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TIIT SALUM

Similarity and difference of temperature-dependence of the brain sodium pump in normal, different neuropathological, and aberrant conditions and its possible reasons

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II Roots K, Kairane C, **Salum T**, Kõks S, Karelson E, Vasar E, Zilmer M (2006) Very low levels of cholecystokinin octapeptide activate Na-pump in the cerebral cortex of CCK2 receptor-deficient mice. Int J Dev Neurosci 24: 395–400.

III Roots K, Kõks S, Kairane C, **Salum T**, Karelson E, Vasar E, Zilmer M (2003) Na-pump kinetic properties are differently altered in the brain regions of the cholecystokinin 2 receptor-deficient mice. Ann NY Acad Sci 986: 644–645.

IV Zilmer M, Tähepõld L, **Salum T**, Sillard R, Kask R (1991) The role of conformational changes in the functioning of brain Na,K-ATPase. Cytology 33: 55–60.

V **Salum T**, Zilmer M, Vihalemm T, Kullisaar T, Tähepõld L, Randvere T, Sinisalu V (1989) Features of temperature-dependence of the Na^+,K^+–ATPase reaction in normal and tumorous brain tissue. Ukr Biokhim Zh 61: 65–69.

Contribution of author:

1. The author performed all of the experiments (enzyme preparation isolation from brain, enzyme activity measurements, temperature-dependence measurements, oxidative stress related measurements), analyzed the results and participated in writing of the manuscript (Paper I).
2. The author performed the enzyme preparation isolation, enzyme activity and cooperativity measurements and participated in writing of the manuscript (Papers II;III;IV).
3. The author performed all of the experiments (enzyme preparation isolation from brain, enzyme activity and cooperativity measurements, temperature-dependence measurements) analyzed the results and participated in writing of the manuscript (Paper V).

ABBREVIATIONS

ADP	adenosine diphosphate
AMOG	adhesion molecule on glia
Aβ	amyloid-β peptide
ATP	adenosine triphosphate
BTT	brain tumor tissue
CAT	catalase
CAV1	caveolin 1
CCK	cholecystokinin
CCK2R	cholecystokinin 2 receptor
CCK2R+/–	heterozygous CCK2 receptor deficient
CCK2R–/–	homozygous CCK2 receptor deficient
CM	cell membrane
CNS	central nervous system
CTS	cardiotonic steroids
DOC	desoxycholate
EGFR	epidermal growth factor
ER	endoplasmatic reticulum
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
HAD	human Alzheimer disease
HBTT	human brain tumorous tissue
NAKA	Na,K-ATPase, sodium pump
NBT	normal brain tissue
n_H	Hill coefficient
OxS	oxidative stress
OSI	oxidative stress index
P_i	inorganic phosphate
PI-3	phosphatidylinositol 3 kinase
PKA	protein kinase A
PKC	protein kinase C
PUFA	polyunsaturated fatty acids
SDS	sodium dodecyl sulfate
ROS	reactive oxygen species
SOD	superoxide dismutase
Src	non-receptor protein tyrosine kinase
SU	subunit
TAR	total antioxidant response
TM	transmembrane
TMB	tetramethylbenzidine
TPX	total peroxide

INTRODUCTION

The Na,K-ATPase (sodium pump, NAKA (EC 3.6.3.9)) is an integral plasma membrane protein and its main task is active transport of Na^+ and K^+ across the cell membrane (CM) using energy released from the hydrolysis of ATP (Skou 1965). A number of essential processes in the organism are based on the function of active transport, including conduction of nerve impulses, muscle activity, maintenance of intracellular pH and cell volume, transport of glucose and amino acids, and protein synthesis. In conjunction with other proteins of the CM and cytosol the NAKA also participates in signal transduction by transmitting the signal to the cell organelles (Xie and Askari 2002). Alteration in the enzyme function causes changes in Na^+ and K^+ gradients which serve as basis for development of various disturbances.

NAKA activity depends on several physical CM properties, including membrane thickness/fluidity (Johannsson et al. 1981), phospholipid composition (Vemuri and Philipson 1989), fatty acyl chain length (Marcus et al. 1986), and membrane fluidity (Kimelberg and Papahadjopoulos 1974). Membrane fluidity is one of the most important parameters that are affected by the lipid status of CM. The function of proteins is also substantially influenced by the status of membrane lipids. In general, alterations in the lipid environment that increase membrane fluidity also tend to increase NAKA activity (Kimelberg and Papahadjopulus 1972; 1974). Membrane lipid status and compositions play an important structural and functional role for plasma membrane enzymes. They have an effect on the conformation, function and regulation of CM enzymes and receptors (Cornelius 2001, Cornelius et al. 2003; Harikumar et al. 2005). The complete removal of lipids surrounding the NAKA rapidly inactivates it and activity is restored by restoring proper lipid environment (Stekhoven and Bonting 1981). The large variability and flexible changes in the composition of the CM lipids enable the cell to easily adapt to changes in intracellular and extracellular environments. It has been shown that changes in the composition and condition of CM lipids have an important role in the etiology of several diseases, including hypertension, cardiac hypertrophy, Alzheimer's disease, schizophrenia, and tumors (Maxfield and Tabas 2005).

Study of sodium pump function at different temperatures (the Arrhenius plot) is accepted and used for estimation of CM lipid status and CM lipid-protein interaction (Priestland and Whittam 1972; Boldyrev 1980, 1988; Esmann and Skou 1988). Regarding the latter, it is known that the nonlinear Arrhenius plot (the breakpoint at 19–22°C) is typical for the NAKA in animal tissue, reflecting sensitivity of the enzyme to the phase reconstructions of CM lipids (Boldyrev 1988). Graphical Arrhenius method is used to study the fluidity that is conditioned by the lipid status and composition of the CM. It means that CM enzyme activity is measured at different temperatures and the relationship between temperature and activity is plotted on a graph. The phase of the CM lipids (more fluid or more solid) is altered at a certain temperature and,

therefore, the dots on a graph are not placed on a straight line and a so-called breakpoint appears on the graph. This typically occurs between the temperatures 19–22°C for the enzyme preparation isolated from the nervous tissue. Under the influence of different factors (e.g. prostaglandins) and in the case of pathological conditions the classical breakpoint can be shifted (Karelson et al. 1985).

The present study is focused on the next aspects: a) to measure temperature-dependence of nervous tissue NAKA in the case of different pathological and aberrant conditions; b) to analyze this information for the established nature (similarity or non-similarity) of changes in lipid status and cationic cooperativity in the case of different pathological and aberrant conditions in the brain (brain tumor, Alzheimer's disease, genetic invalidation of CCK2 receptors) and to analyze the possible reasons underlying such changes. We hypothesize that one of the common unique phenomena of these pathologies and aberrant conditions is a change in sodium pump lipid environment status which, therefore, results in a change of sodium pump functioning. Thus, in this work for the first time the data collected from experiments involving different brain pathologies and aberrant conditions are compiled. The data obtained from experiments have fundamental value for understanding basic relationships in sodium pump regulation and may have practical value for the development of new diagnostic or therapeutic strategies and tools for management of neuropathologies.

REVIEW OF LITERATURE

1. General molecular structure and functioning of Na-pump

The NAKA protein complex consists of α, β and γ subunits in multiple isoforms and the minimal functional unit consists of non-covalently linked α and β subunits.

The expression of each subunit is controlled by its own gene, which is expressed in a tissue- and cell-specific manner. The concentration of NAKA in tissues varies largely with around a 160,000 fold difference between the lowest (erythrocytes) (Wiley and Shaller 1977) and the highest (brain cortex) (Schmidt et al. 1996) concentrations. Cellular regulation of the pump expression can be controlled by the rate of synthesis of the pump subunits and delivery to the CM. The studies showed that α and β subunits assemble during or very soon after synthesis in the ER (Geering et al. 1996) and both of the subunits are mutually dependent on each other to be transported out of the ER (Ackermann and Geering 1990). Environmental and hormonal factors can increase the sodium pump activity per cell by increasing the turnover of pumps that are already present in the CM (short term regulation), insertion of more pumps into the CM and increasing the synthesis of pump subunits (long term regulation), resulting in an increased number of pump sites in the CM (Nemoto et al. 1997; Songu-Mize et al. 1996).

The α subunit of NAKA (an integral protein with about 1018 residues, 100–113 kDa) is ubiquitously expressed in all tissues and has four isoforms in mammalian cells that are expressed in a cell type- and tissue-dependent manner. Its chain traverses the membrane 10 times, forming the domains M1 to M10 and 2 large intracellular loops (Fig. 1). α1 and α2 are expressed in glial cells and neurons may express α1, α2, α3 or any combination of these isoforms (McGrail et al. 1991). The larger cytoplasmic loop is the site for ATP binding (Lingrel and Kuntzweiler 1994) and phosphorylation (an aspartate residue, D369) (Ohtsubo et al. 1990). The N- and C- ends of the chain are located on the cytosol side. The crystal structures of the NAKA show two potassium ions coordinated between transmembrane helices 4, 5, and 6 (Morth et al. 2007; Shinoda et al. 2009). These ion-binding residues will rearrange to coordinate two sodium ions, whereas other residues in M5, M8 and M9 have been proposed to bind the third sodium ion (Ogawa and Toyoshima 2002; Li et al. 2006).

Knockout of two of the isoforms of the catalytic α subunit of NAKA has been reported to be lethal (James et al. 1999). The α subunit C terminus of NAKA controls Na^+ affinity on both sides of the membrane through Arg935.

The β subunit is located close to the transmembrane domain M7/M10 (Fig. 1) of the α subunit (Morth et al. 2007). The β subunits (about 300 residues, about 36–38 kDa proteinic part and about 60 kDa in glycosylated form) of

NAKA are glycoproteins with a single transmembrane segment, a short cytoplasmic tail (N-terminus), and a large extracellular domain (ectodomain), with three conserved S-S bridges and conserved glycosylation sites (Skou and Esmann 1992). These three 3 S-S bridges and 3 to 7 N-linked sugar chains on extracellular domain are necessary for the proper folding and functioning of β subunits as well as their interaction with the α subunit (Beggah et al. 1997). Beta 1 is expressed in all tissues; in the brain the β1 subunits are found in neurons, β2 predominantly in glial cells (Watts et al. 1991), and β3 in oligodendrocytes (Martin-Vasallo et al. 2000). The different isoforms of the β subunit have been found with moderate differential effects on NAKA activity (Geering 2001).

