Different Aspects of Electrophysiological and Quantitative Electron Microscopic Investigations in the Rat and the Human Neocortex

Theses of PhD Dissertation

Estilla Zsófia Tóth

Neurosciences ("János Szentágothai") Doctoral School
Semmelweis University

Supervisor: Lucia Wittner, D.Sc
Official reviewers: Márk Kozsurek PhD
Hajnalka Ábrahám PhD
Head of the Complex Examination Committee:
Alán Alpár D.Sc
Members of the Complex Examination:
Árpád Dobolyi, PhD
Borhegyi Zsolt, PhD

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1. Introduction

Nowadays, we have the opportunity to choose from a wide range of methods for investigating the brain. With technological advances, more and more rather complicated methods became widely available. It is crucial to choose the proper method for the right questions or combine multiple approaches.

In the two studies I presented in my dissertation, we have used a rodent model for testing the in vivo biocompatibility of a novel material that can be used for preparing electrodes used in neuroscience research and clinical application on human patients. This project was also deemed perfect for testing our automated cell counting method and quantitative electron microscopy method, which can be proven helpful in many of our studies regarding the human brain.

Animal models are highly valuable in research since many methods can't be applied to human patients in vivo or the samples in vitro. But they are also limited in nature since the human brain differs from the most widely used rodent models and shows numerous unique qualities. Because of this, the translation of findings from animal models of diseases to the human equivalent is often unsuccessful.

One possibility to reduce the failure ratio of the translation is not just the development of novel models but the use of human brain samples in research. Our research team has the opportunity to receive surgically removed human brain samples from the National Institute of Mental Health, Neurology and Neurosurgery (OMIII) as part of a long-standing collaboration. The most significant value of these samples compared to post-mortem human samples is that we can carry out in vitro electrophysiological investigations followed by post hoc anatomical examinations.

We have the opportunity to receive samples from patients with drug-resistant epilepsy and those who had to undergo surgeries due to a brain tumour but did not show any sign of epilepsy or a seizure. With these two groups, we can discover the changes possibly caused by epilepsy.

2. Objectives

2.1 Su8 Biocompatibility in vivo

In vivo and vitro electrophysiological recordings are essential both in research and, in many cases, in clinical applications.

Our Objectives in this study were to investigate the changes caused by a chronic 2-month long in vivo implantation of a device made from the SU-8 material into rat neuronal tissue. This material can be a good candidate for manufacturing micro electrodes because of its cost-effectiveness, photoactivity (for easy manufacturing), presumed non-toxicity, high flexibility and transparency.

Our research group carried out anatomical investigations on the light microscopic level.

Based on these findings, we realised the need for a quantitative electron microscopic study.

My objectives in this study were:

How does the general ultrastructure of the tissue change? Are the synapses preserved around the track in close vicinity to allow us to record neuronal signals?

Is the glial scar that was observed at the light microscopic level actually as profound as it seems at the light microscopic level or not? How many problems would this encapsulation cause in chronic implantations?

2.2 Examination of the perisomatic inhibition of Layer 2/3 pyramidal cell in the epileptic and non-epileptic human brain

As a continuation of previous studies in our lab, we aimed to investigate the Spontaneous Population Activities (SPAs) in the human neocortex in vitro, with a focus on the involvement of perisomatic inhibitory cells.

We investigated two non-overlapping perisomatic inhibitory cell types, the parvalbumin-positive (PV) and the Cholecystokinin/Type 1 Cannabis Receptor (CCK/CB1R)-positive inhibitory cells.

We carried out extracellular in vitro electrophysiological recordings and pharmacological modification with 3 drugs to investigate the PV+ and CB1R+ cells' role in the generation of SPAs. We used the non-selective acetylcholine receptor agonist carbachol to block perisomatic inhibition, then selectively removed the two cell types from the effects of it. To target the PV+ cells, we used AFDX-116 to block the M2 receptors on them. To target the CCK/CB1R+ cells, we used AM-251, a CB1R antagonist, to block the indirect effect of Cch on these cells.

We aimed to investigate how these pharmacons affect the SPAs in epileptic and non-epileptic samples.

To further explore the possible changes in the perisomatic inhibition in the epileptic samples and the areas presenting SPAs, we carried out quantitative electron microscopic investigations with PV and CB1R immunostained samples separately. The SPAs were most often present in the supragranular layers of the neocortex. Hence, we chose layer 2/3 as our region of interest.

