

UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA
DOCTORAL SCHOOL

**“A CLINICAL AND EXPERIMENTAL STUDY ON THE
PERIPHERAL NERVOUS SYSTEM’S EXCITABILITY IN
NERVOUS PATHOLOGY ”**

-ABSTRACT-

**SCIENTIFIC COORDINATOR:
Prof. Univ. Dr. Maria Iancău**

**PhD CANDIDATE:
Ciorbagiu Mihai-Călin**

**Craiova
2015**

Table of Contents

INTRODUCTION.....	3
1. THE CURRENT STATE OF KNOWLEDGE	4
1.1. Morphologic and functional characters of the peripheral nervous system	4
1.2. Electrophysiological aspects of peripheral nervous system excitability.....	4
1.3. Involvement of voltage – gated sodium channels in neuronal excitability	4
2. PERSONAL CONTRIBUTION	5
2.1. OBJECTIVES	5
2.2. RESEARCH METHODOLOGY	5
2.2.1. Work methods in the clinical stage.....	5
2.2.2. Work methods in the experimental stage	6
2.3. RESULTS AND DISCUSSIONS.....	7
2.3.1. Clinical stage	7
2.3.2. Clinical stage	7
2.4. CONCLUSIONS.....	11
2.5. SELECTIVE BIBLIOGRAPHY	13

KEY WORDS:

Peripheric neuroexcitability, rheobase, chronaxie, compound motor action potential, voltage gated sodium channels, sodium channels blocking agents, carbamazepine.

INTRODUCTION

The complex range of modern morbidity include diseases whose share is increasing gradually, giving medical professionals profound concerns both on the diagnosis and treatment.

In this series we can frame diabetes, pathologic entity which has boosted the research into early diagnosis of the disease and especially in its treatment.

Through my daily activity I come in contact with the frequent complications of this disease, which are strongly disabling, involving professional, socio-economic and familial consequences.

The above mentioned particularly motivated me in choosing this theme's study, highlighting aspects which are hardly addressed in the specific research area.

The field of neurophysiological exploration captivated me since college, performing research on electroneurogram and visual evoked potentials, their modifications in various pathologies, together with the teaching staff and students in the university's department of physiology.

Considering both the observed clinical aspects and the testing possibilities for neuropathic complications of diabetes, which were poorly explored worldwide, we established as purpose the study of peripheral nervous excitability through electroneurography completed with the experimental research of its modifications under the influence of sodium channel blocking drugs.

We consider that the results of this study can establish a diagnosing protocol to help in early detecting of the nervous damage in diabetes context, before clinical manifestations occur. We also want to highlight the role of voltage gated sodium channels in the variation of peripheral nervous excitability's parameters.

I could not accomplish my research without the help of my colleagues in the Neurologic Functional Exploration's laboratory from the Emergency County Hospital Craiova, which facilitated the access to the patients presented for investigations, whom I am most thankful. I also thank my colleagues from the Physiology department in our university, Cătălin Bogdan and Tudor-Adrian Bălșeanu, who helped me during the whole study, both in the clinical and experimental phase. I thank my family and my friends which supported me during the study, and last but not least, I thank Mrs professor Dr. Maria Iancău, the teacher which, without hesitation

accepted me, agreeing to coordinate me through this study, guided my steps with professionalism, tact, patience and kindness, like a true parent in the difficult process to accomplish this PhD thesis.

1. THE CURRENT STATE OF KNOWLEDGE

1.1. Morphologic and functional characters of the peripheral nervous system

Driving the electric pulse depends on the structure of the nerve fiber in which the pulse is conducted. Thus, the quality of the electrical insulation provided by the myelin sheaths, as well as the number and function of voltage-gated ion channels from the axonal membrane play a key role in the propagation of nerve impulses (Hille, 2001).

Extensive electrophysiological studies on peripheral nerves were able to identify many types of ion channels, differently distributed along the structure of peripheral nerves, with significant variations between nodal and internodal location (Salzer et al.2008). Moreover, differences were found between conductance properties of various areas in the same axon, suggesting that there is a possibility that the impulse transmission may vary depending on the axonal segment in which the current is conducted (Waxman and Ritchie,1993).

