REVIEW

From Frog Muscle to Brain Neurons: Joys and Sorrows in Neuroscience

František VYSKOČIL[1]

[1]Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic

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Summary

One element, potassium, can be identified as the connecting link in the research of Czech neurophysiologist Prof. František Vyskočil. It accompanied him from the first student experiments on the frog muscle (Solandt effect) *via* sodium-potassium pump and quantum and non-quantum release of neurotransmitters (e.g. acetylcholine) to the most appreciated work on the reversible leakage of K^+ from brain neurons during the Leao´s spreading cortical depression, often preceding migraine. He used a wide range of methods at the systemic, cellular and genetic levels. The electrophysiology and biochemistry of nerve-muscle contacts and synapses in the muscles and brain led to a range of interesting findings and discoveries on normal, denervated and hibernating laboratory mammals and in tissue cultures. Among others, he co-discovered the facilitating effects of catecholamines (adrenaline in particular) by end-plate synchronization of individual evoked quanta. This helps to understand the general effectiveness of nerve-muscle performance during actual stress. After the transition of the Czech Republic to capitalism, together with Dr. Josef Zicha from our Institute, he was an avid promoter of scientometry as an objective system of estimating a scientist´s success in basic research (journal *Vesmír*, 69: 644-645, 1990 in Czech).

Key words

Skeletal muscle • Neuromuscular end-plate • Neuropharmacology • Excitable membrane • Acetylcholine release • Ion sensitive microelectrodes • Synaptic delay • Brain potassium • Na^+, K^+-ATPase

Corresponding author

F. Vyskočil, Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic. E-mail: kinacka@seznam.cz

Early times

My first research project in the high school was about alopecia. To this day, I can move the subcutaneous muscles and thus improve blood circulation and nutrition in the hair follicles, so at the age of 82 years, I have a lush, minimally gray mane. I used to stop people on the street, bald and hairy alike, asking if they could also move their hair. It turned out that 80 % of bald individuals could not. However, I truly discovered real science at the Department of Physiology of the Faculty of Science, Charles University in Prague. During the summer holidays between the third and fourth semester of my pregradual studies, on the recommendation of my supervisor, Dr. Ivan Novotný (1931-2021), I started experimenting with a Fenn micromanometer made by a skillful Faculty glassblower. It measured oxygen consumption in an isolated frog muscle. A supposed weak depolarization by application of 10 mM K^+ increased oxygen consumption up to 10 times without any muscle contraction. We demonstrated that this "Solandt effect" could be inhibited by several substances affecting the internal concentration of Ca^{2+} ions. Years later, I asked Ivan why he believed unconditionally my measurements. Answer was “I secretly asked the lab technician Jane to measure it again after you for extra money in the evenings”. The work was published a few months later in the journal Nature [1]. However, how much the muscle fibers were depolarized without contracting was an important question. Dr. Novotný had a friend at the Institute of Physiology of the Czechoslovak Academy of Sciences (IPHYS), Dr. Radan Beránek, who was experimenting to introduce a method

of measuring the transmembrane potential of cells using glass ultramicroelectrodes. I began to visit the institute, and together with Drs R. Beránek and L. Vyklický Sr. we implemented this method [2,3]. Stimulators and high-resistance input amplifiers were manufactured directly in the laboratory by Ing. Evžen Ujec, using high-quality microtransistors smuggled into Czechoslovakia in the pockets of random institutional travelers with Communist Party credentials. We directly measured the depolarization of muscle fibers by impaling microelectrodes into muscle fibers and found that the depolarization by potassium remained unchanged despite the blockade by physostigmine, ouabain, and caffeine [2-5].

At the student research competition in 1961, I won a second position nationally behind a team of radiophysiologists from Hradec Králové, Czech Republic. In 1963, after graduating with honors, I began my postgraduate studies when Dr. Beránek returned from a year-long stay with Nobel Prize laureate Prof. Bernard Katz at University College London. For my dissertation, I was tasked to measure dose-response curves of curare and atropine inhibition – antagonists of acetylcholine receptors (nAChR). Using microelectrodes this was done on standard end-plate potentials of innervated rat diaphragm muscle after stimulation of the phrenic nerve and then after a week of denervation when new acetylcholine receptors are formed along the entire length of the sarcolemma. An iontophoretic dose of acetylcholine (ACh) replaced the nerve. I was deeply engrossed in this work; we were a generation hungry for new information. Thus, I often slept in the lab, conducting experiments and also helping with the institute's relocation from Dejvice to the new campus in Prague's Krč district.

When I compared the effect of curare on native and denervated nAChRs, I found no difference. However, I noticed that the lab assistant, Mrs. Petrtýlová, had prepared a stock solution of curare for experiments on denervated nAChRs that exhibited peculiar opalescence. Upon inquiry, it turned out that the concentration of curare was by mistake 10 times higher than for innervated rat fibers. Nearly missing this discovery, I corrected the concentration error and found that denervated nAChRs receptors (similar to those in embryonic muscles) had 10 times lower sensitivity to this drug and likely had a different composition. This was confirmed by molecular biology methods. One of the first findings of two different subtypes of the same receptor was thus made. After my first publication in *Nature*, Radan Beránek and I wrote two articles together, which were virtually unchanged when published in the iconic *Journal of Physiology London*, focusing on this surprising discovery [6,7].

The second paper described how atropine reduces and shortens synaptic end-plate potentials. This was the basis for the use of atropine in anticholinesterase poisoning (sarin, soman, etc.) (Fig. 1) and soldiers carry it with them at all times in case of chemical attack.

