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Glutathione-S-Transferase Induction of Tranquilizers in Certain Organs of Laboratory Animals and Livestock and New Neuroinformatic Methods in Effect Analysis

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I. Introduction

Tranquilizers that have a strong blocking effect on the central nervous system of vertebrata, reduce stress and aggression, anxiolytics and sedatives that decrease anxiety have a very significant role in human therapeutics, veterinary practice, animal experiments as well as in stock-raising. They are the most important medicines used for the treatment of schizophrenia in human therapeutics. The anxiety and excitement of psychotic patients decrease, hallucinations and delusions remit or cease as the effect of the treatment with neuroleptic (antipsychotic) medicine.

The application of a small dose sedates wild and aggressive animals and makes them treatable. It turns off the natural or artificially evoked anger reactions and fighting, aggressive behavior. Therefore, they are used in the case of laboratory animals (rodents, rabbits, monkeys, etc.) in order to be able to perform various operations.

They are also widely used in both veterinary science and breeding. In the case of smaller, pain-free but unpleasant surgery it makes animals treatable, or – combined with analgetics – even painful operations can be carried out (neurolepanalgesia). With their use, the application of the more dangerous hypnotics or narcotics can be avoided.

In the case of livestock breeding they are used mainly to reduce the negative effects of transportation stress, to protect valuable animals and as a tool in medical taming. Stress can have a serious negative effect on homeostasis, therefore on reproduction, growth, capability of resistance and various other breeding parameters. Therefore, tranquilizers have economic significance and a *raison d'étre* in the field of livestock breeding as well.

At the same time, tranquilizers – and neuroleptics in particular- have a significant number of, sometimes dangerous, side effects (dyskinesia, Parkinsonism, akatisia, aggressiveness, endocrine-, cardiovascular-, anticholinergic- or haematologic side effects, etc.). On the long run, this can result in the alteration of the fat and muscle ratio, may cause behavioral problems, may decrease the index of reproduction, etc. Their application is also limited because the quantity of chemical agents remaining in livestock is strictly controlled by the considerations of consumer protection. Statutes of the Ministry of Health (2/1999. (II.5) EüM.) and the European Parliament and Council (2377/90/EGK) regulate the maximum quantity of animal medicament remains in food, including that of tranquilizers.

The applied medicaments used to achieve various biological effects – including the compounds that I have examined – are xenobiotics (are not present in the body normally.) For their removal from the organism special metabolism enzymes and transporting mechanisms developed during phylogenesis. Increased metabolism encumbers the detoxification mechanisms of particular organs to a varying degree. As a result of this, there is a decreased capacity for protection against other harmful phenomena. It reduces the immune protection performance of animals which may influence the quality and quantity of useful production.

A significant number of metabolites of antipsychotic and tranquilizing medicaments have been identified. However, not all the intermediers are known and it is not known either what kind of biological effect they have or whether they play a role in the almost always observable development of side effects or in the evolution of individual capacity for endurance and toxic effects (DAHL and STRANDJORD, 1977; DOLLERY, 1991; YEUNG et al., 1993; JAVAID, 1994).

I have supposed that some of the above mentioned medicaments (primarily halogen substituent compounds) with the help of Glutathione-S-Transferase enzyme (GST), through Glutathione S- Conjugation can metabolize, or rather can be removed from the organism. If this happens, we can also suppose that the compounds themselves can have an induction effect on the synthesis of enzymes that are involved in their metabolism. Therefore, the

synthesis of the GST enzyme can also intensify. This way the glutathione conjugates of tranquillant compounds can be generated in an increased degree (e.g. in the brain tissue as well). It may also be supposed that the glutathione conjugates can interact in the central nervous system with proteins and receptors that have a role in the effect mechanism of tranquilizers. At the same time, glutathione conjugates, similarly to other γ-glutamine peptides and glutathione analogues, can influence the glutamatergic neurotransmission of the central nervous system. (VARGA et al., 1988; MCMOHAN et al., 2000; JANÁKYet al., 2000; HERMANN et al., 2004). Thus, these compounds can take effect in the central nervous system in another route, through the blocking of the stimulating mechanism as well. This way they can also influence the development of side effects.

One of the main targets of tranquilizers is the formatio reticularis of the brain stem and the neurons of the reticular activating system (RAS). Based mainly on EEG (Electroencephalography) examinations it seems that tranquilizers evoke a typical synchronous firing phenomenon in the cortex which is probably related to the sedative effect on the central nervous system. The mechanism for the development of the synchronization is not known, but we know that the RAS has a crucial role in its regulation. The EEG examination provides rather indirect information about the operation of the nervous system, therefore the investigation of various regions of the central nervous system and the neurons having a role in it is absolutely necessary. To carry this out we need neuroinformatic methods of investigation. With the help of this the firing of particular neurons - that is their depolarization frequency -, the alteration of field potential (developed as a result of the activity of neurons) or the registration or analysis of action potentials can be examined. Little is known about the nature of firing patterns and their relation with tranquilizers and their glutathione conjugates' efficiency, the effect mechanism of certain compounds. It is not known what kind of firing pattern changes do these compounds cause in the formatio reticularis.

The evaluation of the firing pattern can be efficient if we can register with as many electrodes as possible in real time (with an array of 128 electrodes in my investigations.) It provides a number of other possibilities for examination, if a system like this is also capable of stimulation, i.e. it is capable of establishing two directional communication with a targeted part of the central nervous system. An artificial neural network, that shows a surface analogy with the structure of the living nervous system connected to it, can be capable of processing such a large amount of data. The amazing development of digital technology made the practical, neurobiological use of high performance microcontrollers possible, however, up to the present only very few such applications were created. There is no bibliographical data that would show that a microcontroller based artificial neural network would be able to establish two-directional communication through a multielectrode array with the central nervous system connected to it. Therefore, I aimed at developing a neuroinformatic system based on new principles and at writing its algorithms. With the help of this system we can examine the firing patterns in the brain stem, the operation of the nervous system, the communication of particular areas with other, distant regions and the effects of tranquilizers and their metabolites on the electronic activity of the brain stem's formatio reticularis.

Intensive research is being carried out in order to reduce the significant number of side effects and to synthesize new, more efficient compounds; therefore the medicaments are changing from time to time in the medicaments register. It is an important task to understand the metabolism of this chemically diverse group of medicaments and its elimination in animals. The more precise knowledge of the bioelectric activity of the nervous system and the analysis of the tranquillizer effects on it can help us understand their effect mechanism better. My investigations can help in testing the new and more efficient compounds, and in the

examination of their biological effects. Experiments on various animals can provide data regarding which medicaments can be used trustworthily with which animals and can largely contribute to the exploration of biometabolic ways.

In my dissertation I have examined the effects of some of the tranquilizers (only partially) used in human and animal therapeutics on GST induction, in different organs of three animal species. I have tested the possibility of formation of GSH-medicine conjugates and their biological effects. I have used and developed bioinformatic methods for measuring GST enzyme activity. I have developed an original, microcontroller based multiprocessor artificial neural network that integrates both digital and analog characteristics, as well as its algorithms with which I have examined the effect of medicaments and their metabolites on the firing pattern of the brain stem. The results of my investigation are interdisciplinary, from the area of neurobiology and neuroinformatics.

II. Objectives

I examine certain significant medicaments from the compounds with tranquilizing effect. The selection is based on heterogeneous chemical structure with a similar effect-profile, on the traditional and new generational classification, and the extent of practical use in human and animal therapeutics (or in both.)

In my dissertation I am looking for answers and have developed solutions for the following questions:

1. The development of a new, microcontroller-based measuring system, its hardware and software that enables the fast and precise measurement of the enzyme activity of a large number of biological samples, alongside with the monitoring, analyzing and evaluating data gained during enzyme kinetic measurements.
2. Are the examined compounds in vitro substrates of GST enzymes? The 1-chloro-2, 4-dinitrobenzene (CDNB) as a toxic xenobiotic is the substrate of the GST enzyme and the enzyme activity can be measured photometrically by it. Do the examined medicaments block competitively the development of GSH-CDNB conjugates?
3. In which organs and to what extent do some compounds with a tranquilizing effect generate GST enzyme induction, i.e. the protein synthesis of the new enzyme, gene expression?
4. Is there a difference between the GST induction in particular organs Whatof laboratory rats (*Rattus Norvegicus*) widely used in laboratory practice; in rabbits (*Orictolagus Cuniculus*) that are used as the model for mammal farm animals and in the case of fish, silver crucian (*Carassius Auratus*) that holds significance in animal breeding?
5. What is the efficiency and applicability of certain medicaments on farm animals (using rabbits and the silver crucian as models) compared to widespread compounds used in human and animal therapeutics? Special emphasis is put on the role of first- and second-generation neuroleptics.
6. Are the GSH conjugates effective in the case of the above mentioned species? Can they have a role in the appearance of side effects?
7. Developing and working out an original multi-electrode diversion system, a multi-electrode array and an amplifier system based on the newest

technology that enables the examination of the effect of various compounds on the firing pattern of the neurons in the brain stem.

