPSP, hinder the neuron to create an action potential. If a neuron is receiving more IPSPs from different synapses, it is inhibited in signaling for a distinct time period. Once the threshold of excitation is received, the action potential depolarizes the neuron, while the hyperpolarization of the neuron happens when the inhibitory signal dominates.

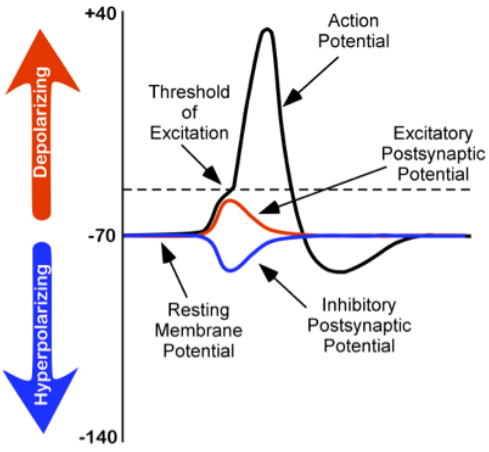


Fig. 4 Changes in membrane potential

Figure from: Furtak, S., *Neurons.* California State University, Sacramento. [12]

Depiction of the part of IPSP to the action potential. While in depolarization, the excitatory postsynaptic potential (red curve) reaches the threshold and triggers an action potential, the membrane potential gets up to 30mV (black curve), while in Hyperpolarization the resting membrane potential of -70mV decrease further to hinder the neuron to create an action potential (blue curve).

GABA is synthesized within the presynaptic terminal. Glutamic acid decarboxylase (GAD) produces GABA from glutamate with the cofactor pyridoxal phosphate. So only the absence or presence of the enzyme glutamate decarboxylase specifies the sort of synapse, either excitatory or inhibitory. Like glutamate, GABA can't pass the blood brain barrier and needs the presence of specific receptors and transporters.

3. Synapses

Neuronal activity runs through synapses. Two forms of synapse exist: chemical and electrical (Gap junctions). Electrical synapses communicate with ion flux, whereas chemical synapses convert electrical impulses of an action potential into chemical signals on the presynaptic membrane of neurons. This triggers an opening of calcium channels and the increase of calcium leads to secretion of neurotransmitter from the synaptic vesicles into the synaptic cleft. These neurotransmitters bind on receptors on the postsynaptic membrane of the synapse from the second neuron and can start a new action potential.

Signals in form of neurotransmitter, like adrenalin, noradrenalin or dopamine, are released from the presynapse into the synaptic cleft and received by receptors at the postsynapse. The received signals on the surface of the postsynapse are tansformed in electrical and biochemical changes in the postsynapse.

Fig. 5 Synaptic Contact in detail

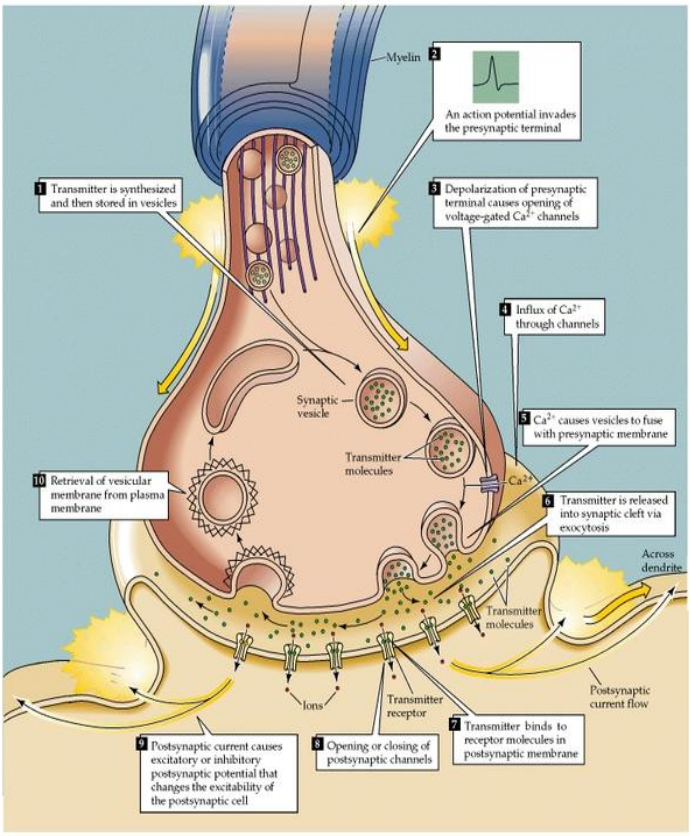


Figure from: Weeks, A., *Synapse Ultrastructure.* NURON Nipissing Univesity Research on Neuroscience, 2001. [13]

Schematical picture of a synaptic contact. The neurotransmitter are stored in vesicles (1). If an action potential is arriving at the presynaptic terminal, (2) voltage-gated calcium channels were opened (3) and Ca2+ Ions will influx into the cell (4). This influx cause vesicle fusion to the membrane (5) and neurotransmitter were released into the synaptic cleft (6). On the opposite site of the presynapse, the postsynapse hold transmitter receptors, where the released transmitters in the synaptic cleft can bind (7). Postsynaptic channels were activated through the neurotransmitter binding (8) and launch out an excitatory or inhibitory potential in the postsynaptic cell (9), while the empty vesicle gets recycled in the presynapse (10).

Whereas excitatory synapses are located on dendritic spines, 70% [14] of the inhibitory synapses are found on the shaft of dendrites and on axon onsets for modulating the excitability locally. Both inhibitory and excitatory synapses differ from the consumption of the neurotransmitter receptors, morphology and the molecular consumption and organization.

Fig. 6 Different Synaptic Contacts

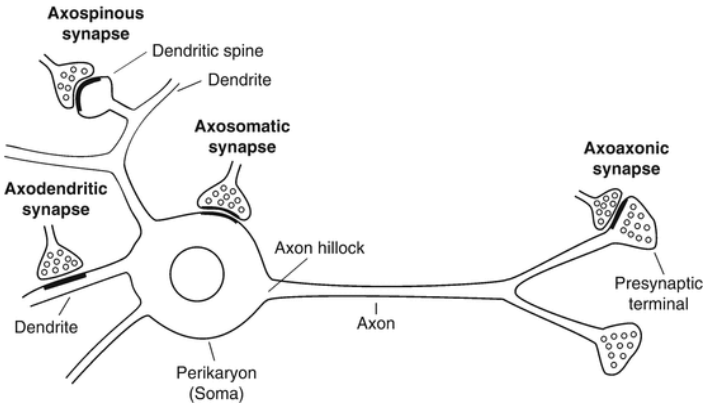


Figure from: Rochelle, S.C., *Neuroscience in the 21st Century.* Springer, 2013. [15]

Schematical picture of different synaptic contacts. Axospinous synapses build from an axon and a dendritic spine. Axosomatic synapses develop from an axon directly on the cell body of the second neuron. Axoaxonic synapses arise from an axon as well of the first neuron as the second neuron. Axodendritic synapses occur on axons directly on dendrites.

Neurons form spines to deliver or receive information. Up to 40.000 spines are present at the largest pyramidal neurons [16], but they can also be formed on the dendritic shaft or the soma of a neuron. Dendritic spines differ in shape and size. The classic one is the mushroom spine, but also stubby and thin spines are represented. They can change their shape within minutes to hours, especially on pyramidal neurons during hippocampal development [17]. The average length of an spine is 2µm [16]. The basis of plasticity is the structural change due to the actin containment in synapses, which indicates an instable structure, which is also because of lack of tubulin. The actin is present in development and in mature spines, pointing to the importance of the plasticity [18]. But the plasticity of spines declines with age [19]. Synaptic proteins like scaffold proteins or ion channels are clustered on spines, while the size of a spine reflects their synaptic connection [20]. Spine morphology determines the stability and strength, in addition it reflects its function: Thin spines are more plastic and the length of the neck controls calcium diffusion [21], the volume of the spine head is direct proportional to the number of the postsynaptic receptors [22] and to the number of the presynaptic vesicle [23]. This reflects why study on spine changes due to synaptic plasticity is important to understand and to find the mechanism behind it.

PSD is the postsynaptic density, an electron rich region opposite to the pre synapse. The protein PSD-95 (DLG4) is located on dendritic spines, characteristic for excitatory synapses and responsible for clustering of postsynaptic receptors like glutamate receptors. There are roughly 400 PSD proteins like DLG4, Shank1, Cadherin-10 and many more, involved in cell adhesion, chaperone activity, cytoskeleton actin binding, GTPases and regulator activity, kinases/ phosphatases activity, membrane trafficking, metabolism, mitochondria activity, motor proteins, receptors and channels activity and stabilization, scaffold and translation [24]. But the consumption of proteins in the PSD varies in different brain regions and is quickly changed via phosphorylation, palmitoylation, ubiquitination, and proteasome mediated degradation. This pre-, post synaptic information transfer is spread all over a dendrite to enable brain activity, which influences motor function, behavior and memory. Mutations in many PSD proteins lead to neurological and psychiatric diseases in humans [25].

The fact that spine density early in development is higher [26], the subsequent loss and the changes in spine structure which underlie learning [27] have been shown before. In some species (e.g. guinea pig [28]) spine formation has completed before birth, pointing to an independent environmental spine formation [29]. So in some species synaptogenesis occurs unrelated to sensory processing or learning. But it has proven that spine formation is regulated by neuronal activity, at least in the proximal region of the dendritic tree [30]. There are many studies that point to a new spine formation during LTP like Trommald et al. [31] and Andersen and colleagues [32] but others showed rather a morphological change of spines [33]. When the second argumentation is true, then learning is limited to structure of the existing synapses built during development. Numerous molecules and proteins play key roles or at least supporting roles in spine activity and morphology. They are present on every spine on a dendritic synapse, where the biochemistry has to be regulated separately “to

provide a subcellular organization or isolation of the biochemical machinery of the synapse" [18]. In addition to spine loss due to disease, there are compensation mechanisms that help to maintain the synaptic contact [34], but also some of the synapses are more vulnerable than others in disease. Like in the Aβ pathology, where the order of synapse types seemed to be firstly the cholinergic, secondly the glutamatergic and finally the GABAergic terminals [35].

There are excitatory and inhibitory synapses, which differs in the neurotransmitter molecules and therefor the receptors, scaffold proteins and other proteins initiating the response signal in the postsynaptic cell.

3.1. Excitatory synapses

Excitatory synapses, such as glutamatergic and cholinergic, depolarize the postsynaptic cell when the neurotransmitters bind to the transmembrane receptors in the synaptic cleft. Further sodium influxes depolarize the neuron from its resting membrane potential and increase the proportion of an action potential.

For the communication between neurons, neurotransmitter receptors play a fundamental role. They can be localized on the postsynaptic membrane at the synaptic cleft to induce an action potential in the post synapse, on the dendritic shaft or even on the presynaptic terminal to receive some feedback information.

3.1.1. Glutamatergic synapse

The glutamate release from vesicles is the major mechanism of excitatory neurotransmission in the mammalian brain and therefore important for neuronal activity. 80-90% of all neurons in the brain are glutamatergic [36]. "20 glutamate receptors have been identified in the mammalian central nervous system" [37]. Whereas glutamate can't pass the blood-brain barrier, it is only derived by local synthesis from glucose. Three ionotropic receptors are voltage sensitive and located on the glutamatergic synapses: Ionotropic receptors, like: N-methyl-D-aspartate (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepriopinoinic acid (AMPA) and kainate, as well as metabotropic/ G-protein-coupled glutamate receptors: mGluR, to start the second messenger cascade like cAMP.

The ionotropic receptors include ion channels, but differ in the permeability of the ions, like Na^+, K^+ and Ca^{2+} [38]. Once an ionotropic receptor is activated it undergoes conformational change and allows the influx of extracellular ions. This process is fast and allow neurons to act fast to signal transmission. NMDAR is highly expressed on neurons as well on astrocytes, is coupled on calcium channels and plays a role in the compensation mechanism of the body during injury [39] and is important for learning and memory.

The glutamate binding on a metabotropic receptor activates the G-Protein, bound to the cytoplasmic tail of the receptor, which activates a second messenger cascade for signal transmission. Metabotropic receptors are slower in response and are responsible for gene expression and protein synthesis, which enhance the excitability of glutamatergic neurons [37], but also for direct modulation of neuronal excitability.

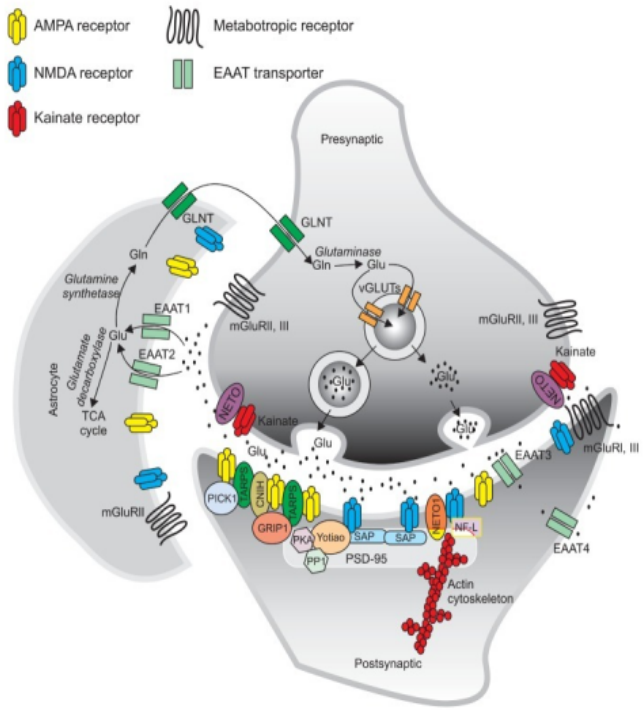


Fig. 7 Glutamatergic Synapse

Figure from: M. D. Rubio, J.B.D., J. H. Meador-Woodruff, *Glutamate Receptor Abnormalities in Schizophrenia: Implications for Innovative Treatments.* Biomol Ther (Seoul), 2012. **20(1)**: p. 1-18. [40]

Schematical picture of a glutamatergic synapse. Glutamate is packed in vesicles in the pre synapse via vGLUT and then transported to the membrane and released at the synaptic cleft. The neurotransmitter can bind on the post synapse at different receptors as AMPA (yellow), NMDA (blue) or kainate (red).

Three transporters are expressed on glutamatergic neurons in mammals: VGlut1, VGlut2 or VGlut3, which mediate the glutamate uptake into the synaptic vesicles. The different types of transporters are present in different regions of the brain, as VGlut1 predominates in the cortex, cerebellum and hippocampus, whereas VGlut2 expression is mainly in the brainstem and the thalamus. Both isoforms are complementary in these regions, but other areas express both of them [41]. The third isoform VGlut3 is homologous to the other two and expressed at a very low level striatum, hippocampus, cerebral cortex and raphe nuclei. VGlut3 is also expressed outside the nervous system, like the liver and eyes, but only a few neurons in the brain express VGlut3. It is present at cholinergic and serotonergic neurons in contrast to VGlut1 and VGlut2 [42]. Why some regions prefer the expression of one isoform is not clear yet. Some suggest that it is a developmental issue.

3.2. Inhibitory synapses

Besides excitatory synapses there are inhibitory synapses, which prevent the cell from generating an action potential. This is due to a hyperpolarization through Cl^- influx. In this short time period there is no excitation of the cell possible.

3.2.1. GABAergic synapse

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the brain and is produced via the precursor glutamine via glutaminase and GAD. Similar to glutamate, both ionotropic and metabotropic receptors exist. GABA can lead to either the influx of negative charged chloride ions via ionotropic receptors or to the flow of positively charged potassium ions out of the cell via metabotropic receptors. The major GABA receptor on the post synapse is the GABA-A receptor, which forms a chloride ion channel. Ionotropic receptors are heteropentamers with the α2 subunit being widely expressed in the mammalian brain. But there are reports showing a presence of both receptor subtypes, GABA-A and GABA-B, on non-GABAergic neurons and also GABA transporter (GAT) on the membrane of non-GABAergic neurons were found, maybe playing a role in co-transportation of other ions or GABA release [16].

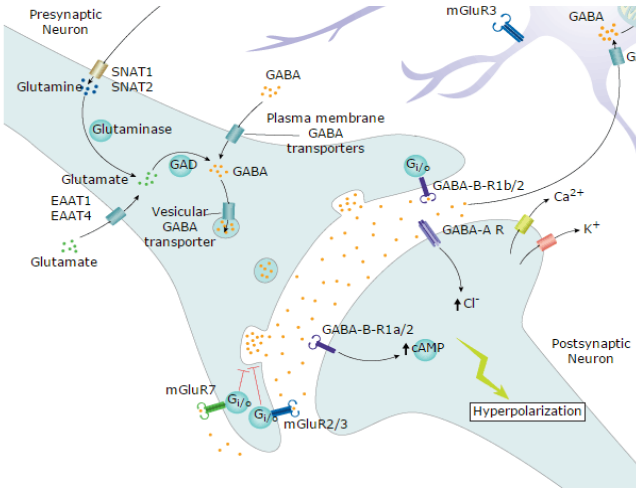


Fig. 8 GABAergic Synapse

Figure from: Systems, R.D., *Overview of Synaptoc Neurotransmission: GABAergic Inhibition.* 2012. [43]

Schematical picture of a GABAergic synapse. Glutamine is transferred into Glutamate by the glutaminase in the shaft of the pre synapse. Further glutamic acid decarboxylase (GAD) produces GABA from glutamate. The product is packed into vesicles via the vesicular GABA transporter, transported to the membrane of the synaptic cleft and released from the vesicles. On the opposite of the presynaptic release, the postsynaptic receptors receive GABA. There are two main receptor subtypes: GABA-A and GABA-B receptors. The first opens Chloride channels, which lead to a Cl^- influx and further to a hyperpolarization of the cell. Whereas GABA-B regulating the cAMP production in the cell.

There are different compositions of the subtypes in one GABA receptor cluster and 16 subunits have been identified yet. This expression of the different subunits are highly variable among individuals and dependent of environmental and genetic factors [44]. The most dominant one is the α1β2γ2, localized in almost all brain regions. The α2 subtype is

most prominent in the cluster of α2β3γ2 [45]. The regulation of the transcription in the different brain regions must be a complex system with a tight controlling part, where the change in the expression might reflect a change in neuronal activity. Also other receptor types (also excitatory ones, like NMDAR) can influence the GABA receptors and its expression. [46]

A few functional partners for GABRA2 in the human brain are ubiquilin 1, gephyrin, $GABA_A$ receptor α4/ β1/ β2/ β3/ γ2/ γ3, $GABA_A$ receptor associated protein and dynamin 1.

3.3. Interaction Inhibitory – Excitatory synapses

Glutamate is a precursor protein in the GABA production in inhibitory synapses, but itself is a neurotransmitter in excitatory synapses. To regulate the production of only one neurotransmitter in a synapse, there is no GAD present in excitatory synapses, to allow only glutamate as neurotransmitter and no further transformation into GABA. Whereas glutamate depolarizes neurons, GABA hyperpolarizes them, so in general those two pools are not co-localized. But in distinct isolated neurons in some brain regions, both neurotransmitters were reported. However the functional significance of such an arrangement is not clear [16].