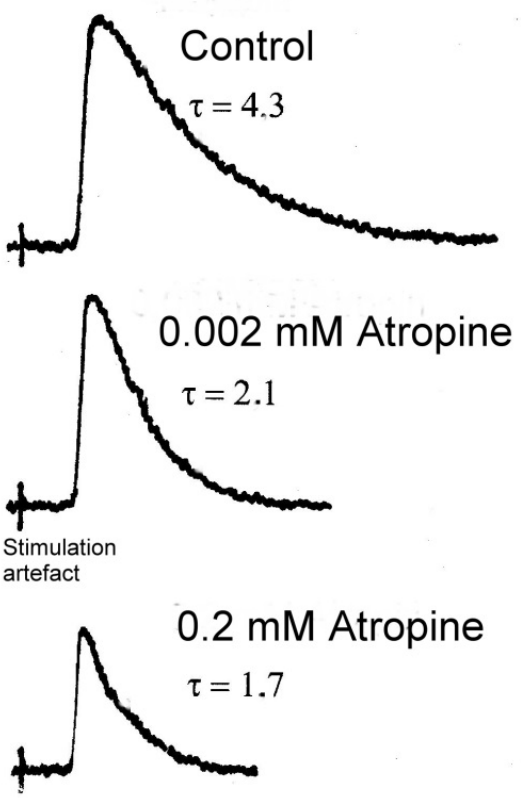

Fig. 1. Atropine reduces the amplitude and shortens the exponential time course (see values of T) of intracellularly recorded end-plate potentials (F. Vyskočil, unpublished).

At that time, we were relatively isolated from the rest of the world except for Russia. It was recommended to maintain contacts there, so Dr. Beránek sent me to Kyiv Ukraine, for a conference. In 1967, I presented our results on adult and embryonic receptor types. I traveled to Kyiv by train, with some time to explore the beaches along the Dnipro and the ancient Ukrainian city with its grand boulevard, Khreshchatyk. One afternoon, before the conference, I was sunbathing on the Dnipro's shore when two young men asked me for a cigarette. They turned out to be thieves and stole my new shoes. The next morning, before the conference, my colleagues took me to a store where I bought some unsightly replacement shoes.

On the conference I met an interesting and smart

man from St. Petersburg named Lev G. Magazanik. We spent the entire night discussing neuromuscular junctions, science, and we clicked instantly. When I returned to Prague, we attended a stage performance of Gogol's play "*The Government Inspector*" in a small theatre. When famous Czech actor (Mr. Jiří Kodet) appeared as Chlestakov, the impostor posing as the inspector, wearing exactly the same shoes I had acquired in Kyiv, I could not contain myself and loudly whispered from the second row, "Those are my shoes!" The actor glanced at me somewhat curiously, wondering what it meant. The costume director had gone to great lengths to ensure authenticity, even procuring genuine Russian ugly army shoes.

After completing my postgraduate studies, I was supposed to go to the USA for a research internship at Prof. Del Castillo's laboratory and then to Prof. Kuffler, both renowned neurophysiologists. However, there was an incident typical of the communistic time. In the Institute of Physiology, like everywhere else, there was a local Communist Party organization. Its chairman, Dr. Hrůza, left for the West and wrote to the Academy's president Prof. Šorm, that he would not return, intending to emigrate and denounce the socialist regime. Punishment fell not on the guilty but on the innocents. Despite being a good organic chemist, Prof. Šorm issued a ban on travel to Western countries for Institute of Physiology staff. Thus, my trip to the USA was cancelled. Then, suddenly, a call came from St. Petersburg. Dr. Lev Magazanik had received a special six-month stipend for a foreign guest and remembered me. We could conduct experiments together, just as we had discussed in Kyiv. Frustrated by my thwarted trip to America, I accepted his invitation, and my wife and I traveled to St. Petersburg. I found that we had to either make many technical setups in the laboratory ourselves or somehow modify other devices. For example, we converted a camera from a captured wartime German aircraft into an oscilloscopic camera, which we used to record cell potentials and currents. In this Russian intellectual environment, there were personalities like the biophysicist Sergei Kovalyov, a well-known Russian dissident who vehemently protested after the 1968 invasion of troops to Prague. He was imprisoned, and went through the Gulag. When we met years later at President Havel's Forum 2000, we reminisced about those years that were essentially taken from us by communist regimes, preventing us from realizing ourselves in life as we might have imagined.

Together with my colleague Lev Magazanik, we made several significant discoveries about nAChRs desensitization, the effects of toxins on neuromuscular synapses, and the influence of ethanol on glutamatergic fly synapses (Fig. 2) [8-19]. One of them [10] was cited about 170 times in literature today, the other [15] about 70 times. At that time, we were nominated for a joint Award by the Academy of the Soviet Union and the Czechoslovak Academy of Sciences. The problem was that I had a career halt status because I was involved in the all-academic Committee against the Russian invasion in 1968. However, a directive came from Moscow that we should receive the award regardless of how inconvenient or uncomfortable Dr. Vyskočil might be. This demonstrates that good scientific results can be appreciated independently of the political system.

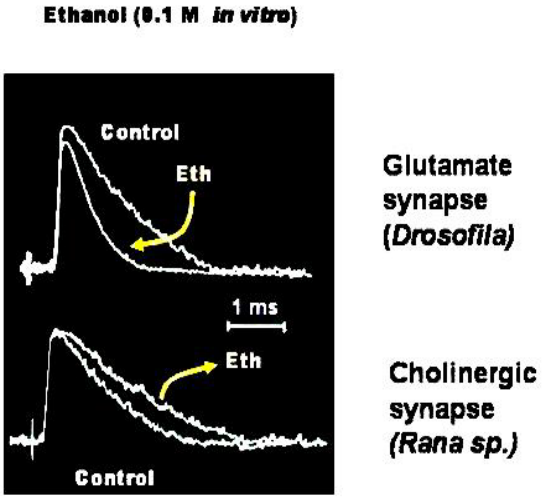

Fig. 2. Ethanol shortens excitatory postsynaptic potentials to the glutamatergic synapse (where glutamate is the neurotransmitter, mediator), but prolongs cholinergic EPSP, the mediator is acetylcholine and the receptors are nicotine-type. The resulting confusion contributes to drunkenness (after Magazanik and Vyskočil, unpublished).

