Quantitative analysis of lipid debris accumulation caused by cuprizone induced myelin degradation in different CNS areas and Superficial neurogliaform cells activation leads to different summation of ionotropic and metabotropic GABA receptor mediated postsynaptic responses

Ph.D. Thesis summary

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1. Introduction

In my doctoral dissertation, I present two independent research topics:

1. The implementation of a newly developed coherent anti-Stokes Raman scattering (CARS) microscopy system for imaging lipid content in the brain. In these experiments, the process of myelin sheath degradation was investigated in living brain tissue.

2. The investigation of the integration of convergent ionotropic and metabotropic inhibitory signals induced by the neurogliaform cell population.

1.1 Quantitative analysis of lipid debris accumulation induced by myelin degradation in the central nervous system areas

In the central nervous system, the axons of nerve cells are covered by the myelin sheath. The myelin sheath is formed by oligodendrocytes, which spirally encircle the axon sections with their fine projections. The myelin sheath thus forms an intermittent electrical insulating layer along the axon. The exposed axon segments between the myelinated sections are known as the nodes of Ranvier. The uninsulated nodes of Ravier are the only places along the axon where ions are exchanged across the axon's membrane. This increases the conduction velocity of action potentials compared to the non-myelinated fibers.

In some neurodegenerative diseases, such as multiple sclerosis (MS), the myelin sheath is damaged. In terms of pathomechanism, demyelinating and inflammatory processes occur in myelinated areas. Because the myelin sheath acts as an electrical insulator along the axon, the electrical conduction of the myelin is significantly impaired in the early stages of the disease due to myelin damage. As a result of myelin degradation, neurodegeneration processes take place in the later stages of the disease. Currently, the underlying causes of the disease are unknown, but certain environmental and genetic factors may be implied. As with the underlying causes, the exact pathomechanism of the disease is unknown. The most accepted view is that MS is an autoimmune disease initiated by an autoimmune system dysregulation. The dysregulation causes autoreactive T-cells to enter the central nervous system, which is followed by microglia and macrophages attacking the myelin sheath, resulting in selective oligodendrocyte death.

In contrast, there is an alternative theory for the development of the disease, since in some cases, lesions can be observed in the early stages of the disease without the presence of lymphocytes. It was suggested that the death of the oligodendrocyte may be the primary

problem of disease and autoreactivity against it due to the release of myelin debris causing more inflammation and further demyelination. According to this theory, the appearance and distribution of myelin debris in the central nervous system is important for the development of subsequent inflammatory processes. Today, however, we have no concrete data on the incidence of myelin debris. In my dissertation, I used a newly developed imaging microscope system to study the demyelination processes. The microscope system operates on a nonlinear optical method called coherent anti-Stokes Raman scattering (CARS). CARS microscopy identifies molecules based on their vibrational spectra. This method provides chemical selectivity for certain molecules. We focused femtosecond pulsed laser beams combining 796 nm "pump" and 1028 nm "Stokes" beams to acquire excitation of vibration resonance of CH2 bonds of lipid molecules. The advantage of this method over other known methods is that the examination of biological structures does not require either natural fluorescence or fluorescent labeling. Measurements can be performed on living tissue without chemical modification/destruction of the biological structure.

1.2 Superficial neurogliaform cells activation leads to different summation of ionotropic and metabotropic GABA receptor mediated postsynaptic responses

The human brain is the most complex and widely investigated biological structure, the pinnacle of evolutionary development. The human brain accounts for only 2% of the body's total weight, weighting on average 1.3 kilograms; yet it consists of approximately $8.6 * 10^{10}$ neurons and $8.4 * 10^{10}$ glial cells, and 10^{12}-10^{14} synaptic connections established between neurons. A stratified cortex constitutes the surface of the brain, which is 1-2 mm thick in rodents and 3 mm in humans. The neocortex plays a critical role in the execution of higher-order cognitive functions, such as processing sensory inputs, associative thinking, memory, motor command development, and consciousness. These tasks require a complex operation of the neocortical network.

Information processing in the neocortex is defined by a dynamic micro-network of two large groups of neurons. One of the groups consists of the excitatory glutamatergic neurons: the so-called principal pyramidal cells. Typically, they are present in large numbers; 85-90% of cortical neurons are pyramidal cells. The other large group of neurons is the GABAergic interneurons, which are present in much smaller numbers, about 10-15% of the cells in the cortex. Their role is important in controlling the flow of neuronal signals and shaping the

dynamic network-level functioning of the nervous system. Effective signal processing requires diverse regulation of excitatory signals by the highly diverse interneuron population in the cortex.

