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# NEURONAL FUNCTION AND ALPHA3 ISOFORM OF THE Na/K-ATPase

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## 1. ABSTRACT

The Na/K-ATPase is a complex of integral membrane proteins that carries out active transport of sodium and potassium across the cell plasma membrane, and maintains chemical gradients of these ions. The alpha subunit of the Na/K-ATPase has several isoforms that are expressed in a cell type- and tissue-dependent manner. In adult vertebrates, while kidney cells express mostly alpha1, muscle and glial cells - alpha1 and alpha2, and sperm cells - alpha1 and alpha4 isoforms of Na/K-ATPase, neurons may express alpha1, alpha2, alpha3 or any combination of these isoforms, and evidence suggests that neuronal type is the determining factor. The functional significance of multiple isoforms of the Na/K-ATPase and their non-uniform expression, and the link between neuron function and expression of a given isoform of the Na/K-ATPase in particular, remains unknown. Several hypotheses on this account were introduced, and in this work we will review the present status of these hypotheses, and their standing in application to recent data on the expression of isoforms of the Na/K-ATPase in the peripheral nervous system of vertebrate animals.

## 2. INTRODUCTION

Neuronal activity is intrinsically linked to strong passive fluxes of $Na^+$ and $K^+$ across the cell plasma membrane. Therefore, restoration and maintenance of chemical gradients that support these ionic fluxes is of critical importance for the neuron. This function is performed by Na/K-ATPase. Signifying the important role of Na/K-ATPase, neurons of the CNS and PNS use up to 50% of the energy of ATP hydrolysis just to maintain the operation of this enzyme and transporter. Another critical feature of the organization of active ionic transport in neurons relates to the presence and non-uniform expression of multiple isoforms of the Na/K-ATPase (1, 2).

The functional Na/K-ATPase complex is composed of $\alpha$ and $\beta$ protein subunits. The $\alpha$ subunit determines all major enzymatic and transporter properties of the Na/K-ATPase and binds ouabain and other cardiac glycosides that act as specific inhibitors (3, 4, 5). Four isoforms of the $\alpha$ subunit are known to exist, and are expressed in a cell type-specific manner in higher

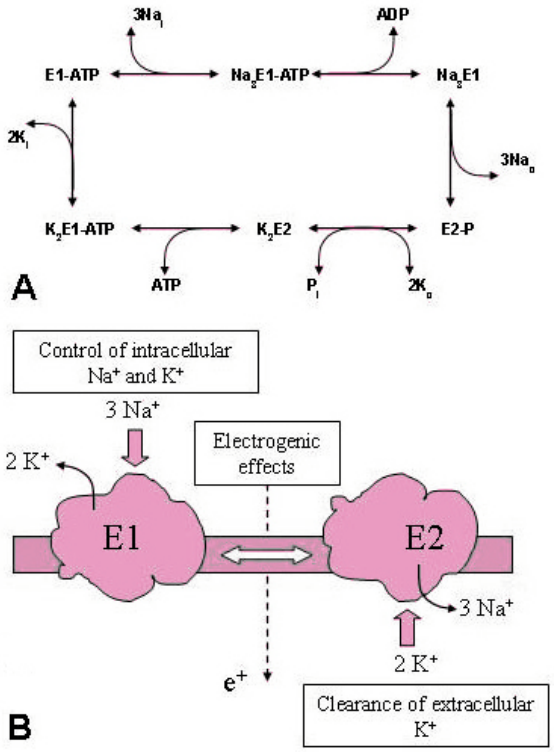


**Figure 1.**Variant of Post-Albers reaction scheme (A) and cartoon depicting some reaction steps and associated functions of the Na/K-ATPase (B). See Chapter 1 and references (224, 25, 23, 225, 2, 226) for detailed description of kinetic and electrogenic properties and functions of the Na/K-ATPase.

vertebrates (6, 4, 5). Thus, adult rat kidney and liver cells express the $\alpha_1$ isoform, glial and skeletal muscle express both $\alpha_1$ and $\alpha_2$, and sperm cells express both $\alpha_1$ and $\alpha_4$. Unlike most other cells, neurons may express $\alpha_1$, $\alpha_2$, or $\alpha_3$, or any combination of these $\alpha$ isoforms. Furthermore, with rare exceptions, the $\alpha_3$ isoform of Na/K-ATPase is only detected in neurons of adult vertebrates (7, 6, 8, 9, 4, 10). The reason for this neuron-specific expression of the $\alpha_3$ isoform of Na/K-ATPase is not known.

Over time, a variety of hypotheses on the role of $\alpha_3$ Na/K-ATPase in neurons have been introduced. One common and essential component of many of these hypotheses is that the function and activity of the neuron may be important in regulating isoform expression. Indeed, studies in the CNS have shown that although the $\alpha_3$ Na/K-ATPase isoform is expressed by many central neurons, its abundance varies greatly between different groups of neurons, and there are neuronal populations in which this isoform is not detectable (11, 12, 13). However, because of the complexity of the CNS, the relationship between expression of isozymes of the Na/K-ATPase and neuronal function remains elusive (14, 15, 16, 17, 13). On the other hand, parallel efforts in peripheral nervous system have recently resolved the functional identification of at least two groups of peripheral neurons expressing $\alpha_3$ Na/K-ATPase, the skeletal muscle stretch receptor afferent neurons, and γ-motoneurons (18).

Because of the comparative simplicity of PNS, the peripheral neurons are much better characterized than are their central counterparts. Specifically, a wealth of information is available about function, electrophysiology, structure and biochemistry of stretch receptor afferents in both developing and mature vertebrates (see (19, 20, 21)). In this work we will review the present status of existing hypotheses on functional significance of $\alpha_3$ Na/K-ATPase and in relation to recent data on the expression of isoforms of the Na/K-ATPase in the peripheral nervous system of vertebrate animals.

## 3. PHYSIOLOGICAL ROLES OF THE Na/K-ATPase IN NERVOUS SYSTEM

The Na/K-ATPase is a complex integral membrane protein that hydrolyses ATP and transports $Na^+$ out and $K^+$ into the cell against existing chemical gradients of these ions. The transport cycle of Na/K-ATPase is conventionally described on the basis of Post-Albers reaction scheme suggesting that the enzyme shuttles between two major conformational states, E1 and E2 (Figure 1). In the dephosphorylated E1 state, the enzyme has high affinity for intracellular $Na^+$ and ATP. Hydrolysis of ATP and phosphorylation of the enzyme are followed by a conformational change to E2. During this transition, three $Na^+$ become temporary occluded in the protein moiety and transported across the plasma membrane, out of the cell. In the E2 state, the enzyme has high affinity to external $K^+$. Also, ouabain and other cardiac glycosides specifically inhibit the Na/K-ATPase by binding to the E2 state. Two $K^+$ become occluded in a manner similar to $Na^+$ and are transported into the cell when the enzyme dephosphorylates and changes conformation back to the E1 state. One additional and important feature of the Na/K-ATPase cycle is that because of unequal exchange of $Na^+$ and $K^+$, one net positive charge per cycle is expelled from the cell. Thus, Na/K-ATPase is an electrogenic mechanism that generates outward current. (22, 23, 24, 25).

There are three known functions of the Na/K-ATPase. The transporter maintains $Na^+$ and $K^+$ gradients across the cell plasma membrane. In addition, as an electrogenic mechanism it directly hyperpolarizes the cell. Finally, the Na/K-ATPase is one of mechanisms for extracellular $K^+$ clearance. All three functions are of extreme importance for the activity of the nervous system (Figure 1B, and see (2)).

### 3.1. Intracellular ion concentrations

The major function of Na/K-ATPase is to offset passive fluxes of $Na^+$ and $K^+$ associated with the activity of a variety of plasma membrane ion channels and transporters, thus maintaining intracellular concentrations of $Na^+$ low and $K^+$ high with respect to extracellular concentrations of these ions. Besides the Na/K-ATPase, there are some additional mechanisms allowing the cell to take up $K^+$ (K:Cl cotransport, Na:K:2Cl cotransport).

**Table 1.** Concentration of intracellular $Na^+$ ($[Na]_i$) and its changes in neurons

| Preparation | Basal $[Na]_i$, mM | Treatment / time of treatment | Post-treatment $[Na]_i$, mM | Reference |
|---|---|---|---|---|
| Mice cortex neurons | 10 | Zinc, 50 μM or ouabain, 500 μM / 10 min | 30 | 193 |
| Rat substantia nigra neurons | 11.8 to 13.5 | | | 194, 195 |
| Rat hippocampus neurons | 7 to 9 | Ouabain, 1 μM | 12 | 109, 196 |
| | | Ouabain, 1 mM / 5 min | 59 | |
| Rat hippocampus neurons | 8.9 | Spontaneous burst | Rise by 5 mM | 197 |
| | | Veratridine, 50 μM | 10 | |
| | | Potassium-free solution | 50 | |
| | | Ouabain, 0.5 mM / 10 min | 50 | |
| Rat neurohypophysis nerve endings | 10 to 15 | Stimulation 40 Hz /30 s | Rise by 2.4 mM | 198 |
| Rat superior cervical ganglion neurons | 5[1] | | | 199 |
| Mice DRG neurons | 10 | | | 141 |
| Crayfish motor axons | 7 to 8 | Stimulation 20 Hz / 10 min | 80 | 200 |

[1] Denotes experiment in which electron microprobe technique was used. In all other cases sodium concentration was measured using fluorescent probe technique

However, the Na/K-ATPase is the only known mechanism of active extrusion of excess cellular $Na^+$.

Like many other cells, the basal concentration of $Na^+$ in neurons is about 10 mM (Table 1), while plasma $Na^+$ concentration is about 140 mM (26)). Unlike other cells, the intrinsic electrical activity, abundance of ion channels and Na-coupled transporters and generally high surface-to-volume ratio of neurons makes the task of controlling intracellular $Na^+$ a challenge for the Na/K-ATPase. In neurons with active Na/K-ATPase, a short burst of action potentials raises intracellular $Na^+$ by as much as 5 to 10 mM. When the Na/K pump is inhibited, the activity of ion channels and transporters of the resting neuron may increase intracellular $Na^+$ up to 30 – 50 mM within minutes (Table 1). Not only should intracellular $Na^+$ be brought down by the Na/K-ATPase after the neuronal discharge, but in some neurons this should be done within milliseconds to ensure their uninterrupted signaling.

Failure of the Na/K-ATPase to maintain $Na^+$ and $K^+$ gradients leads to a decrease in $Na^+$ and $K^+$ currents through the voltage-dependent channels, a decrease in membrane potential, action potential and eventual loss of neuronal excitability. Moreover, an additional consequence is a reduced driving force for a variety of Na-coupled co- and counter-transport mechanisms, including impairment of $Na^+/H^+$ exchange and $Na^+/Ca^{2+}$ exchange, resulting in intracellular acidification and $Ca^{2+}$-overload. These events have been implicated in the pathogenesis of several neurodegenerative disorders, spongiform encephalopathy, and neuronal injury following exposure of neurons to ouabain or non-specific inhibitors of Na/K-ATPase in experimental animals (27, 28, 29, 30, 31, 32, 33).