The β SU has an essential function as a molecular chaperone. Association of the β subunit, mediated by multiple interaction sites, facilitates the correct CM integration and packing of the newly synthesized catalytic α subunit, which is necessary for its protection against cellular degradation, acquisition of functional properties, and routing to the plasma membrane (Geering 2001). In addition to its chaperone function, β subunit influences the transport properties of mature NAKA. Alpha subunits associated with different β isoforms exhibit different apparent potassium affinities and the β structure influences the apparent sodium affinity of NAKA (Geering 2001). A conformational rearrangement observed between α and β subunits during the catalytic cycle (Dempski et al. 2005) is consistent with the coupling of the β subunit to the function of NAKA. In addition, the β subunit influences the K^+ activation kinetics and may be required for stabilization of the K^+ transporting conformations of mature NAKA (Geering 2001; 2008). Normal glycosylation of the β1 subunit also appears to play an important role in cell-cell contacts (Vagin et al. 2006; 2008). Beta 1 subunit mediates cell-cell contacts directly or via interactions with other proteins (Shoshani et al. 2005; Vagin et al. 2006). The β2 isoform has long been known to act as a cell adhesion molecule between astrocytes and neurons in the brain (Gloor et al. 1990). However, the mechanism of β-mediated cell adhesion or specific protein partners of β are not known.

The γ subunit has a certain importance in the enzyme function. It has 7 isoforms and has been identified primarily in renal tissue and is mainly responsible for the regulation of the enzyme activity, stabilization, and interaction with Na^+ and K^+ ions (Geering 2005). It is exclusively expressed in the brain both in neurons and glial cells and it decreases the apparent affinity for extracellular K^+ which may be essential for proper neuronal excitability (Beguin et al. 2002).

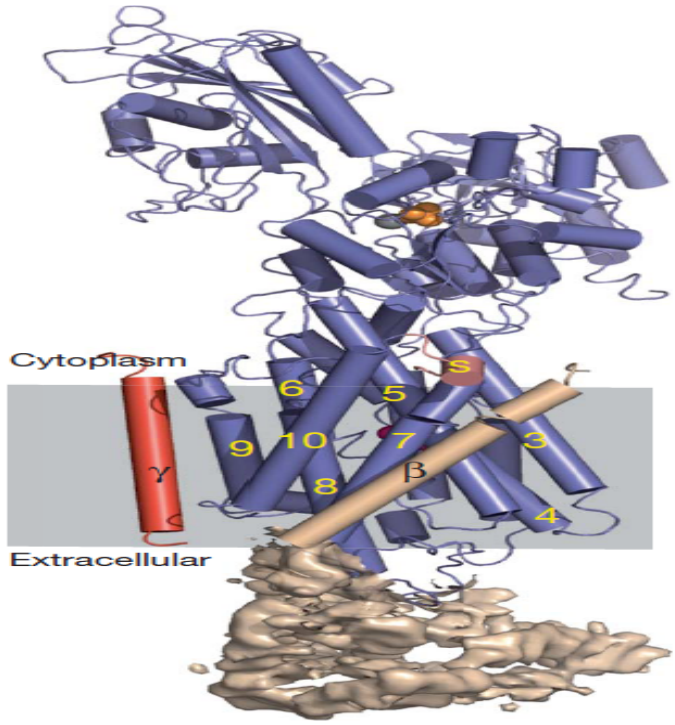

Figure 1. Architecture of the Na^+,K^+-ATPase α β γ complex and the K^+/Rb^+ sites. The α, β and γ subunits are coloured blue, wheat and red, respectively. The transmembrane segments of the α subunit are numbered (yellow) starting with the most N-terminal. The small C-terminal helix (S, for switch) is light red. Mg^{2+} and Rb^+ ions are grey and pink, respectively (Morth et al. 2007).

The crystal structures which have been published over the last few years of P-type ATPases in different conformational states (Morth et al. 2007; Pedersen et al. 2007; Toyoshima 2008) provide undisputable evidence for an ion translocation pathway through the centre of a single catalytic unit of α-subunit of NAKA. Therefore, there is no structural reason for P-type ion pumps to necessarily aggregate into dimers or higher oligomers in order to translocate ions. Concerning ion transportation function the first basic information was given by the pioneering work of Albers (Albers 1967) and Post (Hegyvary and Post 1971; Post et al. 1972) that presented the most widely accepted working hypothesis of the reaction cycle of P-type ATPases which is the Albers–Post or E_1–E_2 model. During the working cycle there are generally two conformational states E_1 and E_2 (Fig. 2). In the first step of the reaction sequence, Na^+ and ATP bind with very high affinity to the E_1 conformation of the enzyme, during which phosphorylation at an aspartate residue occurs via the transfer of the γ – phosphate of ATP (Fig. 2, step 2). Magnesium is essential for this reaction.

Thereafter, three Na^+ ions are occluded while the enzyme remains in a phosphorylated condition. After the E_2-P3 Na^+ conformation is attained, the enzyme loses its affinity for Na^+ and the affinity for K^+ is increased. Thus, three Na^+ ions are released to the extracellular medium (Fig. 2, step 3) and K^+ ions are taken up (Fig. 2, step 4). The binding of K^+ to the enzyme induces a spontaneous dephosphorylation of the E_2-P conformation. The dephosphorylation of E_2-P leads to the occlusion of two K^+ ions, leading to $E_2(2K^+)$ (Fig. 2, step 5). Intracellular ATP increases the extent of the release of K^+ from the E_2 $(2K^+)$ conformation (Fig. 2, step 6) and thereby also induces the return of the $E_2(2K^+)$ conformation to the E_1ATPNa^+ conformation. The affinity of the $E_2(2K^+)$ conformation for ATP is very low (Skou 1988).

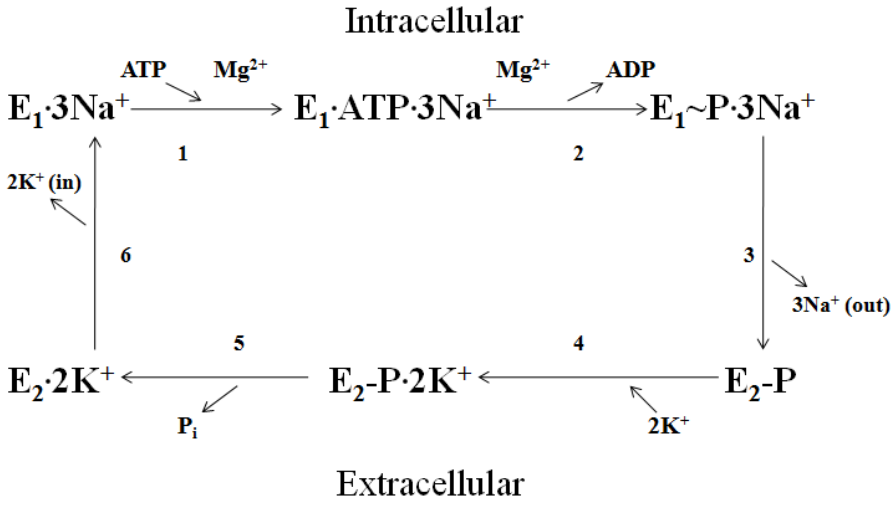

Figure 2. Kinetic scheme for the active transport of Na^+ and K^+ by Na,K-ATPase

These signal transduction functions of NAKA are mediated by the interactions between NAKA and other signaling proteins – intracellular soluble enzymes and other CM proteins. Pump inhibition causes an increase in the cellular Na^+ concentration, which can lead to changes in intracellular pH (via the Na/H exchange system) or intracellular Ca^{2+} (via the Na/Ca exchange system) and such changes could obviously produce their own consequences to cell physiology. Several functional domains are involved in the binding of the enzyme to protein kinases and cytoskeletal proteins. The central loop CD3 of the α subunit contains the highly conserved P (phosphorylation) domain and the nucleotide-binding domain (N). The P domain is buried close to the CM whereas the N domain is highly exposed and has been shown to interact with other proteins. The NAKA serves as a native negative Src regulator (Liang et al. 2006). This

interaction forms a functional receptor complex for cardiotonic steroids (CTS) (Li and Xie 2009).

Binding of CTS to the receptor complex activates the NAKA associated Src and other tyrosine kinases which results in the activation of protein kinase cascades and the generation of second messengers (Liang et al. 2006; Li and Xie 2009). Subpopulation of NAKA needed for signaling is proposed to be colocalized with caveolin 1 and concentrated in caveolae (Liu et al. 2003; 2004; Wang et al. 2004). The α1 isoform contains two conserved caveolin-binding motifs and in vitro assays have shown that the purified NAKA can bind to the amino-terminus of caveolin 1. CM lipid status has probably a significant influence on NAKA receptor functioning. In astrocytes the α2 is structurally (Lencesova et al. 2004) and functionally (Golovina et al. 2003) linked to the Na^+/Ca^{2+} exchanger and thereby helps to modulate Ca^{2+} transport and Ca^{2+} signaling. In neurons, the α3 likely plays a similar role (Lencesova et al. 2004).

Cardiotonic steroids are modulators of all molecular forms of the NAKA expression and activity. Complexes containing α2 and α3 are more sensitive and those involving α1 are less sensitive to cardiac glycosides (Urayama and Sweadner 1988; Munzer et al. 1994). Several studies have suggested their possible use in oncology (Haux 1999; Stenkvist 2001). Novel cardenolide UNBS1450 displays an increased affinity and improved anti-tumor activity for overexpressed α1 in non-small cell lung cancers (Mijatovic et al. 2007; 2009) and gliomas (Lefranc et al. 2008).