Within this heterogeneous interneuron population, neurogliaform cells form a distinct group. Approximately 20% of the total amount of inhibitory interneurons in the cerebral cortex are neurogliaform cells. They have a round morphology of 10-20 μm in diameter. The multipolar dendrites starting from the soma are short and branch quickly. The dense axon arborization generally follows the somatodendritic location, being closely located to it. The axons can extend up to 1000 μm from the soma, but the majority of axons are concentrated at a distance of ~ 100-150 μm around the soma and they have a total length of ~ 20000 μm. This degree of axon density is unique among interneurons. However, since the spatial extent of the neurogliaform cell axon is limited to a small area, the density of the presynaptic bouton is the highest among the interneurons. On average, there are ~ 30 presynaptic boutons in the 100 μm axon section (this represents a distance of ~ 3 μm between consecutive boutons). Compared to parvalbumin-positive basket cells, the distribution of presynaptic boutons is 5-6 times denser on neurogliaform cells. In recent decades, numerous studies have focused on the output of cortical interneuron types. Rapid $GABA_A$ receptor-mediated inhibition from certain interneuron groups have been identified with multiple patch-clamp recordings, however, the source of biphasic $GABA_A$ and mostly extrasynaptically located $GABA_B$ receptor-mediated slow inhibition remained unknown. Each GABAergic interneuron specifically targets certain subcellular subunits of pyramidal cells, suggesting that $GABA_B$ receptor-mediated dendritic inhibition may also occur by the activation of a specific GABAergic interneuron group. Also, several studies have shown that $GABA_B$ receptor activation can be accomplished by the co-activation of multiple interneurons, or by the prolonged activation of single neurons. During such $GABA_A$ receptor activation, a large amount of excess GABA neurotransmitter leaks from the synaptic space to the extracellular space. In contrast, subsequent research conducted by our laboratory has shown that combined $GABA_A$ and $GABA_B$ receptor activation can be triggered by a single action potential of a single interneuron, the neurogliaform cell. Synaptic and extrasynaptic receptor activation suggest that synaptic and non-synaptic signaling may occur simultaneously.

Opening and closing of postsynaptically located ion channels can take place in a manner dictated by two large receptor protein families. One of the large receptor families is the ligand-gated ion channel. These integrated transmembrane proteins have two functional subunits: an extracellular specific binding site that binds endogenous ligands, neurotransmitters, and another

subunit, a transmembrane protein, that creates a channel for ion flow. The major inhibitory ligand-gated ion channel in the central nervous system is the $GABA_A$ receptor. The $GABA_A$ receptor is predominantly localized in the synapse, but some isoforms have also been observed extrasynaptically. As a result of GABA binding, the receptor undergoes a conformational change to open the channel through which Cl^- ions selectively pass.

Another large family of receptors is metabotropic receptors. They got their name from the fact that the opening of the channel is conditional on the occurrence of indirect metabotropic signaling interactions. Unlike the ligand-gated ion channels, these receptors do not have a direct ion channel structural subunit but instead, activate the surrounding proteins via G-protein. For this reason, metabotropic receptors are also called G-protein coupled receptors. Structurally, the metabotropic proteins comprise a neurotransmitter-binding extracellular subunit and an intracellular G-protein binding subunit. Upon neurotransmitter binding, the first components of signal transduction, the heterotrimeric G-proteins, have three subunits (α, β and, γ) and binds GDP in its inactive form. During $GABA_B$ activation, the GTPase subunit of the G-protein binds GTP, followed by conformational change. The α-subunit dissociates from the heterotrimeric complex and participates in a further molecular cascade. Meanwhile, the dimeric βγ subunit diffuses laterally on the intracellular side of the plasma membrane. The βγ subunit may also bind to the nearby G-protein-coupled inward rectifier potassium channel (GIRK), which causes a change in the potassium conductance. Overall, activation of the two postsynaptic receptor families can result in radically different postsynaptic responses in the neuron. Ligand-gated ion channels usually transmit fast post-synaptic effects within a few tens of milliseconds. In contrast, metabotropic receptors, by their association with multiple molecular interactions, produce hundreds of milliseconds of slower effects.

In the central nervous system, nerve cells may receive thousands of inhibitory and excitatory synaptic inputs. The sum of these inputs will determine whether the nerve cell's membrane potential reaches the threshold potential to generate an action potential. Understanding how the brain functions and how nerve cells process input information has always been a central issue in brain research. Most synaptic inputs come from the dendritic segment of nerve cells. Cortical GABAergic inhibitory interneurons provide regulation of timing and efficacy of information flow by inhibition of various structural subunits of principal cells. GABAergic interneurons play an important role in enhancing the accuracy of cell firing, synchronizing network activity, or temporal and spatial regulation of excitability through cortical networks. These cortical processes occur through the integration of inhibitory synaptic inputs on the dendrites.