We investigated the cell bodies of the pyramidal cells located in these layers and measured the synaptic contacts made on them. We measured the amount of inhibitory contacts coming from a stained and unstained terminal, the overall coverage, the lengths of synaptic active zones and their number in 100 μm of soma perimeter.

3. Methods

3.1 Biocompability of The SU-8 material

The in vivo biocompatibility of the SU-8 probes was tested by a two-month-long implantation into both hemispheres of the brains of 31 Wistar rats. After the implantation period, the animals were anaesthetised and perfused with 4% paraformaldehyde. The samples were cut into 60μm thick horizontal sections perpendicular to the electrode track. The slices were immunostained against either NeuN (Neuronal Nuclei) or GFAP (Glial Fibrillary Antibody Protein) to visualise the neurones or glial cells, respectively.

Automated cell counting (NeuN) and pixel density measurements (GFAP) were carried out at the light microscopic level.

I carried out the subsequent electron microscopic investigations. We measured the thickness of the glial scar around the electrode track and the overall quality of the tissue. Based on the distance from the track, we have also measured the number of synaptic connections in the neuropil and the amount of glial elements.

3.2 Investigations of the Human Neocortex

We have received surgically removed neocortical tissues from patients undergoing brain surgery (ETT TUKEB 20680-4/2012/EKU).

In this study, we used tissue from 13 non-epileptic patients (ages: 32-81 (60.5±14.2, n=4 frontal, n=5 temporal, n=3 parietal and n=1 occipital lobes), who had undergone surgeries to treat their brain tumours. These patients did not have a history of epilepsy or seizures. Based on preoperative imaging studies (MR), the obtained tissue was outside of the tumour zone in every case.

We have used tissue from 12 epileptic patients (ResEpi), ages: 28-53 (mean±st.dev: 37.8±7.7) (Table 2), suffering from pharmaco-resistant epilepsy for 22±15 years on average. One sample was from the frontal cortex and 11 from the Temporal lobe.

We have cut 500 μm thick slices from tissue blocks using a Leica VT1000S vibratome in an ice-cold oxygenated sucrose-based cutting solution. We only used slices that contained all 6 cortical layers.

We have recorded the extracellular Local Field Potential gradient (LFPg) with a 24-channel laminar microelectrode in ACSF (Artificial Cerebrospinal Fluid).

When we were able to record stable SPAs, we applied different pharmacological agents targeting the perisomatic inhibitory cells.

First, we applied carbachol (5 μM in ACSF), a non-selective acetylcholine receptor agonist. After a washout period with ASCF, we used different pharmacons targeting the various subtypes of perisomatic inhibitory cells. For investigating PV-positive inhibitory cells, we used AFDX-116 (10 μM), an M2 muscarinic acetylcholine receptor agonist. To target CB1R/CCK positive cells, we used AM-251(1 μM), a CB1R antagonist.

After the pharmacological experiments, we fixed the samples in 4% paraformaldehyde. The fixed samples were recut into 60μm thick samples for post hoc anatomical examinations.
We have carried out immunohistochemistry against Parvalbumin or Type 1 Cannabis Receptor (CB1R) proteins.
We have investigated the samples at light and electron microscopic levels.
For the quantitative electron microscopic investigations, we chose the L2/3 since the SPAs are more often present in the superficial layers.
We have measured the average perisomatic coverage (μm active zone/100μm soma perimeter), the average length of the synaptic active zones and the average number of synapses.

4. Results

4.1 Biocompability of The SU-8 material

At the light microscopic level, we have analysed the data based on its distance from the probe. As expected, the astroglia cells showed a relatively uniform distribution across the different cortical layers. Meanwhile, the neurons have shown a rather characteristic layered distribution.
The decrease in neuronal density was considerable in the two closest ROIs, 24% at 20-40 μm and 74% between 40-60 μm compared to the control region (200-400 μm, 1.00±0.22). At a further distance, the neuronal density was not significantly different.
Regarding the glial cells, we found a difference based on the cortical depth in the glial scar. We examined layers 1-3 and 4-6 in two different groups. We have found a significant difference between the superficial and deep layers up to 240 μm from the track, and it was more pronounced in the superficial layers. At longer than 240 μm distance, there were no significant differences between layers 1-3 and layers 4-6.