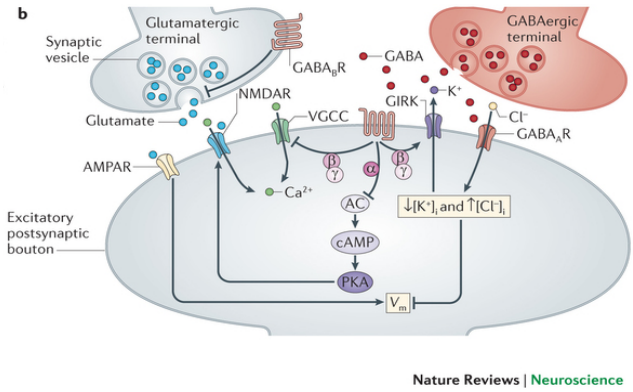


Fig. 9 Interaction of GABAergic and Glutamatergic terminal

Figure from: Higley, M.J., *Localized GABAergic inhibition of dendritic Ca2+ signalling.* Nature Reviews Neuroscience, 2014. **15**(567-572). [47]

Schematical picture of the response mechanism of a post synapse, receiving both GABAergic and glutamergic presynaptic input.

While on glutamatergic terminal, AMPAR and NMDAR gets activated through glutamate and open calcium channels, for a Ca2+ influx into the postsynaptic cell, the GABAergic terminal address to GABAAR (Cl ion influx), and G-Protein coupled receptors, that can lead to a K+ efflux via GIRK. The both pathways can influence each other, e.g. inhibits the G-Protein coupled receptor the NMDAR. But also $GABA_B$R on the glutamatergic terminal inhibits the glutamate release.

4. Disease

All neurodegenerative disorders have one thing in common: pathological accumulations of different proteins and peptides, specific for the different disease types. The most frequent are neurofibrilliary tangles and β-Amyloid plaques, which are hallmarks of Alzheimer's disease.

The molecular mechanism behind many neurodegenerative disorders is largely unknown. The main reason is certainly the complex interaction of disease supporting factors. Neurodegeneration is defined as neuron loss and astrogliosis, as well as synapse loss, neuro inflammation and misfolding of specific proteins that cause toxicity. Each of these alterations has a complex mechanism and many pathways for activation. For example the secretion of interleukin 1β causes Inflammation, which blocks neurotrophic factors, that are necessary for neuron stability, maintenance and growth, leading to spine loss [48]. But also the direct alteration of ion channels by inflammatory cells, like the phosphorylation of AMPA receptors may cause spine loss [49].

As Herms and Dorostkar depict nicely, there are three different structural spine alterations: The change of the number in a specific brain region and reduced spine density because of reduced dendrite length or nerve cell loss. Spine shape modification (stubby, mushroom and thin) as well as spine head enlargement and third: Pathological changes like altered endoplasmic reticulum or enlarged PSD [50]. All these abnormalities appear in neurodegenerative diseases.

Dementia is common the elderly population and may be caused by many different diseases like Alzheimer's disease (AD), dementia with Lewy bodies (DLB), Parkinson disease dementia, frontotemporal lobar degeneration, Huntington's disease and cerebrovascular disease.

Alzheimer's disease is one of these neurodegenerative disorders. It is a slowly progressive loss of neuronal functions, including impairments in processing of information in the brain, synapse reduction and loss of neurons in certain brain areas. The hippocampus and entorhinal cortex are affected very early in the disease, followed by the limbic system and the neocortex, and finally the brainstem and the cerebellum. This loss of important neuronal processes and cell bodies in the brain results in memory loss, problems in fluent speech, changed cognition and behavior.

Early onset, familial forms of AD and sporadic forms can be distinguished. While gene mutations in the amyloid precursor protein or presenilin 1 or 2 genes cause early onset, familial AD, mutations and variants in a large number of different genes, such as apolipoprotein E, can increase the risk for sporadic AD. The main risk factor for sporadic AD, however, is age.

Minimal cognitive impairment (MCI) is a prodromal stage, reflecting the earliest clinical signs of Alzheimer's disease. Affected individuals show cognitive deficits, atypical for normal

aging, but are not clinically dement. In MCI, there is typically loss of presynaptic markers like Synapsin-1, a phosphoprotein located in the cytoplasm of small synaptic vesicle, which plays an important role in the neurotransmitter release. Other presynaptic markers, which may be lost are synaptophysin, a membrane protein of synaptic vesicle, VAMP2 and SNAP25. Also postsynaptic markers such as DLG4, which is responsible for the scaffolding in postsynaptic elements, Shank1 and SAP-87, may be lost [51, 52].

Further Parkinson's disease (PD) is the most common movement disorder and after AD the most common neurodegenerative disease. The hallmark of PD is the loss of dopaminergic neurons, first in the substantia nigra and the striatum. Not only motor dysfunctions appear, but also autonomic dysfunctions, sleep disturbances, depression and cognitive impairment. The lesions of the brain are characterized by the appearance of Lewy bodies (described later) [53].

Synapse loss is not specific to predict AD, because it is present in many dementia diseases, as well as common in normal aging. But it is the main predictor for cognitive impairment in those diseases. According to World Alzheimer Report 2015, a published statistic shows more than 46,8 million cases of dementia worldwide. The most frequent form is Alzheimer's disease with 65% [54], but also dementia with Lewy bodies accounts for 20% of all dementia cases [55]. Most importantly, aging of the general population caused an increasing incidence of AD that continues to escalate over the next several decades. The trend predicts a significant increase to 131,5 million (68%) people worldwide in 2050, who will be affected with AD. This means almost a doubling every 20 years. [56] There are regional differences with Germany, China, USA, India and Japan being most strongly affected. There are also gender differences: Over 65 years, every second woman and every third man is affected with dementia. This is due to the fact, that woman become older and so the amount of affected people is greater [57]. The worldwide estimated cost of dementia in 2015 is 818 US$ [54].

This numbers indicate how urgent the studies in neuroscience are. The reduction of synapses might be the main trigger in changed behavior and recognition. The aim of neuroscience is to understand how long term stability of synaptic contact can be ensured and to develop therapies to work against the loss of synapses in disease and in age.

Many factors might be responsible for spine loss, like changed presynaptic input or external factors. And it is not clear if the presynaptic dysfunction or the postsynaptic change appear first [50]. Till today many different mechanisms have been suggested to declare the alteration in spine shape and functions leading to dysfunction and lost in AD. Therefor many factors have to be considered.

4.1. Genetics

Many genes are involved in the pathogenesis of AD. The main genetic risk factor for developing sporadic AD is homozygosity for the ε4 allele of Apolipoprotein-E [58, 59], which is correlated with an increase of amyloid plaque deposition in the brain [60], by decreasing the clearance of amyloid β from the brain [61]. Two genes involved in familial AD, presenilin 1 (PS1) on chromosome 14 [62] and presenilin 2 (PS2) on chromosome 1 [63], increase the levels of neurotoxic forms of amyloid β (Aβ42) by processing the amyloid precursor protein to amyloid β [64, 65] as a part of the enzymatic complex. While some authors argue that the genetic contribution to develop AD is small in total AD cases [66], others argue that the Aβ aggregation is the causative element in developing AD [50]. But not only Alzheimer's disease is related with accumulation of Aβ42 in the brain, also Down's syndrome Individuals show these depositions [67].

In sporadic and familial Parkinson's disease, mutations in genes regulating mitochondrial functions (described later) [53] are known. Also mutations on the synuclein gene may cause dementia in the form of Lewy body disease.

4.2. Proteins and indirect factors involved in Dementia

The hallmarks of AD, which have been discovered by Alois Alzheimer in 1906, are intraneuronal neurofibrially tangles (NFT), consisting of hyperphosphorylated and misfolded tau, as well as extracellular plaques caused by accumulation of Amyloid-β [52]. Amyloid plaques can be diffuse and are usually not associated with synaptic loss, but common founded in brains of cognitively intact elderly people. Plaques can also contain a dense core, and these forms typically occur with cognitive impairment [68]. Further, α-synuclein, can accumulate into Lewy bodies. This protein can bind ubiquitin and initiate cell damage.

These neuropathological changes are used to determine the stage of the disease. But for a clear AD diagnosis, a postmortem histological analysis of the brain is required to document the presence of characteristic plaques and tangles in specific brain regions.

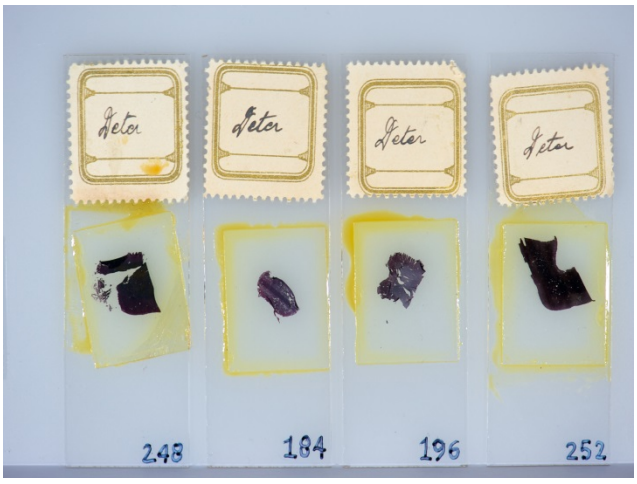


Fig. 10 Brain sections of Auguste Deter († 1906)

Figure from: M. Dorostkar

Brain sections of Auguste Deter, the first person diagnosed with Alzheimer's disease by Alois Alzheimer.

T

pathogenesis for AD, where the accumulation of neurotoxic Aβ in the cerebral cortex occurs initially [69]. The amyloid precursor protein (APP) is produced by a single copy gene on chromosome 21 and cut by β- and γ-secretase, resulting in variable length Aβ cleavage products, with the major products being Aβ40 and Aβ42. In healthy Individuals the proportion of Aβ40 is about 90 % [70]. But in AD cases the ratio of Aβ40 and Aβ42 shows a trend to increase Aβ42 by a factor of 1,5 - 1,9 [69]. β-APP is cut first by the β-secretase to a ~12 kDa C-terminal fragment that is further cleaved on the carboxyl side by γ-secretase to release Aβ. An isomer of ~9 kDa is formed by α-secretase, that can be cut by γ-secretase afterwards to build p3, a small C-terminal fragment of Aβ [70].

Fig. 11 Amino acid sequence and cleaving sites of APP

Figure from: Findeis, M.A., *The role of amyloid beta peptide 42 in Alzheimer's disease.* Pharmacol Ther, 2007. **116**(2): p. 266-86. [66]

Above is a short section of the APP sequence depicted, with the cleaving sites of β-secretase before the aspartate, the α-secretase cuts 15 amino acids after this aspartate, after glutamate. Finally γ-secretase cut after 40 (valine) or 42 (alanine) amino acids after the β-secretase cutting position on APP.

Aβ monomers are soluble with a short β-sheet region and a large α-sheet portion. When the concentration rises, Aβ undergoes a conformational change with at the end a β-sheet rich part, which aggregates into fibrils. These fibrils diffuse into the extracellular space and form AP. There can be diffuse plaques without fibrils, cored plaques with fibrillary cores or core-only plaques with intracellular compartments, like cell bodies, axons and dendrites [50].

The distribution progress of β-Amyloid is one of the diagnostic criteria for the diagnosis of AD and calling “Thal phasing scale”. Thal described a stereotypic pattern of five phases, with no/minimal pathology “0” beginning in the neocortex, up to high pathology “5” ending in the cerebellum [71].

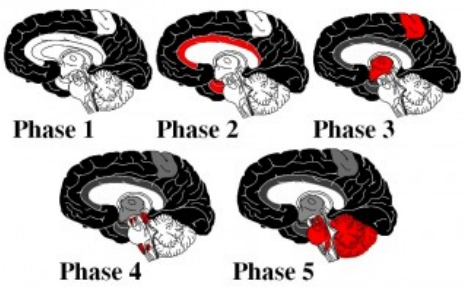


Fig. 12 Thal phase scaling

Figure from: Thal DR, R.U., Orantes M, Braak H, *Phases of Aβ-deposition in the human brain and its relevance for the development of AD.* Neurology, 2002. **58**(1791-800). [71]

Figure 12 gives a general distribution overlook of β-Amyloid in disease progression. β-Amyloid in **phase 1** occurs in the neocortex, further in **phase 2** in the allocortex. Lesions spread into the striatum (**phase 3)** and the brainstem (**phase 4)**. Finally at **phase 5** the cerebellum gets affected with plaques.

Amyloid β can have posttranslational modifications like oxidation, phosphorylation, nitration, racemization, isomerization, pyroglutamylation and glycosylation, where some forms aggregate more likely [50]. The accumulation of Aβ starts in the neocortex, spread to the hippocampus and finally get to the brainstem and the cerebellum [71].

In Down's syndrome and Alzheimer's disease the intra-neuronal amyloid decrease in later stages, whereas the extracellular concentration rises [67, 72]. The accumulation of Aβ in the extracellular space is toxic and lead to neuron and spine degeneration. However, the concentration of amyloid β in the extracellular space increases with synaptic activity [73]. Teng et al. [74] and Atwood et al. [75] suggested that Aβ40 is produced as a cellular antioxidant and "the real culprit within the cell, particularly in human neurons, is $A\beta_{1–42}$ […]" [74] which has a stronger tendency for accumulation than $A\beta_{1–40}$. It has been shown by Busche et al., that rather Aβ oligomers (dimers) than Aβ monomers are responsible for disease and increase the population not only of silenced neurons, but also of hyperactive neurons in the vicinity of AP, due to an increase in action potential firing [76]. However, it has remained unknown what comes first, neuronal silencing or hyperactivity. Busche et al. saw hyperactivity before plaques formation, indicating that soluble Aβ is the driving force. [77]

Aβ amyloid plaques are extracellular and in part soluble and insoluble. Gouras et al. [72] also detected intraneuronal Aβ in AD Individuals. From the different types of AP, the soluble pool of Aβ42 is the best in correlating with the degree of cognitive loss and intra neuronal Aβ accumulation is an early event in AD, suggesting that it is the driving force. High levels of "Aβ42 in vulnerable distal neurites and synapses lead to their destruction, from within" [78]. Further the formation of plaques activates inflammatory cells leading to more destruction. Furthermore, Aβ sligomers have negative effects widely spread on synapses with many targets (Prion protein, EphB2, RAGE, α7 nicotinic acetylcholine receptor, glutamate transporter, the Wnt receptor frizzled, Insulin receptor, presynaptic P/Q channels, neurotrophin receptor P75, mGluR5, β2 adrenergic receptor, calcineurin). Oligomers destroy the neuronal scaffold, accelerate DNA damage and may form ion-permeable pores, that lead to irregular calcium influx and afterwards to synapse toxicity [78]. Garcia-Marin et al. found that neurons in contact with plaques form less synapses as neurons without contact to plaques [79].

But vaccination against Aβ declined AP numbers without improvement of cognitive functions points not to a significant role of Aβ in Alzheimer's Disease [80]. Further immunotherapy against oligomeric amyloid showed that plaques are not primarily mediated by oligomers [81]. Therefore Aβ is not a good indicator for the progress in disease. There are also naturally occurring oligomers like Aβ56 that promote age dependent memory loss [82]. Although none of this oligomers is the main contributor in AD [78], they can change the consumption of synaptic proteins and consequently influence synaptic functions. For instance, is there a reduction of DLG4, Shank1 and Shank 3, due to accumulated Aβ dimers and particularly pentamers [51]. In addition there are some receptors for Aβ oligomers that are involved in

signaling cascades, as mGluR5 [83] and ephrin [84]. Aβ oligomers bind on NMDA receptors and are responsible for abnormal calcium influx that induces oxidative stress [85]. White et al. showed that cells without APP were resistant to the neurotoxic effects of Aβ [86].

Importantly, it has been shown before, that the activity of Aβ oligomers is directly linked to tau hyper phosphorylation [87], which is the second major hallmark of Alzheimer's disease.

4.2.2 Tau tangle

While the accumulation of AP are well correlated with synapse loss [88] and therefore with severity of AD, neurofibrially tangles have a clear influence in the cognition but are less specific for AD [80]. Also NFT formation is not necessary for plaque accumulation but can disrupt cell function and participate in neuronal death and degeneration [89].

Tau proteins are usually associated with cytoskeletal cell elements, like microtubules, which are stabilized by tau. As many proteins, tau gets regulated through phosphorylation. In AD patients, tau detaches, gets hyper phosphorylated and forms tangles.

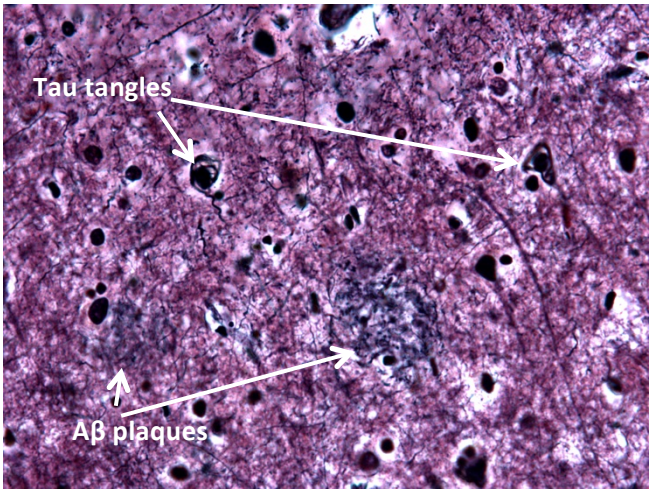


Fig. 13 Hematoxylin and Eosin stained brain tissue (Tau tangles – Aβ plaques)

Figure from: M. Dorostkar

Light microscopy image of a brain with Tau tangles and Aβ accumulation into plaques. Bielschowsky's silver stain.

Hyper phosphorylated tau aggregates are not specific for AD, but is present in nearly all forms of dementia. The knock-down argument by SantaCruz et al. is that memory deficits appears two months bevor NFT formation [90], indicating that NFT are a secondary respond to injuries and intracellular [91] spread from the brainstem to the hippocampus and last to the neocortical regions [92].

4.2.3 α-synuclein - Lewy bodies

A number of clinically different entities, such as Parkinson's disease (PD), dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD) show neuropathologically indistinctible alterations. These are based on the presence of α-synuclein aggregates, so called Lewy bodies (LBs), therefore the neuropathological term encompassing all previously mentioned clinical entities is Lewy body disease (LBD). The distribution of LBs is different than the one of tau or Aβ. It starts from the periphery, in the olfactory bulb and in the lower brainstem, followed by the substantia nigra, the mid- and forebrain and finally the neocortex. Patients with α-synuclein aggregates are characterized as LBD, when the dementia occures before motor disruption, otherwise the patient is diagnosed as Parkinson's disease. If a patient with Parkinson's disease later develops dementia, this is called Parkinson's disease dementia.

