



UNIVERSITAT ROVIRA i VIRGILI

Facultat d'enologia

ANALYSIS OF NEURODEGENERATION, T AND B CELL INFILTRATION IN GAD ENCEPHALITIS

Bachelor's Degree Final Project | Biotechnology degree

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thank you

2. CENTER FOR BRAIN RESEARCH

The Center for Brain Research (CBR) is a young institution devoted to the advancement of translational neurosciences. It is located in Spitalgasse 4, 1090 Vienna, Austria. The CBR is part of the Medical University of Vienna, and is a non-profit organization which receives founding mainly by the Austrian Science Fund (FWF), but also by other institutions such as Biomedical Primate Center (BPRC), ELA-Deutschland, the European Commission (EC), National Multiple Sclerosis Society of the USA and the Oesterreichische National Bank.

The CBR has six different departments: Neuroimmunology Department, Neurophysiology Department, Molecular Neurosciences Department, Neuronal Cell Biology Department, Cognitive Neurobiology Department and the Pathobiology of the Nervous System Department. The members of each department share their knowledge through regular oral presentations in which they talk about the current projects that are being carried out.

This project has been performed in the Neuroimmunology Department and has been supervised by Ao. Univ. Prof. Dr. Jan Bauer. The Department of Neuroimmunology was the first Department to be fully established at the CBR in 1999 with Hans Lassman being appointed Full Professor and Head of the Department. Research within the Department focuses on brain inflammation and inflammatory diseases of the nervous system. This involves basic research on immune surveillance of the nervous system and mechanisms of immune mediated tissue injury as well as applied research on inflammatory disorders of the nervous system, such as multiple sclerosis.



3. ABSTRACT

In the past years, several types of antibody-associated encephalitis haven been discovered. This group is also named autoimmune epilepsies since patients reveal prominent epileptic seizures (Bauer and Bien, 2016). Antibody-associated encephalitis can be classified according to serum antibodies and their specific targets: Antibodies against intracellular antigens, Antibodies to extracellular epitopes, antibodies against intranuclear epitopes and antibodies against neuronal membrane antigens (Blümcke, Sarnat and Coras, 2015).

Glutamic acid decarboxylase (GAD) antibody-associated encephalitis is a type of autoimmune encephalitis with antibodies against intracellular antigens. GAD encephalitis is characterized by the presence of anti- GAD65 antibody in patient's serum and/or cerebrospinal fluid (CSF) (Daif et al., 2018). Since GAD antibodies target intracellular antigens it is thought that T cell cytotoxicity might be responsible for the neuronal damage. Furthermore, histopathological studies in GAD encephalitis have shown T and B cell infiltration in the hippocampus of patients. More in detail, some patients have shown GrB⁺ lymphocytes in apposition to single neurons suggesting that these cells might be responsible for the neuronal loss (Bien et al., 2012). Thus, in this study this hypothesis has been tested using more cases.

In addition, the pathogenic role of anti-GAD antibodies is still unknown since there is no clear evidence that GAD antibodies can bind to GAD65 in situ neurons (Daif et al., 2018). In this study it has been hypothesized if there is a loss of GAD65 in the hippocampus of patients with GAD encephalitis.

Finally, there are no studies supporting correlation between neuronal loss, T, B and plasma cell infiltration and the disease duration. Here it has been hypothesized if there is actually a correlation between these factors in GAD encephalitis.

Keywords:

Antibody-associated encephalitis; Epilepsy; Cytotoxic T cells; GAD65; neuropathology.

4. INTRODUCTION

The innate immune system develops at an early stage of life and provides the first line of defence against pathogens (Matin et al., 2015). Tissue-resident macrophages, dendritic cells, mast cells, phagocytes and complement molecules are the main components of the innate immune response (Waisman et al., 2015). The adaptive immune system is activated when the innate immune response is not enough to defend the host. The adaptive immune response specifically targets antigens on different pathogens. Moreover, immunological memory is incorporated through a mechanism of gene re-arrangement (Matin et al., 2015). In this case, specific B- and T- cells are activated for pathogen clearance (Waisman et al., 2015).

The major way by which the innate immune system deals with infections and tissue injury is to stimulate acute inflammation, which is the accumulation of leukocytes, plasma proteins, and fluid derived from the blood at an extravascular tissue site of infection or injury. Acute inflammation can develop from minutes to hours and last for days. Chronic inflammation is a process that takes over from acute inflammation if the infection is not eliminated or the tissue injury is prolonged. Although innate immune stimuli may contribute to chronic inflammation, the adaptive immune system is also involved as cytokines are produced by T cells and are powerful inducers of inflammation (Abbas, Lichtman and Pillai, 2015).

4.1 Epilepsy

Epilepsy is a disorder of unprovoked seizures affecting individuals of all ages, with a particular predilection for the very young and old. In addition to seizures, cognitive and psychiatric is often reported in patients (Shirven 2015).

A “seizure” is an alteration of the neurologic function occurring when there is a distortion of the normal balance between excitation and inhibition in the brain. This imbalance can result from an alteration at many levels of the brain function, from genes and subcellular signalling cascades to widespread neuronal circuits. The factors that alter the balance between excitation and inhibition can be genetic or acquired. Brain development is particularly prone to seizures for physiological reasons. Excitatory synaptic function develops before inhibitory synaptic function even in normal brain development, favouring excitation and seizure generation. Moreover, early in life, the neurotransmitter GABA (γ -aminobutyric acid) causes excitation rather than inhibition. These

findings partly explain why the very young brain is especially susceptible to seizures (Stafstorm and Carmant, 2015).

In 1989, the International League Against Epilepsy (ILAE) divided epilepsy aetiology into idiopathic, cryptogenic/symptomatic and unknown. Idiopathic epilepsies are genetic, cryptogenic/symptomatic epilepsies have an identifiable cause such as chromosomal disorders, malformations or hippocampal sclerosis among others (Shirven, 2015).

Inflammatory mechanisms have been increasingly involved in seizure and epilepsy origin. These mechanisms are implicated in the genesis of encephalitis in which seizures are a common complaint. Epilepsies in which seizures develop after infiltration of adaptive immune cells in the central nervous system (CNS) are a broad group of disorders with different etiologies (Bauer et al., 2017).

Encephalitis-associated epilepsies account for 1.7% of patients collected at the German Neuropathological Reference Centre for Epilepsy Surgery, with seizure onset at a mean age of 11.3 years (Blümcke, Sarnat and Coras, 2015). Previous studies suggest the adaptive as well as innate immune system are directly involved in pathomechanisms of epileptogenesis¹ (Bien et al., 2012).

Immunomodulatory therapies are currently under consideration for patients with drug-resistant epilepsy. In order to identify which patients would benefit most from these immunomodulatory therapies, subsets of epilepsy must be defined, including autoimmune conditions, which would be the ideal candidates for immunomodulation. Identifying biomarkers is the first challenging step towards personalised care for epilepsy patients (Bien et al., 2012).

Despite the fact that current immunotherapies can offer great benefit to the correctly identified patient, there are limitations to their efficacy, and they may have considerable side effects. Thus, the identification of new immunomodulatory compounds remains of great importance (Bien et al., 2012).

¹Epileptogenesis: The term encompasses the cascade of cellular events, following which a brain develops spontaneous seizures or epilepsy (Thom, 2014).

4.1.2 Temporal lobe epilepsy with hippocampal sclerosis (TLE-HS)

Temporal lobe epilepsy (TLE) is the most common type of adult focal epilepsy. For about 30% of patients who suffer TLE cannot be cured, fact that proves it is a serious neurological disorder (Santyr et al., 2017). The main feature of this disease are the seizures which can involve regions of hippocampus, amygdala, entorhinal cortex, lateral temporal neocortex, medial thalamus and inferior frontal lobes (de Lanerolle and Lee, 2005). Temporal lobe epilepsy is often associated with Hippocampal sclerosis (HS). Hippocampal sclerosis is a neuropathological condition described by atrophy, neuronal loss and astrogliosis in the field of the hippocampus (Lanerolle and Lee, 2005).

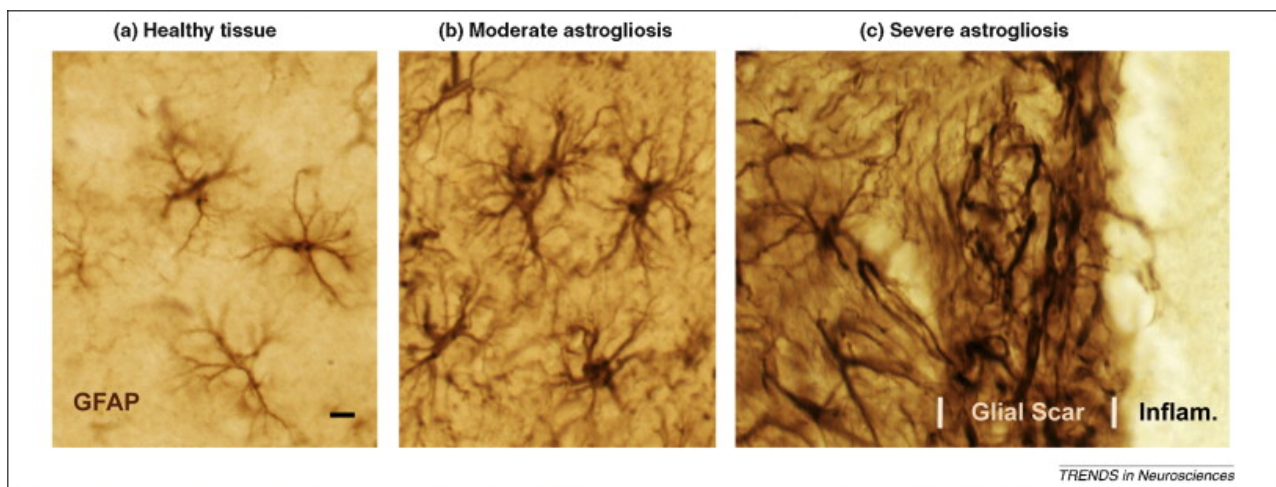


Figure 1. Astrogliosis. A: Astrocytes in healthy tissue. B: Moderate astrogliosis. C: Severe astrogliosis. GFAP (Anti-glial fibrillary acidic protein) staining. Image taken from *Trends in Neurosciences*.

It is thought that seizures induce neuronal injury and subsequent loss results from excitotoxic, glutamatergic neurotransmission, excessive Na^+ and Ca^{2+} , resulting in osmolytic stress and cellular free-radical production, leading neurons to necrosis (Thom, 2014).