Compared to the control areas found, significantly stronger glial staining up to 560 μm from the track in the supragranular layers (0.29±0.14 at 20-40 μm and 0.98±0.06 at
520–560 μm from the probe).
In the case of the deep layers, we found a significantly stronger staining up to 480 μm from the probe compared to the control areas (0.44±0.21 at 0–40 μm and 0.99±0.18 at 440–480 μm distance, $p<0.05$).

To gain a deeper understanding of the structural changes around the probes, we carried out transmission electron microscopic investigation.
We found a 2.5-15 μm thick glial scar around the probe in all 3 samples. In close vicinity to the probe, we have seen a high number of damaged elements in the neuropil. The characteristics of these elements were disrupted membranes with a low number of cellular organelles or a complete lack of them. This suggests that these elements were not able to function properly.
We found numerous caverns in the tissue up to 30-50 μm from the track as a sign of tissue damage. Up to a 10-40 μm distance, we could find neuronal cell bodies, but they had a distorted shape (oval somas, presumably from the compression caused by the implant). At distances greater than 50-100 μm, we found round, seemingly healthy-looking NeuN-stained cell bodies. The caverns were sporadic at this distance. The tissue has also shown a great number of healthy elements, such as dendrites, axons, and synapses.
Notably, the caverns were not detectable at the light microscopic level. At distances lower than 5 μm, we did not see synapses (due to the glial scar), from 6-12 μm, the contacts were visible occasionally. At this distance (0-12 μm), the number of synaptic elements was significantly lower, at 0-12 μm, it was 6.44±13.05/100 μm^2 while at 180–192, it was 36.45±10.19/100 μm^2 ($p<0.005$). Between 12-24 μm from the probe, the density was still decreased, but this was not significant. At

distances from 24 μm to 192 μm, the number of synaptic elements was comparable to the control area.

Notably, close to the track (less than 30 μm distance), some synapses had shown signs of damage as incomplete membranes of the pre/post-synaptic element, but the synaptic cleft was preserved. We did not find any damaged synapses over 60 μm from the track.

We quantified the amount of glial elements in the neuropil based on the distance from the probe.

We found a significant increase up to 24 μm from the probe. At 0-12 μm, it was more pronounced, with 0.16±0.004 μm^2 area of glial elements / μm^2 area and 0.009±0.003 μm^2 at 12-24 μm ($p<0.05$ in both cases when compared to the control area at 160-180 μm 0.003±0.002 μm2), between 24 μm and 180 μm the amount of glial elements were comparable.

Notably, even at close distances, the space taken up by glial elements was only 16% of the neuropil.

4.2 Investigations of the Human Neocortex

We have investigated neocortical samples from NoEpi (12 slices from 8 patients) and ResEpi patients (8 slices from 6 patients). We have recorded SPA in ACSF as expected based on previous research.

SPA events can occur in all cortical layers. In this study, in 11 out of 12 NoEpi and 6 out of 8 ResEpi cases, we have recorded the SPA in the supragranular layers (Layers 1-3) and in the remaining cases in the granular-infragranular layers.

The recurrence frequency of the events was 1.37 [0.74 – 1.76] Hz in NoEpi and 0.88 [0.67 – 1.84] Hz in ResEpi samples. It did not differ significantly.

The LFPg amplitude was significantly higher in the ResEpi samples ($p=0.035$), in the NoEpi cases, it was 36.30 [25.01 – 46.44] μV, and in the ResEpi, it was 66.01 [36.46 – 106.49] μV.

The MUA activity was also significantly different ($p=0.035$), it was 0.67 [0.56 – 1.10] μV in NoEpi and 3.12 [1.82 – 5.36] μV in ResEpi, the latter being significantly higher.

First, we applied carbachol (Cch, NoEpi n=12, ResEpi=8 slices) in 5 μM concentration (in ACSF) to decrease the GABA release from the perisomatic inhibitory cells.

In NoEpi slices, we have recorded a slight decrease in the recurrence frequency of the SPAs (1.24 [0.59 – 1.57] Hz) to 80 [72 – 95] % of the baseline, although this was non-significant. In the case of ResEpi slices, the recurrence frequency of the SPAs was significantly lower, only 9 [3 – 27] % of the baseline (0.10 [0.02 – 0.37] Hz, p=0.006).