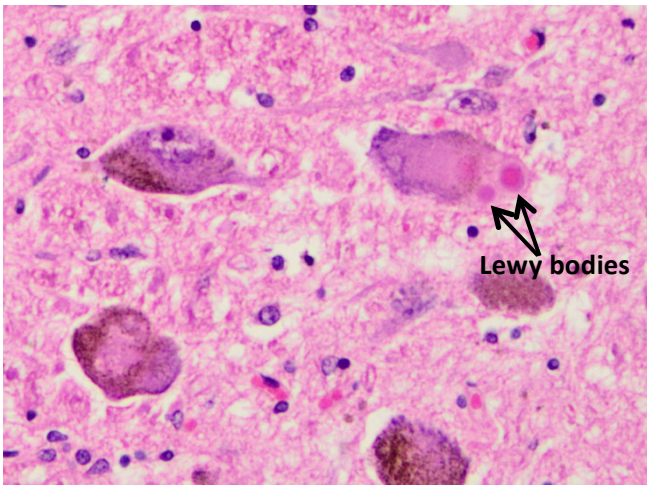


Fig. 14 Hematoxylin and Eosin stained Locus coeruleus with Lewy-bodies

Figure from: M. Dorostkar

Light microscopy image of an AD brain with Lewy bodies (arrows).

In disease the appearance of Lewy bodies starts in the locus coeruleus, followed by the substantia nigra. The best categorizing method with focus on the α-synuclein pathology is the braak staging. Severity starts at "I", which is the very low stage, up to stage "VI", with the highest density of protein accumulations. Roughly the stages of the transfected regions can be integrated into stage I-II: Trans-entorhinal, classified as prodromal AD; stage III-IV: Limbic, which is the early-moderate stage in AD and stage V-VI: Isocortical, categorized as moderate-late AD.

Fig. 15 Braak stages

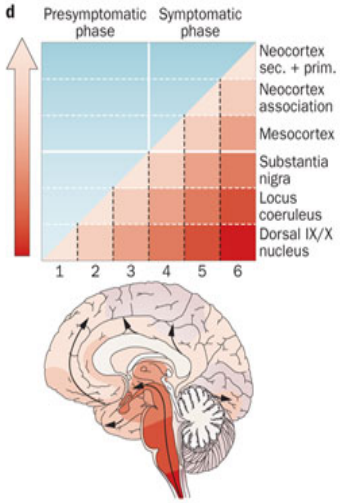


Figure from: M. Goedert, M.G.S., K. Del Tredici, H. Braak, *100 years of Lewy pathology.* Nature Reviews Neurology, 2013. **9**: p. 13-24. [93]

In figure 15 are the Braak stages from I to VI. The distribution of α-synuclein starts in the Dorsal IX/X nucleus, go further to the locus coeruleus, the substantia nigra and the meso cortex, ending up into the neocortex. The lesions at **stage I** occur in the olfactory bulb and the dorsal motor nuclei. At **stage II** lesions observed in the locus coeruleus, magnocellular nucleus and the lower raphe nuclei. **Stage III and IV**: α-synuclein spreads into the pedunculopontine nucleus, the cholinergic magnocellular nuclei of the basal forebrain and the substantia nigra (stage III). In stage IV the hypothalamus and the first cortical regions were affected. **Stage V and VI**: Lesions reach neocortical areas (stage V) as well as primary fields of the neocortex (stage VI).

Down below is an overview of the different distributions pathways of neuritic plaques, neurofibrillary tangles and Lewy bodies.

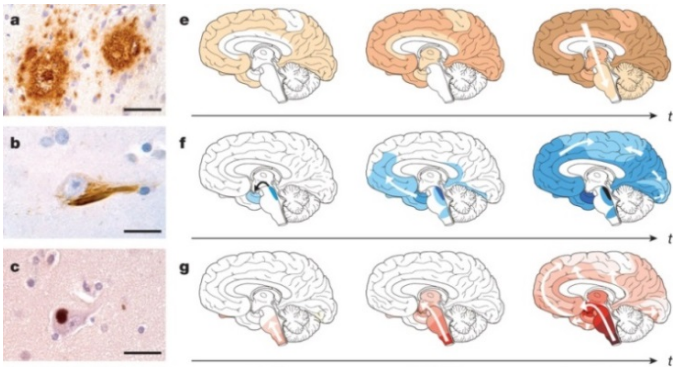


Fig. 16 Distribution of deposits in different neurodegenerative diseases over time

Figure from: M. Jucker, L.C.W., *Self-propagation of pathogenic protein aggregates in neurodegenerative diseases.* Nature, 2013. **501**: p. 45-51. [94]

On the left different protein accumulations were depicted, while on the right different stages of diseases were shown. In **a)** senile plaques (Amyloid-β) were imaged. Further shows **e)** the distribution of Aβ over the time, starting neocortical, spreading over the Allo cortex and ending up in the subcortical regions. **b)** Depicts a tau inclusion as a neurofibrillary tangle. **f)** Represents the accumulation of NFT from the locus coeruleus and transenthorhinal area, distribute further to the amygdala and interconnected neocortical brain regions. **c)** Is an example of an α-synuclein inclusion (Lewy body) which is typical for Parkinson disease/ Lewy body dementia. **g)** The accumulation of α-synuclein starts in the peripheral and autonomic nervous system, followed by olfactory bulb and lower brainstem. LB spread through the substantia nigra, over areas of the midbrain and basal forebrain, and finally to the neocortex.

4.2.4 Indirect factors

Neuropsychiatric diseases are the result of multilevel neuronal dysfunction. [52] Synaptic protein levels are just one aspect in disease, other important factors like neuro inflammation, mitochondrial dysfunction and oxidative stress should also be considered.

Mitochondria

Mitochondria dysfunction is thought to be one of the main causes of neuron loss. Mitochondria are the batteries of a cell, producing ATP out of oxygen and pyruvate, split between astrocytes and neuron, results in 30-fold higher energy consumption than in glycolysis. 20 % of the oxygen requirements of the human body and 25 % of the glucose consumption are dedicated to the brain [9]. Mitochondria are moving from the cell body to the axons, dendrites and synapses and back to the cell body. This transport is called "anterograde transport, when the translocation toward microtubule plus ends with kinesin motors are involved, whereas retrograde transport requires translocation toward minus ends, using dynein motors" [95]. Neurons are cells with high energy demands and therefore need mitochondria for the axonal transport of many proteins and molecules.

When Aβ accumulates in synapses and synaptic mitochondria, the transportation of life important proteins and substances gets interrupted. In AD patients also radicals were found. These radicals were also formed by mitochondria, but are not produced in health. Aβ is toxic, impairs mitochondrial events and causes synaptic degeneration.

In Parkinsonism mitochondria dysfunction plays an important role. The key functions of mitochondria are the regulation of the energy metabolism as mentioned above, but also cellular processes, like the calcium homeostasis, stress response and cell death pathways are functionally controlled by mitochondria [53].

Neurodegeneration in the substantia nigra and PD symptoms were also found with the abuse of drugs, like MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). This neurotoxic substance can pass the blood-brain-barrier, is taken up by dopaminergic neurons via dopamine transporter [96] and reaches mitochondira, where it inhibits the energy consumption [97].

Environment

Further factors are environment influences like head injuries or malnutrition, leading to increased oligomerization of intra-neuronal Aβ42 in distal neurites and synapses, contributing to their destruction.

There are findings of NFT and plaques in Individuals without dementia, with similar densities to cases with dementia [98]. As well as frontal cortex cell loss not only in AD but also in normal aging indicates that a certain level of neuropathology is a characteristic of normal aging. However aging is one of the most important risk factors in AD. [99] Scheff et al. showed that the loss of synapses is not evenly distributed in all brain areas, meaning that age dependent changes appear not overall, but in distinct areas, like the cortex and the hippocampus. He also pointed that the loss of synapses in Alzheimer's disease is a consequence of the disease and not of normal aging. [100] And also differences from human to human hinder comparisons and development of therapies to prevent AD.

Further may be possible that education plays a role in neurodegenerative disease [89].

All this phenomena mentioned above run in parallel and underlie a yet not known primary source. It is a complex interaction of proteins in Alzheimer's disease, which make it difficult to differ between disease specific and non-specific. A number of possible mechanisms could be responsible for synapse dysfunction. Many molecules, like hormones [101], cadherines [102], ephrins [103], neurotrophins [104], actin-related [105], PSD [106], cocaines and amphetamines [107], are involved in spine dynamics. The combination of them makes it very hard to identify one unique pathway to explain all problems.

AD has become a serious public health care problem that will become increasing concern in the future. It is important to understand molecular and cellular mechanism of synaptic degeneration to develop new biomarker and therapy.

4.3 Previous findings

Scheff and collegues measured pre- (synapsisn-1 and synaptophysin) and postsynaptic (PSD-95/DLG4, drebrin and SAP-97) protein levels and showed a decline in synaptic numbers with AD progression. [51, 52] But in another study in 2001, they claimed that the synapse loss is not distributed in all brain areas and might be due to the disease process and not a consequence of normal aging [51, 52].

The mRNA level of excitatory and inhibitory markers of hippocampal neurons were analyzed by Loreth et al. and indicated an unchanged level of GABRA1 in TauPS2APP mice, but a significantly reduced level of Gad67, which is glutamate decarboxylase and necessary for GABA production, and Y1R, which is a G-Protein coupled neuropeptide receptor. For the excitatory synapses, NMDAR1, mGluR1 and Ryr3 were measured, but showed no alterations [108].

In Alzheimer's disease GABAergic neurons are very well preserved in comparison to the other neurotransmitter systems [109]. But in other publications the focus is on the GABAergic neurons, because the inhibitory neurons are supposed to be the targets of neurodegeneration [108].

In latest papers the focus of synapse loss lie on the inhibitory synapses.

Garcia Marin wrote in 2009: "Membrane surfaces of neurons in contact with AP lack GABAergic perisomatic synapses". The author suggests that the GABAergic loss is due to the specific selectivity of AP. Further they described that "plaques in the neuropil will produce different alterations to synaptic circuits than those associated with neuronal somata", because of the fact that pyramidal cells with synapses in the perisomatic are in general GABAergic, whereas other dendrites in the neuropil build synapses with all kind of axons (glutamatergic, GABAergic, noradrenergic, dopaminergic, serotonergic and cholinergic). They also claimed, that not the cell body, but dendrites are more susceptible to the toxic effect of Aβ. [79]

Busche et al. used two photon Ca^{2+} imaging in the mouse model APP23 x PS45, overexpressing Aβ precursor protein and presenilin1, and found that the neuronal activity decreased, as well as synaptic inhibition, whereas the spontaneous Ca^{2+} transients, due to action potential firing in neurons, increase in the proximity of AP. The question whether there is an increased excitability of these hyperactive neurons near to plaques, was answered through a glutamate receptor inhibitor and the outcome was no increase in excitability of neurons, but maybe an impairment of inhibition. This thesis was supported by diazepam treatment, a $GABA_AR$ channel activator, which reduced the activity of hyperactive neurons and a $GABA_AR$ antagonist, increasing the Ca^{2+} transients, indicating an impaired synaptic inhibition. The study showed a decrease in neuronal activity in 29 %, what they named "silent" neurons and an increase of 20 % in "hyperactive" neurons, mostly in the vicinity of AP. [65]

In a study of mouse models of AD amyloidosis, Busche and colleagues found that the "impairment can be rescued by enhancing $GABA_A$ergic inhibition". Further $GABA_AR$ inhibition is important for slow Waves development, necessary to support synaptic plasticity underlying cortex and hippocampus communication and memory consolidation during sleep. Busche et al. wrote in 2015, that Aβ applied into the cortex, results in an impaired inhibition of $GABA_AR$. The balance of excitatory and inhibitory can be rescued through $GABA_AR$ function repair [77], means that $GABA_AR$ is the main mediator in AD.

In another study, Busche and colleagues claimed that there is probably a change in function, not in excitation of neurons, in the $GABA_AR$ in Alzheimer's disease. So the question is if there is a change in the subunits of the GABAergic receptors in disease, which support the impaired inhibition in synapses. [77]

Garcia-Marin and colleagues claimed, that in the vicinity of plaques neurons lack of GABAergic synapses and further lead to increased hyperactivity. They suggested, that Aβ is toxic to the synapses on dendrites and not on the cell body itself, with sprouting effects to function impairment [79]. But they only labeled for GAT1 and VGAT, which are both representatives for the pre synapse.

These results indicate not a loss of inhibitory neurons, but a loss of the GABA neurotransmission itself, which fits in our hypothesis, that there is a change in the GABAergic postsynaptic receptors, due to the changed GABA level and changed synaptic input of the pre synapse. To put Busche's and Garcia-Marin's findings together, it could be possible, that in disease the pre synapse vanishes, maybe through the presence of Aβ, and the $GABA_AR$ on the post synapse therefore change its subunits to react to the loss of synaptic connections.

4.4 Stage of disease severity

There are different methods to differentiate between the disease severities. However, a certain diagnosis of the type of the disease and the actual stage can only be made through an autopsy. Yet, a proper clinical diagnosis is important to enroll patients in studies. Diagnose methods were invented, for example diagnose-interviews (CAMDEX, SIDAM), general dementia scales (FAST, BRAAK), neuropsychological instruments (CERAD, AKT), psychopathological and behavior scales (BRSD, BEHAVE-AD) or function scales (DAD, Barthel index). Some of them were used usually together to get a better output of the condition of a patient. But all of them test the cognitive performance (verbal and figure memory, frontal-executive function, attention, speech, visuperceptive performance and areal notion).

CERAD (Consortium to establish a registry for Alzheimer's Disease)

This test aims to differentiate between the neuropsychological features of Alzheimer's disease. It is based on the complete clinical evaluation, the neuropsychological evaluation of the manifestation and the standardization of the neuropathological evaluation of AD and related diseases [110, 111]. The composition of the CERAD test is verbal fluency (Isaacs & Kennie 1973), modificated Boston naming test (Kaplan, Goodglass & Weintraum, 1983), mini mental state examination (Folstein, Folstein, McHugh, 1975), word memory (Atkinson & Shiffrin, 1971; Rosen, Mohs & Davis 1984), constructive praxis (Rosen et al., 1984), recognition of words (Mohs et al., 1986), constructive praxis recall, trail marking test and phonetic fluency. The test results were evaluated age-, education- and gender-dependent and are an important instrument for early diagnostics. The lowest stage of AD is marked with "A", further severity with "B" and high AD with "C".

5. Objective

Since loss of cognitive function has been shown to result from loss of functional synapses, we quantified markers for excitatory and inhibitory synapses. We aimed to determine how synapse loss in specific brain regions correlates with neuropathological disease stages and clinical presentation.

6. Material

67 human brains, fixed with paraffin were derived from Prof Johannes Attems, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, UK. The donors of the brains had been neuropathologically classified as minimal pathology (controls), Alzheimer's disease (high, intermediate or low) and Lewy body disease.

Case	Braak	CERAD	Thal	LBD	
729_10	0		0		Controls no/minimal pathology (n=8)
272_10	0		1		
891_11	0		0		
400_13	1		0		
353_14	1		0		
1903_14	0		0		
1904_14	0		0		
1916_14	1		0		
67_09	1		2		Low AD Neuropath change (n=10)
150_10	2		3		
359_10	2		1		
682_10	2		1		
139_13	2		2		
1187_13	2		3		
523_14	1		2		
1901_14	2		1		
676_15	2		3		
2915_15	2		3		
1085_11	3	B	4		Intermediate AD Neuropath change (n=9)
340_12	3	B	3		
9994_12	4	B	4		
388_14	4	B	4		
767_14	4	B	4		
1097_14	4	B	4		
125_07	4	B	5		
2905_15	3	B	5		
310_15	4	B	5		
98_10	6	C	5		High AD Neuropath change (n=20)
129_12	6	C	5		
426_10	6	C	5		
557_10	6	C	5		
714_12	6	C	5		
793_10	5	C	5		
987_10	6	C	5		High AD Neuropath change (n=20)
1026_10	6	C	5		
1151_10	6	C	5		
135_11	6	C	5		
9991_12	6	C	5		
205_11	6	C	5		
681_11	6	C	5		
825_11	6	C	5		
1149_11	6	C	5		
151_12	6	C	5		
474_12	6	C	5		
486_12	6	C	5		
530_12	6	C	5		
598_12	6	C	5		
81_08	4	B	3	Neocortical	Dementia with Lewy bodies i.e. neocortical Lewy body disease (n=20)
1032_12	3	B	3	Neocortical	
27_09	3		2	Neocortical	
82_10	3		0	Neocortical	
575_10	3		0	Neocortical	
745_10	4	B	3	Neocortical	
979_10	3	B	4	Neocortical	
105_07	2	A	1	Neocortical	
403_11	3		4	Neocortical	
17_07	2		0	Neocortical	
1050_12	3		1	Neocortical	
696_14	1		3	Neocortical	
1164_14	3		4	Neocortical	
1902_14	0		5	Neocortical	
61_15	3	A	4	Neocortical	
159_15	3		1	Neocortical	
639_15	3	A	5	Neocortical	
691_15	3	A	5	Neocortical	
2919_15	2		5	Neocortical	
2920_15	2		3	Neocortical	

Table 1 Classification of patients

Summary of all patients involved in the project. The first column shows the case numbers, the second indicates the Braak stages, followed by CREAD and Thal. The fifth column gives information about the LBD status. Different groups were highlighted; controls no/minimal pathology with 8 cases; low AD (10 cases); intermediate AD (9 cases); high AD (20 cases) and dementia with Lewy bodies (20 cases).

6.1 Tissue Microarrays

From each of the following regions, a small punch with ~5mm diameter had been taken from the paraffin embedded tissue. All these samples were then assembled in a new paraffin block, which then contains all samples from a single patient.

The prefrontal cortex is one of the most complex brain regions with many functions, e.g. important for planning, decision making, personality expression and social behavior. The midfrontal cortex and amygdala are both responsible for the organizing and controlling of the memory circuit, as well as the processing of emotions, while the amygdala is further responsible for decision making. For the emotion handling, cingulate is also important brain region, but also for learning and memory formation. The main memory network region is the entorhinal cortex, which also connects the information processing from the hippocampus and the neocortex. Further putamen has interconnection functions and so has many different functions, like controlling motor learning and movement process. Movement is also controlled by globus pallidus and the motor cortex. Functions like perception, motor control, self-awareness, cognitive functioning were controlled by the insular cortex. The regulation of consciousness, sleep and alertness is handled by thalamus. Next the temporal cortex is responsible for language reception and processing, as well as recognition of colors, words, bodies and faces. This region is one of the first affected in Alzheimer's disease. For cognition, language and mathematics the parietal lobe is a modulator. Last the occipital cortex is necessary for the visual information processing.

Location	Punch
Prefrontal cortex	1 - 3
Mid frontal cortex	5 - 7
Cingulate	9, 10
Caudate	11
Putamen	12
Globus Pallidus	13
Insular cortex	14, 19
Amygdal	15
Motor cortex	17, 18
Thalamus	20
Entorhinal cortex	16, 21 - 25
Temporal cortex	26 - 30
Parietal cortex	31 - 34
Occipital cortex	36 - 39
White matter	4, 8, 35, 40

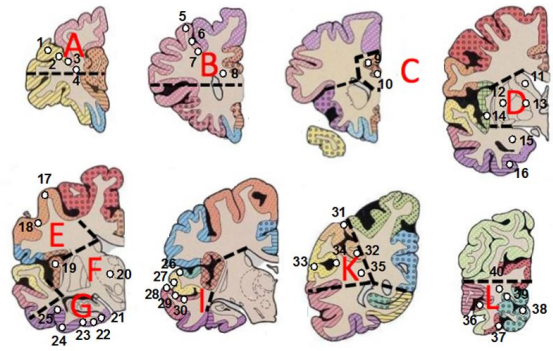


Fig. 17 Tissue Microarrays

Figure from: J. Attems

In Figure 17 are the microarrays from the different brain regions listed on the left and marked on the right. While the white matter has the number 4, 8, 35 and 40; basal ganglia has 11, 12 and 13. Amygdala (15) and thalamus (20). The cortex is further divided into prefrontal- (1-3), midfrontal- (5 - 7), insular- (14, 19), motor- (17, 18), entorhinal- (16, 21 - 25), temporal- (26 - 30), parietal- (31 - 34) and occipital cortex (36 - 39).