Moreover, there is evidence that both innate and adaptive immune responses are activated since $\text{IL-1}\beta$ and IL-1 receptor upregulation has been noted in astrocytes, microglia and neurons. In addition, B and T cell infiltrates are not usually found in the parenchyma of the CNS but are mainly present in a perivascular location (Thom, 2014).

There have been several schemes to classify subtypes of HS, based on the region of the hippocampus affected with neuronal loss and gliosis (Thom, 2014). The hippocampus is formed

by three subregions: the subiculum (Sub), Ammon’s Horn (CA1-CA4) and dentate gyrus (DG), which are structurally and functionally different from each other (Santyr et al., 2017).

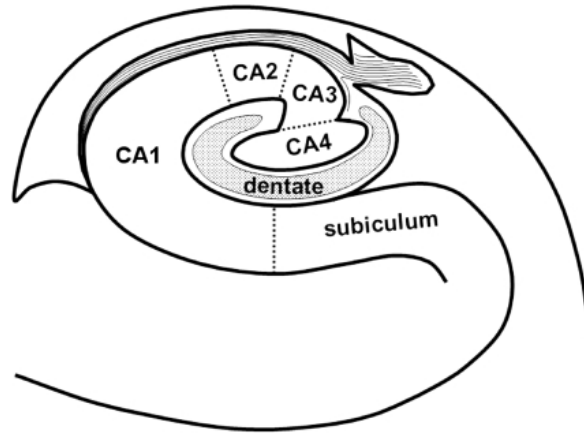


Figure 2. Coronal schematic drawing of the subfields in the hippocampus.

According to the International League Against Epilepsy (ILAE), hippocampal sclerosis can be classified into 4 groups:

Table 1. International consensus classification system of HS (ILAE¹ 2013)

Region	HS type 1	HS type 2	HS type 3	No-HS / gliosis only
DG	0 - 2	0 - 1	0 - 2	0 - 1
CA4	2	0 - 1	1 - 2	0 - 1
CA3	0 - 2	0 - 1	0 - 2	0 - 1
CA2	0 - 2	0 - 1	0 - 1	0 - 1
CA1	2	1 - 2	0 - 1	0 - 1
SUB	0 - 1	0 - 1	0 - 1	0 - 1

The scoring system refers to neuronal loss and is defined for CA1 to CA4 as following: 0 = no obvious neuronal loss or moderate astrogliosis only; 1 = moderate neuronal loss and gliosis; 2 = severe neuronal loss and fibrillary astrogliosis. Scores for dentate gyrus (DG): granule cell layer is normal (score = 0), dispersed (score = 1) or shows severe granule cell loss (score = 2). Scores for the subiculum (SUB): 0 - 1 = the subiculum shows no neuronal loss or moderate. ¹ILAE: International League Against Epilepsy (Blümcke, Sarnat and Coras, 2015).

4.2 Inflammation in the CNS

The central nervous system (CNS) has a broad spectrum of responses called “neuroinflammation”, which allows it to fight pathogens, toxins, trauma and degeneration. Neuroinflammation is the culmination of the immune system, vascular cells, and CNS-resident cells, and can be activated by both pathological conditions and increased neuronal activity.

When the CNS is injured, there is an accumulation of glial cells. Astrocytes and microglia responses start immediately after the injury occurs. In this response, cellular and molecular immune components such as cytokines, complement and pattern-recognition are contributing players, and they can lead to the activation of the glial cells, i.e., microglia and astrocytes. Moreover, inflammation in the CNS can also be chronic rather than acute. If the stimulus persists, then the entrance of macrophages, monocytes, lymphocytes and plasma cells is extended leading to uncontrolled inflammation. These events are able to result in neuronal degeneration, cell damage and eventually might induce the development of neurodegenerative diseases (Morales et al., 2014).

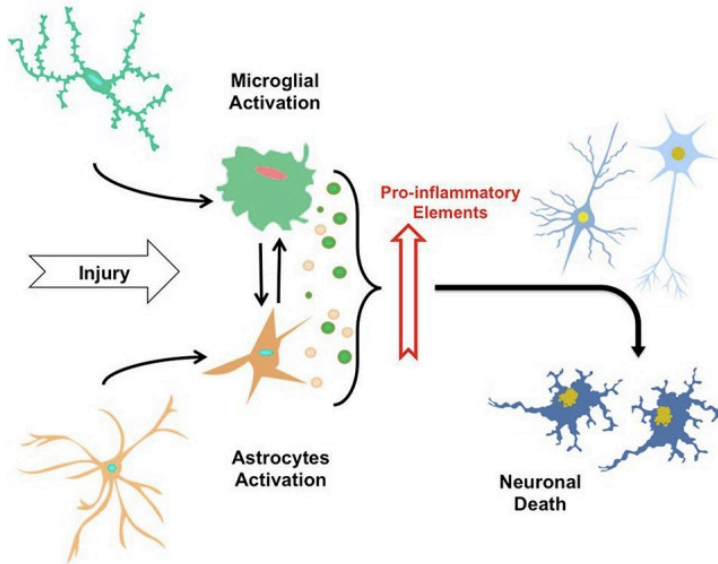


Figure 3. The neuroinflammatory process. By sensing signals of damage or injury, astrocytes and microglia suffer a gradual activation process, leading to morphological changes and secretion of pro-inflammatory elements (i.e., cytokines, cytotoxic elements, ROS). Thus, the constant exposure of astrocytes and microglia to factors causing injuries and secretion of these elements induce mutual activation of microglial cells and astrocytes along with neuroinflammatory process that eventually trigger neuronal death (Morales et al., 2014).

4.3 Encephalitis

Encephalitis is an inflammation of the brain parenchyma with neurological dysfunction associated. It can result from an infection or from an autoimmune response. Patients usually show memory loss, psychiatric changes, seizures and fever among others (Venkatesan et al., 2019).

The leading identified causes of encephalitis are viral, followed by the syndrome of acute disseminated encephalomyelitis (ADEM), which is typically a post-infectious or para-infectious condition. The Japanese encephalitis virus (JEV) and herpes simplex virus (HSV) are the most commonly identified causes. Overall, approximately 40-50% of all identified cases are caused by infectious agents. Autoimmune conditions, which are increasingly recognised and tested since the initial description of anti-N-methyl-D-aspartate (anti-NMDA) receptor encephalitis, account for approximately 20-30% of cases, and the remaining cases have an unknown cause (Venkatesan et al., 2019).

Clinical diagnosis of encephalitis

- Acute or subacute onset of altered level of consciousness, lethargy, and personality change. Short-term memory deficits or psychiatric symptoms also support clinical diagnosis, particularly for autoimmune encephalitis.
- Supportive elements:
 - New focal CNS findings
 - Seizures not explained by previous seizure disorder
 - Cerebrospinal fluid (CSF) white blood cell pleocytosis
 - Imaging features of encephalitis
 - Focal or diffuse abnormality on electroencephalography (EEG) consistent with encephalitis and not attributable to another cause (Venkatesan et al., 2019).

In order to find out the specific cause of encephalitis, testing of CSF, magnetic resonance imaging (MRI), ancillary investigations of blood and EEG is essential. EEG is a key step in the diagnosis of encephalitis. First of all, because the cerebrospinal fluid (CSF) and magnetic resonance imaging (MRI) can be normal in patients with encephalitis, particularly in cases of autoimmune encephalitis. Secondly, in patients with altered consciousness, EEG can identify patients in subtle

motor or non-convulsive status epilepticus, which might present de novo or evolve from a convulsive to a non-convulsive picture in the setting of encephalitis (Venkatesan et al., 2019).

Treatment for encephalitis

Because of the frequency and high mortality associated with delayed treatment of Herpes simplex virus (HSV) encephalitis, empirical acyclovir should be initiated and continued until the diagnosis of HSV has been excluded. When the initial evaluation does not support an infectious cause and an autoimmune cause is suspected, treatment is directed toward systematic immunosuppression. Such treatment is usually started presumptively because of the time required for diagnostic results. Drugs such as rituximab are in some cases used as early treatment with the aim to reduce the risk of clinical relapse (Venkatesan et al., 2019).

For the autoimmune encephalitis associated with antibodies to neuronal cell surface antigens, rapid clearance of antibodies can result in rapid improvement. However, even in such cases, T-cell mediated mechanisms could additionally contribute to disease pathogenesis. When the target is intracellular, T cell or other mechanisms are thought to be driving disease pathogenesis. Thus, cyclophosphamide or other broad-spectrum chemotherapeutic drugs might be more effective (Venkatesan et al., 2019).

In patients with no pathogen or autoantibody identified but in whom there is suspicion of autoimmune encephalitis, immunotherapy with corticosteroids and intravenous immunoglobulin is recommended (Venkatesan et al., 2019).

4.3.1 Autoimmune encephalitis

Antibody-associated encephalitis are an expanding group of brain disorders in which autoantibodies against neural antigens can be identified in serum and/or cerebrospinal fluid (CSF). This group is also referred as autoimmune epilepsy since many of the patients reveal epileptic seizures (Bauer and Bien, 2016).

Autoimmune encephalitis is difficult to diagnose due to the similarities in the clinical, imaging and laboratory findings of many forms of autoimmune and infectious encephalitis. A broad approach to testing for infectious diseases and various neuronal autoantibodies can lead to the correct

diagnosis. Treatment usually involves escalating immune therapies. In most of the cases, patients respond well to immune therapies, however, powerful immune suppression for weeks or months may be needed in some cases. Follow-up care is important as autoimmune encephalitis may relapse (Lancaster, 2016).

Autoimmune encephalitis involves several types of diseases with different pathophysiology. Understanding the pathophysiology of these diseases is important to choose the right therapies and to diagnose it properly. Autoimmune encephalitis can be divided into four different groups:

1. Classic paraneoplastic disorders² with antibodies against intracellular antigens, such as anti-Hu. These disorders are strongly cancer-associated and involve T-cell responses targeting neurons. The antibodies in this case are useful tumour markers. The antibodies themselves are not directly pathogenic.
2. Autoantibodies to extracellular epitopes of ion channels, receptors and other associated proteins, such as the NMDA receptor. That group can be paraneoplastic or non-paraneoplastic. The antibodies in these disorders are thought to be directly pathogenic, causing reversible effects on synaptic function in neurons with relatively little neuronal death.
3. Autoantibodies to intracellular synaptic proteins such as GAD65. It is unclear if these antibodies are directly pathogenic or not.
4. Other forms of autoimmune encephalitis in which precise antigens are less clearly established, such as lupus cerebritis or ADEM (Acute disseminated encephalomyelitis) (Lancaster, 2016).