Changes in the LFPg amplitude were significant in both patient groups. In NoEpi cases, it has decreased to 68 [63 – 77] % (23.42 [20.00 – 31.01] μV, p=0.002). While in the ResEpi samples, it was 37 [25 – 45] % (18.10 [12.20 – 31.21] μV, p=0.004). It is visible that the changes in LFPg amplitude were more pronounced in the ResEpi samples, but this was non-significant compared to the NoEpi slices (p>0.05).

We have found that the MUA also decreased significantly in Cch compared to the baseline in both groups. In the NoEpi group, it was 81 [57 – 107] % (0.67 [0.46 – 0.85] μV), while in the ResEpi one, it was 43 [22 – 85] % (1.21 [0.41 – 3.03] μV,).

We have been able to wash out the effects of Cch to the levels of the baseline recordings regarding all three aspects of the events.

For the investigation of the PV-positive cells, we have used AF-DX 116 (NoEpi n=6, ResEpi n=4 slice) since these cells express the M2 type of mAChRs on their axon terminals, so blocking these should prevent the effects of Cch on them. We have used AF-DX 116 in 10 μM concentration combined with 5 μM Cch on 6 NoEpi and 4 Res Epi slices.

In the NoEpi group, the recurrence frequency decreased non-significantly, which is similar to the effect of Cch alone. In the case of the ResEpi samples, the AF-DX116 were more effective in preventing the impact of Cch 83 [71 – 96] % (0.90 [0.87 – 1.07]) compared to the baseline.

We have found that the LFPg amplitudes were less affected than in applying only Cch. It was 92 [75 – 96] % (34.28 [28.52 – 39.61] μV)

in the NoEpi group and 87 [83 – 91] % (63.87 [53.55 – 76.51] μV) in the ResEpi one. The effects were only significant in the case of the Res Epi group (p=0.028).

The changes in the MUA amplitude were not significant.

To prevent the effects of Cch on the CB1Rs, we have applied their selective agonist AM-251 in 1 μM concentration together with 5 μM Cch (n=5 slices both in NoEpi and ResEpi).

We have found that the recurrence frequency of SPAs in NoEpi slices remained the same as in ACSF 88 [84 – 97] % (1.47 [1.03 – 1.50] Hz). In the case of the ResEpi samples, we did not find significant changes compared to the application of Cch alone.

Regarding the LFPg amplitudes, the effects were not significantly different from the Cch application alone. We also investigated the effects of AM-251 on the LFPg amplitude of the single SPA events, we found that it was significantly higher in both patient groups compared to the application of only Cch ($p < 0.0001$), although the effect was still significant compared to the baseline ($p < 0.0001$).

When we investigated the MUA amplitudes, we found no significant changes.

In summary, regarding the recurrence frequency, we only found a tendency to recover by the application of AM-251 in the ResEpi samples compared to the effects of only the Cch.

We investigated 12 NoEpi and 12 ResEpi neocortical slices with parvalbumin immunostaining. The PV antibody stained non-pyramidal-shaped cells in all 6 layers of the neocortex, with a significant axonal cloud around layer 3, as expected.

The CCK/ CB1R positive interneurons are a non-overlapping perisomatic inhibitory cell type (compared to PV-positive cells). In the neocortex, CB1R can only be found in symmetric (presumably inhibitory) synapses in rodents and primates.

We carried out CB1R immunostaining in samples from 9 NoEpi and 8 ResEpi patients. We have found the axonal clouds to be homogenous in both groups and in all cortical layers. In the parietal cortex, we

found a denser axonal cloud in layer 4, while in the temporal cortex, it was located in layers 1-2.

Since we have been able to record the SPAs most frequently from the supragranular layers, we have chosen layers 2-3 for the quantitative electron microscopic investigations.
We have investigated the PV- and the CB1R-stained samples separately to determine the ratio of the two groups. We were also interested in the changes related to the ability to generate synchronous activity, so we have chosen to investigate two regions of interest (ROIs) from each slice, one where we recorded SPA and one where it was not present. We have used 6 slices from both PV- and CB1-stained samples (3-3 slices NoEpi and ResEpi), investigating two ROIs in each slice (24 ROI from 12 slices).
We have found only symmetric (presumably inhibitory) synapses terminating on the cell bodies. We calculated each pyramidal cell's synaptic coverage (µm synaptic active zone/100 µm soma perimeter). The overall synaptic coverage did not change, it was 1.07 [0.62 – 1.50] µm synapse/100 µm soma perimeter in NoEpi and 1.12 [0.64 – 1.67] µm synaptic active zone/100 µm soma perimeter in ResEpi.
We found that the average length of the synaptic active zones was not significantly different when we compared the NoEpi and ResEpi samples. The same can be said about the region generating or lacking SPAs.
The proportion of PV-positive terminals did not differ significantly. In the case of CB1R staining, the ratio also did not show significant changes.
In NoEpi and ResEpi samples and ROIs generating or lacking SPAs, the perisomatic synaptic coverage from PV+ or CB1R+ terminals was similar.
We found no significant differences in the overall synaptic coverage regarding the ability to generate SPAs (NoEpi and ResEpi were analysed separately).

