The Na,K-ATPase $\alpha_2$ Isoform Is Expressed in Neurons, and Its Absence Disrupts Neuronal Activity in Newborn Mice*

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Na,K-ATPase is an ion transporter that impacts neural and glial physiology by direct electrogenic activity and the modulation of ion gradients. Its three isoforms in brain have cell-type and development-specific expression patterns. Interestingly, our studies demonstrate that in late gestation, the $\alpha_2$ isoform is widely expressed in neurons, unlike in the adult brain, in which $\alpha_2$ has been shown to be expressed primarily in astrocytes. This unexpected distribution of $\alpha_2$ isoform expression in neurons is interesting in light of our examination of mice lacking the $\alpha_2$ isoform which fail to survive after birth. These animals showed no movement; however, defects in gross brain development, muscle contractility, neuromuscular transmission, and lung development were ruled out. Akinnesia suggests a primary neuronal defect and electrophysiological recordings in the pre-Bötzinger complex, the brainstem breathing center, showed reduction of respiratory rhythm activity, with less regular and smaller population bursts. These data demonstrate that the Na,K-ATPase $\alpha_2$ isoform could be important in the modulation of neuronal activity in the neonate.

Na,K-ATPase is a plasma membrane enzyme necessary for maintaining the sodium and potassium ion gradients in the cell, and it drives the sodium-dependent transport of calcium and amino acids as well as the reuptake of neurotransmitters. The ion gradients generated by Na,K-ATPase are also used to regulate the volume of the cell and to support and modulate electrical activity through direct (electrogenic) and indirect effects on membrane potential.

Na,K-ATPase is a heteromeric protein composed of an $\alpha$ catalytic subunit that binds sodium and potassium ions, $\text{ATP}$, and cardiac glycosides, and $\beta$ and $\gamma$ (FXYD) subunits that can modulate substrate affinity. There are different genes that code for multiple $\alpha$, $\beta$, and $\gamma$ isoforms. Four $\alpha$ isoforms ($\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_4$) have been identified, and all but $\alpha_4$ are expressed in the brain (1, 2). Examination of the enzymatic properties of the $\alpha$ and $\beta$ isoforms in different expression systems revealed that the $\alpha$ isoforms have differences in substrate affinity and kinetic properties (3–7). In most adult mammals, the $\alpha_2$ isoform is expressed most abundantly in skeletal muscle and brain and in lower abundance in heart, adipocytes, and eye (8–11). In situ hybridization performed on sections of embryonic days 9.5–16.5 mouse brain revealed that the Na,K-ATPase $\alpha_2$ isoform is expressed throughout most regions of the brain (12). In the adult brain it has been found in astrocytes, pia/arachnoid, and a few types of neurons (13–15).

To understand further the specific roles of individual Na,K-ATPase isoforms, we have analyzed mice in which the $\alpha_2$ isoform gene has been knocked out. The animals died shortly after birth but did not display obvious gross morphological defects in any tissue, including the brain. Lack of motor activity was significant, but muscle contractility was not found to be critically impaired. Consequently we investigated the cellular distribution of $\alpha_2$ in the newborn brain and the function of an intrinsic neuronal circuit that could contribute directly to immediate death: generation of the breathing rhythm.

**EXPERIMENTAL PROCEDURES**

*Genotyping and Blood Analysis—Mice heterozygous for the Na,K-ATPase $\alpha_2$ isoform were generated as described previously (16). Heterozygous females were mated with heterozygous males, and the resulting offspring were genotyped by Southern blot as described previously (16). Blood was taken from decapitated mouse pups immediately after birth, collected in capillary tubes, and measured for carbon dioxide and oxygen content using a Chiron blood gas analyzer (Norwood, MA model 384). Blood glucose levels were analyzed using an Accudata GTS Glucose Test Station (Roche Molecular Biochemicals).

*Lung Histology*—Within 15–30 min after birth, newborn mouse pups were sacrificed, and the lungs were carefully removed and immersed in 10% formalin. The tissues were then embedded in paraffin and sectioned at 5 µm. Sections were stained with hematoxylin and eosin, and digital pictures were taken using a microscope setting on ×10 magnification.