### 3.2. Electrogenic effects

The activity of the Na/K-ATPase generates a net outward current due to the unequal exchange of Na and K during the pump cycle (22, 24). The electrogenic activity of the Na/K-ATPase contributes from 9% to 45% of resting membrane potential (RMP) of neurons (Table 2). The signaling function of neurons critically depends on resting membrane potential and rate of recovery of the resting potential after periods of neuronal activity. These characteristics determine excitability of the neuron and its capacity to properly code signals related to action potential frequency and burst duration. Furthermore, activation of electrogenic Na/K-ATPase by $Na^+$ entering the neuron during its repetitive discharge is an important mechanism of post-burst or post-tetanic hyperpolarization. This phenomenon plays a central role in determining the frequency pattern of neuronal activity. Partial inhibition of the Na/K-ATPase by low concentrations of cardiac glycosides causes depolarization of neurons and suppression of post-burst hyperpolarization. This has been shown to result in conversion of spontaneous bursting into persistent tonic firing in ventral midbrain neurons, in guinea pig trigeminal neurons, and in rat spinal cord interneurons (34, 35, 36, 37, 38). Similar mechanisms have been implicated in the shaping of synaptically induced burst response of medial geniculate body neurons, slow spike frequency adaptation of guinea pig hippocampal pyramidal neurons, and conduction of high frequency signals through the rat spinal cord sensory-motor circuits (39, 40, 41).

### 3.3. Extracellular $K^+$ clearance

Normal extracellular concentration of $K^+$ in the nervous system of vertebrate animals is 3 to 4 mM, which is lower than in blood plasma (5.5 ± 0.2 mM in rat; (26, 42, 43, 44, 45)). Because of the intrinsically small volume of interstitial space in the nervous system, potassium leaving the neurons, particularly during periods of intense electrical activity, may accumulate in this space with an initial rate of several millimoles per second. However, under normal physiological conditions and physiological range of neuronal activity, variations of $K^+$ in the extracellular space of the nervous system do not exceed a few millimoles (46, 47, 48). Experiments in rabbit vagus nerve, rat optic nerve, and rat hippocampal sections have shown that both glial and neuronal Na/K-ATPase are heavily involved in the control of extracellular potassium in these brain structures (43, 49, 50). Failure of the Na/K-ATPase to control extracellular $K^+$ below 8 - 12 mM can cause hyperexcitability of the neurons and pathological states such as recurrent epileptiform activity and spreading depression developing in dentate gyrus of rat hippocampus (51, 52).

### 3.4. Conclusion

Because of intrinsically intense electrical activity of neurons, and generally high surface-to-volume ratios of both neuronal and extracellular compartments of the nervous system, neurons are critically dependent on the

**Table 2.** Neuronal resting membrane potential (RMP) and its electrogenic component

| Preparation | RMP, mV | Electrogenic fraction of RMP | | Reference |
|---|---|---|---|---|
| | | mV | % to RMP | |
| Rat neostriatum interneurons | -67 | 12 | 18% | 201 |
| Rat medial geniculate body | -68 to -74 | 6 to 9 | 10.5% | 112 |
| Rat hippocampus dentate interneurons | -65 | 18 | 28% | 202 |
| Rat hippocampus pyramidal neurons | -55 to -70 | 5 to 6 | 9% | 203 |
| Rat cerebellum, Purkinje cells | -70 | 25 | 36% | 204 |
| Rat cerebellum, Purkinje cells | -58 | 27 | 45% | 205 |
| Guinea pig myenteric neurons | -54 | 16 | 29% | 206, 207 |
| Rat spinal cord interneurons | -49 to -55 | 12 - 20 | 30% | 208 |

**Table 3.** Expression of protein subunit isoforms of Na/K-ATPase in adult rat tissues

| Tissue | α-Subunit | | | | β-Subunit | | | References |
|---|---|---|---|---|---|---|---|---|
| | $\alpha_1$ | $\alpha_2$ | $\alpha_3$ | $\alpha_4$ | $\beta_1$ | $\beta_2$ | $\beta_3$ | |
| Kidney | + | | | | + | | | 7 |
| Thymus | + | | | | | + | | |
| Lung | + | | | | + | | + | |
| Liver | + | | | | + | | + | 7, 209 |
| Heart | + | + | | | + | | | 210, 7 |
| Skeletal muscle | + | + | | | + | + | + | 211, 212, 213, 214 |
| Pineal gland | + | | + | | | + | | 215 |
| Testis/Germ cells | + | | | + | | | + | 216, 217, 218 |
| Brain | + | + | + | | + | + | | 7, 8 |

active transport of $Na^+$ and $K^+$ by Na/K-ATPase. As a transporter, an electrogenic mechanism and a mechanism of extracellular $K^+$ clearance, the Na/K-ATPase is involved in practically every aspect of neuronal function and homeostasis. Therefore, understanding the organization and regulation of neuronal active transport is an obligatory requirement in studies of neurophysiology that is too often neglected.

## 4. MULTIPLE ISOFORMS AND "NEURONAL" $\alpha_3$ ISOFORM OF THE Na/K-ATPase

The form of the Na/K-ATPase that is functionally capable of ATP hydrolysis and ion transport is a heterodimer of α and β integral membrane protein subunits (Figure 2). The larger, catalytic α subunit of Na/K-ATPase determines all major properties of the αβ enzymatic complex; its capacity to bind ligands, hydrolyze ATP, occlude and transport ions. It also contains the binding site for cardiac glycosides. The β subunit, together with core lipids associated with the Na/K-ATPase in the plasma membrane, are required for enzyme activity. However, the major role of the β subunit is to ensure proper folding, targeting and delivery of α subunit from the endoplasmic reticulum to the membrane. Smaller, PXYD-family protein subunits may also join the complex of Na/K-ATPase (for review see (53, 54)).

Currently, four isoforms of the α ($\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_4$) subunit and three isoforms of the β ($\beta_1$, $\beta_2$, and $\beta_3$) subunit of Na/K-ATPase of higher vertebrates are known to exist. These subunit isoforms are coded by different genes and are expressed in a tissue- and cell type-dependent manner. Since any combination of the α and β subunit isoforms produces functional Na/K-ATPase, the reason for such complexity of the organization of active ion transport in the tissues of vertebrates is not understood. A relevant question is what is the functional significance of neuron-specific expression of the α3 isoform of the Na/K-ATPase (9, 2, 4, 55, 56).

Neurons express $\alpha_3\beta_1$ and rarely $\alpha_3\beta_2$ forms of the Na/K-ATPase; however, $\beta_1$ and $\beta_2$ subunits are also expressed in other tissues and cell types. In this review it is the α3β1 form that will be referred to as α3 Na/K-ATPase, which demonstrates obvious neuron-specificity (Table 3 and 4).

### 4.1 Discovery and neuron-specific expression of $\alpha_3$ Na/K-ATPase

The $\alpha_3$ isoform of the Na/K-ATPase ($\alpha_3$ Na/K-ATPase) was discovered and sequenced in 1986 as a result of analysis of the cDNA library of rat brain for genes coding the catalytic subunit of the enzyme (57). Subsequently, the full amino acid sequences for human and avian Na/K-ATPase $\alpha_3$ subunits were deduced from the cDNA sequence (58, 59). One important outcome of the $\alpha_3$ Na/K-ATPase sequencing was that it allowed development of isoform-specific hybridization probes and antibodies to determine tissue and cellular distribution of this subunit (Figure 2). As a result of these studies, a striking feature of $\alpha_3$ Na/K-ATPase is that it is abundantly and selectively expressed in neurons. In the adult rat, the only tissues where $\alpha_3$ Na/K-ATPase can be detected by Western blot are nervous system, retina and pineal gland (Table 3). Within the nervous system $\alpha_3$ Na/K-ATPase appears as a "neuronal" isoform of the transporter. Different types of central and peripheral glia (astrocytes, oligodendrocytes and Schwann cells) express $\alpha_1$, or $\alpha_1$ and $\alpha_2$, but not $\alpha_3$ Na/K-ATPase (60, 61, 62, 63, 15, 64, 62, 65, 66, 67). In retina, photoreceptor and all neuronal-type cells express $\alpha_3$ Na/K-ATPase, while Muller cells (retinal glia) express $\alpha_1$ and $\alpha_2$ isoforms (68).

Perhaps the only examples of non-neuronal expression in adult rat brain of $\alpha_3$ Na/K-ATPase are pineal gland cells and cerebral microvessels that express this

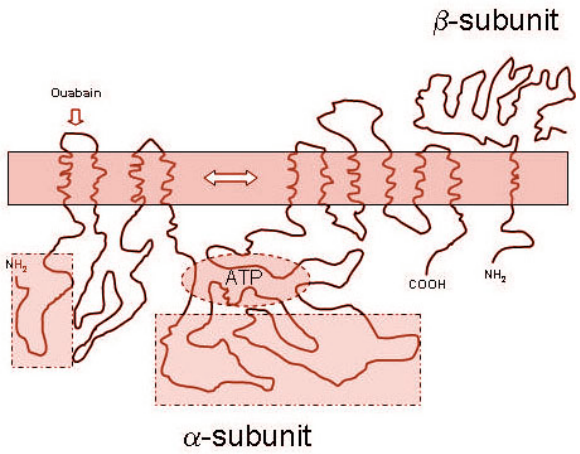


**Figure 2.** Schematic representation of molecular structure of α and β subunits of the Na/K-ATPase. The catalytic α subunit is a sequence of about 1020 amino acid residues (~110 KDa) that has ten membrane spanning segments and intracellular N- and C-termini. The segments of the sequence that are most conserved between isoforms and different species are the transmembrane segments, including 4th and 5th segments that are involved in ion binding (double headed arrow), and parts of the sequence involved with ATP binding and hydrolysis (shaded oval). Most of the α isoform-specific sequences are found at the N-terminus and parts of the third intracellular segment of the molecule (shaded rectangles). Cardiac glycoside binding is strongly determined by the sequence of first extracellular loop of the enzyme (arrow). For review of the molecular structure of the Na/K-ATPase see (9, 5, 3, 56).

isoform together with $\alpha_1$, or both $\alpha_1$, and $\alpha_2$ Na/K-ATPase, respectively (69). There are only a few exceptions for nervous system-specific expression of $\alpha_3$ Na/K-ATPase known. Interestingly, most of these exceptions associate with cardiac muscle tissue. While adult rat heart expresses $\alpha_1$ and $\alpha_2$, in early neonatal rat heart $\alpha_1$ and $\alpha_3$ Na/K-ATPases are expressed (7, 70). Canine atrial and ventricular myocytes express $\alpha_1$ and $\alpha_3$ isoforms of the Na/K-ATPase (71, 72). Human heart expresses $\alpha_1$, $\alpha_2$, and $\alpha_3$ isoform proteins, and reticulocytes have message for $\alpha_1$, $\alpha_3$, $\beta_2$, and $\beta_3$ isoforms (73, 74, 75).

### 4.2. Phylogenetic preservation of $\alpha_3$ Na/K-ATPase

Analysis of dendrograms of amino acid sequence similarity suggests that $\alpha_3$ and other α isoforms appeared some time in the very beginning of evolution of vertebrates, probably as a result of several duplications of one parent gene. This analysis also suggests that selective pressure prevented significant modifications of these genes during subsequent vertebrate evolution, suggesting the biologically important role of isoforms of the Na/K-ATPase (76, 77, 59).

While interspecies similarity of amino acid sequences of different α Na/K-ATPases ranges from 78% to 87%, the cross species similarity of $\alpha_3$ Na/K-ATPase sequences is about 96% (59). In both rat and chicken, $\alpha_3$ mRNA was detected only in brain, but not in skeletal muscle or kidney (59). One of the "signature" regions of rat $\alpha_3$ Na/K-ATPase consists of the amino acid sequence near the phosphorylation site of the α subunit. This sequence was detected by specific antibodies from brain, but not kidney, of opossum, guinea pig, dog, prairie dog, bovine and chicken, and in catfish brain, but not gill (78). Recently, two genes with 90 and 92% homology to genes of higher vertebrate $\alpha_3$ subunit of Na/K-ATPase were discovered in Zebra fish genome. Interestingly, the gene with 92% similarity was expressed only in brain and eye of the fish, the gene with 90% similarity was also expressed in brain, eye and gut (77).