I further divided the optical cortex into the primary visual field Brodmann's area 17 (Punch 38), and the secondary visual field Brodmann's area 18 (Punch 37, 39).

Tissue microarrays from all patients were immunofluorescence labelled with antibodies against DLG4 (also known as PSD-95 scaffold protein, which "is a hallmark of a mature synapse" [18]) to stain excitatory synapses and γ-Aminobutyric acid receptor A subunit α2 (GABRA2) to stain inhibitory synapses.

6.2 Chemicals

- Xylol 100 %
- EtOH 100 %, 96 %, 70 %
- Citrate Puffer PH 6
- dPBS
- Normal Goat Serum (NGS)
- Triton x-100
- Sudan Black
- Sodium Azide
- Dako (Fluorescence mounting medium)

ATB

Primary

- Anti PSD-95 (Mouse monoclonal ATB, SYSY 124011)
- Anti $GABA_A$-R α2 (Guinea Pig polyclonal ATB, SYSY 224104)

Secondary

- Anti-Mouse A488 (Goat, Molecular probes; A11029)
- Anti-Guinea Pig A488 (Goat, Molecular probes; A 11073)

Nuclei staining

- DAPI (Roth, 6335.2)

Puffer and solutions

- Blocking Buffer (for Blocking and primary ATB Solution)

  DPBS + 10 % NGS + 1 % Triton x-100 (Stock with dPBS and Triton x-100 with 0,05 % Sodium Azide)

- Secondary ATB Solution

  DPBS + 3 % NGS + 1 % Triton x-100 (Stock with dPBS and Triton x-100 with 0,05 % Sodium Azide)

- Sudan Black Solution

  0,3 % Sudan Black in 70 % EtOH, filtered (prepared freshly every week, stored in fridge at 4°C)

Equipment

Confocal microscope	Images were taken with Zeiss LSM 780
Software	Processed in IgorPro7, detecting single puncta, based on published algorithms [81, 112].

7. Methods

Given below is a schematic process of the staining. The procedure is constructed on deparaffinization steps, antigen retrieval, blocking and antibody and Sudan Black incubations.

Day 1

- Deparaffinization
- Antigen retrieval (5x 4 min)
- Blocking (1 h)
- Primary ATB incubation 4°C, Over Night (ON)

Day 2

- Washing with dPBS (3x 5 min)
- Secondary ATB incubation (2 h)
- Washing with dPBS (3x 5 min)
- Sudan Black incubation (1,5 min)
- Washing with ddH_2O (3x 5 min)
- Drying
- Mounting (ON)

Deparaffinization

First paraffin slices were brought to Xylol and EtOH to hydrophilize the tissue and get rid of the paraffin.

- 2 x 15 min Xylol 100 %
- 2 x 5 min EtOH 100 %
- 1 x 5 min EtOH 96 %
- 1 x 5 min EtOH 70 %

For removing the alcohol, the slides were washed 3 times with H_2O.

Antigen retrieval

Because of tissue paraffinization for fixation, crosslinking appear and modify the tissue. But the antibodies are not specific for this crosslinking, so it has to be removed. Therefor the slides were cooked 5 x 4 min in the microwave oven (700 Watt) in citrate puffer pH 6. The liquid level was holding constant with warm dH_2O to prevent the slices from drying out. After that the sections were cooled to RT for 20 min.

Blocking

Next the slides were masked to avoid unspecific binding. 10 % NGS in dPBS with 10 % Triton x-100 were used as blocking agent and the slices were kept in a humidity chamber with the blocking solution for 1 h.

Primary ATB incubation

Further the primary ATB were diluted in blocking solution and kept in the humidity chamber at 4°C ON.

Dilutions

ATG	Host	Dilution
Anti PSD 95	Mouse	1 : 1000
Anti $GABA_A$-R α2	Guinea Pig	1 : 200

Washing

The slides were washed with dPBS for 3 times, each 5 min.

Secondary ATB incubation

The secondary ATB were diluted in 3 % NGS in dPBS with 10 % Trition x-100 and incubated on the slices for 120 min at RT, dark.

Dilutions

ATG	Host	Conjugated	Dilution
Anti-Mouse	Goat	A488	1 : 500
Anti-Guinea Pig	Goat	A488	1 : 500

Washing

The slides were washed with dPBS for 3 times, each 5 min.

DAPI incubation

For visualize the nucleus of the neurons, DAPI were used to stain the DNA in the nucleus. The incubation was 5 min at RT, dark.

Washing

The slides were washed with H_2O for 2 times, each 5 min.

Sudan Black incubation

Further auto-fluorescence and the noise of the background-staining were reduced by Sudan Black. The incubation in the solution was 1,5 min.

Washing

The slides were washed with H_2O for 5 times, each 5 min.

Drying

Afterwards the slides were dried at a dark Place.

Mounting

Finally DAKO mounting medium were used to fix the staining. A coverslip were put on top and the slices dried ON at RT, dark.

Image acquisition and Analysis

The slices were imaged on a Zeiss LSM 780 confocal microscope using the following settings:

ATB	*Laser*	*Objective*	*Gain*	*Scaling*	*Dimensions*	*Digital Offset*	*Digital Gain*
DAPI	1,5	Plan-Apochromat 40x/1.4 Oil DIC M27	600	0.052 µm x 0.052 µm	1024 x 1024 16-bit	0	1
PSD 95	10		700				
$GABA_A$-R α2	10		700				

The images were analyzed with IgorPro7, detecting single puncta, based on published algorithms [81, 112].

First the synapse densities were quantified, excluding nuclei, which were stained with DAPI, blood-vessels, which are dark regions and auto fluorescence. Also a filter was used to exclude puncta smaller than 4 pixels, which are caused by unspecific noise, as well as those brighter than the 90th percentile, which are caused by auto-fluorescence. Second the ROI (region of interest) was calculated, excluding nuclei, blood-vessels and auto fluorescence. With this two bench marks, the synapse number per region was calculated.

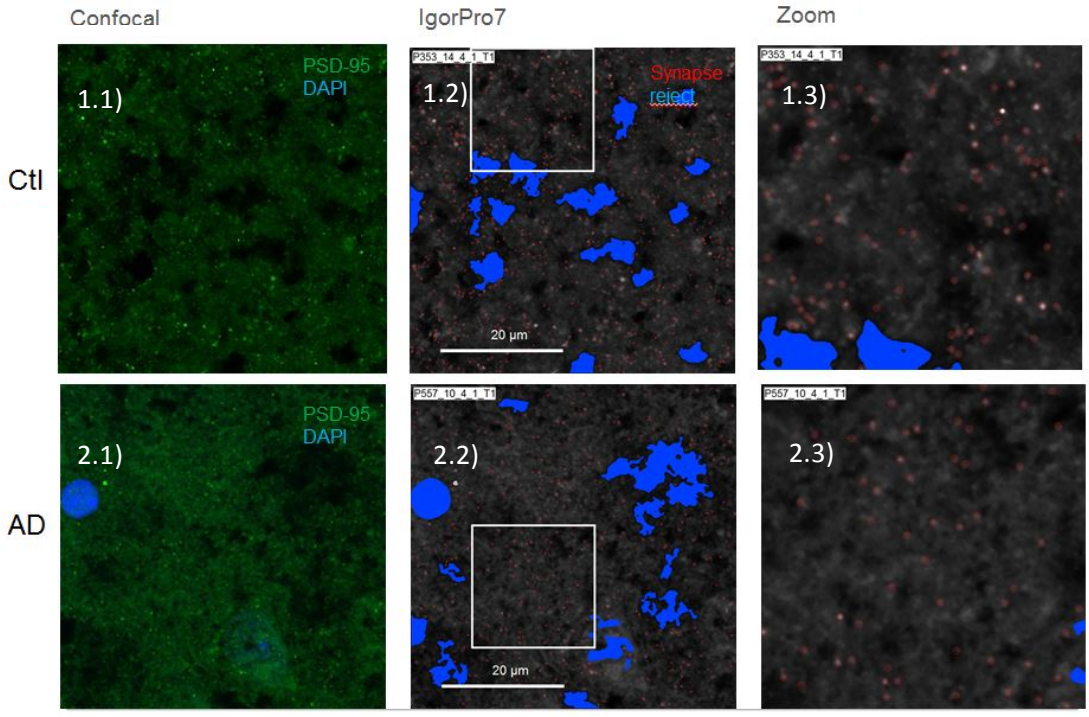


Fig. 18 Analysis Steps

In Figure 18 is the order of the analysis shown. Analysis of a control picture (above) and an AD picture (bottom). Starting with the confocal image (1.1; 2.1) the DLG4 (PSD-95) staining is shown in green dots, whereas the DAPI nuclei staining is depicted in blue. After analysis in IgorPro7 (1.2; 2.2) synapses detected in red and the rejected area in blue (including nuclei, background and blood vessels). In 1.3 and 2.3 is the zoom of the white box of 1.2 and 2.2 shown.

The statistical analysis was determined by a two-tailed unpaired Student's t-test with confidence intervals of 95%. All calculations were performed with Graph Pad Prism and for $p < 0,05$ the data was accepted as statistically significant.

The classification of *AD* and *no AD* was due the Braak stages. *AD*: Braak 5, 6 and *no AD*: Braak 0, 1, 2.

9. Results

Prefrontal Cortex – Midfrontal Cortex – Motor Cortex – Cingulate – Parietal Cortex – Thalamus – Brodmann's area 17 – Brodmann's area 18

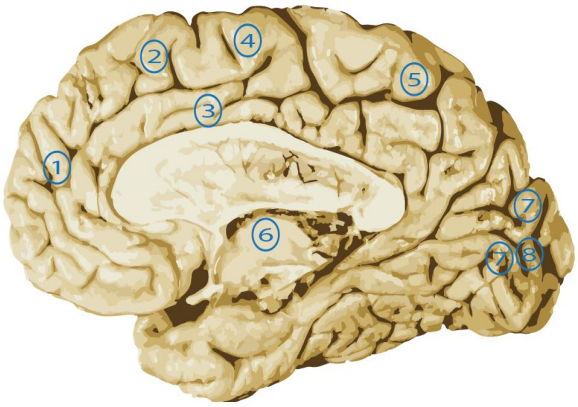


Fig. 19 Sagittal view of the brain

Figure 19 gives a sagittal view of the brain. Followed marked regions were analyzed: Prefrontal cortex (1), midfrontal cortex (2), cingulate (3), motor cortex (4), parietal cortex (5), thalamus (6), Brodmann's area 18 (7) and Brodmann's area 17 (8).

Temporal Cortex – Insular Cortex – Putamen – Caudate – Putamen – Globus Pallidus – Amygdala – Entorhinal Cortex

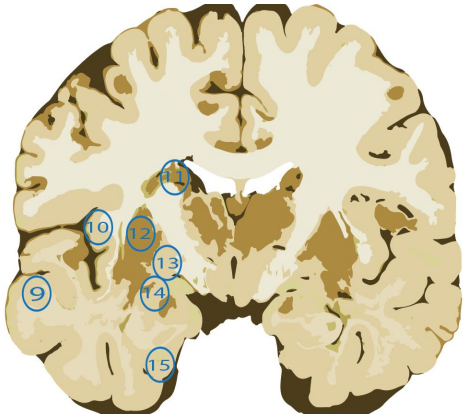


Fig. 20 Frontal view of the brain

Figure 20 gives a frontal view of the brain. The numbers. Followed marked regions were analyzed: Temporal gyrus (9), insular cortex (10), caudate (11), putamen (12), pallidum (13), amygdala (14) and entorhinal cortex (15).

9.1 Statistical Analysis

AD vs *no AD*

	GABRA2											DLG4										
				Mean		Median		Δ	SD		n				Mean		Median		Δ	SD		n
Region	Significant		P-Value	AD	no AD	AD	no AD		AD	no AD	AD/no AD	Significant	P-Value		AD	no AD	AD	no AD		AD	no AD	AD / no AD
Cingulate	Yes	***	0.0002	0.1484	0.1937	0.1447	0.1985	0.0538	0.03165	0.03329	18 / 18	Yes	< 0.0001	****	0.108	0.1573	0.1076	0.1514	0.0438	0.02909	0.02675	17 / 14
Entorhinal	Yes	****	< 0.0001	0.147	0.1859	0.146	0.1787	0.0327	0.01953	0.03355	20 / 18	Yes	< 0.0001	****	0.1311	0.1842	0.1262	0.1731	0.0469	0.03072	0.03141	19 / 16
Putamen	Yes	****	< 0.0001	0.1415	0.1882	0.1376	0.1921	0.0545	0.03048	0.03309	17 / 16	No	0.1526		0.1806	0.2124	0.1812	0.218	0.0368	0.06034	0.06418	16 / 17
B17	Yes	****	< 0.0001	0.1373	0.1983	0.1258	0.1951	**0.0693**	0.02898	0.0405	16 / 14	No	0.6506		0.1003	0.09484	0.09787	0.09727	-0.0006	0.03673	0.03933	25 / 17
B18	Yes	**	0.0068	0.1579	0.1914	0.1647	0.1926	0.0279	0.03539	0.03412	19 / 17	No	0.9822		0.1315	0.1218	0.134	0.1335	-0.0005	0.0351	0.05135	7 / 6
Caudate	Yes	***	0.0002	0.1383	0.1961	0.1378	0.1972	**0.0594**	0.03134	0.03832	13 / 16	No	0.1593		0.1574	0.1816	0.1464	0.1877	0.0413	0.05788	0.03515	15 / 17
Prefrontal	Yes	**	0.0043	0.1479	0.1757	0.1477	0.1727	0.025	0.0266	0.0279	18 / 18	No	0.3977		0.1118	0.1243	0.1092	0.1135	0.0043	0.03179	0.04252	13 / 14
Globus Pallidus	Yes	**	0.0009	0.1553	0.2104	0.1556	0.2015	0.0459	0.0413	0.04445	18 / 15	Yes	0.0239	*	0.09217	0.1231	0.09483	0.1308	0.03597	0.024	0.04762	15 / 8
Midfrontal Ctx	Yes	**	0.005	0.1524	0.1836	0.1383	0.1906	0.0523	0.03545	0.02784	20 / 18	No	0.6249		0.1139	0.1202	0.1092	0.1135	0.0043	0.02993	0.03911	15 / 14
Motor Ctx	Yes	*	0.0386	0.1565	0.1804	0.1642	0.1797	0.0155	0.03316	0.03117	18 / 17	No	0.0615		0.1207	0.1522	0.1182	0.1377	0.0195	0.04	0.05381	17 / 17
Amygdala	Yes	***	0.0009	0.1396	0.1818	0.1405	0.1685	0.028	0.03342	0.03233	18 / 15	No	0.1362		0.1386	0.1639	0.1239	0.1593	0.0354	0.05519	0.04009	17 / 17
Thalamus	Yes	*	0.0163	0.16	0.2009	0.1591	0.1931	0.034	0.0497	0.04361	18 / 16	No	0.329		0.1171	0.1349	0.1171	0.1349	0.0178	0.1171	0.1349	15 / 16
Parietal Ctx	Yes	**	0.0014	0.1525	0.1889	0.1548	0.1856	0.0308	0.03231	0.03172	19 / 18	No	0.936		0.1502	0.1515	0.1456	0.1443	-0.0013	0.03539	0.03943	10 / 10
Insular Ctx	Yes	**	0.0017	0.1486	0.182	0.1469	0.1818	0.0349	0.0279	0.03545	22 / 19	Yes	0.0152	*	0.1388	0.1712	0.1364	0.1703	0.0339	0.02553	0.04533	17 / 17
Temproal Gyrus	Yes	***	0.0006	0.15	0.1914	0.1475	0.1838	0.0363	0.02903	0.02972	18 / 13	Yes	0.0025	**	0.1379	0.1678	0.1393	0.1618	0.0225	0.01224	0.03257	15 / 15

LBD vs Ctl.

	GABRA2											DLG4										
				Mean		Median		Δ	SD		n				Mean		Median		Δ	SD		n
Region	Significant		P-Value	LBD	Ctl	LBD	Ctl		LBD	Ctl	LBD / Ctl	Significant	P-Value		AD	no AD	AD	no AD		AD	no AD	AD / no AD
Cingulate	No		0.1802	0.1599	0.1799	0.1716	0.1915	0.0199	0.03804	0.02724	25 / 8	No	0.5754		0.1333	0.1486	0.1145	0.1407	0.0262	0.06944	0.02254	19 / 7
Entorhinal	Yes	*	0.0267	0.151	0.1865	0.1466	0.1843	0.0377	0.03314	0.04022	18 / 8	No	0.5685		0.1512	0.1651	0.1539	0.1651	0.0112	0.06248	0.007003	17 / 7
Putamen	No		0.729	0.1684	0.1727	0.1764	0.1833	0.0069	0.02768	0.0308	18 / 8	No	0.2983		0.1715	0.1998	0.1781	0.2201	0.042	0.06574	0.05588	19 / 8
B17	No		0.8039	0.1721	0.1786	0.1675	0.1739	0.0064	0.05894	0.03956	19 / 6	No	0.0943		0.1027	0.07046	0.1078	0.06655	-0.0413	0.04704	0.01999	20 / 7
B18	No		0.2237	0.161	0.1833	0.1471	0.1796	0.0325	0.04644	0.02832	18 / 8	No	0.593		0.1144	0.1036	0.1108	0.1105	-0.0003	0.02612	0.03356	7 / 3
Caudate	No		0.6027	0.1822	0.1723	0.1796	0.1662	-0.0134	0.049	0.02541	16 / 8	No	0.1152		0.1516	0.1786	0.1447	0.1836	0.0389	0.07694	0.02962	16 / 8
Prefrontal	No		0.6711	0.1622	0.1692	0.1617	0.1644	0.0027	0.04209	0.02735	18 / 8	Yes	< 0,0001	***	0.1333	0.0909	0.132	0.0857	-0.0463	0.009246	0.01708	7 / 7
Globus Pallidus	No		0.0881	0.1795	0.2057	0.1871	0.2019	0.0148	0.03501	0.03406	18 / 8	No	0.207		0.1101	0.1308	0.1094	0.1231	0.0137	0.0304	0.04762	16 / 8
Midfrontal Ctx	No		0.6922	0.1809	0.1738	0.1824	0.183	0.0006	0.04467	0.03146	17 / 8	Yes	0.0085	**	0.1232	0.08842	0.1305	0.08513	-0.0454	0.02368	0.0191	8 / 7
Motor Ctx	No		0.4978	0.1632	0.1758	0.1619	0.1698	0.0079	0.04895	0.02328	18 / 8	No	0.7234		0.1351	0.1421	0.1485	0.1327	-0.0158	0.04595	0.04569	17 / 8
Amygdala	No		0.2944	0.1707	0.1884	0.1741	0.1685	-0.0056	0.0352	0.04024	17 / 7	No	0.4582		0.166	0.1476	0.1678	0.1529	-0.0149	0.06266	0.04187	17 / 8
Thalamus	Yes	*	0.0196	0.1606	0.2021	0.1534	0.1924	0.039	0.03612	0.04219	16 / 8	No	0.9749		0.1231	0.1238	0.1238	0.1189	-0.0049	0.04821	0.05846	18 / 8
Parietal Ctx	Yes	*	0.0359	0.1568	0.1815	0.1655	0.1787	0.0132	0.0279	0.02108	18 / 8	No	0.3707		0.1432	0.1292	0.1393	0.1249	-0.0144	0.02853	0.02693	11 / 5
Insular Ctx	No		0.2399	0.1629	0.1773	0.1661	0.1781	0.012	0.03079	0.0222	21 / 8	No	0.6322		0.1458	0.1556	0.1223	0.1492	0.0269	0.05138	0.02053	18 / 7
Temproal Gyrus	No		0.4012	0.173	0.183	0.1788	0.1796	0.0008	0.0268	0.02281	16 / 7	No	0.3078		0.1424	0.1624	0.1196	0.1472	0.0276	0.04697	0.02871	18 / 7

Table 2 Statistical analysis

Overview of p-value, mean, median, SD and n of GABRA2 (blue) and DLG4 (yellow) stainings for the AD vs. *no AD* (above) and LBD vs. Ctl (bottom, no bar graphs shown).