*Microsome Preparation and Western Blot Analysis—Tissues from at least four embryonic day 18.5 pups of the same genotype ( $\alpha_2^{+/+}$, $\alpha_2^{+/-}$, or $\alpha_2^{-/-}$) were pooled and microsomes prepared as described (16). The pellet was resuspended in 1 mM imidazole, 1 mM EDTA, pH 7.4, then aliquoted and stored at −80 °C. Protein concentration was determined using the BCA assay (Pierce Chemical Co.). The microsomal membranes were used for Western blot analysis. SDS-PAGE was performed as described (17). The Western blot procedure was performed as described (18).
Approximately 5–10 µg of microsomal membrane protein was loaded per lane. The primary antibodies used were a α2 isoform-specific monoclonal antibody, Mcb2, and an α3 isoform-specific monoclonal antibody, MA3-915 (Affinity BioReagents, Golden, CO). The signal was detected using an ECL system (Amersham Biosciences).

**Diaphragm Immunohistochemistry**—For whole mount diaphragm preparations, diaphragms from embryonic day 18.5 mice were prepared as described previously (19). Diaphragms were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS), blocked in 0.1 M glycine in PBS, and then permeabilized in 2% bovine serum albumin in PBS overnight. The tissues were cryoprotected in 30% sucrose in PBS overnight and then embedded in OCT for cryosectioning. Cryostat sections (12 µm thick) were immersed in 30% sucrose in PBS overnight and then embedded in OCT for cryosectioning. Cryostat sections (12 µm thick) were dried on slides and then postfixed, prehybridized, hybridized, and developed as described previously (20, 21).

**Antisense and Sense RNA Probes Were Synthesized With 35S-Labeled UTP From Plasmids That Contain Either α1 or α2 Na,K-ATPase Isoform-Specific Sequences** (9).

**Immunofluorescence**—Slices used for immunofluorescence were prepared in an ice-cold artificial cerebrospinal fluid containing (in mM) 118 NaCl, 3 KCl, 1.5 CaCl2, 1 MgCl2, 25 NaHCO3, 0.026 EDTA, and 11 glucose, equilibrated with 95% CO2 and 5% O2. The diaphragm was cut to obtain a muscle strip from the central region with ribs attached to the tendon at each end. Triangular clips were attached at each end of the muscle strip, and the muscle was held in the clip against the ribs. Muscles were mounted in a constant temperature PBS over 30 min. For all subsequent incubations, the temperature was maintained at 37 °C.

**Slices were incubated with rabbit antibodies to synaptophysin (Zymed Laboratories, San Francisco) and βIII-tubulin (mouse) for one hour each at 4 °C in anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) and tetramethylrhodamine-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR) in 2% bovine serum albumin in TPBS overnight at 4 °C. After washing in TPBS, diaphragms were mounted on coverslips in glycerol-paraphenylenediamine to retard fading, viewing with epifluorescence and filters that were selective for rhodamine or fluorescein, and evaluated with an Axioplan2 microscope (Zeiss, Thornwood, NY). Images were captured with a digital camera (Hammatsu, Bridgewater, NJ) and imaging software (QED Imaging, Pittsburgh, PA).

**Diaphragm Contractility**—Diaphragms with ribs attached were removed from embryos (day 18.5) and placed in Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 25 NaHCO3, 2.5 CaCl2, 1.2 MgSO4, 1.2 Na2HPO4, 0.026 EDTA, and 11 glucose, equilibrated with 95% CO2 and 5% O2. The diaphragm was cut to obtain a muscle strip from the central region with ribs attached to the tendon at each end. Triangular clips were attached at each end of the muscle strip, and the muscle was held in the clip against the ribs. Muscles were mounted in a constant temperature PBS over 30 min. For all subsequent incubations, the temperature was maintained at 37 °C.

**RESULTS**

**Na,K-ATPase α2−/− Mice Appear Normal but Do Not Breathe**—Mice lacking the α2 isoform are born and display no gross anatomical or histological abnormalities. However, these mice appear limp and do not respond to pinch. By opening the chest cavity of newborn pups immediately after birth we established that the hearts from the α2−/− mice were beating, indicating that the mice were alive when born. Several minutes after birth the wild-type and α2−/− mice breathe and turn pink, but the α2−/− mice do not breathe. Therefore we measured blood gas levels in newborn mouse pups within 15–30 min after birth. Both the wild-type and α2−/− mice showed normal levels of oxygen and carbon dioxide (26), whereas the α2−/− newborn pups displayed very low oxygen and high carbon dioxide levels consistent with failure to breathe (Table 1). Blood glucose levels and body weight were normal for the α2−/− mice (Table 1).