²Paraneoplastic disorders: autoimmune disorders that are triggered by tumours.

Table 2. Spectrum of antibody-associated epileptic encephalitis.

Antibody-target	Epitopes	Neuropathology
Intracellular	GAD65, AMP	CD8 positive T-cells and neuronal cell loss preferentially in hippocampus
Intranuclear	Hu, Yo, Ma2	CD8 positive T-cells attacking neurons
Voltage-gated potassium channel complex (VGKC)	LGI1 Caspr2	CD8 positive T-cells attacking neurons, severe cell loss preferentially in hippocampus
Glutamate receptors	NMDA R1	Few T-cells, only mild neuronal cell loss

GAD: Glutamic acid decarboxylase; AMP: Amphiphysin; LGI1: Leucine-rich glioma-inactivated 1; Caspr2: contactin-associated protein-like 2; NMDA: N-methyl-D-aspartate (Blümcke, Sarnat and Coras, 2015)

4.3.2 GAD encephalitis

GAD encephalitis is an autoimmune disease characterized by the presence of anti-GAD65 antibody in serum and/or cerebrospinal fluid (CSF). Since this type of encephalitis causes both acute seizures and chronic epilepsy it is also referred to as GAD65 antibody-associated epilepsy. GAD antibodies were detected in 1.7 to 12.5 % of adult-onset focal epilepsy. The adult-onset epilepsy is predominantly of temporal lobe onset, usually with high GAD65 Ab titers (Daif et al., 2018).

Glutamic acid decarboxylase (GAD) is an enzyme which is highly expressed in the presynaptic terminal of inhibitory neurons and pancreatic β -cells. This enzyme converts glutamate to γ -aminobutyric acid (GABA), and it is the major inhibitory neurotransmitter in the CNS (Daif et al., 2018). Imbalanced levels between glutamate and GABA can trigger excitotoxicity, one of several neuronal death mechanisms (Hampe, Mitoma and Manto, 2017). The brain contains two forms of GAD: GAD65 and GAD67. Both of them are expressed in regions of the brain containing neuro-inhibitory cells, but only GAD65 is also expressed in the pancreas. They differ in molecular size, amino acid sequence and also in the location where they synthesize GABA (Daif et al., 2018). Although GAD65 and GAD67 antibodies have been commonly reported to coexist in patients with GAD Ab-mediated neurological autoimmune conditions, GAD65 has greater antigenicity than GAD67 (McKeon et al., 2017).

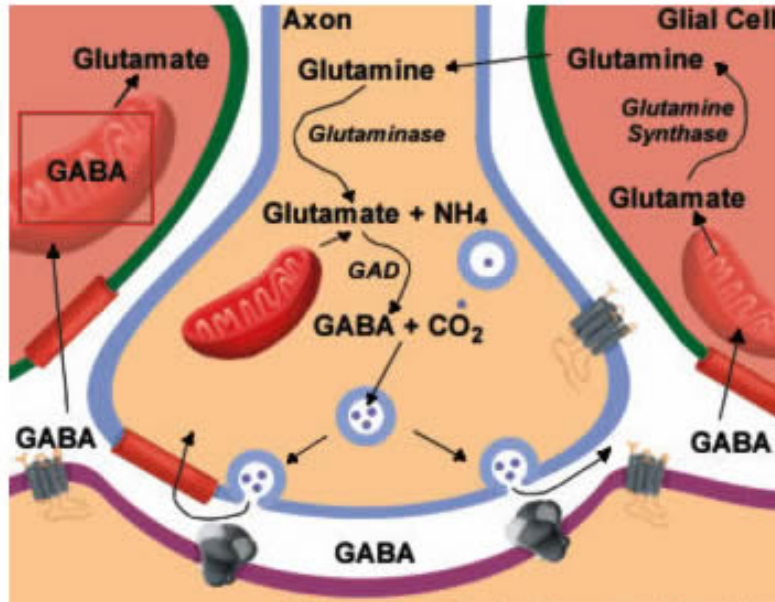


Figure 4. GABA synthesis. GABA is synthesized from glutamate in a reaction catalysed by glutamic acid decarboxylase (GAD). GABA is recycled by glial cells.

Detection of these antibodies is clinically relevant to characterize patients correctly. These antibodies can be detected by different methods including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and screening serum by immunohistochemistry (IHC) followed by RIA or Western Blot (WB) to confirm the positive cases (Daif et al., 2018).

GAD antibodies have a wide range of different disease manifestations i.e. stiff-person syndrome (SPS), cerebellar ataxia, limbic encephalitis (LE), epilepsy and other less common disorders. One possible explanation for this could be the differences in tissue distribution and epitope specificities of anti-GAD reactivity in various conditions. Since GAD antibodies target intracellular antigens it is thought that T cell cytotoxicity is responsible for the pathologic damage. However, the pathogenic property of GAD antibodies is still debated (Daif et al., 2018). Some experimental conditions have provided evidence that GAD antibodies might contribute to a loss of GABAergic inhibition (Hampe, Mitoma and Manto, 2017).

Nevertheless, to be pathogenic these antibodies have to pass the blood-brain barrier and also through the cell membrane. Therefore, it is hard to imagine that antibodies to intracellular antigens are able to overcome all these obstacles (Bauer and Bien, 2016).

Studies have shown T-cell mediated immune response in both paraneoplastic and non-paraneoplastic GAD cases, where the majority of infiltrating T cells were CD3⁺ cells. Moreover, the presence of T cells with cytotoxic GrB⁺ granules in close apposition to neurons has been documented, suggesting that these cells are involved in neuronal cell death (Bien et al., 2012).

In addition to cytotoxic T cells, other components of the inflammatory infiltrate have been investigated. Several CD68⁺ cells have been found forming microglial nodules. Also, CD20⁺ B cells and CD138⁺ plasma cells were found in the meninges and in perivascular cuffs but rarely infiltrated brain parenchyma. Also, in GAD encephalitis there is no evidence of immunoglobulin or complement deposition like in NMDAR encephalitis (Bien et al., 2012).

Finally, patients with GAD encephalitis often do not respond well to antiepileptic drugs (AEDs) and only show a moderately response to immune therapy with steroids, intravenous immunoglobulin (IVIG), or plasma exchange (PLEX). Other more aggressive treatments such as rituximab (RTX) are often needed (Daif et al., 2018).

5. HYPOTHESIS

Neuropathological studies in GAD encephalitis have shown T, B and plasma cell infiltration in the hippocampus of the patients, suggesting that these cells might be involved in the pathology of GAD encephalitis. More in detail, studies in small number of cases have shown GrB⁺ lymphocytes in apposition to single neurons. In addition, IgG immune and complement staining were performed in these cases and no IgG or complement deposition was shown. Because of this and because GAD65 is thought to be an intercellular protein, it has been suggested that a cytotoxic T cell mediated immune response is responsible for neuronal cell death in this disease (Bien et al., 2012). For this study a total of ten GAD cases were analysed in order to find out if there is evidence for a CTL or antibody-mediated neurodegeneration.

Until now, there is no clear evidence that GAD antibodies can bind to GAD65 in situ neurons. However, it is speculated that GAD antibodies may bind to GAD65, leading to a decrease of the enzyme concentration (Daif et al., 2018). In order to test this hypothesis, the presence or absence of GAD65 was analysed in patients with GAD encephalitis.

Furthermore, there are no studies supporting correlation between the duration of the disease and the amount of T, B and plasma cells in the brain's parenchyma, or disease duration with neuronal loss. Consequently, in this study it has been hypothesized if in patients with a short disease duration there are larger numbers of T, B and plasma cells but less neurodegeneration than in patients with a long duration of the disease.

Hippocampal neuronal loss, gliosis and seizures are features of GAD encephalitis, which also can be found as typical features of Temporal Lobe Epilepsy with hippocampal sclerosis (TLE-HS). Pathologically, TLE can be classified in specific types as shown in the classification by the International League Against Epilepsy (ILAE) (See Table 1). Here it has been hypothesized that in GAD encephalitis, loss of neurons and gliosis in the various areas of the hippocampus is similar and thus, this leads to the formation of a specific ILEA type.

6. AIMS

The aim for this study is to answer the following questions to confirm or reject the hypothesis previously mentioned:

- Are different amounts of T, B and plasma cells found in the hippocampus of patients with GAD encephalitis depending on the disease duration?
- Are GrB⁺ and CD8⁺ cells in close apposition to neurons in GAD encephalitis?
- Do the GAD encephalitis brains show a specific ILAE classification type?
- Is there a correlation between neuronal loss and disease duration?
- Is there a loss of GAD65 in the hippocampus of patients with GAD encephalitis?
- Is there complement activation in GAD encephalitis?

7. MATERIALS AND METHODS

7.1 Samples

For this study a collection of formalin-fixed, paraffin-embedded (FFPE) human brain tissue from patients was used. Ten GAD cases were used for this study. As a control, samples from patients with Temporal Lobe Epilepsy with a ganglioglioma (TLE-GG) were used (n=7). Those patients had a ganglioglioma outside the hippocampus, so the area of interest was not affected. The samples were provided by the Bethel Epilepsy Center in Bielefeld-Bethel (Germany) and also by the Department of Epileptology at the University Hospital of Bonn. In general, patients were from both genders, male (m) and female (f). The duration of the disease was estimated between the first seizure recorded until the date of the surgery.

Table 2. Cases of patients used in the study.