In addition to similarity of tissue-specific expression of the $\alpha_3$ Na/K-ATPase, the pattern of expression within the tissue of $\alpha_3$ Na/K-ATPase also appears to be similar between different animal species. Thus, the $\alpha_3$ Na/K-ATPase in monkey cerebellum shows an expression pattern that is virtually identical to that observed in rat (15). The distribution of $\alpha_3$ Na/K-ATPase in rat, guinea pig and human dorsal root ganglion (DRG) and ventral roots also appears to be identical (18).

### 4.3. Ontogenesis and $\alpha_3$ Na/K-ATPase

Mice with homozygous genetic knockout of $\alpha_1$, $\alpha_2$, or $\alpha_3$ Na/K-ATPase are either never born ($\alpha_1$), or do not survive first postnatal day (personal communication with Dr. Jerry Lingrel of Cincinnati University and (79, 80)), indicating important roles of these isoforms and the lack or failure of mechanisms allowing the substitution of one α isoform of the Na/K-ATPase with another.

Studies in mice detect accumulation of $\alpha_3$ mRNA and protein as early as between four-cell and the blastocyst stages. At that time, however, $\alpha_3$ Na/K-ATPase is not at the cell plasma membrane, and $\alpha_1$ Na/K-ATPase appears to be mainly responsible for the active transport of $Na^+$ and $K^+$ in cavitating mammalian embryo (81). Starting with early organogenesis (E10 - E11), the $\alpha_3$ Na/K-ATPase mRNA expression is already demonstrating clear non-uniformity within the mouse embryo, with highest levels located in the marginal regions of developing neural tube (82). Cellular distribution of the Na/K-ATPase isoforms in developing embryo has yet to be addressed. However, from the analysis of ouabain sensitivity of membrane preparations of Na/K-ATPase it follows that the $\alpha_1$ Na/K-ATPase is the major transporter in rat brain between E14 and E18 stages of embryo. The strong shift in favor of ouabain-sensitive isoforms in the rat brain happens sometime between E18 and P10 (83). After that, all three isoforms increase their abundance in the brain (8).

In our studies of $\alpha_3$ Na/K-ATPase expression in peripheral neurons, the first lumbar DRG neurons with membrane location of $\alpha_3$ Na/K-ATPase are observed on E21. Interestingly, about 2 days later (P0) rat DRG have the full complement of $\alpha_3$ Na/K-ATPase expressing neurons (10 - 15% of all DRG neurons). In the mouse retina the adult distribution of $\alpha_3$ Na/K-ATPase is achieved by P13, while a quantitative increase continues

**Table 4.** Expression of α-isoforms of the Na/K-ATPase in nervous system

| Region | Cells | mRNA | | | protein | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | $\alpha_1$ | $\alpha_2$ | $\alpha_3$ | $\alpha_1$ | $\alpha_2$ | $\alpha_3$ | |
| Neocortex: | Pyramidal neurons | | | + | | | +[1] | 11, 60, 12, 13 |
| Hippocampus: | Pyramidal neurons | + | + | + | | | + | 11, 60, 12, 16, 13 |
| | Granule cells | + | | | | +[1] | | |
| Cerebellum: | Purkinje cells | | | + | | +[1] | + | 60, 12, 15, 13, 86 |
| | Granule cells | + | +[1] | +[1] | + | | +[1] | |
| | Stellate cells | | | + | | | + | |
| | Basket cells | | | + | | | + | |
| Spinal cord: | Motor neurons | +[2] | | + | +[1] | +[1] | + | 66, 60, 13, 219 see also Figure 3A and B |
| Retina: | Photoreceptors | | | | | | + | 68, 220 |
| | Horizontal cells | | | | + | | + | |
| | Bipolar cells | | | | | | + | |
| | Amacrine cells | | | | | | + | |
| | Ganglion cells | | | | + | | + | |

[1]: Weakness of the signal, the complexity of neuronal networks, close opposition of neuronal and glial membranes, or neurons and synaptic inputs, made possible only provisional assignment of individual isoforms and cell types see reference 13. [2]: Noticeable non-uniformity of the signal within the population of neurons

until P22 (68). In rat cerebellum a strong increase in expression of Purkinje cell membrane $\alpha_3$ Na/K-ATPase is observed between 13 and 19 postnatal days (84). An interesting feature of rodent development is expression of $\alpha_3$ Na/K-ATPase mRNA and protein in the heart cells of newborn animals, which is replaced by the $\alpha_2$ isoform in P10 animals (82).

Thus, it appears that up-regulation and tissue- and cell type-specific arrangement of $\alpha_3$ Na/K-ATPase protein expression in neurons is a relatively late event on the developmental scale. This is supported by the observation made in cultures of mouse embryonic stem cells induced to differentiate into neurons. The expression of $\alpha_3$ Na/K-ATPase in these cells is prominent only after they have achieved full differentiation (85).

### 4.4. Conclusion

Phylogenetic preservation of $\alpha_3$ Na/K-ATPase and its tissue- and cell-specific expression across vertebrate species, early developmental shaping of its non-uniform expression, and early postnatal lethality of the $\alpha_3$ gene knockout, argues strongly that the existence of this isoform is not a mere result of redundancy of organization of active ionic transport in animal tissues. Alternatively, there may be some functional significance of the expression of this isoform in neurons, possibly associated on the one hand with specific kinetic or regulatory properties of $\alpha_3$ Na/K-ATPase, and on the other hand with the neuron-specific requirements for properties and regulation of the active transport of $Na^+$ and $K^+$ in these cells.

## 5. FUNCTIONAL IDENTIFICATION OF NEURONS WITH $\alpha_3$ Na/K-ATPase

One important implication of the hypothesis linking multiplicity and non-uniform expression of α isoforms of the Na/K-ATPase to neuronal function is that not only glial cells and neurons, but also functionally different groups of neurons should differ in expressed isoforms of the enzyme.

### 5.1. Central nervous system

Indeed, this implication has found strong support in results from a number of studies of vertebrate CNS. Application of both *in situ* hybridization and immunostaining techniques have clearly demonstrated that mRNA and proteins of α isoforms of the Na/K-ATPase, including $\alpha_3$ Na/K-ATPase, are non-uniformly distributed in various populations of CNS neurons (Table 4). However, the structural and functional complexity of CNS has made it almost impossible to identify neurons expressing a particular set of isoforms of the Na/K-ATPase.

Table 4 lists the groups of central neurons best characterized in terms of function and isoforms of the Na/K-ATPase. However, even for these cases several concerns remain. First, the level of mRNA is not always proportional to the respective protein level and therefore results of *in situ* hybridization experiments in general need to be supported by immuno-detection of protein. This has not been unequivocally demonstrated in all situations (e.g., cerebellar neurons, Table 4). Furthermore, most conclusions about protein expression of isoforms are generalizations of immunohistochemical observations of the staining of neuronal somata seen in histological sections. This ignores the potential problem of non-uniform expression of different isoforms of the Na/K-ATPase between different neuronal compartments, which has been addressed in several studies of isolated and cultured neurons. In cultures of rat telencephalic neurons, expression of $\alpha_1$, $\alpha_2$, and $\alpha_3$ Na/K-ATPase was detected with evidence of $\alpha_3$ targeting to the axonal compartment and $\alpha_1$ to dendrites (17). However, in hippocampal neurons expression of $\alpha_1$, $\alpha_2$, and $\alpha_3$ proteins exhibited no signs of compartmentalization (64, 15, 16). In all these studies, cells were isolated from neonate or early postnatal brains and cultured for one to several weeks before study. Therefore, the possible differences between neurons of developing and mature animals, and effects of the artificial culture conditions on the expression of the Na/K-ATPase isoforms should be kept in the mind. Thus, for example, both immunoblot (86) and immunochemical staining (87) of cultured rat cerebellar granule cells detected the presence of $\alpha_1$, $\alpha_2$, and

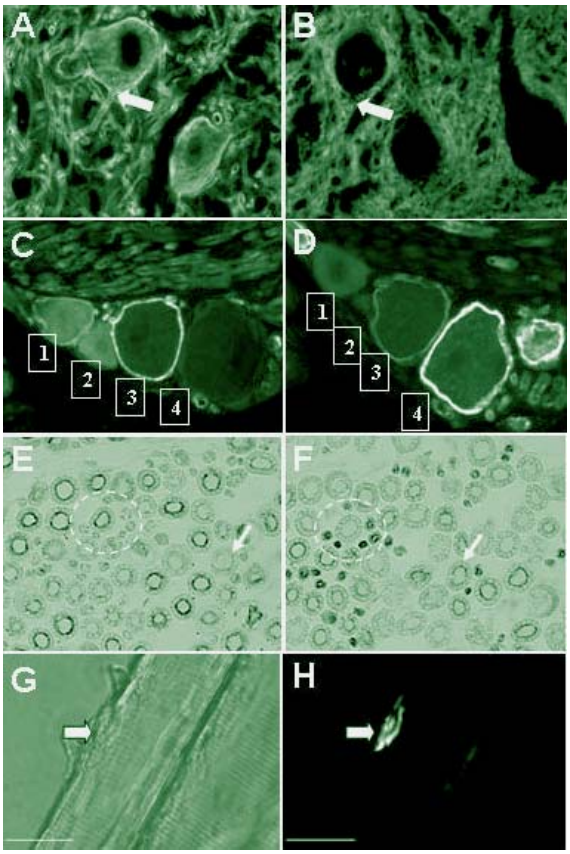


**Figure 3.** Examples of immuno-detection of $\alpha_1$ and $\alpha_3$ Na/K-ATPase in sections of rat paraffin-embedded spinal cord (A,B), DRG (C,D), ventral root (E,F), and in teased skeletal muscle preparation (G,H).In the rat spinal cord, antibodies specific for $\alpha_1$ Na/K-ATPase detect this isozyme in plasma membrane of large, presumably α, motoneurons and their processes (A, arrow). Antibodies specific for $\alpha_3$ Na/K-ATPase produce diffuse labeling that cannot be assigned to any specific cellular structure or plasma membrane of large motoneurons (B, arrow). Studies of parallel sections (C and D) from lumbar DRG demonstrate that some neurons express only $\alpha_1$ (neuron #1, and possibly #2), while other neurons express $\alpha_3$ or both $\alpha_1$ and $\alpha_3$ isoforms of the Na/K-ATPase (neurons #4 and #3, respectively). Only largest DRG neurons express $\alpha_3$ Na/K-ATPase, indicating that it is a marker of mechanoreceptive neurons. Evaluation of parallel sections of L5 ventral root (E and F) show, that small myelinated fibers express $\alpha_3$, while large myelinated fibers express either $\alpha_1$ or $\alpha_3$ Na/K-ATPase (large fiber within the dashed oval area and fiber indicated by arrow, respectively). Since rat ventral root contains only processes of motoneurons at studied spinal level, these data indicate that $\alpha_3$ Na/K-ATPase is a feature of γ-motoneurons (small myelinated fibers) and some of large motoneurons. In teased lower limb muscle preparations, occasionally, $\alpha_3$ Na/K-ATPase could be observed in structures morphologically resembling the motor nerve terminals on the extrafusal muscle fibers (arrows in G and H, phase contrast and fluorescent illumination, respectively). Since percentage of $\alpha_3$ Na/K-ATPase positive nerve endings could hint whether these are nerve endings of α- or β-motoneurons, this question was given more consideration (see next Figure). See (91, 18) for detailed description of methods and antibodies used.