After 1968 and the sudden death of Dr. Radan Beránek, our cellular neurophysiology laboratory underwent little personnel change. I remained alone to study intracellular nerve-muscle electrophysiology. I enjoyed collaboration with colleagues from the Faculty of Science, Charles University in Prague, notably Prof. Ladislav Janský and Dr. Jan Moravec in hibernation neurophysiology [20-22]. Of course, many collaborators were from the IPHYS and its electrophysiological laboratory. It was Dr. Pavel Hník working on physiology of the musculoskeletal system using long-term implanted electrodes. Pavel was also the English editor of our local journal *Physiologia bohemoslovaca*, now *Physiological*

Research. Furthermore, I made a couple of experiments with Dr. Ladislav Vyklický Sr., otherwise studying pain pathways [23], and electrical engineer Evžen Ujec, who constructed amplifiers, stimulators and studied biophysics and morphology of glass microelectrodes [24]. A useful cooperation was also with physical chemist Norbert Kříž and with excellent biochemists ing. Jan Teisinger and Dr. Petr Svoboda, as well as with some other members of the *Spinal Cord Physiology and Neuromuscular System Department*. I have collaborated with histological and electron microscopy laboratory led by Dr. Jiřina Zelená [25] biochemical laboratory of Dr. Ivo Syrový, who studied slow and fast muscle myosin [26,27], neurochemist Dr. Stanislav Tuček [25] and some other specialists and Institute colleagues. Another fruitful interdisciplinary works emerged from my contact with the lead scientist that time and founder of the concept of trophic influence of nerves on muscles, Prof. Ernest Gutmann. We studied together the aging neuromuscular junction and hormonal effects on skeletal muscle and muscle drafts continuously until his death in 1978 [28-35].

Numerous studies were performed – as indicated later –, while visitors came to our laboratory from USA (Prof. Charles Edwards, University of Tampa, Florida), Hungary (Prof. Peter Illes and the president of the Hungarian Academy of Sciences Prof. Sylvester Vizi), Italian and Swedish neurophysiologists Dr. Alfredo Gorio and Prof. Stephen Thesleff, Algerian specialist Dr. Nasira Tabti, and – after 1989 – Prof. Gerta Vrbová (London), Dr. Martin Ward (Newcastle upon Tyne), and Dr. Rosemary Jones (Cambridge) [36]. I was also host and visitor of many Russian colleagues, primarily from Kazan University and the Academy of Sciences under the leadership of Prof. Eugeny Nikolsky (Republic of Tatarstan, joint State Prize in 1994). Those were the only off-line professional relationships when my Western personal contacts were denied for political reasons. One example of such a successful study across the Iron Curtain is an article from the Prague laboratory on non-quantum release of a mediator on the motor end-plate by Prof. C. Edwards (USA), E. Nikolsky (Kazan, SU) and me from 1983, cited 110 times [37].

As reasons for the ban on trips to the West, the directors (first L. Vyklický Sr. and then Z. Drahota) recommended me to write to the inviters that I am seriously ill or that my grandfather would have a funeral at the time of the conference. Occasionally, however, I managed to go out for a short period of time, when the secretaries of the local Communist Party in our Prague district were changing and chaos reigned in their centers. This happened, for example, in 1985, when we have conducted a study for *Journal of Physiology (London)* in the laboratory of Prof. Thesleff in Lund, Sweden, within a few weeks [38,39] or a leak to an Italian laboratory in Abano Terme studying gangliosides [40,41]. On the way to Lund, Sweden, I arrived in Malmö from Copenhagen as the only passenger on board Boeing 747, as I didn't possess some 12 Swedish crowns to cross the Öresund Strait on a boat like other travelers. Alone at the small airport in Malmö, I was looking for some kind of custom or other declaration sheet to fill out, as educated by "socialist" airports. Sure enough, there was a small table with a form in the corner. I started to fill in the fields: Name, nationality, birth, where I am going, what I came from, by what type of airplane (Boeing 747, I wrote, I saw it through the window), what fuel it flies on... That struck me, such Swedish thoroughness! There was an attendant leaning against the doorposts. So I asked him why I should mention it, as I didn't know any type of aviation fuel. With a kind smile, he said, "We need to know what fuel to fill on your personal plane". When I laughingly said that this Boeing is not my personal possession because I am from the East country, where no one had such personal aircraft, he asked me "Well, why not? Why can't you have your private large-capacity plane right in Prague?"

Ion-selective glass microelectrodes

In 1972, we introduced as a second laboratory a new technique called ion-selective glass micro-electrodes for direct measurement of concentrations of K^+, Na^+, Ca^{2+} and Cl^- ions in the cellular environment. Liquid ion-exchanger "membranes" in the tiny tip of glass microelectrode were developed by J.L. Walker for chloride and potassium in 1971 [42]. These liquid ion exchangers were smuggled by Dr. Pavel Hník from Walker´s laboratory in Salt Lake City to Prague that year. Norbert Kříž and me, having already mastered the production of the microelectrodes, put together the necessary equipment and developed many modifications of the technique for recording intra- and extracellular ion concentrations [43]. We decided to use the potassium-specific microelectrodes for testing the hypothesis that potassium is released from cells in working animal and human muscles to their veins [44-48] and also during self-propagating wave of Leao´s spreading depression of rat brain EEC activity due depolarized neurons probably

by extracellular potassium $[K^+]_e$ [49]. This was studied in the cooperation with Dr. Jan Bureš from *Memory Department* of our institute. Such high $[K^+]_e$ has already been expected, but its absolute magnitude remained unknown. The actual experiments on rat cortex were performed in my small, crowded laboratory during two weeks of intensive work in the late summer of 1972. Already the first results confirmed our expectations: intercortical $[K^+]_e$ rose in a few seconds from the resting level of 3 mM to over 60 mM during spreading depression and up to 100 mM during terminal anoxia (downward drop in Fig. 3). The wave of propagating depression was accompanied by a 30-fold increase in the extracellular concentration of potassium, which flows from the depolarized cells. After a few minutes, K^+ was pumped back into the cells and the depression moved further. Spreading depression is therefore a reversible process, ATP for potassium reuptake by the ATPase is plentiful.

We were fascinated by the reproducibility of results and by the power of the method, which offered definitive answers to speculation about brain microenvironment. It was also surprising that the normal $[K^+]_e$ concentration in rat brain was not the same as in blood plasma (5 mM), but significantly lower (3 mM). Anoxic total release of K^+ led to irreversible brain death within 2-3 min [50] (Fig. 3).