8. The development of a new multiprocessor artificial neural network (and related algorithms) that is able to process in real time the multi-electrode firing sample matrixes and to establish two-directional communication with the nervous system.
9. Using the above described system, do the examined tranquilizing compounds cause firing pattern changes -and if yes, what kind – in the brain stem's formatio reticularis? Is there a difference in the case of the firing pattern changes caused by the GSH conjugates of the same compounds?

Between 1998 and 2007 I have worked in the following institutions (in chronological order):

- University of Debrecen Medical School, Institute of Physiology
- Kossuth Lajos University, Faculty of Sciences, Department of Animal Anatomy and Physiology
- University of Debrecen, Faculty of Engineering, Department of Electrical Engineering
- University of Debrecen, Centre of Agricultural Sciences, Faculty of Engineering, Department of Environmental- and Chemical Engineering. Laboratory of Electrophysiology and Bioinformatics.

III. Materials and methods

Tranquilizer Treatments

For the examination of GST induction I treated the animals with s.c. medicaments. I considered various aspects while choosing the tranquilizers. Primarily, I wanted to examine neuroleptics, as they are widespread compounds with great efficiency and have similar effects and side-effect profiles even though they have a heterogeneous chemical structure. It is also noteworthy that some drugs are used only in animal or human therapeutics, while some compounds are widespread in both. For the sake of comparison I also examined medicaments from the group of anxiolytics and sedatohypnotics that are also widely used in practice.

For determining the dose for various species I used the related literature (TÓTH, 2004) as well as medicine descriptions (PERÉNYI, 2002). In the case of those drugs that are not applied for the particular species I used the value of human data's weight converted to kilograms (BORVENDÉG, 2002). I corrected these and made the doses more precise based on the results of my own tests.

Type	Compound	Human dose (agent/day)	Animal dose (agent/kg/day)
Typical neuroleptic	chlorpromazinum	*Hibernal* 3 x 25 mg = 75 mg Maximum: 600 mg	*Hibernal* dog : 1-5 mg im. horse : 0,15-0,25 mg im.
	acepromazine	–	*Ventranquil A.U.V.* horse, bovine, hog : 0,05-0,1 mg iv. horse, bovine, hog : 0,1-0,2 mg im. dog, sheep, goat : 0,005 mg iv. dog, sheep, goat : 0,01 mg im. Nutrition – health awaiting time: meat: 24 hours, milk: 12 hours
	thioridazinum	*Melleril* 100-600 mg Maximum: 800 mg	–
	haloperidolum	*Haloperidol* 5 mg Maximum: 60 mg	–
Atypical neuroleptic	tiapridum	*Tiapridal* 200-1200 mg Maximum: 1800 mg	–
	risperidone	*Risperdal* 4-6 mg Maximum: 10 mg	–
anxiolytic	diazepam	*Seduxen, Stesolid* 5-15 mg Maximum: 40 mg	*Seduxen, Valium* horse : 0,02-0,1 mg iv. colt : 0,1-0,25 mg iv. dog, cat : 0,1-0,5 mg im.
Sedato-hypnotic	xylazine	–	*Xylazin A.U.V., Primazin A.U.V., Rometar A.U.V.* dog : 1-3 mg im. cat : 2-4 mg im. horse : 0,6-1 mg iv. vagy 1,5-3 mg im. bovine : 0,05-0,3 mg im. 0,025-0,15 mg iv. sheep, goat : 0,3-0,5 mg im. CP-Xylazine: cannot be given to animals providing food. In the case of other, Xylazine containing products awaiting time is: meat: 3-7 days, milk: 3-7 days

Table 1. Names of examined compounds in the pharmacopoeia as well as their dose and usage

Laboratory rats

In the first part of investigations I examined neuroleptics only. Data concerning the metabolism of human medicaments is provided mainly as a result of experiments with rats, thanks to pharmaceutical tests. Therefore, in this species I aimed at examining whether the above mentioned medicaments cause GST induction at all in certain organs, and if yes, to what extent.

The following drugs were given to SPF (specific pathogen-free) Wistar rats intraperitoneally during in vivo treatment through 10 days, in the maximum dose used in

human therapy (body weight converted into kg): Risperidonum, Tiapridum, Thioridazinum Hydrochloricum, Chlorpromazinum Hydrochloricum and Haleperidolum.

In all the groups I treated 10 animals using 60 of them this way. The animals were given general mouse-rat laboratory food (normal –CRLT/N, Charles River), tap water and natural lighting. They received the medicaments at the same time of the day, once a day. The control received the same amount of physiological salt solution.

Rabbits

In the second round I did not use laboratory animals but rabbits that have a significant role in animal breeding. The rabbit is used as the model for our mammal farm animals and was used less often in similar research.

After the results of the experiments on rats I carried out extended investigations, using all the compounds listed in Table 1. I vaccinated 5 animals in each group for 8 days, this way, in the 9 groups I used 45 animals. Animals were given standard rabbit food (CRLT/NY, Charles River) with self dosage, tap water and natural lighting. They received the medicaments at the same time of the day, once a day intraperitoneally. The control received the same amount of physiological salt solution.

Silver Crucian Carp

In the case of fish, medicaments were not given intraperitoneally but were dissolved in the water of the aquarium. While breathing, the animal takes in the dissolved compounds through the gill. It is also more practical to examine the effect in the case of dissolved substance because this is how it will be used – for example to reduce the loss resulting from flapping or to reduce the need for oxygen during transportation. From the examined compounds only diazepam is used (0,2-0,4 mg/kg im.) in the case of fish. The standard method in the case of anesthetic dissolved in water involves tricaine methane sulphonate (MS-222 Sandoz), in a concentration of 25-100 ppm (0,1-0,3%). (TÓTH, 2004).

We have to consider several issues in the case of medicine dissolved in water. Apart from the quantity of water, we have to consider the weight of the animal, temperature of water and its oxygen content. It takes longer for the agent to get into the organism, the plasma concentration grows consistently, but the effect is longer and long-drawn. The concentration gradually decreases as the animal takes it in.

The average weight of young fish was 18 g. Based on the test measurements, approximately one tenth of the dose used in the case of rabbits proved effective (in a 5 liter aquarium), and was not lethal even on the longer run.

I treated 5 animals in each group, this way using 45 animals in 9 groups. They were fed with freeze dried red mosquito larvae (bio-lio) in aerated tap water environment, with natural lighting, continuous air pumping, in even temperature. They received the medicaments once a day, at the same time of the day. The control did not receive medicaments.

Preparation of Samples for the Examination of GST induction

The animals were killed in the form of professional, physical euthanasia. (KÁLLAI, 2000; Close et al., 2000; MOLNÁR, 2001; TÓTH, 2004.) The laboratory animals were dissected right away. Insides (brain- with the brain stem, spinal cord, liver, spleen, kidney, heart, lungs) were put on stainless steel plates of -70 ^{o}C. The heat capacity of the surface takes care of the immediate cooling of samples. It is fundamental that samples are continuously kept cold during all the processes. This blocks the operation of proteases and the negative effects of other processes on GST enzyme activity. Certain operations (e.g. centrifugation) can be carried out only above melting point, on a temperature between 0-4 ^{o}C. But all samples

were kept on the necessary temperature required for various work processes for the same amount of time. Samples were frozen on -20 ^{o}C in closed, sample keeping glasses, until further processing.