2. Cell membrane lipid status and functioning of sodium pump under normal conditions

Since the original formulation of the mosaic fluid model by Singer and Nicolson (Singer and Nicolson 1972), it has become evident that the role of membrane lipids is not just to provide a bidimensional solvent for membrane proteins: they are also involved in key biofunctions linked to the spatial organization of the cells, e.g. cellular adhesion (Hakomori 2002), toxin and pathogen attachment (Taieb et al. 2004), signal transduction (Berridge 1984), or protein trafficking (Anderson and Jacobson 2002)

Wide heterogeneity and variety of membrane lipids (more than 2000 different lipids in mammalian cell membranes) suggest that the lipid bilayer existence must have functional consequences. One of them is non-random mixing of lipid molecules in a bilayer, resulting in phase separation and formation of lipid domains. The lipid bilayer is an active factor in the formation of the membrane structure and the lipid composition is responsible for the presence of domains in the membrane. PUFAs are not randomly distributed within CM. The external and cytoplasmic leaflets of the CM differ in the phospholipids headgroup composition, cholesterol content, as well as fatty acid composition, and this non-symmetric composition results in a transbilayer

fluidity gradient regulated by unsaturated fatty acids (increase fluidity) (Kier et al.1986), in particular the amount of docosahexaenoic acid (DHA; 22:6 n-3) (Else and Hulbert 2003; Cornelius et al. 2003) and cholesterol (reduces fluidity at higher temperatures and maintains it at lower temperatures). In order to function, a lipid bilayer must maintain its fluidity. At lower temperatures, organisms use phospholipids containing increasing degrees of unsaturation in their fatty acids. At higher temperatures cholesterol serves to impede phospholipid fluidity and at lower temperatures cholesterol interferes with solidification of membranes (e.g. in the latter case, cholesterol functions similarly to the effect of unsaturated fatty acids on lipid-bilayer fluidity). Alterations in membrane fluidity usually reflect changes in its lipid composition or phospholipid distribution (Zachowski 1993), which are often dependent on nutrition and metabolism. Membrane fluidity can also be influenced by redox status, phosphorylation state of membrane components, local pH, calcium concentration, or cytoskeleton proteins. The CM also contains lateral domains (lipid rafts/ caveolae as well as non-raft domains), which differ in their lipid and cholesterol composition and protein distribution (Pike et al. 2002; Pike 2003).

Lipids are the most abundant organic compounds found in the brain, accounting for up to 50% of its dry weight (Woods and Jackson 2006). As in other mammalian tissues, brain lipids consist mainly of three major categories: phospholipids, sphingolipids, and cholesterol (a lipid-like compound). Nervous tissue CM have a high concentration of PUFAs, especially n-3.

Cholesterol is an essential membrane component and is abundantly present in the nervous tissue, where it is involved in signal transduction, synapse formation, and neurotransmitter release (Dietschy and Turley 2004). Caveolin 1 (CAV1) is a cholesterol-binding integral CM protein with an important role in cholesterol transport and homeostasis (Frank et al. 2006). Low-density and detergent-resistant microdomains of the CM are particularly rich in cholesterol and CAV1 (Wang and Paller 2006), which are critical to the formation and stabilization of membrane lipid rafts (Silvius 2003).

The activity of NAKA is sensitive to the composition of its surrounding CM environment. Less than 100 lipid molecules surrounding the pump and their cooperative action could be sufficient to result in significant conformational effects. The complete removal of lipid molecules inactivates NAKA, but activity is restored by reintroducing it to a proper membrane environment (Ottolenghi 1975; Steckhoven and Bonting 1981). NAKA activity has been correlated to several physical membrane properties including membrane fluidity (Kimelberg and Papahadjopoulos 1974), membrane thickness (Johannsson et al. 1981), phospholipid composition (Vemuri and Philipson 1989), fatty acyl chain length (Marcus et al. 1986), and the degree of saturation of fatty acids (Cornelius 2001; Cornelius et al. 2003). CM proteins are more active in membranes that are more polyunsaturated. In general, lipids that promote bilayer formation of physiological thickness and increased fluidity tend to promote optimal NAKA activity (Kimelberg and Mayhew 1975; Johannsson et al. 1981; Marcus

et al. 1986), as do negatively charged lipids, such as phosphatidylserine and phosphatidylglycerol (Kimelberg and Papahadjopoulos 1972). In general, alterations in the lipid environment that increase membrane fluidity tend to also increase NAKA activity (Therien and Blostein 2000). Free fatty acids present in the membrane or as products of the phospholipase A_2-dependent regulatory pathway tend to inhibit NAKA (Oishi et al. 1990). Certain composition of fatty acids (length of the chain, degree of saturation) and certain concentration of cholesterol are required for optimal catalytic function of the pump (Cornelius 2001; Cornelius et al. 2003).

Cholesterol affects both the pumping and signaling functions of NAKA (Yeagle 1983; Cornelius 1995; Wang et al. 2004; Liang et al. 2007). Several early studies have suggested that cholesterol directly interacts with NAKA in three potential binding sites, one at the first transmembrane domain and two at the last transmembrane domain. It has been demonstrated that a specific phospholipid acyl chain length and the presence of cholesterol (Johannson et al. 1981; Yeagle et al. 1988; Cornelius 1995) are essential to support optimal hydrolytic activity of NAKA. Furthermore, cholesterol significantly increases the maximum steady-state phosphoenzyme level and decreases the apparent cytoplasmic Na^+ affinity (Cornelius 1995). In conclusion, the lipid environment, including the cholesterol content, affects the hydrolytic activity of NAKA by affecting several of the reaction steps in the overall reaction mechanism of the NAKA and not just a few rate-determining steps. Indeed, both rate-limiting steps, the $E_2 \rightarrow E_1$ and the $E_1 \rightarrow E_2$-P reactions are accelerated by cholesterol. The $E_2 \rightarrow E_1$ reaction includes binding of the cytoplasmic ligands ATP and Na^+, as well as deocclusion of K^+, the reactions which all seem to be affected by the lipid environment. The effect on the apparent affinities of ATP and Na^+ may both be caused by the lipid effect on the E_1/E_2 equilibrium. It has been shown that the membrane phospholipid and cholesterol composition have an influence on Na^+ binding to the enzyme but not on K^+ binding (Giraud et al. 1981). The detailed molecular mechanisms of these effects are still largely unknown, but hydrophobic matching between the bilayer and the integral protein seems to be important. Furthermore, change in bilayer hydrophobic thickness induced by either increasing the phospholipid acyl chain length or by inclusion of cholesterol does not always affect the enzyme equivalently. Thus, effects on the conformational mobility of NAKA by the lipid status seem to be important as indicated by the effects on the activation energy of the reactions accompanying the conformational changes associated with the phosphorylation-dephosphorylation reactions. Such effects could be direct or induced by phospholipid and cholesterol effects on the intramembrane charge distribution or result from certain lipid combinations that stabilize oligomeric interactions of NAKA (Cornelius et al. 2003). Membrane proteins and lipids may both be subjected to regulatory processes in response to pathophysiological situations or nutritional-pharmacological interventions which, in turn, may alter the activity and functions of the membrane.

Summarizing the aforementioned: there is no doubt that CM lipid status has an influence on NAKA functioning. A gold standard in studies of NAKA function dependency of CM lipid fluidity is the analysis of NAKA kinetic properties at different temperatures. Depending on the results at different temperatures the Arrhenius plot is constructed. The graph is not linear and typically the breakpoint in the graph appears at temperature 20°C (Kimelberg and Papahadjopoulos 1974).

3. Different neuropathologies and aberrant conditions and regulation of function of Na-pump by lipid status and cations

Generally, various neuropathological processes have an influence on sodium pump function. A central role for NAKA in pathogenesis has been widely implicated, particularly in cardiovascular, neurological, renal, and metabolic diseases (Rose and Valdes 1994). Several regulators (sodium and potassium as the principal ones) can directly regulate the activity of NAKA, which satisfies the functional roles of the enzyme in different conditions and makes the pump protein vulnerable to pathogenic insults and a potential target for therapeutic treatments (Therien and Blostein 2000). Major regulators of the membrane architecture are lipid status, membrane potential, intracellular Ca^{2+} and pH, protein composition, cell-to-cell contact, and membrane coupling with the cytoskeleton or extracellular matrix.

A number of studies have shown changes in sodium pump activity under different neuropathological processes. For example the NAKA activity was 34% lower in ischemic brain cortex and 40% lower in ischemic basal ganglia after 30-min ischemia. After 60-min ischemia, both the NAKA activity and K^+ concentration were decreased in the ischemic hemisphere (Jamme et al. 1997). It has been shown that NAKA molecules isolated from the ischemic brain lost their ability to interact with one another (the Hill coefficient fell to 1) (Dobrota et al. 1999). This is consistent with the observations that ischemia or hypoxia induce energy crisis, increase the production of ROS (Kako et al. 1988; Johnson and Weinberg 1993) and release endogenous inhibitors of NAKA (Hennings et al. 1983). The inhibition of NAKA secondary to cellular energy depletion might contribute to delayed membrane depolarization of cortical neurons after traumatic brain injury (Tavalin et al. 1997). The NAKA activity was reduced or insufficient to maintain ionic balances during and immediately after episodes of ischemia, hypoglycemia, epilepsy, and after administration of glutamate agonists. It has been proposed that the reduction and/or inhibition of NAKA contributed to the central neuropathy found in those disorders (Lees 1991). Inhibition of NAKA activity leads to neuronal death and is related to pathological conditions in the CNS (Hattori et al. 1998). In this context, it has

been demonstrated that cerebral edema and neuronal death associated with a decrease in NAKA activity are mediated by intracellular depletion of K^+ and accumulation of Ca^{2+} and Na^+ (Xiao et al. 2002). Decreased NAKA activity has been associated with excitotoxity. Thus, it has been demonstrated that striatal neurons are more vulnerable to glutamate neurotoxicity when the activity of this enzyme is reduced (Brines and Robbins 1992). In agreement with these findings, other studies indicated that NAKA inhibition, in the presence of a non-lethal insult, activates the apoptotic cascade and neuronal injury probably by amplifying the disruption on K^+ homeostasis (Wang et al. 2003). Dysfunction or deficiency of NAKA has been identified in chronic neurodegenerative diseases (Chauhan et al. 1997). The possibility that the deficiency in the NAKA activity might be a common mechanism in pathogenesis of central nervous system disorders has been tested in patients with CNS glioma, multiple sclerosis, systemic lupus erythematosus, subacute sclerosing panencephalitis, primary generalized epilepsy, Parkinson's disease, Down syndrome, syndrome X with multiple lacunar state, and several other neurodegenerative disorders.