$\alpha_3$ isoforms, while *in situ* immunostaining indicate strong $\alpha_1$ and light $\alpha_3$ immunoreactivity only (see Table 4). Finally, even at the level of the neuron somata, the close opposition of glial cells, terminals and processes of other CNS neurons makes only provisional assignment of a given isoform of the Na/K-ATPase to the plasma membrane of a given neuron possible (Table 4,; see also (13)).

**5.2. Peripheral nervous system**

The relative simplicity of peripheral nervous system of vertebrate animals makes it a natural alternative to CNS when attempting to identify neurons expressing different α isoforms of the Na/K-ATPase. Three major groups of peripheral motoneurons are located in the ventral horn of the spinal cord. Motoneurons with small somata and relatively thin myelinated processes, called γ-efferents, innervate muscle stretch receptors. Larger motoneurons send myelinated axons to innervate extrafusal skeletal muscle fibers, or both extrafusal and intrafusal muscle fibers of the stretch receptors (α and β motoneurons, respectively). Primary afferent neurons reside in dorsal root ganglia (DRG) and are comprised of large neurons with myelinated processes (various mechanoreceptors and cold receptors) and small neurons, most of which are pain and heat-receptors with thin unmyelinated processes (see (88, 89)).

The function and activity of these groups of peripheral neurons differ strongly in many aspects (90, 89, 20). Therefore, to suggest a close link between expression of α isoforms of Na/K-ATPase and neuronal function, implies that there are multiple isoforms expressed in the PNS and these isoforms are expressed non-uniformly. In support of this implication, both *in situ* hybridization and immunohistochemical studies have demonstrated that rat peripheral neurons may express $\alpha_1$ and $\alpha_3$, and expression of these isoforms of Na/K-ATPase is not uniform in peripheral nerves.

*In situ* hybridization detected mRNA of $\alpha_3$ Na/K-ATPase in all DRG neurons and $\alpha_1$ Na/K-ATPase in some lumbar rat DRG neurons (66). Labeling for $\alpha_1$ and $\alpha_3$ mRNA was also observed in another study for most but not all neurons of cervical DRG, and non-uniform expression of $\alpha_1$ and $\alpha_3$ protein subunits of the Na/K-ATPase in rat DRG was confirmed in immunohistochemical experiments (60, 91). In rat sciatic nerve Western blot detected $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$ and $\alpha_3$ Na/K-ATPase (92, 93, 67). Histological experiments have shown $\alpha_1$ expression in some axons and Schwann cells, $\alpha_3$ expression in axons, and $\alpha_2$ and $\beta_2$ expression in Schwann cells only (67). The $\alpha_3$ Na/K-ATPase was found also in presynaptic terminals in rat skeletal muscles and in a sub-population of the largest DRG neurons (71, 91). It has been shown that large motor neurons (α or β, or both) and some mechanoreceptive neurons are $\alpha_3$ Na/K-ATPase-positive (Figure 3 C-H, and (71, 91)). Otherwise, no information identifying neurons or axons expressing either $\alpha_1$ or $\alpha_3$ Na/K-ATPase has been derived from these studies.

Recently, we have identified two groups of neurons, primary muscle spindle type I afferent neurons

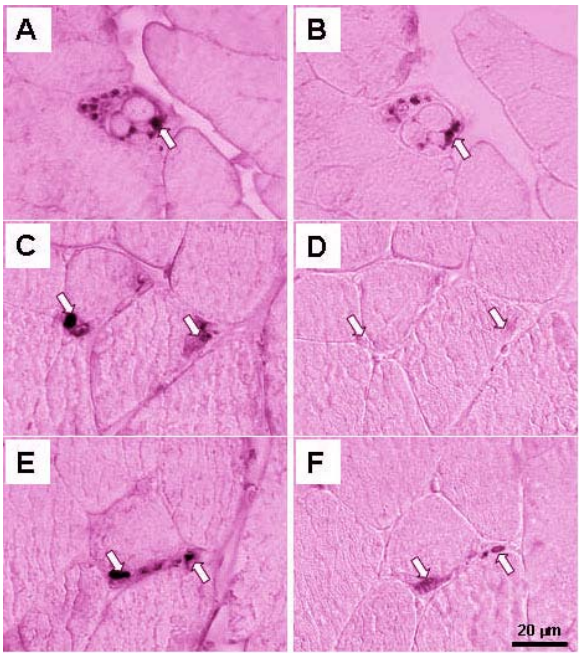


**Figure 4.** Expression of $\alpha_3$ Na/K-ATPase in rat *extensor hallicus longus* muscle. All images shown demonstrate fields captured in one experiment with two parallel sections labeled with antibody to 200 kDa neurofilaments to detect myelinated axons (A, C and E) or with antibody for $\alpha_3$ Na/K-ATPase (B, D and F). In the skeletal muscle $\alpha_3$ Na/K-ATPase labels nerve terminals on intrafusal fibers in the muscle spindles (A and B, arrows; see also (18)). Axonal profiles identified in neurofilament-stained sections and profiles resembling nerve endings on extrafusal muscle fibers may or may not have detectable $\alpha_3$ Na/K-ATPase (E and F, and C and D, respectively; arrows). Overall 31 nerve endings were identified in five pairs of sections studied in this experiment (unpublished data), and $\alpha_3$ Na/K-ATPase was detected in nerve terminals in 17 ± 6% of synapses. We have previously shown that 14% of large myelinated ventral root axons express $\alpha_3$ Na/K-ATPase (18). Both of these numbers are well within the range of relative number of β motoneurons innervating both intrafusal and extrafusal fibers of the rat hind limb skeletal muscles (0 to 40%, (227, 228)). Thus, these data provide further support to the suggestion that expression of $\alpha_3$ Na/K-ATPase in rat PNS is determined by the peripheral target (intrafusal fibers) of the neuron.

and γ-efferents, as $\alpha_3$ Na/K-ATPase-positive (18). In general there is strong evidence that all peripheral neurons that contact muscle spindle (above indicated groups as well as secondary afferents and β-motoneurons), and only those, express $\alpha_3$ Na/K-ATPase (Figures 3, 4 and 5). All of the other numerous somatic neurons, α-motoneurons and neurons innervating skin, joint, and ligaments appear to have very little or no $\alpha_3$ Na/K-ATPase. There is also no evidence that any non-myelinated peripheral nerves of rat express $\alpha_3$ Na/K-ATPase, suggesting that sympathetic and parasympathetic neurons do not express this pump (91), which corroborates previously published data of superior cervical ganglia neurons expressing only the $\alpha_1$ isoform (see (83)).

### 5.3. Stretch receptor

Stretch receptors, or muscle spindles, are slowly adapting mechanoreceptors that transmit to the central nervous system information about skeletal muscle length and the velocity of length changes (reviewed in (19, 20)). Muscle spindles first appear on the phylogenetic scale in amphibians, suggesting that this receptor evolved in association with the transition of vertebrate animals to terrestrial life. Thereafter, the basic structure and functional characteristics of muscle spindles appear to be well preserved among different species of vertebrates, including humans (20, 94, 95, 96).

The receptor consists of a spindle-shaped connective tissue capsule surrounding several (usually four to ten) thin muscle fibers, which are called "intrafusal fibers" to distinguish them from regular, "extrafusal" skeletal muscle fibers (Figure 5B). In the equatorial and juxta-equatorial regions of the stretch receptor capsule, intrafusal fibers are innervated by type Ia and type II stretch receptor afferents, respectively. In the polar regions, the intrafusal fibers receive their efferent inputs from either γ-motoneurons, which are spindle-specific efferent neurons, or β-motoneurons which innervate both intrafusal and extrafusal skeletal muscle fibers.

Within the muscle, the stretch receptors are located in parallel with extrafusal fibers in a way that their intrafusal fibers are always stretched in proportion to the muscle length. This stretch is "sensed" by terminals of spindle afferents, encoded into the frequency of afferent discharge, and conveyed to the central nervous system (CNS). There are two components of type Ia spindle afferent discharge: dynamic and tonic. The dynamic component encodes the velocity of the muscle length changes, while the tonic component encodes the static muscle length. Unlike type Ia afferents, type II spindle afferents discharge only tonically and report only the static muscle length. Efferent innervation of intrafusal fibers serves to prevent slacking of intrafusal fibers and the loss of receptor sensitivity under conditions of strong and maintained contraction of the muscle.

Type Ia spindle afferents and their monosynaptic connections to α-motoneurons innervating homonymous muscle constitute the afferent limb of the tendon reflex pathway (Figure 5B). Type II afferents do not have terminations on motoneurons, but establish numerous contacts with the spinal cord interneurons. These connections, together with analogous "type Ia afferent - spinal interneuron" connections, direct sensory inputs from stretch receptors of different muscles for the segmental and supra-segmental integration required for proper control of body posture, and execution of rhythmic and fine voluntary movements (97, 98, 99, 100, 101, 102).

### 5.4. Conclusion

Functional identification and comparison of physiological characteristic of neurons expressing different

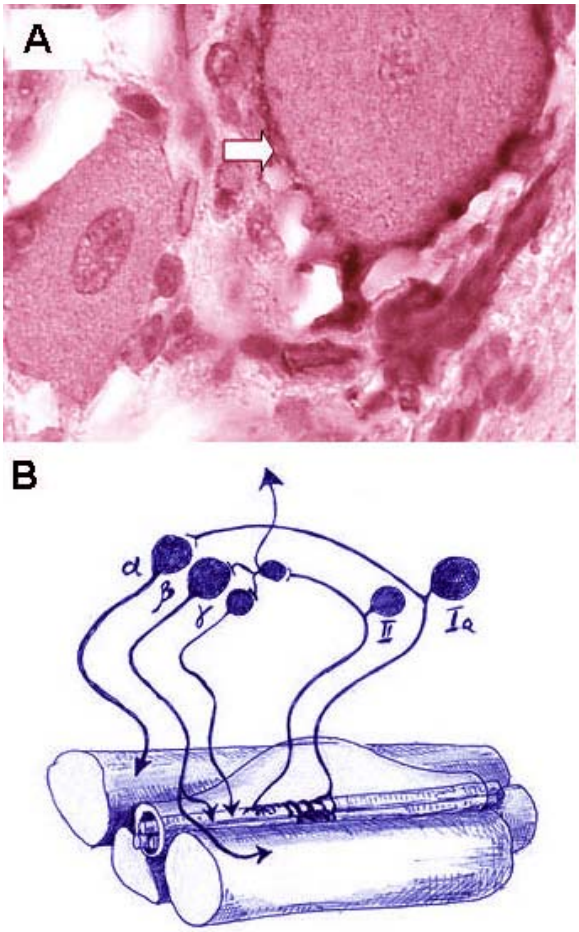


**Figure 5.** Expression of $\alpha_3$ Na/K-ATPase in human DRG (A) and schematic representation of innervation of the stretch receptor and stretch reflex loop (B). Similar to that observed for rat and guinea pig (91, 18), expression of $\alpha_3$ Na/K-ATPase in human DRG is not uniform, and confined to a sub-population of large neurons (A). This, and detection of $\alpha_3$ Na/K-ATPase in some large frog DRG neurons, and in axonal terminals on intrafusal fibers of human muscle spindles (unpublished observations), suggest that stretch receptor (B) -associated expression of $\alpha_3$ Na/K-ATPase is a feature of PNS that is preserved across different vertebrate species. Furthermore, our data suggest that all neurons that contact intrafusal fibers of the muscle spindle (type Ia and II afferent neurons, and γ and β motoneurons, but not α-motoneurons; see this figure panel B) express $\alpha_3$ Na/K-ATPase, suggesting target-determined regulation of this isoform.

sets of isoforms of the Na/K-ATPase is an important step in understanding the specific roles these isoforms may play in neurons. The complexity of central nervous system prevents reliable conclusions on expression of isoforms of Na/K-ATPase in central neurons. The studies in structurally and functionally simpler peripheral nervous system provide a useful alternative to CNS in this respect.