In frozen state, I detached samples of 3 x 0,2 g weight from the organs and homogenized them separately in 10 ml, 0,32 M, 0 ^{o}C saccharoze solutions with a Teflon potter for 1 minute, in icy water (CARBERG and MANNERVICK, 1985). I centrifuged the homogenizate on 4 ^{o}C for 10 minutes with 9000 g. Then I separated the supernatant from the precipitate and measured a 5 x 50 µl sample for protein quantitative determining. The rest of the sample (~5ml) was frozen again, until enzyme kinetic examinations.

Determining the Protein Content

For the correct comparison of the enzyme activity of the GST, the protein concentration of particular samples has to be considered. Lowry's method for determining the protein content provided the proper solution. (Lowry, 1951)

Reagents:

1. 2% NA-carbonate in 0,1 N NaOH
2. 0,5 % $CuS0_4 \cdot 5H_20$ solution in 1% Na-tartarate solution
3. (C reagent): 1 ml $CuS0_4$ solution (2) was mixed with 50ml Na-carbonate solution (1)
4. Folin-Ciocalteu (D reagent) 100 g $Na_2Wo0_4 \cdot 2H_20$ + 25 g $Na_2Mo0_4 \cdot 2H_20$ + 700 ml distilled water + 500 ml 80 % orto-phosphoric acid solution + 100 ml concentrated HCl after 10 hours of cooking, 150 g Li_2S0_4, 50 ml distilled water and 3 drops of Bromine was added to the solution. After cooling, it was diluted to 1000ml with distilled water. The reagent is photosensitive therefore I kept it in brown glass. As acid content was approximately 2 N, the reagent was attenuated with distilled water in 1:1 ratio at determining.
5. As standard protein solution, I used Bovine Serum Albumin (BSA, SIGMA) for calibration. I dissolved 20mg in 100ml of distilled water and kept it refrigerated.

Samples and standards were diluted to 1ml with distilled water. Then 2 ml of the "C" reagent was added. After 10 minutes 200µl was added from the "D" reagent and waited for another 30 minutes until the stabilization of color. The samples were photometrized on 600 nm opposed to a blind sample that did not contain protein.

Calibration was carried out in 0,01-0,1 mg/ml BSA concentration with 10 standards. I fitted a line on the received extinction values with the use of the least squares method.

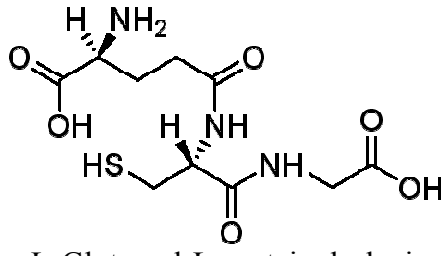
The protein content of each sample had to be determined and on the basis of the measured Δ absorbance the GST activity had to be counted in Units.

species	no. of animals	organ	sample/organ	measurement/ sample	Total
rat	60	4	3	3	2 160
rabbit	45	6	3	3	2 430
silver crucian carp	45	3	3	3	1 215
				Total number of protein quantitative determinations	5 805

Table 2. Number of protein quantitative determinations

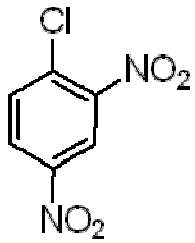
Measuring the GST activity

One of the substrates of the GST enzyme is the 1-chloro-2,4-dinitrobenzene (CDNB). Although it is hard to imagine that the organism would meet with this toxic xenobiotic under natural circumstances, the enzyme still shows a great affinity to it. As a result of the conjugation with GSH –SH group a colorful complex is created



γ-L-Glutamyl-L-cysteinyl-glycine
GSH (307.32 g/mol)

+



1-chloro-2,4-dinitrobenzene
(202.55 g/mol)

The quantity of the product generated under standardized circumstances is directly proportional to the GST enzyme activity that can be measured photometrically (HABIG et al., 1974; MAUCH and DUDLER, 1993).

Reagents:

- 0,1 M sodium phosphate puffer (6,5 pH) with 1 mM EDTA- Na_2
- 20mM GSH dissolved in tridistilled water
- 20 mM CDNB dissolved in 95 % ethanol

Measuring method:

For each measurement I measured 850 µl puffer solution into Eppendorf tubes and in 37,5 ^{o}C water - stabilized with thermostat – incubated it until it reached the required temperature. Following this, I added 50 µl 20mM GSH and 50 µl 20mM CDNB solutions. I started the reaction by adding 50 µl sample and after shaking them together they were put into the photometer immediately. The change in the concentration of the conjugate generated from 1-chloro-2,4-dinitrobenzene (ε_{340} = 9,6 mM^{-1} cm^{-1}) was measured for 3 minutes on 340nm wave-length. The control mixture did not contain a sample. The rate of GST activity is expressed in Unit.

$$\frac{(\Delta A_{340})/\min \times V(ml) \times dil}{\varepsilon_{mM} \times V_{enz}(ml)} = \mu mol/ml/\min$$

Where "dil" is the dilution factor, "V" is reaction quantity, "Ɛ" is the extinction coefficient and "V_{enz}" is the quantity of the sample. The result was corrected based on the protein content of the sample and was converted to µmol / kg protein/ sec.

I measured and represented in the graphs how much the values differ from the expected values (from the mean.) Where x is the mean of the average of values and n is the size of the sample.

$$\sqrt{\frac{\sum (x-\bar{x})^2}{(n-1)}}$$

The 6,5 pH of the phosphate puffer –according to data in related literature – hinders the spontaneous (without enzymes) reaction of CDNB-GSH. I tested this with test measurements. Without the addition of GST, the absorbance did not change in 3 minutes to a significant degree.

species	no. of animals	organ	sample/organ	measurement/sample	Total
rat	60	4	3	3	2 160
rabbit	45	6	3	3	2 430
silver crucian carp	45	3	3	3	1 215
				Total no. of enzyme kinetic photometric measurements :	5 805
				In 3 minutes 19 data is generated, therefore the total no. of data	**110 295**

Table 3. Number of enzyme kinetic measurements.

In vitro GST substrate examination

For the examination of competitive blocking I tried to hinder the generation of GSH – CDNB complex by medicaments of molar equivalent amount. Following this, GST (retrieved from horse liver, SIGMA) was added. The enzyme's performance according to the catalog: 1 μmol CDNB – reduced GSH conjugation per minute pH 6,5 and on 25 ^{o}C. 1mg freeze dried enzyme was dissolved in 5 ml of distilled water and 50 μl was added to the samples. Considering the factors of dissolution I used a 50 μl quantity of 5 mM solution of the substrates. On standard temperature, in phosphate puffer (6,5 pH). The CDNB, however, connects to the GST with great affinity. Therefore, I also created a concentration sequence from the medicaments, with the help of which the determination of the performance of blocking was also possible.

Then I examined the extent of blocking in the dose given to laboratory animals, comparing the medicaments to one another. This way affinity to the GST can be examined in a ratio similar to the concentration that develops in the organism.

GSH Conjugate Synthesis

I have examined the efficiency of conjugates in the case of rabbits and silver crucian carp. I calculated the GSH equivalent for the synthesis of the drug conjugate with the following formula on the basis of molar mass:

GSH equivalent = (((medicament daily dose mg / 1000) / medicament molar mass) x GSH molar mass) x 1000

Where the molar mass of GSH: 307,32 g/mol

The matching of the medicament and the GSH in molar equivalent amount in physiological salt solution was followed by the addition of GST retrieved by horse liver (SIGMA). The performance of the enzyme according to the catalog: 1 μmol CDNB –reduced GSH conjugation per minute pH 6,5 and on 25 ^{o}C. 1mg freeze dried enzyme was dissolved in 5 ml of tridistilled water and 150 μl was added to the samples. I incubated the reaction on room temperature for 5 hours. Meanwhile I was monitoring product formation.

The formation of the product is shown by the decrease of reduced glutathione, while complete conjugation is shown by its disappearance. This way the CDNB added to the sample

taken from the reaction showed less photometrically measurable (340nm) color reaction, as during product formation less and less GSH can be found in it.

Motility Measurement

The measurement of motility provides a possibility for the objective examination of the efficiency of compounds having a blocking or stimulating effect on the central nervous system. At present, webcam measurement systems are often used (e.g. EXPERIMETRIA), but modern and precise results are given by piezoelectric pulse sensors or infrared movement detector systems as well. For the measurements I have developed my own motility measuring system, controlled by microcontroller and using the two techniques mentioned above.