Tumor growth in brain is accompanied with severe changes in brain tissue and metabolism which may have impact on alteration of the CM sodium pump function leading to changes in cell homeostasis. The largest group of primary brain tumors are gliomas. The growth of a glioma results in destruction of the normal brain parenchyma by the glioma cells. This is achieved by the cellular release of glutamate into the peritumoral space (Ye and Sontheimer 1999) in the absence of functional Na^+ dependent glutamate transporters in glioma cells, resulting in consequent accumulation of excitotoxic glutamate in the extra-cellular glial space (Takano et al. 2001). Signaling through the glutamate receptors is also involved in glioblastoma cell proliferation (Arcella et al. 2005). Glioma cells are self-propelled (Merzak et al. 1994) and are able to adjust their shape and volume rapidly as they invade the brain parenchyma. The activity of NAKA can be modulated by glutamate and its receptors (Munhoz et al. 2005). Several studies have reported changes in NAKA activity in the course of malignant transformation, with evidence that these changes occur at the very early stages of tumorigenesis (Kaplan 1978; Shen et al. 1978; Weidemann 2005) . These may result from altered NAKA density in the plasma membrane of tumor cells, as well as differences in isozyme expression. NAKA is also directly involved in the migration of cancer cells in general (Mijatovic et al. 2007) and glioma cells in particular (Lefranc et al. 2008).

The downregulation of NAKA β subunit expression has been found in carcinomas of epithelial origin (Akopyanz et al. 1991; Blok et al. 1999; Rajasekaran et al.1999; Espineda et al. 2003; 2004), while α subunits seem to be up-regulated in certain malignant cells (Sakai et al. 2004). This induces an increase in the expression of the transcription factor Snail, which is known to downregulate E-cadherin (Espineda et al. 2004) thereby facilitating the spread of cancer cells from the primary tumor (Barwe et al. 2005). The β2 isoform of the sodium pump is in fact a homolog of the adhesion molecule on glia

(AMOG) which is a recognition element for cell adhesion that subsequently links cell adhesion and ion transport (Gloor et al. 1990; Schmalzing et al. 1992; Senner et al. 2003). The AMOG/β2 and the α1 subunits of the sodium pump come together to form functional sodium pumps (Schmalzing et al. 1992). The AMOG is downregulated in human and mouse gliomas (Senner et al. 2003). It is found that the α1 subunit of the sodium pump is located at the lamellipodia of the human glioblastoma cell line U373-MG, where it colocalizes with CAV 1. Caveolae functions rely on CAV-1, their major protein, which drives the formation of plasma membrane caveolae and anchors them to the actin cytoskeleton. In addition, caveolin 1 modulates cell interaction with the extracellular matrix and brings together and regulates the interaction of different signaling molecules, with significant roles in cell movement. Importantly, CAV 1 depletion results in the loss of focal adhesion sites and overall cell adhesion (Navarro et al. 2004).

Cell membrane lipid/cholesterol composition, fluidity is changed and lipid peroxidation is increased in the brain tumor tissues (Hattori et al. 1987; Kokoglu et al. 1992; Martin et al. 1996; Cirak et al. 2003; Zajdel et al. 2007).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which affects higher cognitive functions, memory, and learning. In AD brains, there is an increased deposition of amyloid plaques together with the increased number of activated microglial cells in the parenchyma and monocytes in the vessel wall (McGeer et al. 1987; Maat-Schieman et al. 1997; Uchihara et al. 1997; Dickson 1999). Amyloid-β peptide (Aβ) derived from the amyloidogenic pathway of amyloid precursor protein (APP) processing is the primary component of amyloid plaques (Haass and Selkoe 1993; Selkoe 1997; 2000). Aβ monomers aggregate into oligomers, fibrils, and plaques which have different impacts on cellular functions (Cleary et al. 2005; Resende et al. 2008). Deposition of Aβ in AD brains and cerebral vessels results in neurovascular dysfunction and chronic neurodegeneration (Hardy and Higgins 1992). It is known that NAKA activity is decreased in the case of AD (Kairane et al. 2002). The decreased NAKA activity in AD brain is correlated with the specific reduction of the NAKA protein level (Liguri et al. 1990). The decrease in NAKA activity may result from the cytotoxic effects of Aβ proteins. Impaired NAKA activity reportedly results in an increase in Na^+ influx, leading to membrane depolarization, and Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Mark et al. 1995). Thus, impaired NAKA activity may also play an important role in the pathophysiology of neuronal excitotoxicity in AD. The reduction in NAKA activity in AD brain may cause excessive excitatory responses in neurons resulting in neuronal death. Aβ proteins can intercalate into the CM lipid bilayer, leading to protein, lipid, carbohydrate, and nucleic acid damage via different processes.

The CCK2 receptor is a G-protein-coupled integral CM protein and one of the two receptors that bind cholecystokinin (CCK2 and CCK1). It is widely expressed in the gastrointestinal tract and central nervous system. The highest

densities of CCK2 receptors are found in the cerebral cortex, nucleus caudatus, and anterolateral part of nucleus accumbens (Noble et al. 1999). In the CNS it is associated with the control of pain and anxiety, nociception, and memory process (Crawley and Corwin 1994). Brain CCK2 receptor has a 447 amino acid sequence (P32239 7TM) and possesses seven transmembrane domains. The homozygous (–/–) CCK2 receptor deficient (CCK2R–/–) mice were first generated in 1996 (Nagata et al. 1996). The mutant vector replaced a part of exon 2 and exons 3–5 of the CCK2R gene. This replacement deleted most of the seven membrane spanning CCK2 receptor except for the first 108 amino acids, containing the first membrane spanning region. Mice are viable, fertile, and appear to be grossly normal in the adulthood (Langhans et al. 1997). However, they display disturbances in the learning abilities and performance (Dauge et al. 2001), as well as the altered function of the brain dopaminergic system (Kõks et al. 2001).

If a question „what kind of lipid-related processes might underlie the alterated activity and regulation of NAKA in case of brain tumors, AD, and CCK2 receptor deficient animals?" should rise, there are following answers. CM lipids are known to play major structural and functional roles in the plasma membrane, where they can have potential effects on the conformation, function, and regulation of receptors and their signaling pathways. Most important components are cholesterol and sphingolipids, both relatively enriched in the plasma membrane and further concentrated in specialized domains called rafts. In a number of papers it has been shown that plasma membrane cholesterol, sphingolipids and other lipids can affect CCK receptor function (Harikumar et al. 2005). There are no data about membrane lipid status changes in case of brain tumors, AD, and CCK2 receptor deficiency animals. For example, it is remarkable that NAKA interacts with CAV 1 and knockdown of the NAKA α1 SU likely redistributes cholesterol from the cell membrane to other cellular compartments (Chen et al. 2009; 2011). Next, integral CM proteins are especially sensitive to changes in lipid, cholesterol environment and lipid oxidative modification. Alterations in brain cholesterol and lipid homeostasis have been linked to neurodegenerative diseases, such as Niemann-Pick's, AD, Parkinson's, and Huntington's disease (Simons and Ehehalt 2002; Valenza et al. 2005).

All these neuropathologies and aberrant conditions (brain tumor, AD, and CCK2 receptor deficiency) have a relation to quite easily vulnerable brain lipid status of CM. Aldehydic lipid peroxidation of cells in the brain appear to be at a particular risk from ROS damage, because of their high content of PUFAs and of their high metabolic activity. Neurons are non-replicating cells, characterized by a high ratio of CM surface area to cytoplasmic volume (Evans 1993). Neurotransmitters such as dopamine, glutamate, and nitric oxide represent a source of oxygen radical production. Moreover, neurons contain relatively low concentration of CAT, SOD, GSH, and tocopherol, i.e. substances engaged in protection against ROS (Evans 1993). On the other hand, local PUFA defi-

ciencies in glioma cells may limit tumor ROS generation. This deficiency may cause alterations in signal transduction and an interruption of normal cellular events (Martin et al. 1996). Recently, a ROS-based hypothesis has been proposed, in which glioma cells resistance to radiation and other anticancer therapies is linked to a decreased ROS generation following treatment (Leaver et al. 2004). Next, it is well known that oligomeric Aβ protein can induce oxidative stress (OxS), abnormal calcium homeostasis, and long-term potentiation and can self-assemble into large, voltage-independent, and nonselective ion channels at CM. In addition, several elevated indices of OxS, including protein carbonyls 3-nitrotyrosine (markers of protein oxidation), 4-hydroxy-2-nonenal (HNE) (marker of lipid peroxidation) and 8-hydroxy-2-deoxyguanosine (marker of DNA oxidation) (Hensley et al. 1995; Aksenov et al. 2001; Castegna et al. 2003; Sultana et al. 2006) can induce dysfunctional proteins (Aksenov et al. 2001). HNE binds to proteins, including CM proteins, leading to altered structure and function of the target protein (Lauderback 2001; Sultana and Butterfield 2004; Butterfield et al. 2007). Aβ can also perturb the molecular packing of CM, resulting in subsequent alterations of biophysical properties of membranes, such as membrane microviscosity, membrane molecular order, membrane potential, and permeability. Altered membrane properties, in turn, may disrupt membrane functions, activities of membrane-related proteins, and many cellular pathways.

In addition to atherosclerosis, OxS and inhibition of NAKA activity have been found in various neuropathological conditions, including cerebral ischemia (de Souza Wyse et al. 2000), epilepsy (Grisar 1984), and neurodegenerative disorders such as Parkinson's disease, AD, multiple sclerosis, schizophrenia and amyotrophic lateral sclerosis (Hattori et al. 1998; Yu 2003; Pisani et al. 2006). Thus, the cell membrane NAKA is one of the cellular oxidative stress targets (Rose and Valdes, 1994) via lipid peroxidation and alterated redox status. ROS can directly damage cellular proteins, DNA, and lipids and thereby affect cellular functions (Cochrane 1991). The brain is especially vulnerable to free radical-induced damage because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes (Olanow 1992; Metodiewa and Koska 2000; Halliwell 2006). Inhibition of NAKA by ROS has been demonstrated *in vitro* by Thomas and Reed (1990), Kurella et al. 1999) and *in vivo* by Mintorovitch et al. (1994) and Andreoli et al. (1993). Simultaneous lipid peroxidation products increase lipid bilayer disordering and the suppression of NAKA activity is noted (Kako et al. 1988). Recent experiments have shown that membrane protection by hydrophobic antioxidants was accompanied by obligatory preservation of NAKA activity (Thomas and Reed 1990).