Cases	Disease	Gender	Duration of the disease (years)
1722-1723/02	GAD encephalitis	f	0.7
201-203/08	GAD encephalitis	f	6.3
44/16	GAD encephalitis	f	36.2
1733/09	GAD encephalitis	f	59.6
945/98	GAD encephalitis	f	11.1
2277/10	GAD encephalitis	m	4.8
455/11	GAD encephalitis	?	?
1031/11	GAD encephalitis	f	4.5
540/13	GAD encephalitis	?	?
1103/12	GAD encephalitis	?	?
N1344/02	Control (TLE-GG)	m	7.9
N526/03	Control (TLE-GG)	f	9
N1535/04	Control (TLE-GG)	m	24
N2127/03	Control (TLE-GG)	m	0.4
N234/06	Control (TLE-GG)	f	2.4
N971/02	Control (TLE-GG)	f	25.9
N1543/03	Control (TLE-GG)	f	0.4

7. 2 Immunohistochemistry – ABC system with peroxidase activity for detection

Materials

- ▲ 3-5 μm thick FFPE sections mounted on normal glass slides
- ▲ Xylene
- ▲ Ethanol (EtOH) 96%, 70% and 50%
- ▲ TBS-buffer:
 - TBS-stock solution pH 7.1 (1L): 60.75 g of 25 mM Tris Buffer, 180 g of 150 mM NaCl and 400 ml of 1M HCL were dissolved in deionized water; pH was adjusted to 7.5 by adding 1M HCl.
 - TBS working solution: the stock solution was diluted 1:20, 50 ml of stock buffer were diluted in 950 ml of deionized water.
- ▲ PBS-buffer:
 - PBS-stock solution pH 7.4 (2.5 L): 13.8 g of 0.04 M NaH_2PO_4 , 71.2 g of 0.16 M NaH_2PO_4 and 90 g of NaCl were dissolved in deionized water. The pH was adjusted to 7.4.
 - PBS working solution: the stock solution was diluted 1:4; 250 ml stock solution were diluted in 750 ml of deionized water.
- ▲ H_2O_2 / Methanol: 150 ml of Methanol and 1 ml H_2O_2 (30%) were mixed.
- ▲ 10% FCS/DAKO-Buffer: commercial DAKO-buffer solution (Dako Corporation) was diluted 1:10 with deionized water.
- ▲ EDTA-buffer:
 - EDTA 20x stock solution: 1.21 g of 10 mM Tris-Buffer and 0.37 g of 1 mM EDTA solution were dissolved in 50 ml of deionized water. The pH was adjusted to 9.0.
 - EDTA working solution: 2.5 ml of stock solution was diluted in 50 ml of deionized water.
- ▲ Citrate-buffer pH 6.0: 2.10 g citric acid in 1 L deionized water.
- ▲ Proteinase Type XXVI (digestion to unmask epitopes): 0,03 % Proteinase solution in PBS - > 50 ml PBS + 0,015 g Proteinase. Digestion is done in an incubator at 37°C for 15 min; The reaction was stopped with ice-cold PBS.

- ▲ Peroxy conjugated Streptavidin 1:500
- ▲ DAB (3,3'-Diamminobenzidine): 1 ml DAB stock solution was mixed in 50 ml of PBD. The mixture was filtered with filter paper into an Erlenmeyer and 16.5 µl of H₂O₂ was added.
- ▲ Mayer's Hemalaun: Mayer's Hematoxylin (Merck®)
- ▲ HCl- ethanol: 100 ml of 70% ethanol were mixed with 0.5 ml concentrated HCl (37%).
- ▲ Scott's solution: 2 g of KHCO₃ with 20 g of MgSO₄ were dissolved in 1000 ml of H₂O.
- ▲ Coverslips
- ▲ Eukitt®
- ▲ Primary and secondary antibodies (Table 3, Table 4)

Table 3. Primary antibodies used in the study.

Antibody name	Antibody type	Target	Pre-treatment	Dilution
APP (Millipore #MAB348)	Monoclonal	Amyloid protein	Citrate	1:1000
C9neo (Abcam #17931)	Monoclonal	C9 protein	Citrate	1:400
CD3 (DAKO #A0452)	Monoclonal	T cells	EDTA 9.0	1:1000
CD8 (Neomarkers #RM-9116-50)	Monoclonal	Cytotoxic T cells	EDTA 9.0	1:500
CD20 (Thermoscientific #MS-340-50)	Monoclonal	B cells	EDTA 9.0	1:500
CD138 (Serotec #MCA681H)	Monoclonal	Plasma cells	EDTA 9.0	1:500
GAD65 (Chemicon, #AB5992)	Monoclonal	GAD65	Citrate	1:2000
GFAP (DAKO #Z0334)	Monoclonal	Astrocytes	EDTA 9.0	1:3000
Granzyme B (Abcam #ab4059)	Polyclonal	Cytotoxic T cells	EDTA 9.0	1:50
IgG (Abcam #ab97160)	Monoclonal	IgG	Citrate	1:400
IgG 4 (Abcam #ab109493)	Monoclonal	IgG4	Proteinase	1:100
NeuN (Chemicon #AB1981)	Polyclonal	Neurons	Citrate + CSA	1:500

APP: Anti-Alzheimer precursor protein A4. EDTA: Ethylenediaminetetraacetic acid. GFAP: Glial fibrillary acidic protein. HLA: Human leukocyte antigen. CSA: Catalysed signal amplification. MHC-II: Major histocompatibility complex class II. IgG4: Immunoglobulin type 4. All antibodies were pre-diluted 1:2.

Table 4. Secondary antibodies used in the study.

Antibody	Target	Dilution
Bi-donkey- α -mouse (Jackson #715-065-150)	IgG (H+L)	1:500
Bi-donkey- α -rabbit (Jackson #711-065-152)	IgG (H+L)	1:1000

All antibodies were pre-diluted 1:2.

Method

First of all, the FFPE tissue was cut into 3-5 μm thick sections. The samples were deparaffinized two times for 15 minutes in xylene and rinsed twice in 96% ethanol. To block the endogenous peroxidase activity, the samples were incubated 30 minutes in H_2O_2 - methanol. Then rehydration was performed gradually with 96% ethanol 70% ethanol, 50% ethanol and deionized water. Next, antigen retrieval was done by heating the slides in EDTA (pH 9) or citrate-buffer (pH 6) in a household food steamer device for one hour. After one hour the slides were rinsed with TBS buffer 3 to 5 times. The following step was blocking unspecific background reactions by incubating the samples with 10% FCS-DAKO buffer for 15 minutes. Then the primary antibody (see Table 3) was added into the samples and these were incubated at 4°C overnight. Next day, the slides were rinsed with TBS buffer 3 to 5 times. Then, the secondary antibody (see Table 4) was added and the slides were incubated for one hour. After one hour, the slides were rinsed with TBS-buffer 3-5 times, then, the slides were incubated with the peroxidase-conjugated streptavidin (1:500) in 10% FCS-DAKO buffer for one hour. The slides were rinsed again with TBS-buffer 3-5 times and were developed under microscopic control in DAB. The enzymatic reaction was stopped with deionized water. Counterstaining was done by incubating the slides for 15-20 seconds in Mayer's haematoxylin, washed two times with water, rinsed with HCL-ethanol, incubated 4 minutes in Scott's solution and rinsed with water. Next, dehydration of the samples was performed gradually with ethanol (50%, 70%, 95%, 95%, 95%) ending with n-butyl acetate. Finally, the samples were mounted with cover slips using Eukitt® for embedding.

CSA (Catalysed signal amplification)

Signal enhancement with biotinylated tyramine.

Materials

- ▲ Borate Buffer
- ▲ NaOH
- ▲ Sulpho-NHS-LCS-Biotin
- ▲ Tyramine
- ▲ Filter
- ▲ Small aliquots (10-20 µl)

Preparation

- ▲ Borate buffer: Add 0.1545 g boric acid in 50 ml deionized water, adjust pH to 8.0 with NaOH.
- ▲ Mix 6 ml borate buffer with 15 mg sulfo-NHS-LC-Biotin (Sulfosuccinimidyl 6-(biotinamido) Hexanoate).
- ▲ Add 4.5 mg Tyramine.
- ▲ Leave the mixture at room temperature overnight.
- ▲ Filter the mixture.
- ▲ Put the solution into the small aliquots and store at -20°C

Method

The immunostaining was performed according the protocol until the step with Avidin-peroxidase after the secondary antibody. Once the samples have been incubated with the Avidin-peroxidase with 60 min, one freeze aliquot of CSA was mixed with 50 ml PBS and 50 µl H₂O₂. The mixture was added into a cubet with the slides for 20 min. Next, the slides were rinsed with PBS and incubated again with avidin-peroxidase 1:500 in 10% FCS DAKO buffer for 30 min. Finally, the slides were rinsed with TBS and usual development with DAB was performed.

7.3 Quantitative analysis

For this analysis CD3, CD8, CD20, CD138 and GrB stained sections of all samples were used. First of all, they were scanned at 20x magnification with a slide scanner (NanoZoomer Digital Pathology, Hamamatsu Photonics). Next, using NDP.view2 Viewing Software (NanoZoomer Digital Pathology, Hamamatsu Photonics), the hippocampus was analysed by taking 6-10 images at a standard 10x magnification as in the figure 5. The number of images depended on the size of the section. For the GAD cases in which no hippocampus was present in the slide (540/13, 1103/12 and 1031/11), randomly areas were selected.

The Image-Pro Premier 9.2 64-bit program (Media Cybernetics) was used to perform cell counting automatically. Firstly, a spatial calibration was created and saved for all the sections (250 μm). The counting process requires to set the optimal darkness and cell size so the program could be able to differentiate between cells. A double check of all images was done in order to confirm that the program counted the cells of interest. All the images had the same size of 1.91 mm^2 . All data was exported in an Excel file.

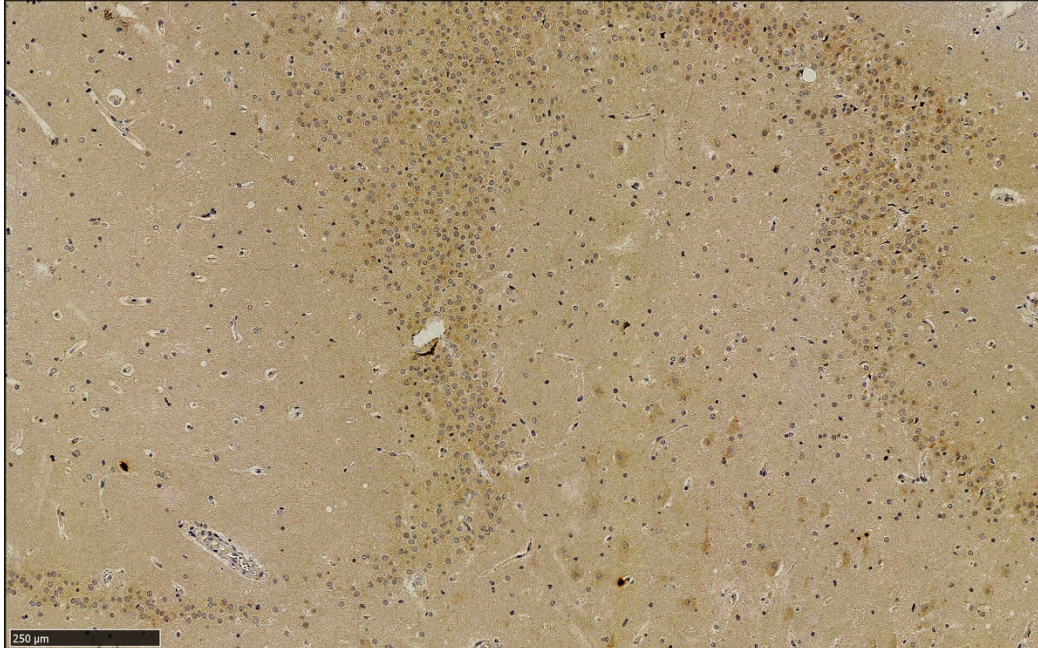


Figure 5. Analysis of scanned sections with NDP.view2 Viewing software. Small region of the hippocampal area with 10x magnification. The slide belongs to the GAD case 44/16. Scale bar: 250 μm .