I measured motility always before administering the drug. Thus, approximately 24 hours after administering the first dose. This way I registered the long run changes in motility as a result of chronic treatment and not the acute effect of the medicament. This causes less expressed alterations but during the 8 day long treatment it showed more unambiguous and stable tendencies.

In the case of single dose examinations I registered the results one hour after administering the medicaments and their conjugates, and after the passing of the acclimatization time.

Animals were given 10 minutes of acclimatization time after they were put in the measuring system, then I started the measurements and monitored them for 5 minutes. A microcontroller (BS2SX) supervising system collected and forwarded the data to the PC, where it was received and processed by a software that I have developed.

Hot-Plate Test

The hot plate is a test that serves as the standardized measurement for the scale of analgesia and sedation. I gave 10 minutes of acclimatization time before the measurement in the device I have built. Then I placed the animals on a 25 x 25 cm constant surface kept on 55 ^{o}C in the container closed on all sides, in which there was a possibility to leave the plate. I controlled the temperature with a 0,1 precision microcontroller controlled digital thermostat. (MALMBERG and BANNON, 1999; MOGIL et al., 1999; WILSON and MOGIL, 2001). Then I have measured the time elapsed until jumping off or climbing down from the plate. If the animal did not react to the thermal stress for 90 seconds I aborted the measurement.

Measuring Body Temperature and Weight

I measured the body weight with a SOEHNLE digital scale (5g precision), body temperature with a SUNLIFE digital thermometer (0,1 ^{o}C precision) rectally in the case of rabbits. I carried out the measurements always exactly before the treatment, this way I did not examine the acute but the chronic effect of the medicament on body temperature.

Choice of Microcontroller

In the artificial neural network and in the enzyme kinetic measurement systems I used the BS2/SX microcontroller that contained the world's fastest SX based processor at the time of its building. The 50 Mhz of clock frequency makes the execution of 10,000 machine code operations per second possible (http://parallax.com). It uses the multi-level "pipe line" technology so its apparent machine cycle is 1 clock signal altogether. It contains 16 I/O lines.

Development of Multi-electrodes

The object oriented neural network of my own making (ADNC – Analog Digital Neural Computer) requires a special electrode array that cannot be bought. Therefore for the production of the multi electrode arrays I have developed my own technology. Several units were made of copper, copper-zinc and wolfram alloys which have different electronic characteristics.

First the coating is put on the wires (d = 50-100 μm). It is followed by folding, this way they multiply to 2, 4, 6, 8, 16, etc. units. After the 7th folding I ensure the cohesion of the 128 units with a silicon matrix. It is put among the electrode wires in its gel form and then I unify them by straining and twisting them at the same time until the silicon polymerizes. As a result of its features on the short run it does not cause tissue irritation that would influence the measurements. At the same time it holds the electrode array flexibly and provides additional insulation between the units.

After this, the prepared bundle of wires is frozen. After reaching the optimal temperature I cut one end of the array in 90, 45, 20 degrees or in another angle. This way we can get a flat or sharp edged electrode array. The smaller the angle the larger will be the surface of units; therefore, they will touch the targeted tissue's cells on a larger surface. Excessive cooling can result in the breaking or rupture of the wire or the insulation. At the same time, in the case of insufficient cooling the material can remain too soft, which happens mainly in the case of copper wires. In the case of wolfram or copper-zinc alloy wires I managed to achieve a finer cutting surface.

The other end is disassembled and the coating is taken off. Following this, the wires have to be soldered to the 64 pole connecting array one by one. It is received by an analog switch IC system of my own making.

IV. Results

For the complex enzyme kinetic analysis of the huge amount of data (more than 5,000 enzyme kinetic measurements, the same amount of protein content determinations, that is more than 110,000 data lines) I have developed a microcontroller based processing system with its algorithms. The 14 (or in the 2nd version 24 bit) A/D converter quantized the detector's analog signals with an increased precision. In order to correct the problems causing inaccuracy I have developed a correcting algorithm, I have tested it and applied it. For the analysis of kinetic curves I have developed various algorithms (linear section search, biggest netto product formation section search) for the BS2/SX microcontroller. The system proved to be successful during processing the great amount of data and during comparative examinations.

Under In Vitro circumstances all the examined drugs competitively hindered the formation of the CDNB-GSH conjugates in the presence of GST. All compounds proved to be the substrates of the enzyme with different affinity and catalyzed the formation of the GSH drug complex. Thioridazin showed the most significant blocking in treatment dose, followed by tiapridum, xylazin, etc. During the treatment, a concentration of similar ratio develops in the organism, which implies the possibility of the formation of the conjugates.

In all the treated groups of the laboratory rats the GST activity in the liver increased. The same tendency was observable in the brain stem's homogenizate as well, although GST activity was only one-20th or one-30th of that of in the liver. Traditional neuroleptics – with more side effects – caused less GST induction in general, than new generational medicaments – causing fewer side effects. In the brain, haloperidol and chlorpromazine caused the biggest GST induction. Therefore, the appearance of their conjugates is the most likely.

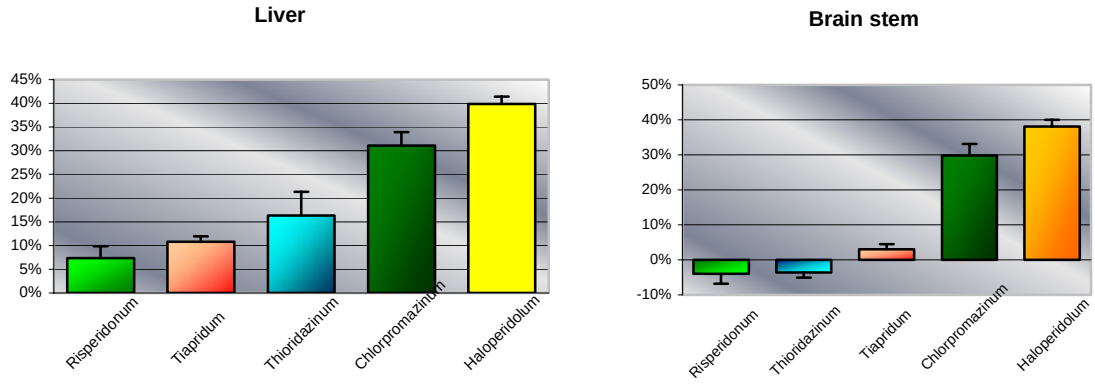


Fig. 1. Laboratory rat. GST induction in brain stem. Controll : 491 Unit, and in liver. Controll : 7 563 Unit

The compounds, except for the new generational risperidone, caused significant increase in the kidney as well. In the spleen, GST activity typically decreased compared to the control. The increase of enzyme activity signals activation of the GSH detoxification system. The larger scale it is, the more the substance burdens the defense capacity of the cells in the given organ. The negative value may show that other detoxification mechanisms came to the foreground, which distracted the capacity from the GST gene expression, for which there was no need to a large scale in these cases.

For the decrease of motility in rabbits the diazepam, the thioridazine, and tiapridum were the most effective. The tiapridum, as a new generational neuroleptic with few side effects did not cause significant GST induction in brains of rats either (as opposed to thioridazin.)

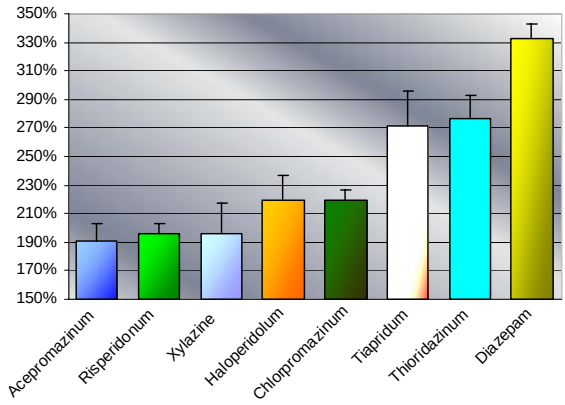


Figure 2.The ratio of the decrease of motility in rabbits compared to the change in the control

In the case of Hot Plate Tests, only risperidon, haloperidol and diazepam performed badly. The treatments caused hibernation usually on the 3rd or 4th day; for these the body compensated the following days. There was no body weight gain observable during treatments. In the case of risperidonum, thioridazin and chlorpromazine weight loss was observed. Thioridazin and chlorpromazine are high efficiency, traditional neuroleptics, so in these cases this result is not surprising. However, risperidonum is second generational, so a more positive side effect profile would be expected (this is the case regarding humans.) Tiapridum and haloperidol caused the least body weight gain leeway.

