Investigations into the Pathogenic Antibody-Antigen-Interference of Glycine Receptor Autoantibodies

Untersuchungen zur pathogenen Antikörper-Antigen-Interferenz von Autoantikörpern gegen den Glycinrezeptor

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Table of Contents

6

Abbreviations

°C degree Celsius

A Ampère

A.d. distillated water

ACSF artificial cerebrospinal fluid

bp base pairs

BSA bovine serum albumine

cm centimetre

CNS central nervous system

CSF cerebral spinal fluid

DM1 type 1 diabetes mellitus

DNA desoxyribonucleic acid

ECD extracellular domain

EGFP enhanced green fluorescent protein

EMG electromyographie

Fab antigen-binding fragment

g gramm

$GABA_AR$ GABAA receptor

$GABA_BR$ GABAB receptor

GAD65 glutamic acid decarboxylase isoform 65

GlyR glycine receptor

GlyT1 glycine transporter 1

GlyT2 glycine transport 2

hc healthy control serum

hpf hours post fertilization

HUWE1 HECT, UBA, WWE domain containing 1

IgG immunoglobulin G

L litre

m milli-

MAC membrane attack complex

MEM minimum essential medium

MRI magnetic resonance imaging

n nano

nAChR nicotinic acetylcholine recepor

NCS neuronal cell-surface

NGS normal goat serum

NHS normal horse serum

pat 1 serum sample of patient 1, patient 1 serum

PBS phosphate-buffered saline

PCR polymerase chain reaction

PERM progressive encephalomyelitis with rigidity and myoclonus

PEx plasma exchange

PFA paraformaldehyde

PKA protein kinase A

PKC protein kinase C

PNS paraneoplastic neurologic syndromes

RT room temperature

SCLC small cell lung cancer

SPS stiff person syndrome

SPSD stiff person spectrum disorder

ss DNA single-stranded DNA

TMS tricaine methanesulfonate

8

TSI thyroid stimulating immunoglobulin

U unit

V Volt

wt wild type

µ micro-

Abstract

Anti-glycine receptor (GlyR) autoantibodies belong to the novel group of autoantibodies that target neuronal cell-surface antigens (NCS), which are accompanied with various neurologic and neuropsychiatric conditions. The inhibitory ionotropic GlyR is one of the major inhibitory neurotransmitter receptors and therefore involved in maintaining homeostasis of neuronal excitation levels at brain stem and spinal cord. Anti-GlyR autoantibodies are associated with progressive encephalomyelitis with rigidity and myoclonus or stiff person syndrome. These neuromotor disorders are characterized by exaggerated startle, muscle stiffness, and painful spasms, leading to immobility and fatal outcome in some cases. It was hypothesized that imbalance of motoneuronal inhibition by functional impairment of GlyR and receptor internalization are direct consequences of antibody-antigen interference. Here, serum samples of four patients were tested for anti-GlyR autoantibodies and were used for the analysis of the functional impact on the electrophysiological properties of recombinant GlyRs, transiently expressed in HEK293 cells. Furthermore, the recognition pattern of anti-GlyR autoantibodies to human, zebrafish and chimeric GlyRα_1 located the epitope to the far N-terminal region. The pathogenicity of anti-GlyR autoantibodies and thereby the autoimmunologic etiology of the disease was confirmed by passive transfer of patient serum to zebrafish (*Danio rerio*) larvae, that yielded an abnormal escape response – a brain stem reflex that corresponds to the exaggerated startle of afflicted patients. The phenotype was accompanied by profound reduction of GlyR clusters in spinal cord cryosections of treated zebrafish larvae. Together, these novel insights into the pathogenicity of GlyR autoantibodies confirm the concept of a novel neurologic autoimmune disease and might contribute to the development of innovative therapeutic strategies.

1 Introduction

1.1 Neuronal Cell-Surface Autoantibodies in Autoimmune Encephalitis

Encephalitis is defined by an inflammation of brain tissue, which can be driven by an infection, most frequently with herpes simplex virus type 1, or by an autoimmune reaction, which has been generally observed to be paraneoplastic (Granerod, Ambrose et al. 2010). Paraneoplastic neurologic syndromes (PNS) occur with specific autoantibodies targeting intracellular neuronal antigens, so called onconeural autoantibodies, which are considered as an immunological epiphenomenon of a predominant role of T-cell-mediated inflammation (Bien, Vincent et al. 2012). Nevertheless, in clinical praxis onconeural antibodies serve as valuable diagnostic markers (Dalmau and Rosenfeld 2008). However, during the last two decades a novel group of encephalitis associated with autoantibodies targeting NCS autoantigens such as ion-channels or neurotransmitter receptors (e.g. glycine receptor, NMDA-R, LGI1, CASPR2, contactin-2) (Dalmau, Geis et al. 2017). In contrast to PNS, NCS autoantibodies are less frequently associated with a tumor disease (Dalmau and Graus 2018). The broad spectrum of inflammatory neurologic syndromes associated with neuronal surface antibodies are referred to as "autoimmune encephalitis". Whereas a T-cell-mediated inflammation is regarded to underlie the pathology of most classic PNS, there is evidence for intrinsic pathogenicity of most of the NCS autoantibodies (Dalmau, Geis et al. 2017, Dalmau and Graus 2018). The epidemiologic relevance of this novel disease entity has been demonstrated in a British multicenter, prospective study, in which NCS autoantibodies were found in eight percent of patients diagnosed with encephalitis (Granerod, Ambrose et al. 2010). Generally, NCS autoantibodies are associated with a corresponding specific clinical syndrome (Dalmau and Graus 2018). GlyR autoantibodies were discovered in serum of a 54-year-old man diagnosed with progressive encephalomyelitis with rigidity and myoclonus (PERM), which is regarded as a "plus" variant of stiff person syndrome (SPS) (Hutchinson, Waters et al. 2008). And subsequently conducted retro- and prospective studies revealed an association of GlyR autoantibodies with stiff person spectrum disorders (SPSD) and notably with the SPS "plus" (McKeon, Martinez-Hernandez et al. 2013, Carvajal-Gonzalez, Leite et al. 2014).

1.2 Stiff Person Spectrum Disorders and Glycine Receptor Autoantibodies

1.2.1 Introduction to a Complex Neurologic Movement Disorder

SPS is a rare and complex neurologic movement disorder with predominant muscle stiffness, frequently leading to disabling conditions and fatal progress in some cases (McKeon, Robinson et al. 2012). The former term "stiff man" was introduced by F. Moersch and H. Woltman at the Mayo Clinic in 1956, when they reported 14 patients suffering from an unrecognized syndrome characterized by fluctuating truncal and limb muscular rigidity and painful muscle spasms, which could be triggered by external stimuli (Fig. 1.1). Muscle involvement was rather symmetrical, with predominant affection of lower extremities, mainly proximal segment, and involvement of axial muscles (Moersch and Woltman 1956). Due to ignorance of frequently affected female patients in the term "stiff man" the disease name "stiff person syndrome" was proposed in 1991 (Blum and Jankovic 1991). Estimated prevalence of SPS is one to two cases per million and an annual incidence of one per million (Dalakas 2009). Median age at onset is 40 years, but infrequently children are afflicted as well. Women are affected by SPS about two times more often than men (McKeon, Robinson et al. 2012, Clardy, Lennon et al. 2013).

Clinical observations in the last decades revealed different disease patterns leading to a subdivision in classical SPS and SPS variants, summarized as SPSD (Barker, Revesz et al. 1998). A partial phenotype of this disorder is referred to as stiff limb syndrome (SLS) with muscle rigidity anatomically confined the lower extremities, which may extend to classical SPS (Brown, Rothwell et al. 1997, Saiz, Graus et al. 1998). In contrast, PERM or SPS "plus", also assigned to SPSD, is characterized by a more severe and rapid disease progress – in some cases fatal – and additional brain stem involvement with disturbances in ocular motility (opsoclonus, nystagmus) and cranial nerve failures. Further clinical features comprise hyperekplexia, pyramidal signs, central paresis, cerebellar ataxia and autonomic dysfunction (Hufschmidt, Hermann Lücking et al. 2017). PERM was first described by A. Campbell and H. Garland, when they recorded three patients presenting a similar clinical syndrome of involuntary, painful muscle contraction and high fever, leading to fatal respiratory failure in two cases (Campbell and Garland 1956).
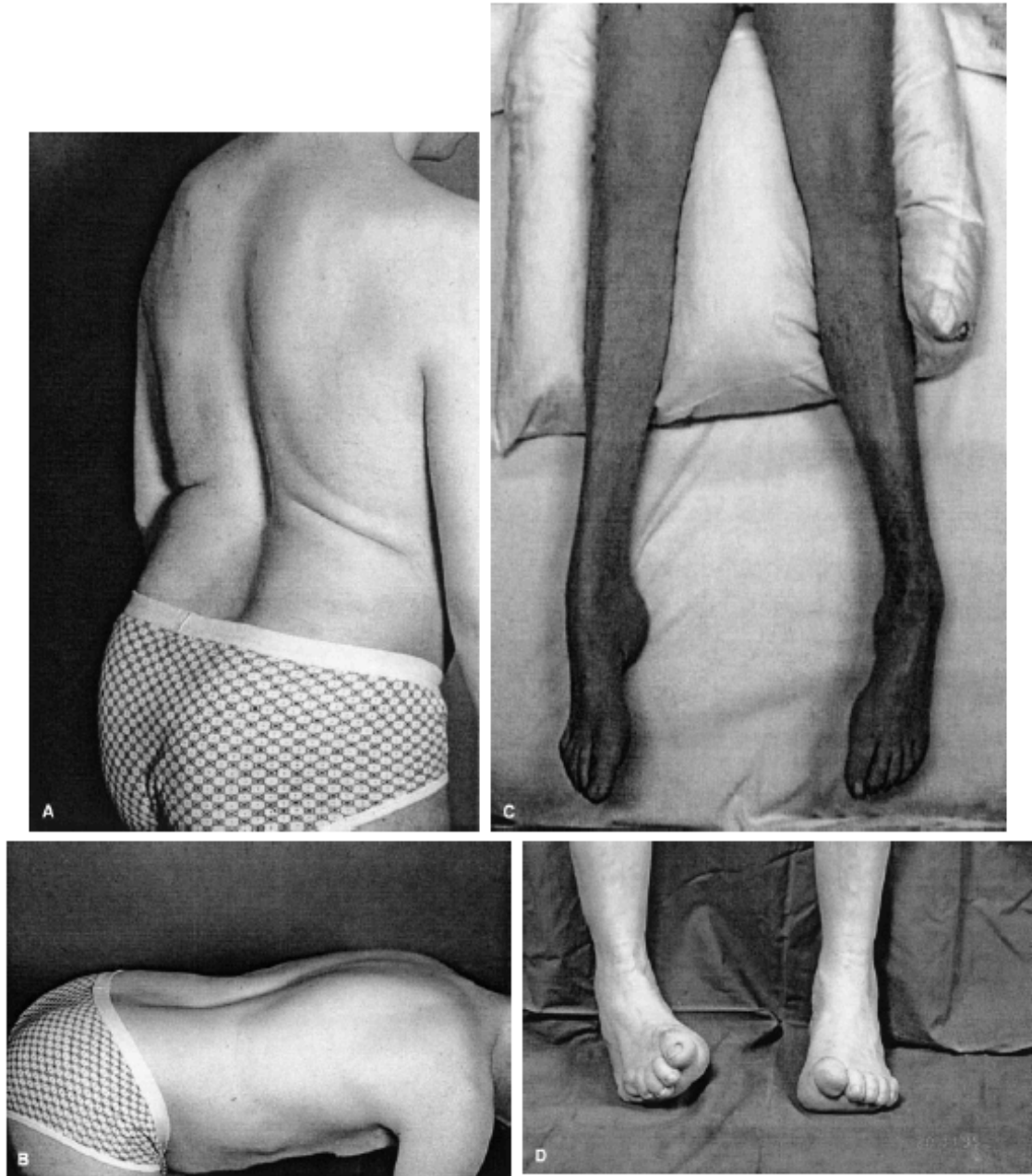
Figure 1.1: Clinical phenomena of pathological muscle innervation. (A) Exaggerated lumbar lordosis in the stiff man syndrome with autoantibodies to glutamic acid decarboxylase isoform 65 (anti-GAD65). The contour of the lumbar paraspinal muscles is accentuated and there is a transverse skin crease across the back reflecting contraction of the lateral oblique truncal muscles. (B) Same patient bending forward, demonstrating the restriction in range of truncal movement caused by the axial muscle contraction. (C) Leg spasms with extensor posturing. (D) Foot posturing in the stiff man syndrome with anti-GAD65. Adopted from Meinck and Thompson 2002 with kind permission from John Wiley and Sons.

1.2.2 Stiff Person Spectrum Disorder – An Autoimmune Disease

1.2.2.1 Glutamic Acid Decarboxylase Autoantibodies

Little was known about the pathogenesis of SPSD until in 1988, an antibody targeting the intracellular enzyme glutamic acid decarboxylase isoform 65 (GAD65) has been discovered in serum of a patient with SPS, type 1 diabetes mellitus (DM1) and epilepsy (Solimena, Folli et al. 1988) Hence, the assumption arose that SPS has an autoimmune etiology. GAD65 is the rate-limiting enzyme of γ-aminobutyric acid (GABA) synthesis, the predominant inhibitory neurotransmitter in the mammalian CNS (Buddhala, Hsu et al. 2009). Regarding the fact that SPSD are characterized by clinical features of exaggerated neuronal excitation like muscle rigidity an impairment of GABAergic neuronal pathways potentially driven by an autoimmune process was proposed. Pronounced effect of $GABA_A$ receptor ($GABA_AR$) agonistic benzodiazepines, usually diazepam, and $GABA_B$ receptor ($GABA_BR$) agonistic baclofen on rigidity and muscle spasms was in line with an assumed major role of deranged GABAergic networks in the disease (Miller and Korsvik 1981, McKeon, Robinson et al. 2012). Now, there is *in vitro* as well as *in vivo* evidence for a pathogenic role of GAD65 autoantibodies by inhibition of GABA synthesis and disturbance of supraspinal structures related to motor control (Dinkel, Meinck et al. 1998, Hansen, Grunewald et al. 2013). Despite an assumed autoimmune pathogenesis immunomodulatory treatment in SPSD had only moderate effects, which might be limited due to irreversible neuronal damage at the moment of treatment initiation (McKeon, Robinson et al. 2012). According to a retrospective study GAD65 autoantibodies are present in around 80% of SPSD patients. Three in four patients with GAD65 autoantibodies were diagnosed with classic SPS, one in four with SLS and only one patient with SPS "plus" (McKeon, Robinson et al. 2012).

1.2.2.2 Amphiphysin Autoantibodies

In 1993, autoantibodies directed to amphiphysin were discovered in the serum of three women with paraneoplastic SPS (De Camilli, Thomas et al. 1993). Whereas anti-GAD65-positive SPSD patients often suffer from DM1 (about 40%), amphiphysin autoantibodies almost always come along with breast cancer or small cell lung cancer (Pittock, Lucchinetti et al. 2005, Murinson and Guarnaccia 2008, McKeon, Robinson et al. 2012). Additional neurologic symptoms found in anti-amphiphysin positive SPS

patients are sensory neuropathy and myelopathy (Balint and Meinck 2018). Amphiphysin is a neural protein critically involved in retrieval of neurotransmitters in the axon terminal's plasma membrane by clathrin-mediated endocytosis. Intrathecal application of anti-amphiphysin IgG derived and purified from paraneoplastic SPS patients induced SPS-like symptoms in rats, most likely caused by interference of GABAergic synaptic neurotransmission in spinal cord circuitries (Sommer, Weishaupt et al. 2005, Geis, Weishaupt et al. 2010). However, histopathology reports point out towards predominance of CD4+ and CD8+ T cells, suggesting a combined pathogenesis of antibody-effects and T-cell-mediated cytotoxicity (Wessig, Klein et al. 2003).

1.2.2.3   Dipeptidyl-Peptidase-like Protein 6 Autoantibodies and others

Dipeptidyl-peptidase-like protein 6 (DPPX) autoantibodies are rarely found, but turned out to be specifically related to SPS-like conditions with agitation, myoclonus, tremor, seizures and startle (Boronat, Gelfand et al. 2013). Occasionally, they are accompanied by an underlying B cell neoplasm (Balint and Meinck 2018). Hallmarks of DPPX autoantibodies besides the neurologic features are preceding diarrhea or other gastrointestinal symptoms (Hara, Ariño et al. 2017). DPPX is a cell surface auxiliary subunit of the Kv4.2 potassium channels and autoantibodies cause decreased clusters of DPPX and Kv4.2 *in vitro* (Hara, Ariño et al. 2017). In the last decade other antigens like $GABA_AR$, $GABA_AR$ associated protein ($GABA_ARAP$), glycine transporter 2 (GlyT2) and gephyrin have been identified to be associated with SPSD, though their diagnostic and pathogenic significance remains to be clarified (Fig. 1.2) (Balint and Bhatia 2016, Balint, Vincent et al. 2017). However, it is remarkable that all antigens apart from DPPX are proteins typically located at GABAergic or glycinergic inhibitory synapses.
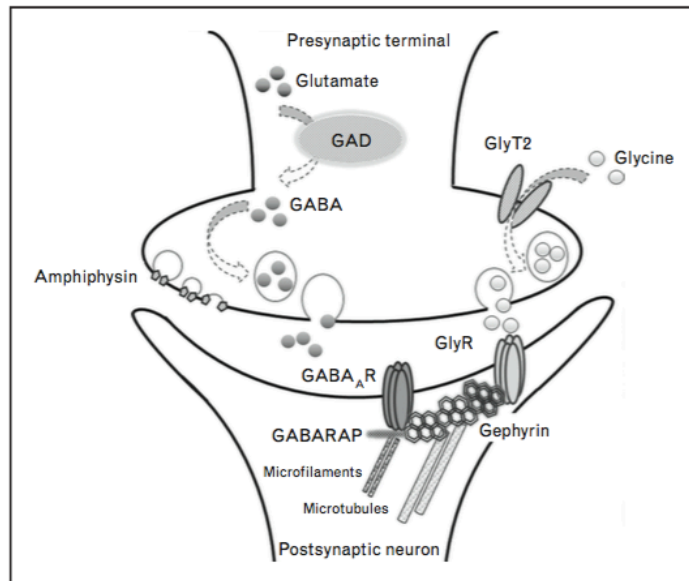
Figure 1.2: Inhibitory synapse with the main targets of antibodies in stiff person spectrum disorders (for illustrative purposes with elements from GABAergic and glycinergic synapses). GABA$_A$R, gamma-aminobutyric acid type A receptor; GABARAP, gamma-aminobutyric acid type A receptor associated protein; GAD, glutamic acid decarboxylase; GlyR, glycine receptor; GlyT2, glycine transporter 2. Adopted from Balint and Bhatia 2016 with kind permission from Wolters Kluwer Health, Inc.

1.2.3 Stiff Person Spectrum Disorder with Glycine Receptor Autoantibodies

1.2.3.1 Clinical Characterization

The index patient presented with violent muscle jerks, spontaneously or triggered by sensory or acoustic stimuli as well as brain stem symptoms like bilateral horizontal gaze palsies and bilateral ptosis, therefore he was diagnosed with PERM (Hutchinson, Waters et al. 2008). Until now, a series of case reports were published, reporting from patients with PERM and associated GlyR autoantibodies (Stern, Howard et al. 2014). The clinical presentation described in the case reports was fairly consistent with core features of rigidity and painful muscle spasms. Most patients also had spontaneous or stimulus-triggered myoclonus, diplopia and autonomic dysfunction (tachycardia, hyperhidrosis, urinary dysfunction). A combined retrospective and prospective study by Carvajal-Gonzalez et al. increased the understanding of the clinical spectrum of anti-GlyR positive patients (Carvajal-Gonzalez, Leite et al. 2014). The prospective study identified 52 patients with GlyR autoantibodies in a cohort of 779 patients, whose serum samples were referred to the Oxford Neuroimmunology service for GlyR autoantibody testing. No gender predominance was detected and age at onset varied

between one and 75 with a median age of 50 years. The vast majority – around two third – of anti-GlyR positive patients were diagnosed with PERM, only two with SPS, five with limbic encephalitis (LE) or epileptic encephalopathy, two had only brain stem features and two had demyelinating optic neuropathies. In contrast to the low number of SPS and SLS diagnoses in anti-GlyR positive patients, the retrospective cohort of 56 SPSD serum samples revealed GlyR autoantibodies in 19 patients with SPS, in two patients with SLS, in six patients with acquired hyperekplexia and in 24 patients with PERM.

A retrospective case-control study at Mayo Clinic (Rochester; USA) tested serum or CSF of SPSD patients, neurologic control subjects and healthy controls for anti-GlyR positivity. GlyR autoantibodies were found in 12% of patients with stiff person phenotype, whereas serum samples of neurologic control patients or healthy controls was detected only in one patient with optic atrophy. In CSF GlyR autoantibodies were exclusively found in SPSD patients (McKeon, Martinez-Hernandez et al. 2013, Martinez-Hernandez, Arino et al. 2016). These data suggest that GlyR autoantibodies are associated with the whole spectrum of SPSD, but they occur predominantly with PERM. Some patients diagnosed with anti-GlyR associated SPS or SLS might have progressed to PERM, if they were not treated with immunosuppressive therapy early enough.

1.2.3.2 Diagnostic Findings, Related Diseases and Clinical Course

Diagnostic investigations in anti-GlyR positive conditions revealed infrequent and unspecific abnormalities in cranial and spinal magnetic resonance imaging (MRI) (Carvajal-Gonzalez, Leite et al. 2014, Martinez-Hernandez, Arino et al. 2016). Electromyography (EMG) displayed continuous motor unit activity in every second patient reflecting hyperexcitation of spinal cord motor circuitries (Carvajal-Gonzalez, Leite et al. 2014). Another EMG finding was spontaneous or stimuli-induced activity in some patients. CSF abnormalities are pleocytosis, raised protein levels or oligoclonal bands, which put further weight on an inflammatory pathogenesis. Notably, about a third of patients with GlyR autoantibodies is also afflicted by other autoimmune diseases, suggesting a general tendency in these individuals for autoimmune conditions. Also of interest, in some cases there is an association with malignancies of the immune system such as thymoma, Hodgkin and non-Hodgkin lymphoma, with substantial improvement or even complete remission of PERM symptoms by tumor

resection or chemotherapy (Clerinx, Breban et al. 2011, Carvajal-Gonzalez, Leite et al. 2014, Borellini, Lanfranconi et al. 2017).