1.2. Electrophysiological aspects of peripheral nervous system excitability

This subchapter contains data on the SNP excitability parameters, including the modalities of their determination. In this section some terms as excitability threshold and minimal electrotonus are defined, filling the gaps in classic axonal excitability testing, contouring some complex investigation protocols useful in medical practice.

1.3. Involvement of voltage - gated sodium channels in neuronal excitability

Aspects on the voltage-gated sodium channels functions were revealed by Cox et al. 2006, 2010 and Eijkelkamp et al., 2012, mentioning that gene mutations may lead to channelopathies with clinical expression, in nervous tissue, heart and lung diseases, but also in neoplastic diseases.

2. PERSONAL CONTRIBUTION

2.1. OBJECTIVES

1. Evaluation of peripheral nerve excitability parameters and comparing the values obtained with a control group;
2. Highlighting the excitability characteristic parameters with the highest variability, placing them in the category of those loyal to the severity of nerve disease;
3. Correlating the values obtained with neurophysiological testing and the values of the main parameters defined in the diabetics.
4. Investigate the possibility of translating the paradigm used in clinic and experimental electrophysiological testing.
5. Validating the animal model used in order to obtain some experimental data.
6. Using the experimental model to investigate how the medication administered in a variety of central nervous system disorders affects the functionality of peripheral nerves.
7. Rating dose-effect relationship of unspecific sodium channels inhibitors in correlation with the electrophysiological properties of the peripheral nervous system.

2.2. RESEARCH METHODOLOGY

The entire study was to identify how the electrophysiological properties of the peripheral nervous system are affected by degenerative biological processes within some systemic diseases, and how specific treatments directed towards organ-specific diseases can affect these properties.

To achieve this goal, the study comprised an experimental phase, carried out on laboratory, animals in order to properly quantify the dose-effect relationship and a clinical study in patients with type II diabetes.

2.2.1. Work methods in the clinical stage

This study was prospective. We included in the lot patients with diabetes, arrived in the functional exploration laboratory situated in the Emergency County Hospital Craiova, for confirmation and/or evaluation of their clinically suspected neuropathy. All the recordings were performed following the internal laboratory protocol for investigating diabetic neuropathy.

The investigated group was composed by 29 patients with type II diabetes. To compare the recorded values of these patients, we recorded also the same parameters in 29 healthy volunteers.

For reference values, all the tests were based on electro-neuro-physiologic investigations such as:

- Motor nerve conduction velocity (VCM);
- Mathematical methods for determining the rheobase;
- Mathematical methods for determining the chronaxie

2.2.2. Work methods in the experimental stage

Using an experimental model of induced diabetes was not approved due to the following reasons:

- Reduced duration for the model exploitation;
- The need for more active substances required to maintain the experimental model, which may influence the results;
- Genetic or surgical induced diabetes in experimental models are cumbersome, expensive and with a high rate of mortality. Chemical induced diabetes with aloxan or streptozocin has a high risk of tumor genesis and induces changes in other metabolisms besides glucose, requiring also their follow up.

For conducting the experiments, we used 21 Sprague Dawley (SD-CD) male and healthy rats, aged between 7 and 9 months, weighing about 660-750 grams. Animals were housed in an animal care complex with food and water ad libitum.

We recorded nerve conduction velocities in the right tibial nerve, using four straight, monopolar isolated electrodes. Two of these electrodes were used for nerve stimulation, and the other two for recording the nervous response. The stimulating anode was placed at the base of the tail, while the cathode was placed proximal to the calcaneus, in the area of the Achilles tendon. Recording the compound action potential (CMAP) was performed from the right lumbricali muscles using a set of monopolar electrodes with a length of 2 cm and a diameter of 5 mm. Also, we mention that the procedure was repeated for different values of stimulus duration for determining chronaxie and rheobase from the tibial nerve.

After the initial recording which was used as reference, carbamazepine was injected in the peritoneum (5mg/kg). Using the same technique additional recordings were made, at 1,2,3,4, and 5 hours after the injection of carbamazepine. The initial values of CMAP components (latency and amplitude) also rheobase and chronaxie were compared to the data recorded from the treated animals.