## 6. HYPOTHESES ON FUNCTIONAL ROLE OF NEURON-SPECIFIC EXPRESSION OF THE $\alpha_3$ Na/K-ATPase

Since the discovery of nonuniform expression of Na/K-ATPase isoforms, the search for a neuron-specific role of $\alpha_3$ Na/K-ATPase has led to multiple hypotheses that broadly can be separated into four categories according to whether they are focused on the Na/K-ATPase kinetics, its regulation by endogenous factors, cellular compartmentalization or regulation of gene transcription.

### 6.1. $\alpha_3$ Na/K-ATPase as a "reserve" pump

Hypothesis: With small variations, this is probably the most frequently mentioned hypothesis in the relevant scientific literature. The essence of the hypothesis is the suggestion that in neurons, like in any other animal cell, the role of "housekeeping" transporter, maintaining background ionic homeostasis, is played by $\alpha_1$ Na/K-ATPase, while the $\alpha_3$ Na/K-ATPase serves as a "reserve" transporter activated only when $Na^+$ concentration is high, such as following repeated action potentials (103, 104, 105, 106, 107, 108, 109). This hypothesis is attractive because the kinetic differences between isoforms of the Na/K-ATPase are used to explain several different facts and observations relevant to specific requirements of neurons for active transport of $Na^+$ and expression of $\alpha_3$ Na/K-ATPase.

Multiple studies of the properties of isoforms of Na/K-ATPase expressed in several different cell systems have suggested the existence of several important kinetic differences between $\alpha_3$ and $\alpha_1$ and $\alpha_2$ isoforms of the enzyme. First, and the most consistently reported feature of $\alpha_3$ Na/K-ATPase, is its relative insensitivity to activation by $Na^+$. While $\alpha_1$ and $\alpha_2$ isozymes are half active at 11 ± 2 and 14 ± 3 mM $Na^+$, the half activation of $\alpha_3$ Na/K-ATPase requires 28 ± 8 mM $Na^+$ (mean values of half activation constants from cited works are given (104, 105, 106, 110, 107, 108)). In addition, studies of voltage dependences of human or rat Na/K-ATPase expressed in frog oocytes show that in both species the rate of $\alpha_3$ Na/K-ATPase, unlike that of $\alpha_1$ or $\alpha_2$ enzymes, is almost independent of voltage (108, 111). Shallow voltage dependence of the pump generated current was also described for rat auditory thalamus cells that express $\alpha_3$ Na/K-ATPase and no detectable $\alpha_1$ or $\alpha_2$ isoforms (112, 39). Also, in two studies a higher ATP affinity of $\alpha_3$ Na/K-ATPase was found; about 400 μM ATP was required for half-activation of $\alpha_1$ Na/K-ATPase, while only 90 to 210 μM was required for $\alpha_3$ Na/K-ATPase (104, 105). These measurements corroborate the early findings that the range of sensitivity to ATP of rat kidney Na/K-ATPase is 200 to 400 μM (depending on concentration of K in the assay), and it is 100 to 200 μM for the enzyme isolated from axolemma (113). Finally, no consistent differences in $K^+$ affinity of different isoforms were detected with half activation for each isoform being: 0.9 ± 0.2, 2.2 ± 1.0, and 1.4 ± 0.7 mM ($\alpha_1$, $\alpha_2$, and $\alpha_3$ Na/K-ATPase, respectively (104, 106, 110, 105, 114, 111, 108)).

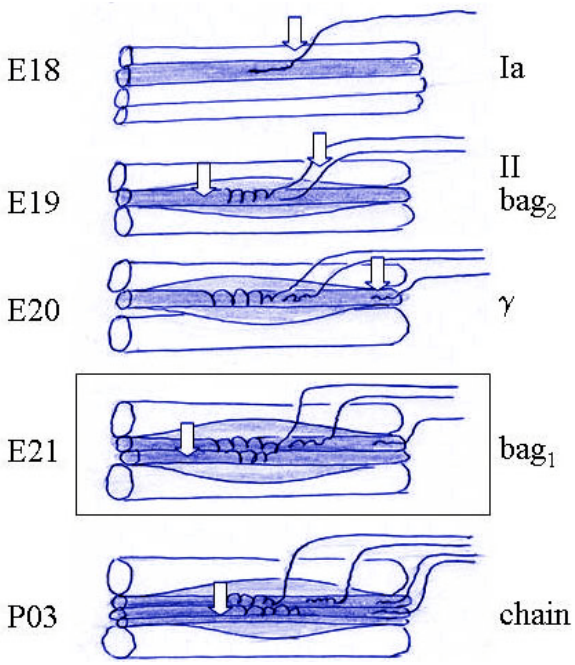


**Figure 6.** Development and innervation of rat hind limb muscle stretch receptor (based on (229)). Development and innervation of the rat stretch receptor occurs in several sequential steps. At embryonic day 18 (E18) first Ia afferents arrive at the rat lower hind limb muscles and establish contacts with as yet undifferentiated muscle fibers. About one day after this original contact, the first intrafusal fiber of the spindle (bag2) is formed, type II afferents start to arrive and the spindle capsule starts to develop. The first γ-efferents innervate the spindle on E20, and the second intrafusal fiber to be formed on E21 is bag1 fiber. By the end of first postnatal week muscle spindles have full complement of intrafusal fibers and all morphological features of adult spindles. Our unpublished observations demonstrate that first lumbar DRG neurons with $\alpha_3$ Na/K-ATPase detectable in their plasma membrane appear at E21 (boxed in figure). By first postnatal day DRG have an adult complement (10 - 15%) of $\alpha_3$ Na/K-ATPase expressing neurons.

Review of these findings suggests that different isoforms of Na/K-ATPase may be complementary to each other in terms of active ion transport in neurons. Thus, $\alpha_1$ Na/K-ATPase, which has $K_{0.5}$ of activation by Na of about 10 mM, appears to be well suited for control of intracellular $Na^+$ in resting neurons (10 mM, see Table 1). The steep voltage dependence of its transport rate also allows the $\alpha_1$ isoform to respond with fast activation during short bursts of AP associated with depolarization and an entry of additional $Na^+$ through the voltage-dependent channels. However, in most neurons a burst of AP and particularly prolonged and high frequency neuronal discharge is immediately followed by post-tetanic hyperpolarization (PTH). During PTH the intracellular $Na^+$ is still above normal, but because of voltage-dependence the capacity of $\alpha_1$ Na/K-ATPase to expel this $Na^+$ may be severely limited. Theoretically, the $\alpha_3$ Na/K-ATPase that has low affinity and shallow voltage dependence is an ideal transporter to control intracellular $Na^+$ under these conditions (Figure 7A and B). Furthermore, relatively high ATP affinity of $\alpha_3$ Na/K-ATPase may be an additional advantage ensuring the activity of this isoform even under conditions when ATP reserves may be depleted as a result of prior neuronal firing and continuous work of $\alpha_1$ Na/K-ATPase. It can be argued that many problems associated with the control of intracellular Na in highly active neurons could be resolved just by increasing the density of $\alpha_1$ pump sites in the cell plasma membrane. However, taking into consideration the role of electrogenic Na/K-ATPase in frequency accommodation and PTH suggests that increasing density of Na pump sites sensitive to intracellular Na is not an option for neurons that code their messages in high frequency and continuous series of AP. The neurons abundantly expressing Na/K-ATPase with high affinity to Na are unlikely to be capable of maintaining a prolonged high-frequency discharge, because of activation of electrogenic pump and development of PTH in the very beginning of neuronal firing. This "negative" effect of early activation of electrogenic pump may be avoided by increasing the membrane conductance to "shunt" the pump-generated current or having expressed the Na/K-ATPase with low affinity to Na and tolerating increased intracellular Na (Figure 7C).

The data and theoretical considerations suggest the $\alpha_1$ Na/K-ATPase is suitable for controlling intracellular $Na^+$ under resting conditions. These conditions are not likely to be very different in neurons and cells of other types, which explains ubiquitous expression of $\alpha_1$ Na/K-ATPase throughout the tissues and cells of animals. This role also correlates with the finding that in cultured rat cerebellar granule neurons under basal conditions, almost 90% of active transport is carried by the Na/K-ATPase with low affinity to ouabain (presumably $\alpha_1$), and Na/K-ATPase with high affinity to ouabain does behave like a "reserve" isoform being substantially activated (40% of total activity) only after cell stimulation with glutamate (87, 115).

View of the hypothesis from the PNS perspective: The hypothesis discussed above predicts that among different neurons only those featuring high frequency and prolonged discharges are likely to express $\alpha_3$ Na/K-ATPase. This prediction is difficult to verify in CNS; however, it does seem to be very compatible with data on stretch receptor-associated distribution of $\alpha_3$ Na/K-ATPase in rat (Figures 3 and 5; (91, 18)). One outstanding and common functional characteristic of γ-efferents and stretch receptor afferents expressing $\alpha_3$ Na/K-ATPase is a capacity of these neurons to generate a relatively long, slowly adapting series of high-frequency action potentials. In response to tonic stretch, cat and rat type Ia and type II spindle afferents may discharge impulses with a frequency of 25 to 100 AP/sec for at least several seconds (116, 117, 118, 119). Cat γ efferents may also discharge at a rate of 30 to 50 AP/s for up to 1 second during animal locomotion with little frequency adaptation (120, 121). Skin Pacinian corpuscle has comparable or higher rates of AP; however,