In addition to the automatic cell counting, for CD8 and GrB staining, a manual cell counting was performed to find out how many cells were in close apposition to neurons. The counting was done using 10x magnification objective. The area of the grid was 1 mm² so the number of positive cells per mm² was calculated.

For statistical analysis, GraphPad Prism7 was used to process and interpret the data. Mann-Whitney U-test was performed for comparison between GAD cases and controls. All graphical data is represented as median with the interquartile range. P-value below 0,05 was considered to be significant. Moreover, the correlation between the disease duration and the number of CD3, CD8, GrB, CD20 and CD138 cells found in each GAD case was tested (This analysis was not performed for the control group).

In order to test if there was complement activation, C9neo staining was performed. Also, IgG and IgG4 staining was done. The slides were analysed under the microscope (This analysis was not performed for the control group).

7.4 Semiquantitative analysis

For this study, different semiquantitative analysis were done in order to test our hypothesis. These analyses were performed using the immunohistochemistry staining system as mentioned above. Thus, NeuN, GFAP, APP and GAD65 stainings were performed and analysed.

In order to classify the GAD cases following the ILAE classification for TLE-HS (Table 1), NeuN and GFAP staining were used to identify the neuronal loss (NeuN) and the presence of astrogliosis (GFAP). Since for this classification all structures of the hippocampus have to be present, only the cases with the complete hippocampus could be used. Therefore, only three (44/16; N455/11; 203/08) out of ten GAD cases were analysed under the optical microscope.

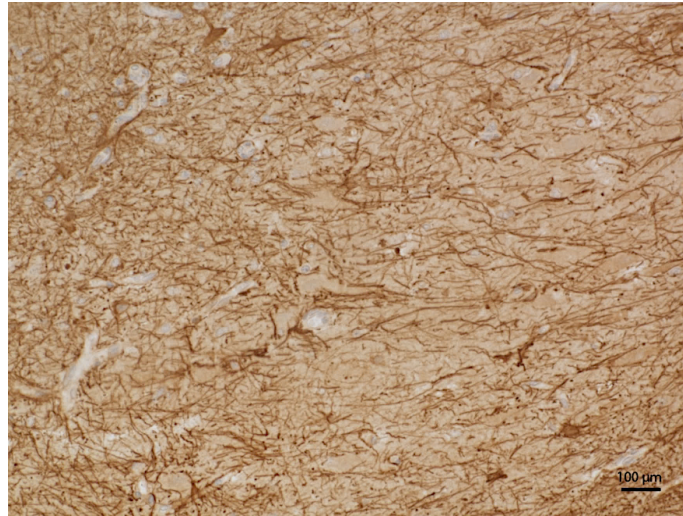


Figure 6. Astroglialosis in the hippocampus. GFAP staining of the GAD case N455/11. Image taken with NIS-Elements D 3.10 software at 10x magnification. Scale bar: 100 μm.

Furthermore, another semiquantitative analysis was done with the aim to correlate the acute neurodegeneration to the duration of the disease in GAD encephalitis. Amyloid precursor protein (APP) staining was performed in order to detect the axonal damage in the neurons of the hippocampus. APP is produced in neurons and accumulates at sites where there is recent axon damage, so it's related to the acute stage of the disease. The slides were analysed under the microscope and classified depending on the numbers of axonal spheroids visualized. The following score system was used: 0 = no axonal spheroids present; 1 = a few axonal spheroids present; 2 = quite a lot of axonal spheroids present. All GAD cases were used for this analysis (n=10).

In addition, neuronal loss was also correlated to the duration of the disease in GAD encephalitis. For this analysis, NeuN staining was performed. The samples were analysed with the optical microscope and classified depending on the amount of neuronal loss. The following score system was used: 0 = no neuronal loss; 1 = moderate neuronal loss; 2 = severe neuronal loss. All GAD cases were used for this analysis (n=10).

Lastly, the amount of GAD65 in the hippocampus was analysed using GAD65 staining for seven GAD cases: 1723/02, 203/08, 44/16, 1733/09, 2277/10, 540/13 and 1103/12. For this analysis all controls were used (Table 2). The following score system was used: 0 = no GAD65 loss; 1 = moderate GAD65 loss; 2 = severe GAD65 loss.

8. RESULTS

In this study, the presence of CD3 and CD8 T cells, CD20 B cells and CD138 plasma cells was analysed in the hippocampus of GAD patients (n=10).

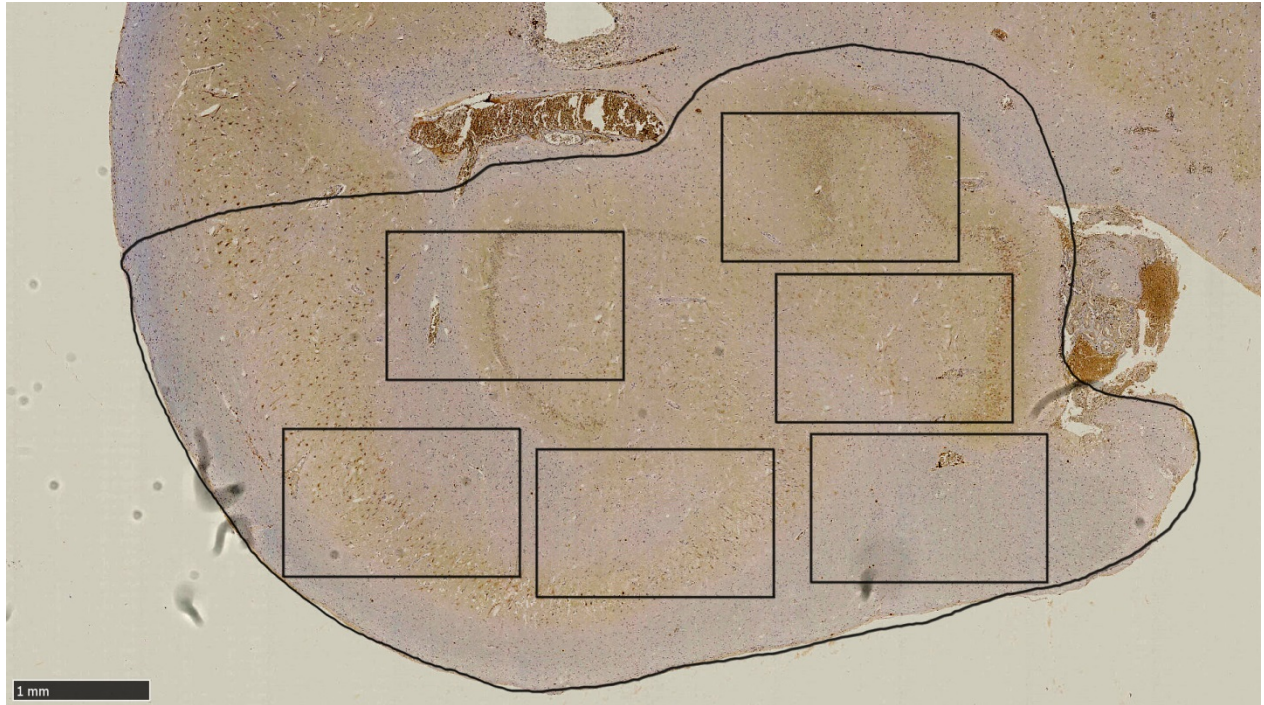


Figure 7. Selected regions of the hippocampal area for quantitative analysis. Image imported from NDP.view2 Viewing Software. Scale bar = 1mm.

8.1 Analysis of T cell infiltration

In this study the presence of CD3 and CD8 cells was investigated in the region of the hippocampus in GAD encephalitis (n=10) (including the cases where there was no hippocampus). All GAD cases were compared with the controls (n=7).

With regard to the CD3 infiltration, the statistics do not show significant difference between both groups (Figure 8).

Regarding CD8 inflammation, results show again significant difference between both groups. Furthermore, for GAD cases the number of CD8⁺ cells were compared with the number of CD8⁺ cells in close apposition to neurons, significant difference was also found (Figure 9).

Comparing the CD3 and CD8 stainings, it was noticed that in many cases the numbers of CD3 and CD8 cells were close to each other, suggesting that most of the CD3⁺ cells were also CD8⁺.

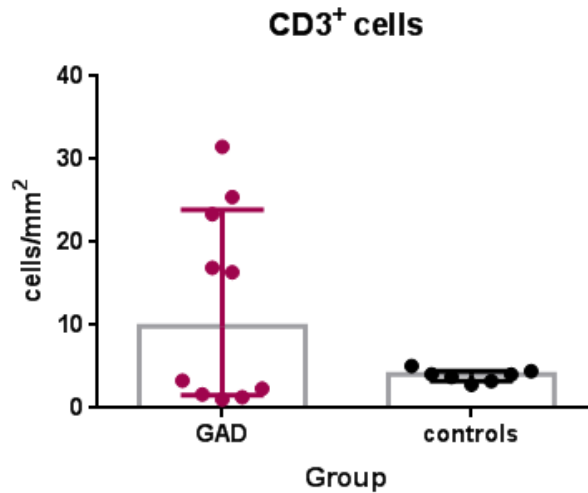


Figure 8. Quantification of the number of CD3 cells. Quantification of CD3 cells per mm² in patients with GAD encephalitis (n=10) and in the control group (n=7). Data is shown as median ± interquartile range (IQR).