2.3. RESULTS AND DISCUSSIONS

2.3.1. Clinical stage

The average rheobase for the median nerve registered in our laboratory is comparable with other values reported in literature.

Like the rheobase, all the values obtained in our study fits perfectly within the existing studies.

We analyzed the raw response obtained for nerve stimulation, to exclude associated disorders. The first analyzed parameter was the latency of motor response for the DM patients and the control group, with no significant differences, $p=0.3228$.

The second characteristic parameter for the response generated by motor nerve stimulation, amplitude, showed a significant, high difference between the control and patient group studied, $p=0.001$.

Analysis of motor electrophysiological properties in these patients revealed a "tendency" of decay, not statistically significant of rheobase comparing with the control group, $p=0.865$.

Calculation of average chronaxie in the patients population, revealed a statistically insignificant increase of its values comparing to the control group, $p=0.848$.

I tempted the comparison of rheobase values obtained for the median nerve stimulation in our patients with values reported in the literature, noticing the absence of similar data.

Although in 2006, Misawa determined the electrophysiological parameters in patients with inadequately controlled diabetes, he reports data only for the chronaxie values obtained.

Comparing to other existing studies, the values for rheobase in patients with an inadequate control of diabetes, obtained in our study, we report the lowest value of rheobase.

The average value obtained in our study represents the highest value reported in literature for diabetic patients with inadequate glycemc control.

2.3.2. Clinical stage

While there is a great variability in neurotropic side effects on different classes of drugs, we wanted, to achieve the objectives proposed in this part of the study, to identify only the changes in the function of SNP emerged as a consequence of the drugs administered.

For this reason we proposed that this part of the study to concede in animal testing, where we can properly and accurate asses the effects of medication. The three experimental groups were investigated for five hours, hourly. For each step was determined, in addition to chronaxie and rheobase, the amplitude and latency of the motor response, which functioned as the internal control parameters of the obtained values.

In the initial phase, we analyzed the latencies of the three groups investigated and could not find any statistically significant difference, $p=0.741$. There were no statistically significant differences between motor response amplitudes in the three groups in the first stage of the experiment, $p=0.860$. The values determined for the rheobase of the tibial nerve for the three groups of animals immediately after injection of the active substance had no statistically significant difference, $p=0.969$. Also we couldn't find any difference in the calculated value of chronaxie at the beginning of the experiment, $p=0.682$.

The results were the last internal control, eliminating the possibility that some of the animals used to present subclinical peripheral nervous lesions, influencing the results of the study.

In the experimental stage of this study we analyzed the components of CMAP (latency and amplitude) for the three experimental groups in 60,120,180,240 and 300 minutes after the drug administration, noticing a trend of decay which appears at the range of 120 minutes and is maintained through all the determinations, (p for latency varies between 0.741 and 0.918, for amplitude between 0.578 to 0.860).

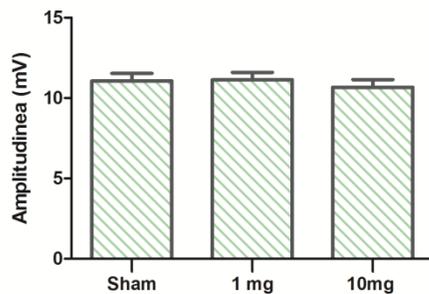


Fig. 1. The average amplitude of motor responses recorded for the three batches analyzed at 60 minutes after injection.

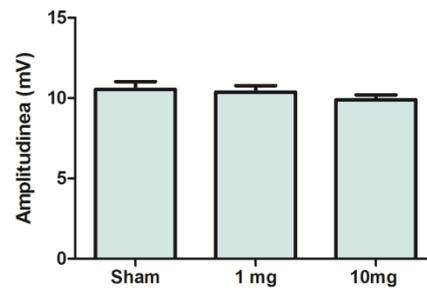


Fig. 2. The average amplitude of motor responses recorded for the three batches analyzed at 300 minutes after injection.