Usually, haloperidol and thioridazin caused the biggest GST induction in organs and tiapridum the lowest. The liver, as the most important place of detoxification mechanisms,

reacted with significant GST induction in the case of all treatment, except for risperidon. The kidney, similarly to the liver and heart showed strong GST induction, except for tiapridum, or in the case of the heart for diazepam. This substance caused significant increase only in the liver.

Apart from the organs' detoxification load, the brain deserves special attention, where glutathione conjugates can influence neurotransmission the most.

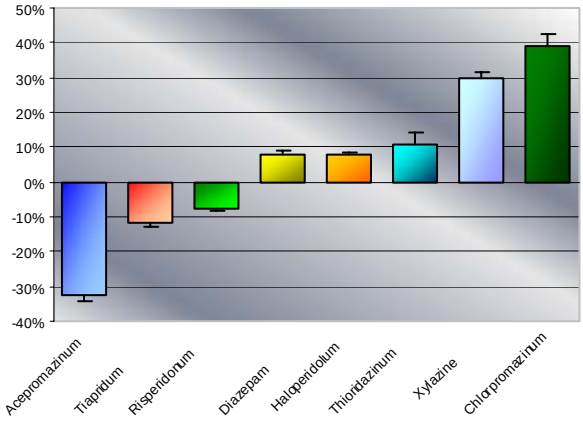


Fig. 3. GST induction in rabbits brain stem. Controll : 15 260 Unit

In the brain stem samples all examined compounds with the exception of acepromazin, tiapridum and risperidonum caused GST induction, which could cause the appearance of GSH conjugates in the brain tissue. This can influence the process of glutamatergic neurotransmission, as it has been seen in the case of various γ-glutamine peptides and glutathione analogs (VARGA et al., 1988; MCMOHAN et al., 2000). The two new generational neuroleptics- that are not used in animal therapeutics so far – did not increase GST induction. It is possible that the lack of accumulation of GSH conjugates can be one of the reasons for the fewer side effects of these substances. In the brain stem of rabbits chloropromazine and xylazin caused the biggest GST induction.

I have carried out the same efficiency tests on the synthesized glutathione conjugates of the medicaments as well. From among the conjugates risperidonum and chlorpromazine proved to have the weakest, while xylazin and thioridazin the strongest effect. The order is almost the same as in the case of the scale of GST induction in the liver. In the hot plate test, however, xylazin, chlorpromazine and tiapridum showed smaller values. This, however, can be connected to the smaller degree of analgesia as the test is based on pain stimulus. Body temperature one hour after the treatment was decreased most significantly by chlorpromazine and acepromazin. While in the case of base compounds it was the haloperidol and diazepam. From the new type of substances, the conjugate of risperidon caused less, that of tiparidum more significant hibernation than the base compound.

It is interesting that the GST activity of rabbits was significantly (sometimes 10 times) larger than that of laboratory rats. This is probably the result of the fact that SPF Wistar rats have been bred in laboratory circumstances for several generations; therefore they are less affected by the environment than rabbits are. Therefore, their detoxification system is less active too.

In the case of silver crucian carp breathing was slowed down most significantly by acepromazin and thioridazin and their GSH conjugates were similarly effective. Risperidonum and haloperidol did not slow down breathing but their glutathione conjugates did so significantly. The diazepam conjugate increased breathing compared to the based

compound. GST induction was evoked significantly by acepromazine; in the brain also by diazepam and thioridazin. New type neuroleptics showed low induction, and risperidon a negative value. Supposedly in the case of silver crucian carp it is not the GSH that is the main metabolic path's first step.

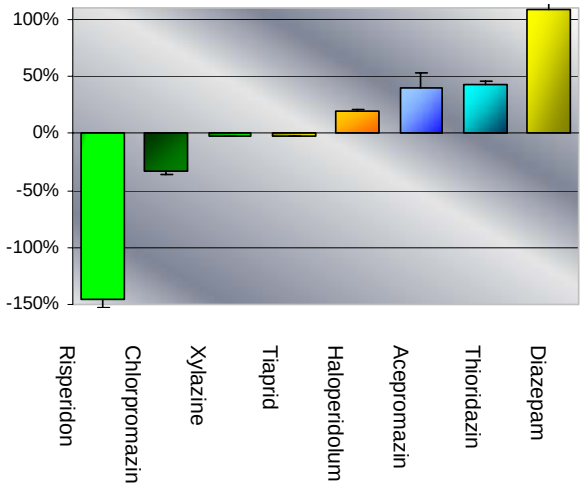


Fig. 4. GST induction in silver crucians brain. Controll : 3 183 Unit

GST induction data have been compared in compound grouping as well.

I also examined the effect of medicaments and their glutathione conjugates on the bioelectric activity of the brain stem. To achieve this I have developed an original multiprocessor artificial neural network incorporating analog and digital features (ADNC: Analog Digital Neural Computer) and its related algorithms, with the help of which we can establish two-directional communication with a targeted section of the living nervous system through a 128 multi electrode array (MEA). The system offers a new approach towards the interaction of the neural network and the nervous system; it treats it as its analog part connected to it in an unknown way.

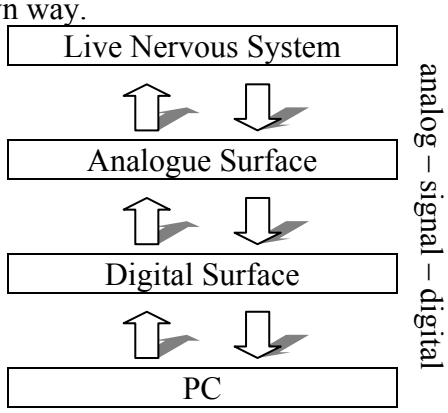


Fig. 5. The way of analog and digital signal. The connection of surfaces from PC towards nervous system area aimed by microelectrodes.

The analog and digital surface of the ADNC integrates microelectronic hardware of the newest technology. The digital surface consists of 9 high performance BS2/SX microcontrollers. Apart from detecting firing matrixes it can also perform algorithmic operations on them and by re-stimulating its result it can communicate with the nervous system.

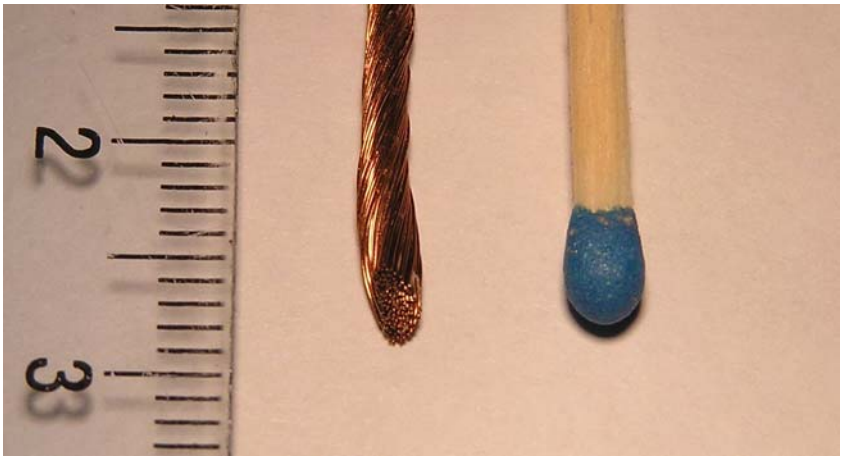


Fig. 6. 45° sharp-edged MEA consisting of 128, 100 µm diameter units (Own Photo)