Lungs removed from pups −15–30 min after birth as well as from day 18.5 embryos were fixed and stained with hematoxylin and eosin. As shown in Fig. 1, lungs from the α2−/− mice
there have been several reports that phenotypic changes from ATII-like to ATI-like in culture (46). However, it itself could be unable to contract. In agrin-deficient mice, for it could be caused by either a defect in the neuromuscular junction (NMJ) in which the signal was not functioning properly it could be caused by either a defect in the neuromuscular junction (NMJ) in which the signal was not functioning properly.

It has been reported that α2 is expressed in alveolar cells when their phenotype changes from ATII-like to ATI-like in culture (46). However, there have been several reports that α2 mRNA is lacking in lung (9, 47–49), consistent with the absence of the protein reported here.

Table I

| Genotype | Body weight | Blood gas | Blood glucose |
|----------|-------------|-----------|---------------|
|          | g           | PCO2 mm Hg | pO2 mm Hg | mg/dl |
| α2−/−    | 1.38 ± 0.09 (4) | 46.1 (1) | 87.7 ± 42 (4) | 31 ± 17 (8) |
| α2+/−    | 1.53 ± 0.17 (8) | 63.9 ± 28 (7) | 68.8 ± 16 (9) | 62 ± 20 (16) |
| α2−/−    | 1.48 ± 0.01 (4) | 138.9 ± 4.8 (3) | 19.4 ± 1.8 (3) | 38 ± 24 (8) |

Fig. 1. Hematoxylin and eosi n staining of lungs of Na,K-ATPase α2−/− and wild-type mice at embryonic day 18.5 and 15–30 min after birth. Note that the alveoli in the homozygous mice (α2−/−)(B and D) are not as dilated compared with wild-type (wt)(A and C). Scale bar, 1 mm.

Fig. 2. The Na,K-ATPase α2 isoform is expressed in brain and diaphragm at embryonic day 18.5. The expression of each of the α isoforms of Na,K-ATPase was examined in microsomes prepared from wild-type (wt) and Na,K-ATPase α2 homozygous (−/−) mice in brain (Br), heart (Ht), skeletal muscle (Sm), diaphragm (Dia), kidney (K), liver (Lu), and lung (Lu). 10 μg of protein was loaded in each lane for each antibody blot except for the kidney sample, which contained 5 μg of protein for the α1 blot.

Density at the NMJ, and these mice die at birth from an inability to breathe (19). We used rhodamine-labeled bungarotoxin to detect acetylcholine receptors as a marker for NMJ development. Synaptophysin, a synaptic vesicle-specific membrane protein, is expressed abundantly in nerve terminal synaptic vesicle boutons, and we used synaptophysin antibody and fluorescein isothiocyanate-labeled secondary antibody to detect this protein as a marker for the nerve terminal. Whole mount immunohistochemistry revealed normal NMJ development in α2−/− mice (Fig. 3). We then tested whether the NMJ was functional by electrically stimulating the phrenic nerve. The diaphragm was able to contract, indicating that the synaptic connection between muscle and nerve was functional.

To test whether the absence of the α2 isoform in diaphragm altered contractility we developed a method of electrically stimulating and measuring isometric contractility in muscle preparations from day 18.5 embryos. Because of the small size of the diaphragm muscle as well as the presence of the attached ribs, accurate weights were difficult to obtain. Thus, assuming that the thickness of each diaphragm was the same in all preparations we normalized the tension data to muscle area (length times width) of the diaphragm strips. No significant differences in maximum twitch force of contraction were observed between wild-type and α2−/− mice (Fig. 4). Two other normalization routines were evaluated: force normalized to length and force normalized to width. In all cases of normalization (Fig. 4A) as well as the raw tension data (Fig. 4B), a similar trend was observed, with the α2−/− muscle producing a force within 10% of the wild-type muscle with no statistically significant difference (p > 0.05, Student’s t test). Together, these results demonstrate that embryonic diaphragm muscle without the α2 isoform is able to contract both by direct electrical stimulation and by stimulation via the phrenic nerve with a force similar to that of wild-type. Therefore the brain,
which also showed expression of the α2 isoform at embryonic day 18.5, was examined further for physiological defects associated with the absence of the α2 isoform.