Analyzing the nuroexcitability parameters the rheobase values obtained by stimulating the tibial nerve, at 120 minutes after the injection begin to change, drawing an increasing trend, not statistically significant ($p = 0.285$), in the group injected with the therapeutic dose of sodium channel blocker (10 mg), growth which is highly significant at 240 minutes post-injection ($p = 0.002$). At 300 minutes after injection rheobase values keep the rising trend, which now becomes very highly significant ($p = 0.0013$).

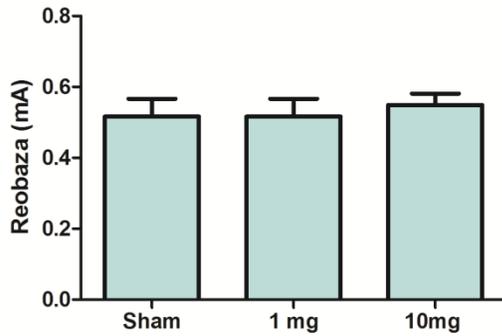


Fig. 3. Rheobase values recorded in the three groups analyzed within 60 minutes after injection

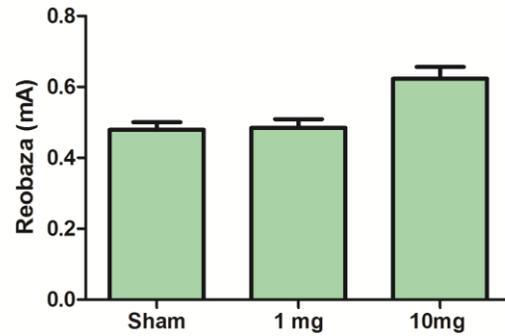


Fig. 4. Rheobase values recorded in the three groups analyzed within 300 minutes after injection

By assessing the changes in the values of chronaxie 60, 120, 180, 240 and 300 minutes after injection of the therapeutic substance, we found a downward trend, which is highly significant ($p = 0.007$) in the range of 120 minutes, a trend maintained and strengthened to three ($p = 0.0004$), and four hours ($p = 0.0002$) after drug administration. At the end of the tests after five hours from the drug administration, the downward trend of chronaxie becomes boundary insignificant ($p = 0.06$).

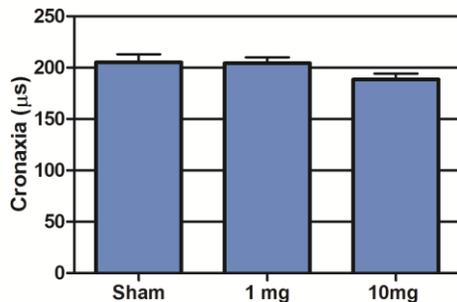


Fig. 5. The average values of chronaxie recorded in the three groups analyzed at 60 minutes post-injection

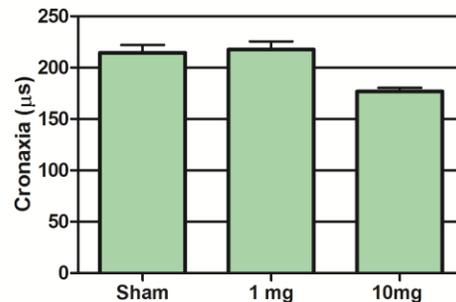


Fig. 6. The average values of chronaxie recorded in the three groups analyzed at 240 minutes post-injection

The correlation between peripheral nerve excitability parameter values, with plasma concentrations of sodium channel blockers, was done with data reported for carbamazepine blood concentrations (Malhotra et al., 2002).