2. Aims

Myelin sheath injury is a major cause of some neurodegenerative diseases, such as MS, but our knowledge of the process of myelin degeneration is lacking. Recently, the theory that the disease is of autoimmune origin is being questioned. Some studies ask whether the immune response is triggered by selective damage to oligodendrocytes. According to the latter theory, the accumulation of lipid debris from degraded myelin sheath may be critical during disease progression. Therefore, in my dissertation we sought to answer the following questions:

1. Is the CARS method suitable for the examination of lipid-containing structures of living, unstained brain tissue?

2. Is the CARS method capable of monitoring selective damage to the myelin sheath? What is the distribution of the lipid debris accumulation in different brain area?

Summation of ionotropic receptor-mediated responses is extensively studied in the neocortex. However, to date, there has been no experimental analysis of how neurons integrate electrical signals that are linked to inhibitory metabotropic receptors. Among cortical interneurons, neurogliaform cells (NGFC) are especially effective in recruiting metabotropic $GABA_B$ receptors in addition to ionotropic $GABA_A$ receptors using non-discriminatory volume transmission to cover extrasynaptic and synaptic locations of the neuronal microcircuit.

In my doctoral thesis the main questions were the following:

1. Is it possible to characterize the quantal properties of the GABAergic connection established by individual neurogliaform cells?
2. What is the effective signaling range of volume transmission from the presynaptic neurogliaform bouton?
3. What are the structural characteristics of the GABAergic connection established by the population of layer 1 neurogliaform cells?
4. Under physiological in vivo condition, do co-activation of layer 1 neurogliaform cells occur?
5. How do ionotropic and metabotropic receptor-mediated signals integrate from converging neurogliaform connection?
6. What possible mechanisms affect the integration of metabotropic signals?

3. Methods

3.1. Slice preparation

Experiments were conducted to the guidelines of the University of Szeged Animal Care and Use Committee. We used young adult (19 to 46-days-old, (P)23.9±4.9) male Wistar rats for the electrophysiological experiments. Animals were anaesthetized by inhalation of halothane, and following decapitation, 320 µm thick coronal slices were prepared from the somatosensory cortex with a vibration blade microtome (Microm HM 650 V). Slices were cut in ice-cold (4 ^{0}C) cutting solution (in mM) 75 sucrose, 84 NaCl, 2.5 KCl, 1 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 4 $MgSO_4$, 25 d (+)-glucose, saturated with 95% O_2 and 5% CO_2. The slices were incubated in 36^{0}C for 30 minutes, subsequently the solution was changed to (in mM) 130 NaCl, 3.5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$, 10 d (+)-glucose, saturated with 95% O_2 and 5% CO_2, and the slices were kept in it until experimental use. The solution used for recordings had the same composition except that the concentration of $CaCl_2$ and $MgSO_4$ were 3 mM and 1.5 mM unless it is indicated otherwise. The micropipettes (3-5 MΩ) were filled (in mM) 126 K-gluconate, 4 KCl, 4 ATP-Mg, 0.3 GTP-Na_2, 10 HEPES, 10 phosphocreatine, and 8 biocytin (pH 7.25; 300 mOsm).

3.2. In vitro Electrophysiology and Pharmacology

Somatic whole-cell recordings were obtained at ~37 ^{0}C from simultaneously recorded triplets and doublets of NGF and PC cell visualized by infrared differential interference contrast video microscopy at depths 60-160 µm from the surface of the slice (Zeiss Axio Examiner LSM7 (Carl Zeiss AG, Oberkochen, Germany)), 40x water-immersion objective (1.0 NA; Carl Zeiss AG, Oberkochen, Germany) equipped with Luigs and Neumann Junior micromanipulators (Luigs and Neumann, Germany) and HEKA EPC 10 patch-clamp amplifier). Signals were filtered 5 kHz, digitalized at 15 kHz, and analyzed with Patchmaster software.

Presynaptic cells were stimulated with a brief suprathreshold current pulse (800 pA, 2-3 ms), derived in >60 s interval. In the case of 100 Hz presynaptic burst stimulation the interval was increased >300 s. During the stimulation protocol, the order of triggering a set of 1 to 4 APs on the NGFCs were randomized. The postsynaptic responses were normalized to the single AP in each individual set. During postsynaptic current-clamp recording, -50 mV holding current was set. The experiments were stopped if the series resistance (Rs) exceeded 35 MΩ or changed more than 20%. During postsynaptic voltage-clamp recordings, Rs and whole-cell capacitance were monitored continuously. The experiment was discarded if the compensated Rs change reached 20% during recording.