Co-existing NCS autoantibodies of anti-GlyR positive patients were anti-GAD65, anti-NMDAR and anti-VGKC (voltage-gated potassium channel). An overlap with anti-GAD65 has been observed in a subset of SPSD patients and might be regarded as a rather unspecific immunoreaction to inhibitory synaptic proteins. Interestingly, anti-GAD65 and anti-GlyR positive SPSD patients exhibited prominent anxiety and hyperexcitability, potentially displaying a SPS subform (Alexopoulos, Akrivou et al. 2013). Anti-NMDAR and anti-GlyR were found coincidentally in a 28-year-old patient with clinical features of NMDAR encephalitis and PERM, maybe representing a hybrid form of both syndromes (Turner, Irani et al. 2011).

In general, anti-GlyR associated SPSD patients respond well to immunotherapy, with substantial improvement concerning the degree of disability (Carvajal-Gonzalez, Leite et al. 2014). At disease maximum, about three-fourths of patients with anti-GlyR positive PERM were requiring constant nursing care. Though at last follow-up the majority of patients were no longer significantly disabled (Carvajal-Gonzalez, Leite et al. 2014). Compared with anti-GAD65 associated SPSD, those with anti-GlyR tend to have better outcome and respond better to immunotherapy, despite the higher symptom severity at disease maximum (Martinez-Hernandez, Arino et al. 2016).

1.2.4 Glycine Receptor Autoantibodies in Other Neurologic Conditions

For the sake of completeness it remains to be mentioned that anti-GlyR were also detected in serum or CSF of patients with epilepsy, optic neuritis, cerebellar ataxia and opsoclonus-myoclonus-syndrome (Brenner, Sills et al. 2013, Ariño, Gresa-Arribas et al. 2014, Zuliani, Ferlazzo et al. 2014, Martinez-Hernandez, Sepulveda et al. 2015, Armangue, Sabater et al. 2016). Their diagnostic and pathogenic relevance in these disorders has not yet been determined due to insufficient quantity of data.

1.3 Glycine Receptor

1.3.1 Glycine Receptor – A Ligand-Gated Ion Channel

The mammalian CNS relies on balanced levels of neuronal excitation and inhibition. GlyRs induce fast inhibitory postsynaptic currents (IPSC) by a conformational change upon glycine binding, leading to an influx of anions – predominantly chloride ions – and consecutive hyperpolarization of postsynaptic membrane potential (Singer, Talley et al. 1998). GlyRs are part of the group of ligand-binding ion-channels and belong to the family of Cys-loop receptors, which comprises the nicotinic acetylcholine receptor (nAChR), the serotonin type 3 receptor (5-HT$_3$R) and the GABA$_A$R. Four vertebrate genes (*Glra1-4*) encoding for α subunits (α1–α4) and one gene (*Glrb*) encoding for the β subunit have been identified (Lynch 2004). Physiologically GlyRs are thought to be configured as heteropentamers composed by α and β subunits (Lynch 2004). Different stoichiometries have been proposed: 2α:3β, 3α:2β (Burzomato, Groot-Kormelink et al. 2003, Yang, Taran et al. 2012, Patrizio, Renner et al. 2017), but a recent study favored a 3α:2β ratio by quantitative photoactivated localization microscopy (Patrizio, Renner et al. 2017). GlyRα$_1$ and α$_3$ form homopentamers *in vitro* (Fig. 1.4), and recombinant expression of GlyRα$_1$ homopentamers in expression systems like *Xenopus oocyte*s or in human embryonic kidney cells 293 (HEK293) yields in functional ion channel formation (Mohammadi, Krampfl et al. 2003, Grudzinska, Schemm et al. 2005, Huang, He et al. 2007). Electrophysiological characteristics of homopentamers are slightly different from GlyRs containing the β subunit in terms of lower EC$_{50}$ and a decreased slope in dose-response curve (Mohammadi, Krampfl et al. 2003). In contrast to the β subunit, GlyR α-subunits lack the essential motif for the protein-protein-interaction with the scaffolding protein gephyrin, which facilitates synaptic clustering of GlyRs (Meyer, Kirsch et al. 1995, Kneussel, Hermann et al. 2008). Therefore, GlyRα homopentamers are thought to be extrasynaptically located. There is evidence, that these extrasynaptically located GlyRs are involved in the modulation of presynaptically localized neurotransmitter (GABA, glutamate, glycine) release in various brain regions (Turecek and Trussell 2001, Jeong, Jang et al. 2003, Ye 2004).
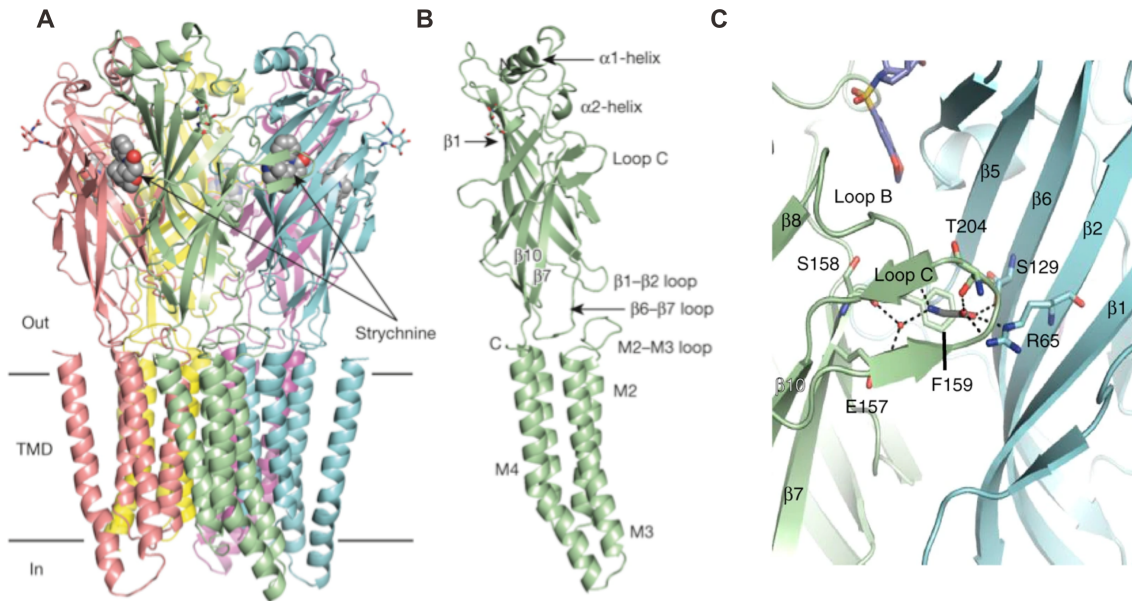
Figure 1.3: (A) The GlyRα₃–strychnine complex integrated into the plasma membrane. Each subunit is depicted in a different color. (B) A single subunit of the GlyRα₃ is displayed. Important structure elements are marked. (C) Stereo view of the neurotransmitter binding site. Glycine is represented as gray sticks. The principal and complementary subunits are pale green and cyan respectively. Secondary-structure elements and important residues are noted. Hydrogen bonds are indicated as black dashed lines. Amino acid positions correspond to the mature protein. A and B are adopted from Huang, Chen et al. 2015 and C is adopted from Huang, Shaffer et al. 2017, both with kind permission from Springer Nature. TMD: transmembrane domain

1.3.2 Molecular Structure and anatomical distribution of Glycine Receptor

The overall GlyR topology has been reviewed by Dutertre et al.: GlyR subunits consist of an aminoterminal large extracellular domain (ECD) composed by ten β-sheets and a short C-terminal extracellular tail (Fig. 1.3 B). The aminoterminal ECD of one subunit forms together with an adjacent subunit the ligand-binding pocket (Fig. 1.3 C). Between the two terminal extracellular domains four transmembrane α-helices (M1-M4) (Fig. 1.3 B) are interposed, displaying the transmembrane domain (TMD). Two small domains connect the first three transmembrane domains (M1-M2 loop, M2-M3 loop). The cytoplasmatic M3-M4 loop harbors amino acid motifs for posttranslational modifications such as phosphorylation and probably ubiquitination (Ruiz-Gomez, Vaello et al. 1991, Buttner, Sadtler et al. 2001, Dutertre, Becker et al. 2012)

GlyRs are mainly expressed within the brain stem – particularly within the brain stem nuclei – and the dorsal and ventral horn of the spinal cord (Probst, Cortés et al. 1986, Waldvogel, Baer et al. 2010). Notably, low expression levels were found in

20

phylogenetically younger brain structures like the forebrain, diencephalon or cerebellum (Probst, Cortés et al. 1986). Corresponding to the widespread existence of GlyRs in brain stem nuclei, patients with GlyR autoantibodies present frequently symptoms, which reflect brain stem dysfunction like dysphagia, dysarthria, trismus and facial numbness (Carvajal-Gonzalez, Leite et al. 2014, Doppler, Schleyer et al. 2015).

The predominant subunit during embryonic neurodevelopment is $GlyR\alpha_2$, but it's the expression level declines during postnatal development (Watanabe and Akagi 1995). In the adult brain, $GlyR\alpha_1$ shows the most abundant expression. It is assumed that $GlyR\alpha_1\beta$ heteromers is the most frequent subunit-composition (Malosio, Marqueze-Pouey et al. 1991). $GlyR\alpha_3$ is fairly less expressed in the CNS compared to $GlyR\alpha_1$, but exists on nociceptive neurons of the murine dorsal spinal cord and modulate chronic inflammatory pain (Harvey, Depner et al. 2004). Also its presence has been reported in cerebellum, hippocampus, frontal and parietal cortex, but the physiological function remains elusive (Meier, Henneberger et al. 2005). The *Glra4* gene is regarded to be a pseudogene in humans, since there is a stop codon within the M4 presumably disabling a normal receptor function (Simon, Wakimoto et al. 2004). Interestingly, the β subunit mRNA is expressed already at early developmental stages and persists in various brain regions during adulthood (Malosio, Marqueze-Pouey et al. 1991). In contrast to the abundance of GlyRβ mRNA, the synaptic protein distribution has found to be restricted to spinal cord, brain stem and olfactory bulb by immunostaining of mouse brain sections (Weltzien, Puller et al. 2012). The discrepancy between distribution of mRNA expression and surface protein levels might be explained by proteasomic degradation of non-assembled GlyRβ subunits due to lacking co-expression of GlyR α-subunits. All GlyR subunits are known to play a certain role of retinal processing of visual information, since they are located on postsynaptic site of retinal bipolar, ganglion and amacrine cells (1.3.3.3) (Wassle, Heinze et al. 2009).

1.3.3 Consequences of Disrupted Glycinergic Neurotransmission Can Explain the Clinical Syndrome of Anti-GlyR SPSD

The important role of adequate glycinergic neurotransmission is reflected by humans and animals carrying naturally occurring mutations in *GLRA1* and *GLRB*, which encode for GlyR subunits α1 and β respectively (Lynch 2004, Harvey, Topf et al. 2008, Bode and Lynch 2014). Therefore, knowledge of the physiological role of GlyRs arose from clinical assessment of afflicted patients (hereditary hyperekplexia) or animals like mice

(*oscillator, spasmodic, spastic*), cows (*myoclonus*) and zebrafish (*bandoneon*), and neurobiological characterization of the respective mutants (Bode and Lynch 2014). Altered GlyR function and diminished or abrogated receptor expression turned out to be the most commonly observed consequences of genetically defective GlyR subunits (Bode and Lynch 2014). On the behavioral level, hallmarks of disrupted glycinergic neurotransmission are in any species an exaggerated startle reflex to unexpected stimuli (acoustic, tactile) and muscle stiffness (Lynch 2009). Patients suffering from hereditary hyperekplexia show these symptoms straight after birth, but muscle rigidity declines and normalizes usually during the first six months, arguing for compensatory mechanisms that take place during postnatal neurodevelopment. This is potentially enabled by substitutional GABAergic neurotransmission. In contrast, stimulus-sensitive exaggerated startle reflexes persist during adulthood, which might be explained by a not-replaceable role of glycinergic neurotransmission in the corresponding neuronal circuitries (Harvey, Topf et al. 2008, Dreissen, Bakker et al. 2012, Bode and Lynch 2014, Hufschmidt, Hermann Lücking et al. 2017). Regarding the pathogenicity of anti-GlyR, it is intriguing that patients with anti-GlyR have several neuromuscular clinical features in common with patients or animals with genetically disrupted GlyR function.

1.3.3.1 Neuromotor Control

Physiologic muscle tonus and movement execution require accurate dosage of muscle innervation by α-motoneurons and coordinated innervation of antagonistic muscles. These prerequisites are facilitated by (i) reciprocal and (ii) recurrent inhibition of α-motoneurons, which are both mediated by glycinergic inhibitory neurotransmission (Callister 2010). (i) Ia afferents originate from muscle spindles (e.g. of a flexor) and activate inhibitory interneurons resulting in inhibition of the antagonistic muscle (e.g. an extensor). The mechanism is called 'reciprocal inhibition' and prevents simultaneous activity of antagonistic muscles. (ii) Physiological role of recurrent inhibition is presumably to limit motoneuron discharge, which might result in spasticity or muscle spasms. This inhibition is mediated by an inhibitory interneuron named Renshaw cell. Activation of the Renshaw cell by α-motoneuron collaterals results in auto-regulation of motoneuronal excitation level. Core symptoms of anti-GlyR-positive patients like stiffness, rigidity and (often painful) spasms can be well explained by autoantibody-mediated glycinergic dysfunction (Carvajal-Gonzalez, Leite et al. 2014). The derogation of neuromuscular activity regulation leads frequently to disabling conditions with severe walking difficulties and numerous falls (Carvajal-Gonzalez, Leite et al. 2014).

1.3.3.2 Brain Stem Functions

The startle reflex is a physiologic reflex consisting of forced closure of eyes and flexion of neck, trunk, elbows, hips and knees. In hereditary hyperekplexia, latencies from stimulus to motor response are shorter and extent of motor response is stronger compared to physiological startle reflex (Dreissen, Bakker et al. 2012). The functional neuroanatomy of the acoustic startle is represented by a brain stem neuronal circuit. The pontomedullary reticular formation processes synaptic input form the cochlear nucleus and innervates brain stem and spinal motoneurons (Valls-Solé, Kumru et al. 2008). Abundance of GlyR in the respective brain stem areas and a pathological exaggerated startle reflex in individuals with genetically disrupted glycinergic neurotransmission indicate an important role of GlyRs in the regulation of the startle reflex (Waldvogel, Baer et al. 2010). About 50% of anti-GlyR-positive SPSD patients suffer from an exaggerated startle reflex (spontaneous or triggered by sensory stimuli like noise or touch) (Carvajal-Gonzalez, Leite et al. 2014). Furthermore, oculomotor disturbances like diplopia and nystagmus are common clinical features patients with anti-GlyR-positive SPSD (Carvajal-Gonzalez, Leite et al. 2014). Given the fact that GlyR are expressed in brain stem nuclei like abducens nucleus and vestibular nuclei, these symptoms could also be attributed to an anti-GlyR pathology (Waldvogel, Baer et al. 2010).

There is evidence for the important role of glycinergic mediated inhibition within the Bötzinger and preBötzinger complex (ventrolateral medulla oblongata), brain stem structures that are crucial for the generation of the respiratory rhythm pattern (Janczewski, Tashima et al. 2013, Sherman, Worrell et al. 2015). In mice, optogenetic photostimulation of glycinergic neurons of the preBötzinger complex is able to produce apnea and ceases when stimulation is stopped (Sherman, Worrell et al. 2015). Apnea attacks occur frequently in hereditary hyperekplexia patients (Thomas, Chung et al. 2013) and respiratory failure displays a life-threatening complication in patients with anti-GlyR-positive SPSD (Thomas, Chung et al. 2013, Carvajal-Gonzalez, Leite et al. 2014).

Various signs of autonomic disturbance (hyper- and hypohidrosis, xerostomia, brady- and tachycardia, hypo- and hypertension, bladder, bowel or sexual dysfunction) are seen in anti-GlyR-positive SPSD (Carvajal-Gonzalez, Leite et al. 2014). GlyRs are known to be present in brain stem nuclei (reticular formation, nucleus tractus solitarii and dorsal nucleus of vagus nerve) that are involved in autonomic regulation (Probst,

Cortés et al. 1986, Waldvogel, Baer et al. 2010, Trepel and Dalkowski 2017). On the functional level, there is evidence for glycinergic inhibition in brain stem neuronal networks, which contribute to the central regulation of blood pressure (Gao, Korim et al. 2019).

Bracketed together, brain stem symptoms (exaggerated startle, respiratory failure, oculomotor disturbances) that are pathophysiologically related to dysfunction of neuronal circuitries with possible or known GlyR involvement put further weight on the theory of autoantibody-induced disruption of glycinergic neurotransmission.

1.3.3.3 Hippocampal Glycine Receptors and Epilepsy

Due to GlyRα_2, α_3 and β hippocampal expression, GlyR play a putative role in learning, memory and epilepsy. There is increasing evidence for an essential role of tonic inhibition meditated by extrasynaptically located GlyR in regulation of hippocampal network activity (Zhang, Gong et al. 2008, Keck and White 2009). Concerning the pathophysiological role in epilepsy originating from hippocampal hyperexcitation, it has been shown that post-transcriptional modifications (alternative splicing, C-to-U-Editing) of *GLRA2* and *GLRA3* lead to altered receptor function and their transcription levels are associated with severity of mesial temporal lobe epilepsy and excitotoxicity (Meier, Henneberger et al. 2005, Eichler, Kirischuk et al. 2008, Eichler, Förstera et al. 2009). Moreover, the pathophysiological contribution of impaired glycinergic inhibition to epilepsy is supported by an *in vitro* study revealing the pro- and anticonvulsive effects of GlyR activation in immature hippocampal neurons (Chen, Okabe et al. 2014). A shift in homeostatic hippocampal activation towards excitation as a consequence of hindered glycinergic inhibition might underlie the fact, that 13% of anti-GlyR-positive SPSD patients had epileptic seizures as part of their complex neurological syndrome (Carvajal-Gonzalez, Leite et al. 2014).

1.3.3.4 Pain Perception

Glycinergic neurotransmission is critically involved in processing of somatosensory signals, in particular of pain perception (Zeilhofer 2005). Prostaglandin E$_2$-mediated inhibition of GlyRα_3, which is expressed in the dorsal horn of the spinal cord, causes pain sensitization during chronic inflammation (Harvey, Depner et al. 2004). Interference with glycinergic neurotransmission by pathogenic anti-GlyR might explain that up to 50% of patients with anti-GlyR-positive SPSD suffer from a bunch of sensory

symptoms like pruritus, dysasthesia, hyperaesthesia or pain (Carvajal-Gonzalez, Leite et al. 2014).

1.4 Pathogenicity of Glycine Receptor Autoantibodies

1.4.1 Physiological, Pathophysiological and Therapeutic Effects of (Auto-) Antibodies

Immune responses in general are initiated by contact of immune cells to foreign antigens, e.g. fragments of bacterial or viral proteins, and lead to their differentiation to antigen-specific cytotoxic T cells or/and to antibody-producing plasma cells (Hoth and Wischmeyer 2016). Physiologically, autoimmune reactions are prevented by negative selection during B and T cell maturation that induces apoptosis of self-reactive immune cells, differentiation into regulatory T cells or B cell receptor editing. Defects in these mechanisms can lead to breakdown of self-tolerance, causing autoimmune diseases (Theofilopoulos, Kono et al. 2017). In general, it is assumed that the pathogenesis of autoimmune disorders is either exerted predominantly by autoantibodies or by autoreactive T cells (Murphy and Weaver 2018). Immunological consequences of autoantibodies are (i) complement-activation and (ii) antibody-dependent cytotoxicity. Initiation of the complement cascade leads to proteolytic cleavage and activation of complement factors and assembly of the membrane attack complex (MAC) that shapes a pore into the plasma membrane yielding lysis of the targeted cell or germ. Furthermore, upon complement binding the mononuclear phagocyte system reacts with phagocytosis, exerted by microglia cells within the CNS compartment. Considering the toxicity of complement-activation in brain tissue a profound role may also be attributed to chemotaxis and activation of inflammatory leukocytes (Hoth and Wischmeyer 2016, Murphy and Weaver 2018). (ii) Antibody-dependent cell-mediated cytotoxicity is driven by natural killer cells that detect antibodies via their membrane-bound $F_c\gamma$ receptors and cause cell death via release of perforins and granzymes (Bournazos, Wang et al. 2017). Cell toxic enzymes released by complement-dependent attracted leukocytes and antibody-dependent stimulated natural killer cells are potential key players of autoantibody-induced inflammation and tissue damage.

In addition to the autoimmunologic effects of autoantibodies, direct interactions with the respective autoantigen are regarded to contribute to the pathogenesis. For example, modified receptor function by antibody-antigen-interference is generally accepted for

anti-nAChR autoantibodies in Myasthenia gravis (receptor blockade) or autoantibodies targeting the thyroid stimulating receptor (also referred to as thyroid stimulating immunoglobulin; TSI) in Graves's hyperthyroidism (receptor overstimulation) (Drachman, Adams et al. 1982, Epstein, Bahn et al. 1993). Since the discovery of NCS autoantibodies vast investigations into the direct effects have been undertaken. Thus, the following pathogenic mechanisms of NCS autoantibodies became apparent: receptor internalization (e.g. NMDAR), functional receptor blockade (e.g. $GABA_BR$), disrupted synaptic protein-protein interactions (LGI1 and CASPR2) and disturbed synapse formation (Dalmau, Geis et al. 2017). In contrast to autoantibody-induced inflammation and irreversible tissue damage, autoantibody effects on the respective targeted antigen are regarded to be reversible, allowing marked clinical improvement or even full recovery after antibody removal by plasmapheresis or B cell depletion (Dalmau and Graus 2018).

1.4.2 Etiology of Antineuronal Autoimmunity

1.4.2.1 Tumor

There is evidence, that tumors are triggers of neuronal autoimmune phenomena (Dalmau, Geis et al. 2017). NMDAR encephalitis is associated with tumors in about 40% of all cases, mainly with ovarian teratoma (97%) (Titulaer, McCracken et al. 2013), and an ectopic NMDAR subunit (GluN1) expression by tumor tissue as well as inflammatory infiltrates by B and T cells has been demonstrated (Tüzün, Zhou et al. 2009, Chefdeville, Treilleux et al. 2019). If a cancer-driven immunopathogenesis applies also for GlyR antibody-mediated encephalopathy has not been studied yet. Given that up to ten percent of patients with anti-GlyR have a coexisting or preexisting malignancy (e.g. thymoma, lymphoma, small cell lung cancer (SCLC) and breast cancer) and cancer treatment improves the neurologic condition dramatically, an etiologic role of cancer might account to a subgroup of patients (Carvajal-Gonzalez, Leite et al. 2014, Armangue, Spatola et al. 2018, Swayne, Tjoa et al. 2018). In this context, it is interesting that *GLRA1* mRNA transcripts were found in histologic SCLC samples, suggesting that SCLC might be a potential trigger for humoral autoimmunity against GlyRs (Gurrola-Diaz, Lacroix et al. 2003). But further research is required to reveal the contribution of ectopic GlyR expression to anti-GlyR neurologic syndromes.