When we analysed the length of the synaptic active zones, we found that in regions generating SPAs, the PV-positive active zones were significantly longer in the ResEpi compared to the NoEpi slices (NoEpi: 0.231±0.037, 0.23 [0.20 – 0.25], ResEpi: 0.26±0.035, 0.26 [0.24 – 0.27], p=0,01). We found that the non-stained terminals in the CB1R stained tissue were also significantly longer in the epileptic tissue (NoEpi: 0.224±0.050, 0.22 [0.20 – 0.25], ResEpi: 0.253±0.053, 0.25 [0.23 – 0.28], p=0.05) in the ROIs where SPAs were present. This data suggests that since the synaptic coverage was not changed as described above, the number of synapses given by PV-positive terminals might be lower. We did find a significant difference in the number of PV+ terminals/100 μm soma perimeter between NoEpi and ResEpi slices in the region generating SPA (NoEpi: [1.36 – 3.51], ResEpi: [0.00 – 1.90], p=0.01).

5. Conclusions

In the first study, we focused on investigating the chronic in vivo biocompatibility of SU-8 implants in the CNS and obtained quantitative electron microscopic data with a large sample size. Notably, we observed good preservation of neurons as close as 20-40 μm from the SU-8 devices, and the density of synapses returned to control levels as close as 24 μm from the track. These findings indicate that the SU-8 material has the potential to facilitate the generation of reliable neuronal signals.

Furthermore, through electron microscopic examination, we discovered that the glial scar thickness ranged between 5-10 μm and the presence of glial cells in the neuropil did not exceed 16% in close proximity to the probe (0-12 μm), decreasing to less than 5% beyond 24 μm from the probe.

In line with previous studies conducted in cell culture models, muscle and subcutaneous implantations, our results demonstrate the biocompatibility of SU-8 in the CNS.

Considering the low-cost fabrication and more flexible nature of SU-8-based electrodes, they hold potential as an alternative to highly biocompatible and widely used silicon probes in future clinical and research applications.

In our human research, based on our previous findings, we formed a hypothesis, namely that the perisomatic innervation of the layer 2/3 neocortical pyramidal cells participate in the formation of spontaneous populational activities. We investigated our hypothesis on two levels. In vitro extracellular electrophysiological measurements and pharmacological modulation followed by an anatomical investigation, including quantitative electron microscopy.

Our data shows that rescuing the PV+ or the CB1R+ perisomatic inhibitory cells both at least partially restored the SPA from the effects of Cch, both in epileptic and non-epileptic samples (LFPg amplitudes in ACSF > in Cch + AFDX-116/AM-251 > in Cch). Notably, when we compared the averages rescuing the PV-positive cells only had a significant elevating effect on the LFPg amplitude in the epileptic samples. This change is further emphasised by the fact that the only significant difference that we found in the epileptic patients in the SPA-generating ROIs was that the PV-positive synaptic active zones were significantly increased in length and lower in number on the pyramidal cell somas. The longer synapses are associated with a stronger transmitter release and, consequently, higher synaptic strengths. The modified synapses can be more efficient in inhibiting their targets, therefore making certain areas more susceptible to generating synchronous events.

In our previous studies, we described that both excitatory and inhibitory cells participate in the initiation of hypersynchronous events, both spontaneous and induced cases. Possibly the more substantial effect of carbachol in the epileptic samples can be an effect throughout the excitatory cell activation, which further supports that both excitatory and inhibitory cells are required for the generation of SPAs.

Therefore, we suggest that the spontaneous population activities in vitro in the human neocortex can result from a complex interplay between various cell types, one of which is the perisomatic inhibitory cells.

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