*Na,K-ATPase α2 Isoform Is Expressed in Neurons at Embryonic Day 18.5*—Previous reports on Na,K-ATPase α2 isoform expression have shown that it is expressed primarily in astrocytes of adults (for review, see Ref. 28); however, little data exist on the expression of α2 at the time of birth in mice. Therefore, as an initial step toward the evaluation of the α2⁻/⁻ mice, we determined the expression profile of the α2 isoform from embryonic day 18.5 wild-type mice by *in situ* hybridization and immunofluorescence analysis of mRNA and protein, respectively, to determine the cell type and the regions of the brain that express it. Fig. 5 shows *in situ* hybridization of sagittal brain sections in which signals for α1 and α2 isoforms can be compared. In Fig. 5, A and B, the choroid plexus is shown. This highly elaborated secretory epithelium that emerges from the ventricular lining shows strong hybridization for α1 isoform mRNA with the antisense probe, but little or no hybridization above background with the α2 antisense probe. In contrast, in Fig. 5D, it can be seen that the α2 isoform antisense probe showed extremely heavy hybridization over the pia mater, a tissue that is known to express the α2 isoform, whereas the α1 isoform antisense probe showed less (Fig. 5C). Neither the α1 isoform antisense nor the α2 isoform sense probes labeled the pia. These data validate the methods by confirming the known distribution of the α1 isoform in choroid plexus and the α2 isoform in pia. In the cortical layer, the α1 isoform shows stronger expression than the α1 isoform. Scale bar, 100 μm.

![Fig. 3](image1.png) **Fig. 3.** Pre- and postsynaptic differentiation of diaphragm is normal in Na,K-ATPase α2⁻/⁻ mice. Whole mounts of diaphragm muscle from a wild-type (+/−) and Na,K-ATPase α2⁻/⁻ mutant (−/−) mouse were stained simultaneously with antibodies against synaptophysin (Syn) to label vesicles in presynaptic nerve terminals and α-bungarotoxin (αBGT) to label acetylcholine receptors on the postsynaptic muscle membrane. The whole mounts were viewed with optics selective for either fluorescein (A and D) or rhodamine (B and E) or both fluorescein and rhodamine (C and F). Presynaptic sites in wild-type muscle (A and B) are located adjacent to the main intramuscular nerve (arrow in A), and the nerve terminals are characterized by an accumulation of clustered acetylcholine receptors (B) underlying the terminals (C). Na,K-ATPase α2⁻/⁻ mice exhibited normal synaptic differentiation with presynaptic sites (D) localized adjacent to the nerve (arrow in D) and the nerve terminals showing clustered acetylcholine receptors (E) underlying the terminals (F).

![Fig. 4](image2.png) **Fig. 4.** Maximal twitch force of contraction in diaphragm of α2⁻/⁻ mice is similar to wild-type mice. Diaphragm muscle strips from embryonic day 18.5 mice were used for isometric force of contraction measurements. A, maximal twitch force was normalized to muscle length, muscle width, or to area (length times width) for both wild-type (wt) and α2⁻/⁻ (−/−) mice. B, maximal twitch force without normalization. Values are the means ± S.E. (n = 7 in the wild-type group, n = 6 in the α2⁻/⁻ group).