1.4.2.2 Viral infection

Autoimmune phenomena are frequently observed following a viral or bacterial infection that are thought to be driven by a cross-reaction against autoantigens (Khitrov, Shogenov et al. 2007). Regarding neuronal autoimmunity, it has turned out that about one third of patients with herpes simplex encephalitis develop a subsequent immunotherapy-responsive anti-NMDAR encephalitis or autoimmune encephalitis (Armangue, Spatola et al. 2018). Moreover, coincidence of CSF positivity for herpes virus DNA (HSV-1/-2, EBV, VZV, HHV-6, CMV) and known (anti-NMDAR, anti-AMPAR, anti-GABA$_B$R) or unknown NCS autoantibodies has been found in encephalitis patients (Linnoila, Binnicker et al. 2016). Investigations into the pathogenic properties of NMDAR-IgM autoantibodies coexisting in herpes encephalitis patients revealed downregulation of the receptor. This suggests a pathogenic role of autoantibodies also in herpes encephalitis patients (Pruss, Finke et al. 2012). However, so far, no investigations into parainfectious autoimmunity to GlyR have been undertaken.

1.4.2.3 Propensity to Autoimmunity

Given the fact, that a considerable number of patients (around 30%) with GlyR have additional autoimmune diseases (thyroid disease, diabetes among others), an predisposition for self-reactive inflammatory responses can be assumed to play a role in some patients (Carvajal-Gonzalez, Leite et al. 2014, Theofilopoulos, Kono et al. 2017).

1.4.3 Pathophysiologic Relevance of Glycine Receptor Autoantibodies

1.4.3.1 Neuroinflammation

The pathophysiological concept of autoantibody-associated encephalitis is divided into autoantibodies that target intracellular autoantigens and those, who bind to NCS autoantigens. Whereas the latter are regarded to exhibit pathogenic properties, autoantibodies towards intracellular autoantigens are generally considered to represent an epiphenomenon of T cell inflammation. This is supported by investigations into the immunopathology of brain specimens derived from patients with autoantibody-mediated encephalitis harboring NCS autoantibodies (anti-VGKC, anti-NMDAR) or with autoantibodies to an intracellular antigen (anti-Ma2, anti-Hu). Complement- and

autoantibody-mediated mechanisms are responsible for neurodegeneration in anti-Caspr2 and anti-LGI1 cases whereas CD8+-T-cell-mediated cytotoxicity plays a predominant role in AE with intracellular antigens (Bien, Vincent et al. 2012). Interestingly, cases with NMDAR encephalitis lacked any neuronal pathology and showed only low infiltrated of inflammatory cells (Bien, Vincent et al. 2012).

To date, there is no available pathology data from GlyR-autoantibody-positive patients. Though there is a former pathologic report dating back to the time when PERM has not yet been recognized as an autoantibody-associated disease, where extensive perivascular lymphocytic infiltrations as well as neuronal loss and astrocytic gliosis in brain stem (e.g. reticular formation and abducens nucleus) and spinal cord were described (Whiteley, Swash et al. 1976). Back then the etiology of inflammation and neurodegeneration was unknown. From today's perspective, the neuropathological picture could be interpreted as an autoinflammatory process in brain stem nuclei. The neuronal loss could be either attributed to T cell- or NK cell-mediated cytotoxicity or to complement activation. Anti-GlyR autoantibodies consist of complement-activating IgG1 and IgG3 subtypes and their ability to fix complement has been proven on HEK293 cells (Carvajal-Gonzalez, Leite et al. 2014).

1.4.3.2 Internalization

Decades ago, receptor internalization upon autoantibody-binding and cross-linking of adjacent receptor subunits via the two antigen-binding fragments of IgG has been demonstrated to contribute to pathology of the neuromuscular disorder Myasthenia gravis (Drachman, Angus et al. 1978, Drachman, Angus et al. 1978). Patients with Myasthenia gravis suffer from progressive muscle weakness, which is explained by the diminished density of postsynaptic receptors and the reduced synaptic strength.

In vitro investigations into the pathogenesis of AE revealed, that some NCS autoantibodies induce protein internalization (anti-NMDAR, anti-AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid-receptor) and anti-dopamin-2-receptor) (Dalmau, Geis et al. 2017, Dalmau and Graus 2018). That internalization accounts also for anti-GlyR autoantibodies has been shown in HEK293 cells expressing recombinant GlyRα$_1$. Upon autoantibody-exposure, GlyRα$_1$ was downregulated in a time- and temperature-dependent manner and subsequently directed to lysosomal degradation (Carvajal-Gonzalez, Leite et al. 2014). Reduced receptor expression as a consequence of an impaired receptor biogenesis has been found to cause hereditary hyperekplexia,

the genetic phenocopy of SPSD, underlining the pathogenic potential of reduced GlyR surface levels (Schaefer, Roemer et al. 2018). Interestingly, there is one study proposing that testing for GlyR internalization elevates the diagnostic specificity of immunologically treatable SPSD compared to solely detection of anti-GlyR IgG (Hinson, Lopez-Chiriboga et al. 2018). One aim of this study is to reproduce GlyR receptor internalization as a pathogenic effect of anti-GlyR autoantibodies. Therefore, patient samples with anti-GlyR IgG were added to GlyRα1-expressing HEK293 cells and immunofluorescent analysis of leftover membrane-integrated GlyRα1 after incubation at 37° C.

1.4.3.3 Functional Impairment

Taken into consideration that anti-nAChR and TSI alter the function of the respective targeted receptor, it is feasible that NCS autoantibodies exhibit also functional effects. It has been shown on primary hippocampal neurons, that $GABA_BR$-mediated currents are significantly reduced upon exposure to purified IgG derived from a patient with limbic encephalitis and seropositivity for anti-$GABA_BR$. Since the anti-$GABA_BR$ failed in internalizing the receptor, the electrophysiological impact of anti-$GABA_BR$ is attributed to an impairment of receptor function (Nibber, Mann et al. 2017). A recombinant monoclonal NMDAR autoantibody derived from single cell cloning of a patient's B cell suppressed NMDAR channel activity, which however was accompanied by NMDAR cluster downregulation (Kreye, Wenke et al. 2016). Just recently a profound disruption of glycinergic currents of anti-GlyR IgG was observed on cultured rat spinal motor neurons in whole-cell patch-clamp recordings, and, intriguingly, the same effect was also seen after digestion of IgG to antigen-binding fragments (F_{ab}) (Crisp, Dixon et al. 2019). Since internalization relies usually on dimerization by divalent antibodies, it is unlikely that the abolishment of glycinergic currents is caused by receptor endocytosis. In the present study, whole cell currents of GlyRα1-transfected HEK293 cells were recorded after incubation in anti-GlyR IgG patient samples. It could be demonstrated, that patient anti-GlyR autoantibodies have a potent effect on GlyR channel activation.

1.4.3.4 Autoantibodies' Epitope

To the author's knowledge there is no existing information about the epitope of anti-GlyR autoantibodies, but differences in subunit-specificity of anti-GlyR argues for miscellaneous epitopes (Carvajal-Gonzalez, Leite et al. 2014). Localization of the

epitope would allow correlating the individual binding sites with the clinical presentation and observed *in vitro* effects. Furthermore, from the treatment of inflammatory diseases (e.g. inflammatory bowel disease or rheumatoid arthritis) we know that therapeutic monoclonal antibodies (e.g. infliximab) can antagonize cytokines (e.g. TNFα), peptides that play key roles in the immunopathogensis. A novel treatment strategy for anti-GlyR-associated autoimmune encephalitis could be the individual development of patient and epitope specific peptides that antagonize anti-GlyR IgG. Here, it could be shown for one patient's serum, that the far N-terminus of the mature receptor is detected by the autoantibodies.

1.4.3.5 *In Vivo* Effects of Glycine Receptor Autoantibodies

Phenotype of mutations in GlyR-encoding genes has been studied intensively in human and mammalian animal models, but also in phylogenetically older organisms like zebrafish (Lynch 2009, Ogino and Hirata 2016). Glycine transporter 1 (GlyT1) and GlyRβ zebrafish mutants have been characterized and named *bandoneon* and *shocked* respectively due to their typic phenotype (Cui 2005, Hirata, Saint-Amant et al. 2005). Upon a tactile stimulus, which provoked an escape response, *bandoneon* mutants showed contraction of bilateral trunk muscles, in contrast to alternating contractions that enable swimming behavior in wild type zebrafish (Hirata, Saint-Amant et al. 2005). *Bandoneon* turned out to be a phenocopy of wild type zebrafish that is treated with the GlyR antagonist strychnine (Hirata, Saint-Amant et al. 2005). Whereas the *shocked* phenotype was caused by potentiated glycinergic transmission, defective glycinergic transmission underlay the pathology of GlyRβ mutants (*bandoneon*) (Cui 2005, Hirata, Saint-Amant et al. 2005). Furthermore, decreased expression levels of GlyRα$_1$, α$_3$ and α$_4$a mRNA due to a mutated DEAH-box RNA helicase or morpholino knockdown caused a deficient motor response to touch, analogous to what has been observed in *bandoneon* mutants (Hirata, Ogino et al. 2013). In this study, it was intended to give proof of the pathogenicity of anti-GlyR IgG. A patient-derived serum sample was transferred to the CSF of zebrafish larvae and their escape response upon a tactile stimulus was analyzed subsequently. Indeed, treated zebrafish larvae were unable to initiate swimming behavior. Corresponding to the neuromotor phenotype, reduced GlyR surface clusters were observed in spinal cord sections of treated zebrafish.

2 Material and Methods

2.1 Patient Samples

2.1.1 Acquisition of Patient Material

Patient serum samples were gratefully received from Prof. C. Sommer (Department of Neurology; University of Würzburg; Germany) Prof. emeritus H.-M. Meinck (Department of Neurology; University of Heidelberg; Germany) and Prof. emeritus A. Vincent (Nuffield Department of Clinical Science; University of Oxford; United Kingdom). Samples were taken from patients with SPSD either via standard blood withdrawal or via therapeutic plasmapheresis or immunoadsorption. Therapeutic plasmapheresis and immunoadsorption are common procedures to eliminate pathogenic autoantibodies. In case of plasmapheresis, blood plasma is removed and replaced by a specific substitution solution, whereas in immunoadsorption exclusively antibodies and antigen-antibody-complexes are eliminated, and patient plasma is returned. Control serum was obtained from a patient with suspected optic nerve schwanomma.

Blood serum and in some cases cerebral spinal fluid (CSF) were beforehand analyzed for anti-GlyR and other autoantibodies (e.g. anti-Amphiphysin, anti-GAD65) at diagnostic laboratory EUROIMMUN AG (Lübeck, Germany).

2.1.2 Ethical Statement

Permission of the study was obtained from the ethic committee of the Medical Faculty of the University of Würzburg (Germany) for the project "Autoantibodies and Glycinergic Dysfunction – Pathophysiology of Associated Motor Disorders" (20190424 01).

2.1.3 Purification of Serum IgG

Purification of serum IgG was performed by medical technical assistant Susanne Helmig (Department of Neurology, University Hospital Würzburg, Germany). All IgG fractions were purified from plasma filtrate obtained during therapeutic plasma exchange (PEx) as part of standard patient care by separation on exchange

chromatography as described previously (Sommer, Weishaupt et al. 2005). The IgG fractions were dialyzed, freeze-dried and stored at −20 °C until use. Lyophilized IgG was dissolved in normal saline at a concentration of 100 mg/ml just before use.

2.2 Chemicals, Solutions and Enzymes

2.2.1 Chemicals

All chemicals applied in this study were produced by Sigma-Aldrich (St. Louis, MI, USA), AppliChem (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany) if not otherwise indicated.

2.2.2 Cell Culture Media and Solutions

Medium/Component	Manufacturer/Composition
MEM (minimum essential medium)	Life Technologies, Darmstadt, Germany (10270-106)
Dulbecco´s PBS (1x phosphate buffered saline)	PAA laboratories, Cölbe, Germany (H15-002)
L-glutamine	Life Technologies, Darmstadt, Germany (25030-024)
FCS (fetal calf serum)	Life Technologies, Darmstadt, Germany (10270-106)
Penicillin/Streptomycin (Pen/Strep)	Life Technologies, Darmstadt, Germany (15140-122)
Trypsin 0.05%	PAN Biotech, Aidenbach, Germany (P10-023500)
HEK293 medium	MEM, 10% FCS, 1% L-glutamine, 1% Pen/Strep

2.2.3 Transfection Solutions

Buffer/Solution	Composition
2x HBS (HEPES buffered saline)	50 mM Hepes, 12 mM dextrose, 10 mM KCl, 28 mM NaCl, 1.5 mM Na_2HPO_4; pH 6.95
TE buffer	10 mM Tris base, 1 mM EDTA; pH 7.4
$CaCl_2$	2.5 M; pH 7

2.2.4 DNA Standard Solutions

Buffer/Solution	Composition
1x TE buffer (Tris-EDTA)	10 mM Tris base, 1 mM EDTA; pH 7.4
1x TBE buffer	89 mM Tris base, 89 mM boric acid, 2 mM EDTA; pH 8.3

2.2.5 Buffer and Solutions for Immunocytochemistry

Material	Manufacturer/Composition
4% paraformaldehyde (PFA) 4% sucrose	2 g, 2 g PFA, 2 drops NaOH (1 M), 10 ml 10x PBS, dH_2O ad 100 ml
Normal goat serum	PAA Laboratories, Cölbe, Germany
Normal horse serum	PAA Laboratories, Cölbe, Germany
Mowiol	Carl Roth, Karlsruhe, Germany

For the production of 4% PFA 4% sucrose 2 g PFA and 2 g sucrose were dissolved in 50 ml dH_2O. 2 drops of NaOH (1 M) were added, and reagent mixture was heated (55°C) until PFA has been completely dissolved. Then, 10 ml 10x PBS was added and the solution was filled up with dH_2O ad 100 ml.

2.2.6 Buffer for Electrophysiological Experiments

Buffer	Composition
External buffer	137 mM NaCl, 5.4 mM KCl, 1 mM MgCl, 5 mM HEPES; pH adjusted to 7.4 with NaOH
Internal buffer	120 mM CsCl, 20 mM $N(Et)_4Cl$, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES; pH adjusted to 7.2 with CsOH

2.2.7 Enzymes

Enzyme	Manufacturer	Application
High Fidelity Taq-Polymerase incl. buffer	Roche, Basel, Switzerland	PCR
Pfu-Polymerase incl. buffer	In-house production	PCR
Antarctic Phosphatase incl. buffer	NEB, Ipswich, MA, USA	Ligation
T4 DNA-Ligase incl. buffer	NEB, Ipswich, MA, USA	Ligation
Restriction enzymes incl. buffer	NEB, Ipswich, MA, USA/Promega, Madison, WI, USA	DNA digest

2.2.8 Commercial Kits

Kit	Manufacturer	Application
Gel Extraction Kit	Qiagen, Hilden, Germany	Gel extraction
Gel and PCR Cleanup	Macherey-Nagel, Düren, Germany	DNA purification
NucleoBond® Xtra Maxi EF	Macherey-Nagel, Düren, Germany	DNA isolation

2.2.9 Bacterial Culture Media

Medium	Composition
2x YT-medium (yeast/tryptone)	15 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl

2.3 Plasmids

Vector/Plasmid	Insert/Application	Producer/Origin
pRK7	High-copy vector, CMV and Sp6-promotor, ampicillin-resistance, expression in HEK293 cells; integration of GlyRα_1^{ch} insert	P. Seeburg, MPI for Medical Research, Heidelberg
hsa1_pRK5	*Homo sapiens* GlyRα_1 (GlyRα_1^{hs})	AG Villmann
dra1_pBSrGLRa1	*Danio rerio* GlyRα_1 (GlyRα_1^{dr}) in pBS vector	H. Hirata, Aoyama Gakuin University,

34

Tokyo

dra1_pBSrGLRa1_PpuMI	GlyRα$_1^{dr}$ carrying AC mutation at DNA position 177 (GlyRα$_1^{dr}$ (177AC); vector for N-terminal GlyRα$_1^{hs}$ fragment)	Generated in this study
dra1_pBS_GLRa1ch	Chimeric GlyRα$_1$ (GlyRα$_1^{ch}$) in pBS vector	Generated in this study
dra1_pRK7_GLRa1	GlyRα$_1^{ch}$ in pRK7 vector	Generated in this study
GlyRα$_1^{c\text{-}myc}$	*Rattus norvegicus* GlyRα$_1$; c-myc tag (amino acid sequence: EQKLISEEDL) introduced at the N-terminus after amino acid position four of mature protein	Robert J. Harvey, UCL School of Pharmacy, London
eGFP	Transfection of GFP as an indicator for successful transfection and detection of transfected HEK293 cells for patch-clamp recordings	Clontech Laboratories Inc., Mountain View, CA USA

2.4 Primer

Name	Sequence (5' to 3')	Application	T (°C)
278_dra1_pBSrGLRa1_BGH_For	TAA TAC GAC TCA CTA TAG GGC GAA TTG G	PpuMI recognition site	58
zebraa1_parental_AS	ATTATACAGGTCTGCACATCCATAGGG	PpuMI recognition site	58
zebraa1_PpuMI_S	CCTAATTTCAAAGGTCCCCCTGTGAA	PpuMI recognition site	58
zebraa1_PpuMI_AS	GTTACGTTCACAGGGGGACCTTTG	PpuMI recognition site	58
hsa1_parental_KpnItail	CGGGGTACCATGTACAGCTTCAATACTCTTCGA	KpnI recognition site	57
384-long	GTA ACC ATT ATA AGC TGC AAT AAA CAA GTT	KpnI recognition site	57

2.5 Experimental Organisms

2.5.1 HEK293 Cells

In this study, the human cell line HEK293 (human embryonic kidney cells, ATCC®CRL-1573™, Wesel, Germany) was taken for cell-based experiments. Cells were grown in Minimum Essential Medium (MEM, Life Technologies, Darmstadt, Germany) supplemented with 10% fetal calf serum, L-glutamine (200 mM) and 50 U/mL penicillin and streptomycin at 37°C and 5% CO_2 in cell culture incubator.

2.5.2 *Escherichia Coli* Bacteria

For transformation of DNA into *Escherichia coli* chemically competent cell lines DH5α (Thermo Fisher Scientific) or NEB5α (New England Biolabs) were utilized.

2.5.3 Zebrafish

Fertilized eggs were obtained by mating of wildtype zebrafish (*Danio rerio*), which were held at 28.5°C on a 14 h light/10 h dark cycle. Zebrafish larvae were raised in breeding water (28,5°C) in an incubator (IC602, Yamato Scientific, Tokyo, Japan). On day 1, zebrafish larvae were treated with pronase (Roche, Basel, Switzerland) to remove embryonic chorion. All experimental procedures were approved by Animal Care and Use Committee in Aoyama Gakuin University.

2.6 Commercially Acquired Antibodies

2.6.1 Antibodies Applied for Immunocytochemistry with transfected HEK293 Cells

Antibody	Specifications/Manufacturer
mAb2b	Mouse monoclonal IgG1 specific for GlyRα_1 subunit, 146 111 / Synaptic Systems, Göttingen, Germany
mAb4a	Mouse monoclonal IgG1 specific for all GlyR subunits, 146 011 / Synaptic Systems, Göttingen, Germany
Anti-c-Myc-tag	mouse monoclonal IgG specific for human 9E10 peptide of c-Myc, MA1-980 / Santa Cruz Heidelberg, Germany
Anti-human IgG (Cy3)	Conjugated goat anti-human IgG (Fc)-Cy3 109-167-008 / Dianova, Hamburg, Germany
Anti-mouse IgG (Cy3)	Conjugated goat anti-mouse IgG (Fc)-Cy3 115-165-008 / Dianova, Hamburg, Germany
Anti-goat IgG (DyLight488)	Conjugated polyclonal donkey anti-goat IgG (H+L)-DyLight 488, DkxGt-003-E488NHSX / Dianova, Hamburg, Germany

2.6.2 Antibodies Applied for Immunohistochemistry on Zebrafish Spinal Cord Cryosections

Antibody	Specifications/Manufacturer
Anti-GlyR	mAb4a mouse monoclonal IgG1 (specific for all GlyR subunits), 146 011/Synaptic Systems, Göttingen, Germany mAb4a
Anti-Synapsin1	Rabbit polyclonal IgG, 106103/Synaptic Systems, Göttingen, Germany
Anti-GluR 2/3	EP929Y, rabbit monoclonal IgG/Abcam, Tokyo, Japan
Anti-synaptic vesicles	SV2, mouse IgG$_1$/DSHB, Iowa City, USA
Anti-mouse IgG (Alexa 488)	Conjugated anti-mouse IgG/Life Technologies, Tokyo, Japan
Anti-mouse IgG (Alexa 555)	Conjugated anti-mouse IgG/Life Technologies, Tokyo, Japan

Anti-rabbit IgG (Alexa 488)	Conjugated anti-rabbit IgG/Life Technologies, Tokyo, Japan
Anti-rabbit IgG (Alexa 546)	Conjugated anti-rabbit IgG/Life Technologies, Tokyo, Japan
Anti-human IgG (Cy3)	Conjugated anti-human IgG (Fc-specific), C2571-1ML, Sigma-Aldrich, St. Louis, MO, USA

2.7 Cell Biological Methods

2.7.1 Acetone Treatment of Coverslips

Via acetone treatment coverslips surface gets roughened and facilitates cell adhering. Therefore, coverslips were incubated for 10 min in 99.5% acetone, dried at RT and sterilized.

2.7.2 Cultivation of HEK293 Cells

HEK293 cells were cultivated in HEK293 medium at 37°C, 5% CO_2 and 95% humidity in cell culture incubator. All procedures were performed in sterile conditions under a tissue culture hood. All media and solutions were pre-warmed in a water bath at 37°C. Cells were split at a confluence density of 70-90%. In a first step, cell medium was aspirated off and cells were washed using PBS. Then PBS was aspirated, and 1-3 ml 0.05% trypsin was added. Cells were incubated for 3 min at 37°C to detach adhering cells. Trypsin digest was ceased by addition of cell medium. The cell solution was then centrifuged for 6 min at 1400 rpm. Cell medium was replaced again, and the cell number was determined in use of a hemocytometer (Neubauer chamber). For immunocytochemical experiments 1.8×10^5 cells, whereas for electrophysiological experiments $1.0-1.5 \times 10^5$ cells were plated on 3 cm cell culture dishes, filled up with 2 ml cell medium.