Pharmacological experiments were carried out on NGFC-PC pairs using ACSF with the following drugs: 10 µM SR 95531 hydrobromide (Tocris), 10 µM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) (Tocris), 10 µM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) (Tocris), 10 µM 4-(N-Ethyl-N-phenylamino)-1,2 dimethyl-6-(methylamino) pyrimidinium chloride **(**ZD7288) (Sigma-Aldich), 10 µM 1-[2-[[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride hydrochloride (NO711) (Sigma-Aldrich).

We performed Bayesian quantal analysis (BQA) by altering the extracellular Ca^{2+} and Mg^{2+} in two different conditions. During BQA experiments the ACSF solution contained the following substances: 10 μM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) (Tocris), 10 μM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) (Tocris). Each epoch of the BQA experiment contains a stable segment of 28 up to 42 unitary IPSCs (mean 32.75±4.15). BQA experiments required at least 60 min of recording time (up to 90 minutes). We tested all epochs for possible long-term plasticity effect by measuring the linear correlation between IPSCs amplitude and elapsed time during the experiment, and we found no or negligible correlation (Pearson's r values from all of the experiments (n= 8) were between -0.39 and 0.46, mean -0.01±0.29).

3.3. Immunohistochemistry and anatomical analysis

After electrophysiological recordings slices were fixed in a fixative containing 4% paraformaldehyde, 15% picric acid and 1.25% glutaraldehyde in 0.1 M phosphate buffer (PB; pH= 7.4) at 4°C for at least 12 hr. After several washes in 0.1 M PB, slices were cryoprotected in 10% then 20% sucrose solution in 0.1 M PB. Slices were frozen in liquid nitrogen then thawed in PB, embedded in 10% gelatin and further sectioned into slices of 60 μm in thickness. Sections were incubated in a solution of conjugated avidin-biotin horseradish peroxidase (ABC; 1:100; Vector Labs) in Tris-buffered saline (TBS, pH= 7.4) at 4°C overnight. The enzyme reaction was revealed by 3'3-diaminobenzidine tetrahydrochloride (0.05%) as chromogen and 0.01% H_2O_2 as an oxidant. Sections were post-fixed with 1% OsO_4 in 0.1 M PB. After several washes in distilled water, sections were stained in 1% uranyl acetate, dehydrated in ascending series of ethanol. Sections were infiltrated with epoxy resin (Durcupan) overnight and embedded between glass slides. Three dimensional light microscopic reconstructions were carried out using Neurolucida system with a 100x objective.

3.4. Surgery for imaging experiments

Experiments were conducted to the guidelines of the University of Szeged Animal Care and Use Committee. Young adult (22 to 28 days old, (P)24.75±2.75) male Wistar rats were initially anaesthetized with halothane before urethane anaesthesia (1.4 g/kg of body weight) was administrated intraperitoneally. Body temperature was maintained at 37^0C with a heating pad (Supertech Instruments, Hungary). Before surgery dexamethasone sodium phosphate (2 mg/kg of body weight) was administrated subcutaneous, and carprofen (5 mg/kg of body weight) was administrated intraperitoneally. Anaesthetized animals head were stabilized in a stereotaxic frame and headbars were attached to the skull with dental cement (Sun Medical, Japan). Circular craniotomy (3 mm diameter) was made above the primary somatosensory cortex, centered at 1.5 mm posterior and 2.2 mm lateral from the bregma with a high-speed dental drill (Jinme Dental, China). Dura mater was carefully removed surgically. Finally, the craniotomy was filled with 1.5% agarose and covered with a coverslip to limit motion artifacts. The craniotomy was then submerged with HEPES buffered ACSF recording solution containing (in mM) 125 NaCl, 3.5 KCl, 10 HEPES, 1 $MgSO_4$, 1 $CaCl_2$, 0.5 d (+)-glucose, pH= 7.4.

3.5. Two photon calcium imaging in L1

Before covering the craniotomy with the coverslip calcium indicator Oregon Green 488 BAPTA-1 AM (10mM) (OGB-1 AM, Thermo Fisher Scientific), and astrocytic marker sulforhodamine 101 (1 μM) (SR101, Thermo Fisher Scientific) were pressure-injected with a glass pipette (1-2 MΩ) in L1 cortical region under the visual guide of Zeiss Axio Examiner LSM7 (Carl Zeiss AG, Oberkochen, Germany) two-photon microscope using 40x water immersion objective (W-Plan, Carl Zeiss, Germany). Subsequently, the craniotomy was filled with agarose and covered with a coverslip. Imaging experiments were performed 1 hour after preparation. The activity of L1 interneurons was monitored during ipsilateral hindlimb electrical stimulation (Digimeter, United Kingdom, 200 mA, 10 ms). OGB-1 AM was excited at 800nm wavelength with a femtosecond pulsing Ti:sapphire laser (Mai Tai DeepSee (Spectra-physics, Santa Clara, USA)). In the somatosensory hindlimb region, Z-stack image series (volume size 304μm x 304μm x 104 μm) were acquired. Calcium signals from interneurons were obtained within this volume in full-frame mode (256x100 pixel), acquired at a frequency of ~20 Hz.