![Fig. 5](image3.png) **Fig. 5.** *In situ* mRNA hybridization analysis revealed that both the Na,K-ATPase α1 and α2 isoforms are expressed throughout the brain in wild-type embryonic day 18.5 mice. Embryonic day 18.5 brain sections from wild-type mice were incubated with antisense α1 and α2 isoform-specific probes. A, C, and E represent darkfield photomicrographs of sections hybridized with the α1 antisense probe. B, D, and F represent sections hybridized with the α2 antisense probe. The α1 sense control is shown in G, and the α2 sense control is shown in H. A and B, sections through the hippocampus. A, the dentate gyrus shows uniform expression (bright white grains) of the α1 isoform, and the star designates robust expression for the α1 isoform but not the α2 isoform in the choroid plexus. The adjacent layers also show uniform expression but overall less intensity because of the cellularity differences. B, in contrast, the α2 pattern is punctate although not uniform across all cells in that region. The ependymal lining is also more intense (arrow) for the α2 isoform. C and D, sections through the cortex. C, the α1 signal shows a mostly uniform pattern throughout the cortex but no expression in the pia mater. D, in contrast to C, the α2 isoform pattern is much more nonuniform in the neuronal layers of the cortex because some neurons are more intense than others in this layer. The pia mater shows strong α2 isoform expression (arrow). E and F, brainstem near the ventral respiratory group; the inset shows a brightfield view of hematoxylin and eosin staining with the larger (neuronal) nuclei purple, and the hybridization signal is the small black grains. The α2 isoform shows stronger expression than the α1 isoform. Scale bar, 100 μm.
Fig. 6. The Na,K-ATPase α2 isoform protein is localized predominantly in neurons in the respiratory center of the brain. Immunofluorescent localization of the Na,K-ATPase α2 subunit in wild type (A–D) and α2−/− (E) embryonic day 18.5 mouse brains is shown. A, α2 stain in the ventral respiratory group area. B, control section (primary antibody omitted) showing nonspecific stain. C, α2-labeled processes (arrowheads) crossing the midline in the region of the raphe pallidus nucleus. D, α2 stain in large presumptive neuronal cell bodies (arrows). E, α2-specific antibody (Fig. 6E) (25). The outcome was that rhythmic activity showed abnormal properties.

Spontaneous neural activity was observed in all genotypes including the α2−/− mice, indicating that the absence of the Na,K-ATPase α2 isoform does not result in a complete loss of neuronal function. Rhythmic activity was observed in the ventral respiratory group in 26 of 31 preparations examined. Of the five preparations from which activity could not be recorded, two were from α2−/−, three from wild-type animals, and none from α2−/−. Of the 26 active slices, 19 produced two clearly distinct patterns of activity (Fig. 7, A and B). Larger amplitude bursts occurring at intervals of about 40 s to several minutes represent fictive sighs, whereas smaller amplitude bursts oc-
Na,K-ATPase α2 Isoform and Neuronal Function

Table II
Summary of respiratory rhythm patterns measured in brain slices from Na,K-ATPase α2 homozygous (−/−), α2 heterozygous (+/−), and wild type (+/+)

| Genotype            | Sighs | Eupnea |
|---------------------|-------|--------|
|                     | Frequency ± S.E. | Period CV | Frequency ± S.E. | Period CV | Burst duration | Amplitude |
| α2−/−               | 0.67 ± 0.16 | 0.18    | 12.02 ± 2.31 | 0.68° | 0.42 ± 0.03 | 38.5 ± 5.3° |
| α2+/−               | 0.99 ± 0.16 | 0.15    | 10.29 ± 1.72 | 0.57 | 0.35 ± 0.02 | 55.3 ± 2.5° |
| α2+/+               | 1.08 ± 0.17 | 0.26    | 12.57 ± 2.17 | 0.48 | 0.40 ± 0.02 | 61.9 ± 5.8° |
| α2−/− and α2+/+     | 0.86 ± 0.14 | 0.21    | 11.43 ± 1.37 | 0.43 | 0.37 ± 0.02 | 58.9 ± 3.4° |

* Denotes significant difference from pooled control (p < 0.01).

Although the absence of the Na,K-ATPase α2 isoform did not result in a complete loss of neural activity, the regularity and the amplitude of the respiratory rhythm of normal breathing (eupnea) were affected profoundly (Table II, Fig. 7). Table II shows that neither the burst duration nor the mean frequency was significantly different (p = 0.1659 and p = 0.8225, respectively). However, the regularity of the eupneic rhythm was significantly lower in the α2−/− mice compared with α2+/− and wild-type mice. This is reflected by the coefficient of variation of cycle periods of eupnea (designated as period CV in Table II and Fig. 7C) from 0.43 for the α2−/− and α2+/− group to 0.66 for the α2+/+ group (p = 0.0011). Note that in this case, a higher number means lower regularity of rhythm. We also examined the amplitude of the eupneic rhythm. Generally, the amplitude of the integrated population burst is not directly comparable between preparations because of differences in recording quality and the lack of an absolute measurement scale. However, sighs apparently reflect a maximal activation of inspiratory cells within the respiratory network (25), and this enabled us to assess the amplitude of the eupneic burst as a percentage of the sigh amplitude for each individual recording. The amplitude of the eupneic burst was significantly lower in slices obtained from α2−/− embryos (98%) than in slices from α2+/− and wild-type animals (59%) (p = 0.0056) (Table II and Fig. 7, A and D). This appears to be a major factor leading to the failure of the animals to breathe effectively.