2.7.3 Transfection of HEK293 Cells

For efficient and gentle transfection of plasmid deoxyribonucleic acid (DNA) calcium phosphate precipitation was applied. This technique was first developed by Frank L. Graham und Alex J. van der Eb in the beginning of the 1970's (Graham and van der Eb

1973). In the following decade the calcium phosphate transfection protocol was further optimized to achieve highly efficient transfection results. In this thesis, a protocol based on a publication of Claudia Chen and Hiroto Okayama was applied (Chen and Okayama 1987). Transfection of mammalian cells is attained by endocytosis of complexes consisting of calcium phosphate and plasmid DNA.

HEK293 cells were seeded on cover slips in a 3 cm cultural dish and transfected after 24 h. In the sterile environment of a tissue culture hood 88 µl 1x TE buffer, 1 µl of the respective plasmid DNA (1 µg/µl stock concentration) and 10 µl 2.5 M $CaCl_2$ were pipetted into a reaction tube. To achieve a homogenous concentration the reaction mixture was stirred by a vortex mixer. Then, 100 µl 2x HBS buffer was added, vortex-mixed again and centrifuged. Following a 20 min incubation step at room temperature (RT) the reaction mixture was dropwise pipetted to HEK293 cells grown on cover slips. By moving the culture dish in a figure of eight a homogenous distribution of the transfection mixture was achieved. 6 h of reaction time in the cell culture incubator has been observed as an adequate duration to minimalize negative stress of cells, though allowing sufficient endocytosis of plasmid DNA. To stop transfection the reaction buffer was removed by a vacuum pump. In order to remove calcium phosphate complexes, cells were washed two times by 500 µl MEM. Finally, 2 ml of the latter was added again into the cultural dish and cells were placed back into incubator.

2.8   Indirect Immunocytochemistry of HEK293 Cells

Immunocytochemical staining take advantage of strong chemically bonds of antibodies to their antigen to visualize a specific protein. So-called primary antibodies bind to the protein of interest. In this study, primary antibodies were monoclonal antibodies as well as patient autoantibodies. Antibodies can also serve as an antigen of so-called secondary antibodies, which are linked to a reporter like a fluorochrome. Fluorochromes are molecules characterized by their ability to re-emit light of a certain wavelength (color), when they are excited itself by light of a specific wavelength. To match the characteristics of the applied fluorochrome, fluorescence microscopes hold an excitation filter next to a light source and an emission filter above the specimen.

2.8.1 General Remarks

In the following descriptions of immunocytochemical staining, HEK293 cells on cover slips were located in a dark chamber to avoid bleaching of fluorescence labeled antibodies. Using forceps, cover slips were transferred from the cell culture dish to a parafilm inside the dark chamber. A water-soaked paper towel was placed underneath the parafilm to avoid drying-out of cells.

Any solution pipetted on cover slips had a volume of 50 µl and each incubation step was stopped by washing-off the respective material. Therefore, cover slips were dipped three times into a bath of PBS. In contrast, after a blocking procedure – to reduce nonspecific secondary antibody binding –, instead of a washing step blocking solution was removed by carefully approaching cover slips onto paper towel. Thereby, a wash-off of epitope blocking antibody was prevented. The last washing step before fixation of cover slips on microscope slides was followed by soaking cover slips in A.d. in order to rinse off salt crystals, which would disturb otherwise analysis under fluorescence microscope. Cover slips were fixed by Mowiol on microscope slides with caution to ensure absence of air bubbles. Then microscope slides were stored overnight at RT to allow proper adhering of cover slips before being placed in the cold room for long-term storage.

For analysis and generation of digital images of stained HEK293 cells a confocal fluorescence microscope (Olympus, Fluoview1000ix81) was used.

2.8.2 Testing for Glycine Receptor Autoantibodies

An established diagnostic tool in detection of GlyR autoantibodies is to screen for binding of patient samples to HEK293 cells, expressing GlyRα_1 homopentamers tagged with enhanced green fluorescent protein (EGFP) (Hutchinson, Waters et al. 2008, Damasio, Leite et al. 2013, Carvajal-Gonzalez, Leite et al. 2014). EGFP is a modified variant of the original green fluorescent protein (GFP), a protein – first isolated from the jellyfish *Aequorea victoria* – that exhibits bright green fluorescence when excited by blue to ultraviolet light. In this study HEK293 cells were co-transfected with GlyRα_1 and eGFP instead of using EGFP-tagged GlyRα_1.

Cells were subjected to immunocytochemical experiments 24-48 h post-transfection. Patient serum (1:50), purified IgG (1:50), CSF (1:10) mAb2b (1:500) or healthy control

40

serum (hc; 1:50) were diluted in MEM and carefully pipetted over cells. MEM was used to create ideal conditions for living cells during incubation period. Cells were afterwards fixed by 4% PFA 4% sucrose (20 min. on ice) and as a blocking step 5% normal goat serum (NGS, PAA Laboratories, Cölbe, Germany) diluted in PBS (30 min. at RT) was applied. Then, cells were incubated for 1 h at RT in anti-human IgG (Cy3) or anti-mouse IgG (Cy3) diluted in blocking solution (1:250).

2.8.3   Internalization Assay

2.8.3.1   Immunocytochemistry

A modified technique of 2.8.2 was applied to investigate the effect of GlyR autoantibodies on receptor internalization. In brief, a second antibody was used to visualize GlyRs remaining at cell membrane after a certain period (0.5 h, 1 h, 2 h, 4 h) of incubation at 37°C and 5% CO_2, which promoted endocytosis of membrane-bound receptors. At temperatures below 37°C endocytosis is not very effective in mammalian cells (Tomoda, Kishimoto et al. 1989). Cells were transfected with $GlyR\alpha_1^{hs}$ or $GlyR\alpha_1^{c-myc}$.
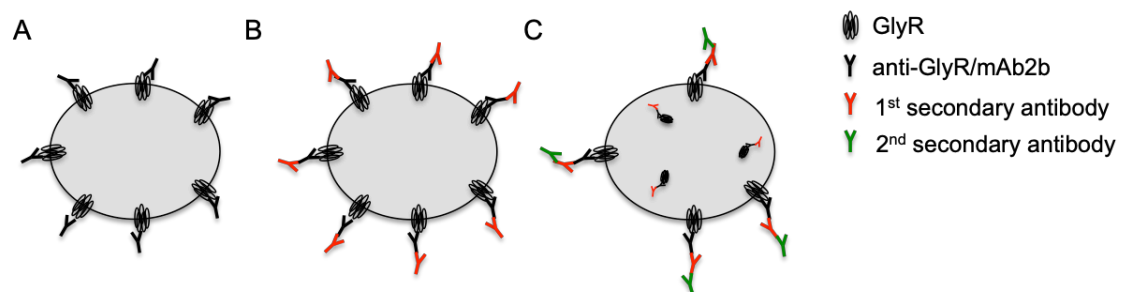
Figure 2.1: Scheme of antibodies used for internalization assay. HEK293 cells were first transfected with the $GlyR\alpha_1$ subunit. (A) Patient anti-GlyR IgG or mAb2b bound to GlyR on the cell surface. (B) Anti-GlyR autoantibodies or mAb2b were subsequently labeled by the 1st secondary antibody (goat anti-mouse IgG or goat anti-human IgG, conjugated with Cy3). (C) After incubation at 37°C, HEK293 cells were fixed by 4% PFA 4% sucrose and non-internalized GlyR were labeled by a 2nd secondary antibody (donkey anti-goat IgG, conjugated with DyLight 488)

As primary antibodies served patient 1 serum (pat 1) sample (1:50), anti-mAb2b (1:500) or anti-c-Myc (1:500) diluted in MEM (1 h on ice). Patient 1 serum sample and mAb2b were applied on HEK293 cells transfected with $GlyR\alpha_1^{hs}$ and anti-c-Myc-tag on those transfected with $GlyR\alpha_1^{c-myc}$. Incubation was performed on ice to conserve cells

and to inhibit metabolic cell processes like receptor degradation. Secondary fluorescent antibodies (goat anti-human IgG or goat anti-mouse IgG, conjugated with Cy3; 1:250 in MEM) were subsequently applied to label membrane-bound GlyRα$_1$^{hs}/ GlyRα$_1$$^{c-myc}$ (1 h on ice). Then, one cohort of cells on cover slips was gently transferred into a 16 well plate containing 500 µl MEM and placed into cell culture incubator for various durations (0.5, 1, 2, 4 h) (Fig. 2.1 A). The other cohort of cells was used to set up a baseline at time point 0 h. Cells of both cohorts were fixed by 4% PFA 4% sucrose (20 min. on ice), blocked by 5% normal horse serum (NHS) diluted in PBS (30 min. at RT) before second secondary antibodies (donkey anti-goat IgG, conjugated with DyLight 488; 1:250 in blocking solution) were used to label GlyRs, which remained attached to the cell-membrane (Fig.2.1 B). Cell nuclei were stained by 4′,6-Diamidin-2-phenylindol (1:5000 in PBS; 5 min).

2.8.3.2   Image Data Analysis and Quantification

Data analysis was first performed in a large scale comprising all time points 0 to 4 hours, but only one representative stack layer was included in quantification. Then, the time point 2 hours was chosen for detailed analysis and for each cell up to four representative stack layers – depending on cell body diameter – were analyzed. Between two layers was a minimum distance of 1 µm to ensure that fluorescent dots were not counted twice. Quantification of internalization was performed in usage of Fiji image analysis software (Schindelin, Arganda-Carreras et al. 2012). Color threshold for analysis of fluorescent dots was defined by the 'default' algorithm. Single dots were counted and measured automatically by the 'Analyze Particles' function. Total area of fluorescent dots was calculated, and the following equation was implemented to assess percentage of internalized glycine receptors dependent on time for every stack of images:

$$Internalization\ (\%) = \frac{Total\ area\ (red) - Total\ area\ (green)}{Total\ area\ (red)}$$

For each cell, the mean value of all respected stack layers was then determined.

The internalization calculation postulates that the entirety of red fluorescence signal corresponds to the amount of GlyR integrated in cell membrane before incubation at 37°C as well as the entirety of green fluorescence signal corresponds to the amount of GlyR left integrated in cell membrane after incubation period. A two-sided *t*-Test was applied to prove statistical significance.

42

2.8.4 Immunocytochemistry after Patch Clamp Recordings

Some samples of cells were stained with anti-human IgG (Cy3) subsequent to patch clamp recordings to give proof of anti-GlyR IgG binding during measurements.

First, cells were washed three times in PBS and then secondary antibody (goat anti-human IgG or goat anti-mouse IgG, conjugated with Cy3; 1:250 in external buffer; 1 h on ice) was added.

2.8.5 Immunocytochemistry for Epitope Localization

In order to identify the epitope of glycine receptor antibodies, immunocytochemical staining as described in 2.8.2 was performed. Here, HEK293 cells were transfected with $GlyR\alpha_1^{hs}$, $GlyR\alpha_1^{dr}$ or $GlyR\alpha_1^{ch}$ and then subjected to staining procedure. Due to a specific pattern of antigen detection by GlyR autoantibodies and monoclonal GlyR antibodies with known epitopes, an anti-GlyR autoantibody epitope could be localized.

For immunocytochemical staining with mAb4a, the technique was modified according to manufacturer's recommendations. Cells have been fixed by 4% PFA 4% sucrose and blocked by 5% NGS, before primary antibodies were added.

2.9 Electrophysiology

To address the question whether $GlyR\alpha_1$ function is affected by anti-GlyR autoantibodies glycine-evoked currents were recorded applying the patch clamp technique. This technique has been developed by Bert Sakman and Erwin Neher to measure transmembrane currents of ion channels (Sakmann and Neher 1984) and in 1991, their achievements were appreciated by Nobel Prize for Physiology or Medicine.

2.9.1 Patch Clamp Technique and Procedure

Electrophysiological recordings were performed in a whole-cell configuration using the patch clamp technique. HEK293 cells were co-transfected with $GlyR\alpha_1^{hs}$ and GFP, and cells were subjected to electrophysiological recordings 24-72 h post-transfection. GFP was applied to allow easy identification of transfected HEK293 cells during patch clamp experiments. Confluency and transfection efficiency were controlled using a fluorescent microscope prior to experiments. Then, cover slips were transferred in a dark chamber

and HEK293 cells were pre-incubated in mAb2b (1:500; dilution in external buffer), patient sera or healthy control samples (1:10) for 1 h at 22°C. Incubation was ceased by immersion of cover slips into PBS and cover slips were transferred into a measurement chamber filled up with a bathing solution (external buffer), which was set under a fluorescence microscope with 40-fold optical magnification.

The recording pipette was maneuvered in use of a micromanipulator to the cell membrane of a transfected HEK293 cell. A U-tube system, which was navigated above the respective cell, was used to add glycine (1 mM or 50 μM, diluted in external buffer) in a spatial and temporal controlled manner. The perfusion system was controlled by Pulse Software (HEKA Electronics, Göttingen, Germany). Electric signals were amplified with an EPC-9 amplifier (HEKA, Göttingen, Germany). Recording pipettes were fabricated from borosilicate capillaries (World Precision Instruments, Berlin, Germany) with a pipette resistance of 4-6 MΩ and were connected to the same Pulse Software. Cells were held at -60 mV at room temperature (~22°C) during current measurements. Cells with input resistances of 50 MΩ-1.5 GΩ were determined as healthy and used for data analysis. To diminish background noise signal, electrophysiological experiments were performed on a vibration-damped table and inside of a Faraday cage. For data analysis the following values were calculated by Microsoft Excel:

Mean value: $\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$

Variance: $V(x) = \frac{1}{(n-1)} \times \sum_{i=1}^{n}(xi - \bar{x})^2$

Standard deviation: $\sigma = \sqrt{V(x)}$

Standard error of the mean: $\text{SEM} = \frac{\sigma}{\sqrt{n}}$

Statistical significance was determined by two-sided t-Test. Marking of significance is displayed as follows: *p-value < 0.05; **p-value < 0.01; ***p-values < 0.001. For graphic presentation Microsoft Excel, Origin 5 (OriginLab Corporation, Northampton, Massachusetts, USA) and Corel Draw (Corel Corporation, Ottawa, Ontario, Canada) were used.

2.10 Zebrafish Experiments

This part of the study was performed in cooperation with K. Ogino at the laboratory of Prof. H. Hirata at Aoyama Gakuin University in Tokyo, Japan. Together with K. Ogino the author conducted the pilot studies, which leaded to the development of the final protocol of passive transfer of patient serum or purified IgG to zebrafish larvae. However, it was K. Ogino who executed the experiments that yielded the final results.

2.10.1 Passive Transfer of Glycine Receptor Antibodies

2.10.1.1 Transdermal Application

Initially, it was aimed to enable transdermal application of patient autoantibodies by adding 1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) to the water bath in order to increase dermal permeability (Pope and Oliver 1966). Larvae at age of 24 hours post fertilization (hpf) were set into a 96 well plate. Fresh water was enriched with 10% of patient serum or purified IgG (150 mg/ml). Strychnine (75 µmol; Sigma-Aldrich, St. Louis, MO, USA) served as a positive control and NGS, hc serum and fresh water were negative controls. To reduce toxicity, zebrafish larvae were exposed to the antibody-enriched solution for eight hours on three consecutive days at 28.5°C. In the meantime, the solution was exchanged to fresh water. Each day zebrafish larvae were analyzed for their touch-evoked escape response after exposure time by tapping the tail fin with a fine steel needle (see 2.10.2.)

2.10.1.2 Intraventricular Microinjection

Prior to operation, tricaine methanesulfonate (TMS; Wako Pure Chemical Industries Ltd., Osaka, Japan) was used to anesthetize zebrafish. TMS is a sodium-channel blocker and thereby inhibits sensory input as well as muscle-contraction. This muscle relaxant effect is important to enable precise manipulation. Afterwards zebrafish larvae were handled by plastic transfer pipettes into a drop of 3% methylcellulose, which is semisolid at RT, in a dorsal-up posture. This state of matter allows adequate positioning of zebrafish and also quick removal of methylcellulose coat. Patient serum (1:2), purified IgG (75 µg/ml), bovine serum albumin (75 µg/ml, BSA, Sigma Life Science, St. Louis, MO, USA) together with the fluorescent dye sulforhodamine B, (1:100 in PBS, Sigma Life Science, St. Louis, MO, USA) or Alexa 555 goat anti-mouse

IgG (LifeTechnologies, Darmstadt, Germany) was injected into the 4th ventricle due to its large size and accessible position during early developmental stages. Microinjections were performed under a microscope (SZX16-ILLT, Olympus, Tokyo, Japan) using glass capillaries (Narishige Scientific Instrument Lab., Tokyo, Japan), which were produced via a micropipette puller (P-97/IVF, Sutter Instrument Co., Novato, CA, USA). The injected volume (≈50 nl) was controlled by a microinjector (P=20 psi, t=8 ms; Picospritzer III, Parker Hannifin Co., Pine Brook, NJ, USA). Behavior of microinjected embryos was analyzed three hours after treatment, if they showed no evident injury caused by the injection procedure and if microinjection was effective. Microinjection was guaranteed by the presence of sulforhodamine at the intraventricular space (applied fluorescence microscope: M205 C, Leica, Wetzlar, Germany).

2.10.1.3 Skin Lesion

Zebrafish were anesthetized and postured in semisolid methylcellulose as described in 2.10.1.2 before a skin lesion above the 4th ventricle was made using a glass capillary. Methylcellulose coat was washed away by artificial CSF (ACSF) buffer (in mM: 100 NaCl, 2.46 KCl, 1 $MgCl_2$, 0.44 NaH_2PO_4, 1.13 $CaCl_2$, 5 $NaHCO_3$, 10 glucose, pH 7.2), and treated larvae were transferred in ACSF enriched by either 1% pat 1 serum or 1% hc serum. Zebrafish larvae were incubated in this GlyR autoantibody containing solution for 16 h at 28.5°C. Escape behavior was studied before and after treatment as described in 2.10.2. to evaluate the impact of GlyR autoantibodies in stimulus-induced locomotion. In order to analyze if receptor internalization also occurs *in vivo*, immunostaining of cryosections obtained from treated zebrafish larvae was performed (see 2.10.3.).

2.10.2 Video Recording of Escape Behavior

A tactile stimulus on tail fin with fine steel needles caused escape behavior of zebrafish larvae. In use of a high-speed camera (HAS-220, Ditect, Tokyo, Japan) set on a stereomicroscope (MZ16, Leica, Wetzlar, Germany) escape behavior was recorded at 200 frames per second.

2.10.3 Immunohistochemistry of Spinal Cord Cryosections[1]

Pre-treatment larvae and human serum (pat 1 and hc) permeated larvae were embedded in O.C.T. compound (SAKURA, Tokyo, Japan) and subsequently frozen by liquid nitrogen. The frozen blocks were sectioned at 20 µm by cryostat (CM3050S Leica, Wetzlar, Japan). Sequential cryosections were affixed on two MAS-coated slide glasses (Matsunami Adhesive Slide, S9441, Matsunami, Osaka, Japan) and then fixed by 4% paraformaldehyde at room temperature for 30 min. One glass slide was used for GlyR immunostaining and the other one was used for AMPAR immunostaining. The sections were immunostained using the following primary antibodies anti-GlyR (1:1000), anti-Synapsin1 (1:1000), anti-GluR 2/3 (1:1000), anti-synaptic vesicles SV2 (1:200) and secondary antibodies Alexa 488 conjugated anti-mouse IgG, Alexa 555 conjugated anti-mouse IgG, Alexa 488 conjugated anti-rabbit IgG, Alexa 546 conjugated anti-rabbit IgG (each 1:1000). In addition to these antibodies, NucRed Dead 647 (Thermo Fisher Scientific, Tokyo, Japan) was used to stain nuclei of spinal neurons in accordance with manufacturer's instruction. Fluorescent images were captured by a confocal microscope (SP5, Leica, Wetzlar, Germany). Five fluorescent images were obtained in each larva. Fluorescent images were quantified on ImageJ (https://imagej.net/Welcome). Number of GlyR or AMPAR clusters was counted in the lateral regions of spinal cord that were stained by antibodies to pre-synaptic markers. A mean number of the clusters of each larva was calculated based on the observation in five fluorescent images. The mean value of cluster numbers from pre-treatment larvae was used as control value. A mean cluster number of each larva was divided by the control value. The quantitative data was shown as mean ± 2 SE in the graph. Significance was examined by student's t-Test. Level of significance are displayed for all experiments as follows: * p-value < 0.05; ** p-value < 0.01; *** p-values < 0.001.

2.11 Molecular Biological Methods

The methods described in this chapter were used to produce the chimeric glycine receptor gene construct $GlyR\alpha_1^{ch}$, built up by a large C-terminal $GlyR\alpha_1^{dr}$ part and a short N-terminal $GlyR\alpha1^{hs}$. Via transfection with $GlyR\alpha_1^{ch}$, HEK293 cells express the recombinant protein and allow epitope analysis in use of specific binding patterns to

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[1] The immunohistochemical staining was done by K. Ogino and therefore this subchapter was written by him.

GlyRα$_1$^{dr}, GlyRα$_1$^{hs} and the recombinant GlyRα$_1$^{ch} in indirect immunofluorescence staining (2.10.5).

Figure 2.2: Chimeric GlyRα$_1$^{ch} is composed of the N-terminal part (M^1-G^{62}) of GlyRα$_1$^{hs} (grey bar) and the C-terminal part (P^{59}-Q^{444}) of GlyRα$_1$^{dr} (black bar). Cutting site of PpuM I is indicated by a vertical dotted line.

2.11.1 Experimental Design of GlyRα1^{ch} Construction

It was aimed to replace the N-terminal fragment of GlyRα$_1$^{dr} by the corresponding part of GlyRα$_1$^{hs}. Therefore, a GlyRα$_1$^{hs} DNA fragment (insert) was created, which could be consecutively introduced into the GlyRα$_1$^{dr} including expression vector. The respective DNA sequences needed to carry a recognition motive for the same restriction enzyme at both outer ends of the regarding fragment, to allow cleavage and ligation at the identic site. Lacking recognition sites within GlyRα$_1$^{dr} and GlyRα$_1$^{hs} DNA sequence were introduced by site-directed mutagenesis. Via overlap PCR a silent mutation (177A→C) was generated in a DNA fragment of GlyRα$_1$^{dr}, to establish the recognition pattern for the restriction enzyme PpuM I and via a so-called tailed-primer the lacking Kpn I recognition motive was added on a linear DNA fragment ahead of the GlyRα$_1$^{hs}'s start-codon.