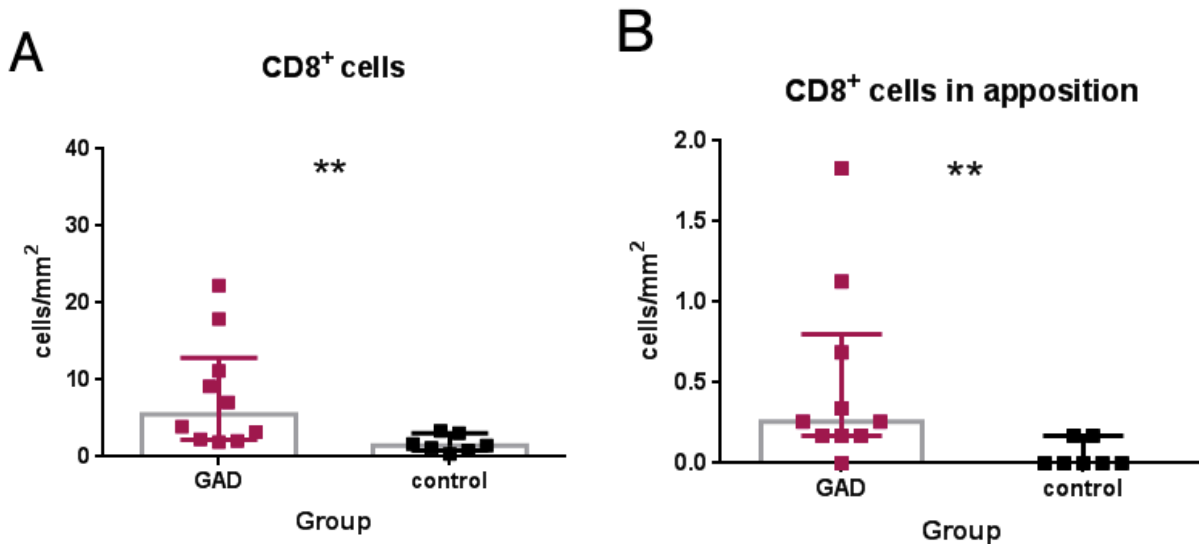


Figure 9. Quantification of the number of CD8 cells. Quantification of CD8 cells per mm² in patients with GAD encephalitis (n=10) and in the control group (n=7). Data is shown as median ± interquartile range (IQR). **A:** all CD8 cells. **B:** CD8 cells in close apposition to neurons. (**p<0.01).

8.2 Analysis of B cell infiltration

Besides the presence of T cells, CD20 B cell infiltration was also analysed. Despite B cells were found in the GAD cases, on average the numbers of B cells were much lower than the numbers of CD3 and CD8 T cells. No significant difference was found between the GAD and the control group (Figure 10).

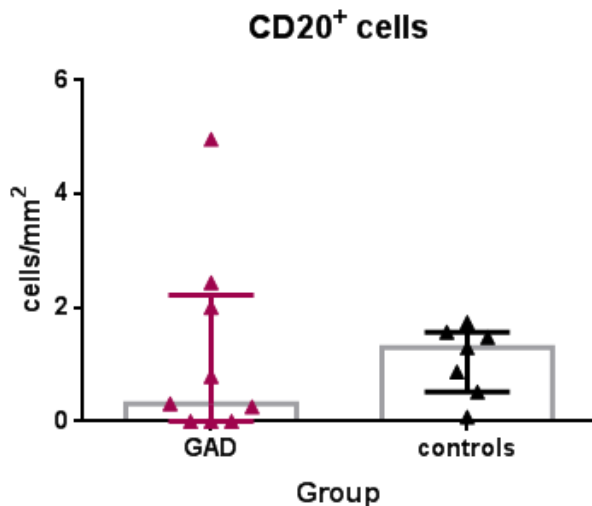


Figure 10. Quantification of the number of CD20 cells. Quantification of CD20 cells per mm² in patients with GAD encephalitis (n=10) and in the control group (n=7). Data is shown as median \pm interquartile range (IQR).

8.3 Analysis of plasma cell infiltration

The presence of plasma cells was studied by staining for CD138. The numbers of plasma cells were much lower than the numbers of T cells, but in comparison to the controls, clear differences have been shown. Whereas in control tissue very few plasma cells were found in the perivascular space of blood vessels, in GAD cases high numbers of plasma cells were found in the parenchyma. Moreover, comparing CD138 staining with IgG and IgG4 staining, those plasma cells were identified in the IgG staining but not in the IgG4 one. This comparison suggests that CD138 plasma cells shown in the GAD cases were either IgG1, IgG2 or IgG3 subclass.

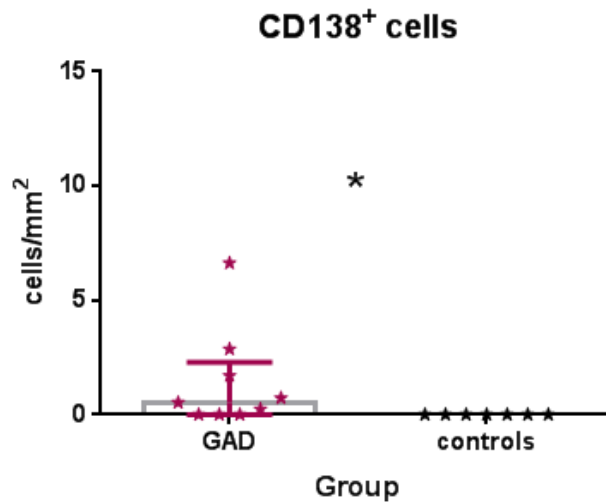


Figure 11. Quantification of the number of CD138 cells. Quantification of CD138 cells per mm² in patients with GAD encephalitis (n=10) and in the control group (n=7). Data is shown as median ± interquartile range (IQR).

8.4 Analysis of granzyme B infiltration

In order to analyse GrB infiltration in GAD encephalitis, cell counting was performed as in the other cell types. In addition, GrB in close apposition to neurons was also studied in order to see the amount of T cells which might be causing neuronal death. No significant difference was found between GAD patients and control group (Figure 12).

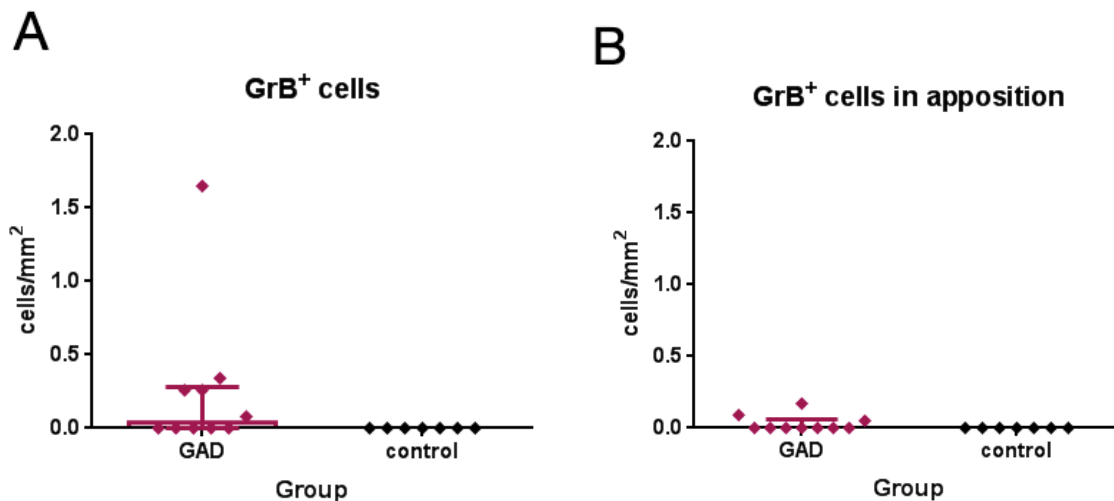


Figure 12. Quantification of the number of GrB+ cells. Quantification of GrB+ cells per mm² in patients with GAD encephalitis (n=10) and in the control group (n=7). **A:** All GrB+ cells. **B:** GrB+ cells in close apposition to neurons. Data is shown as median ± interquartile range (IQR).

8.5 Evaluation of T, B and plasma cell infiltration in relation to the disease duration

Since one aim of this study is to see if there is a correlation between the numbers of T and B cells in the hippocampus of GAD patients and the duration of the disease, a comparison was made for all cell types as mentioned in methods (this analysis was not performed for the control group).

For all cell types tested, the results shown that the higher numbers of cells correspond to the patients with a short disease duration. Conversely, in patients with the longest disease duration (36.2 and 59.6 years) low numbers of T and B cells were found (Figure 13).