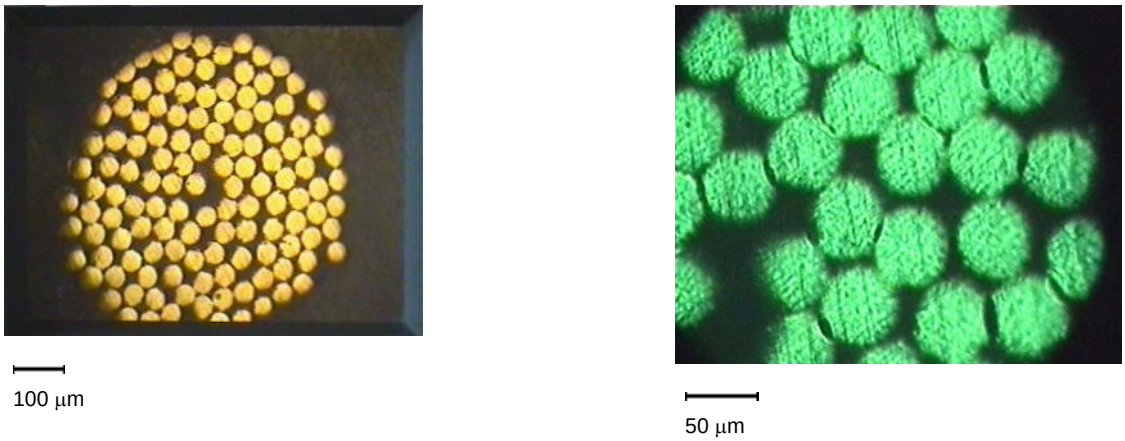
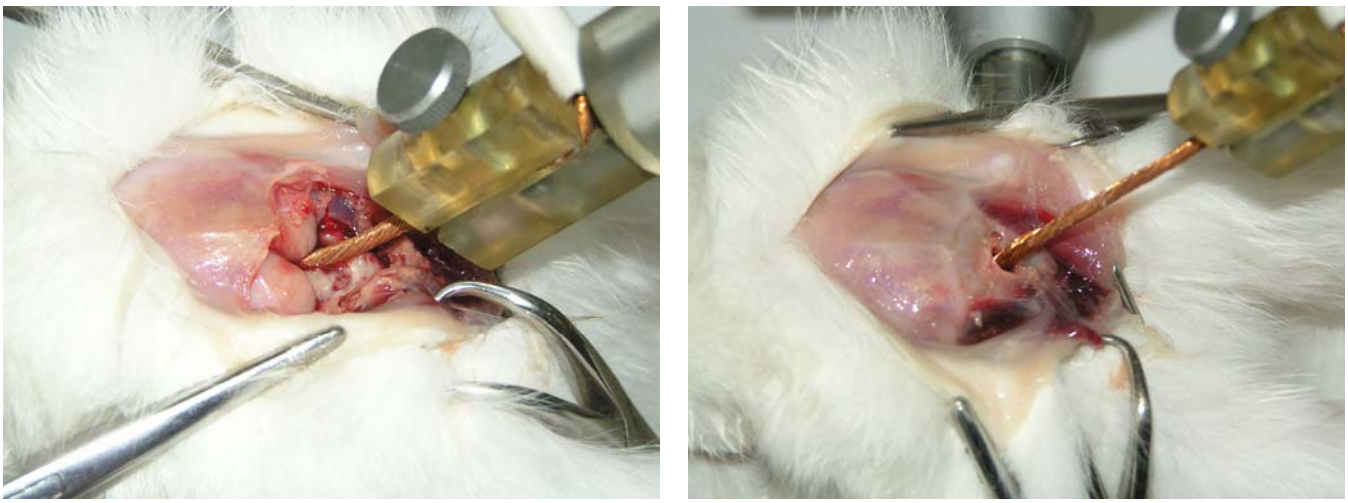


Fig. 7. Bluntedged electrode array consisting of 128, 50 µm diameter electrodes (microscope, own photo)



With the partial removal of cranial bone Through a bore hole

Fig. 8. Electrode implanting with the help of stereotaxic aiming equipment (Own photo)

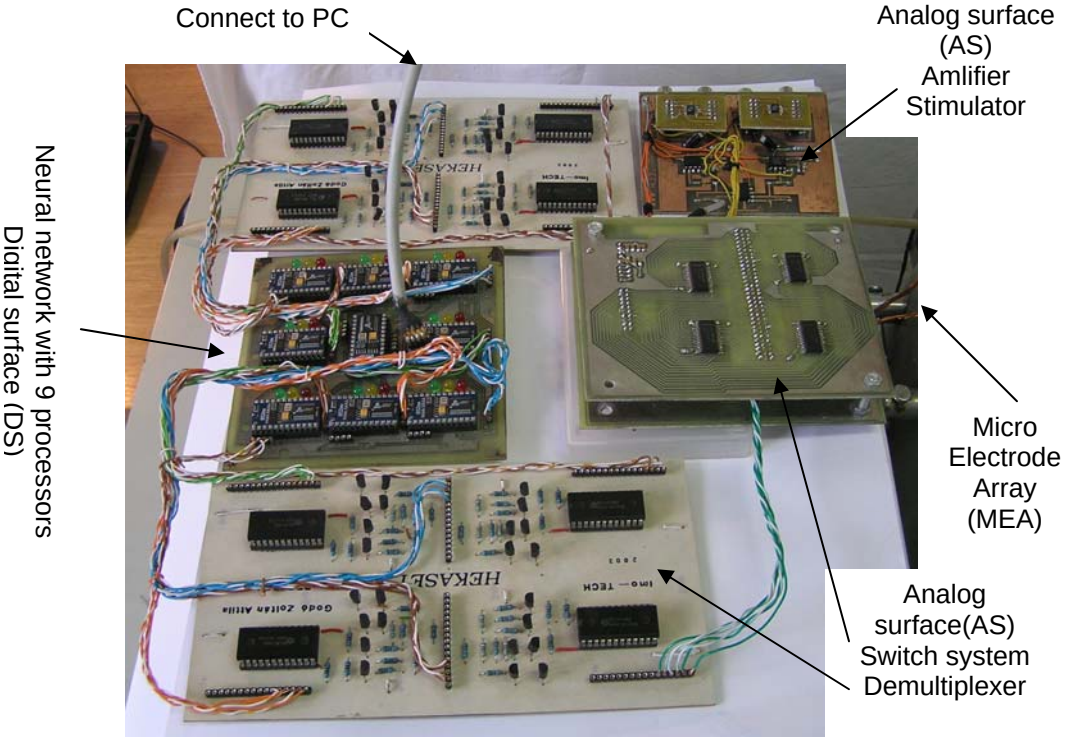


Fig. 9. The ADNC

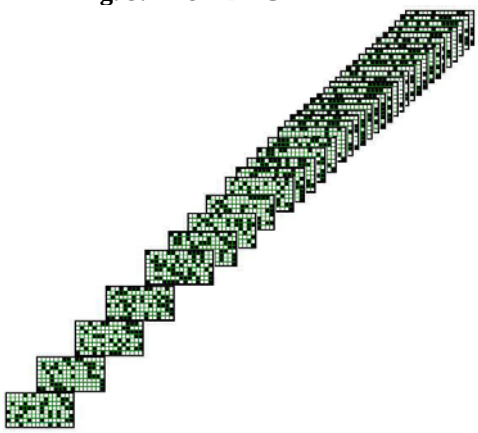


Fig. 10. 0,16 second firing pattern from rabbits brain stem
(32% inactive unit, 46% prevalence)

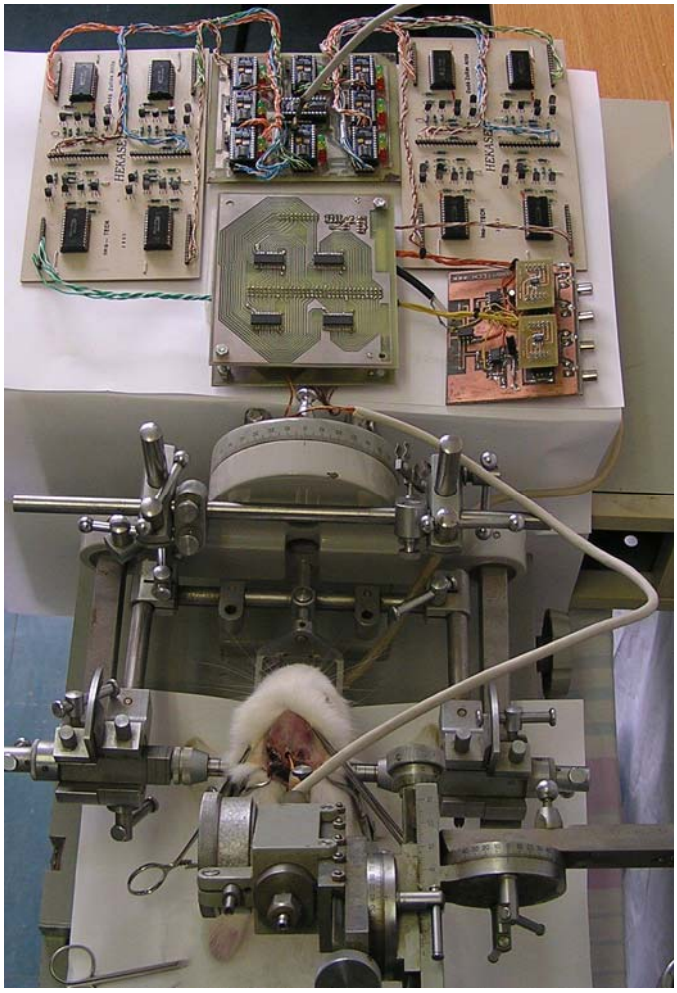


Fig.11. The entire system in operation. (Own photo)