Linear DNA fragments can be integrated in expression vectors by molecular cloning. The part of GlyRα$_1$^{dr}, where the PpuM I recognition site was required, was replaced by a novel GlyRα$_1$^{dr} fragment, which harbored the PpuM I recognition pattern. It was introduced into the GlyRα$_1$^{dr} plasmid by restriction digest and subsequent ligation, thereby yielding a novel GlyRα$_1$^{dr} plasmid named GlyRα$_1$^{dr} PpuM I. At this point, it was possible to excise the N-terminal part of GlyRα$_1$^{dr} by restriction digest with Kpn I and PpuM I. Following gel extraction, the GlyRα$_1$^{dr} was substituted by the N-terminal GlyRα$_1$^{hs} fragment (also digested by Kpn I and PpuM I), thus providing the desired GlyRα$_1$^{ch} construct.

Gene expression in cultured mammalian cell lines – like the human HEK293 cell line used in this study – requires presence of a promoter sequence within the expression

vector. By reason of absence of an appropriate promoter in dra1_pBSrGLRa1 and dra1_pBS_GLRa1ch, it was necessary to transfer the respective inserts ($GlyR\alpha_1^{dr}$ and $GlyR\alpha_1^{ch}$) into the vector *pRK7*, which contains a cytomegalovirus promoter that is suitable for mammalian cell lines. The molecular cloning was performed by Michael Enders.

2.11.2 Polymerase Chain Reaction (PCR)

PCR is an essential method in molecular biology to amplify DNA *in vitro* by use of an enzyme called DNA polymerase. It was developed by Kary Mullis, and his work was rewarded with the Nobel Prize in Chemistry in 1993 (Mullis, Faloona et al. 1986).

The PCR procedure consists of three consecutive parts of incubation at different temperatures: denaturation ($\cong 94°C$), annealing (55-65°C) and elongation (68-72°C). These steps are usually repeated within 20-50 cycles in a thermocycler (2720 Thermal Cycler, Applied Biosystems, Waltham, MA, USA). The high temperature during denaturation causes breaks of hydrogen bonds between complementary bases of double-stranded DNA (ds DNA) templates, generating two single-stranded DNA (ss DNA) molecules. To synthesize the new complementary DNA strand by adding deoxyribonucleotide triphosphates (dNTPs), DNA polymerase requires a free 3'hydroxy group, which is supplied by oligonucleotides, called primers. Primers are short ss DNA molecules corresponding to the end points of the DNA template, which is aimed to be amplified. Then, reduction of temperature (annealing) allows creation of new hydrogen bonds between complementary base pairs (bp) of primer and single-stranded DNA molecules. The right annealing-temperature, which is crucial to obtain specific and effective results, depends on the primers' length and on the GC content. Hence, to calculate annealing temperature the following homepage was consulted: *http://biotools.nubic.northwestern.edu/OligoCalc.html.* By re-elevation of temperature to a certain degree – depending on the characteristics of the applied DNA polymerase –, the new complementary DNA strand gets elongated, yielding again a ds DNA that is identical to the DNA template. In this study, Taq- (*Thermus aquaticus)* and Pfu- (*Pyrococcus furiosus)* polymerase were used.

For standard PCR reaction mixture and PCR procedure see Tab. 2.3 and Tab. 2.4 respectively.

Table 2.3   Standard Reaction Mixture (PCR)

Reagent		Volume
DNA template	(100 ng/µl)	1 µl
dNTPs	(10 mM)	1 µl
Taq-Polymerase	(5 U/µl)	0.5 µl
Forward-primer	(100 pmol/µl)	1 µl
Reverse-primer	(100 pmol/µl)	1 µl
5x Taq buffer		20 µl
dH$_2$O		ad 100 µl

Table 2.4   PCR Procedure

Step	Temperature (°C)	Time (min.)
1. Initialization	95	5
2. Denaturation	95	1
3. Annealing	58	2
4. Extension	72	2
		25-30 cycles of steps 2-4
5. Amplification (last cycle)	72	10

When Pfu-DNA polymerase (2 µl) was chosen for PCR, 2 µl of corresponding Pfu 10x buffer was used.

2.11.3 Overlap Extension PCR

For the introduction of mutations into a DNA sequence, overlap extension PCR is a widely applied technique (Ho, Hunt et al. 1989). In a first step, two PCR fragments were created including the identical mutation and overlapping by 12 base pairs. Therefore, two parental primers (a and d) flanking the desired PCR fragments and two internal mutagenic primers (b and c) are needed, i.e. one sense (5' to 3') and one antisense (3' to 5') primer (Fig. 2.3).

Here, primer combinations 278_dra1_pBSrGLRa1_BGH_For (a; parental/sense) and zebraa1_PpuMI_AS (b; internal/antisense), as well as zebraa1_PpuMI_S (c; internal/sense) and zebraa1_parental_AS (d; parental/antisense) were used together

50

with the plasmid dra1_pBSrGLRa1 as template DNA, yielding two PCR products named AB and CD. For the first PCR, the following incubation steps were applied: 5 min, 95°C Denaturation, 5 min, 58°C Annealing, 5 min 72°C Elongation, 28 cycles.

In a second PCR, AB and CD are template DNAs after denaturation and DNA polymerase hybridize at their complementary parts (5 min 95°C denaturation, 5 min 55°C, annealing, 5 min 72°C Elongation, 2 cycles).

At last, parental primers a and d are added to allow exponential production of the mutated DNA fragment. The PCR conditions were set as in the first PCR.
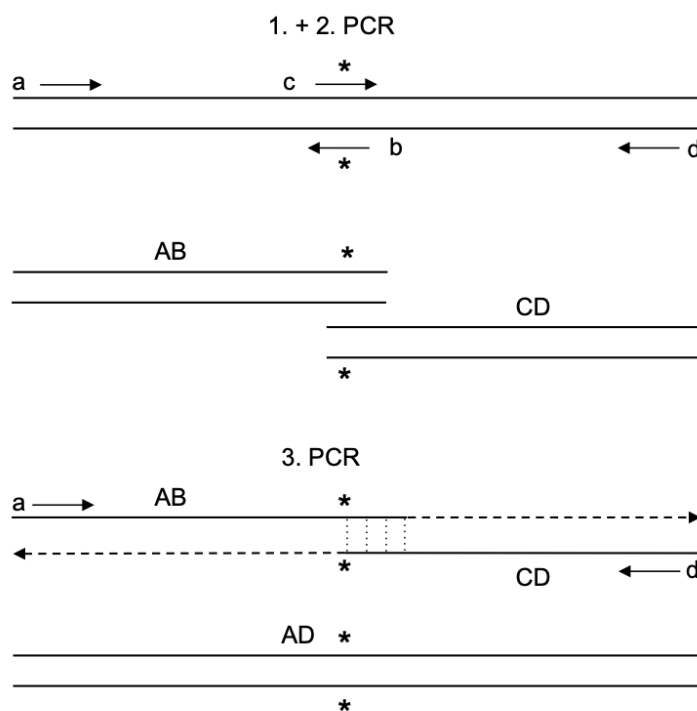
Figure 2.3: Scheme of an overlap extension PCR. Primers are displayed as arrows in continuous line (a-d), asterisk marks site of mutagenesis. New synthesized DNA strands are marked in capital letters.

2.11.4 Tailed-Primer

In order to generate a PCR fragment containing a novel Kpn I restriction site ahead of the cDNA encoding for the GlyRα$_1$^{hs} (hsa1_pRK5), the forward tailed-primer hsa1_parental_KpnItail was used together with the reverse primer 384-long. PCR setting were: 1 min 94°C, denaturation, 1 min 57°C annealing, 5 min 72°C elongation, 30 cycles).

2.11.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a standard method to separate mixed macromolecules such as DNA depending on their size. At neutral pH conditions DNA molecules are negatively charged, because of their negatively charged phosphate groups. Thus, they are electrostatically attracted by an anode inside an electric field. Agarose molecules built up a matrix representing a filter for migrating molecules, so that smaller molecules advance faster than larger within the electric field. Absolute size of DNA fragments can be estimated by comparison with a DNA standard.

In this study, gels with 0.5 – 1.0 % agarose concentration were made by melting 0.5 – 1.0 g LE-Agarose in 100 ml TBE buffer (Tab. 2.2.4) in a microwave. For the visualization of DNA bands, 5 µl Midori Green (Nippon Genetics, Düren, Germany) was added to agarose solution and poured into a gel chamber. Midori Green binds to DNA or RNA molecules and functions as a fluorescence dye by UV-light excitation ($\approx$ 490 nm). By cooling down gels polymerize and become ready for use. Then DNA probes, mixed with a blue loading buffer (1:1 ratio) were pipetted into wells, created by a comb set into the gel during polymerization. The electric field was generated for 30 – 60 min by voltage of 140 – 180 V with an electric current of 100 – 140 mA. At last, gels were analyzed by UV transillumination and photographed for documentation purposes.

2.11.6 Gel Extraction

DNA molecules obtained by PCR or restriction digest were separated by agarose gel electrophoresis and subsequently extracted in application of *NucleoSpin Gel and PCR clean up Kit*s (Macherey-Nagel, Düren, Germany).

First, DNA probes were loaded in 4 wells of agarose gel next to each other. Alongside the vertical boundaries of each well, agarose gel was cut by a scalpel. To prevent DNA damage caused by mutagenic UV light, only the first piece was analyzed under UV light to excise the band of interest. At same level, the other bands were excised blindly, i.e. without UV transillumination. Gel fragments were stored in Eppendorf reagent reservoirs and isolated and purified with *NucleoSpin Gel and PCR clean up Kit*s (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Hereupon, isolated DNA was used for molecular cloning.

2.11.7 Molecular Cloning

Molecular cloning is a bunch of methods, which enables the assembly of recombinant DNA and their replication by host organisms. The assembly of recombinant DNA requires an insert, a DNA fragment of interest, as well as an expression vector. Essential steps of molecular cloning are: preparation of vector and DNA fragment (restriction digest), creation of recombinant DNA (ligation), introduction of recombinant DNA into a host organism (transformation), identification of populations expressing the recombinant DNA, preparation of purified plasmid DNA and verification of recombinant DNA sequence.

2.11.7.1 Restriction Digest

Restriction digest is based on the ability of bacterial restriction enzymes to cleave DNA molecules at a specific recognition site. Under natural conditions restriction enzymes are part of the bacterial defense system preventing insertion of hostile DNA. For molecular cloning insert DNA and vector DNA are digested by the same restriction enzymes, to be connected by ligation afterwards. After mini plasmid preparation a control digest was performed, to proof successful insert integration.

For the digest restriction enzymes, their required buffer and BSA were applied according to manufacturer's instructions. Reaction mixture (Tab. 2.5) was incubated at 37°C for 1 h.

Table 2.5:   **Reaction Mixture for Restriction Digest**

Digest (insert)	Digest (vector)	Control digest
11 µl PCR product	1 µl plasmid DNA (1 µg/µl)	5 µl DNA
0.5 µl enzyme no. 1	0.5 µl enzyme no. 1	0.2 µl enzyme no. 1
0.5 µl enzyme no. 2	0.5 µl enzyme no. 2	0.2 µl enzyme no. 2
1.5 µl 10x buffer	1 µl 10x buffer	1 µl 10x buffer
1.5 µl BSA	1 µl BSA	1 µl BSA
	6 µl dH$_2$O	0.2 µl dH$_2$O

2.11.7.2 Phosphatase Treatment

In order to prevent a digested DNA fragment from self-ligation, its 5' phosphate group was dephosphorylated by treatment with an enzyme called phosphatase. Therefore, 8 µl DNA were incubated with 1 µl Antarctic Phosphatase and 1 µl of its appropriate NEB buffer for 30 min at 37°C. Temperature was then elevated to 65°C for 5 min to inactivate phosphatase.

2.11.7.3 Ligation

For ligation of vector and insert T4 ligase was used with the corresponding NEB buffer. Reaction mixture contained 0.5 µl vector DNA (digested and dephosphorylated), 7 µl insert DNA (digested), 1 µl T4 ligase, 1 µl 10x buffer and 0.5 µl H_2O and was incubated overnight at 14°C.

2.11.7.4 Transformation

Transformation is a method to introduce recombinant DNA into bacteria, with the objective of DNA amplification. In this study, artificial competent *Escherichia coli* (DH5α) bacteria were used. 5 µl of ligation reagent mixture was incubated together with 50 µl of thawed competent bacteria for 30 min on ice. Subsequent thermal shock for 30 s in a water bath with 42°C was performed to enhance uptake efficiency. Then, heat damage of bacteria was prevented by cooling on ice for 2 min. 200 µl 2x YT media was added and an incubation step at 37°C for 45 min followed. In the following bacterial cells were plated on agar, containing the antibiotic ampicillin, and incubated overnight at 37°C. Only bacterial colonies expressing the ampicillin resistance protein, which is part of the recombinant DNA, survive on ampicillin-containing agar.

2.11.7.5 Plasmid Preparation

In order to obtain purified plasmid DNA from bacterial cultures, plasmid preparation was performed by application of a commercial plasmid DNA extraction kit (Qiagen, Hilden, Germany). In a first step, a so-called mini preparation was performed to isolate small amounts of plasmid DNA (up to 25 µg). After verification of successful DNA recombination and transformation by DNA sequencing a maxi preparation was undertaken to gain larger amounts (up to 1 mg). Preparation techniques are analog and differ only in amounts of isolation reagents and DNA result. The procedure

comprises growth of bacterial culture, alkaline lysis of bacteria and plasmid DNA purification by isopropanol (Sigma-Aldrich, St. Louis, MI, USA).

In this study, single transformed bacterial colonies were picked by a pipette tip and set into 2 ml (mini preparation) or 250 ml ampicillin-including 2x YT media including. For sufficient cell growth bacteria were incubated overnight at 37°C while rotating (220 rpm). The following day, DNA preparation was executed according to manufacturer's instructions of the respective reagent kit.

For storage of bacteria carrying the recombinant DNA 700 µl of overnight culture were mixed with 300 µl glycerin (86%) and conserved in cryo tubes (Thermo Fisher Scientific, Waltham, MA, USA) at -80°C.

2.11.7.6 Measurement of DNA Concentration

Nanodrop ND-100 (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine concentration and to assess purity of DNA samples by spectrophotometry. Concentration was adjusted by TE buffer to 1 µg/µl.

2.11.7.7 DNA Sequencing

For DNA sequencing 15 µl DNA (10 ng/µl) and 15 µl of the respective primers (150 pmol) were sent to Eurofins Genomics (Ebersberg, Germany).

3 Results

3.1 Clinical Data of SPSD Patients

In this study, four sera (patient 1-4; pat 1-4) and two CSF (from pat 1 and pat3) samples of SPSD patients, as well as purified total-IgG (pat 1) were used (Fig. 3.1 and Tab. 3.1). A serum sample taken from a healthy person served as a negative control. There were three male and one female patients, their age varied from 37 to 63. All patients were positive for anti-GlyR autoantibodies, pat 1 had additional anti-GAD65 and islet-cell autoantibodies and pat 2 had additional anti-gephyrin and thyreoglobulin autoantibodies. Pat 2 and 3 were diagnosed with PERM, whereas pat 1 and 4 received the diagnosis of SPS by the treating clinician.
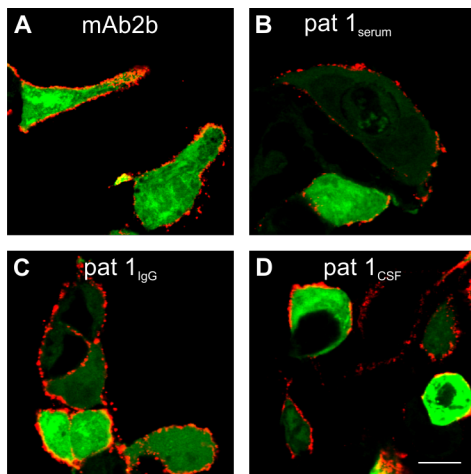
Figure 3.1:   GlyRα1 autoantibodies are detectable in serum, CSF and purified IgG of the same patient. GlyRα1^{hs} and GFP co-transfected HEK293 cells were incubated with GlyRα1-specific mAb2b (A), pat 1 serum (B), pat 1 CSF (C) and purified IgG of pat 1 (D). Red fluorescent signals correspond to IgG bound to GlyRα1. Scale bar refers to 20 µm. This figure was reused with permission from John Wiley and Sons (Rauschenberger, von Wardenburg et al. 2020).

Examining clinicians reported as major symptoms stiffness and myoclonus, startle (spontaneous or triggered by acoustic or sensory stimulus), paresis and spasticity, walking difficulties or falls. Sensory symptoms like pain or allodynia was seen in two patients. Pat 3 developed autonomic failure and was respiratory-dependent for several weeks. All patients improved by immunotherapy (plasma exchange, corticosteroids, azathioprine, rituximab, intravenous immunoglobulins) and received benzodiazepines (clonazepam, diazepam) as supportive treatment to control motor symptoms. EMG abnormalities were found in three patients reflecting disinhibition of brain stem or spinal

motoneuronal activity. Neuroradiologic investigations yielded no significant alterations in any patient. CSF analysis revealed lymphocytic pleocytosis in two patients indicating intrathecal inflammation. One patient had a tumor history of pleomorphic adenoma of parotid gland. The same patient was first diagnosed with cervical spinal stenosis, because of tetraparesis and progressive spasticity, and therefore underwent surgery. A few days later, he developed myocloni and seizures, showing then the full clinical picture of PERM. Disease courses were monophasic, relapsing or chronic.

Table 3.1:   Clinical Data of Patients

	patient 1	**patient 2**	**patient 3**	**patient 4**
Sex/Age at presentation	f/54	m/52	m/63	m/37
Diagnosis	SPS	PERM	PERM	SPS
Symptoms	lower limb stiffness, spasticity and paresis (4/5), walking difficulties, falls, pain, startle	myoclonic jerks of trunk and legs, exaggerated head retraction and acoustic startle reflexed violent, allodynia of anterior trunk	myocloni, tetraparesis, stiffness, dysphagia, autonomic failure	lock jaw, limb stiffness, falls
Electrophysiology	orbicularis oculi blink reflex: myoclonic synchronization	SEP, BAEP, MEP, masseter reflex inhibition normal, EMG polymyography: tactile reflex myoclonus	not done	lack of silent period in masseter inhibitory reflex
Neuroradiology	DaTSCAN™, 123I-IBZM-SPECT: unclear alterations	FDG-PET CT normal	cMRI: old right-sided infarct	cMRI normal
CSF abnormalities	none	45 lymphocytes per µl	30 lymphocytes per µl	mild increase in protein and albumin
GlyR autoantibodies	positive	positive	positive	positive
Other autoantibodies	Islet cell, GAD65	thyreoglobulin, gephyrin	none	none
Cancer history	none	none	pleomorphic adenoma of parotid gland	none
Immunotherapy	PEx, St	PEx, St	St, IVIG, PEx, RTX, AZA	PEx
Supportive therapy	clonazepam	clonazepam	diazepam	clonazepam
mRS maximum/final	4/3	4/1	4/2	3/2
Course of disease	chronic	relapsing	monophasic	relapsing

Modified Rankin scale (Farrell et al., 1991): 0 - No symptoms; 1 - No significant disability 2 - Slight disability 3 - Moderate disability 4 - Moderately severe disability 5 - Severe disability 6 - Dead. PEx: plasma exchange; St: steroids; IVIG: intravenous immunoglobulins; RTX: Rituximab; AZA: azathioprine;

3.2 Glycine Receptor Internalization upon Autoantibody-Binding

In several neurological autoimmune diseases autoantibodies induce internalization and depletion of the respective antigen (Drachman, Adams et al. 1981, Hughes, Peng et al. 2010). Hence, it was assumed, that internalization of GlyR leads alike to diminished levels of receptor density via endocytosis caused by autoantibody-antigen interaction, which was demonstrated in a recent study (Carvajal-Gonzalez, Leite et al. 2014). Three different antibodies were applied in this study: (i) monoclonal GlyR antibody mAb2b and (ii) serum of patient 1 (pat 1), containing anti-GlyR IgG, interacting with wild type $GlyR\alpha_1^{hs}$; (iii) c-Myc tag antibody (anti-c-Myc-tag) interacting with the construct $GlyR\alpha_1^{c-myc}$. $GlyR\alpha_1^{c-myc}$ is a genetic construct carrying c-Myc tag between amino acid positions four and five of mature $GlyR\alpha_1$. In contrast to anti-$GlyR\alpha_1$ and patient anti-GlyR autoantibodies, anti-c-Myc does not interact with GlyR. Thereby, the question was addressed, whether GlyR internalization requires immediate antibody-antigen-interaction or whether antibody binding to a peptide introduced into GlyR is sufficient to trigger internalization.

First, GlyR internalization was determined after incubation at 37°C, comparing dynamics after incubation with mAb2b or pat 1 (Fig. 3.2 C). Analysis revealed no significant differences in internalization dynamics in both conditions. After 1 h incubation, 47±10% (mAb2b, n=12) and 32±10% (pat 1; n=8, p=0.31) of GlyR, after 2 h 73±12% (mAb2b, n=10) and 85±8% (pat 1; n=7; p=0.42) of GlyR were internalized. At time point 4 h, no GlyR were left on the cell surface.