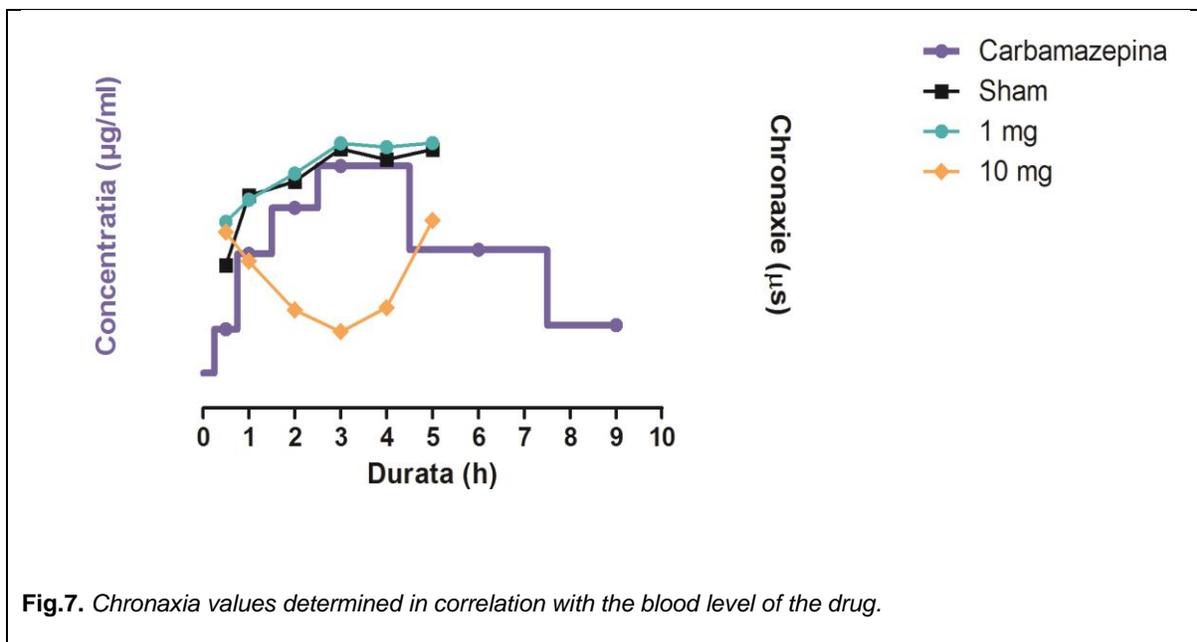
Due to the propagation of nerve impulses, existed the suspicion that, in the group with therapeutic drug concentration, a slowdown in impulse propagation might appear. Paradoxically, conduction latency for maximum drug concentration is minimal, but not with statistical

significance, $r=0.26$. We could not register a significant correlation between plasmatic drug concentration and the modifications in latencies.

For maximum drug concentration, registered after three hours, we record the lowest amplitude for motor nervous response, at the animals in this group. Although the correlation coefficient is high enough, it is at the limit of statistical significance, imposing the increase of the number of animals investigated, $r=0.46$.

Correlating carbamazepine concentration with rheobase values we can see a direct link between drug concentration and changes in peripheral excitability present only with therapeutic doses. The correlation coefficient, in this case, is significant for the injected group with therapeutic dose, $r=0.62$ and not significant for the other two groups.

Certainly in to the inverse relationship between plasma concentrations and changes in SNP is nowhere more evident than in chronaxie where the correlation coefficient has a significant value ($r = -0.94$) in the group treated with therapeutic doses and is insignificant for others two groups (Fig. 7)



It outlines a good point of view in which the peripheral nerve excitability significant changes have a minimal functional correspondent.

Assuming that changes in chronaxie reflect the conduction activity of persistent sodium channels and the passive properties of the cell membrane at the nodes of Ranvier (Mogyoros et al., 1996 and Bostock and Rothwell, 1997), the mutual relationship between the level of HbA1c and chronaxie reflect alterations in sodium channels conductance due to hyperglycemia in human subjects with diabetes.

Results from this study are consistent with the literature, which reported an increase in chronaxia in pathological entities that affect the lower motor neurons / axons, such as amyotrophic lateral sclerosis, spinal muscular atrophy, and peripheral axonal neuropathy (Mogyoros et al. , 1998). In these circumstances, the percentage of persistent sodium channels in the nerve segment can be greater than normal, resulting in increased persistent sodium conductance, so a higher value of chronaxia.