DISCUSSION

Mice deficient in the Na,K-ATPase α2 isoform are born but die soon after birth. By gross examination all organs appear normal; however, the mice are akinetic. This study shows for the first time that the α2 isoform is highly expressed in neurons throughout the brain at the time of birth, with lower levels of expression in glia. This would not have been predicted from the literature. A previous study showed that α2 mRNA is present quite early in brain development, but without cellular resolution it could not be certain whether the signal was in neurons or glia (12). In another study, embryonic stem cells and aggregates of undifferentiated cells expressed α1 and α2 isoform mRNA, but only α1 protein. In vitro neuronal induction of embryonic stem cells was accompanied by induction of the α3 isoform at late stages of differentiation, but α2 isoform mRNA or protein was not induced at any stage (29). Embryonic fetal forebrain examined after several days in culture either as aggregates or as plated cells expressed the α3 isoform in neurons and α2 isoform in glia, as assessed either by selective elimination of cell types by toxic agents (30) or by immunofluorescence microscopy (31). Different results have been reported for cultures of hippocampal neurons: one paper reported finding only the α1 and α3 isoforms by immunofluorescence (32), whereas two papers reported finding the α2 and α3 isoforms or all three isoforms by Western blots (33, 34), although the latter result could have reflected contamination with astrocytes (35). Cerebellar granule neurons have been reported by Soga et al. (36) to express all three isoforms in culture, although the presence of the α2 isoform was found exclusively in cerebellar astrocytes in prior studies both in situ and in granule neuron cultures (13, 14, 28), raising the possibility that the commercial antibodies used by Soga et al. were cross-reactive. Inspection of the published stain for the α2 isoform in the developing mouse retina, however, suggests that it may have been present at birth in the nascent inner nuclear layer, but subsequently lost over the next few days (37).

We show in this study by in situ hybridization that large diameter neuronal cell bodies expressed the α2 isoform at all levels in the brain, and diffuse signal characteristic of glia was also observed. By immunofluorescence, most of the α2 isoform stain had a reticular pattern, with outlining of presumptive neuronal somas in places and many fine processes characteristic of neurons. At this age, the brainstem lacks the bundles of ascending and descending myelinated axons and well developed neuronal nuclei of the adult, but the distribution of stain clearly differs from the purely astrocytic pattern for the α2 isoform in the adult rat brainstem (13). Taken together with the published evidence, it seems likely that the α2 isoform is expressed transiently in many central nervous system neurons during development but that with phenotypic maturation either in vivo or in culture, the expression is lost. At the time of birth it is expressed mainly in neurons, whereas in adult it is expressed mainly in astrocytes, which proliferate and differentiate mostly postnatally.

The lack of α2 isoform expression in mice results in a gross defect in that the α2−/− mice are unresponsive to pinch and do not breathe. Although the α2−/− mice may have widespread functional disturbances throughout the brain, we were able to document the effect of the absence of α2 on the integration of neuronal activity in the brainstem respiratory center. We observed some basic neuronal activity in the α2−/− mice; however, the fictive eupnea breathing rhythm recorded in vitro was disrupted significantly in the α2−/− mice compared with wild-type littermates. Population bursts were both lower in amplitude and much less regular, and in some α2−/− animals this was altered to such an extent that this rhythm was nearly absent (Fig. 7). To obtain rhythmic activity in slice preparations it is necessary to raise the extracellular potassium concentration (25). Thus it is conceivable that the centrally generated rhythm would be even weaker in vivo and insufficient to support a normal motor output under physiological conditions.