To check for differences in internalization dynamics at time point 2 h, a more detailed analysis was performed (Fig. 3.2 D). Larger number of cells and four image-layers throughout the cell body were analyzed for internalization. In addition, antibody-effect of anti-c-Myc-tag on $GlyR\alpha_1^{c-myc}$ was also included. Again, internalization dynamics did not vary significantly among subgroups (pat 1: 83±3%, n=16; mAb2b: 80±5%, n=18; anti-c-Myc: 79±4%, n=15). It is not clear, whether anti-c-Myc antibody binding to the c-Myc-tag integrated into the N-terminal part of $GlyR\alpha_1$ causes conformational changes in the receptors tertiary structure. In consideration of an epitope that lies outside the native protein structure at far N-terminal end, it can be assumed that antibody-antigen-interaction has a minor conformational effect.
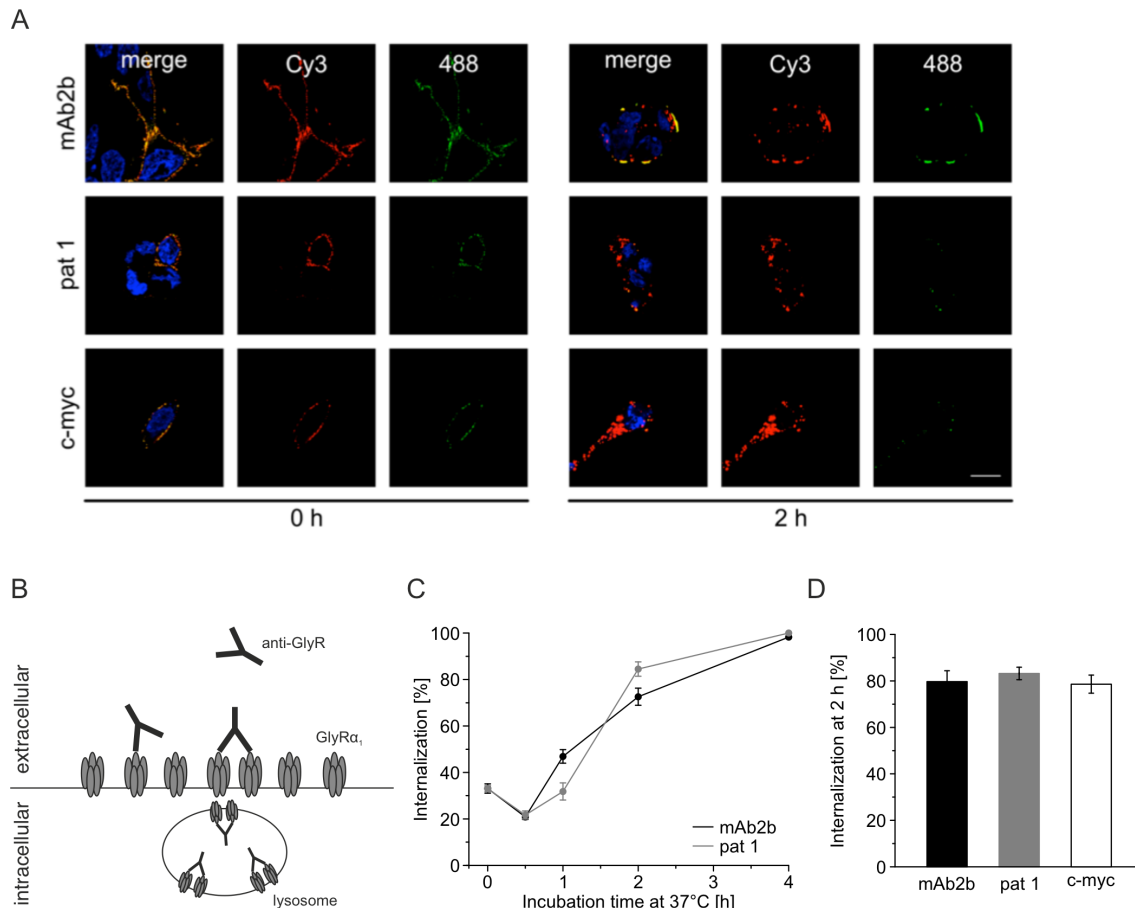
Figure 3.2: Anti-GlyR autoantibodies provoke receptor internalization *in vitro*. GlyRα_1^{hs} or GlyR$\alpha_1^{c\text{-myc}}$, a GlyR construct with N-terminal c-myc tag, were expressed in HEK293 cells following transfection and incubated with pat 1 serum (1:50) or monoclonal antibody [mAb2b (1:500), anti-c-myc (1:500)]. (**A**) Membrane attached or internalized GlyRs are displayed at time point 0 h and 2 h. Internalized GlyRs appear in red, membrane integrated GlyRs in yellow (merged images). DAPI was used to stain cell nuclei (blue). Scale bar represents 10 µm. (**B**) Scheme of the postulated internalization mechanism with GlyRα_1 homopentamers (grey) cross-linked by anti-GlyR autoantibodies (anti-GlyR, black). (C) Curve chart of internalized GlyRs. (D) Percentage of internalized receptor after 2 h of incubation at 37°C.

3.3 Functional Impairment of Glycine Receptor as a Ligand-Gated Ion Channel by Autoantibody Interference

GlyRα_1 homopentamers are ligand-gated ion channels, which respond to glycine with a conformational change allowing the diffusion of monovalent anions across the cell membrane. Appropriate neurotransmitter binding as well as precise structural transformation is crucial for GlyR function (1.3.2). It was hypothesized, that antibody-exposure interferes with GlyR function as an ion channel. In this study, HEK293 cells overexpressing GlyRα_1 subunits, which assemble to homopentamers, were used to

prove anti-GlyR autoantibodies impact on glycine-induced whole cell currents by the patch clamp technique. Before the conduction of electrophysiological recordings, cells were incubated with either monoclonal antibody mAb2b, anti-GlyRα$_1$ containing serum samples (pat 1-4) or human control serum (cs). Antibody presence during measurements was confirmed by subsequent immunocytochemical staining of human or murine IgG (data not shown). Ion currents were evoked by 50 µM glycine, which is close to the half maximal effective concentration of homomeric GlyRα$_1$ in HEK293 cells, or 1 mM, representing a saturating concentration (Atak, Langlhofer et al. 2015).
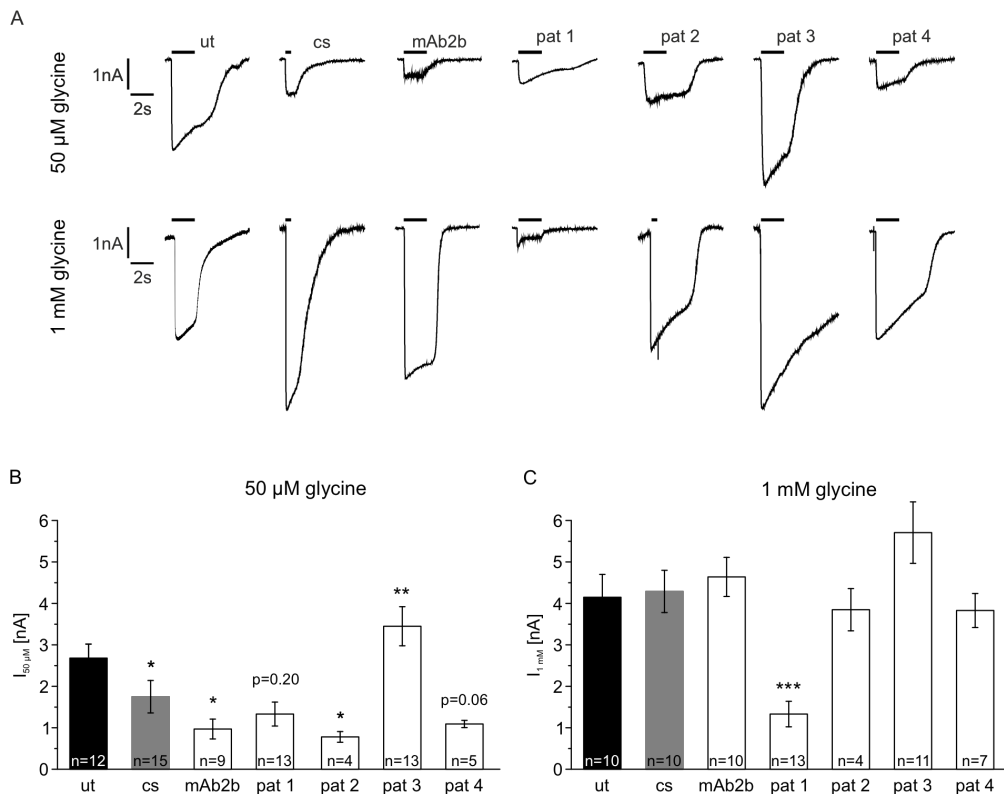
Figure 3.3:   **Whole cell currents of GlyRe$_1$[hs] are affected following pre-incubation of transfected HEK293 cells with patient sera.** (A) Glycine-evoked currents recorded using whole cell configuration of HEK293 cells following incubation with either patient sera (1:10) or mAb2b (1:500). Currents evoked by 50 μ M glycine were significantly reduced after treatment with mAb2b and pat 1, pat 2 and pat 4 sera. Currents evoked by 1 mM glycine were only reduced after treatment with serum of pat 1. Bold bar above trace indicates duration of glycine application. (B), (C) Mean current values of whole cell recordings ± standard error of the mean. Statistical significance for mAb2b and pat 1-4 was determined against cs by unpaired *t*-test.

By application of 50 µM glycine a reduction of glycine-evoked whole cell currents was observed (pat1: 1.3±0.3 nA, p=0.20; pat 2: 0.8±0.1 nA, n=4, p=0.015; pat 4: 1.1±0.1 nA; n=5; p=0.06), (Fig. 3.3 A, B). The current amplitude was reduced up to 53% (pat 2) by anti-GlyR-containing serum compared to cs. Notably, pat 3 serum had an

augmentative effect on whole cell currents by increasing glycine efficacy and potency ($I_{50\mu M}$: 3.3±0.5 nA, n=14, p=0.005; I_{1mM}: 5.7±0.7 nA, n=11, p=0.06). Equivalent to anti-GlyR autoantibodies, GlyR impairment was also seen upon incubation with mAb2b (1.0±0.2 nA; n=12; p=0.05). Surprisingly, control serum (cs) had also a minor unspecific effect on GlyR function (1.8±0.4 nA; n=15; p=0.04). After activation with saturating glycine concentration only pat 1 serum showed diminished current amplitude (pat 1: 1.3±0.3 nA; n=13; p=0.0008), which reflects a blockade of receptor function by interference with glycine binding sites. In contrast, GlyR function was not affected by pat 2 and 4 sera under saturating glycine concentrations. This indicates a rightward shift of the dose-response curve due to a competitive antagonistic effect of patient autoantibodies.

Table 3.2:   Electrophysiological properties of GlyRs following incubation with patient sera

Serum	Dilution	n	$I_{50\mu M}$ ± SEM [nA]	n	I_{1mM} ± SEM [nA]
Untreated	-	12	2.7 ± 0,3	10	4.2 ± 0.6
Control Serum	1:10	15	1.7 ± 0.4	10	4.3 ± 0.5
mAb2b	1:500	9	1.0 ± 0.2	10	4.6 ± 0.5
Patient 1	1:10	13	1.3 ± 0.3	13	1.3 ± 0.3
Patient 2	1:10	4	0.8 ± 0.1	4	3.9 ± 0.5
Patient 3	1:10	13	3.5 ± 0.5	11	5.7 ± 0.7
Patient 4	1:10	5	1.1 ± 0.1	7	3.8 ± 0.4

n = number of measured cells; $I_{50\mu m}$, I_{1mM} = mean current after application of 50 µM or 1 mM glycine respectively; SEM = standard error of the means

3.4   Defining of Glycine Receptor Autoantibodies' Epitope

For the standard detection of anti-GlyR autoantibodies in suspected patients a cell-based assay is generally exerted. Therefore, patient serum or CSF is added to living HEK293 cells overexpressing $GlyR\alpha_1^{hs}$ (McKeon, Martinez-Hernandez et al. 2013, Carvajal-Gonzalez, Leite et al. 2014). Since anti-GlyR autoantibodies bind to GlyR expressed on cell surface of living cells, it is assumed that the epitope is located at the ECD (Fig. 3.5 C). Pronounced differences in $GlyR\alpha_1^{hs}$ and $GlyR\alpha_1^{dr}$ amino acid sequence are located at the far N-terminus (A^{29}-P^{38}) (Fig. 3.5 B). At the remaining ECD only single amino acids are different. To check, whether anti-GlyR autoantibodies epitope is at the far end of the N-terminal domain (NTD), a chimeric construct was generated. The N-terminal $GlyR\alpha_1^{dr}$ fragment was substituted by the respective N-terminal $GlyR\alpha_1^{hs}$ fragment (M^1-G^{62}) (Fig. 3.5 A, B). Due to the identical amino acid sequence in mAb4a epitope, monoclonal antibody mAb4a detected $GlyR\alpha_1^{dr}$, $GlyR\alpha_1^{hs}$

as well as $GlyR\alpha_1^{ch}$ (Fig. 3.5 C), demonstrating that all three proteins are integrated into plasma membrane. In contrast, the epitope of mAb2b (^{29}ARSAPKPMSP^{38}) is not present in $GlyR\alpha_1^{dr}$. Indeed, mAb2b failed in recognition of $GlyR\alpha_1^{dr}$ as expected. However, mAb2b showed clearly detection of $GlyR\alpha_1^{ch}$ indicating that exchange of the equivalent human N-terminal fragment reconstitutes the mAb2b epitope. (Fig. 3.5 C). To note, pat 1 serum resembled the mAb2b recognition pattern, and bound to $GlyR\alpha_1^{ch}$ (Fig. 3.5 C). Hence, the exchanged fragment contributes to autoantibody binding to such an extent, that absence yields low or no affinity and binding cannot be detected in immunocytochemistry. Since the signal peptide (M^1 to A^{28}) is cleaved after membrane integration, it is impossible to be part of the epitope. Considering discrepancy in amino acid alignment between mature $GlyR\alpha_1^{hs}$ and mature $GlyR\alpha_1^{dr}$ at A^{29} to P^{38}, D^{40} as well as at positions R^{48} and T^{49}, these parts represent likely candidates for pat 1 autoantibodies epitope. Similar side chains of D^{40}, R^{48} and T^{49} ($GlyR\alpha_1^{hs}$) and the equivalent $GlyR\alpha_1^{dr}$ amino acids argue for A^{29} to P^{38}, the very first ten amino acids of the mature protein, to be an indispensable element of the autoantibodies epitope.

A

GlyRα$_1$^{hs}

GlyRα$_1$^{dr}

GlyRα$_1$^{ch}

B

```
                                              *
GlyRα₁ʰˢ  01 MYSFNTLRLYLWETIVFFSLAASKEAEAARSAPKPMSPSDFLDKLMGRTSGYDARIRPNFKGPPVNVSCNIFINS 75
GlyRα₁ᵈʳ  01 MSA   LGIYLWETIVFFSLAASQQAAARKAA SPMPPSEFLDKLMGKVSGYDARIRPNFKGPPVNVTCNIFINS 71
GlyRα₁ᶜʰ  01 MYSFNTLRLYLWETIVFFSLAASKEAEAARSAPKPMSPSDFLDKLMGRTSGYDARIRPNFKGPPVNVTCNIFINS 75

GlyRα₁ʰˢ  76 FGSIAETTMDYRVNIFLRQQWNDPRLAYNEYPDDSLDLDPSMLDSIWKPDLFFANEKGAHFHEITTDNKLLRISR 150
GlyRα₁ᵈʳ  73 FGSIAETTMDYRVNIFLRQQWNDPRLAYSEYPDDSLDLDPSMLDSIWKPDLFFANEKGANFHEVTTDNKLLRISK 146
GlyRα₁ᶜʰ  76 FGSIAETTMDYRVNIFLRQQWNDPRLAYSEYPDDSLDLDPSMLDSIWKPDLFFANEKGANFHEVTTDNKLLRISK 150

GlyRα₁ʰˢ 151 NGNVLYSIRITLTLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWQEQGAVERQ.................. 247
GlyRα₁ᵈʳ 147 NGNVLYSIRITLVLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWDEKGAVERQ.................. 243
GlyRα₁ᶜʰ 151 NGNVLYSIRITLVLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWDEKGAV.................ERQ 247
```

C

Figure 3.5: The far GlyRα$_1$^{hs} N-terminus is part of the autoantibody epitope. (**A**) Chimeric GlyRα$_1$^{ch} is composed of the N-terminal part of GlyRα$_1$^{hs} (grey bar) and the C-terminal part of GlyRα$_1$^{dr} (black bar). Cutting site is indicated by a vertical dotted line. (**B**) Amino acid sequence alignment of the extracellular domains of the immature GlyRα$_1$^{hs}, GlyRα$_1$^{dr} and GlyRα$_1$^{ch}. Deviations in amino acid alignment are displayed in bold letters. The known epitopes of mAb2b and of mAb4a are underlined and double-underlined respectively. Start of mature protein is marked by an asterisk. Restriction site of the enzyme PpuMI is shaded in yellow. Extracellular domain is shown according to Du *et al.* 2015. (**C**) HEK293 cells where co-transfected with GFP (green) and GlyRα$_1$^{hs}, GlyRα$_1$^{dr} or GlyRα$_1$^{ch}. All recombinant expressed constructs were detected by mAb4a. In contrast, mAb2b and pat 1 bound only to GlyRα$_1$^{hs} and GlyRα$_1$^{ch}. White bar refers to 10 μm. This figure was partially reused with permission from John Wiley and Sons (Rauschenberger, von Wardenburg et al. 2020).

3.5   Passive Transfer of GlyR Autoantibodies to Zebrafish Larvae

Our previous experiments showed, that GlyR internalization and functional derogation are solely caused by autoantibody-receptor-interaction. Thus, autoantibodies themselves are probable agents in the pathogenesis of SPS spectrum disorders. A respected assay to give proof of the pathogenic potential of autoantibodies is the passive transfer of autoantibodies into an experimental animal (Koch-Witsebsky postulates)(Rose and Bona 1993). Here, zebrafish larvae were selected for the animal tests, since zebrafish with mutated GlyRs present with a specific phenotype of impaired escape response, which can be seen as an equivalent of the exaggerated startle reflex in hereditary hyperekplexia (Hirata, Saint-Amant et al. 2005). To elaborate the most appropriate way of autoantibody transfer the following approaches were tested.

3.5.1   Transdermal Application of GlyR Autoantibodies

Initially, it was aimed to enable transdermal application of patient autoantibodies by adding of 1% DMSO to the water bath in order to enable their dermal penetration. Zebrafish larvae (24 hpf) were exposed for three consecutive days (eight hours per day) to purified IgG or patient serum (Fig. 3.6A). As negative controls NGS and hc serum were used. Strychnine was applied to give proof that transdermal application of a pharmacological agent can be effective. Indeed, strychnine treated zebrafish showed a typical phenotype with strong reduced or abolished locomotion (data not shown). Other treated larvae showed no specific phenotype. Immunohistochemistry revealed that increased dermal permeability allowed penetration of IgG into the dermis but did not access the circulation and particularly not the spinal cord (Fig 3.6B).
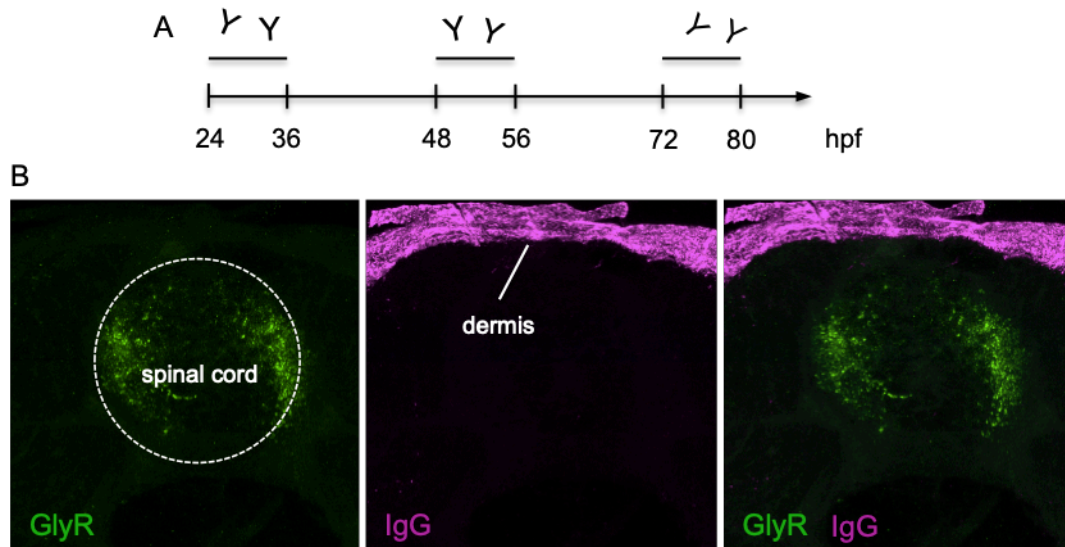
Figure 3.6:   Passive transfer of autoantibodies by enriching the bathing solution with purified IgG and increasing dermal permeability with 1% DMSO. (**A**) Time arrow depicting the experimental schedule. Horizontal bars indicate period of autoantibody exposure. In the meantime, bathing solution was changed to fresh water to reduce toxicity. Numbers represent age of zebrafish larvae. (**B**) Immunohistochemistry of spinal cord cryosections after bathing exposure to pat 1 IgG. GlyRs (mAb4a) are located at lateral parts of the spinal cord. IgG (anti-human-IgG) penetrated the zebrafish dermis but did not reach circulation or spinal cord.

3.5.2   Microinjection of GlyR Autoantibodies into the Fourth Ventricle

Since transdermal application of autoantibodies failed in reaching the CNS, the next step was to evaluate microinjection of patient serum or purified IgG into the fourth ventricle of zebrafish larvae. Different stages of development were used for microinjection to determine the suitable age (data not shown). Since larvae at 24 hpf are very short and vulnerable, this stage turned out to be rather impractical. Beginning from 72 hpf the fourth ventricle becomes less accessible for injection. Therefore, 48 hpf was found to be the most suitable period for passive transfer of autoantibodies directly into the intraventricular space.

The fluorescence dye sulforhodamine B was added to injected solution to be able to verify successful injection into the targeted region (Fig. 3.7A). Immunohistochemistry subsequent to larvae treatment showed that patient IgG could enter the CNS and reach the spinal cord, when transferred by microinjection into the fourth ventricle. However, no clear overlap of IgG with GlyR was detectable, which might be explained by too less proportion of GlyR autoantibodies among the total IgG and so not sufficient

amounts of GlyR autoantibodies get in contact with their target structure to become detectable in immunohistochemistry (Fig. 3.7B). On the behavioral level there were large differences within each group, most likely due to diverse extents of injury caused by the injection procedure. Hence, this technique was assessed to be not appropriate to see a group-dependent and significant effect specifically attributed to the GlyR autoantibodies pathogenicity.
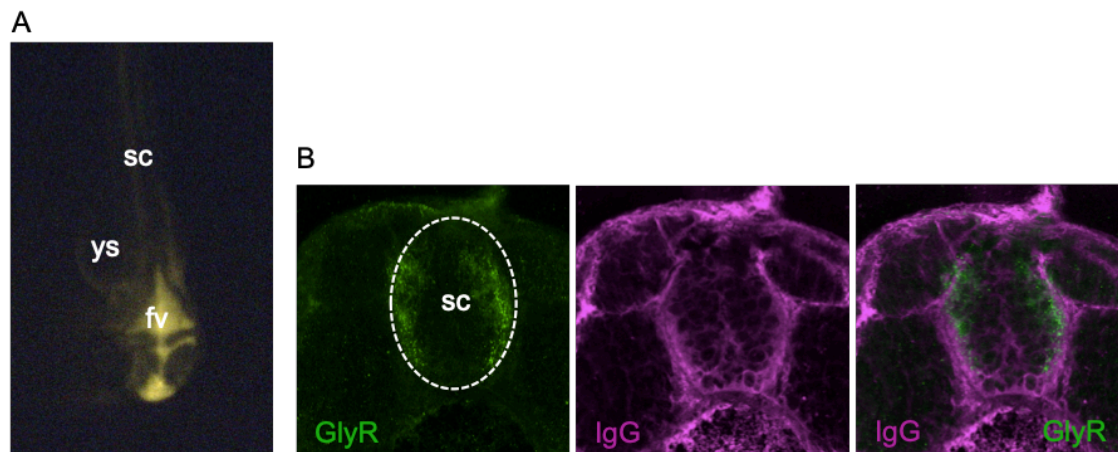
Figure 3.7: Passive transfer of autoantibodies by microinjection of serum or purified IgG. (**A**) Zebrafish larvae after microinjection of pat 1serum. (**B**) Immunohistochemistry of spinal cord cryosections after microinjection of pat 1 IgG. GlyRs (mAb4a) are located at lateral parts of the spinal cord. IgG (anti-human-IgG) reached the spinal cord but did not overlap with GlyR. sc: spinal cord; ys: yolk sac; fv: fourth ventricle.