Undoubtedly, other groups were hot on the same trail. The feeling that we were participating in a race contributed to the exhilarating atmosphere of those days as well as to the decision to expedite publication by submitting the results in the short-communication format [49]. Indeed, results of similar research in Munich, 400 kilometers from Prague, were published only a year later, and publications from six other laboratories followed in 1974-1975. Our paper thus became the first of a series marking a wave of renewed interest in the mechanism of spreading depression, which was recognized as a dramatic example of the failure of ionic homeostasis in the central nervous system. It was used in numerous later studies employing ion-selective microelectrodes to demonstrate transmembrane shifts not only of K^+, but also of Cl^-, Na^+, H^+ and Ca^{2+} during various physiological and pathophysiological states. This wave crested in the early 1980s, when anoxic depolarization started to be used for testing the role of excitotoxic amino acids and their antagonists in ischemic brain damage.

The high impact of this paper [49] was already obvious in the late 1970s as it won the contest for the most-cited week paper in *Current Content* published by the Institute of Scientific information (ISI) in Philadelphia. Besides ISI, this study did not receive any particular recognition from the national or international academic establishment. Spreading depression has recently been linked with the onset of migraine, which predominantly affects women. Using a potassium-selective microelectrode technique, we confirmed in the next paper [50] that the threshold $[K^+]_e$ concentration for the initiation of spreading depression in the extracellular space of the cerebral cortex in the female rat brain is lower more than half compared to the male one.

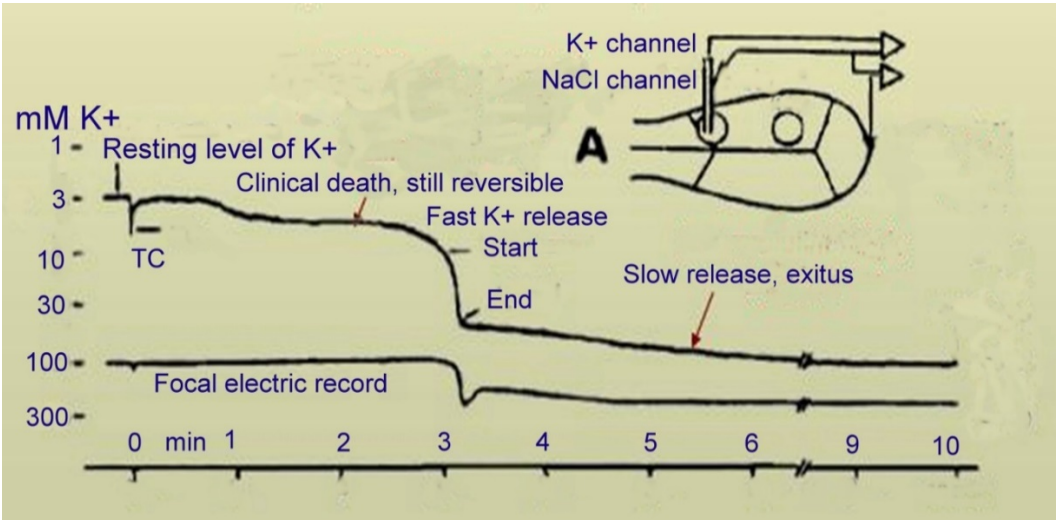

Fig. 3. Terminal anoxia. Failure of the sodium/potassium pump in the cerebral cortex of a narcotized rat is evidently due to deficiency of ATP during anoxia. (**A**) Diagram of a rat skull with holes for scanning $[K^+]_e$ in mM using double-barrel microelectrode. A second channel filled with 100 mM NaCl was used for simultaneous recording of the electrical focal potential at the measuring point of the cerebral cortex. For the first 3 min after down-stopped breathing by tubocurarine injection (TC), the potassium is still mostly in the cell, the pump is more or less working and animal can be resuscitated. After a sudden fast K^+ release, (downward drop), this clinical death turns into exitus (after Vyskočil *et al.* [49]).

Non-quantal release of acetylcholine at the neuromuscular junction

In the next part of this article, I will describe a number of other activities in the research of neuromuscular junction and its physiology, pharmacology and biochemistry in *Cellular Neurophysiology Department* of IPHYS and adjacent laboratories. With the exception of non-quantal release of neurotransmitters, especially of acetylcholine (ACh), other aspects will be mentioned only in shortened form. There are two principal mechanisms of ACh release from the resting motor nerve terminal: quantal (miniature and stimulation evoked end-plate potentials, QR) and non-quantal (NQR); the former being only a small fraction of the total, at least at rest [51]. The first demonstrations of non-quantal transmitter release on mouse and rat diaphragm and analysis of the mechanism release, action mechanism and physiological significance were obtained in 1977 [52,53]. In the series of original articles we then described basic research about the NQR which we quantified at end-plate zone as hyperpolarization due to a removal of the slight depolarization by NQR in anticholinesterase-treated skeletal muscles by curare. In mammals, it exceeded ten times the similar effect found in frog muscle [cf. 52]. Possible mechanisms of the non-quantal release were suggested and proved by the inhibition of NQR using vesicular ACh-transporter inhibitors, mostly vesamicol [37,54,55]. QR means that vesicular ACh-transporter (transferring normally Ach into vesicles during their intracellular refilling) is incorporated into the presynaptic membrane in the moment of the release of quanta, when the vesicular membrane spline with the membrane of the nerve endings (Fig. 4). This creates an outward directed pathway for the non-quantal escape of ACh into the synaptic cleft [56].

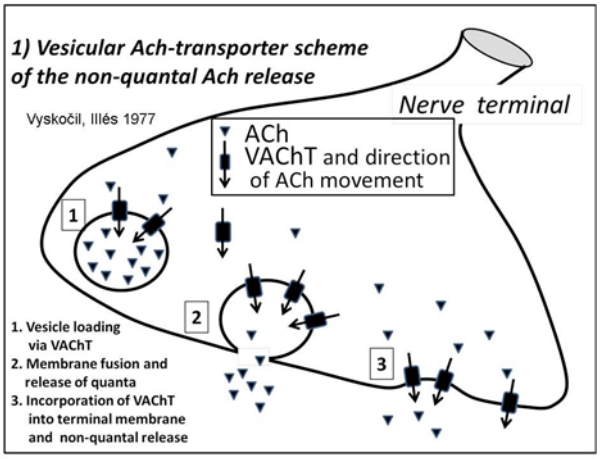

Fig. 4. Scheme of possible mechanism of non-quantal Ach release. Follow numbers from 1 to 3. ACh molecules are schematized as the full triangles. VAChT – Ach transporter, arrows show the direction of Ach movement (F. Vyskočil, unpublished).