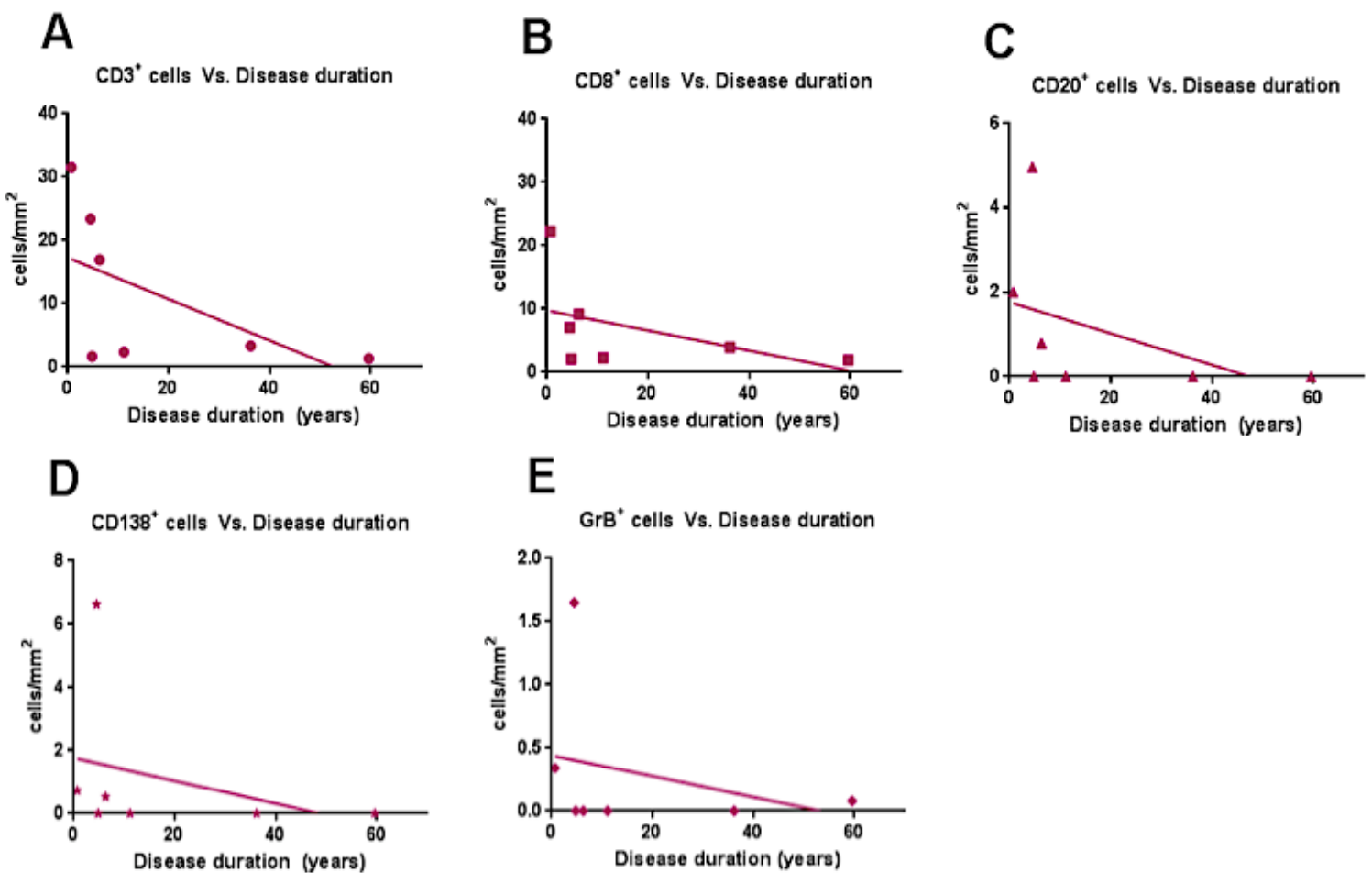


Figure 13. T, B and plasma cell infiltration and disease duration. Numbers of CD3 cells (A), CD8 cells (B), CD20 cells (C), CD138 cells (D) and GrB cells (E) found in the hippocampus of patients with GAD encephalitis (n=10) in relation with the disease duration of each patient (years).

8.6 Evaluation of neuronal loss and acute neurodegeneration in relation to the disease duration

Semiquantitative analysis was performed in all GAD cases (n=10) in order to see if there is a correlation between acute neurodegeneration and the disease duration. Results are shown in Table 5; Our data do not show a clear correlation, however, three cases with a short disease duration shown acute neurodegeneration.

Table 5. Semiquantitative analysis for acute neurodegeneration in GAD encephalitis.

Case	Duration of the disease (years)	Axonal spheroids present
1733/09	59.6	1
44/16	36.2	2
945/98	11.1	1
203/08	6.3	0 - 1
2277/10	4.8	2
1031/11	4.5	2
1723/02	0.7	2
455/11	?	2
540/13	?	0 - 1
1103/12	?	1

Semiquantitative microscopic examination based on APP staining. The scoring system refers to the presence of axonal spheroids as following: 0= absence of axonal spheroids; 1= moderate number of axonal spheroids; 2= large number of axonal spheroids.

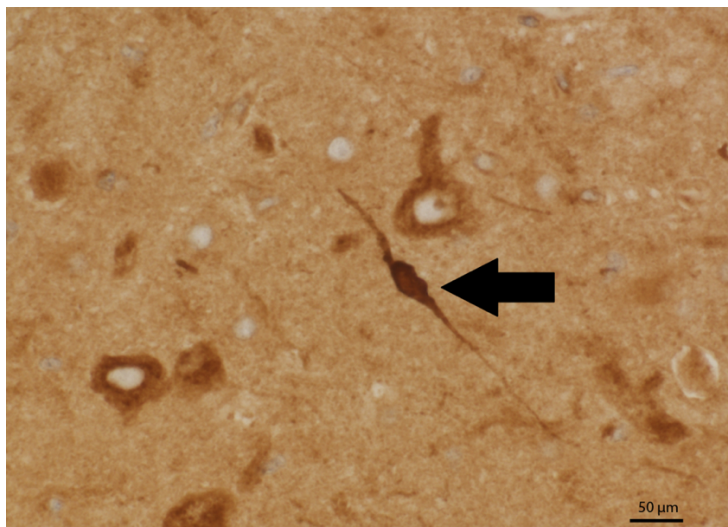


Figure 14. Axonal spheroid. Axonal spheroid found in the hippocampus of a GAD patient (44/16) using APP staining. Photo taken with Nis-Elements D 3.10 software. Scale bar = 50 μm.

Moreover, another semiquantitative analysis was performed to compare the amount of neuronal loss with the duration of the disease. Again, the data do not show correlation between neuronal loss and disease duration (Table 6).

Table 6. Semiquantitative analysis for neuronal loss in GAD encephalitis.

Case	Duration of the disease (years)	Neuronal loss
1733/09	59.6	1 - 2
44/16	36.2	1
945/98	11.1	1 - 2
203/08	6.3	1 - 2
2277/10	4.8	0 - 2
1031/11	4.5	1 - 2
1723/02	0.7	2
455/11	?	1
540/13	?	0 - 1
1103/12	?	0 - 1

Semiquantitative microscopic examination based on NeuN staining. The scoring system refers to the neuronal loss as following: 0= absence of neurodegeneration; 1= moderate neurodegeneration; 2= severe neurodegeneration.

8.7 Analysis of neuronal loss and gliosis

By performing staining for NeuN and GFAP it was possible to identify neuronal loss and gliosis in the GAD cases. In those cases where the entire hippocampus was present (n=3), the ILAE classification system for TLE-HS was used in order to classify the GAD cases. Three cases were successfully classified. Results shown that in GAD encephalitis it seems to be different neuronal loss and gliosis patterns in the areas of the hippocampus as it happens in TLE-HS (Table 7).

Table 7. ILAE classification of TLE-HS in GAD encephalitis.

Region	GAD CASE		
	44/16	203/08	455/11
DG	0 - 1	0 - 2	0 - 2
CA4	0 - 1	1 - 2	2
CA3	0 - 1	0 - 2	0 - 2
CA2	0 - 1	0 - 2	0 - 2
CA1	1 - 2	0 - 2	2
SUB	0 - 1	0 - 1	0 - 1
Classification	Type 2	Type 3	Type 1

The scoring system refers to neuronal loss and is defined for CA1 to CA4 as following: 0 = no obvious neuronal loss or moderate astrogliosis only; 1 = moderate neuronal loss and gliosis; 2 = severe neuronal loss and fibrillary astrogliosis. Scores for dentate gyrus (DG): granule cell layer is normal (score = 0), dispersed (score = 1) or shows severe granule cell loss (score = 2). Scores for the subiculum (SUB): 0 - 1 = the subiculum shows no neuronal loss or moderate. ¹ILAE: International League Against Epilepsy (Blümcke, Sarnat and Coras, 2015)



Figure 15. Neuronal loss in the hippocampus. NeuN staining of one GAD case (44/16). Image taken with NDP.view viewing software. Scale bar = 1 mm.

8.8 Analysis of IgG and complement deposition

Regarding the IgG and complement deposition, IgG, IgG4 and C9neo stainings were performed for this analysis. IgG staining shown that there is a lot of IgG leakage in the hippocampus but there was not clear deposition. In addition, C9neo staining shown absence of complement deposition. These results suggest that there is no antibody-mediated complement deposition and complement-mediated degeneration in GAD encephalitis.

8.9 Analysis of GAD65 loss

The amount of GAD65 was studied in GAD encephalitis (n=6)* and in the control group (n=7) to see if there is a loss of the enzyme in GAD encephalitis. Results are shown in table 8.

For this analysis, GAD65 staining was performed and studied in the hippocampal area (Figure 16). More in detail the GAD65 enzymes were seen (as small dark dots) around neurons because of the synapses.

NeuN stainings were also used for this analysis. Comparing both stainings, GAD65 loss was found where there also was neuronal loss.

Table 8. Semiquantitative analysis for GAD65 loss in GAD patients and in controls.

	CASE	GAD65 loss
GAD cases	1723/02	1 - 2
	203/08	0 - 2
	44/16	0
	1733/09	1 - 2
	945/98	1 - 2
	N455/11	0
Controls	1344/02	0
	N234/06	0
	N526/03	0
	1543/03	0
	N1535/04	0
	971/02	0
	1134/03	0

Semiquantitative microscopic examination based on GAD65 staining. The score system refers to the GAD65 loss as following: 0 = no GAD65 loss; 1 = moderate GAD65 loss; 2 = severe GAD65 loss.

*4 GAD cases were not used for this analysis due to technical reasons.