3.5.3 Intrathecal Administration of GlyR Autoantibodies via a Superficial Skin Lesion

To overcome the problems of a harmful technique together with not satisfactory quantity of transferred autoantibodies to the spinal cord, a method was evaluated where a small skin lesion was set above the fourth ventricle followed by a transfer of larvae into a CSF-like bathing solution enriched with purified IgG. Thereby, the violation of zebrafish larvae was diminished and according to the long-lasting exposure to autoantibodies, it was expected that sufficient transfer could be attained. Indeed, a pilot study demonstrated that this technique was adequate (not shown).

3.5.4 Abnormal Escape Response after Intrathecal Passive Transfer of GlyR Autoantibodies

Physiologically 72 hpf zebrafish larvae initiate swimming after a tactile stimulus to the tail fin. Pre-treatment as wells as hc permeated larvae (n=11) responded normally to touch (Fig. 3.8 A). In contrast, zebrafish larvae treated with pat 1 serum (n=11) showed abnormal escape behavior with only weak convulsions or no response to touch instead of swimming initiation (Fig 3.8 B). Among the GlyR autoantibody treated group, only one larva exhibited a physiological escape response.

3.5.5 SPSD-Like Phenotype is Accompanied with a Decrease in GlyR Clusters

To reveal if less GlyR numbers account for the GlyR pathomechanism in the *in vivo* situation and thus interfered the swimming initiation, GlyR internalization was investigated. Subsequent to behavioral analysis of GlyR autoantibody treated larvae, cryosections of their spinal cord were subjected to immunohistochemistry and the number of GlyR clusters as wells as AMPAR clusters were quantified in the lateral region of the spinal cord. Indeed, the number of GlyR clusters was reduced in pat 1 serum permeated larvae (0.51 ± 0.10, n = 11) by half in comparison to untreated larvae (1.00 ± 0.23, n = 12) (Fig. 3.8 C, D, G, H, O). Relative number of GlyR was not decreased after treatment with hc serum (0.87 ± 0.15, n = 11) (Fig 3.8 E, F, O)*.* Neither pat 1 serum nor hc serum had an impact on the number of AMPAR clusters, indicating that autoantibodies specifically decrease the number of GlyR clusters (Fig. 3.8 I-N, P). Taken together these results demonstrate that GlyR autoantibodies not only provoke internalization of recombinant GlyR expressed in HEK293 cells, but also the internalization of synaptic GlyR of the spinal cord.
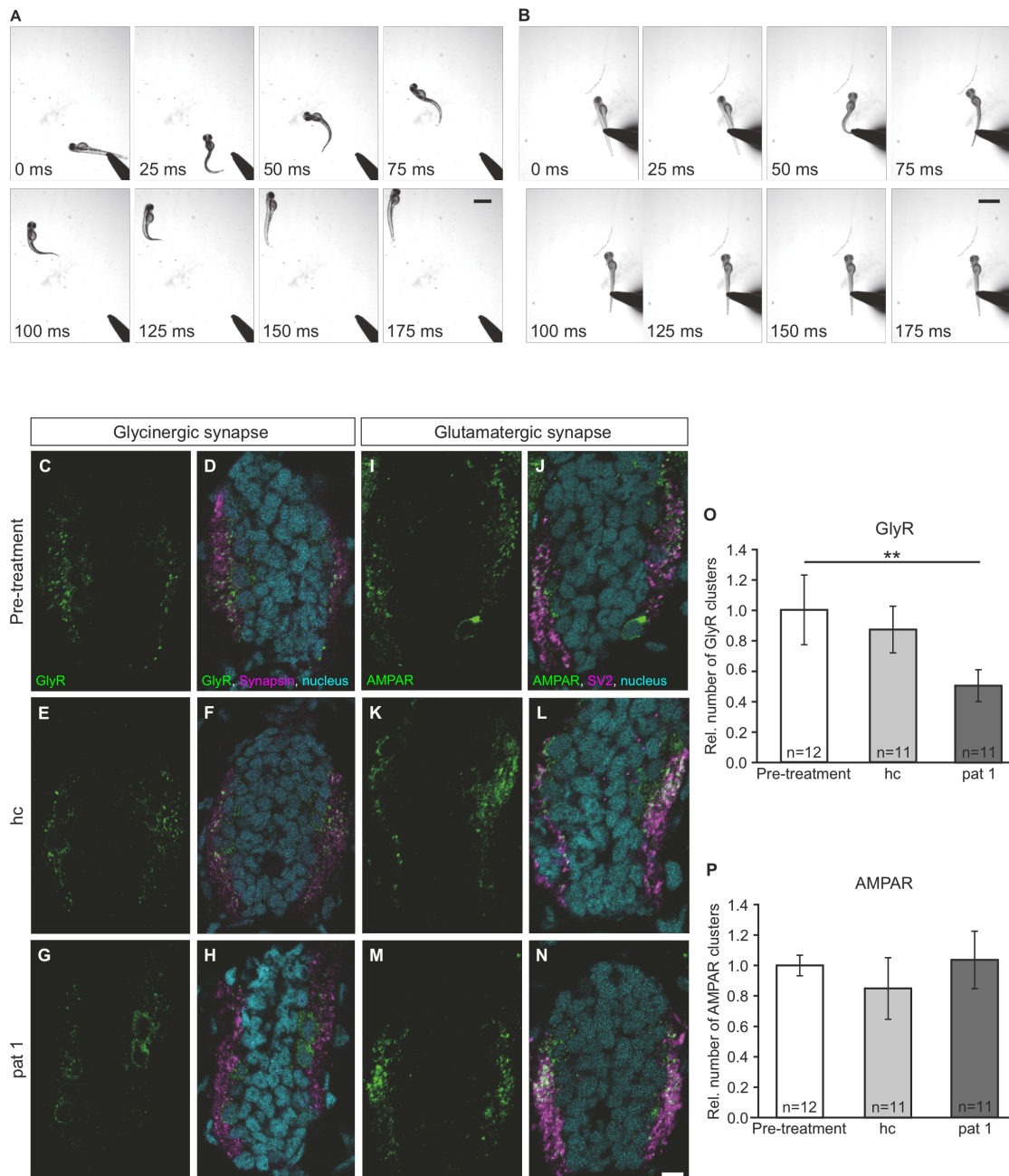
Figure 3.8: Sequence photographs of escape response of pat 1 serum and hc serum treated zebrafish larvae. The moment of tactile stimulus is indicated as 0 ms. Normal escape response of hc serum permeated larva (**A**). Escape response of pat 1serum permeated larva. This larva showed a weak convulsion instead of initiating of swimming (**B**). Scale bars represent 1 mm. Immunostaining of glycinergic synapse. Anti-synapsin1 antibody was used as primary antibody to detect presynaptic terminals (**C-H**). Immunostaining of glutamatergic synapse. Anti-synaptic vesicles antibody was used as primary antibody to detect pre-synaptic terminals (**I-N**). Relative number of GlyR clusters in the lateral region of spinal cord. Pre-treatment larvae (n = 12, 1.00 ± 0.23), hc serum larvae (n = 11, 0.87 ± 0.15), pat 1 serum larvae (n = 11, 0.51 ± 0.10) (**O**). Relative number of AMPAR clusters in the lateral region of spinal cord. Pre-treatment larvae (n = 12, 1.00 ± 0.07), hc serum larvae (n = 11, 0.85 ± 0.20), pat 1 serum larvae (n = 11, 1.03 ± 0.19) (**P**). Scale bar represents 5 μ m. This figure was reused with permission from John Wiley and Sons (Rauschenberger, von Wardenburg et al. 2020).

4 Discussion

This study expands the knowledge about the contribution of anti-GlyR autoantibodies to the pathogenesis of SPSD. Internalization of GlyR upon incubation with anti-GlyR-autoantibody-containing serum was shown previously and was confirmed in the present study. Just recently, alterations of GlyR's properties as an ion channel by autoantibody-interference have been reported on primary spinal cord neurons. Here, it could be demonstrated that pre-treatment of HEK293 cells with patient serum had pronounced effects on receptor function in a patient-specific fashion. Furthermore, by generation of a chimeric GlyR construct, an epitope of anti-GlyR autoantibodies could be localized to the far N-terminus of GlyR's ECD.

4.1 Antibody-Induced Glycine Receptor Internalization

As it has been shown in a previous study, recombinant GlyRα1 expressed on the plasma membrane of HEK293 cells internalize upon exposure to anti-GlyR autoantibodies. The internalization dynamics were comparable, i.e. significant loss occurred after 2 h of incubation at 37°C. Here, it could also be demonstrated that monoclonal antibody mAb2b represents a trigger for receptor endocytosis alike. This is not surprising, since NMDR mouse monoclonal antibody (M68 IgG) shows comparable effects on receptor internalization as serum derived antibodies (Castillo-Gómez, Oliveira et al. 2017). And even a monoclonal antibody, targeting an epitope artificially integrated into the receptor at the far N-terminus, showed undistinguishable results. Due to peripheral location outside the native GlyRα$_1$, it can be assumed that internalization is triggered rather by receptor dimerization – carried out by the two antigen-binding fragments (Fab) of IgG – then by a conformational change. Although this interpretation remains fairly hypothetic, it is supported by the observation that dimerization of nAChR, "labeled" with α-bungarotoxin, via an anti-α-bungarotoxin-antibody accelerate endocytosis like patients anti-nAChR autoantibodes (Drachman, Angus et al. 1978).

4.1.1 Informative Value and Limitations of Applied Internalization Assay

An evident weakness regarding the informative value of this experiment is, that it was not possible to include a proper negative control in application of the identical protocol. In this assay visualization of GlyRα$_1$ relies on antibody binding. Therefore, it was not

possible to analyze receptor internalization using control serum from a healthy person, which does not harbor anti-GlyR autoantibodies. In a pulse-chase radiolabeling assay GlyRα$_1$ expressed in HEK293 cells were detectable even after 24h (Villmann, Oertel et al. 2009). Thus, reduction of GlyR remaining on the cell surface, when incubated with patients antibodies, to about one third after 2 h and absence of any membrane bound GlyRs after 4 h, strongly argues for enhanced internalization compared to homeostatic turnover of GlyRα$_1$ in HEK293 cells. Since the same effect was observed by monoclonal antibodies, diluted in cell culture medium, it is less likely that an undetected component of human serum accelerated receptor internalization.

Potential compensatory mechanisms that counteract loss of surface GlyRα$_1$ are on the transcriptional level, i.e. neurons synthesize larger amounts of GlyR, extrasynatptic receptor molecules move to synaptic sites or internalized receptors are directed to recycling pathways and reintegrated to the plasma membrane (Dumoulin, Triller et al. 2009, Kneussel and Wagner 2013, Kneussel and Hausrat 2016). The internalization assay performed in this study did not include newly synthesized and membrane integrated GlyRα$_1$ into the analysis, since by application of an overexpression model, it would be misleading to look for cellular compensatory mechanisms to counteract loss of surface GlyRα$_1$.

It has to be considered that results derived from HEK293 cells do not necessarily account for cultured neurons or the *in vivo* situation, although many insights into GlyR assembly, maturation and degradation originated from studies performed on HEK293 cells (Huang, He et al. 2007, Villmann, Oertel et al. 2009, Schaefer, Kluck et al. 2015). Experiments were performed on HEK293 cells expressing homomeric GlyRα1 without co-transfection of gephyrin or the β-subunit. In the spinal cord and brain stem, synaptic GlyR are thought to be mainly heteromeric GlyRα$_1$β (Lynch 2004). The β-subunit carries the essential binding motif for the attachment to the synaptic scaffolding protein gephyrin (Meyer, Kirsch et al. 1995), which facilitates stabilization of GlyR at synaptic sites (Meier, Vannier et al. 2001). It is possible, that stabilization by gephyrin can counteract the internalization effect of anti-GlyR autoantibodies.

4.1.2 Potential Mechanisms Underlying Autoantibody-Associated Endocytosis

From early investigations into the pathology of Myasthenia gravis and Lambert-Eaton syndrome, we know that the capacity to cross-link the target of autoantibodies (nAChR, voltage-gated calcium channel) via the two F$_{ab}$-arms is a mechanism, which triggers

receptor internalization (Drachman, Angus et al. 1978, Nagel, Engel et al. 1988). This disease mechanism has been proved to account also for the more recent discovered anti-NMDAR-autoantibody-mediated encephalitis (Hughes, Peng et al. 2010). Patients' autoantibodies lead to a significant reduction of NMDAR clusters on dendrites of cultured hippocampal neurons, with a plateau after 24 h (Ladépêche, Planagumà et al. 2018). A nanoscale analysis revealed that prior to internalization, NMDAR form enlarged and densely packed clusters and move to extrasynaptic sites. It is possible, that endocytosis processes itself are not enhanced, but that receptor cross-linking leads to an increased number of receptors in each endocytosis vesicle, because of an receptor agglomeration as observed in nanoscale analysis of NMDAR clusters.

Another possibility is that aggregated receptors might be recognized by the cell membrane due to size or mobility and selected for endocytosis. Different processes are known to be involved in the regulation of GlyR turnover. A popular signal for receptor endocytosis is ubiquitination, followed by lysosomal or proteasome-mediated degradation (Lin and Man 2013). It is known, that GlyRα$_1$ homopentamers are directed to the lysosomal pathway and cleaved into fragments as a consequence of ubiquitination (Buttner, Sadtler et al. 2001). GlyRα$_1$, which were endocytosed upon anti-GlyR binding, co-localized with the late endosomal marker LAMP2 indicating a lysosomal degradation of internalized receptors (Carvajal-Gonzalez, Leite et al. 2014). Several enzymes are involved in ubiquitination of proteins. E3 ubiquitin-protein ligases are substrate-specific enzymes, which execute the "labeling" of a certain membrane protein for internalization and degradation (Ardley and Robinson 2005). HECT, UBA, WWE domain containing 1 (HUWE1) has recently been identified to be the E3 ligase specific for GlyRα$_1$ ubiquitination (Zhang, Guo et al. 2019). Ubiquitin mono- or polymers are transferred to an intracellular lysine residue of the respective target protein (Pickart 2001). In HEK293 cells, protein kinase C (PKC) stimulates GlyRα1 endocytosis and creation of endocytic vesicles relies on the interaction of the GTPase dynamin with amphiphysin (Huang, He et al. 2007). On spinal cord neurons, stimulation of endocytosis is mediated by activation of PKC or protein kinase A (PKA) (Velazquez-Flores and Salceda 2011). Thus, PKA- or PKC-mediated phosphorylation or ubiquitination display potential mediators of autoantibody-induced internalization. Albeit, details how these mechanisms can take place in detail are not simple to conceive.

4.1.3 Effect of Internalization on Glycinergic Neurotransmission

Obviously, pronounced decrease in GlyR surface quantity limits synaptic strength. This is reflected by the pathophysiology of hereditary hyperekplexia with mutations in *GLRA1*, where reduced surface integration of GlyRs is frequently found (Schaefer, Roemer et al. 2018). Albeit, *in vitro* whole-cell recordings of HEK293 cells expressing a T162M GlyRα_1 mutant with a reduced surface expression of 53% exhibited normal maximum current amplitudes (Schaefer, Kluck et al. 2015). However, if quantity of surface integrated receptors is diminished to less than 25%, as it accounts for S231R or R392H mutations, marked effects on maximum currents values were observed (Villmann, Oertel et al. 2009). Depending on the neuronal ability to compensate antibody-induced GlyR loss, an impact on glycinergic neurotransmission by enhanced internalization can be assumed.

4.2 Individual Effects on Glycine Receptor Function

4.2.1 Results in Consideration of Limitations

Just recently, it has been demonstrated for the first time that anti-GlyR autoantibodies have the capability to alter the intrinsic function of its target as a ligand-gated ion channel (Crisp, Dixon et al. 2019). In this study, primary spinal cord neurons were incubated with patient IgG or F_{ab} fragments prior to whole-cell patch-clamp recordings. Here, GlyR functional impairment has been assessed by pre-incubation of transiently transfected HEK293 cells with patient serum. This approach benefits from an isolated stimulation and recording of activated GlyRs.

The individual effects of anti-GlyR autoantibodies might vary among the polyclonal antibody repertoire of a single patient, but particularly from patient to patient as it is the case for nAChR autoantibodies (Vincent, Whiting et al. 1987). The functional properties of autoantibodies depend largely on the respective epitope, since direct blockade of ligand-binding or allosteric modulation via induction of conformational shifts depend on the antibodies binding site. In this study, serum containing polyclonal GlyR autoantibodies were applied. Therefore, only the compound effect of the existing polyclonal autoantibodies of one patient could be analyzed and compared with other patients' autoantibodies. Indeed, different functional effects were observed.

Glycine-evoked currents were lowered at saturating conditions solely after pre-treatment with pat 1 serum, reflecting reduced glycine efficacy due to noncompetitive antagonism. In contrast, monoclonal mAb2b, pat 2 and pat 4 sera had no significant influence on glycine efficacy but on glycine potency, showing the pharmacodynamic properties of competitive antagonists. Since they acted as competitive antagonists, it can be assumed that autoantibodies' epitope of pat 2 and 4 is close to the glycine-binding site or alternatively autoantibodies hinder sterically glycine to reach the binding site in a way that it can be counteracted by sufficient glycine concentration. Interestingly, lowered glycine sensitivity – in a comparable scale – is frequently found to be a functional effect of *GLRA1* mutations found in hereditary hyperekplexia, the genetic phenocopy of SPSD (Bakker, Van Dijk et al. 2006). This indicates strongly that the observed modulatory effects can cause SPSD symptoms. The ability to enhance receptor function was seen by pre-treatment with pat 3 serum. This is line with previous observations, that autoantibodies can exert not only inhibitory, but also stimulatory effects (Epstein, Bahn et al. 1993). Albeit, to the author's knowledge, there are no existing reports about autoantibodies to ligand-gated ion channels with properties of an allosteric potentiator. On the other hand, it is questionable, whether potentiation of glycinergic neurotransmission contributes to the pathology of pat 3, but potentially other mechanisms such as internalization might be more relevant in this patient.

GlyR and nAChR belong to the family of Cys-loop receptors, since they share several structural similarities (Lynch 2004). Taken this into account, antibody-antigen-interference might be alike and presumptions regarding pathogenicity of GlyR autoantibodies can be transferred from insights into nAChR autoantibody-effects. Receptor blockade and degradation are hallmarks of nAChR autoantibodies pathogenicity, but their contribution to the clinical severity seems be different in every patient due to heterogeneity of the individual epitope repertoire (Drachman, Adams et al. 1982, Vincent, Whiting et al. 1987).

Surprisingly, control serum had also a moderate but significant effect on whole-cell currents, when 50 µM glycine concentration was applied. Serum incubation was followed by washing with saline solution, therefore only molecules with strong affinity to GlyR or other molecules attached to the plasma membrane of HEK293 cells should remain in the measuring chamber at relevant amount. Thus, it is unlikely that serum components such as zinc, that has known modulating effects on GlyR, are responsible for the reduced glycine potency after control serum incubation (Laube, Kuhse et al. 1995). Since in application of saturating concentrations current amplitude resembled

untreated cells, reduced receptor expression due to unspecific cell stress is also not a plausible explanation.

4.2.2 Interplay of Impaired Receptor Function and Receptor Internalization

It is questionable, whether internalization of GlyRs contributes to the observed effect in whole-cell recordings. Since pre-incubation with patients sera was conducted at room temperature and endocytosis seems not to occur efficiently in mammalian cells at this temperature, a major impact is rather unlikely (Tomoda, Kishimoto et al. 1989). Another study showed that monovalent Fab fragments of anti-GlyR, that lack the capability of dimerization and therefore presumably internalization, result in reduced glycinergic currents (Crisp, Dixon et al. 2019).

However, receptor internalization and receptor dysfunction should have a synergistic impact on impairment of glycinergic neurotransmission, when number of receptors is significantly diminished and, in addition, the remaining surface-integrated receptor are hindered in their function. Though it is possible that reduced glycinergic synaptic activity is counteracted to some extent by slow-down of receptor turnover induced by activity-deprivation (Ehlers 2003).

4.3 Antibody-Effects in Consideration of the N-Terminal Epitope

GlyR autoantibodies recognize their epitope in native folded GlyR attached to the plasma membrane in living cells arguing for an epitope at the N-terminal domain (NTD) according to antigen-accessibility. The present results demonstrated that the human N-terminal fragment A^{29}-G^{62} of the human GlyRα$_1$ is indispensable for antigen-recognition of pat 1 autoantibodies in immunocytochemistry, since failure in detection of the zebrafish GlyRα$_1$ could be restored by exchange of the respective fragment. Discrepancy in sequence alignment indicated that the first ten amino acids (A^{29} to P^{38}) are required for the antigen-formation. Serum of pat 1 showed the ability to enhance receptor endocytosis and to alter glycine efficacy in transiently transfected HEK293 cells. Taken into account that a superficial and peripheral location of an epitope facilitates receptor crosslinking – the most probable internalization mechanism –, it seems to be plausible that the far N-terminal epitope of pat 1 autoantibodies is accompanied by internalization as a disease mechanism. However, pat 1 serum had also a relevant impact on glycine efficacy as observed in electrophysiological

recordings. Glycine-evoked current amplitudes have been lowered to less than 50%, when stimulated with a saturating concentration. This indicates a noncompetitive allosteric mechanism that cannot be overcome by sufficient elevation of agonist presence. This is in line with the elsewhere localized glycine binding-site, which is composed by β-strands further downstream on the alignment than the here determined epitope (Huang, Shaffer et al. 2017).

Future studies should identify the autoantibodies' epitope for other patients' sera, since autoantibodies in general are heterogeneous regarding their functional activity and their binding sites (Vincent, Whiting et al. 1987, Drachman 1994), and should define epitopes more precisely.