Another candidate can be a choline transporter, the inhibition of which also does suppress the NQR. But this may be an indirect consequence of choline deficiency for ACh production in the nerve terminal [57]. In general, the permanent NQR release and hydrolysis of ACh in the cleft, together with the quick uptake of the newly produced choline, could keep the synthetic machinery within the terminal ready for prompt fulfillment of different physiological demands when quantal release is augmented for example during exhaustive physical work [58] and ionic changes around the synapse [58-61].

We found further that NQR is undoubtedly an important trophic factor in adult neuromuscular contacts [62-64] and during end-plate development. It helps to eliminate the polyneural innervation of developing muscles, supports higher excitability of the end-plate subsynaptic membrane by surplus polarization and protects the resting membrane potential from postdenervation depolarization. NQR might shorten the end-plate potentials by promoting postsynaptic receptor desensitization when acetylcholine esterase (AChE) is inhibited during anti-AChE poisoning [65,66]. It ensures higher excitability of the adult subsynaptic membrane by surplus polarization and protects the resting membrane potential from depolarization by regulating the NO cascade and chloride transport [69]. In adult synapses, it can also activate the electrogenic Na^+/K^+-pump, change the degree of synchronization of quanta released by the nerve stimulation and affects the contractility of skeletal muscles *via* purinergic effects [52,62-71].

Apparently NQR is not restricted to the cholinergic neuromuscular junction only, since massive non-quantal release was shown also at the glutamatergic neuromuscular junction of the blowfly larvae and in calyx-bearing fibers of the turtle ampula posterior crista. Similar transmitter release ("tonic" release) mediated by a transporter was also described in certain brain GABAergic synapses playing the role in perinatal changes of $GABA_A$ receptors from excitatory to inhibitory mode [cf. 67].

In adult vertebrates, some of the ACh released from the nerve terminal might escape hydrolysis by AChE if it is released perisynaptically, and might then act as a "local hormone" on more remote parts of muscle fibers, for example, activating the electrogenic Na^+/K^+ pump. It can also change the degree of

synchronization of quanta released by the nerve stimulation [68]. Non-quantal ACh release can also alter the ovalbumin-induced functional properties of postjunctional ACh receptors and contribute to the disturbance of carbachol-induced contractility of skeletal muscles as reported by Teplov *et al.* [70a].

Other molecular mechanisms of interaction between excitable cells

Over the past decades, we have been interested in several molecular mechanisms of chemical interaction between excitable cells and factors determining the excitability of nerve cells and regeneration, including NO pathway. For this purpose, we used tissue cultures of dissociated nerve cells, spinal cord of the rat, mouse and frog synapses and, of course, neuromuscular junctions. Explored techniques were mostly glass microelectrodes, ion-sensitive microelectrodes, voltage and current clamp including patch clamp and systems for rapid application of drugs to particular cell area. Besides open-channel blockade**,** desensitization of nicotinic ACh receptors is a classical model of functional fatigue of ion channels. We proved as the first that nAChR desensitization is dependent on postsynaptic fiber voltage, temperature and Ca^{2+} ions as well as on some otherwise biologically inactive substances [10,15,17]. Role of negatively charged amino acids in beta 4 F-loop in activation and desensitization of alpha 3 beta 4 rat neuronal nicotinic receptors was demonstrated together with our students and coworkers [69-76]. Occasionally, synaptic events were mathematically modeled in respect to NO effect on denervated muscle resting potential, ionic changes and space conditions in the nerve ending possessing also glutamatergic auto-receptors of NMDA-type calcium channels [77-83]. We found that muscle NMDA receptors regulate the resting membrane potential through NO synthase [79]. The structural and functional similarity between imidazole derivatives and the known NO synthase inhibitor, 7-nitro-indazole suggests that imidazole, carnosine and anserine might act by inhibiting NO production which is stimulated by glutamate and carbachol [84]. Interestingly, an early postdenervation depolarization develops faster at end-plates of hibernating golden hamsters where spontaneous quantal and non-quantal acetylcholine release is very small [85]. On the other hand, acetylcholine and carbachol prevent muscle depolarization in denervated rat diaphragm [86]. This coincides nicely with immunocytochemical demonstration of M_1 muscarinic acetylcholine receptors at the presynaptic and postsynaptic membranes of rat diaphragm end-plates [87,88].

Synchronization over time of evoked quantal release

Another mechanism for regulating synaptic transmission is the time delay between the presynaptic nerve spike and the release of individual quanta, which accumulate over time to form the final postsynaptic potential. Improved synchronization of individual delays is one of the ways to make synaptic signal transmission much more efficient without any extra energy requirements. Extracellular miniature and nerve-evoked end-plate currents were measured in the studies of synaptic delays between nervous stimulation and the outpouring of quanta. It is worth of noting, given the long-term non-quantal release of ACh into the end-plate cleft, that the long release latencies are even increased by acetylcholine [87]. This desynchronization is in contrast with synchronizing positive effect of catecholamines, adrenaline in particular, on neuromuscular latencies. First demonstration and subsequent explanation of the beta-adrenergic receptor mediated action on synchronization and thus better time synchronization of the quantal release was done during my visits in Kazan in 1998 and 1999 in the laboratory of Prof. E.E. Nikolsky [89-92]. Better synchronization increased the amplitude of end-plate potentials by up to 20 %. These findings were further elaborated and the somewhat complicated relationships between the sensitivity of different types of skeletal muscles to catecholamines were gradually specified. The truth remains, that adrenaline increases the number of spontaneous and nerve-evoked quanta and improves synchronization on the mammalian neuromuscular end-plate of the skeletal muscles by up to 40 % [93].