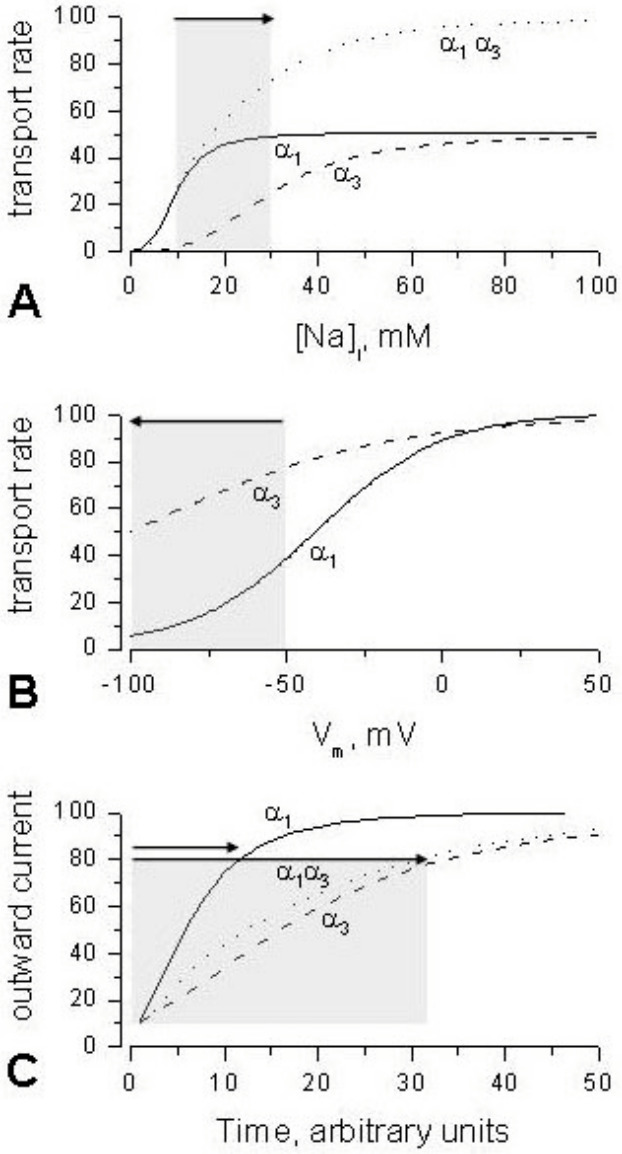


**Figure 7.** Functional significance of putative differences between $\alpha_1$ and $\alpha_3$ Na/K-ATPase. (A) Low intracellular $Na^+$ affinity of $\alpha_3$ Na/K-ATPase: A hypothetical neuron expresses $\alpha_1$ and $\alpha_3$ Na/K-ATPases at a ratio of 1:1. Activation of Na/K-ATPase by $Na^+$ is described by the Hill equation with a Hill Coefficient equal to 3 to account for cooperative binding of three $Na^+$ ions, and $K_{0.5}$ of activation equal to 10 mM for $\alpha_1$ Na/K-ATPase and 30 mM for $\alpha_3$ Na/K-ATPase (solid and dashed lines, respectively). The activation by Na of total neuronal Na/K-ATPase (both $\alpha_1$ and $\alpha_3$ isoforms) is shown by dotted line. Comparison of calculated activation curves shows that active transport in neurons expressing isoforms of the Na/K-ATPase with different affinities to intracellular $Na^+$ can respond to a wider range of intracellular $Na^+$ changes (shaded area and arrow). (B) Shallow voltage-dependence of $\alpha_3$ Na/K-ATPase: A regular, featuring the cardiac and glial cell $\alpha_1$ Na/K-ATPase dependence (see (230, 231)) of the transport rate on membrane voltage is shown by the solid sigmoidal line. A hypothetical voltage-dependent transport curve resembling that described for rat $\alpha_3$ Na/K-ATPase expressed in frog oocytes (232) is depicted by the dashed line. Comparison of these curves shows that hyperpolarization from -50 to -100 mV that may be expected to follow neuronal high frequency discharge (shaded area and arrow) will suppress activity of $\alpha_1$ Na/K-ATPase, while having only minor effect on $\alpha_3$ Na/K-ATPase. (C) Development of electrogenic effect of the Na/K-ATPase: It can be calculated that under conditions of continuous firing and steady accumulation of intracellular $Na^+$, the activity of Na/K-ATPase and associated outward current will reach a peak earlier after the beginning of discharge in a neuron expressing $\alpha_1$ Na/K-ATPase as compared to a neuron expressing $\alpha_3$ Na/K-ATPase only, or both $\alpha_1$ and $\alpha_3$ pumps at 1:1 ratio (solid, dashed, or dotted lines, respectively; sodium-dependencies of isoforms of Na/K-ATPase were as used in panel A of this figure).

this receptor is rapidly adapting, displaying much shorter bursts of AP than spindle receptor. Discharge of skin slowly-adapting mechanoreceptors generally never exceeds 20 Hz, and both types of receptors apparently express $\alpha_1$ Na/K-ATPase (18, 122). Therefore, the pattern of expression of $\alpha_3$ Na/K-ATPase in PNS seems to fit the hypothesis that this isoform of the Na/K-ATPase works under conditions of strong $Na^+$ accumulation and ensures the capacity of neurons to maintain tonic, high-frequency activity. Indeed, electrogenic activity of the Na/K-ATPase is an important determinant of the frequency discharge parameters in lobster, crayfish, and frog stretch receptors (123, 124, 125, 126, 127). Inhibition of the Na/K-ATPase with cardiac glycosides does not immediately affect receptor potential or dynamic discharge, but it does inhibit tonic discharge of cat stretch receptors (128, 129, 130). During rat development, tonic discharge of muscle afferent could be evoked only after birth and according to our unpublished observations, only after $\alpha_3$ Na/K-ATPase was up-regulated in DRG neurons (Figure 6 and (131,132)). Finally, if $\alpha_3$ Na/K-ATPase is an obligatory requirement of the neuronal capacity to sustain tonic high-frequency activity, then the isoform should be expressed throughout the site of generation of the frequency- and duration-encoded message to the site of destination of this message. Our data on expression of $\alpha_3$ Na/K-ATPase in the body, peripheral and central processes and peripheral terminals of spindle afferents are in agreement with this supposition (18).

Difficulties and unresolved questions: Even though the hypothesis of a "reserve" role of the $\alpha_3$ Na/K-ATPase explains many observations, it has several problems that if not resolved may either dismiss the hypothesis or call for a serious modification of it. First, the backbone of the hypothesis, the observation of relatively low affinity of $\alpha_3$ Na/K-ATPase to $Na^+$, remains a controversial issue. Unlike that for $\alpha_1$ Na/K-ATPase, which is the only α isoform in kidney and the major isoform in the adult rat heart, there are no natural sources of pure $\alpha_2$ or $\alpha_3$ Na/K-ATPases (8). Therefore, most of the data on kinetic properties of $\alpha_2$ and $\alpha_3$ Na/K-ATPase is from experiments in transfected cell systems. Even though most of these studies support the difference in Na affinity of $\alpha_3$ and $\alpha_2$ or $\alpha_1$ isoforms, no such differences were found for rat Na/K-ATPase isoforms expressed in frog oocytes or yeast cells (104, 105, 106, 107, 108, 110, 114, 111). Furthermore, the general problem of transfection system approach is the assumption that changes in membrane environment and possible relationships with cellular proteins will not affect the function of isoforms of expressed protein or will affect the function of each isoform similarly. However, it was noted that when kidney enzyme is expressed in Sf-9 insect cells its apparent affinities for $K^+$ decrease and affinities for $Na^+$ and ATP increase (133). Furthermore, a number of observations indicate that changes in lipid environment and/or in some cell-specific membrane-bound or cytosolic factors may in fact have isoform-specific effects on kinetic characteristics of the Na/K-ATPase, including the enzyme affinity to $Na^+$ (110, 134, 133, 135, 103, 136, 137). Thus, studies of α Na/K-ATPases in natural host cells are imperative for further progress in understanding the functional roles that these isozymes may play.

In patch-clamp studies of rat auditory thalamus neurons expressing $\alpha_3$ Na/K-ATPase or horizontal cells from carp retina, expressing $\alpha_1$ and $\alpha_3$ Na/K-ATPase the $Na^+$ dependence of measured currents was not evaluated. In our patch-clamp studies of rat DRG neurons, we did not find components of pump current with high affinity to ouabain that could be attributed to $\alpha_3$ Na/K-ATPase (39, 112, 138, 139). However, in a recent patch-clamp study of small DRG neurons two components of the Na/K-ATPase-generated current were identified. The major component (93% of total current) had low affinity to ouabain and apparent $K_{0.5}$ of activation by $Na^+$ of about 7 mM, while the smaller component was inhibited by micromolar concentrations of ouabain and activated by $Na^+$ with apparent $K_{0.5}$ of 68 mM (140). If confirmed, this would be the first data from a system more closely approaching the natural situation supporting observations made previously in transfected cells.

Another aspect of the hypothesis that the $\alpha_3$ Na/K-ATPase acts as a "reserve" transporter requiring more study is a definitive assignment of the role of "housekeeping" transporter to the $\alpha_1$ Na/K-ATPase. This assignment assumes that $\alpha_1$ Na/K-ATPase should be present in all neurons, regardless of whether they express $\alpha_3$ Na/K-ATPase. However, no $\alpha_1$ or $\alpha_2$ isoforms of Na/K-ATPase were found in $\alpha_3$ Na/K-ATPase positive neurons of medial geniculate body (112). Also, we have previously demonstrated by immunohistochemistry that at least 30% of large DRG neurons expressing $\alpha_3$ Na/K-ATPase do not have detectable $\alpha_1$ Na/K-ATPase ((91) and Figure 3C and D). These data suggest that there are neurons in which the $\alpha_3$ Na/K-ATPase serves both housekeeping and "reserve" roles. However, it is possible that the $\alpha_1$ Na/K-ATPase is present in these neurons, but at a level below the detection limits of immunohistochemistry. Thus, while immunohistochemistry does not reveal $\alpha_3$ Na/K-ATPase in small DRG neurons, patch-clamp recordings suggest it represents about 10% of pump sites present in these neurons (18, 91, 140). However, it is difficult to imagine that if the $\alpha_1$ Na/K-ATPase is responsible for background ionic homeostasis in both large and small neurons, it would be detectable in small neurons but would have site density below detection in large neurons.

It has been shown that HeLa cells transfected with rat $\alpha_3$ Na/K-ATPase maintain intracellular Na concentrations in the range of 28 to 50 mM (106, 107). Therefore, it can be suggested that muscle spindle neurons and γ-motoneurons may tolerate high intracellular Na and that the $\alpha_3$ Na/K-ATPase is sufficient to meet the housekeeping needs of these neurons for Na transport. This suggestion is not consistent with the observation that an average of 10 mM Na was measured in dissociated mouse DRG neurons (141). However, the size of studied neurons

was not specified and if there were any sub-populations of neurons with high intracellular sodium this was not indicated in this work. Evaluation of indirect signs of increased intracellular Na and decreased Na gradient, duration and amplitude of AP, also does not provide an unequivocal answer. Muscle spindle afferents have shorter AP than morphologically similar, but presumably expressing $\alpha_1$ Na/K-ATPase, large myelinated skin afferents (low threshold mechanoreceptors; LTM), arguing against decreased Na gradient in the former group of neurons (142, 143). Also, amplitude of AP was not different between LTM and muscle spindle afferents with the soma size > 40 μm in diameter, 57 ± 3 mV and 54 ± 3 mV, respectively. However, the AP of spindle afferents is smaller than that of LTM (51 ± 2 and 65 ± 2 mV, respectively) when medium size neurons are compared (31 to 45 μm in diameter; (143)).

One final but important question relevant to this discussion is the possibility suggested by Gerbi and co-authors that the $\alpha_3$ isoform of the Na/K-ATPase may change between states with low and high affinity for Na in response to some unknown trigger (144).

### 6.2. Sub-cellular targeting and compartmentalization of $\alpha_3$ Na/K-ATPase

Hypothesis 1: Compared to other cells an outstanding feature of most neurons is the existence of two morphologically and functionally different compartments: axonal and dendritic. Dendrites collect and integrate the input information. The axon conducts and transmits the signal to a postsynaptic cell. The complement of ion channels and electrical activity may differ sharply between neuronal axons and dendrites (145). Therefore, the idea of non-uniform distribution of isoforms of Na/K-ATPase between neuronal compartments could explain the need for multiple isoforms. The original hypothesis that $\alpha_3$ Na/K-ATPase may exist to be directed into the axons to work at distant synaptic membranes was inspired by observations of high levels of expression of this isoform in large projection neurons of CNS (14, 13).

In studies of axonal transport of Na/K-ATPase in the rat optic nerve, the $\alpha_2$ and $\alpha_3$, but not $\alpha_1$, enzymes were the predominant isoforms transported from retinal ganglion cells with fast axonal transport (>240 mm/day; (146)). This could ensure the delivery of $\alpha_3$ Na/K-ATPase down the long axon of projection neurons. Furthermore, evidence of selective $\alpha_3$ and $\alpha_1$ Na/K-ATPase targeting to axonal and dendritic compartments was obtained in cultures of rat telencephalic neurons (17).