4.4 Affected Escape Response Underscores anti-GlyR Autoantibodies' Pathogenicity

Glycinergic neurotransmission is crucial for the generation of motor pattern and processing of sensory stimuli in zebrafish (Hirata, Takahashi et al. 2011). Reticulospinal neurons named "Mauthner cells" integrate sensory input and excite spinal cord networks inducing a behavioral response (Berg, Björnfors et al. 2018). Generation of the adequate motor response relies on glycinergic neurotransmission and therefore a passive transfer of pathogenic GlyR autoantibodies should yield abnormal escape behavior (Takahashi, Narushima et al. 2002, Hirata, Saint-Amant et al. 2005, Hirata, Ogino et al. 2013). Indeed, zebrafish larvae permeated with anti-GlyR-autoantibody-containing serum caused impaired escape response upon a tactile stimulus comparable to the pathological phenotype of the well-characterized GlyRβ mutant *bandoneon* (Hirata, Saint-Amant et al. 2005)*.* Surprisingly, pat 1 did not recognize $GlyR\alpha_1^{dr}$ expressed in HEK293 cells. However, impaired escape response and GlyR internalization might be mediated by binding to $GlyR\alpha_2^{dr}$, $GlyR\alpha_3^{dr}$ and/or $GlyR\alpha_4^{dr}$. In contrast to $GlyR\alpha_1^{dr}$, the other α subunits of the zebrafish bear a sequence identity at ^{37}S, ^{40}D, ^{48}R and ^{49}T with $GlyR\alpha_1^{hs}$ within the A^{29}-G^{62} fragment that is inevitable for *in vitro* binding of pat 1 GlyR autoantibodies. Assuming that $GlyR\alpha_2^{dr}$, $GlyR\alpha_3^{dr}$ and/or $GlyR\alpha_4^{dr}$ display the autoantibody targets *in vivo*, ^{37}S, ^{40}D, ^{48}R and ^{49}T are essential components of the epitope.

Disturbed locomotion attributed to pathogenic GlyR autoantibodies was accompanied by decreased numbers of GlyR cluster at the spinal cord, probably as a consequence of internalization as it has been shown *in vitro* in this study and also previously

(Carvajal-Gonzalez, Leite et al. 2014). The reduced detection of anti-GlyR (mAb4a) of GlyR could also be an effect of competitive binding with patients' autoantibodies. However, a binding competition is unlikely regarding the distance between the binding sites of anti-GlyR (residues 96-105 of GlyRα_1^{hs}) and pat 1 autoantibodies (located between residues 29 and 62).

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                                          29                                    62
GlyRα₁ʰˢ   01 ·······MYSFNTLRLYLWETIVFFSLAAS·KEAEAARSAPKPMSPSDFLDKLMGRTSGYDARIRPNFKGPPV 65
GlyRα₁ᵐᵐ   01 ·······MYSFNTLRFYLWETIVFFSLAAS·KEAEAARSAPKPMSPSDFLDKLMGRTSGYDARIRPNFKGPPV 65
GlyRα₁ᵈʳ   01 ·······MSA···LGIYLWETIVFFSLAAS·QQAAARKAA·SPMPPSEFLDKLMGKVSGYDARIRPNFKGPPV 61
GlyRα₁ᶜʰ   01 ·······MYSFNTLRLYLWETIVFFSLAAS·KEAEAARSAPKPMSPSDFLDKLMGRTSGYDARIRPNFKGPPV 65
GlyRα₂ᵈʳ   01 MT··RPSVKLLTTLLACLMEMLNFRVSSGKDPDLLS···SSSSMSPSDFLDKLMGRTSGYDARIRPNFKGPPV 68
GlyRα₃ᵈʳ   01 MT··RKRLRSVAARGFIAWEIGILLSLVAS·KEPESPRRRAAPMSPPDFLDKLMGRTSGYDARIRPNFKGPPV 70
GlyRα₄ᵈʳ   01 MTHFSLIITDCINCSFCHVYFSGFFSISLPLCFLYIWCK···PMSPSDFLDKLMGRTSGYDARIRPNFKGPPV 70

GlyRα₁ʰˢ   66 NVSCNIFINSFGSIAETTMDYRVNIFLRQQWNDPRLAYNEYPDDSLDLDPSMLDSIWKPDLFFANEKGAHFHE 13
GlyRα₁ᵐᵐ   66 NVSCNIFINSFGSIAETTMDYRVNIFLRQQWNDPRLAYNEYPDDSLDLDPSMLDSIWKPDLFFANEKGAHFHE 13
GlyRα₁ᵈʳ   62 NVTCNIFINSFGSIAETTMDYRVNIFLRQQWNDPRLAYSEYPDDSLDLDPSMLDSIWKPDLFFANEKGANFHE 13
GlyRα₁ᶜʰ   66 NVTCNIFINSFGSIAETTMDYRVNIFLRQQWNDPRLAYSEYPDDSLDLDPSMLDSIWKPDLFFANEKGANFHE 13
GlyRα₂ᵈʳ   69 NVTCNIFINSFGSVTETTMDYRVNIFLRQKWNDPRLAYSEYPDSSLDLDPSMLDSIWKPDLFFANEKGANFHD 14
GlyRα₃ᵈʳ   71 NVTCNIFINSFGSIAETTMDYRVNIFLRQKWNDPRLAYSEYPDDSLDLDPSMLDSIWKPDLFFANEKGAHFHE 14
GlyRα₄ᵈʳ   71 NVTCNIFINSFGSITETTMDYRLNVFLRQQWNDPRLAYKEYPDDSLDLDPSMLDSIWKPDLFFANEKGANFHE 14

GlyRα₁ʰˢ   139 ITTDNKLLRISRNGNVLYSIRITLTLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWQEQGAV....ERQ 24
GlyRα₁ᵐᵐ   139 ITTDNKLLRISRNGNVLYSIRITLTLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWQEQGAV....QVA 24
GlyRα₁ᵈʳ   135 VTTDNKLLRISKNGNVLYSIRITLVLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWDEKGAV....ERQ 24
GlyRα₁ᶜʰ   139 VTTDNKLLRISKNGNVLYSIRITLVLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWDEKGAV....ERQ 24
GlyRα₂ᵈʳ   142 VTTDNKLLRIFKDGTVLYSIRLTLILSCPMDLKNFPMDVQTCTMQLESFGYTMNDLIFEWLDKGPV....ERQ 25
GlyRα₃ᵈʳ   144 VTTDNKLLRIFKNGNVLYSIRLTLTLSCPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWQEKGPV....ERQ 25
GlyRα₄ᵈʳ   144 VTTDNKLLRIFQNGNVLYSIRLTLILSCPMDLKNFPMDTQTCTVQLESFGYTMNDLIFQWLDEGPV....ERQ 25
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Figure. 4.1 The far GlyRα_1^{hs} N-terminus is part of the autoantibody epitope. Amino-acid sequence alignment of the extracellular domains of the immature GlyRα_1^{hs}, GlyRα_1^{mm}, GlyRα_1^{dr}, GlyRα_1^{ch}, GlyRα_2^{dr}, GlyRα_3^{dr}, and GlyRα_4^{dr}. Deviations in amino-acid alignment are displayed in bold letters. This figure was partially reused with permission from John Wiley and Sons (Rauschenberger, von Wardenburg et al. 2020).

According to the revised Witebsky's postulates the presented results demonstrated that GlyR-mediated SPSD is an autoimmune disease, since a passive transfer to an animal model induced disease-like symptoms (Rose and Bona 1993). Autoantibody-treated larvae showed weak convulsions or no response to touch probably reflecting dysfunction of hindbrain and/or spinal cord networks. Hallmarks of SPSD are muscle stiffness and spasms as well as exaggerated startle as a consequence of spinal cord and hindbrain disinhibition (Khasani, Becker et al. 2004). Taken into account the large differences between human and zebrafish behavioral patterns and complexity of neuronal circuits, the failure in escape response might be regarded as a disease-like symptom and correspond to the dysfunctional startle response in SPSD, since both neuromotor features represent brain stem reflexes that mediate a motor response upon a (threatening) sensory stimulus.

4.5   Relevance for Novel Treatment Strategies

4.5.1   Symptomatic Treatment

Insights into the molecular mechanisms underlying NMDAR internalization led to the discovery of a potential drug counteracting anti-NMDAR effects on receptor density, neuronal plasticity and behavior (memory, depressive-like behavior) in mice (Mikasova, De Rossi et al. 2012, Planaguma, Leypoldt et al. 2015, Planagumà, Haselmann et al. 2016). Hence, identification of key regulators of GlyR turnover might unveil novel targets for drug development to improve symptomatic treatment of SPSD patients. An established strategy to overcome reduced neurotransmission in Myasthenia gravis is to enhance synaptic acetylcholine levels by pharmacological inhibition of acetylcholinesterase, which degrades acetylcholine at the neuromuscular junction (Drachman 2016). Glycine transporter GlyT1 and GlyT2 remove glycine from the synaptic cleft, thereby terminating the synaptic signal, and their inhibition prolongs and extends glycinergic postsynaptic currents (Zeilhofer, Acuna et al. 2018). Ongoing research into the discovery of adequate glycine transporter inhibitors for the treatment of chronic pain might contribute to the development of novel drugs with a potential benefit also in SPSD patients. A treatment strategy yielding elevated glycine levels in the synaptic cleft is also appropriate concerning the decreased glycine potency. The potential to ameliorate patients' symptoms, facilitating mobility in particular, by pharmacological strengthening of inhibitory neurotransmission has been demonstrated by the successful application of $GABA_AR$ agonists (benzodiazepines) (Balint and Meinck 2018). It is assumed that in hyperekplexia patients with inherently compromised glycinergic neurotransmission the relatively moderate phenotype compared with subacute/chronic or acute impairment caused by GlyR autoantibodies, strychnine intoxication (highly potent and specific competitive GlyR antagonist) or *Clostridium tetani* (toxin disables presynaptic glycine release) the deficiency is endogenously counterbalanced to a certain degree by the GABAergic system as a surrogate (Thomas, Chung et al. 2013). However, treatment with benzodiazepines remains unsatisfactory in some cases and pharmacological modulation of glycinergic neurotransmission might yield superior symptom control (Carvajal-Gonzalez, Leite et al. 2014).

Another strategy to overcome impairment of glycinergic neurotransmission comes from the treatment of the Lambert-Eaton-Myasthenic-Syndrome, which is thought to be

caused in most of the cases by autoantibodies to the voltage-gated calcium channel. As a consequence of decreased calcium influx into presynaptic terminals acetylcholine release is diminished. Administration of the reversible potassium channel blocker 3,4-diaminopyridine prolongs depolarization resulting in increased neurotransmitter release (Yoon, Owusu-Guha et al. 2019). Identification of a target structure that elevates glycine release from presynaptic terminals displays another imaginable pharmacological strategy. However, since pat 3 serum pre-treatment caused potentiation of glycinergic currents, pharmacological strengthening of glycinergic neurotransmission might have adverse effects in a subgroup of patients.

4.5.2 Causal Therapy

As this work suggests that pathology is driven by intrinsic pathogenic properties of GlyR autoantibodies future efforts should focus on their specific and effective removal. The benefit from autoantibody-elimination and the reversibility of SPSD with GlyR autoantibodies are reflected by retrospective studies and case studies, which demonstrated the marked response to immunotherapy (McKeon, Martinez-Hernandez et al. 2013, Martinez-Hernandez, Arino et al. 2016), in particular to rituximab (Kyskan, Chapman et al. 2013) (a therapeutic monoclonal antibody that depletes CD20+ B cells yielding marked reduction in antibody synthesis) and antibody-removal by PEx or immunoadsorption (Doppler, Schleyer et al. 2015). However, drawback of rituximab is an unspecific depletion of B lymphocytes and a generalized diminution of antibody serum levels, thereby increasing the risk for opportunistic infections (Pavanello, Zucca et al. 2017). Also, PEx bears risk of potentially life-threatening complication like catheter infection, sepsis and cardiovascular complications (Eyre, Hacohen et al. 2019). Therefore, new therapeutic strategies are required that act more effectively, more specifically and safer than the ones, which are to hand for clinicians at the moment. An interesting approach originates from cell-based therapy in treatment of leukemia, where a chimeric antigen receptor with specificity to CD19 (a B cell marker) is molecularly engineered and transduced to patients' derived T cells, which target and kill very effectively tumor cells when reinfused into a patient (Porter, Levine et al. 2011). Recently, the potential of this therapeutic approach for the treatment of antibody-mediated autoimmune diseases was evaluated in a preclinical study. And indeed, chimeric autoantigen receptor T cells that recognize specifically B cells, which produce autoantibodies to the antigen relevant in the respective autoimmune disease, showed promising results *in vitro* as well as *in vivo* (Ellebrecht, Bhoj et al. 2016).

Clinical studies testing the safety of this novel cell-based therapy in patients are scheduled. The potential of chimeric autoantigen receptor T cells in treatment of autoantibody-mediated encephalitis such as GlyR autoantibody-mediated conditions should be evaluated in future.

5 Summary

GlyR autoantibodies are associated with stiff person spectrum disorders, predominantly with the very serious condition progressive encephalopathy with myoclonus and rigidity. This study aimed to shed light on their pathogenicity and their pathogenic properties by patch clamp analysis and a passive transfer to zebrafish larvae as an animal model.

For one patient serum, fast antigen internalization could be confirmed as one component of the pathomechanism as it has been recently demonstrated (Carvajal-Gonzalez, Leite et al. 2014). To analyze the functional impact of GlyR autoantibodies, ion channel currents from recombinant GlyRα_1 expressing HEK293 cells were recorded following incubation with antibody-containing patient serum. The here applied patient sera exerted diverse effects on glycine-mediated currents. Various effects of GlyR autoantibodies could be observed, comprising reduced glycine efficacy, reduced glycine potency, but surprisingly also enhanced potency. This reflects the heterogeneity of autoantibody effects to their target antigen, depending on the specific epitope, affinity and immunoglobulin subclass (Vincent, Whiting et al. 1987). Additionally, in use of a chimeric GlyRα_1 construct, the N-terminus (A^{29}-G^{62}) could be defined to be a requisite component of the epitope of Patient 1 GlyR autoantibodies. At the structural level, this region corresponds to the first α-helical element that is easily accessible due to its superficial location.

For the first time, the pathogenicity of GlyR autoantibodies could be demonstrated by a passive transfer to zebrafish larvae as an animal model. The touch-evoked escape response in zebrafish is a neuromotor reflex, which relies on intact glycinergic neurotransmission (Hirata, Carta et al. 2009). A transdermal lesion above the fourth ventricle and exposure to autoantibody-containing ACSF resulted in failure in initiation of swimming away from the tactile stimulus. This behavioral consequence of autoantibody transfer was accompanied by a reduction of GlyR expression in the spinal cord of treated zebrafish larvae.

To sum up, this study extends largely the knowledge of the pathogenic capacities and properties of GlyR autoantibodies. The discoveries emphasize their disease relevance in SPSD patients and point out towards treatment strategies that aim specific antibody-removal.

6 Zusammenfassung

GlyR Autoantikörper sind mit dem Stiff-Person-Syndrom assoziiert, insbesondere mit der schwerverlaufenden Variante der Progressiven Enzephalopathie mit Rigidität und Myoklonus. Diese Studie hat sich als Ziel gesetzt, die Pathogenität der Autoantikörper sowie deren pathogenen Eigenschaften mit Hilfe der Patch-Clamp-Methode sowie eines passiven Transfers der Erkrankung auf Zebrafischlarven zu erklären.

Zunächst konnte eine rasche Internalisierung des Antigens als Teil des Pathomechanismus bestätigt werden (Carvajal-Gonzalez, Leite et al. 2014). Um den funktionellen Einfluss der GlyR Autoantikörper genauer zu untersuchen, wurden Ionenkanalströme von HEK293 Zellen, welche die rekombinante GlyRα_1-Untereinheit exprimieren, nach Inkubation mit Autoantikörper-enthaltendem Patientenserum abgeleitet. Die hier untersuchten Patientenseren zeigten unterschiedliche Einflüsse auf durch Glycin hervorgerufene elektrische Ströme. Die beobachteten Effekte umfassen eine reduzierte Agonistenwirksamkeit, eine reduzierte Potenz, allerdings auch eine gesteigerte Potenz des Agonisten Glycin. Diese Tatsache spiegelt die Heterogenität der Autoantikörper-Effekte wider, welche von dem jeweiligen Epitop, der Affinität und der Immunglobulin-Subklasse abhängig sind (Vincent, Whiting et al. 1987). Weiterhin konnte mit Hilfe eines chimären GlyRα_1 Konstrukts, der N-Terminus (A^{29}-G^{62}) als essentieller Bestandteil der Autoantikörperbindung von Patient 1 identifiziert werden. Auf der Strukturebene korrespondiert dieser Abschnitt zum ersten α-helikalen Element, welcher aufgrund seiner oberflächlichen Lage für Autoantikörper gut zugänglich ist.

Zum ersten Mal konnte hier die Pathogenität von GlyR Autoantikörper durch einen passiven Transfer der Erkrankung auf Zebrafischlarven gezeigt werden. Der durch Berührung ausgelöste Fluchtreflex in Zebrafischen ist ein neuromotorischer Reflex, welcher auf eine intakte glycinerge Erregungsübertragung angewiesen ist (Hirata, Carta et al. 2009). Eine transdermale Läsion oberhalb des vierten Ventrikels und Exposition zu Autoantikörper-enthaltendem artifiziellen Liquor cerebrospinalis hatte zur Folge, dass die physiologische Fluchtreaktion in entgegengesetzter Richtung zum taktilen Reiz den behandelten Zebrafischen unmöglich war. Eine anschließende immunhistochemische Aufarbeitung des Rückenmarks zeigte eine damit einhergehende signifikante Reduktion der GlyR-Dichte.

Zusammenfassend lässt sich sagen, dass durch diese Arbeit der Kenntnisstand über die pathogenen Eigenschaften von GlyR Autoantikörpern ausgeweitet werden konnte.

Die hier neugewonnen Erkenntnisse betonen die Relevanz der Autoantikörper für die Entstehung von Stiff-Person-Spektrum-Erkrankungen und unterstreichen die Notwendigkeit von Therapiestrategien, die eine Entfernung der Autoantikörper zum Ziel haben.

7 Outlook

After about a decade of research into GlyR autoantibodies in neurologic conditions a lot of questions regarding the pathomechanism remain unaddressed. To date, there is no data, which gives prove that GlyR internalization is mediated by cross-linking like it is the case in other autoantibody-mediated neurologic diseases, e.g. anti-AChR-mediated Myasthenia gravis or anti-NMDAR-encephalitis (Drachman, Angus et al. 1978, Hughes, Peng et al. 2010). This could lead to novel treatment strategies like the development of small molecules that counterbalance the degradation effect or monovalent Fab fragments that are incapable of cross-linking and internalization, but hinder antigen binding by divalent autoantibodies.

To gain further insights into the detailed pharmacodynamic effects of GlyR autoantibodies with special regards to the impact on GlyR functional properties, it would be of large benefit to clone monoclonal GlyR reactive autoantibodies derived from patients' antibody-producing cells (Kreye, Wenke et al. 2016). This could also lead to the detection of monoclonal antibodies with certain properties on glycinergic neurotransmission, which might be applicable in a therapeutic intention in diseases (epilepsy, autism) or conditions (chronic pain), where an important role of GlyR function has been demonstrated (Pollak, Beck et al. 2016).

Regarding the etiology and prevention of GlyR autoantibody-mediated disorders further research is needed to unravel the trigger of the autoimmunologic reaction, in particular silent virus infections. Since GlyR autoantibodies occur also as a paraneoplastic phenomenon, it would be of interest to determine the initial tumor antigens that provoke the immunologic cross-reaction. Knowledge of the tumor antigens could help to identify patients with a risk of GlyR autoimmunity and could enable clinicians to take preventive measures to circumvent the pathogenesis of SPSD.

Identification of a common epitope like the N-terminus in a larger cohort of patients could help to develop adoptive T cell treatment strategies like chimeric autoantigen receptor T cells that recognize B cell receptor bearing and antibody-producing cells (Ellebrecht, Bhoj et al. 2016). Specific detection and cytolysis of pathogenetic autoantibody-producing cells would avoid general immunosuppression and might have even stronger B cell depleting potentials with better therapeutic outcomes than conventional immunomodulatory treatment.

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Curriculum Vitae

Education

2019	Third part of medical exam, medical approbation
2017	Semester abroad at University of Aix-Marseille (ERASMUS)
2012-2019	Human medicine – Julius-Maximilians-Universität Würzburg
2009-2010	Philosophy (Bachelor) – Munich School of Philosophy
2000-2009	Pater-Rupert-Mayer-Gymnasium (Pullach)

Academic Career

06/2020	"GlyR autoantibodies impair receptor function and induce motor dysfunction" (Rauschenberger et v. Wardenburg et al. 2020) published in Annals of Neurology
10/2019	Research fellow at the Prüß group (DZNE, Berlin, Germany, associated with Charité Universitätsmedizin Berlin)
09/2016:	Attendance and Poster Presentation at the 13th International Society of Neuroimmunology Congress in Jerusalem ("Investigations into the pathogenic properties of glycine receptor autoantibodies using transfected human embryonic kidney cells and zebrafish embryos")
01-02/2016	Research Stay at Laboratory of Prof. H. Hirata (Department of Chemistry and Biological Science; Aoyama Gakuin University Tokyo)
10/2015	Symposium 2015 of the Graduate School of Life Science – University of Würzburg (Poster Presentation: „Pathogenic effects of glycine receptor antibodies associated with stiff-man syndrome")
09/2015	Participation at Summer School 2015 of the Graduate School of Life Science – University of Würzburg

08/2015	Initiation of the doctoral project at the Villmann group, Institute for Clinical Neurobiology, University of Würzburg

Academic Scholarships

Travel Scholarship Homberger-Inheritance, PROMOS Scholarship (DAAD) and Japan Student Services Organization (JASSO) Scholarship; Clinical Traineeship at Nagasaki University Hospital in 2016

Doctoral Scholarship by the Faculty of Medicine (University of Würzburg) and the Graduate School of Life Sciences

Professional Experience

01-04/2019	Clinical Traineeship at Charité Berlin Campus Virchow Klinikum, Emergency Department
09-12/2018	Clinical Traineeship at DRK Berlin-Mitte, Department of Thoracic Surgery
05-09/2018	Clinical Traineeship at University Hospital Hamburg-Eppendorf, Department of Neurology
02-04/2016	Clinical Traineeship at Nagasaki University School of Medicine (Hematology, Neurosurgery, Neurology, Immunology)
02-03/2015	Clinical Traineeship at the Department of Psychiatry at Charité Berlin Campus Benjamin Franklin
07-08/2014	Internship at General Practice Dr. S. Huber (Herrsching)

Affidavit

I hereby confirm that my thesis entitled „*Investigations into the Pathogenic Antibody-Antigen-Interference of Glycine Receptor Autoantibodies*" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date   Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „*Untersuchungen zur pathogenen Antikörper-Antigen-Interferenz von Glycin-Rezeptor Autoantikörpern*" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum   Unterschrift

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