At the end of the experiments, few L1 neurons were filled with biocytin containing intracellular solution to make the immunohistochemical remapping easier.

3.6. Tissue preparation for immunohistochemistry

After imaging experiments rats were deeply anaesthetized with ketamine and xylazine. Subsequently, perfusion was performed through the aorta, first with 0.9% saline for 1 min, then with an ice-cold fixative containing 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH=7.4) for 15 min. The whole brain was extracted and stored in 4% paraformaldehyde for 24 hours, afterward in 0.1M phosphate buffer (pH=7.4) until slicing. Later 60 μm thick sections were cut from the same two-photon Ca^{2+} imaged brain area parallel to the pia mater and washed overnight in 0.1 M PB.

3.7. Fluorescence immunohistochemistry and remapping

After several washes in 0.1 M PB, slices were cryoprotected with 10% then 20% sucrose solution in 0.1M PB than frozen in liquid nitrogen. The sections were incubated for two hours in Alexa-488 conjugated streptavidin (1:400, Molecular Probes) solved in Tris-buffered saline (TBS, 0.1 M; pH=7.4) at room temperature to visualized the biocytin labeled cells. After several washes in TBS, sections were blocked in normal horse serum (NHS, 10%) made up in TBS, followed by incubation in mouse anti-alfa-actinin (1:20000, Sigma-Aldrich) diluted in TBS containing 2% NHS and 0.1% Triton X-100 at room temperature for 6 hours. Following several washes in TBS, Cy3 conjugated donkey anti-mouse (1:500, Jackson ImmunoResearch) secondary antibody was used to visualize the immunoreactions. After several washes in TBS then in 0.1 M PB, slices were counterstained with DAPI (4′ ,6-diamidino-2-phenylindole, ThermoFisher Scientific). Sections were then mounted on slides in Vectashield (Vector Laboratories). Images were taken with LSM 880 confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany) using 40X oil-immersion objective (1.4 NA). Confocal image z-stack was tilted and panned manually to match with the *in vivo* two-photon z-stack, allowing to profile imaged interneurons. During this process, biocytin labeled neurons were used as a reference point.

3.8. Data analysis

Electrophysiological data were analyzed with Fitmaster (HEKA), Origin 7.5 (OriginLab), IgorPro (Wavemetrics). BQA experiments were analyzed using a Python written program, incorporating NumPy and SciPy packages. Two-photon calcium imaging data were acquired with ZEN 2 (Zeiss) and analyzed with MATLAB (The MathWorks), using Statistical Toolbox, Image Processing Toolbox, and custom written scripts.

3.9. Statistics

Data are represented as mean±standard deviation (SD), statistical test were defined for each paradigm; result were considered significantly different if $p<0.05$.

3.10. MCell model construction

Simulation of $GABA_B$ receptor- GIRK channel interaction was carried out with MCell v3.4 (www.mcell.org). We used a 3D reconstruction of a dendritic structure based on a series section of electron microscopic data, available from VolRoverN program. To simulate extracellular space, and achieve proper tortuosity we created an array of cubic cell containing cavities. The cubic cells have 800x800 nm length, containing a cavity that is 400x400 nm wide and 340 nm deep. The cubic cells and the dendritic segment were spaced 32 nm apart. Custom Matlab scripts created the MDL (Model Description Language) file that required for MCell simulation. MCell simulated the release and diffusion of GABA, $GABA_B$ receptors and GIRK channel interaction.

First, to manage a biological like distribution for the receptors and channels a reaction cascade was used. At the beginning of every iteration, primary seed particles were placed on the dendritic membrane. Primary seed particles subdivide into secondary seed particles, that which then produce $GABA_B$ receptor or GIRK channel clusters. Those secondary seed particles that produce the GIRK channel clusters - which contain 1 to 4 channels - were immobile in the membrane. Meanwhile, the secondary seed particles that produce $GABA_B$ receptor clusters can diffuse laterally in the membrane. Optimization algorithm based on simulated annealing technique was written in Matlab for approximating the optimal values for the delay and the forward rate of the reaction, producing $GABA_B$ receptor clusters containing 1 to 8 receptors. Optimal values of delay and the forward rate of the reaction was set to allow secondary seeds, that generate $GABA_B$ receptor clusters to diffuse to a specified distance, resulting in the required $GABA_B$ receptor-GIRK channel cluster distribution as seen in Kulik et al. 2005.