Concerning OxS activity, NAKA is known to be affected by the redox state of the cell, and reduced antioxidants or antioxidant enzymes activities are related to reduced NAKA activity (Morel et al. 1998; Streck et al. 2001; Petrushanko et al. 2006; Wilhelm et al. 2009). Of all the brain cells, neurons are

particularly vulnerable to oxidative insults due to low levels of reduced glutathione (Dringen et al. 2000).

In summary, a number of studies show that NAKA activity is altered in several neuropathological conditions. However, there is limited information regarding lipid status in AD. Furthemore, such information is absent regarding brain tumor tissue and CCK2 receptor deficient mice. A limited number of authors have investigated sodium regulation and its relations to temperature-dependence of NAKA functionality in brain tumors or AD, whereas data about such relations in CCK2 receptor deficiency are absent. In 1989 we demonstrated for the first time, using the Arrhenius plot method to study the sodium pump function, the shift of breakpoint in the Arrhenius plot for NAKA isolated from brain tumor. These findings lead us to use the Arrhenius plot method to investigate the NAKA properties in other neuropathologies and aberrant conditions like AD and CCK2 receptor deficient mice. The latter is regarded as a unique aberrant status and our study was focused on comparing the temperature-dependence of NAKA in CCK2 receptor deficient (homo- and heterozygous) and normal (wild type) mice brain cortex. Until now, there have been no studies concerning CM lipid status in the named neuropathologies and aberrant conditions. Therefore, it was an interesting task to study similar changes concerning NAKA function in such different neuropathologies and aberrant conditions like brain tumor, AD and genetic invalidation of CCK2 receptors and also to establish possible reasons for these changes.

AIMS OF THE STUDY

The general aim of the present study was to provide evidence of the brain sodium pump function and regulation dependency of membrane lipid status in normal, different neuropathologies and aberrant conditions.

The specific aims of the study were as follows:

1. To study the temperature-dependence of sodium pump isolated from human, rat and mouse normal brain cortex cell membrane.
2. To study the temperature-dependence of sodium pump isolated from human brain tumorous tissue and CCK2 receptor deficiency mouse brain cortex cell membrane.
3. To summarize and analyze information about sodium pump temperature-dependence, cation cooperativity and CM lipid status, collected from experiments of normal brain, different neuropathologies and aberrant conditions.

MATERIALS AND METHODS

1. The origin of brain tissues

The enzyme preparations were isolated from the Wistar rat brain cortex, normal human brain cortex and glioma tissue. The frontocortical tissues of AD postmortal human brain were obtained from Huddinge Brain Bank (Karolinska Institute, Stockholm, Sweden. The CCK2 receptor deficient mice were provided by T. Matsui from Kobe University School of Medicine, Japan. From the original background of 129Sv/C57/BL6 mice the CCK2 receptor deficient mice were generated by homologous recombination by replacing a part of exon 2 and exons 3–5 (Nagata 1996). Mutant mice were crossed twelve times to the C57/BL6 background to minimize the possible genetic effects from the 129Sv strain. Breeding and genotype analysis were performed in the Department of Physiology, University of Tartu. Male homozygous (–/–), heterozygous (+/–) receptor-deficient, and wild-type (+/+) mice (90 days old) brain cortex were used in experiments. Mice were kept in the animal house at 20±2°C under 12:12h light/dark cycle (lights off at 19:00h). Tap water and food pellets were available *ad libitum*. All animal procedures were approved by the University of Tartu Animal Care Committee in accordance with the European Communities Directive of 24 November 1986 (85/609/EEC).

2. Isolation of Na,K-ATPase preparations

The NAKA membrane preparations were isolated from the brain tissue by the previously described method (Karelson 1985). The tissues were homogenized at 4°C in a medium containing 0.32 M sucrose, 1 mM EDTA, 0.1% DOC (deoxycholate) and 37.5 mM imidazole–HCl (pH 7.4 at 8°C). The homogenate was centrifuged for 10 min at 10000 × *g*. The supernatant was removed and centrifuged for another 30 min at 24000 × *g*. The enzyme preparation was obtained by resuspension of the final sediment in the buffer described above (without DOC). The protein content of enzyme preparation was determined by Lowry, using bovine serum albumin as a standard (Lowry et al. 1951). The total NAKA activity was measured by incubation of membrane proteins (30–40 μg) in 280 μl of medium containing 100 mM NaCl, 20 mM KCl, 4 mM MgCl2, 4 mM Tris–ATP and 25 mM imidazole–HCl (pH 7.4 at 37°C). The reaction was carried out for 10 min and terminated with 3.5% of SDS. The released inorganic phosphate (P_i) was determined as described earlier (Karelson et al.1985). The NAKA activity was established as a difference between the release of P_i from ATP with and without NaCl + KCl in the incubation medium. The specific activity of the enzyme was expressed as micromoles P_i/min/mg protein. Under

all experimental conditions, the activity of NAKA was linear as a function of incubation time and enzyme amount.

3. Assay of Na,K-ATPase temperature-dependence (the Arrhenius plot)

The temperature-dependence (the Arrhenius equation) of NAKA was studied between 11.5° and 37°C (an enzyme activity measurement) and the data were plotted as log v_0 *vs* temperature as described earlier (Boldyrev 1988).

4. The degree of Na^+ and K^+ cooperativity measurement

The degree of Na^+ or K^+ cooperativity (the Hill coefficient, n_H) for NAKA were determined as described earlier (Salum et al. 1988). The activity of NAKA measured at several concentrations of Na^+ (the K^+ concentration was constant) or K^+ (the Na^+ concentration was constant). The values on n_H were established from the Hill plot: log(v/Vmax-v) *vs* log[Na^+] or log[K^+].

5. Oxidative stress index and the GSSG/GSH ratio measurement

The markers of lipid peroxidation and antioxidant defence were measured in the brain tissue homogenates. To characterize the oxidative stress level the oxidative stress index (OSI) was calculated. For calculation, two parameters, the total antioxidant response (TAR) and total peroxide (TPX) concentration were determined. The measurement of TAR was performed as decribed earlier (Erel 2004). In this method the hydroxyl radical (OH•), the most potent biological radical was produced and the rates of the reactions were monitored by following the absorbance of colored dianisidyl radicals. Ortho-dianisidine (10 mM) and ferrous ammonium sulfate (45 μM) were dissolved in KCl/HCl solution (75 mM, pH 1.8). This mixture was named as Reagent 1 and hydrogen peroxide solution (7.5 mM) as Reagent 2. The OH•, produced by mixing of reagent 1 and reagent 2, oxidized o-dianisidine molecules into dianisidyl radicals, leading to a bright yellow-brown color development within seconds. Antioxidants, present in the sample, suppressed the color formation to a degree that is proportional to their concentrations. The suppression of the color formation was calibrated with Trolox, which is widely used as a traditional standard for TAR measurement assays, so the results are expressed as in terms of millimolar Trolox equivalent

per liter. Total peroxide (TPX) concentrations of samples were determined using OxyStat Assay Kit Cat. No BI-5007 (Biomedica Gruppe, Biomedica Medizinprodukte GmbH & Co Kg, Wien). The kit detects peroxide concentrations based on reaction of the biological peroxides with peroxidase and a subsequent color-reaction using tetramethylbenzidine (TMB) as substrate. After addition of a stop solution, the coloured liquid is measured photometrically at 450 nm, using ELISA platereader Photometer Sunrise (Tecan Austria GmbH, Salzburg). For the assay a calibrator is used to calculate the concentration of biological peroxides in the sample. The concentration is stated as H_2O_2-equivalents (µmol/L). Percent ratio of the total peroxide concentration of plasma (TPX) to the total antioxidant responce of plasma (TAR) was accepted as oxidative stress index (OSI), an indicator of the degree of oxidative stress. OSI = [(TPX, µmol/L)/ (TAR, µmol Trolox/L) x 100)] (Horoz et al. 2006)

Concentrations of reduced and oxidized glutathione were assessed by an enzymatic method for GSH using manufacturer kit Glutathione Assay Kit (Cayman Chemical Company, Ann Arbor, Mich., USA). Briefly, the homogenate was deproteinated by 10% solution of metaphosphoric acid (MPA, Sigma-Aldrich) in water. The equal volume of the metaphosphoric acid was added to the sample and mixed vigorously. The mixture was allowed to stand at room temperature for 5 min and centrifuged at 3000 g for 5 min. In cases where the assay was not performed immediately, the supernatant was carefully collected and stored at –20°C. Glutathione content was measured by adding 0.05 ml of triethanolamine 4 M solution in water per ml of sample and vortexed immediately. The sample was divided into two parts. For assay of oxidized glutathione (GSSG), reduced glutathione (GSH) was derivatised by adding 10 µl of 2-vinylpyridine 1 M solution in ethanol to the first part of the sample, mixing and incubating at room temperature for 1 h. 50 µl of standard or sample was added per well of the plate. The enzymatic reaction was initiated by the addition 150 µl of the freshly prepared assay cocktail, containing nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase and 5,5´-dithio-*bis*-2-nitrobenzoic acid in buffer (pH 6.0) containing EDTA. The change in optical density was measured spectrophotometrically after 25 min at 412 nm. The glutathione content was calculated on the basis of a standard curve. The amount of GSH was calculated as a difference between the total glutathione and GSSG. The glutathione content was expressed as the glutathione redox ratio (GSSG/GSH).

6. Statistics

All experiments were repeated at least eight times and the results were expressed as mean values ± S.E.M.. Student`s t-test for independent samples was applied to establish the significant differences between two groups.