9.2 Alzheimer's Disease

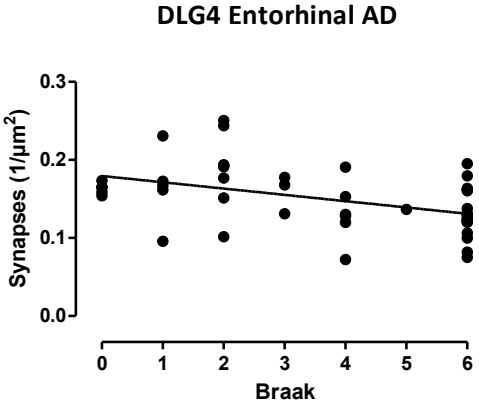


Fig. 21 DLG4 Entorhinal AD/Braak

Synapse density (1/µm²) against the Braak stage is shown in Figure 21. For the entorhinal cortex from AD patients, the synapse loss is not significant higher with further course of the disease.

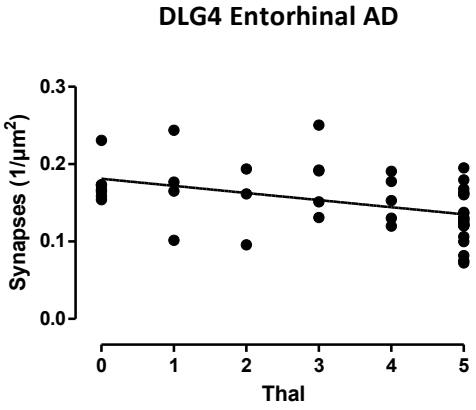


Fig. 22 DLG4 Entorhinal AD/Thal

In Figure 22 the synapse density (1/µm²) of the DLG4 staining in the entorhinal cortex in AD patient is plotted against their Thal values. The curve shows a significant decay with higher Thal levels.

In Figure 21 and 22 the synapse density (1/µm²) of the entorhinal cortex is plotted against Braak (above) and Thal (bottom). The slope of the classification with Braak is -0,01296 and not significant (compared with Braak 0), whereas the slope of the classification with Thal is -0,01490 and significant (compared with Thal 0). The distribution of the cases in each stage is mainly broad and similar in the other brain regions (not shown). The synapse density does not correlate well with the Braak or the Thal stages. With respect to this, another illustration with another classification method was used to analyze and depict the data.

Further down the brain regions prefrontal cortex, midfrontal cortex, cingulate, motor cortex, parietal cortex, thalamus, Brodmann's area 18 (Punch 37, 39 in figure 16) and Brodmann's area 17 (Punch 38 in figure 16) were analyzed.

In the first graph – *Prefrontal cortex*, where no significant change in the DLG4 is depicted and the P-Value from GABRA2 amounts 0,0009. The decrease of synapse density drops from 0.1757 (1/µm²) in the GABRA2 tissue staining's (*no AD)* to 0.1476 (1/µm²) in the AD cases.

Similar results were seen in the *midfrontal cortex*, where only the GABRA2 staining's show a significant decrease from 0.1836 (1/µm²) in the *no AD* cases to 0.1524 (1/µm²) in the AD cases (P-Value: 0,005)

Cingulate, the synapse density in the *AD* cases, analyzed with the DLG4 staining show a significant drop from 0,1573 in the *no AD* cases to 0,108 in the *AD* cases. The p-value is <0,0001 in the DLG4 and 0,0002 in the GABRA2 staining's, where the mean value drops from 0,1938 in the no AD to 0,1484 in the *AD* cases.

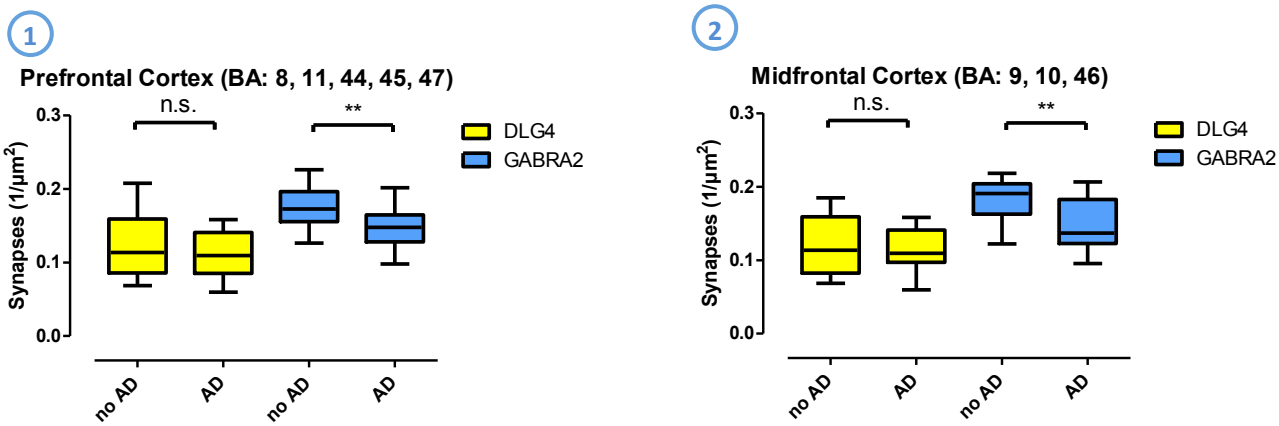
Further the synapse densities in the *motor cortex* depict a small, but significant loss of GABRA2 synapses. The p-value is about 0.0386, and the synapse decay drops from the *no AD* cases 0.1804 (1/µm²) to 0.1565 (1/µm²) in the *AD* cases.

In the *parietal cortex* the synapse loss count with 0.03231, measured with the mean value 0.1856 (1/µm²) in the *no AD* and 0.1548 (1/µm²) in the AD cases.

Next, *thalamus* count with a mean value of 0.2009 (1/µm²) in the *no AD* for the GABRA2 stained tissues and 0.16 (1/µm²) in the AD cases, with a median difference of 0.034 (1/µm²).

Further in *the Brodmann's area 18*, there is no significant change in the DLG4 visible, whereas in GABRA2 the synapse loss is significant with a p-value of 0,0068 and a difference of the mean value 0,0335 (0,1914 in the *no AD* and 0,1579 in the AD cases).

Also in the *Brodmann's area 17* is a very small effect seen in the DLG4 results, but the decrease of synapses in the GABRA2 is with a P-Value of <0,0001 and the highest median difference of 0,0693. Mean values of 0,1983 in *no AD* and 0,1373 in AD were measured and are the biggest effect in the analysis of all brain regions.



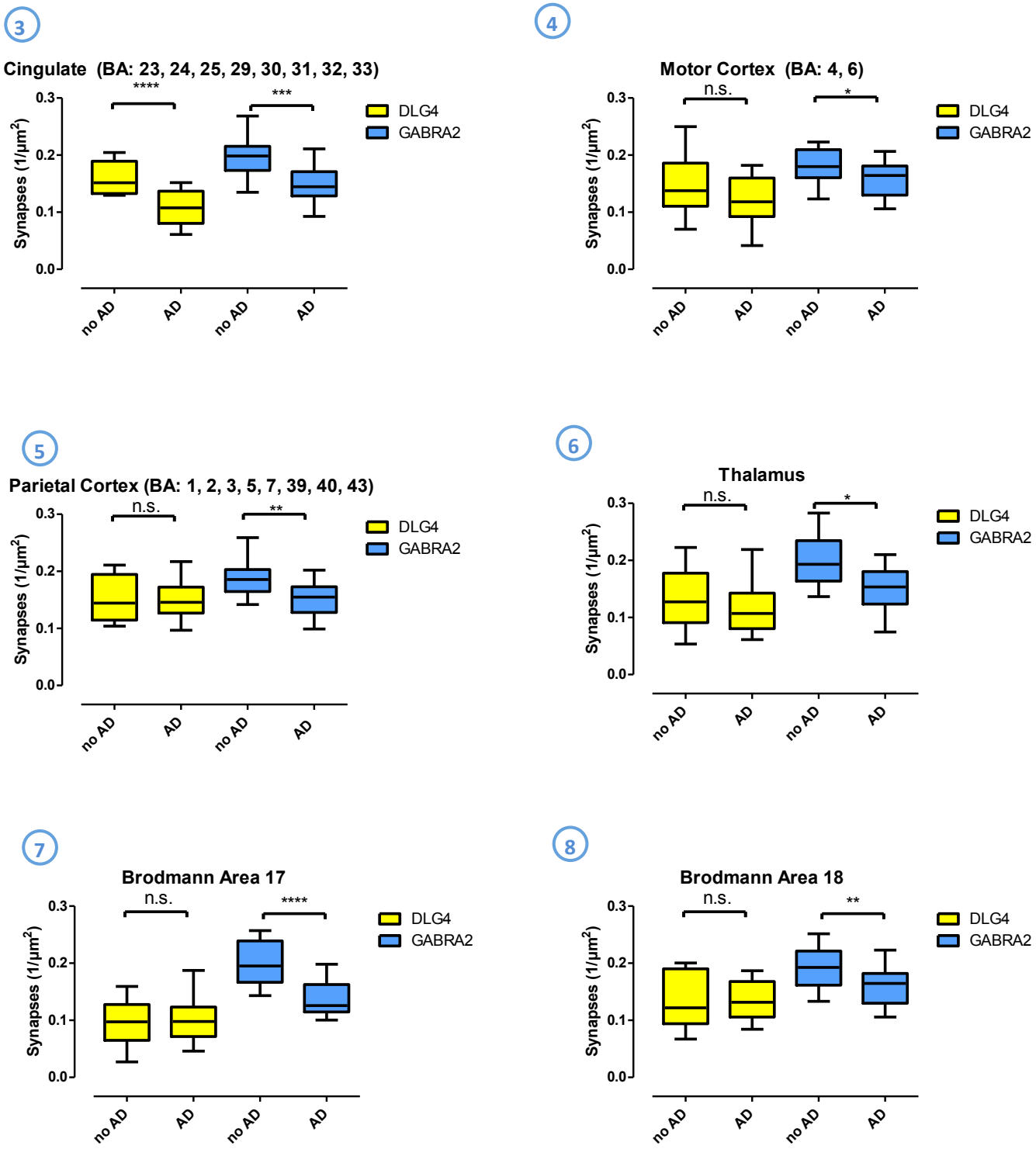


Fig. 23 - 30 Synapse densities (1/µm^2) of DLG4 and GABRA2, *no AD* compared to AD

Each graph represents the synapse density (1/µm^2) from one brain region of controls (no AD) and Alzheimer disease cases (AD). DLG4 (yellow) and GABRA2 (blue).

When looking at the synapse density in the first graph (next page) – *Temporal gyrus,* it is clear, that a significant decrease in synapses for DLG4 and GABRA2 occurs in the AD cases, compared to the *no AD*. The P-Value is 0.0006 for the GABRA2 and 0.0025 for the DLG4. The mean drop from 0.1914 (1/µm²) for the *no AD* cases in the GABRA2 stained tissue to 0.15 (1/µm²) in the AD cases, whereas the mean in the DLG4 drop from 0.1678 (1/µm²) (no AD) to 0.1379 (1/µm²) (AD cases).

In the *insular cortex* the synapse loss is only significant in GABRA2 tissue staining, with a drop from 0.182 (1/µm²) (*no AD*) to 0.1486 (1/µm²) (*AD* cases) for the mean-value.

Also in the *caudate* only the synapse loss measured on the GABRA2 staining tissue was significant, with a mean value of 0.1961 (1/µm²) in the *no AD* cases and 0.1383 (1/µm²) in the AD cases. The median difference of 0.0594 (1/µm²) is the second highest measured in all brain regions. The p-value is about 0.0002.

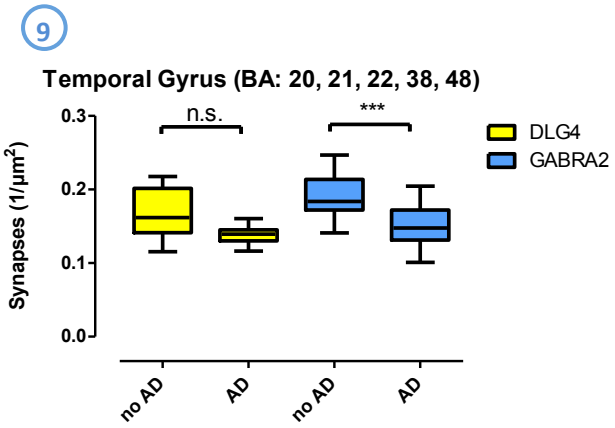
A decrease in synapses occurs in *putamen* in the *AD* cases, compared to the *no AD* for DLG4 and GABRA2. While DLG4 show a not significant decrease, the drop in GABRA2 is significant (P-value < 0,0001). The synapse density drops from 0.1882 (1/µm²) to 0.1415 (1/µm²) in the AD cases. In comparison the DLG4 have a higher median (0,1812 (1/µm²) for *AD* and 0,218 (1/µm²) for *no AD*) than the GABRA2 (0,1376 (1/µm²) for AD and 0,1921 (1/µm²) for *no AD*) in the putamen.

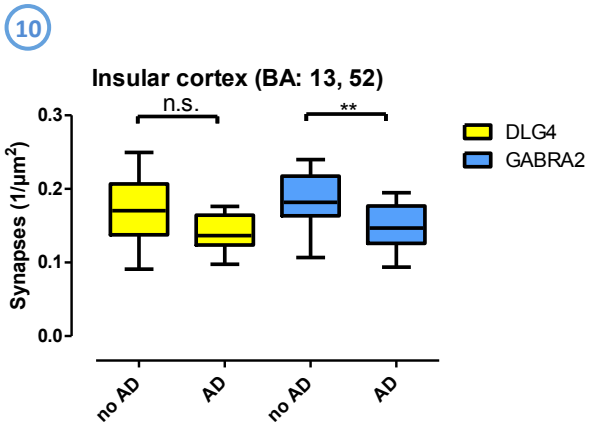
Similar results were seen in the *caudate*, where DLG4 shows a not significant decay of synapses with a median of 0,1464 (1/µm²) in *AD* and 0,1877 (1/µm²) for *no AD*. GABRA2 synapse density drops from a mean of the *no AD* cases: 0,1961 (1/µm²) to the *AD* cases: 0,1383 (1/µm²) and a p-value of 0,0002.

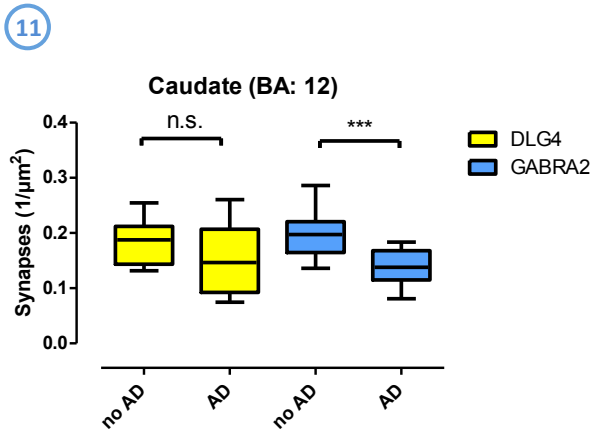
The next brain region – *Globus pallidus* depicts a lower mean value in the DLG4 synapse measurement (0,09217 (1/µm²) for *AD*; 0,1231 (1/µm²) for *no AD*), than in the GABRA2 measurement (0,1553 (1/µm²) in the *AD* cases; 0,2104 (1/µm²) in the *no AD* cases). Both decays were significant from the *no AD* to the *AD* cases (P-Value DLG4: 0,0239; GABRA2: 0,0009).

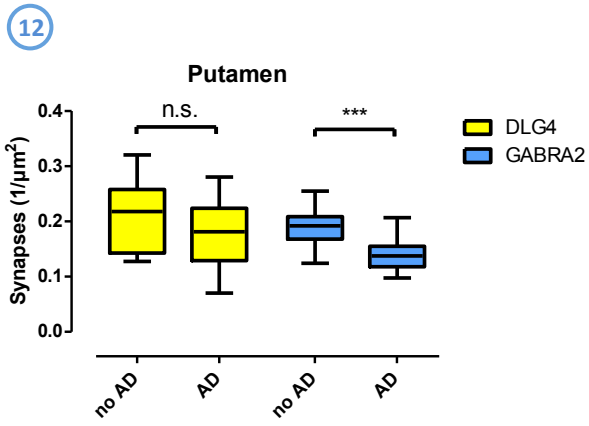
Also a significant drop from *no AD* to *AD* were depicted in the *amygdala* for the synapse loss in the GABRA2 tissues from 0.1818 (1/µm²) in the no AD cases to 0.1396 (1/µm²) in the *AD* cases, but not in the DLG4 tissues.

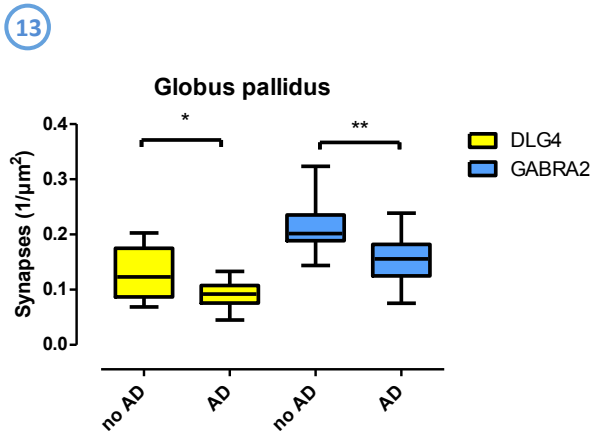
Entorhinal cortex, where the p-value of DLG4 and for GABRA2 was <0,0001. The difference of the median in DLG4 in *no AD* (0,1731 (1/µm²)) and AD (0,1262 (1/µm²)) pointed a greater number with 0,0469 (1/µm²) than the difference of the median in GABRA2, which was 0,0327 (1/µm²) (no AD: 0,1787 (1/µm²) and AD 0,146 (1/µm²)), showing the biggest effect from these four regions. The synapse density drops from 0.1859 (1/µm²), in the no AD to 0.147 in the *AD* cases, in the GABRA2 synapses and from 0.1842 (1/µm²) (*no AD*) to 0.1311 (1/µm²) (*AD* cases) in the DLG4 synapses.