Since we were interested in the interaction between the $GABA_B$ receptors and GIRK channels, our model does not include $GABA_A$ receptors and GABA amino transporters. There is no experimental data regarding possible neurotransmitter concentration from a single release site of NGFCs into the extracellular space, we set the concentration to ~1 mM (3750 GABA molecules) similar to what occurs in traditional synapses.

Up to 6 MCell simulations were run with 1μs time steps in parallel on pc with Intel(R) Core i7-4790 3.6 GHz CPU, 32 GB RAM. Total of 3322 iterations was simulated.

3.11. Animals, cuprizone treatment and brain slice preparation.

All procedures were performed with the approval of the University of Szeged and in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. C57BL/6 male mice were used in this study obtained from Charles River (Germany). Animals had ad libitum access to standard mouse chow and water. 8 weeks old mice were treated with cuprizone (Sigma-Aldrich) to induce demyelination for up to 5 weeks. Cuprizone was mixed to chow for two different groups of mice treated with 0.2% and 0.6% concentrations. To assess disability of treated animals we examined motor behavior and used the following clinical score: 0 - no sign of the disease; 1 - loss of tail tone; 2 - hind limb weakness or partial paralysis; 3 - complete hind limb paralysis and paresis; 4 - hind and front limb paralysis; 5 - death.

Mice were anaesthetized by intraperitoneal injection of ketamine (30 mg/kg) and xylazine (10 mg/kg), and following decapitation, coronal slices (350 μm thick) were prepared from the somatosensory cortex. Slices were incubated at room temperature for 1 hour in a solution composed of (in mM) 130 NaCl, 3.5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$, 10 D(+)-glucose, saturated with 95% O_2 and 5% CO_2. The solution used during recordings differed only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$. During imaging, slices were kept at ~35˚C.

3.12. Coherent anti-Stokes Raman scattering (CARS) Imaging.

A prototype of our experimental setup for CARS imaging was detailed earlier (Haluszka et al., 2015). Our present system included a FemtoCARS Laser Unit (R&D Ultrafast Lasers Ltd., Hungary), Axio Examiner LSM 7 MP laser scanning two-photon microscope (Carl Zeiss, Germany) using 40x water immersion objective (W-Plan, Carl Zeiss, Germany) driven by MaiTai femtosecond pulsing Ti:sapphire laser (SpectraPhysics, Santa Clara, USA). Anti-Stokes frequency was generated by combining 796 nm pump and the 1,028 nm Stokes pulses using a dichroic mirror to excite vibration frequency of CH_2 bond, which is present abundantly in saturated fatty acids offering chemical selectivity to lipids.

3.13. Immunofluorescence Labeling

Slices were incubated in a fixating solution which contained 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH=7.4) at 4°C for 3 hours. After several washes in 0.1M PB, slices were cryoprotected with 10% then 20% sucrose solution in 0.1M PB. 200 μm thick slices were frozen three times in liquid nitrogen then embedded in 10% gelatin and further sectioned to 50 μm thick slices. The sections were incubated for one hour in 1 μg/ml BODIPY 493/503 (Thermo Fisher Scientific) in tris-buffered saline (TBS, 0.1M; pH=7.4) at room temperature. The BODIPY 493/503 stock solution (1 mg/ml) was prepared in DMSO. After several washes in 0.1M PB, all sections were mounted in Vectashield (Vector Laboratories) on slides. BODIPY 493/503 fluorescence was visualized using an LSM 880 confocal laser scanning microscope (Carl Zeiss, Germany) with a x40 objective.

3.14. Image analysis

Intensity measurements in acquired images were analysed in ZEN (Carl Zeiss, Germany) and ImageJ (NIH, USA) softwares using images of 20 μm depth from the surface of the brain slice. G ratio was calculated as the relation between the inner axonal diameter and outer fiber diameter determined CARS images. Distinct longitudinal fibers parallel with the image plane were selected without signs of swelling or degeneration. Z-stack images were made with 1 um step size. Diameters of fibers were acquired from intensity profiles using custom - written script in Igor (Wavemetrics, Lake Oswego, USA) by measuring at the level of 70% of the amplitude. Intensity profile of fiber was measured perpendicular of the middle plane of the fiber. For detecting and measuring number and volume of lipid debris we used a custom-written image stack analysis program using C++ language. The program after preprocessing and image thresholding, performed 3D particle separation considering that particles are distinct and globose shaped. Then the volume of separated particles was determined based on pixel content.