Interestingly, the fictive sigh rhythm was still prominent, despite its apparent origination from the same neural network (25). This suggests that sighs alone may not be sufficient to sustain life, even in the relatively hypoxia-resistant newborn. Alternatively, the apparent failure of the knockouts to take...
even one breath, based on the lack of alveolar expansion, suggests that the first breath of life is not a sigh, but might instead be a gasp. Bursts corresponding to gasps are manifested in slices but are distinct from sighs in that gasps are found only in anoxia and are not intermixed with eupneic breaths (25). The decreased regularity and the decreased amplitude of the eupneic respiratory rhythm, combined with an apparent lack of breathing in the intact animal, indicates that the population level activity we observed in slices is essentially failed central breaths. The individual cells retain some bursting ability but do not synchronize sufficiently, the amplitude being a measure of synchronous activity, to generate a regular rhythm or to give rise to respiratory movements. Fictive sighs are observed in the brain slice preparations of α2−/− animals. However, the data we present here show that the likelihood of observing fictive sighs was actually higher in α2−/− mice (10 of 12, or 83%) compared with α2+/+ mice (4 of 8, or 50%) or wild-type mice (5 of 11, or 45%). The genesis of sighs might be stimulated by the failure of the eupneic rhythm in the knockouts; perhaps the eupneic rhythm ordinarily exerts a negative modulatory influence on the frequency of sighs.

Because the α2 isoform was found to be expressed throughout neurons in the brain of day 18.5 embryos, it is quite possible that the altered neuronal activity we observed in respiratory center neurons may be representative of a more global defect in neurons throughout the rest of the brain in the α2−/− mice. This aberrant neuronal activity could be a result of one or more functional disturbances. A specific role for Na,K-ATPase has been proposed to be in clearance of potassium from the extracellular space to prevent depolarization of neurons during high neuronal activity (38, 39). It was proposed that glial Na,K-ATPase (α2 isoform) contributed to the initial fast uptake of extracellular potassium K, whereas the axonal Na,K-ATPase (α3 isoform) participated in the slower poststimulus recovery from elevated extracellular potassium (39). We would predict that in our homozygous knockout mice, the absence of the α2 isoform might result in delayed removal of extracellular potassium in both neurons and glia and thus affect neuronal excitability.

Another function of Na,K-ATPase in the brain is that the glutamate transporter works in concert with the Na,K-ATPase to maintain neuronal excitability. To test the specific contribution by the α2 isoform in maintaining neuronal excitability versus the contribution from the α3 isoform, however, would require the specific inhibition of the α3 isoform either by pharmacological means (no specific inhibitor exists yet) or by genetic knockout (that animal has not been produced).

Na,K-ATPase has been proposed to regulate intracellular calcium via the Na/Ca exchanger. It has been proposed that the Na,K-ATPase α2 isoform and the Na/Ca exchanger are colocalized in microdomains in the plasma membrane of astrocytes and mesenteric artery smooth muscle cells to work as a functional unit (34). During neuronal activity, sodium must be pumped out of the cell to maintain excitability. If the capacity of the neuron to pump out intracellular sodium is reduced, intracellular sodium would rise, which then would change the driving force for Na/Ca exchange, resulting in elevated [Ca2+]. A recent report by Golovina et al. (44) supports this idea in which it was shown that in our α2−/− mice, astrocytes have elevated levels of intracellular calcium as well as elevated stores of calcium in the endoplasmic reticulum. We show in this study that in wild-type animals the α2 isoform is expressed more abundantly in neurons than in astrocytes at birth. We would predict even higher levels of intracellular calcium in neurons than that shown for astrocytes in the α2−/− mice (44). Tang et al. (45) have reported that a rise in intracellular calcium caused by inhibition of the Na/Ca exchanger can enhance neurotransmitter release in chromaffin cells. It is possible that a similar mechanism could occur in the central nervous system of our α2−/− mice in which glutamate release would be enhanced as a result of Na/Ca exchange reduction. As discussed above, glutamate has been shown to alter neuronal activity as a secondary response to inhibition of Na,K-ATPase. In sum, it is clear from the present work that the absence of the α2 isoform results in altered integration of neuronal activity, and further work will be required to sort out whether the mechanism entails neurotransmitters.

Last, it is also possible that the α2 isoform directly provides a significant contribution to the total Na,K-ATPase activity in the cell and that with its removal, the activity of the other Na,K-ATPase isoforms is simply not sufficient to handle the demand for ion transport in the cell, resulting in membrane depolarization. Because respiratory rhythm generation was not abolished, some basic synaptic and neuronal membrane properties were still intact. Given that the absence of the Na,K-ATPase α2 isoform could lead to a number of different functional defects as proposed above, this work provides a foundation to investigate further specific roles for the α2 isoform in maintaining neuronal excitability. The present work provides a new area of study of the α2 isoform in neurons as this cell type was not recognized previously to express this Na,K-ATPase isoform.

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