Patch-clamp studies on nerve and muscle cells

The patch clamp method is a powerful technique used in electrophysiology to measure the electrical currents through individual ion channels in cell membranes. A fine heat polished glass micropipette (tip diameter about 2 μm) is filled with an electrolyte solution and brought into contact with the cell membrane under microscopic control. The glass microhole has an incredible affinity for membrane phospholipids. Gentle

suction by experimentator´s mouth through plastic capillary is applied to the micropipette to form a tight seal (gigaohm seal, controlled by microohm-meter on the PC screen) between the pipette and the cell membrane, isolating a small patch of membrane. The patch clamp can be configured in different modes. Cell-attached, whole-cell mode, inside-out and outside-out patch and even perforated patch, preventing the outflow of cell plasma into the attached micropipette.

The electrical currents of the nano- to pico-amperes flowing through the ion channels in the patch of membrane provide insights into the channel's conductance, ion selectivity, and gating mechanisms. It is invaluable since the 80's and was soon introduced in our laboratory [94] on a home-made apparatus by Ladislav Vyklický Sr. and Ladislav Vyklický Jr. with the help of other collaborators, namely Dr. Jan Krůšek and Dr. Viktorie Vlachová, who are still combining mathematical modeling with transfection of artificially mutated receptors for pain and other channels. Patch clamp microphysiology and micropharmacology is based on locally focused one-cell targeted and very fast multiple drug application, which was developed by Ing. Ivan Dittert in our laboratory.

We were able to provide first demonstration of K^+ channel subtypes during myotube formation [95], presence of Cl^- channels in neuroblastoma cells [96,97] and evidence that excitatory amino-acids not only activate the receptor channel complex but also lead to use-dependent block [98,99]. Several other joint papers have pointed to the GABAergic effect of cerebrolysin (used to treat vascular dementia), inhibition of glutamate transmission by cobalt, etc. [98-104]. Also interesting was the finding of the inhibitory effect of the standard selective serotonin reuptake inhibitor citalopram on Ca^{2+} currents in cardiomyocytes [105]. With substantial help of Dr. Jan Krůšek I was happy to confirm – using molecular biology and patch-clamp records – previous findings about muscle nicotinic receptors [6], different degree of cooperativity in adult, embryonic and mutated mouse nAChR in particular [106]. The new data provided the basis for mathematical modeling of the course of end-plate currents and prediction of further research directions in this synaptic connection [107-111].

Sodium-potassium membrane pump

Starting with the connection between the non-quantum outpouring of acetylcholine and the membrane Na^+/K^+ pump [52], the functional correlation between Na^+, K^+-ATPase in membrane fractions and electrogenic sodium pump in intact muscle cells was also in the center of our experimental interests. We discovered the direct effect of acetylcholine on the Na^+, K^+-ATPase and surplus postsynaptic hyperpolarization of muscle fibers that can be inhibited by AChR inhibitors such as alpha-bungarotoxin, curare and atropine [112]. A discrepancy has been found between the inhibitory effects of vanadate on the membrane Na^+, K^+-ATPase (reportedly responsible as a pollutant for mental depressions in the industrial areas in England) and the Na^+/K^+ pump of the skeletal muscle. Vanadate in concentrations, which are necessary to block the enzyme Na^+, K^+-ATPase activity of membrane fractions, failed to inhibit the electrogenic Na^+/K^+ pump in intact muscle cells [113], probably due to non-enzymatic reduction of vanadate to the less efficient vanadyl ion [113-115]. We also studied the effects of high calcium and calcium-channel blockers on Na^+/K^+ pump [116] and internal calcium measured electrophysiologically and by the fluorescent indicator [117]. It could be stressed that increase of Ca^{2+} concentration up to 10 mM in bath medium induced in diaphragm muscle tissue an elevation of intracellular $[Ca^{2+}]_i$ accompanied by a depression of sodium pump electrogenic activity and a depression of energy metabolism [118]. These changes may be involved in pathology of muscle tissue during the Ca^{2+} overload. The K^+-induced hyperpolarization of Na^+-loaded mouse diaphragm muscle, enzymatic activity of Na^+, K^+-ATPase and ^{3}H-ouabain binding to rat brain microsomes were also affected by K^+ channel blockers – tetraethylammonium (TEA), tetrabutylammonium (TBA) and apamin. TBA, and to a lesser extent TEA in millimolar concentrations, inhibited the electrogenic effect of the Na^+/K^+ pump, Na^+, K^+-ATPase activity, and ^{3}H-ouabain binding. The site of action of apamin on Na^+, K^+-ATPase is different from that of tetralkylammonium compounds; it apparently decreases the turnover rate of the enzyme [119]. Arachidonate (polyunsaturated fatty acid participating in the regulation of membrane fluidity, axonal growth, development, memory, and inflammatory responses) was also tested on both electrogenicity and ATPase activity [120,121]. When applied to Na^+-loaded muscles without potassium, arachidonate induced an ouabain-sensitive hyperpolarization of the muscle fibers. The arachidonate also increased the rate of hyperpolarization induced in Na^+-loaded mouse diaphragm fibers by 5 mM K^+. The activity of rat brain microsomal Na^+, K^+-ATPase was stimulated by arachidonate in reaction media with reduced amounts of ATP or K^+ and after short-lasting sonication of

the samples. It was concluded that, under particular conditions, arachidonate might serve as a Na^+, K^+-ATPase activator or inhibitor regulating its ion transport and electrogenicity [120,121]. We also found that Na^+, K^+-ATPase of brown adipose tissue and brain responds similarly to higher doses of isoprenaline, norepinephrine and epinephrine. But this stimulation of brown fat Na^+, K^+-ATPase by catecholamines does not have much relevance to the norepinephrine-stimulated thermogenesis in this tissue [122].

We remotely touched on cosmic muscle physiology when we measured some muscle during modeling of hypogravity. Antiorthostatic hindlimb suspension (unloading) of rats decreased the resting membrane potential (RMP) of skeletal muscle fibers in both fast extensor digitorum longus and slow soleus muscle of the rat by about 10 % within 7 days and more [123]. We compared these changes with kinetics of neurotransmitter release in neuromuscular synapses of newborn and adult rats [124].