RESULTS AND DISCUSSION

1. Sodium pump temperature-dependence and cationic regulation in normal brain tissue (in the example of different species)

This part of the dissertation provides data on the sodium pump function dependence of temperature (the Arrhenius plot) and the Na^+ and K^+ binding to NAKA isolated from the normal brain cortex of rat, human, and mouse (Papers I-V). We found that the breakpoint in the Arrhenius plot was at 21.6 ± 0.6; 21.9 ± 0.2, and 20.3 ± 0.4°C in rat, human, and mouse brain cortex, respectively (Table 1). The cooperative binding character (the Hill coefficients of NAKA) for Na^+ in normal rat, human, and CCK2R wild type mouse were 1.7 ± 0.10; 1.5 ± 0.10 and 1.2 ± 0.06, respectively. In the case of K^+ the corresponding values were 1.4 ± 0.10; 1.3 ± 0.10 and 1.3 ± 0.07, respectively (Table 1). We also established the cooperative effect of Na^+ and K^+ for NAKA in the temperature interval 27–29°C, isolated from normal brain cortex of human. The values were 1.3 ± 0.09 and 1.4 ± 0.06 respectively.

Table 1. Breakpoint in the Arrhenius plot of sodium pump preparations isolated from rat, human, and mouse normal brain tissue and the Hill coefficients for Na^+ and K^+ ($n \geq 8$).

Enzyme preparations	Breakpoint in the Arrhenius plot (°C)	The Hill coefficient for Na^+ and K^+
Normal rat brain (cerebral cortex)	21.6 ± 0.6	1.7 ± 0.10 / 1.4 ± 0.10
Normal human brain (cerebral cortex)	21.9 ± 0.2	1.5 ± 0.10 / 1.3 ± 0.10
Wild type mouse (cerebral cortex)	20.3 ± 0.4	1.2 ± 0.06 / 1.3 ± 0.07

Before our experiments it was known that the nonlinear Arrhenius plot (breakpoint at 19–22°C) is typical for NAKA isolated from different tissues (brain, kidney) and inflection on graph reflects sensitivity of the enzyme to the phase reconstructions of CM lipids (Boldyrev 1988). Our study found the first evidence that in the case of NAKA isolated from brain cortex of different species (rat brain, human brain, mouse brain) average values of the breakpoint were between 20.3–21.9°C. Thus, all breakpoints were located in the typical/ classical region. Consequently, different species-specific lipid composition of CM do not have a crucial impact on the temperature-dependence (on the shape of the Arrhenius plot) of NAKA and the latter is more dependent on alterations in CM general lipid status if they occur in different pathologies.

All Hill coefficients for Na^+ were above 1.2 (refers to cooperativity between at least two sites, the allosterical effects of Na^+). However, the Na-cooperativity was lowest in the case of wild-type mice. Probably, the allosterical effect in the case of latter has not that kind of power compared to the results obtained from other NAKA preparations. The Hill coefficients for K^+ were not significantly different. Thus, there were no differences in K^+ allosteric effects power in the case of different species.

Summarizing the aforementioned: our study found for the first time that in the case of NAKA isolated from brain cortex of different species all temperature-dependent breakpoints were located in the typical/classical region. Consequently, different species-specific lipid composition of CM do not have a crucial impact on the temperature-dependence (on the shape of Arrhenius plot) of NAKA, as well as on allosteric regulation by sodium and potassium, but is evidently more dependent on alterations in CM general lipid status. Thus, the results obtained from different control brain tissues guided us to investigate CM lipid status and NAKA function properties in case of different brain pathologies. For these experiments NAKA preparations isolated from brain tumor tissue and Alzheimer diseased brain tissue were used. Third target used in our experiments were NAKA preparations isolated from the fronto-parietal cortex of CCK2R deficient mice.

2. Sodium pump temperature-dependence and cationic regulation in different neuropathologies and aberrant conditions

In the previous part of the results and discussion we established that the breakpoint in the Arrhenius plot and the Hill coefficients of Na^+ and K^+ for NAKA from different brain tissues does have a similar location area and there are no species-specific differences. This section of the results and discussion presents data about temperature-dependence of NAKA isolated from human tumorous brain tissue, human Alzheimer diseased brain tissue (AD), and CCK2R deficient (hetero- and homozygous) mouse brain cortex. We established that in all abnormal cases the breakpoint is not in the interval 19–22°C (Fig. 3). At the same time we established that in the case of tumorous brain tissue and AD the new breakpoint in the Arrhenius plot was at 28.3 ± 0.4°C and 28.0 ± 0.4°C, respectively (Figure 3) (Paper I). In the case of NAKA from brain CCK2 receptor deficient mice (hetero- and homozygous mutant mice) the new breakpoints were at 25.4 ± 0.4°C and 26.0 ± 1.1°C, respectively.

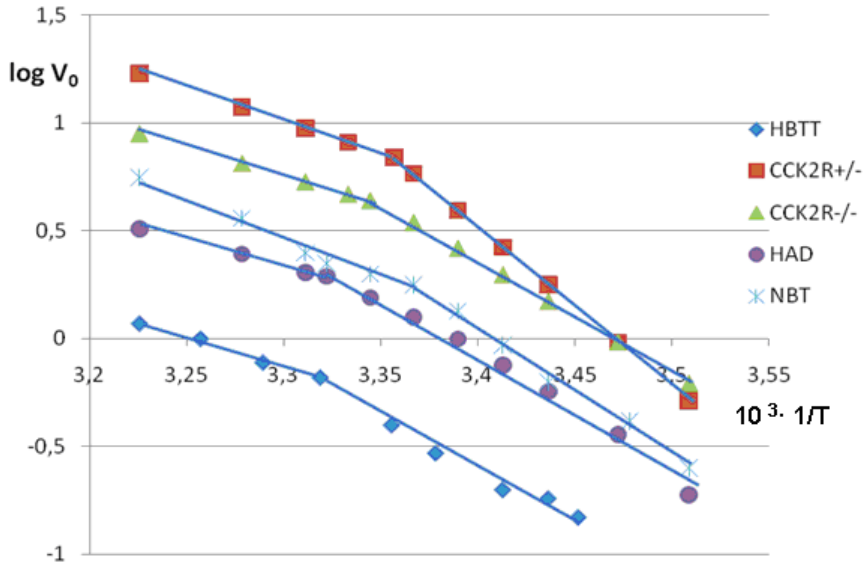

Figure 3. The temperature-dependence of NAKA activity (typical Arrhenius plots) in the different brain tissues.

These changes of temperature-dependence were accompanied by alterations in cooperative effects of sodium as the Hill coefficients for Na^+ were 0.9 ± 0.09, 1.1 ± 0.1 and 1.0 ± 0.09 for human brain tumor tissue, AD brain tissue and CCK2R deficient mice (homozygous), respectively. Interestingly, the Hill coefficient of Na^+ for CCK2R deficient (heterozygous, having 50 % of intact receptors present) was 1.3 ± 0.05. The Hill coefficients for potassium were not different from normal values given in Table 1.

Summarizing the aforementioned: we established the shift of the breakpoint in the Arrhenius plot and lack of cooperativity for Na^+ in case of NAKA preparation isolated from brain tumorous tissue (Papers IV; V). Next we showed that the same phenomenon regarding temperature-dependence and sodium cooperativity appears in the case of AD brain tissue as well as in the case of homozygous CCK2R deficient mice (Papers I; II; III).

It is remarkable that with the appearance of breakpoint in Arrhenius plot at higher temperature area the cooperativity effect of Na^+ is also changed (except in case of CCK2R heterozygous) but not of K^+ for NAKA. Intact Na^+ cooperativity in case of CCK2R heterozygous indicates the dependence from the gene dose. Here we can point out that alterations in general CM lipid status evidently underlie the changes of shape of the Arrhenius plot. Quite similar changes in the Arrhenius plot (the breakpoint shift) at different abnormalities indicate analogous changes in CM status and refer to the existence of a universal phenomenon apart from the actual cause of abnormality. Therefore, we decided to study at least one process/event that occurs in all the cases of abnormalities

(tumor, AD, CCK2R deficiency) and may underlie such a universal phenomenon (practically similarly alterated temperature-dependence of NAKA apart from the cause of abnormality).

3. Some similar characteristics of altered function of the sodium pump in different neuropathologies and aberrant conditions and its possible reasons

Several pathological processes have an effect on sodium pump functioning. In principle this may result in changes in the CM lipid status, oxidative damage of the enzyme, and changes in the enzyme SU expression and mutations.

There are limited data on the changes in functioning of NAKA depending on CM lipid general status in the case of oxidative stress, lipid peroxidation, and altered redox ratio in different pathologies, especially in brain tumor, and no data in case of CCK2R deficiency. Moreover, there are no data about temperature-dependence of NAKA in the case of different brain abormalities. Therefore, our main interest was already in 1989 to establish the character of temperature-dependence in the case of brain tumor and then investigate whether such alterations in temperature-dependence of NAKA are also valid for AD as well as CCK2R deficiency. If such alterations had a universal character then we aimed to establish whether some common reasons underlie such similarities apart from the character of the disease or genetic abnormality.

The Arrhenius plot method indicates the sensitivity of the reaction rate to temperature and, therefore, is the golden standard for investigation of CM lipid fluidity and CM lipid-protein interactions. The break area in the Arrhenius plot for NAKA isolated from human tumor brain tissue, Alzheimer's disease brain tissue, and the fronto-parietal cortex of CCK2R deficient (homo- and heterozygous) mouse was detected at higher temperature area (25.4–30.5°C), but the typical breakpoint at 19–22°C common for normal brain tissue in all studied species was disappeared (Papers I;V) (Table 3).

It is noteworthy that the cooperative binding of sodium show confidential change of sodium cooperativity (the Hill coefficients) in case of all species except in heterozygous CCK2R deficient mice (Table 3). This suggests that the gene dose effect between homo- and heterozygous mice does not appear in the Arrhenius plot. However, this effect appears as a difference between corresponding Hill coefficients. These results lead to the conclusion that the CM lipid status/composition is more sensitive to the gene dose effect than the cooperativity of the sodium pump for sodium. Summarizing the aforementioned: changes in the Arrhenius plot (shift of breakpoint) appear together in change of Na^+ cooperativity with high probability. In other words, CM lipid general status has a crucial role in NAKA functioning.

Table 2. Changes in the Arrhenius plot and Na^+ cooperativity of Na-pump in different pathological conditions.