4. Results and Discussion

4.1.1. Quantitative analysis of lipid debris accumulation induced by myelin degradation in the central nervous system areas

We focused on monitoring myelin sheath breakdown using *in situ,* nondestructive CARS imaging on different brain areas in *ex vivo* brain slice preparations. We analyzed baseline lipid content in different brain areas: subgranular layers of the somatosensory cortex and white matter tracts of the mouse brain: corpus callosum, forceps minor, anterior commissure, and cerebellar white matter. As expected, the corpus callosum, being the most heavily myelinated area, produced the most intense CARS signals compared to other brain areas, and the relatively scarcely myelinated grey matter showed the lowest CARS intensity. Oligodendrocytes have a high lipid content (70–85%) therefore apoptosis of these cells and breakdown of the myelin sheath leads to the accumulation of lipid debris. The method applied here has several advantages relative to earlier approaches. Signal detection with CARS microscopy allows direct and quantifiable lipid measurements. Moreover, CARS microscopy can be performed in living tissue *in situ* without tissue damage due to the use of multiphoton lasers by tuning our setup for the detection of CH_2 molecular bonds which occur predominantly in lipids. Furthermore, this method is free of potentially lipid damaging chemical post processing required for immunocytochemical or histochemical studies. Cuprizone treatment applied to initiate selective oligodendrogliopathy in mice is an effective, well-described and widely used model of MS. Our experiments show cuprizone induced oligodendropathy and myelin destruction using a noninvasive method of detection *in situ*. We also confirmed demyelination by measuring single fiber thickness and calculating G-ratios. Taking advantage of our quantifiable method, the spatial distribution of lipid granules were found less extent in two weeks long treatment. Proportionally in four weeks long treatment, lipid droplets were found to be clustered with a few scattered single particles. Clustered lipid droplets observed here are reminiscent of phagocytosed lipid debris detected by electron microscopy in samples taken at progressed stages of MS and similar to findings documented by immunocytochemistry on biopsies from human patients. Here we show that in the early stage of demyelination the decrease of the thickness of myelin sheath yet was not statistically detectable measure in the corpus callosum, however, debris formation has been already detectable and reached a significant rate. During cuprizone treatment, our results show the gradual accumulation of lipid droplets over time which can turn into a subsequent agglomeration at later stages. Recent observations

demonstrating the lack of immune response against slowly degrading myelin during the first 40 weeks of oligodendrocyte loss and demyelination suggest that the onset of oligodendrocyte death might be independent of the complement system. Clustering of lipid droplets is consistent with the involvement of macrophages and generation of inflammation. Our measurements suggest that oligodendrocyte death may be followed by the gradual accumulation of sporadic lipid droplets, which eventually can lead to phagocyted lipid droplet agglomeration in macrophages and onset of inflammation.

4.2.1. Quantal and structural characteristics of GABAergic connections established by individual neurogliaform cells

First, we characterized the structural and functional properties of the inhibitory connections formed by individual NGF cells. Paired whole-cell patch-clamp recordings were performed *in vitro* on layer 1 NGF and layer 2/3 pyramidal cells in rat somatosensory cortex. Quantal parameters of the inhibitory connection established by NGF cells were estimated. In addition to functional characterization, three-dimensional light microscopic anatomical reconstructions were made for the structural mapping of NGF's inhibitory connections. The results of correlated functional and structural studies confirmed previous theories about the presence of volumetric signal transduction and based on our experimental results we estimated that the effective range of GABA released from the presynaptic terminal of NGF is ~ 2μm.

4.2.2. Structural characteristics of GABAergic connections established by the population of layer 1 neurogliaform cells

Following the characterization of the inhibitory connection of individual NGF cells, we characterized the structural properties of GABAergic connections established by the population of layer 1 neurogliaform cells. With anatomical measurements, we defined the distribution of the axon network and the bouton frequency along the axon and the distribution of NGF cells in the superficial cortex. With this data, the convergent inputs of the entire upper layer NGF cell population were quantified to potential postsynaptic voxel. Our results suggest that, given the relatively large effective signal transduction distances in the cerebral cortex, a maximum of two NGF cell inputs can converge in a significant portion of the extracellular space (83.04 ± 5.56%). Furthermore, only a fraction of the extracellular space (16.95 ± 5.56%) accessible to more than two NGF cells, even if the entire NGF cell population is active at the same time.