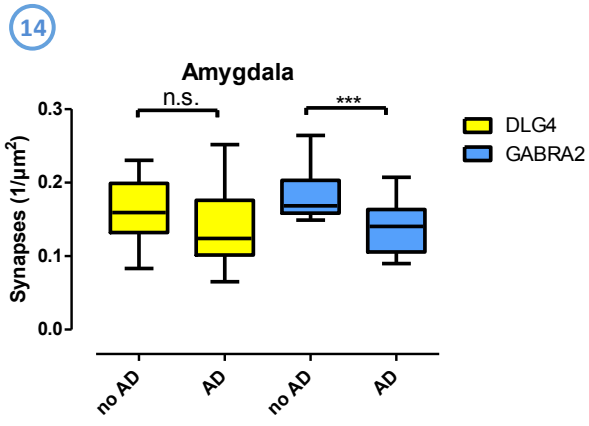












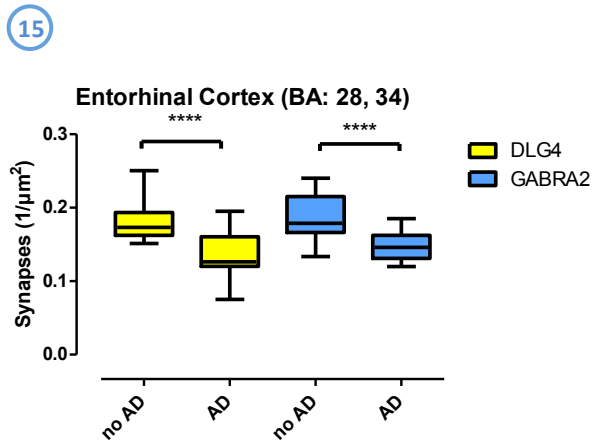


Fig. 31 - 37 Synapse densities (1/µm^2) of DLG4 and GABRA2, *no AD* compared to *AD*

Each graph represents the synapse density (1/µm^2) from one brain region of controls (no AD) and Alzheimer disease cases (AD). DLG4 (yellow) and GABRA2 (blue).

To summarize the bar graphs above, there are only two brain regions, cingulate and entorhinal ctx, where the synapse loss in DLG4 is significant with P<0,0001. Interestingly the synapse density in the *no AD* cases for the entorhinal ctx showing higher numbers than the rest, but also putamen reaches the 0,2/μm^2. On the contrary, globus pallidus have the lowest values for the *no AD* cases in the DLG4 staining and this brain region has higher synapse numbers in the inhibitory synapses in the *no AD* cases. Same count for Brodmann's area 17 and the midfrontal ctx.

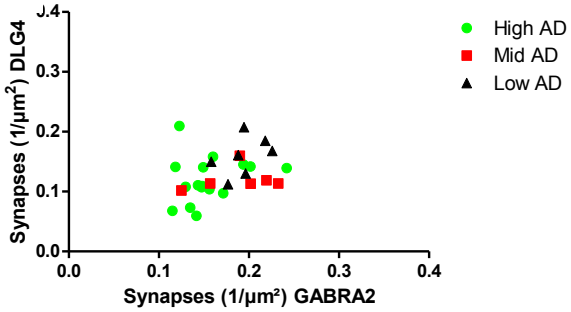
When the *no AD* cases were compare with GABRA2 and DLG4, Brodmann's area 17 count with the highest value in the median difference, next is the globus pallidus in both *AD* and *no AD* cases.

In comparison the GABRA2 analysis shows bigger effects in synapse loss than the DLG4 results. The most significant loss in synapse density for inhibitory synapses came out in Brodmann's area 17 and the entorhinal ctx, followed by cingulate, temporal gyrus, putamen, caudate and amygdala. The higher synapse loss in GABRA2, pointing out that inhibitory synapses were more strongly affected in *AD*, than excitatory synapses.

Next I put the synapse densities (1/µm²) from GABRA2 in comparison to the densities of DLG4 in each brain region.

Each point indicates one patient.

1



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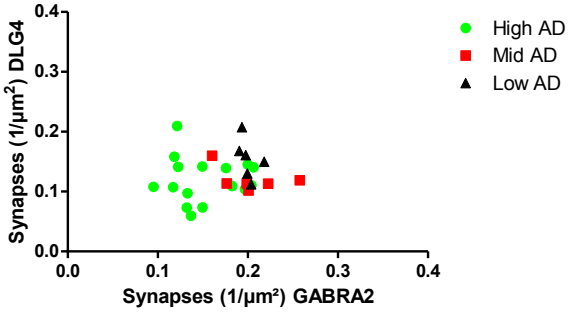

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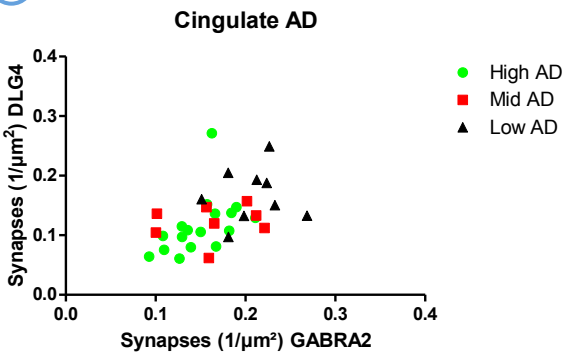

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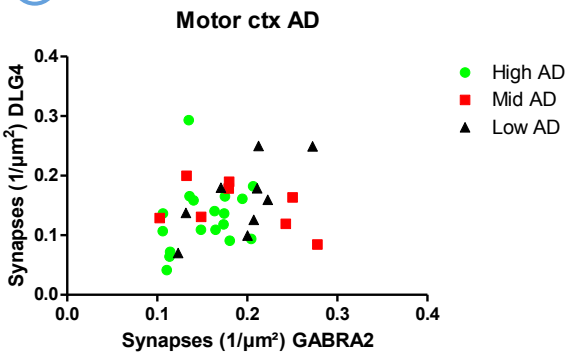

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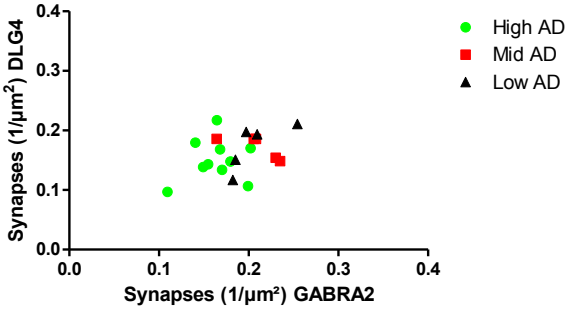

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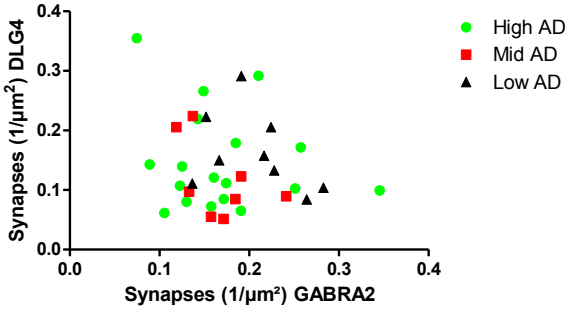

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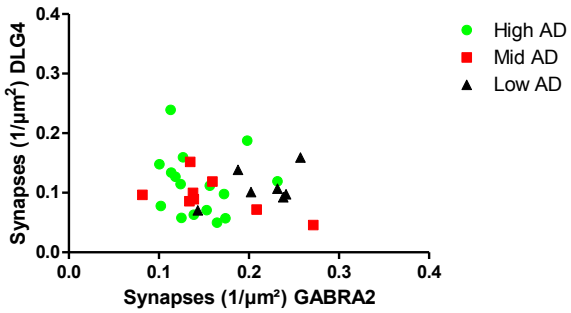

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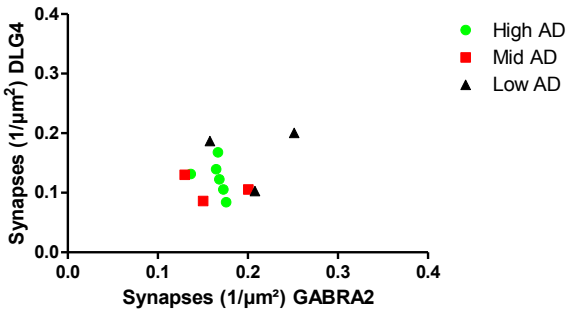

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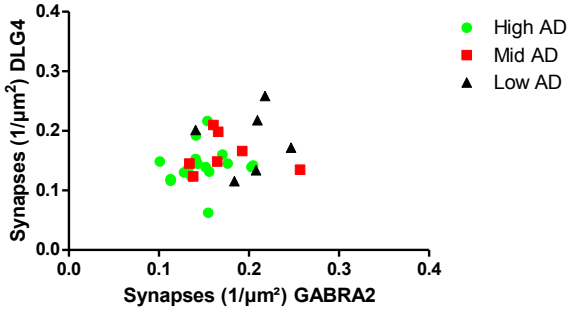

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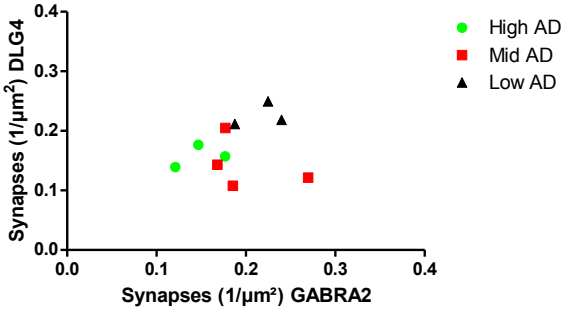

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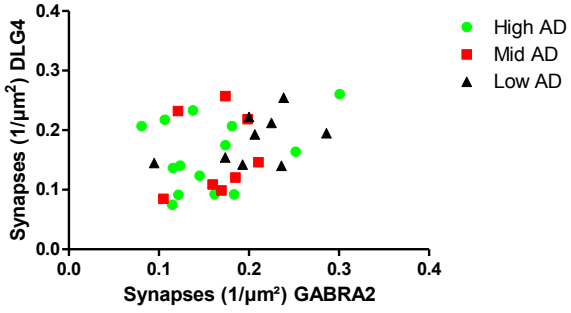

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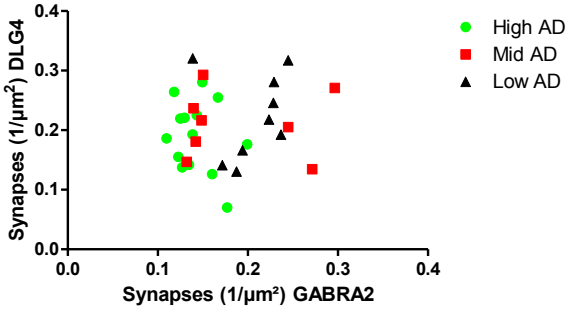

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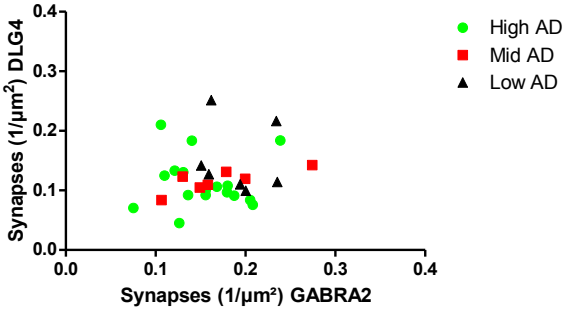

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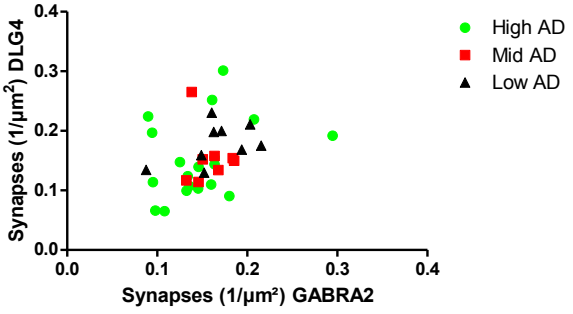


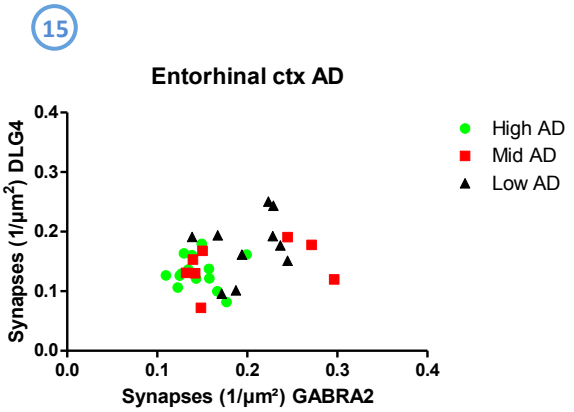


Fig. 38 - 52 Correlation of DLG4 and GABRA2 synapses for the AD cases

Synapse densities ($1/\mu m^2$) of DLG4 plotted against GABRA2 for the AD cases. The patients were divided in high AD (green), mid AD (red) and low AD (black).

This plotting was used as a control for the staining method. What can be seen is no correlation of inhibitory and excitatory synapses in almost all brain regions. However, if certain brains were affected from exogenous artificial changes, such as increased postmortem intervals, one would expect a stronger correlation between excitatory and inhibitory markers. Since this is not the case, I assume that the staining was good and also the brain tissues were well preserved for the synapse staining.

The entorhinal cortex depicts nicely the low synapse numbers for high AD, whereas the numbers for mid AD and low AD are more distributed. This effect is also visible in cingulate and the midfrontal cortex. In the other brain regions the distribution of the synapse densities seemed to follow no line. There are some patients with high AD, showing higher GABRA2 numbers than DLG4 and other showing the opposite, also in one brain region. Brodmann's area 17 has lower DLG4 synapse densities than GABRA2, mostly in the low and mid AD. For Brodmann's area 18 and the insular ctx are the patient numbers too low to point something out.

Further the high AD cases (green) are mostly located in the lower synapse numbers, followed by the mid AD cases (red). The highest synapse densities showed the low AD cases (black). There is no obvious differentiation of high AD, mid AD and low AD, rather a floating threshold. The numbers of the synapse densities are roughly the same in inhibitory synapses and excitatory synapses, between 0.1 /µm² and 0.3 /µm².

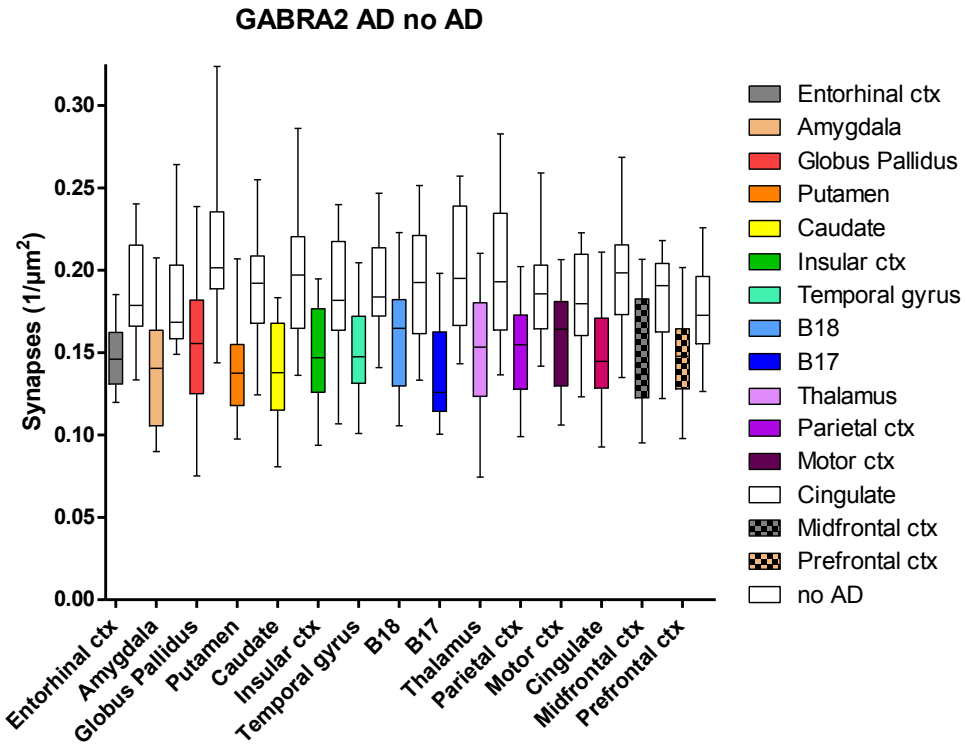


Fig. 53 A) Synapse densities (1/µm^2) of *AD* and *no AD* cases with the GABRA2 staining

Synapse densities (1/µm^2) of GABRA2 for the *AD* and *no AD* (directly behind the AD case, in white) were plotted for each brain region.

Above is a graph with all the Data of GABRA2 synapses in the *AD* cases put together. In color are the bars of the different brain region, with immediately right to them the *no AD* of the same brain region. In all regions the control value is higher in synapse density than the AD case.

The synapse densities lay between 0.1/µm² and 0.18/µm², whereas the controls count between 0.15/µm² and 0.24/µm². Surprisingly putamen and Brodmann's area 17 depicts the lowest synapse densities and not entorhinal ctx, temporal gyrus or frontal ctx, where synapse loss occurs first due to disease. However in the *no AD* cases (white bars) the Globus pallidus got the highest numbers in GABAergic synapse, followed by Brodmann's area 17 and thalamus.

The significant alterations in the inhibitory network recorded in these studies, indicate a direct loss of GABAergic terminals in distinct brain regions and not a compensatory mechanism due to over-excitability in disease, shown before by Palop and colleagues [113].