As a result of treatments, with the decrease of firing frequency, the ratio of synchronous firing grew; therefore the bioelectric tone of the brain stem moderated and became more ordered at the same time. The decrease of the firing rate by certain medicaments, that is the ratio between the base compound and its GSH conjugate revealed a close connection with efficiency tests, in the case of rabbits with motility results, and in the case of fish with the scale of breathing depression.

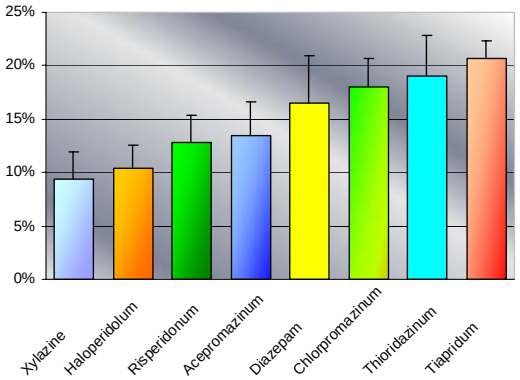


Fig. 12. Decrease of the firing frequency in rabbits after base compound treatment

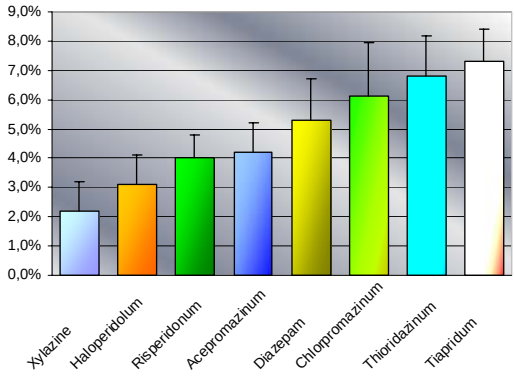


Fig. 13. The change in the ratio of synchronous firing units in rabbits as a result of treatment

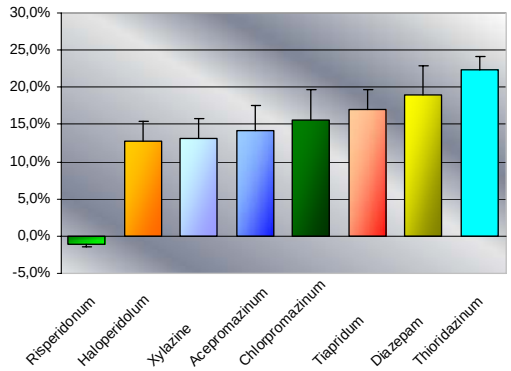


Fig. 14. Decrease of firing frequency of GSH conjugate following treatment

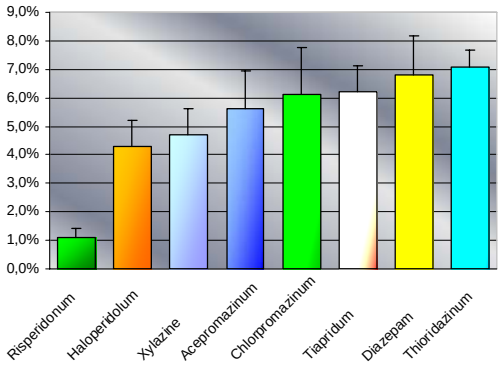


Fig. 15. The change in the ratio of synchronous firing units as a result of treatment

It seems valid that tranquilizing compounds, similarly to narcotics may be effective in the RAS as a result of the depression of conduction. The RAS activity's effect can be the change of the ratio of the "sign-noise relation", with the decrease of absolute irritability. Next to the decrease in the firing rate, the ordered firing activity increased to a certain degree. My investigations also show that the effects of psychopharmacons in the central nervous system's firing matrix still provide new possibilities for analysis. The nature of synchronous firing and its relation with firing activity should be examined in more detail. The nature of response patterns for stimulation could be investigated, comparing the effects of various treatments. Patterns could be analyzed from numerous, new point-of-views and with new algorithms that would bring us closer to the better understanding of the effect of tranquilizers on the central nervous system. Unfortunately, the length of this publication does not allow this so it is part of my future objectives.

My findings show that the examined psychoactive compounds have GST enzyme inductive effect as well. At the same time, as a result of GST activity in the brain stem the glutathione conjugates of the compounds can be formulated in the brain tissue or in other organs as well. The glutathione conjugates, similarly to other γ-glutamine peptides and glutathione analogs can influence the central nervous system's glutamatergic neurotransmission, this way contributing to the development of symptoms of certain side effects, like that of pseudo parkinsonism.

Certain psychopharmacons caused the blocking of GST. Probably other metabolic mechanisms play a role in this. Probably the izoenzymes found in the organs are responsible for the specific tissue effects caused by medicaments.

The results show the differences between the traditional and new compounds. The known side effects are in a supposedly close relation with the appearance of the formed glutathione complexes.

Based on my findings, it seems that it would be useful to examine the applicability of medicaments used successfully in human therapeutics in the case of certain animal species. The new generational neuroleptics have better side effect profiles and do not burden the organism so much. The negative effects of chronic treatment could be more moderate in the case of animal breeding as well. At the same time, their efficiency is not worse than that of traditional medicaments.

The continued examination of GSH conjugates of those medicaments that cause significant GST induction may be necessary. The efficiency of conjugates and their role in the

development of side effects on the level of particular organs and the examination of their possible interaction with the process of glutamatergic neurotransmission.

V. The new and original findings of the dissertation

1. During my investigations I have touched upon various fields of study with my results. In the following, I will summarize my findings according to the nature of the various disciplines.
2. I have developed a novel microcontroller based hardware and its connected algorithms for a more effective GST enzyme kinetic analysis of a great amount of samples. With the help of the system the effect of tranquilizers on GST induction can be detected and analyzed more precisely.
3. I have proven that acepromazin, chloropromazin, diazepam, haloperidol, risperidon, thioridazin, tiaprid and xylazine medicines with a tranquilizing effect, all – to a different degree- competitively block the in vitro development of GST induced CDNB-GSH conjugates. All compounds are substrates of the GST enzyme and catalyze the formation of medicine-GSH conjugates.
4. As regards the above mentioned compounds in the case of rabbits and silver crucian carp I established an efficiency order considering the parameters of motility, hot-plate, body temperature, body weight and breathing.
5. I showed that acepromazin, chlorpromazine, diazepam, haloperidol, risperidon, thioridazin, tiaprid and xylazine compounds resulted in a different scale of GST enzyme induction effect in the case of particular organs (liver, brain, kidney, spleen, lungs, heart) of laboratory rats, rabbits and silver crucian carp.
6. I showed and compared the efficiency of GSH conjugates of the above mentioned compounds in the case of rabbits and silver crucian carp (regarding motility, hot-plate, body temperature, body weight and breathing)
7. I have developed an original multiprocessor artificial neural network incorporating analog and digital features and its connected algorithms that can establish two-directional communication with a targeted section of the living nervous system through a 128 multi electrode array. The system offers a new approach towards the interaction of the neural network and the nervous system; it treats it as its analog part connected to it in an unknown way. With its help – in my present investigations – the efficiency and effect mechanism of psychopharmacons in the central nervous system can be examined.
8. I have found out and compared with the results of an efficiency test the effect of the synthesized GSH conjugates of the examined drugs on the brain stem's firing pattern in the case of rabbits and silver carp.
9. I have found out that the presumption that the tranquilizing compounds cause the alteration of the signal/noise ratio of the firing pattern in the upper part of the brain stem is adequate. Alongside the decrease of the firing rate the synchronized activity increases proportionately.