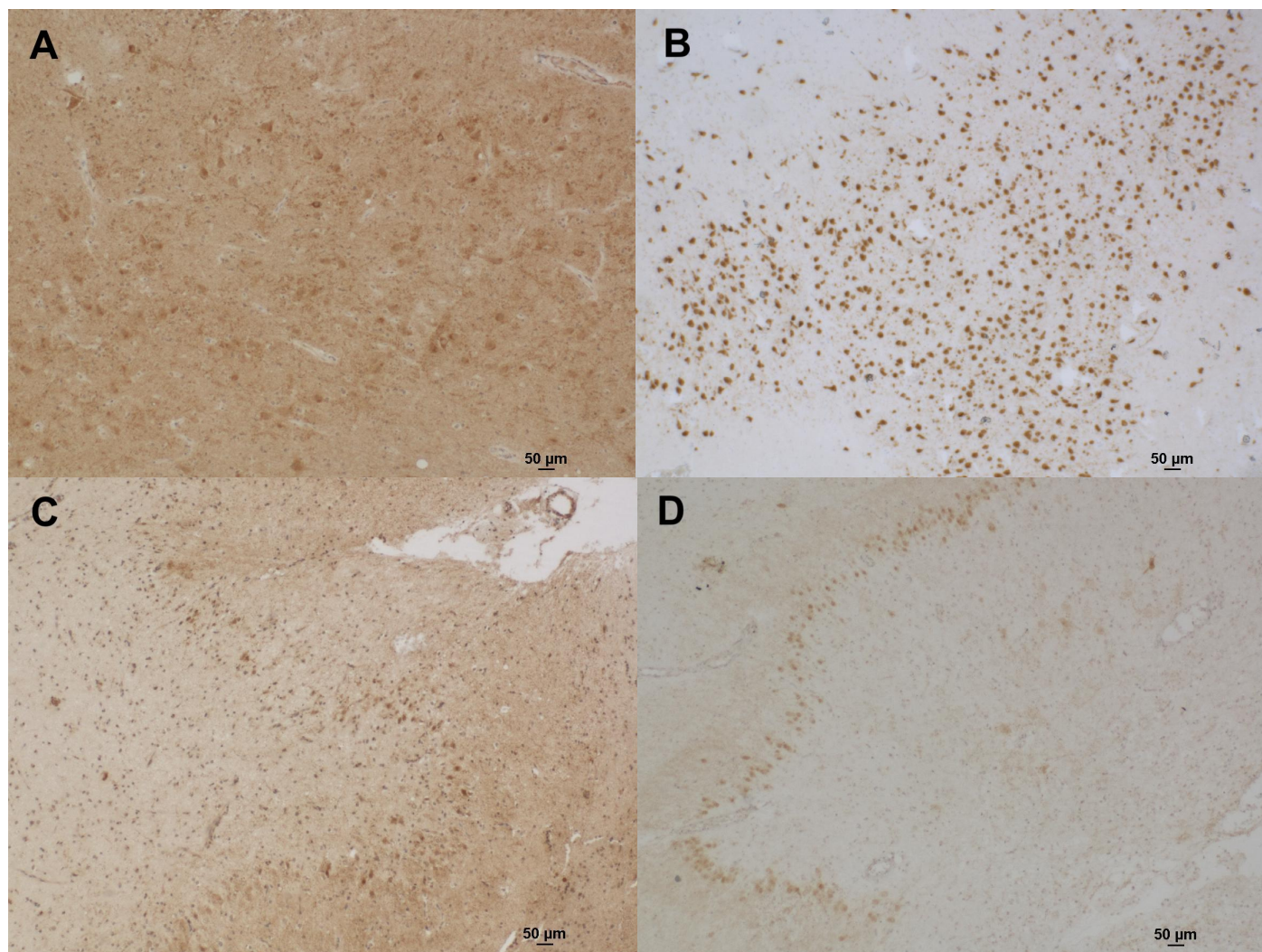


Figure 16. Analysis for GAD65 loss in the CA4 region of the hippocampus. A: GAD65 staining of GAD case 44/16. **B:** NeuN staining of GAD case 44/16. **C:** GAD65 staining of GAD case 203/08. **D:** NeuN staining of GAD case 203/08. GAD65 loss was shown in the CA4 of case 203/08. No GAD65 loss was shown in the GAD case 44/16. All images were taken with Nis-Elements D 3.10 software at 4x magnification. Scale bar = 50 µm.

9. DISCUSSION

Nowadays, it remains a question whether or not the neuronal damage in the hippocampus of patients with GAD encephalitis is mediated by a cytotoxic T cell response. Here we tried to confirm this hypothesis by analysing the numbers of CD8⁺ and GrB⁺ T cells in close apposition to neurons. Another aim of this study was to see if there was a correlation between T, B and plasma cell infiltration and the disease duration in GAD encephalitis. Thus, T, B and plasma cell distribution was studied in ten patients with GAD encephalitis (n=10) and also in the control group (n=7). Special attention was paid to the disease duration of all patients in relation to the number of T, B and plasma cells per mm². Moreover, neuronal loss and gliosis was also studied in relation to the disease duration in our GAD cases, hence, a classification following the ILAE system for hippocampal sclerosis in temporal lobe epilepsy (TLE-HS) was done for patients with GAD encephalitis. Additionally, IgG4 and complement deposition was analysed. Acute neurodegeneration was also tested and compared with the disease duration in GAD patients. Last but not least, GAD65 concentration in the hippocampus was compared between GAD patients and controls.

The CD3 T cell infiltration analysis shown that some of the GAD cases had large numbers of infiltrating T cells in the hippocampus. More in detail, analysis for CD3 and CD8 infiltration shown that in most of the cases, numbers of CD3 and CD8 cells were close to each other, suggesting that most of the CD3⁺ cells also were CD8⁺ cells. Moreover, significant difference was found in CD8 T cell analysis between the GAD and control group. Analysis for plasma cell infiltration also shown significant difference between both groups suggesting that plasma cells could be involved in the pathology mechanisms of GAD encephalitis. Regarding CD20 B cell infiltration, no significant difference was found.

The correlation between T, B and plasma cell infiltration and the disease duration show that the GAD patients with lower numbers of T and B cells are those who had a long disease duration (36.2 and 59.6 years). Conversely, the higher numbers of T and B cells were found in GAD patients with a short disease duration (0.7 and 4.5 years).

On the other hand, higher numbers of GrB⁺ and CD8⁺ cells in apposition to neurons were expected to be found since previous studies have hypothesized that a cytotoxic T cell mediated response

could be the responsible for the neuronal loss (Bien et al., 2012). However, in this study we only found higher numbers of GrB⁺ and CD8⁺ cells in apposition to neurons in a patient with a very short disease duration (0.7 years). These results might mean that a cytotoxic T cell mediated response could take place in the acute stage of the disease. Furthermore, analysis for IgG and complement deposition show no evidence of complement mediated response. Nevertheless, the numbers of CD8⁺ and GrB⁺ cells in close apposition to neurons were low to confirm the hypothesis that T cells are actually causing neuronal death.

On the other hand, analysis for GAD65 loss shows a decrease of the enzyme concentration in four of the GAD cases in comparison with the control group. However, the lack of the enzyme was seen in the areas where there also was neuronal loss. Thus, the low concentration of GAD65 in those GAD patients may be result of the neuronal death. These results cannot confirm our hypothesis that in the hippocampus of patients with GAD encephalitis there is a lack of GAD65. This could mean that the antibody is not responsible for GAD65 decrease. Nevertheless, further studies should be done to test the pathogenicity of the anti-GAD65 antibody.

Here we also hypothesized if the ILAE classification for temporal lobe epilepsy with hippocampal sclerosis could be used for GAD patients. This classification system allows reliable recognition of three types of HS by visual histopathologic examination of surgical specimens. It is hoped that this classification system can be used to uniformly diagnose surgical specimens of patients with TLE and provide a vehicle for collaborative studies across surgical epilepsy centers (Blümcke, Sarnat and Coras, 2015). Since there is no classification system for GAD encephalitis, it could be helpful to find one suitable system to differ between surgical specimens and get a better histopathological knowledge of this disease.

Since just three GAD cases were used, it may be too restricted to say if this classification system is suitable or not. However, three GAD cases were successfully classified into three different types following the ILAE classification patterns. These results might mean that this system could help to differ between patients with GAD encephalitis.

Another point of this study was to analyse if there is a correlation between neuronal loss and disease duration. Since GAD encephalitis is a neurodegenerative disease, it is expected to find

severe neuronal loss at the end of the disease. Our data reveal different patterns between patients. Surprisingly, moderate neuronal loss was shown in the semiquantitative analysis for one patient who had a long disease duration (39.2 years). Conversely, severe neuronal loss was seen in the case 1733/09, which sample was from a patient with also a long disease duration (59.6 years). Another patient with GAD encephalitis with a short disease duration (4.5 years) shown less neuronal loss.

On the other hand, acute neurodegeneration was also analysed. We found high numbers of axonal spheroids in three patients with a short disease duration (4.8, 4.5 and 0.7 years).

In the patient with the shortest disease duration (0.7 years) where a large amount of axonal damage was found, also severe neurodegeneration was seen. Furthermore, this patient shown severe loss of GAD65. This might mean that GAD encephalitis progresses faster in some patients than in others. However, it is difficult to say if there is a correlation or not between neuronal loss and disease duration since it is not known when the GAD65 autoantibodies appear for the first time.

10. CONCLUSION

The analysis performed in this study allow us to answer some of the questions realized in the aims section:

- Different numbers of T, B and plasma cells are found in the hippocampus of patients with GAD encephalitis with different disease duration.
- Small numbers of GrB⁺ and CD8 T cells are found in close apposition to neurons in the hippocampus of patients with GAD encephalitis.
- There is not a clear loss of GAD65 in the hippocampus of patients with GAD encephalitis.
- Complement mediated response seems to be absent in patients with GAD encephalitis.

Some questions remain to be answered; The ILAE classification system seems to be suitable for three GAD cases used in this study. Nevertheless, it is a small number of cases to confirm if GAD encephalitis patients show a specific classification type. Future studies should try to classify more brain specimens in order to confirm our hypothesis.

Secondly, it is difficult to say if there is a correlation between neuronal loss and the disease duration in GAD encephalitis. Since it is a neurodegenerative disease, it is expected to find and increase in the neuronal damage as the disease progresses. However, the results are different from one patient to another.

Moreover, it is still unknown if in GAD encephalitis there is a T cell mediated response or not. Despite the numbers of CD8 T cells were high un the samples used for this study, our results shown small numbers of GrB⁺ and CD8⁺ in close apposition to neurons. Further studies with more GAD cases should be done to answer this question.

Finally, another question arises with the data obtained in this study: which is the role of the plasma cells found in the hippocampus of patients with GAD encephalitis? Future studies should analyse if high numbers of plasma cells are also found in other patients with GAD encephalitis.

11. SELF-ASSESSMENT

First of all, I would like to express my satisfaction after carrying out this project since scientific research has long been of interest to me and I have finally been able to be part of this.

In order to carry out this study, I was given the opportunity to put into practice the knowledge I acquired in my biotechnology degree.

First, I had to depend on databases like PubMed with the aim of getting information from scientific papers about new advances related to the diseases that have been discussed in this project. Moreover, I made use of all the concepts I learnt in Microbial Molecular Biotechnology in order to be able to correctly analyse the content of those articles.

Since this study has been approached from an immunological point of view, I have also been able to put into practice everything I learnt in the subjects of Immunology and Clinical Immunology. My laboratory skills improved too as a result of practicing relevant staining techniques.

Also, I had to apply statistics techniques to provide an adequate interpretation of some of my results.

Moreover, I applied some of the knowledge I gained in the subject of Biochemistry and Molecular biology Techniques when I performed an RNA isolation followed by a qPCR (data not shown).

On the other hand, I have had the opportunity to greatly expand my knowledge in neuroscience and also to learn how to use software like 'Image-Pro Premier' and 'GraphPad Prism' that I have already mentioned in the methodology section.

To conclude, I am really happy, and I feel very proud of having done my external practices in the Center for Brain Research. It was a pleasure to be involved in research for a period of time.

12. REFERENCES

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