Enzyme preparations	Breakpoint in the Arrhenius plot (°C)	The Hill coefficient for Na^+ and K^+
Brain tumor tissue (cerebral cortex)	28.3 ± 0.4*	0.9 ± 0.09*/1.3 ± 0.10
Alzheimer's disease brain sample (frontal cortex)	28.0 ± 0.4*	1.1 ± 0.10*/1.4 ± 0.09
CCK2R homozygous (cerebral cortex)	26.0 ± 1.1**	1.0 ± 0.09*/1.5 ± 0.07
CCK2R heterozygous (cerebral cortex)	25.4 ± 0.4**	1.3 ± 0.05 /1.6 ± 0.08

The cooperativity for potassium remained unchanged compared to control samples.
* $p < 0.05$ values of control samples compared with the affected brain
** $p < 0.01$ values compared with the wild-type mouse

Summarizing all our data, our findings refer to a possibility that the change of the breakpoint in the Arrhenius plot may be a universal phenomenon concerning different neuropathological conditions. Interestingly, CCK2 receptor-deficient mice display reduced mechanical sensitivity by means of von Frey filaments and they do not develop neuropathic pain after peripheral nerve ligation (Kurrikoff et al. 2004). This is an obvious declination from the response of wild-type mice and, therefore, could be stated as a pathological status. These similar findings on the Arrhenius plot and the Na^+ cooperativity for NAKA at different pathological conditions could be explained with two types of identical changes in the enzyme surrounded by membrane lipids.

First, changes in lipid microenvironment – phase transitions, and changes of the hydrophobic volume. Major lipid components of eukaryotic cell plasma membrane are glycerophospholipids, sphingolipids, and cholesterol. Lipids are asymmetrically distributed between and within leaflets, forming lipid microdomains. Glycerophospholipids and sphingolipids contribute to lipid asymmetry, cholesterol and sphingolipids form microdomains. Before our first paper (Paper V) there were no data about changes in the Arrhenius plot, but it was known that membrane phospholipid and cholesterol composition had an influence on Na^+ binding to the enzyme but not on K^+ (Giraud et al. 1981). This effect could be direct or induced by phospholipid and cholesterol effects on the intramembrane charge distribution or result from certain lipid combinations that stabilize oligomeric interactions of NAKA (Cornelius et al. 2003). The NAKA β1 and β2 subunit expression is downregulated in brain tumor (Espineda et al. 2003; 2004; Senner et al. 2003) and, therefore, the α β formation is altered and also the NAKA density in the plasma membrane. It has been shown that NAKA interacts with CAV 1 and knockdown of the NAKA α1 subunit likely redistributes cholesterol from the plasma membrane to other cellular compart-

ments (Chen et al. 2009). Obviously the NAKA subunit changes in brain tumor result in alteration in the cell membrane lipid/cholesterol composition and finally in functioning of NAKA. Genetic invalidation of CCK2R resulted in remarkable changes in receptor composition and structure and it is likely that the altered receptor structure has an influence on cell membrane structure as well. Amyloid β can perturb the molecular packing of CM, resulting in subsequent alterations of biophysical properties of membranes, such as membrane microviscosity, membrane molecular order, membrane potential, and permeability. In several papers it has been shown that changing the membrane fluidity and lipid composition has a direct influence on NAKA activity and function (Johannson et al. 1981; Karelson et al. 2001; Horoz et al. 2006; Bystriansky and Ballantyne 2007; Cornelius 2008) and CCK2 receptor (Harikumar et al. 2005)

Second, all these findings raise one general question concerning the reasons for similar changes in NAKA function in completely different conditions (brain tumor, AD brain, CCK2R deficiency). Possible causes for these changes are oxidative stress, altered lipid peroxidation and redox status. From literature and our original data, it is known that the sodium pump function is impaired by oxidative stress (Dobrota et al. 1999; Kurella et al. 1999; Yang et al. 2003; Valko et al. 2007). Cell membrane lipid/cholesterol composition and fluidity is changed and lipid peroxidation is increased in the brain tumor tissues (Hattori et al. 1987; Kokoglu et al. 1992; Martin et al. 1996; Cirak et al. 2003; Zajdel et al. 2007) and it is known that oligomeric Aβ protein can induce oxidative stress (OxS) as well (Karelson et al. 2001). Overproduction of reactive oxygen species (ROS) under pathological conditions cause profound oxidative stress expressed as elevated lipid peroxidation, DNA oxidation, and carbonylation, leading to impairment of the function of lipids, DNA, and proteins (Marnett 2002). CM components arachidonic and linoleic acid are sensitive to ROS attack, which results in excessive production of fatty acid hydroperoxides and other lipid peroxidation products (Marnett 2002; Yang et al. 2003).

We established for the first time a significant increase ($p < 0.01$) of oxidative stress index in the fronto-parietal cortex of CCK2R deficient homozygous mice (10.9 ± 2.6) compared to wild-type animals (7.7 ± 1.0; $p < 0.01$). Next, we showed for the first time that the redox ratio was altered as our last experiments showed the statistically significant increase of the GSSG/GSH ratio (1.12-fold elevation, $p < 0.05$) in the fronto-parietal cortex of CCK2 receptor deficient mice compared to their wild-type littermates (Fig. 4).

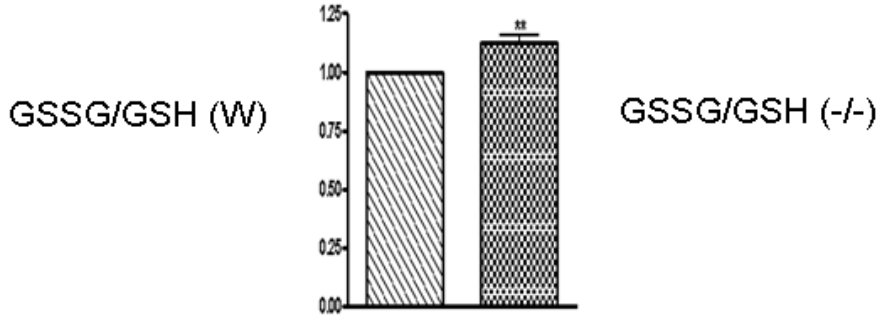

Figure 4. The GSSG/GSH ratio in the fronto-parietal cortex of CCK2 receptor-deficient mice (–/–) compared to their wild-type littermates (W).

These findings were supported by our earlier studies using the human post-mortem brain samples of Alzheimer disease where we established a similar change in the GSSG/GSH ratio (Karelson et al. 2001). Therefore, despite of different pathologies causing the conditions the similar oxidative stress-related changes in lipid peroxidation and redox status are probably the principal reasons for the underlying similarity in changes of temperature-dependence (CM lipid status) concerning functionality of NAKA. The existence of the breakpoint in the Arrhenius plot provides evidence that such kind of alterations in the NAKA temperature-dependence is likely the universal phenomenon for homeostatic adjustment of altered function of the sodium pump in different neuropathologies (brain tumor, Alzheimer's disease, genetic invalidation of CCK2 receptors) and one possible universal factor changing the CM lipid status in different neuro-pathologies is oxidative stress (Fig. 5).

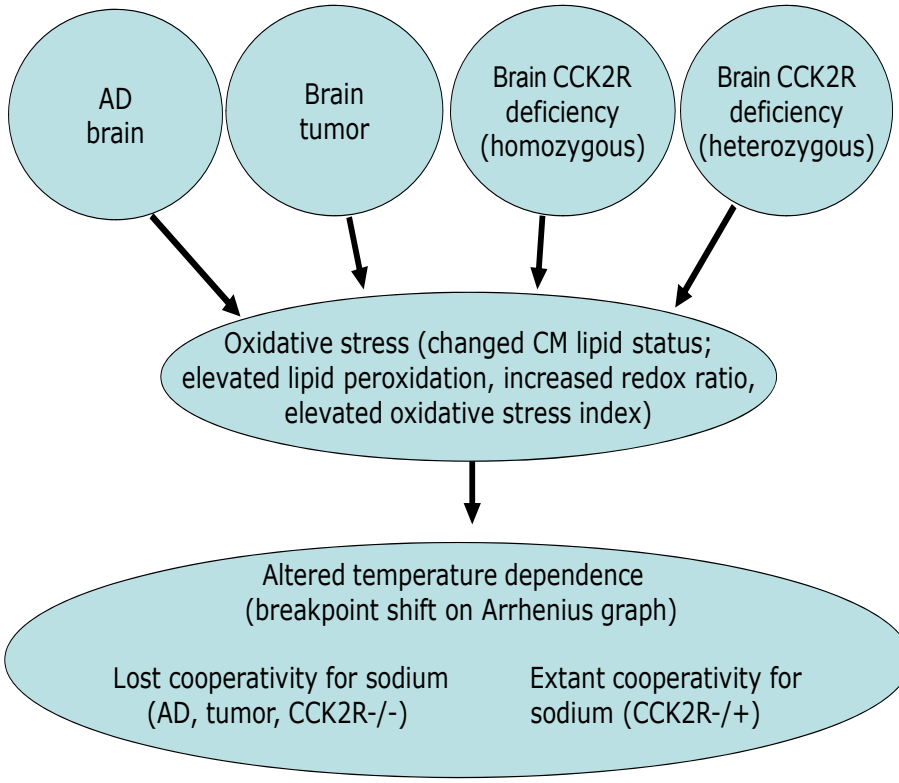

Figure 5. Possible pathway causing changes in Na,K-ATPase regulation in different neuropathologies and aberrant conditions.

CONCLUSIONS

1. It was demonstrated that for NAKA isolated from the normal brain cortex of different species (rat, human, mouse) the breakpoints in the Arrhenius plot were located in the same and typical/classical region. Thus, not the species-specific lipid composition of CM but the general lipid status of CM has a crucial impact on the temperature-dependence (the shape of the Arrhenius plot) of NAKA. The Hill coefficients' values showed cooperative effects both for Na^+ and for K^+ in the case of NAKA from brain of all different species.

2. It was established that the temperature-dependence of NAKA isolated from human tumorous brain tissue, human AD brain tissue, and CCK2 receptor deficient (hetero- and homozygous) mice compared with the results obtained from normal brain tissues of different species has a new breakpoint on the Arrhenius plot that is significantly higher than the classical breakpoint. The alterations also occurred in the cationic regulation as the Hill coefficients for Na^+ were decreased (lack of cooperativity) except in the case of CCK2 receptor deficiency indicating the Na^+ cooperativity dependence from the gene dose. The Hill coefficients for potassium were not altered compared to values in the case of normal tissues. Evidently the changes in general CM lipid status underlie the changes of the shape of the Arrhenius plot as well as sodium cooperativity. Similarity of changes of the Arrhenius plot (the breakpoint shift) in the case of different abnormalities reflects that the analogous changes occur supporting the role of CM lipid status and referring to the existence of an universal phenomenon apart from the nature of abnormalities.