Unlike chronaxia where our study reported changes similar to other studies in the literature, rheobase currents, in our case had a "trend" of decline and no significant change. Unlike cronaxie where our study reported changes similar to other studies in the literature, rheobase currents, in our case had a "trend" of decline and no significant change. Interestingly, also in the case of rheobasic currents in patients with proper glycemic control, they had a slightly higher than normal rheobase, possibly due to geometric modifications suffered by the nerve, such as fibrosis or edema (Nelson et al.,2014).

2.4. CONCLUSIONS

1. The research study was conducted by evaluating parameters of peripheral nervous excitability in a group of diabetic patients with inadequate control of the disease (average blood glucose = 185.7 mg %, average glycosylated hemoglobin – 10.67%) aspect less present in literature.
2. Validation of the control group, in terms of the peripheral nervous excitability parameters was performed by comparing their values to those similar values existing in literature, recording no significant differences, for rheobase $p=0.783$ and for chronaxie $p=0.345$
3. Analyzing the characters of the compound motor response (CMAP) obtained by the median nerve stimulation, we recorded latency values whom compared to the control group do not reveal any statistically significant differences ($p=0.322$). CMAP amplitude values are very highly significant below those from the control group, $p=0.001$.
4. Rheobase, a characteristic nerve excitability parameter, has average values with a decay trend compared to the control group, but the differences are not statistically significant, $p=0.865$.
5. The mean value of chronaxie for the tested group is increased compared to the control group (447,515 μ s and 401,724 μ s), the difference is not statistically significant, $p=0.848$.
6. The presence of few materials related to peripheral nerve excitability parameters in patients with diabetes insufficiently controlled motivated this study, our results fitting perfectly those in literature.

7. By extending our tests through experimental research, we could not use a diabetic experimental model on animals due to the low exploration time and the need for administering other drugs that might interfere with the desired results. So we have chosen healthy SD-CD rats who received a non-selective sodium channel blocker (carbamazepine), tracking the changes of peripheral neuronal excitability under their influence in three groups structured as so: control, with the subtherapeutic dosage and a group injected with the therapeutic dose of the substance.
8. Determining initially the latency and amplitude of CMAP, we found no statistically significant differences (p ranging between 0.741 -latency- and 0.860 – amplitude-). Regarding the values of rheobase and chronaxie determined through the stimulation of the tibial nerve, compared between the three groups, we found no significant differences ($p=0.969$ respectively 0.682) – denoting the absence of additional factors which might influence the results.
9. At 60 and 120 minutes after injection, latency and amplitude characteristics of CMAP show no statistically significant differences between the experimental groups ($p = 0.862$ respectively 0.584). Peripheral neuronal excitability parameters – begin to change, tracing an insignificant increasing trend for rheobase in the group injected with the therapeutic dose (10 mg), after 120 minutes ($p=0.285$). For the same period, chronaxia has low values in the above mentioned group, with highly significant difference ($p=0.007$).
10. Compared to rheobase values, which record an insignificant increasing “trend” which is consolidated after 180 minutes from the drug administration, the average values of chronaxie are decreasing, with a highly significant statistical difference, $p= 0.0004$.
11. As in the other time periods after the drug administration, the components of CMAP (latency and amplitude) did not show significant changes, as opposed to the peripheral nervous excitability parameters recorded from the group who was injected with the therapeutic dose of non-specific sodium channel blocker, that marked highly significant changes in the case of rheobase ($p=0.002$) and very high differences in chronaxie values ($p=0.0002$) after 240 minutes from drug administration.
12. After the 300 minutes interval, the animals injected with the therapeutic dose shows significant changes for rheobase ($p=0.0013$) which increases, but the downward trend of chronaxia is maintained and reaches the limit of significance established ($p=0.06$).
13. Correlating the changes in peripheral nervous excitability parameters value with the plasmatic concentration of sodium channel blockers, we can see that the rheobase values correlate with the therapeutic dose, the correlation coefficient being statistically

significant, $r = 0.62$. For chronaxie values, there is an inverse correlation with plasmatic drug concentration ($r = -0.94$), for the same group injected with 10 mg of carbamazepine.