However, both of these supporting evidences were questioned by later experiments. Western blot analysis of accumulation of isoforms of the Na/K-ATPase at rat sciatic nerve ligature, detected only $\alpha_1$ Na/K-ATPase as carried by fast axonal transport. No changes in $\alpha_2$ or $\alpha_3$ isoforms either proximally or distally to ligature was observed after one day of experiment (93). Also, no signs of axon/dendrite selectivity of $\alpha_1$, $\alpha_2$, or $\alpha_3$ proteins of the Na/K-ATPase were seen in experiments with cultured hippocampal neurons (64, 15, 16). It may be argued that "axons" and "dendrites" of cultured neurons might be very different from those *in vivo*. However, analysis of expression of $\alpha_3$ Na/K-ATPase in adult rat PNS also does not support the idea of $\alpha_3$ Na/K-ATPase as an "axonal" isoform of the enzyme. Both large α-motoneurons and small γ-motoneurons have axons of similar length, but only processes of γ-motoneurons express $\alpha_3$ Na/K-ATPase (18).

Hypothesis 2: The idea of compartmentalization of $\alpha_3$ Na/K-ATPase was revived recently by the observation of apparent "microdomains" in distribution of this isoform of the transporter in the membrane of cultured neurons (64). The image analysis of plasma membrane of hippocampal neurons labeled for isoforms of the Na/K-ATPase demonstrated diffuse distribution of $\alpha_1$ Na/K-ATPase, but expression of $\alpha_3$ Na/K-ATPase was localized to underlying endoplasmic reticulum. It was suggested that while $\alpha_1$ Na/K-ATPase controls global intracellular Na, the $\alpha_3$ Na/K-ATPase controls Na in small cytoplasmic domains of the plasma membrane and cisterns of ER, where it is co-localized with $Na/Ca^{2+}$ exchanger. This arrangement suggests that $\alpha_3$ Na/K-ATPase is involved in intracellular Ca homeostasis in neurons, and perhaps its regulation by endogenous ouabain-like compounds (OLC) (64, 147). Some problems associated with a regulatory role of OLCs will be discussed in the next section of this review. Further studies are required to fully evaluate other aspects of this interesting hypothesis. One question that appears to be difficult to answer within the framework of this hypothesis is how to accommodate the abundant expression of $\alpha_3$ Na/K-ATPase in stretch receptor neurons and the relatively minor role of Ca currents in their AP and probably very moderate Ca homeostasis (148).

### 6.3. $\alpha_3$ Na/K-ATPase as a receptor for endogenous compounds

It was realized long ago that regardless of the existence of kinetic differences between isoforms of the Na/K-ATPase the difference of these isozymes in their sensitivity to regulation by endogenous factors may, on its own, be physiologically important by rendering active transport in certain cells specifically sensitive to regulatory influences (1, 113). Regulation of neuronal $\alpha_3$ Na/K-ATPase may occur by endogenous ouabain-like compounds (OLC) and neuromediators.

Endogenous OLC: Differential sensitivity to the inhibition by ouabain and other cardiac glycosides is one of the earliest known differences of α isoforms of the Na/K-ATPase. It is particularly marked for rodent Na/K-ATPase, in which $\alpha_1$ isoform is least, and $\alpha_3$ is most sensitive to ouabain (ranges of half-inhibiting $\alpha_1$, $\alpha_2$, and $\alpha_3$ isoform of rat enzyme ouabain concentrations are 50 to 450 μM, 60 to 400 nM and 10 to 200 nM, respectively; (149, 150)). Since cardiac glycosides were widely used clinically to treat heart failure this phenomenon has been an object of interest to basic and clinical science. Once OLCs were detected in circulating plasma and various mammalian organs, the differential sensitivity of the pump isoforms to cardiac glycosides became important as a possible explanation of the functional role of ouabain-sensitive isoforms of the enzyme (151, 152, 153)

**Table 5.** Concentration of endogenous ouabain and marinobufagenin in the blood plasma and cerebro-spinal fluid CFS

| Endogenous OLC | Specie/sample | Conditions | Concentration, nM | Reference |
|---|---|---|---|---|
| Endogenous ouabain | Human blood plasma | Control | 0.44 ± 0.20 | 221 |
| | | Heart failure | 1.59 ± 2.2 | |
| Endogenous ouabain | Rat blood plasma | Control | 0.24 ± 0.05 | 222 |
| | | Sympathoectomia | 0.07 ± 0.02 | |
| Endogenous ouabain | Rat blood plasma | Control | 0.74 ± 0.06 | 223 |
| | | Heart failure | 1.50 ± 0.07 | |
| | Hamster, blood plasma | Control | 0.50 ± 0.02 | |
| | | Heart failure | 0.90 ± 0.03 | |
| Endogenous ouabain | Rat blood plasma | Control | 0.1 | 154 |
| | Rat CSF | Control | 0.6 ± 0.2 | |
| Marinobufogenin | Rat blood plasma | Control | 0.2 to 0.5 | |
| | Rat CSF | Control | Below detection | |

However, it remains to be answered if $\alpha_2$ or $\alpha_3$ Na/K-ATPase expressing tissues are specific targets for regulation by endogenous OLCs and what is the physiological significance of this regulation. As of today, two endogenous OLC compounds, endogenous ouabain (EO) and endogenous bufadienolide, marinobufagenin (MBG) are the best-characterized OLC. Unlike the plant-derived ouabain, MBG has a higher affinity to $\alpha_1$ as compared to $\alpha_3$ Na/K-ATPase ($K_{0.5}$ = 2.1 nM vs. 140 nM (154)). Therefore, its functional role may be to regulate $\alpha_1$ Na/K-ATPase - mediated active transport. The $\alpha_3$ Na/K-ATPase, which is the most ouabain-sensitive isoform in the rat, could potentially be a receptor for EO. The functional significance of such regulation if one exists is not clear. In addition, there are several difficulties with this hypothesis that are immediately obvious.

First, comparison of $K_{0.5}$ of $\alpha_3$ Na/K-ATPase inhibition by ouabain and measured levels of circulating EO (10 to 200 nM vs. 0.1 – 2 nM; see Table 5 and text above) suggest that either the affinity of $\alpha_3$ Na/K-ATPase to circulating OLC is higher than to ouabain, or $\alpha_3$ Na/K-ATPase is not a receptor for OLC. It was demonstrated that OLC extracted from the hypothalamus and adrenals of oxen and rats acts on Na/K-ATPase in a similar, but not identical manner to ouabain. Compared to ouabain, OLC has greater capacity to inhibit low affinity enzyme. Furthermore, OLC was more effective inhibiting the enzyme isolated from adult rather than young rats (155). Thus, simple application of the knowledge gained in experiments with ouabain is not appropriate for the OLC cases.

Second, as discussed in Chapter 3, the amino acid sequences for α isoforms of Na/K-ATPase and the tissue- and cell-specific distribution of these isoforms are phylogenetically preserved, implying a common function for each isoform across species. This, however, does not seem to apply to the segment of the sequence responsible for sensitivity of the α isoforms of Na/K-ATPase to cardiac glycosides (Figure 2). Rat, mouse and toad have ouabain-resistant $\alpha_1$ Na/K-ATPase, while $\alpha_1$ Na/K-ATPase of sheep, bovine, human, dog, rabbit, pig, guinea pig, frog and chicken is relatively sensitive to ouabain (83, 156). Furthermore, $\alpha_1$, $\alpha_2$ and $\alpha_3$ human Na/K-ATPase are all sensitive to ouabain within the same concentration range (5 – 35 nM (74, 114)). Again, comparative studies of effects of endogenous OLCs in different species, but not ouabain, are required to address the issue properly.

Finally, the heart $\alpha_2$ Na/K-ATPase is considered to be a receptor for inotropic effect of therapeutic doses of cardiac glycosides and as a potential target for regulation by endogenous compounds. However, the homozygous offspring of mice with cardiac glycoside insensitive $\alpha_2$ Na/K-ATPase are born with no detectable inotropic effect of ouabain, but also without any noticeable abnormality in behavior, postnatal growth and development (157). From these experiments, it is obvious that if OLC regulation of $\alpha_2$ Na/K-ATPase does exist, it is not functionally important in mice over the entire range of embryonic and postnatal development, and maturation. What possible function such regulation could have remains to be elucidated.

Neurotransmitters: Neurons use a variety of synaptic neurotransmitters to communicate signals to each other. After being released, many excitatory neurotransmitters such as glutamate, serotonin, and norepinephrine are removed from the synaptic clefts by Na-coupled uptake mechanisms (158, 89). Thus, coupling of active transport of $Na^+$ by activation of a specific isoform of the Na/K-ATPase to a neurotransmitter could ensure that the strong chemical gradient of $Na^+$ required for efficient reuptake of the neurotransmitter is maintained.

The ability of glutamate to selectively activate $\alpha_2$ or $\alpha_2$ and $\alpha_3$ Na/K-ATPases by 25 - 40% was demonstrated for cerebellar and cerebral neurons and astrocytes (115, 159, 160, 161). Also, there is evidence of selective dopamine-induced inhibition of rat photoreceptor $\alpha_3$ Na/K-ATPase by 50% (162) and rat neostriatum dissociated neurons $\alpha_3/\alpha_2$ Na/K-ATPase by 25% (163). However, to what extent these observations can be generalized for all neurons expressing $\alpha_3$ Na/K-ATPase remains to be established. Furthermore, comparison of the neurotransmitter mode of CNS neurons with the expressed isoforms of Na/K-ATPase makes any association between these characteristics very unlikely. For example, regardless of neurotransmitter $\alpha_3$ Na/K-ATPase is expressed in cerebellar Purkinje neurons (GABA), basket cells (GABA), and retinal photoreceptors (glutamate). The cerebellar granule cells using glutamate, like photoreceptors, express $\alpha_1$ with little or no expression of $\alpha_3$ Na/K-ATPase (see (86)). Studies in PNS emphasize these discrepancies even further. The γ-motoneurons are cholinergic cells, while stretch receptor afferent neurons use glutamate, but both have abundant $\alpha_3$ Na/K-ATPase (18, 89).

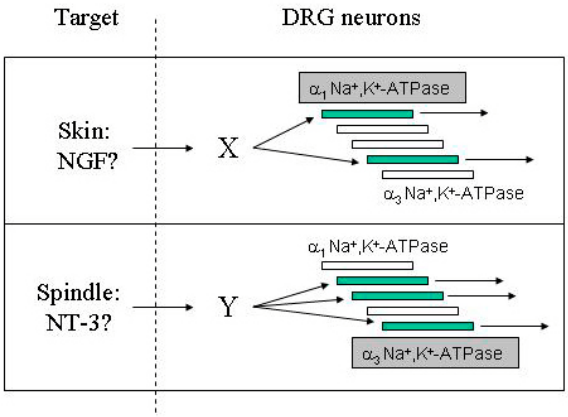


**Figure 8.** Hypothetical scheme of cell-specific expression of α isoforms of the Na/K-ATPase in vertebrate peripheral neurons. Coding essentially similar products genes of $\alpha_1$ and $\alpha_3$ Na/K-ATPase are expressed differentially in different cells, because they are included in different cohorts of constitutive proteins determining the neuronal phenotype and are expressed in accord with the developmental program of the given neuronal population. In this scheme, skin-derived neurotrophic factors acting via transcription factor "X" initiate activation or disinhibition of genes 1 and 4 (counting from the top), with gene #1 being the gene for $\alpha_1$Na/K-ATPase. On the other hand, protein synthesis in neurons that contact muscle spindle is under control of spindle-derived neurotrophins and transcription factor "Y" regulating transcription of genes #2, #3, and #5 ($\alpha_3$ Na/K-ATPase). Several observations support this scheme: 1) Target-determined expression of $\alpha_3$ Na/K-ATPase (see Figure 5). 2) Timing of up-regulation of the $\alpha_3$ Na/K-ATPase in stretch receptor neurons during development (after contact with a peripheral target was established; Figure 6). 3) Finally, sciatic nerve axotomy in adult rats results in the decrease in percentage of $\alpha_3$ Na/K-ATPase - positive DRG neurons from 14 ± 2% to 3 ± 1% within 15 days after surgery (14 and 5 rats, respectively; unpublished observations).