Traveling with older and more recent attractions

After the Velvet Revolution in 1989, I was able to travel and work at several Western universities. I received an invitation to England, for example, where I spent several months at University College London in 1991. Together with Prof. Gerta Vrbová, an emigrant from 1958, we showed how important this non-quantum Ach release is in the formation of synapses during the development of an organism [65, cf.124]. At that time, I was invited to lecture at a number of universities in the UK. At Trinity College, Cambridge, four Nobel laureates were present at one of my lectures. During my speech, some seemed to be falling asleep. But then during the discussion, it turned out that this was only my illusion. They asked precisely targeted and even slightly uncomfortable questions. First of all, there were present Sir Alan Hodgkin and Sir Andrew Huxley, who was already in a wheelchair at that time. Both friends joined the faculty at Cambridge after conducting radar research for the British Air Ministry (1939-1945). They remembered that to disguise their success in aerial combat against Nazi Luftwaffe with a radar lead, the English propagandists claimed that their pilots ate a lot of carrots and had better eyesight than Germans. At Trinity College, Hodgkin and Huxley showed experimentally that the electrical potential of a nerve fiber behaves similarly to submarine electric cables. The third Nobel participant on my session was Sir Bernard Katz, a neuromuscular superstar, and finally Sir John C. Eccels. Interesting, though somewhat sad, was that Sir John was divorced after he was awarded the Nobel Prize in 1963 for synaptic inhibition in the brain. In 1966 he left his wife, four daughters and four sons and married my colleague from the Institute of Physiology, Dr. Helena Táboříková. She was experimentally rather inept, but she cared devotedly for Sir John until his passing at the age of 94.

Despite sharp and relatively long discussion on my topics [125-129] Sir Andrew invited me to Trinity College dinner (I admired his sincere prayer in a medieval cloak for her Excellence Queen Mother) and then to spend the weekend at his house. We discussed the history of the neuroscience and with his grand-daughter we played joyfully her violin. This sweet girl was very proud that in about five minutes she was able to “teach” me how to play the violin, even the virtuoso encore “Canary” by M. B. Polyakin (Figs 5,6).

Fig. 5. A weekend at Sir Andrew Huxley's (right).

Fig. 6. Sir Andrew with his wife Jocelyn in Prague (circa 1997), tasting imported wine from Moravia.

The violin has accompanied me practically all my life. After graduating from high school, I played Beethoven's Romance in F major at a local music school competition, and the present professor Moravec at the Janáček Academy of Music and Performing Arts in Brno told me: “If you don't do well in Prague at Charles University, study violin, you have a talent test with me.” In the end, I was left with violin (as a hobby) and my wife, (who liked my Beethoven) to this day. For me, she did her modern gymnastics and love.

The violin often got me out of a precarious situation. There is a little incident here from the 90's, when my colleague dr. Evžen Amler and I were traveling by car (Pontiac) from the University of Geneva to Prague. There was a car breakdown, we had to call the yellow angel. But we had no money at that time, only 100 DM for gasoline in Germany. Before the repairman arrived, I played cheerfuly melodies on my violin by the parked car, when suddenly a local TV station reporters from Bern arrived and filmed us waiting in peace. I said them: before the yellow angel helps us, Mozart does it: Ta ta, Ta ta, this tata ta... The repairman came, changed the injection fuse for two marks but asked for exactly 100 DM for the trip. But how do we get home? It occurred to me to ask the TV reporters for a fee for half an hour of violin play. And they actually paid in the blink of an eye. I signed the bill with my address and we got to Prague with a full tank. About a month later, I received a cassette from Bern with the recording. I still have it digitized. TV played it to the drivers on Sundays so that they would not be surprised by problems when returning from the weekend in the country and solve any difficulties with a smile as our Czech friends did.

When my travel ban was lifted, I was also invited to give lectures at three Indian universities, Bombay, New Delhi and Bangalore. I have presented a number of our published as well as still unpublished observations on nerve and muscle contacts, from earthworm to rats [130-142]. I had quite uncompromising discussions with a respected muscular physiologist, Prof. Manik Sahani about action potentials of skeletal muscle fibers and their sensitivity to tetrodotoxin during postnatal-development and old age. Our findings documented the gradual exchange of at least two types of sodium channels throughout life [142]. This sodium-channel family eventually expanded to other important subtypes affecting for example the pain sensation.

In Bangalore, in the south of the India peninsula, they have a beautiful university campus. The local students were at one of my lectures, which I ended with some musical interlude – a piece of Bach sonata – and the students at that time won the opportunity to have one more seminar with me, which they held in the large student hall. I said to myself, “That's great, how interested they are in my science, they want even more knowledge”. When I got there, it turned out that most of the students had guitars and other musical instruments such as chikara with them, and they did not want me to tell nothing more about quantum and non-quantum synaptic releases or something like that. They said, “Play us some European melodies again, please”. In the pre-internet era, I played a number of genres on the violin, such as the tango Jalousie, Mozart´s Little Night Music, Dvořák's Humoresque, Monti's Czardas and so on. They liked it very much. So in the evenings I taught several local guitarists to play Monti's Czardas note by note until my departure. Indians love the violin, but they play it along with the zither in a completely different way, usually sitting on the ground, resting it on the instep. In Bangalore, they even have a large concert hall in the shape of a violin and celebrate their famous violinists.

Fig. 7. Prof. Eugeny E. Nikolsky and Dr. Ellya Bukcharaeva receive the Purkyně Prize of the Czech Academy of Sciences from the hands of the President, Prof. Helena Illnerová (left) in 1999.

It is worth mentioning at least one work from our group in collaboration with Kazan scientists (Fig. 7) [141]. New cholinesterase inhibitors were synthesized, based on 1,3-bis[5-(o-nitrobenzylethylammonium) pentyl]-6-methyluracilic unit with selectivity towards mammalian AChE vs. butyryl cholinesterase E8,9,10,11. These inhibitors were found to be efficacious on skeletal muscles with the exception of respiratory muscles such as the diaphragm. The most selective compound, 6-methyl-uracil derivative, C547, was pharmacologically profiled on human AChE and BChE. It can be used for specific treatment of pathological muscle weakness syndromes in

humans of the myasthenia gravis or Alzheimer's disease without any sighs of respiratory muscle failure.