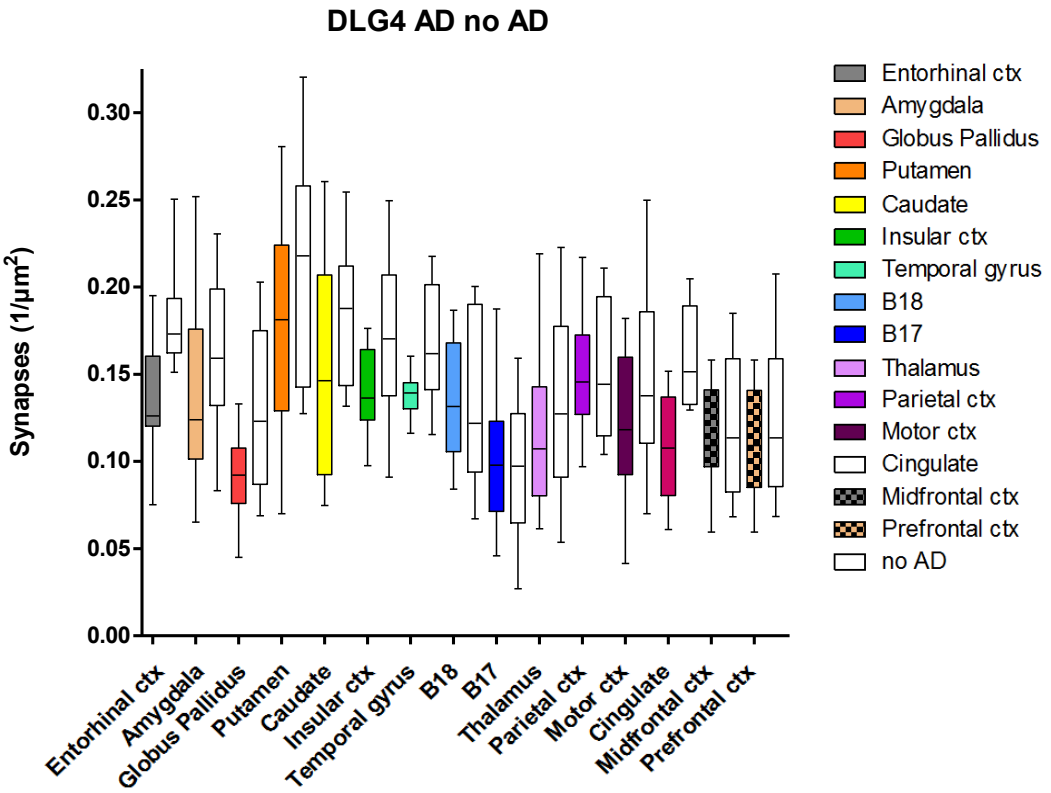


Fig. 53 B) Synapse densities (1/µm^2) of *AD* and *no AD* cases with the DLG4 staining

Synapse densities (1/µm^2) of DLG4 for the AD and no AD (directly behind the AD case, in white) were plotted for each brain region.

In graph 53 B) the DLG4 synapses in the AD cases were put together. In color are the bars of the different brain region, with immediately right to them the no AD of the same brain region. In all regions the control value is higher in synapse density than the AD case.

The synapse densities lay between 0.07/µm² and 0.225/µm², whereas the controls count between 0.08/µm² and 0.25/µm².

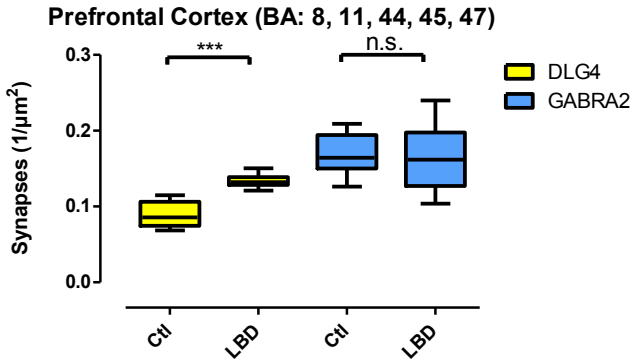
For the DLB4 synapse staining, globus pallidus got the lowest synapse numbers, followed by Brodmann's area 17, thalamus and cingulate. The hightest synapse densities were measured in putamen and caudate. But the loss of synapses in the entorhinal cortex in comparison to the no AD is one of the highest, as in the temporal gyrus and in cingulate. For the *no AD* cases it is a huge distribution visible.

In comparison of the DLG4 (Figure 53 B)) and the GABRA2 (Figure 53 A)) synapse staining, the highest synapse loss is seen in the same brain regions: Entorhinal cortex, temporal gyrus and cingulate, in comparison to the *no AD* case of the same region.

9.3 Lewy Body Disease

Further the LBD cases were analyzed. Here I also plotted the synapse densities (1/µm²) of controls against the densities of LBD cases for DLG4 and GABRA2. Significant graphs were marked with an asterisk (*).

1



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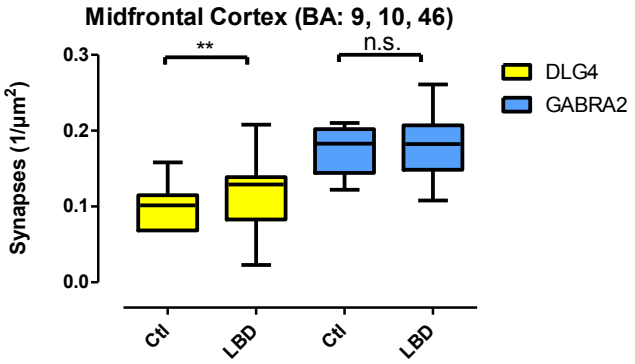

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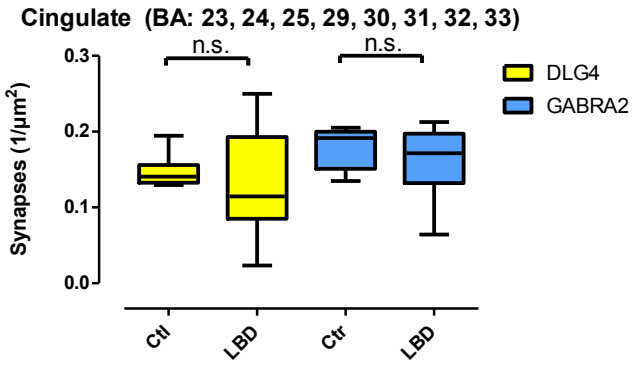

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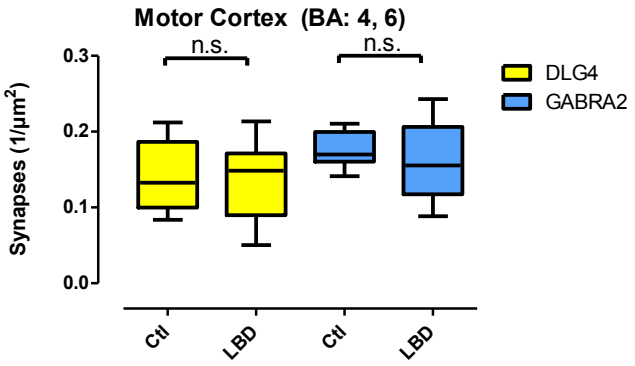

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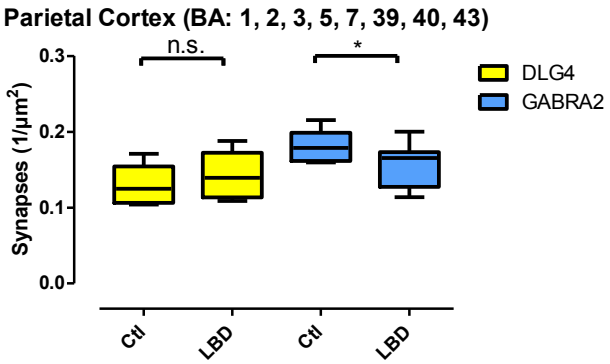

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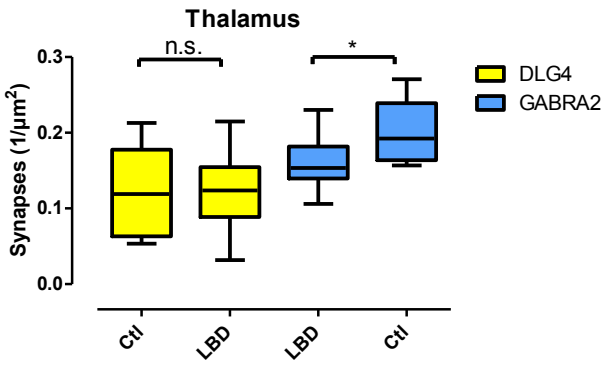
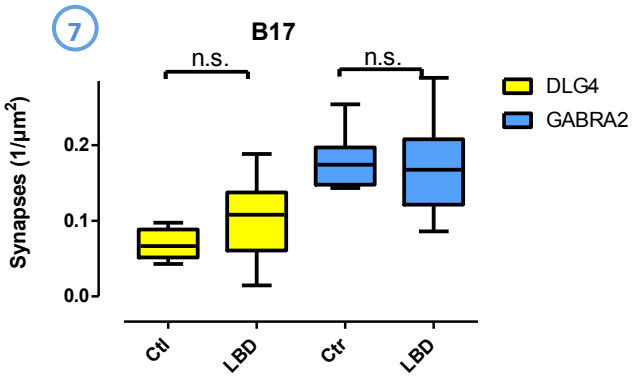
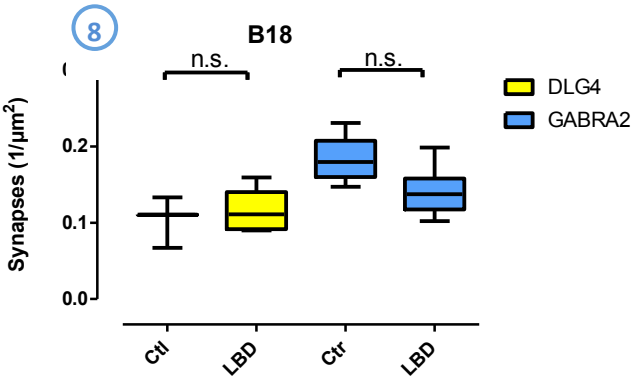
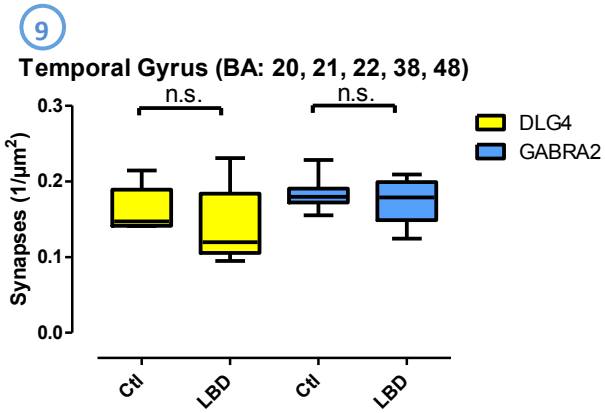
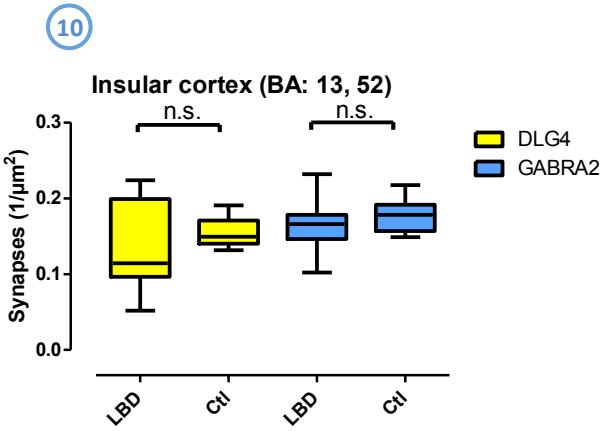
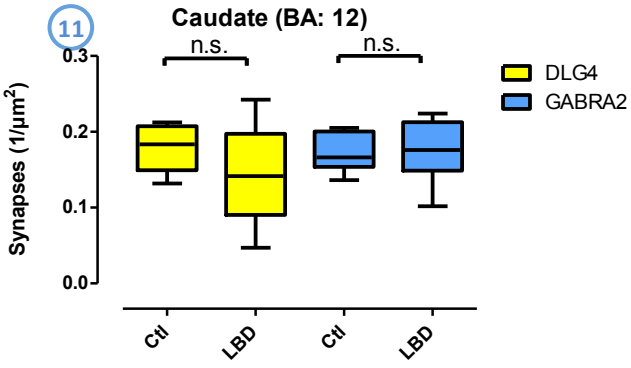


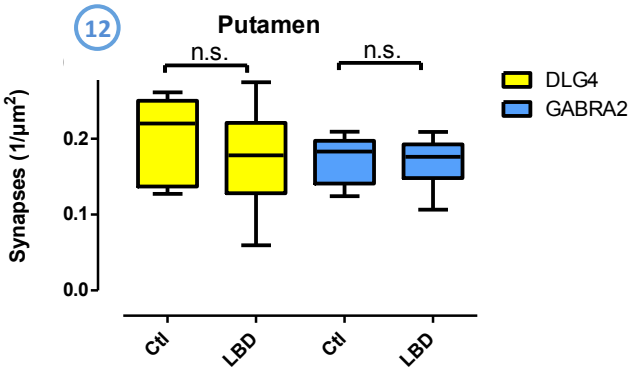

7
B17
n.s.
n.s.
Synapses (1/µm²)
0.0
0.1
0.2
Ctl
LBD
Ctr
LBD
DLG4
GABRA2

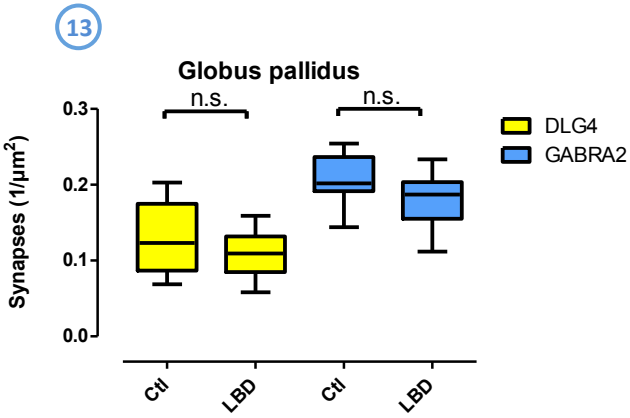

8
B18
n.s.
n.s.
Synapses (1/µm²)
0.0
0.1
0.2
Ctl
LBD
Ctr
LBD
DLG4
GABRA2

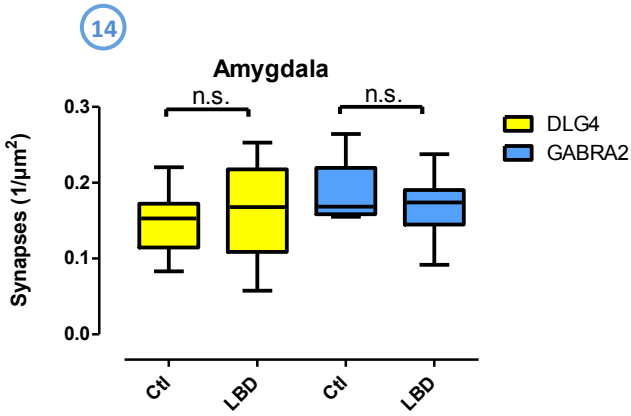

9
Temporal Gyrus (BA: 20, 21, 22, 38, 48)
n.s.
n.s.
Synapses (1/µm²)
0.0
0.1
0.2
0.3
Ctl
LBD
Ctl
LBD
DLG4
GABRA2


10
Insular cortex (BA: 13, 52)
n.s.
n.s.
Synapses (1/µm²)
0.0
0.1
0.2
0.3
LBD
Ctl
LBD
Ctl
DLG4
GABRA2


11
Caudate (BA: 12)
n.s.
n.s.
Synapses (1/µm²)
0.0
0.1
0.2
0.3
Ctl
LBD
Ctl
LBD
DLG4
GABRA2


12
Putamen
n.s.
n.s.
Synapses (1/µm²)
0.0
0.1
0.2
Ctl
LBD
Ctl
LBD
DLG4
GABRA2


13
Globus pallidus
n.s.
n.s.
Synapses (1/µm²)
0.0
0.1
0.2
0.3
Ctl
LBD
Ctl
LBD
DLG4
GABRA2


14
Amygdala
n.s.
n.s.
Synapses (1/µm²)
0.0
0.1
0.2
0.3
Ctl
LBD
Ctl
LBD
DLG4
GABRA2

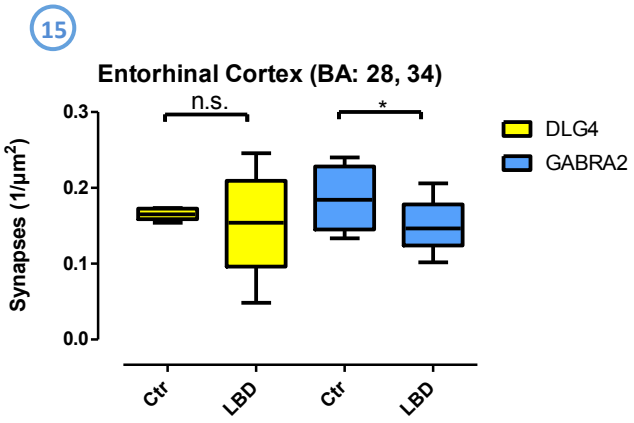


Fig. 54 - 69 Synapse densities (1/µm^2) of DLG4 and GABRA2, control and LBD

Each graph represents the synapse density (1/µm^2) from one brain region of controls (Ctl) and Lewy body disease cases (LBD). DLG4 (yellow) and GABRA2 (blue).

In the bar graphs above were the synapse densities (1/µm²) plotted for the LBD cases and controls for both DLG4 and GABRA2 staining.

There are three brain regions, parietal cortex, thalamus and entorhinal ctx, where the synapse loss in GABRA2 is significant and 2 regions (Prefrontal and midfrontal cortex), where differences in the DLG4 staining were significant

The most striking conspicuity is the control groups, where the levels of the DLG4 and GABRA2 are radical different not only in the LBD cases. Parietal cortex, midfrontal cortex, B17 and B18 depicts very low numbers in the DLG4 synapse staining, compared to the GABRA2 staining.

9.4 α-Synuclein, AT8 and Aβ4G8

Next is the comparison of the different pathologies: α-synuclein, AT8 (Antibody for Tau protein) and Aβ4G8 (Antibody for amyloidpPlaques). The synapse densitiy (1/µm²) is plotted against the Immunepositive area in percentage for the different protein types, while the measurement of each protein was only in the associated disease: α-synuclein is plotted for the LBD cases, AT8 and Aβ4G8 for the Alzheimer's disease patients. The incubation and measurement of the immunopositive area (%) was carried out by Johannes Attems group. The data plotted against the synapse densities of my results are depicted here.

Not all brain regions were analysed.

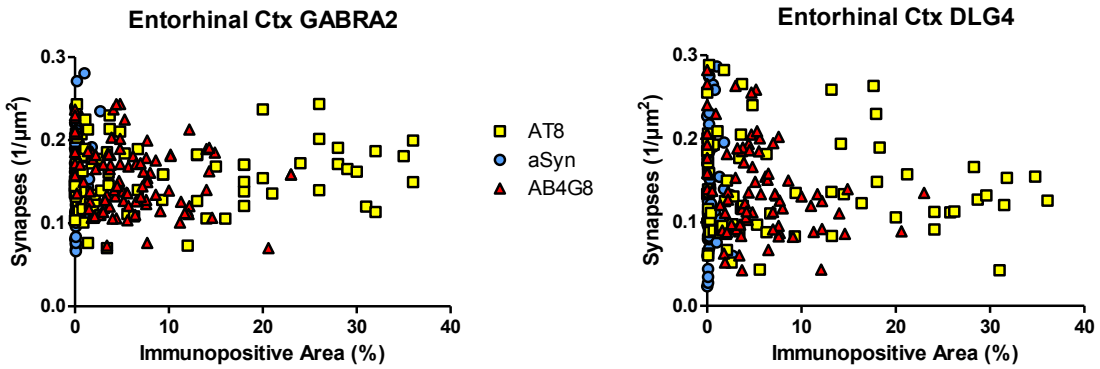


Fig. 70 A and B) Synapse densities (1/µm²) of GABRA2 and DLG of the entorhinal cortex

In the figures 70 A) and 70 B) are the graphs of the different proteins (AT8, α-Synuclein and Aβ4G8) in the Entorhinal cortex depicted for GABRA2 (Figure 70) and DLG4 (Figure 71). Each point represents one patient.