VI. Findings that Can Be Used in Practice

All of the acepromazin, chlorpromazin, diazepam, haloperidol, risperidon, thioridazin, tiaprid and xylazine compounds proved to be the substrates of the GST enzyme to a different degree and formed conjugate with GSH. Considering that other γ-glutamine peptides and glutathione analogs like glutathione conjugated tranquilizers can influence glutamatergic neurotransmission, it would be reasonable to examine the effects of the conjugates of these compounds in more detail. This would hopefully result in the decrease in the number of side effects.

Based on my findings, it would be important to examine the applicability of certain compounds in veterinary science or in animal husbandry. Because of good efficiency and less side effects primarily the second generational tiaprid and risperidon could be considered. The loading of the glutathione conjugated detoxification system - as shown in my work - should be considered in the case of certain medicine and organs. Based on the findings concerning rabbits used as model animals, comparative examination would be necessary in the case of other species as well. I suggest the continued investigation of new generational neuroleptics, in the case of rabbits thioridazin and haloperidol, in the case of silver carp acepromazin, thioridazin and chlorpromazine.

Based on the architecture and algorithms of the microcontroller enzyme kinetic analyzing system the present laboratory measuring systems can be developed.

Based on the hardware architecture and algorithms of the neural network, similar, more complex networks could be built for the analysis of other neural activities as well. I suggest the development of the neural network with the significant increase in the number of processors.

VII. Publications Connected to the Dissertation

Reviewed Scientific Publications

Godó Zoltán Attila, Kocsis István (2004): Mikrokontroller alapú neuronhálózat a központi idegrendszer tüzelési mintázatának elemzéséhez. Debreceni Műszaki Közlemények. 3, 2, pp. 75 - 84.

Godó Zoltán Attila (2004): Microcontroller-based neural network for evaluating the activity change of glutathione conjugates on RAS. IEEE 4th International Conference on Intelligent Systems Design and Application (ISDA), Budapest, Hungary, Proceedings. pp. 547-550.

Godó Zoltán Attila (2004): Use of ADNC in Analyzing the Firing Patterns in Central Nervous System. IEEE 8th International Conference on Intelligent Engineering Systems (INES). Kolozsvár, Romania, Proceedings. pp. 89-92.

Godó Zoltán Attila (2005): Artificial neural network with a multiprocessor for analyzing the changes of the firing pattern of the central nervous system caused by the psychopharmacons. *Abstract*. Clinical Neuroscience. 58, 1, pp. 34-35.

Godó Zoltán Attila (2006): Bioinformatical application of microcontroller in enzyme induced investigation of glutathione-s-transferase. Debreceni Műszaki Közlemények. V., 2, pp. 41-56.

Godó Zoltán Attila (2006): Processing the bioelectric activity of the central nervous system with ADNC-AS. Acta Technica Napocensis. 49, V, pp. 27-34.

Godó Zoltán Attila (2006): Signal amplification of neurons in the Artificial Neural Network for the Effect Analysis of Neurolepticums. Academic Journal of Manufacturing Engineering. 4, 2, pp. 42-48.

Godó Zoltán Attila (2006): Glutathione-S-Transferase induction of certain Neuroleptics. Debreceni Műszaki Közlemények. V., 4, pp. 101-117.

Godó Zoltán Attila, Pirger Zsolt (2007): The Application of Artifical Neural Network in Neurophysiology. International Scientific Conference (microCAD), Miskolc, Hungary, Proceedings. pp. 63-69.

Godó Zoltán Attila (2007): The effectiveness and metabolism of some of the Neurolepticums on the GST pathway. *Abstract*. Clinical Neuroscience. 60, 1, pp. 22-23.

Godó Zoltán Attila (2007): Artificial Neural Network- for the Effect Analysis of Neuroleptics in the Central Nervous System. International Conference on Neural Networks (ICNN), Barcelona, Spain, Proceedings. (Accepted)

Godó Zoltán Attila (2007): Trankvillánsok hatékonysága és toxicitása házinyúlban. Acta Agraria Debreceniensis. *(Közlésre elfogadva)*

Scientific Conference Lectures and Poster Presentations

Godó Zoltán Attila, Pirger Zsolt, Nagy Gábor, Révész Csaba, Varga Vince (2001): Glutation-S-transzferáz enzim indukciója pszichoaktív farmakonokkal. XXXI. Membrane Transport Conference, Sümeg, Hungary p. 84.

Godó Zoltán Attila (2003): Applying microcontrollers to bioinformatics. XXXIII. Membrane Transport Conference, Sümeg, Hungary p.88.

Godó Zoltán Attila (2004): Artificial neural network for analyzing the firing pattern of RAS. XXXIV. Membrane Transport Conference, Sümeg, Hungary, p. 49.

Godó Zoltán Attila (2004): Microcontroller-based neural network for evaluating the activity change of glutathione conjugates on RAS. IEEE 4th International Conference on Intelligent Systems Design and Application (ISDA), Budapest, Hungary.

Godó Zoltán Attila (2004): Use of ADNC in Analyzing the Firing Patterns in Central Nervous System. IEEE 8th International Conference on Intelligent Engineering Systems (INES). Kolozsvár, Romania.

Godó Zoltán Attila (2005): Artificial neural network with a multiprocessor for analyzing the changes of the firing pattern of the central nervous system caused by the psychopharmacons. Hungarian Neuroscience Society XI. Conference (HNS, MITT) Pécs, Hungary, p. 50.

Godó Zoltán Attila (2007): The effectiveness and metabolism of some of the Neurolepticums on the GST pathway. Hungarian Neuroscience Society XI. Conference (HNS, MITT) Szeged, Hungary, p. 14.

Godó Zoltán Attila, Pirger Zsolt (2007): The Application of Artifical Neural Network in Neurophysiology. International Scientific Conference (microCAD), Miskolc, Hungary.

Godó Zoltán Attila, Révész Csaba (2007): Trankvillánsok hatékonyság és toxicitás összehasonlító vizsgálata. XXXVII. Membrane Transport Conference, Sümeg, Hungary *(Közlésre elfogadva)*

VIII. Publications Not Related to the Dissertation

Scientific Conference Poster Presentations:

Godó Zoltán Attila, Nagy Gábor, Pikó Eszter, Varga Vince (2000): A hypertónia etiopatogenézisének rizikófaktorai: testtömeg index (BMI) és testzsír százalék összehasonlító vizsgálata átlag populáció és egyetemi hallgatók körében. XXX. Membrane Transport Conference, Sümeg, Hungary, p. 72.

Nagy Gábor György, Antal Andor, Serfőző Zoltán, **Godó Zoltán Attila**, Komáromi Sándor, Varga Vince (2000): Akut peritonitis kezelése módosított dializáló folyadékkal. XXX. Membrane Transport Conference, Sümeg, Hungary, p. 79.

Révész Csaba, Forgács Zsolt, **Godó Zoltán Attila**, Rajczy Klára, Krizsa Ferenc, Mátyás Szabolcs, Bernard Artur, Lázár Péter, Marcsek Zoltán (2001) : A Ni^{2+} hatása humán primer ovariális granulosa-sejt tenyészet szteroidgenezisére. XXXI. Membrane Transport Conference, Sümeg, Hungary, 72.

Reviewed University Lecture Notes:

Godó Zoltán Attila (2003): Programozás, vezérlések programozása pascal nyelven és a C nyelv alapjai. (p. 66. A/4 ív).

Godó Zoltán Attila (2003): Élettan gyakorlatok biológusoknak I. – A keringés és légzés élettana. (p. 58. A/4 ív).

Godó Zoltán Attila (2004): Élettan gyakorlatok biológusoknak II. – A táplálkozás, hormonális rendszer és az idegrendszer élettana. (p. 64. A/4 ív).

Godó Zoltán Attila (2005): Programozás I. Villamosmérnökök számára. (p. 17. A/4 ív).

Godó Zoltán Attila (2005): Digitális technika számítógéppel. (p. 36. A/4 ív).

Godó Zoltán Attila (2006): Digitális technika mikrokontrollerrel. (p. 42. A/4 ív).