I also obtained a stipendium from Fogarty's extramural program, which provide funding to perform research and to train researchers in a variety of global biomedical areas. I spent nine months in the laboratory of Prof. Zach W. Hall (University of California in San Francisco). He created an interdepartmental neuroscience program, which acted as a model for stimulating cross-disciplinary research. There I learned some molecular neurobiology and biochemistry, DNA sequencing and targeted mutagenesis. Zach was known for biochemistry of the adult, embryonic and brain nAChRs. In the 1970s he came to see me in Prague, „to meet the man who gave direction to my research" as he wrote to me back then [6, cf. 142,143]. We still keep in touch after his retirement from the post of Director of the National Institute of Neurological Disorders and Stroke, an institution that had been in the forefront of brain research since 1950.

Later, I taught at The University of North Carolina at Charlotte, where I informed students, faculty members and former collaborators (Dr. A. Urazaev) about a number of other aspects of cellular excitability that have interested me throughout my career [144-175]. These include, for example, the unexpected membrane anticonvulsive action of diazepam and prostaglandin E_1 [147], calcium-dependent inhibition by prostaglandin-E_1 of spontaneous acetylcholine release from frog motor-nerve [148], dual effect of cortisol on the excitability of the rat muscle fiber membrane and neuromuscular-transmission [149]. On the basis of functional properties of muscle autografts substituted for the rat levator ani muscle [150] a surgical procedure was developed and used for many human patients with anal incontinence. Some results had to be defended in writing due to the ban on travel to Western conferences [151,152].

Our interest in hibernation led to an important observation that within the temperature range between 10 °C and 5 °C the activity of Na^+,K^+-ATPase of hamster preparations was about 2.4 times higher than in the case of the never-hibernating mouse. It demonstrates an adaptation for low-temperature hibernation [153-155] preventing hamsters from cold depolarization and death.

From the experiments on organ level we can also mention primary afferent depolarization and changes in extracellular potassium concentration induced by L-glutamate and presumed antagonist L-proline. It was measured in the isolated spinal cord of the frog in cooperation with Dr. Ladislav Vyklicky Sr. Our results showed that L-glutamate and the hopeful compound L-proline act on different receptors [156]. Post-denervation decrease of intracellular potassium and increase of sodium were estimated first time directly, by ion-selective microelectrodes, in rat soleus and extensor digitorum longus muscle fibers. This explains the decrease of resting potential and the onset of post-denervation fibrillation due to "giant" miniature potentials of degenerating nerve-ending origin [157, cf. 38]. The history of vanadate-vanadyl effectiveness has been further supplemented by the knowledge that vanadyl (VO_2^+) and vanadate (VO_3^-) ions inhibit the brain microsomal Na^+,K^+-ATPase with similar affinities and showed protective abilities of the transferrin and noradrenaline [158-162].

The effects of the replacement of K^+ by Tl^+, Rb^+, and NH_4^+ on the muscle membrane potential confirmed the degree of selectivity of the voltage-dependent K^+ channel (delayed rectifier) in frog nerve and muscle. This similarity suggests that the resting membrane potential is controlled mainly by this channel [163]. This fact should always be taken into account when studying hyperpolarization and depolarization effects, e.g. N-methyl D-aspartate (NMDA), anion-transport inhibitors, catecholamines or venoms and toxins [164-169].

My interest in the physiology of the heart was manifested by measuring the activities of $[K^+]_e$ and $[Ca^{2+}]_e$ during cardiac contraction using suction ion-sensitive electrode. The application of negative pressure of -40 kPa (-300 mm Hg) for 10 min under a suction electrode placed on the surface of the spontaneously beating frog ventricle showed changes the $[K^+]_e$ activity in three phases: a phase of rapidly rising, then a slowly decaying phase and a phase of slowly rising $[K^+]_e$ [170].

In frog muscle we unexpectedly found nAChR desensitization during repetitive end-plate activity with high number of released ACh quanta [171]. But it's not just synaptic activity that's important. The condition of skeletal muscle composed from either red or white fibers might also depend on whether they are stretched or contracted at rest. Therefore wet mass, resting membrane potential, frequency of miniature end-plate potentials and the concentration of [^{3}H]ouabain-binding sites were studied after 7 days of immobilization of the rat soleus (slow) and extensor digitorum longus (fast) muscles in the shortened or stretched position and after 3 and 7 days of remobilization. We observed that the loss of muscle mass by 37 % in the rat soleus immobilized for 7 days in the shortened position is accompanied by a membrane

depolarization of about 5 mV, a decrease in frequency of miniature end-plate potentials by 60 % and a decrease of [^{3}H]ouabain binding by 25 %. Only minor changes were found in stretched soleus as well as in shortened and stretched extensor digitorum longus [172]. But it is possible that it is a combination of external synaptic and contractile systems within the muscle fiber, which determines overall muscle plasticity [173].

The last two publications presented here are the culmination of my collaboration with specialists in binding studies, biochemistry of membrane enzymes and molecular changes in the structure of proteins and masters of the path clamp records. Papers concern chemical modifications of melatonin receptors in chicken brain, ouabain binding, ATP hydrolysis, and Na^+, K^+-ATPase after chemical modification of these ATPases [174,175].

In the United States of America, I was invited several times to join bluegrass musicians. I admired the interesting neuromuscular style, which consists of a rhythmic sequence of solo individuals on a given, often popular, theme and melody. What was nice was that there were no drums in the band, the rhythm is held by a wooden or electric bass. Once I was asked to play a Czech classic. I chose Dvořák's Humoresque, which is the second most famous melody in the world after Beethoven's "for Elise". They soon joined me and we all had a good time with variations of the melody in their style. I started playing as a violinist and ended up as a bluegrass fiddler with the whole band. In a sense, this also applies to my scientific career, as this article attests.

Conflict of Interest

There is no conflict of interest.

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