3. The alteration in NAKA temperature-dependence (shift of breakpoint in the Arrhenius plot) as well as in Na^+ cooperativity are evidently caused by universal changes in CM lipid status which is influenced by oxidative stress-related changes in lipid peroxidation and redox status. We found a significant increase of oxidative stress index in the fronto-parietal cortex of homozygous mice CCK2 receptor deficient mice compared to their wild-type littermates. The redox ratio was altered (an increase of the GSSG/GSH ratio) in the fronto-parietal cortex of CCK2 receptor deficient mice compared to their wild-type littermates. The existence of the shifted breakpoint in the Arrhenius plot provides evidence that such kind of alteration of NAKA temperature-dependence is likely the universal phenomenon for homeostatic adjustment of altered function of the sodium pump in different neuropathologies and aberrant conditions (brain tumor, AD, genetic invalidation of CCK2 receptors) and one possible universal factor changing the CM lipid status in different neuropathologies and aberrant conditions is oxidative stress.

4. The alteration in NAKA temperature-dependence (shift of breakpoint in the Arrhenius plot) appears earlier than changes in Na^+ cooperativity. In other words, the CM lipid status/composition is more sensitive to the gene dose effect than the cooperativity of the sodium pump for sodium. Genetic invalidation of CCK2 receptor in heterozygous mice (50% reduction in the number of intact receptors) results in change of NAKA temperature-dependence but Na^+ cooperative effect is unaffected. CCK2 receptor changes in homozygous mice (the first 108 amino acids from total of 447 are preserved) induce both the shift of breakpoint in the Arrhenius plot and lack of Na^+ cooperativity for NAKA

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SUMMARY IN ESTONIAN

Normaalsest, patoloogilisest ja geneetilise mutatsiooniga ajukoest isoleeritud Na-pumba temperatuurisõltuvuse erinevus ja sarnasus ning selle võimalikud põhjused

Na,K-ATPaas (Na-pump) on raku ja kogu organismi homöostaasi säilitamises hädavajalik integraalne transmembraanne ensüüm, mille põhiülesanne on Na^+ ja K^+ aktiivne transport läbi rakumembraani ATP hüdrolüüsienergia arvel. Pumba töö on aluseks üliolulistele protsessidele nagu närviimpulsi levik, lihastöö, rakusisese pH ja rakumahu säilitamine, glükoosi ja aminohapete transport jt. Koostöös teiste rakumembraani ja tsütosooli valkudega osaleb Na-pump signaali ülekandes rakuorganellidele. Ensüümi funktsioonihäired põhjustavad rakkudes Na^+ ja K^+ gradientide muutusi, mis on seotud mitmesuguste patoloogiliste protsesside tekke ja arenguga.

Enamik Na-pumba teadusuuringuid käsitlevad ensüümi molekulaarset ehitust. Mitmetes töödes on uuritud pumba ensüümreaktsiooni kineetikat erinevate patoloogiliste seisundite korral nagu Alzheimeri tõbi ja kasvaja, samuti muutuste puhul membraani teistes valkudes ja retseptorites. Vähem on uuritud pumba talitluse regulatsiooni ja vaid üksikutes töödes on uuritud võimalikke regulatsioonilisi muutusi normi ja erinevate patoloogiate korral. Seetõttu pööratigi antud töös tähelepanu Na,K-ATPaasi regulatsiooni teatud aspektidele eesmärgiga saada teada, kas võib olla sarnaseid (universaalseid) muutusi regulatsioonis ajukoe erinevate patoloogiate ja geneetilise mutatsiooni puhul.

Mõnedes 1980. aastatel avaldatud töödes väidetakse, et rakumembraani lipiidide koostis ja omadused mõjutavad nii Na-pumba kui ka teiste transmembraansete valkude talitlust. Membraani lipiidide suur varieeruvus ja paindlik koostise muutlikkus võimaldavad rakul kohaneda rakusisese ja rakuvälise keskkonna muutustega. Üks olulisemaid membraani omadusi on voolavus, mida ühe tegurina mõjutavad muutused membraani valkudes. Selliste valkude (ensüümide) tööd mõjutab suurel määral membraani lipiidide seisund, mille muutustel on ilmselt oluline roll mitmete haiguste nagu hüpertoonia, südame hüpertroofia, Alzheimeri tõve, skisofreenia ja kasvajate tekke- ja arengumehhanismis. Membraani lipiidide seisund peaks mõjutama eelkõige integraalsete membraanivalkude, nagu Na-pump, funktsiooni. Klassikalise meetodina membraani lipiidide seisundi ja selle muutuste mõju uurimisel Na-pumba funktsioonile kasutatakse Na,K-ATPaasi temperatuurisõltuvust (nn. Arrheniuse graafiline meetod). Teatud temperatuuril toimub membraani lipiidide faasilisuse (vedelam, tahkem) muutus, mistõttu pole graafik sirge, vaid murdepunktiga (Boldõrev 1988). Katseloomade ajukoest saadud Na,K-ATPaasi puhul on murdepunkt temperatuurivahemikus 19–22°C. Olime esimesed, kes kasutasid inimajust isoleeritud ensüümpreparaati eesmärgiga tuvastada, kas sama leid esineb ka inimese normaalsest ajukoest isoleeritud ensüümi puhul.

Eksperimentide tulemused kinnitasid, et klassikaline murdepunkt esineb ka normaalsest inimajust isoleeritud ensüümi puhul. Järgnevalt leidsime esimestena, et ajukasvajast isoleeritud ensüümpreparaadi korral puudub klassikaline murdepunkt, kuid on olemas uus murdepunkt kõrgemas temperatuurivahemikus (28–29° C). Saadud tulemuste põhjal esitasime järgmise küsimuse, kas avastatud nn mitteklassikaline murdepunkt on olemas ka teiste ajukoe patoloogiliste seisundite korral? Selle hüpoteesi kontrollimiseks kasutasime Karolinska Ülikooli Haiglas Alzheimeri tõvega inimestelt surmajärgselt võetud ajukude, kuna nende proovide puhul oli Alzheimeri tõbi väga korrektselt sedastatud ja saime kasutada ka normaalset ajukude. Katsete tulemused kinnitasid, et normaalsest ajukoest isoleeritud ensüümpreparaadi Arrheniuse graafikul on murdepunkt klassikalises vahemikus, Alzheimeri tõve puhul on nihkunud kõrgemasse temperatuurivahemikku. Geneetilise muundamise arenedes osutus võimalikuks läbi viia järgmised eksperimendid, kasutades selleks TÜ Füsioloogia Instituudis geneetiliselt muundatud CCK2 retseptoriga hiiri (hetero- ja homosügootseid). CCK2 retseptor on rakumembraanis paiknev integraalne valk, mis osaleb kesknärvisüsteemis valu-, ärevuse- ja mäluprotsessides. Retseptori geneetiline muundamine põhjustab muutusi retseptorvalgu primaarstruktuuris ja funktsiooni kadumise. Võrdlusena kasutasime sama liini muundamata hiiri (*wild type*). Leidsime, et geneetiliselt muundatud CCK2 retseptoriga ajukoe puhul on Na,K-ATPaasi Arrheniuse graafik muutunud ning murdepunkt asub temperatuurivahemikus 25–27°C.

Eksperimentide andmetest tulenevad järgmised järeldused. Kõikides meie poolt uuritud normaalsest ajukoest (inimene, rott, hiir) isoleeritud Na-pumba preparaatide korral ei esine liigilist erinevust klassikalise murdepunkti asukohas Arrheniuse graafikul. Järelikult ei oma teatud liigilised erinevused rakumebraani lipiidide koostises olulist tähendust Na,K-ATPaasi talitluse regulatsioonis. Tõenäoliselt on olulisem rakumebraani lipiidse keskkonna üldisem seisund. Andmete analüüsist ilmnes kasvajakoest, Alzheimeri tõvest haaratud ajukoest ja CCK2 retseptori puudusega (hetero- ja homosügootne) ajukoest isoleeritud Na-pumba temperatuurisõltuvuse graafikul murdepunkt ülalpool 25°C, mis viitab muutustele rakumembraani lipiidide seisundis. Sellest avastusest tulenes aga vajadus selgitada, mis võiks olla rakumembraani lipiidide seisundi muutuse selline põhjus, mis omab mõju kõigi kolme väga erineva ajukoe puhul. Varasemad meie grupi tööd (Karelson et al. 2001) on näidanud, et esinevad muutused rakkude antioksüdantses seisundis ja oksüdatiivse stressi näitajates Alzheimeri tõve puhul, mida on näidatud ka kasvajalise protsessi puhul ajukoes (Zajdel et al. 2007). Seetõttu määrasime ka CCK2 retseptori puudusega ajukoest (homosügoot) oksüdatiivset stressi iseloomustavaid näitajaid nagu redoksseisund (GSSG/GSH redoksseisund) ja oksüdatiivse stressi indeks. Need näitajad on statistiliselt usaldusväärselt kõrgemad kui normaalse ajukoes. Saadud ja meie varasemate tööde tulemused lubavad järeldada, et sõltumata konkreetsest patoloogilisest protsessist närvikoes on üheks ühiseks membraani lipiidide staatust muutvaks teguriks *high-grade* oksüdatiivse stress mitmes avaldumis-

vormis, mis oma universaalsuses põhjustab sarnaseid kõrvalekaldeid Na-pumba talitluses, mis on tõnäoliselt aluseks ka naatriumi kooperatiivse toime muutustele. Geneetilise mutatsiooniga ajukoest tehtud uuringud võimaldavad teha veel ühe uudse järelduse, et rakumembraani lipiidse seisundi muutustest põhjustatud murdepunkti nihe Arrheniuse graafikul ei ole sõltuv CCK2 retseptori mutatsiooni korral geenidoosist (homo- ja heterosügoot), küll aga naatriumi ioonide kooperatiivne toime.

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- My family and friends

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Peamised uurimisvaldkonnad

Na,K-ATPaasi alased uuringud normaalses ja patoloogilises ajukoes.

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos.** The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer.** Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
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