For the entorhinal cortex (site before) the distribution of all three proteins is very high. α-synuclein and AT8 are more accumulated in very low percentages of the immunopositive area, means that very low numbers of α-synuclein and AT8 shows also high synapse densities. The highes immunopositive area (%) is measured in the AT8 staining with over 35%, followed by the Aβ4G8 staining with over 22%. The α-synuclein level is under 3% in both inhibitory (Figure 70 A)) and excitatory (Figure 70 B)) synapses. The synapse density reaches at the highest point 0,3/µm²

Next the parietal cortex was analyzed and showed a similar result as the entorhinal ctx (Figure 70 A) and 70 B)). High immunopositive area (%) for the AT8 and Aβ4G8 staining (up to 22%) and low numbers for the α-synuclein staining (under 1%). Synapse density values reaches 0,3µm² but for very low immunopositive area (%).

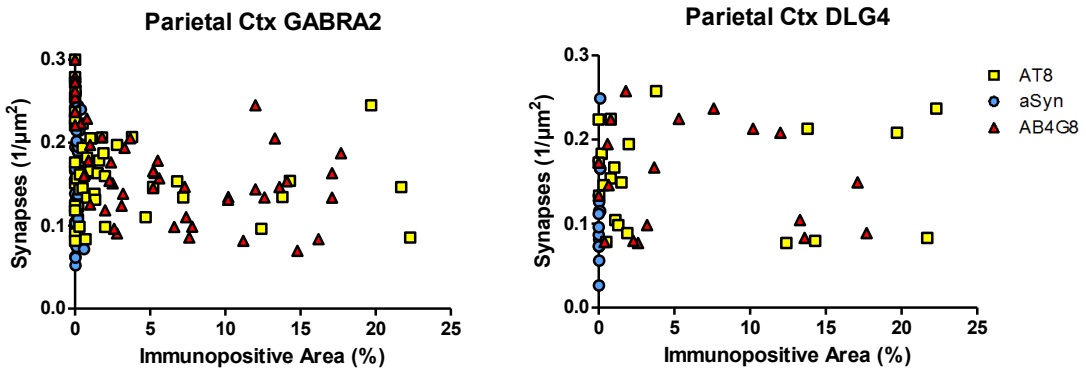


Fig. 71 A), 71 B) Synapse densities (1/µm²) of GABRA2 and DLG of the parietal cortex

In the figures 71 A) and B) are the graphs of the different proteins (AT8, α-synuclein and Aβ4G8) in the parietal cortex depicted for GABRA2 (Figure 71 A)) and DLG4 (Figure 71 B)). Each point represents one patient.

Also the cingulate depicts the same results as mentioned above; high AT8 levels, followed by Aβ4G8 and very low α-synuclein levels. The synapse densities lay between 0,1/µm² and 0,3/µm².

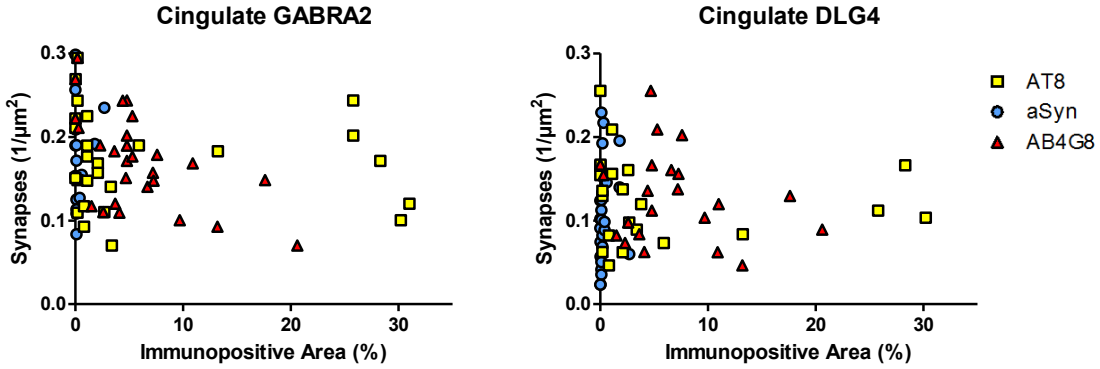


Fig. 72 A), 72 B) Synapse densities (1/µm²) of GABRA2 and DLG of the cingulate

In the figures 72 A) and B) are the graphs of the different proteins (AT8, α-synuclein and Aβ4G8) in the cingulate depicted for GABRA2 (Figure 72 A)) and DLG4 (Figure 72 B)). Each point represents one patient.

Further globus pallidus was analyzed for the GABRA2 (Figure 73 A)) and DLG4 (Figure 73 B)) synapse staining. α-synuclein values come not over 0,3/µm² for both inhibitory and excitatory synapses. Also the AT8 results depicts most patients in a very low immunopositive area (under 1%). Under 5% of the immunopositive area is also accumulated with the Aβ4G8 antibody staining.

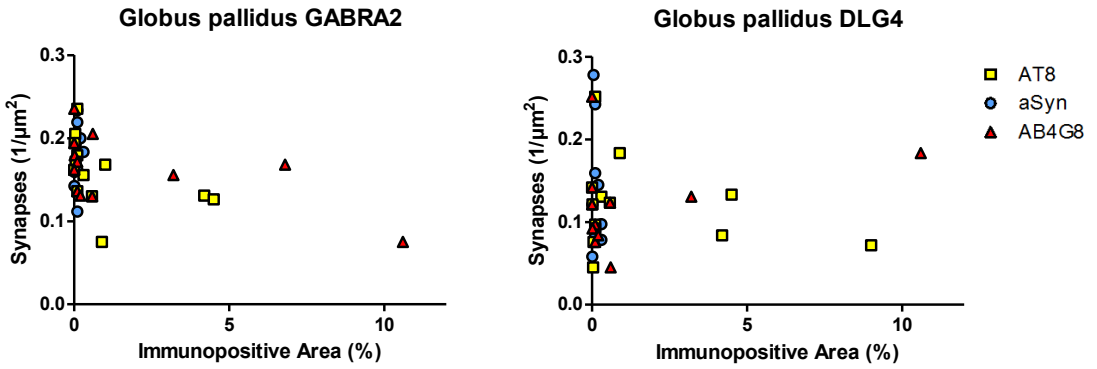


Fig. 73 A), 73 B) Synapse densities (1/µm²) of GABRA2 and DLG of the globus pallidus

In the figures 73 A) and 73 B) are the graphs of the different proteins (AT8, α-synuclein and Aβ4G8) in the globus pallidus depicted for GABRA2 (Figure 73 A)) and DLG4 (Figure 73 B)). Each point represents one patient.

The last brain region depicted in synapse density (1/µm²) versus immunopositive area (%), is thalamus, which fits into the previous findings. α-synuclein values up to 1%, Aβ4G8 levels up to 17% and AT8 mostly under 5%. But here show Aβ4G8 higher Immunopositive area than AT8.

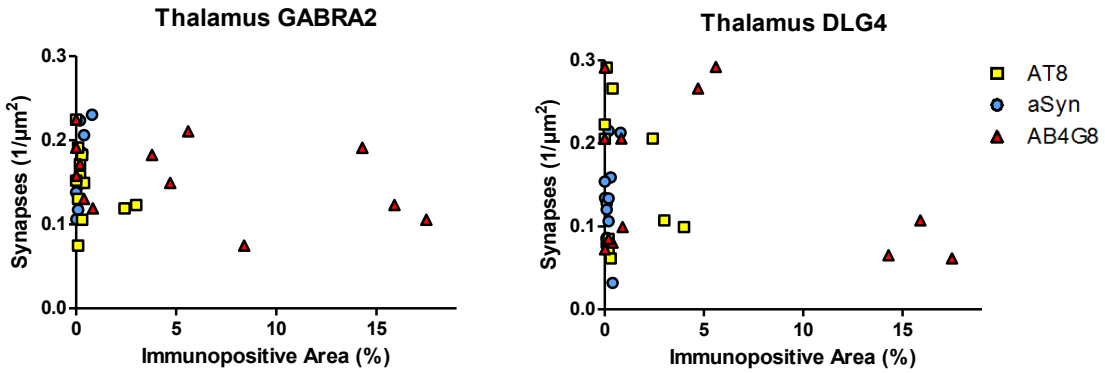


Fig. 74 A), 74 B) Synapse densities (1/µm²) of GABRA2 and DLG of the thalamus

In the figures 74 A) and 74 B) are the graphs of the different proteins (AT8, α-synuclein and Aβ4G8) in the thalamus depicted for GABRA2 (Figure 74 A)) and DLG4 (Figure 74 B)). Each point represents one patient.

10. Discussion

In this study, I have used confocal imaging and the auto-analysis software IgorPro7 to provide a quantitative characterization of inhibitory and excitatory synapses in 15 brain regions, whereby the major finding was a significant loss of GABAergic synapse in all brain regions for the AD cases (Braak 5, 6), compared to the *no AD* cases (Braak 0, 1, 2).

My results demonstrate that the entorhinal cortex, which is usually affected very early in disease (Braak 2-3), show the highest synapse loss in AD in both excitatory (DLG4) and inhibitory (GABRA2) synapses compared to *no AD*.

Synapses in the temporal cortex (affected in Braak stage 4) are in both inhibitory and excitatory synapses significantly lost. GABRA P-Value: 0,0006 and DLG4 P-Value: 0,0025. The Median difference is only the half (0,0363/μm^2) of the highest median difference in Brodmann's area 17.

While the frontal and occipital cortex (Braak 5) are affected very late in disease, the data show very low P-Values and the highest median difference measured (Brodmann's area 17, P-Value: <0,0001, median difference: 0,0693) In comparison of Brodmann's area 17 to 18, it is interesting, that the values are not so similar as estimated. In GABRA2 stained tissue, the synapse densities of the *no AD* cases is almost the same with 0,1983/μm^2 in B17 and 0,1914/μm^2 in B18, but the loss in the *AD* cases is in the B17 higher. With respect to the DLG4 stained tissue, where the numbers differ gross and the median difference is almost 0 in both B17 and B18, the excitatory synapse loss is not seen here.

I can draw two main conclusions from the data generated in this project. Firstly, the synapse loss for the excitatory synapses fits well with the Braak stages and the distribution of Aβ (Figure 16), with the entorhinal cortex as first affected region, next cingulate and the temporal gyrus.

Secondly, the inhibitory synapses fit better with the distribution of tau (Figure 16). With the entorhinal ctx as major affected region, followed by putamen, B17, amygdala and the temporal gyrus.

The fact of the very high synapse loss in the Brodmann's area 17 in inhibitory synapses is new and has not been described before.

Interestingly there is a significant increase in excitatory synapses in the LBD cases in the prefrontal- and midfrontal cortex and also in Brodmann's area 17, whereas the synapse number decrease in the putamen, caudate and insular cortex. The increase in excitatory synapses in the prefrontal region is not surprisingly in LBD, where the action potential firing in the motor neurons is less inhibited than in healthy patients. The decrease of DLG4 for excitatory synapses in LBD was also documented before [114] and fits with my data in the prefrontal and the midfrontal ctx, but not in the motor cxt.

The highest synapse loss for the GABRA2 stained tissues was found in thalamus, entorhinal ctx and the parietal ctx (Figure 12).

Only prefrontal and midfrontal cortex show an excitatory synapse loss in the LBD cases and parietal ctx, tahalmus and entrohinal ctx show significant loss for the inhibitory synapses. The Figures 71 – 74 show very low α-synuclein levels but synapse densities up to 0,3/µm² (Entorhinal ctx, figure 71 A) and B)). But higher immunopositive area values in AT8 and Aβ4G8 staining come with no lower synapse density, pointing that there is no correlation of synapse density (1/µm²) and immunopositive area (%).

For the Alzheimer's disease data, the GABRA2 analysis shows bigger effects in synapse loss than the DLG4 staining. The high synapse loss in GABRA2 stained tissue in AD, indicate that inhibitory synapses were more affected in AD, than excitatory synapses. Maybe the inhibitory synapses are affected earlier during the disease progress, while excitatory synapses are affected later. But the numbers of the synapse densities are roughly the same in inhibitory synapses and excitatory synapses, between 0.1 /µm² and 0.3 /µm².

Another reason for the marked loss of GABRA2 staining may be an exchange of the receptor subtype GABAergic neurons in LBD are functionally altered, as well as a loss in postsynaptic GABA receptor markers, like gephyrin, GABARAP and Kif5A, which leads to decreased GABAergic synaptic activity. There is not a change in synapse density in the LBD in the Brodmann's area 17 [114], as shown in our data. GABRA2 receptors have been related to anxiety, depression and other behavior disorders, like drug dependence and schizophrenia. GABAergic signaling plays an important role in neural stem cell maintenance and renewal. So a change in the receptor subtypes can change the role and the targets of the following cascade.

Finally, in a study of Schizophrenia and Huntington's disease GABA subunits were altered and the mRNA levels of distinct subunits were changed in disease [115, 116]. Mizukami and colleagues reported a decrease of β3 mRNA levels in human brain with Alzheimer's disease [117]. Further other receptor subtypes, like AMPAR are affected in Aβ application and shown a reduced attendance [118]. It is possible, that distinct GABAA receptor subtypes increase in the concentration, while the distribution of GABRA2 and others decreases, so that an exchange of existing α2 subunit occurs. The described compensatory mechanism and with

that come along decreased GABRA2 levels is clearly seen in my data. So I suppose that a changed level of GABRA2 is also due to disease characteristic and in this case in AD patients, it is possible that the α2 subunit is down regulated, disturbed or exchanged by another subunit. In my results, this effect is seen in all brain regions, which promotes this hypothesis.

Maybe it is not the best way to stain and count for synapses, when the marker can be altered during disease and is not reliable to predicate the number of GABAergic synapses. With regard to the changed expression in disease, such marker is not good selected, when in health and disease the consumption of receptors is changed, the comparison of the control and AD patients is not possible.

11. Prospects

Further investigations for the excitatory synapses as well as the LBD cases are needed to provide clarity about the influence of excitatory and inhibitory synapses in Alzheimer's disease and the unexpected increase of DGL4.

Other synapse marker, like marker for cholingergic synapses, should be used to identify the network of synapses and find correlations between them. No easily, in disease substituted protein should be the target of the ATB for quantification of synapses. DLG4 is on one hand a better target than GABRA2, because of its unaltered consumption. But on the other hand DLG4 is not specific for one synapse type as GABRA2, which is representative for GABAergic synapses. New marker should be found to identify each synapse type (GABAergic, glutamatergic and cholinergic) to find the most affected ones in disease and also to identify compensatory mechanisms and the interaction of the different synapse types.

12. Abbreviations

ACh	Acetylcholine
AChR	Acetylcholine receptor
AD	Alzheimer's disease
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepriopinoinic acid receptor
AP	Amyloid Plaques
APP	Amyloid precursor protein
BA	Brodmann's area
β-APP	Beta Amyloid precursor protein
DAPI	4',6-Diamidin-2-phenylindol
DLB	Dementia with Lewy bodies
eAD	early-onset Alzheimer's disease
EPSP	Excitatory postsynaptic potential
GABA	γ-Aminobutyric acid
$GABA_A$-R	γ-Aminobutyric acid receptor A
GAD	Glutamic acid decarboxylase
GAT	GABA transporter
IPSP	Inhibitory postsynaptic potential
K	Thousand
LBD	Lewy Body Dementia
LTP	Long term potention
mAChR	Muscarinic Acetylcholine receptor
MCI	Mild cognitive impairment
MM	Million
nAChR	Nicotinic Acetylcholine receptor
NCI	No cognitive impairment

NFT	Neurofibrially Tangles
NGS	Normal Goat Serum
NMDAR	N-methyl-D-aspartate receptor
ON	Over Night
PD	Parkinsons's disease
PS1	Presenilin 1 gene
PS2	Presenilin 2 gene
PSD	Postsynaptic density
RT	Room temperature
VGlut1	Vesicular Glutamate Transporter1
VGlut2	Vesicular Glutamate Transporter 2
5-HT	Serotonin
5-HT3	Serotonin receptor 3

13. Abstrakt (Deutsch)

Synaptische Plastizität und hohe Synapsen Dichte werden gefördert und aufgebaut während des Lernens, während Spine- und Verbindungsverlust die Kennzeichen für Neurodegenerative Erkrankungen wie die Alzheimer Krankheit sind. Viele Faktoren könnten verantwortlich sein für den Spine Verlust, wie ein veränderter pre synaptischer Input oder externe Faktoren und es ist nicht klar ob die pre synaptische Fehlfunktion oder die Postsynaptischen Veränderungen zuerst auftreten. Dennoch ist der Synapsen Verlust nicht spezifisch um Alzheimer vorherzusagen. Viele neurodegenerative Erkrankungen zeigen diese Verluste, ebenso wie es eine Erscheinung des normalen Alterns ist. Jedoch ist es der Haupt Risikofaktor für Demenz.

Synapsen Verlust ist nicht gleich verteilt in allen Gehirnregionen, was bedeutet, dass alters-abhängige Veränderungen nicht überall auftreten, aber in bestimmten Regionen, wie im Kortex und im Hippocampus. Körpereigene Kompensationsmechanismen, die den Spine Verlust entgegenwirken und synaptische Kontakte erhalten, können nur bedingt gegen die Neurodegeneration halten.

Die Glutamat Freisetzung aus Vesikeln ist der Haupt Signalweg von anregenden Synapsen im Säuger und somit wichtig für die neuronale Aktivität. GABA ist der bedeutendste hemmende Neurotransmitter, bindet auf Rezeptoren der Postsynapse und leitet hemmendes Signal ein.

Doch gibt es Unterschiede in den verschiedenen Synapsen-Typen? Und welche sind in Krankheit eher betroffen? Um diese Fragen zu beantworten, erregende und hemmende Synapsen wurden evaluiert um mittels typischen synaptischen Markern spezifisch markiert: GABAR- α2 für die hemmenden Synapsen und PSD-95 (DLG4) für die Postsynapse der erregenden Synapsen. Antikörper auf menschlichem Paraffin fixierten Schnitten von 15 verschiedenen Hirnregionen, in Alzheimer, Kontrolle und Lewy-Körper-Demenz, wurden mittels Konfokal Mikroskop (Zeiss LSM 780) detektiert und mit Hilfe von IgorPro7 einzelne Synapsen ausgewertet.

Der stärkste Synapsen Verlust war in den hemmenden Synapsen des Entorhinal Kortex von Alzheimer Patienten zu verzeichnen. Für die hemmenden Synapsen waren alle Synapsen Rückgänge signifikant, hingegen die Zahlen bei den erregenden Synapsen wenig signifikant waren und teilweise sogar zunahmen. Für die Lewy-Körper-Demenz konnte auch keine signifikante Korrelation gefunden werden.

14. List of figures

15. List of tables

16. Quotations

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