4.2.3. Coactivation of neurogliaform cell population in layer 1 somatosensory cortex in vivo

At present, there is a lack of information about the NGF cell population's *in vivo* activity. It is not known whether a coordinated activity exists in the cortex that enables the convergent inputs. To investigate this we performed *in vivo* two-photon Ca^{2+} imaging experiments in the somatosensory brain area, during which we monitored the activity of the upper layer NGF cell population during ipsilateral limb stimulation. Our experiments confirmed the active involvement of NGF cells in the interhemispheric inhibition process. After triggering activation of callosal inputs that provide communication between the two hemispheres with sensory stimulation, we observed coordinated activation in ~70% of the total NGF cell population in layer 1. Our results so far suggest that the first layer of NGF cell population can uniformly, and cooperatively inhibit post-synaptic target cells via ionotropic and metabotropic GABA receptors under physiological conditions.

4.2.4. Summation of convergent, unitary IPSPs elicited by NGFC

To investigate the converging responses induced by ionotropic and metabotropic receptors, direct electrophysiological experiments were addressed, simultaneous triplet whole-cell patch measurements were performed on two layer 1 presynaptic NGF cells and a layer 2/3 pyramidal cells that receive converging inhibitory inputs. Our measurements showed sublinear summation of fast, ionotropic $GABA_A$ receptor-mediated postsynaptic potentials. However, slow, metabotropic $GABA_B$ receptor-mediated responses showed different linear summation. Previous studies based on computer simulation have predicted strong supralinear interactions in metabotropic receptor summation due to signal amplification effects on the G protein system. Pharmacological experiments have been performed to understand the possible mechanisms that influence the predicted supralinear integration. Our results suggest that possible HCN1 channel activity or extracellular GABA uptake does not influence the integration of $GABA_B$ signals on the postsynaptic cell.

4.2.5. Subcellular localization of $GABA_B$ receptor-GIRK channel complex determines summation properties

On the postsynaptic cell, the $GABA_B$ receptor and the GIRK channel show a subcellular compartment-dependent distribution, with a dense co-localization on the dendritic spike and a

more segregated arrangement on the surface of the dendritic shaft. Consequently, we aimed to determine whether receptor-channel complex location affects the integration of metabotropic signals. We created a complex computer simulation environment that included an ultrastructural reconstruction of a dendritic section, potential NGF cell neurotransmitter release sites, and appropriately parameterized extracellular space. $GABA_B$ receptor-GIRK channel clusters with compartment-dependent distribution were placed on the membrane surface of the dendritic region. In this realistic spatial simulation environment, the stochastic motion of the particles and their interactions were simulated with MCell v3.4. Our results suggest that the complex distribution of the receptor-channel alone affect signal integration: in the case of a close location, there is a supralinear shift, whereas in a more distant location a sublinear shift is observed.

5. List of scientific publications

MTMT identification number: 10053020

Publication related to the thesis:

Ozsvár A, Szipőcs R, Ozsvár Z, Baka J, Barzó P, Tamás G, Molnár G. (2018) Quantitative analysis of lipid debris accumulation caused by cuprizone induced myelin degradation in different CNS areas. Brain Res Bull.;137:277-284. doi: 10.1016/j.brainresbull.2018.01.003.

IF:3.44

Ozsvár, A., Komlósi, G., Oláh, G., Baka, J., Molnár, G., Tamás, G. (2019) Superficial neurogliaform cells activation leads to different summation of ionotropic and metabotropic GABA receptor mediated postsynaptic responses.

IF: -

Publication not related to the thesis:

Boldog E, Bakken TE, Hodge RD, Novotny M, Aevermann BD, Baka J, Bordé S, Close JL, Diez-Fuertes F, Ding SL, Faragó N, Kocsis ÁK, Kovács B, Maltzer Z, McCorrison JM, Miller JA, Molnár G, Oláh G, **Ozsvár A**, Rózsa M, Shehata SI, Smith KA, Sunkin SM, Tran DN, Venepally P, Wall A, Puskás LG, Barzó P, Steemers FJ, Schork NJ, Scheuermann RH, Lasken RS, Lein ES, Tamás G. (2018) Transcriptomic and morphophysiological evidence for a specialized human cortical GABAergic cell type. Nat Neurosci.;21(9):1185-1195. doi: 10.1038/s41593-018-0205-2.

IF: 19.912

Faragó N, Kocsis ÁK, Braskó C, Lovas S, Rózsa M, Baka J, Kovács B, Mikite K, Szemenyei V, Molnár G, **Ozsvár A**, Oláh G, Piszár I, Zvara Á, Patócs A, Barzó P, Puskás LG, Tamás G. (2016) Human neuronal changes in brain edema and increased intracranial pressure. Acta Neuropathol Commun.;4,78. doi: 10.1186/s40478-016-0356-x.

IF:5.414