The thesis is a comprehensive study of peripheral nervous excitability using functional exploration methods, easily applicable in clinical practice, thereby increasing the efficiency of the follow up and treatment for diabetic neuropathy. Every stage of this study, both clinic and experimental, helped us highlight the specific features for peripheral nervous excitability:

- In diabetic patients, it is possible to identify changes in electrical conductivity even in the case of subclinical neuropathy;
- In the experimental stage, they were identified specific changes in the excitability of peripheral nervous system, both correlated with the dose and blood concentration of sodium channel blockers.

This study phase is contoured as a paraclinical, noninvasive testing protocol of the sodium channel blockers therapies efficiency.

From those mentioned above, the research for this thesis consists in a complex study of the peripheral nervous excitability in patients with tipe II diabetes testing – the first investigation conducted at national level. This character is joined by experimental research which is also carried nationally by us.

Previous claims highlights the original aspects characteristic for the research conducted in this study.

2.5. SELECTIVE BIBLIOGRAPHY

(out of 215 bibliographic titles)

1. Antzelevitch, C.; Nesterenko, V.; Shryock, J. C.; Rajamani, S.; Song, Y.; Belardinelli, L. The role of late I Na in development of cardiac arrhythmias. *Handb. Exp. Pharmacol.* 2014, 221, 137–168.
2. Bennett, D. L.; Woods, C. G. Painful and painless channelopathies. *Lancet Neurol.* 2014, 13 (6), 587–599.
3. Black, J. A.; Waxman, S. G. Noncanonical roles of voltage-gated sodium channels. *Neuron* 2013, 80 (2), 280–291.
4. Burke D, Kiernan MC, Bostock H, Excitability of human axons, *Clinical Neurophysiology* 112 (2001) 1575-1585.
5. Cappelen-Smith C, Kuwabara S, Lin CS, Mogyoros I, Burke D, Activity-dependent hyperpolarization and conduction block in chronic inflammatory demyelinating polyneuropathy, *Ann Neurol.* 2000 Dec;48(6):826-32.
6. Catterall, W. A. Sodium channels, inherited epilepsy, and antiepileptic drugs. *Annu. Rev. Pharmacol. Toxicol.* 2014, 54, 317–338.

7. Eijkelkamp, N.; Linley, J. E.; Baker, M. D.; Minett, M. S.; Cregg, R.; Werdehausen, R.; Rugiero, F.; Wood, J. N. Neurological perspectives on voltage-gated sodium channels. *Brain* 2012, 135, 2585–2612.
8. Kaji Ryuji, Hugh Bostock, Nobuo Kohara, et al., Activity-dependent conduction block in multifocal motor neuropathy. *Brain* (2000), 123, 1602–1611.
9. Kiernan MC, Bostock H. Effects of membrane polarization and ischaemia on the excitability properties of human motor axons. *Brain* 2000; 123:2542-2551.
10. Misawa Sonoko, Satoshi Kuwabara, Kazuaki Kanai, Noriko Tamura, Miho Nakata, Kazue Ogawara, Kazuo Yagui, Takamichi Hattori, Nodal persistent NaC currents in human diabetic nerves estimated by the technique of latent addition, *Clinical Neurophysiology* 117 (2006) 815–820.
11. Nelson, M.; Millican-Slater, R.; Forrest, L. C.; Brackenbury, W. J. The sodium channel β 1 subunit mediates outgrowth of neurite-like processes on breast cancer cells and promotes tumor growth and metastasis. *Int. J. Cancer* 2014, 135 (10), 2338–2351.
12. Ruiz, ML and Kraus RL, Voltage-Gated Sodium Channels: Structure, Function, Pharmacology, and Clinical Indications, *J. Med. Chem.* 2015, 58, 7093–7118.
13. Sahin O, Mandriota N, Molina JJ, Tatem K, Relating Local Nanomechanical Response of Cells to Intracellular Forces and Cell Morphology, *Biophysical J.*, 2015, 108(2):p140a.
14. Srinivasan K. and Ramaro P., Animal models in type 2 diabetes research: an overview , *Indian J Med Res*, 125, 2007, p451-472
15. Waxman SG, Ritchie JM., Molecular dissection of the myelinated axon, *Ann Neurol.* 1993 Feb;33(2):121-36.