Protein kinase-mediated phosphorylation: Many effects of neurotransmitters and hormones are mediated by cellular protein kinases. However, existing data on isoform-specific effects of activation of PKC or PKA do not provide much support for a possible isoform-specific link to these pathways. Phosphorylation by PKC of isoforms of Na/K-ATPase expressed in SF-9 insect cells leads to similar inhibition of α, $\alpha_2$, and $\alpha_3$ Na/K-ATPases by 40%, 30% and 30%, respectively (164). Similarly, in HeLa cells, native $\alpha_1$, and all transfected rat isoforms ($\alpha_1$, $\alpha_2$, and $\alpha_3$) were inhibited by 20 to 30% by PKC activation (165). Also, phorbol ester reduced activity of $\alpha_3/\alpha_2$, and $\alpha_1$, Na/K-ATPase by 30% and 15% respectively, in cultures of acutely dissociated neurons from adult rat neostriatum (163). Phosphorylation by PKA of isoforms of Na/K-ATPase expressed in SF-9 insect cells inhibits $\alpha_1\beta_1$ by 35%, $\alpha_2\beta_1$ by 30% and activates $\alpha_3\beta_1$ by 25% (164)). However, in HeLa cells transfected with $\alpha_1$, $\alpha_2$, or $\alpha_3$ rat isoforms, all were inhibited by PKA activation by 20 to 26% (165).

**6.4. Transcriptional regulation**

Finding isoform-specific kinetic or regulatory differences between the α isoforms of the Na/K-ATPase to provide a functional explanation for the multiple isoforms would be an exciting possibility. However, as discussed long ago, there is an alternative explanation for the multiple isoforms of Na/K-ATPase (5, 59, 166). It is possible that to conveniently organize the regulation of Na/K-ATPase expression during the development of different tissues and groups of cells the multiple isoforms are under unique regulatory control. An increased capacity of active transport seems to be a common requirement for all cells of developing and maturing animals. However, the temporal sequence and exact amount of this increase are cell type and function specific. Therefore, instead of having one gene of the Na/K-ATPase, it might be biologically advantageous to have several genes coding essentially similar products, but placed under control of cell type-specific transcription factors that in addition to Na/K-ATPase may also control expression of genes of other constitutive proteins (5, 59, 166).

Perhaps because it is difficult to prove directly, this hypothesis is currently not frequently mentioned. However, in light of new data on $\alpha_3$ Na/K-ATPase expression in PNS it deserves more consideration in our opinion. Two groups of neurons, stretch receptor afferents and γ-motoneurons, express $\alpha_3$ Na/K-ATPase in PNS. The most obvious trait these neurons have in common is not their discharge pattern, but the target they innervate (Figure 5). Furthermore, our recent studies suggest that β-motoneurons that innervate both extrafusal and intrafusal muscle fibers, but not α motoneurons innervating only extrafusal muscle fibers, also express $\alpha_3$ Na/K-ATPase ((18); see also Figures 3 and 4). These studies also show that during development the $\alpha_3$ Na/K-ATPase appears in the plasma membrane of stretch receptor neurons after they have established contact with their target and the number of $\alpha_3$ Na/K-ATPase expressing DRG neurons decreases if this contact is impaired by peripheral nerve axotomy in adult rat (Figure 6).

It is well known that during development and in mature animals, target-derived factors determine survival of peripheral motor and sensory neurons via the regulation of gene expression, as well as many other morphological, biochemical and functional characteristics of these neurons (167, 168, 169, 170, 171). Therefore, it is reasonable to suggest that expression of genes of α isoforms of Na/K-ATPase is also under control of target-derived influences, and specifically, that muscle spindle-derived factors start the cascade of events for up-regulation or disinhibition of $\alpha_3$ Na/K-ATPase gene expression (Figure 8).

Further studies are required to determine the putative signaling cascade linking muscle spindle receptor to expression of $\alpha_3$ Na/K-ATPase. Large and small DRG neurons have different developmental programs running under control of different neurotrophic factors. Survival of small, mostly nociceptive neurons require NGF during neonatal development and GDNF, NTN and ART (glial

cell-derived neurotrophic factor, neurturin and neublastin/artemin, respectively) postnatally. Proprioceptive afferents, Merkel receptors and D-hair afferents require neurotrophin-3 (NT-3) and GDNF, NT-4 and brain-derived neurotrophic factor (BDNF) during development (172, 173, 174, 175).

NT-3, acting through its specific TrkC receptor and to some extent through BDNF-specific TrkB receptor (176), is potentially involved in $\alpha_3$ Na/K-ATPase regulation. First, our data suggest that a target-derived factor remains an important regulator of $\alpha_3$ Na/K-ATPase throughout the adult life of animals. In agreement with this, and unlike GDNT or NT-4, NT-3 was shown to be important for both sensory and motor neurons not only during development but in mature animals as well (177, 178, 174). In agreement with expression of $\alpha_3$ Na/K-ATPase in γ- but not α-motoneurons, the mRNA of NT-3 receptor, TrkC, is more abundant in γ- than in α-motoneurons (179). Also, over-expression of NT-3 under control of skeletal muscle myosin promoter results in an increased number of both large sensory neurons and fusimotor axons (180). Finally, in diabetic animals both a decrease in target-derived NT-3 levels (181) and a selective decrease in $\alpha_3$ Na/K-ATPase expression in sciatic nerve was reported (93).

Even with all these indirect suggestions for the role of NT-3, some additional factors are likely to be involved in regulation of $\alpha_3$ Na/K-ATPase, since NT-3 is not absolutely stretch receptor-specific. As mentioned above, in developing rat, skin Merkel receptors and D-hair afferents that express $\alpha_1$, but not $\alpha_3$ Na/K-ATPase, are dependent on NT-3 (see also (182)). Similarly, NT-3 supports differentiation of cholinergic sympathetic neurons, that are not likely to express $\alpha_3$ Na/K-ATPase (18, 183).

Further study is necessary to identify transcriptional factors that may be important for control of $\alpha_3$ Na/K-ATPase gene expression during development and maturation of proprioceptive neurons. Several different transcription factors involved with survival and growth of axonal projections of type Ia muscle spindle afferents were described (see (184, 185)). However, relevance of these factors to Na/K-ATPase expression is not known.

While many experiments are still to be conducted, this hypothesis is attractive because the independent developmental control of genes for multiple α isoforms of the Na/K-ATPase allows us to explain some issues that are not easy to resolve within the limits of hypotheses suggesting the importance of kinetic or regulatory differences of isoforms. Thus, the presence of $\alpha_3$ Na/K-ATPase in neurons and in adult human and dog heart, but not in adult rat heart, are difficult to understand taking into account differences in function between neurons and cardiac cells and the similarity in function of cardiac cells from different species. Furthermore, if $\alpha_3$ Na/K-ATPase is a reserve isoform to work under conditions of extra load of Na, why would many different cells respond to such conditions by non-specific up-regulation of isoforms other than $\alpha_3$ Na/K-ATPase? Thus, in cardiac myocytes cultured in the presence of ouabain or monensin, increased intracellular Na induced non isoform-specific transcription and translation of α Na/K-ATPases (186). Similarly, chronic treatment with ouabain of aortic smooth muscle induces up-regulation of both $\alpha_1$ and $\alpha_2$ Na/K-ATPases present in these cells, but not the $\alpha_3$ Na/K-ATPase (187). In brain, the activity-dependent regulation of isoforms of the Na/K-ATPase in neurons of supraoptic and paraventricular nuclei of rat hippocampus was studied after 5 days of salt treatment increasing electrical activity of these neurons. Only the level of $\alpha_1$, but not $\alpha_2$ or $\alpha_3$, mRNA was increased by this treatment (188). In rat cerebral cortex, intracerebroventricular infusion of TTX for 4 days resulted in reduction of Na currents and synapto-neurosomal $Rb^+$ uptake. Both high and low ouabain affinity components were reduced proportionally (189).

### 6.5. Conclusion

With our present knowledge, the hypothesis of $\alpha_3$ Na/K-ATPase acting as a "reserve" pump and the hypothesis implicating convenience of independent transcriptional regulation of multiple genes of the Na/K-ATPase appear to be most consistent with the data on properties and expression of different isoforms of the Na/K-ATPase in peripheral nervous system of vertebrates. These hypotheses are not necessarily exclusive; however, more studies are required to understand the relationships between them.

## 7. CONCLUSION AND PERSPECTIVE

The Na/K-ATPase is a key mechanism of neuronal ion homeostasis that affects signaling function of these cells in a number of direct and indirect ways. Therefore, complete understanding of physiology of nervous cells is impossible without understanding the organization and regulation of Na/K-ATPase in these cells. Neurons express multiple isoforms of the Na/K-ATPase, including $\alpha_3$ Na/K-ATPase, which is rarely expressed anywhere outside the nervous system of adult vertebrates. It is currently unknown if there is any functional significance to the multiplicity of isoforms of Na/K-ATPase. Within the nervous system, the abundance of $\alpha_3$ Na/K-ATPase varies between different neuronal groups, and it is possible that neuronal function determines the need for this isoform of the Na/K-ATPase. Therefore, functional identification and comparative studies of neurons expressing different isoforms of Na/K-ATPase is an important step in determining the specific roles of these isozymes.

In peripheral nervous system stretch receptor neurons and γ-motoneurons express $\alpha_3$ Na/K-ATPase. Because of the relative simplicity and accessibility of PNS to a variety of experimental manipulations the identification of these neurons opens the possibility of determining the role of this isoform of the Na/K-ATPase in neuronal function. Future studies will correlate physiology and Na/K-ATPase activity in peripheral neurons expressing either $\alpha_1$, or $\alpha_3$, or both isoforms of the Na/K-ATPase.

Furthermore, further evaluation of apparent target-determined expression of $\alpha_3$ Na/K-ATPase may bring about important knowledge of cellular signals involved in tissue- and cell-specific expression of this isoform of the enzyme.

## 8. FINAL REMARKS

While this work was under review several articles were published that should be mentioned because of their direct relevance to the topic under consideration. First, providing an additional proof to the critical importance of studies on functional significance of neuronal $\alpha_3$ Na/K-ATPase, evidence of association of missense mutations of the gene coding for this isoform of the transporter and human dystonia-parkinsonism was obtained (190). Another recent publication suggests that in mesencephalic trigeminal neurons $\alpha_3$ Na/K-ATPase may be co-compartmentalized with H-channels (191) to control excitability and integration of synaptic inputs of these neurons. Finally, work published ahead of print in the Biochemical Journal reports Sp1 and NF-4 transcription factors as possibly determining neuron-specific expression of $\alpha_3$ Na/K-ATPase (192).

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