Published in final edited form as:

Eur J Neurosci. 2014 February ; 39(4): 566–578. doi:10.1111/ejn.12415.

Regulation of Na⁺/K⁺-ATPase by neuron-specific transcription factor Sp4: implication in the tight coupling of energy production, neuronal activity and energy consumption in neurons

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Abstract

A major source of energy demand in neurons is the Na⁺/K⁺-ATPase pump that restores the ionic gradient across the plasma membrane subsequent to depolarizing neuronal activity. The energy comes primarily from mitochondrial oxidative metabolism, of which cytochrome c oxidase (COX) is a key enzyme. Recently, we found that all 13 subunits of COX are regulated by specificity (Sp) factors, and that the neuron-specific Sp4, but not Sp1 or Sp3, regulates the expression of key glutamatergic receptor subunits as well. The present study sought to test our hypothesis that Sp4 also regulates Na⁺/K⁺-ATPase subunit genes in neurons. By means of multiple approaches, including *in silico* analysis, electrophoretic mobility shift and supershift assays, chromatin immunoprecipitation, promoter mutational analysis, over-expression, and RNA interference studies, we found that Sp4, with minor contributions from Sp1 and Sp3, functionally regulate the *Atp1a1*, *Atp1a3*, and *Atp1b1* subunit genes of Na⁺/K⁺-ATPase in neurons. Transcripts of all three genes were up-regulated by depolarizing KCl stimulation and down-regulated by the impulse blocker tetrodotoxin (TTX), indicating that their expression was activity-dependent. Silencing of Sp4 blocked the up-regulation of these genes induced by KCl, whereas over-expression of Sp4 rescued them from TTX-induced suppression. The effect of silencing or over-expressing Sp4 on primary neurons was much greater than those of Sp1 or Sp3. The binding sites of Sp factors on these genes are conserved among mice, rats and humans. Thus, Sp4 plays an important role in the transcriptional coupling of energy generation and energy consumption in neurons.

Keywords

cytochrome c oxidase; depolarization; over-expression; RNA silencing; TTX; Sp family of transcription factors

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The authors have no conflict of interest to declare.

Introduction

Neuronal activity depends on a continuous supply of energy, making the brain one of the highest energy-demanding organs of the body (Rolfe & Brown, 1997; Weber *et al.*, 2008). Almost all of the energy is derived from oxidative phosphorylation in the mitochondria, and cytochrome c oxidase (COX) is one of the key energy-generating enzymes in this process. A major energy-consuming enzyme, on the other hand, is Na⁺/K⁺-ATPase, which repolarizes neuronal plasma membrane subsequent to excitatory depolarization (Wong-Riley, 1989, 2010, 2012; Niven & Laughlin, 2008). Failure of Na⁺/K⁺-ATPase activity is implicated in the pathogenesis of several neurodegenerative disorders (Manczak *et al.*, 2006). Of four α and three β subunits expressed in different cell types, most neurons express α 1, α 3 and β 1 subunits, whereas glial cells express α 2, β 2 and β 3 subunits (McGrail *et al.*, 1991; Morth *et al.*, 2009; Poulsen *et al.*, 2010; Bottger *et al.*, 2011). Our previous studies have shown that in the mammalian visual cortex and retina, COX and Na⁺/K⁺-ATPase co-localize in the same sub-regions that receive strong excitatory synaptic input, and both are down-regulated by impulse blockade in deprived visual cortical neurons (Hevner *et al.*, 1992; Wong-Riley *et al.*, 1998a, b). Hence, the expression of COX and Na⁺/K⁺-ATPase are tightly coupled to neuronal activity (Wong-Riley, 1989, 2010, 2012) and may be regulated by the same set of transcription factors.

Specificity proteins belong to a family of zinc-finger transcription factors that bind to GC-rich regions of the proximal promoters of many genes (for a review, see Suske, 1999). Among the members, Sp1, Sp3 and Sp4 bind to and compete for the same *cis*-motif (Letovsky & Dynan, 1989; Suske, 1999). Sp1 and Sp3 are ubiquitously expressed, whereas Sp4 is expressed mainly in neurons and testes (Hagen *et al.*, 1992; Suske, 1999; Safe & Abdelrahim, 2005). Neuronal expression of Sp4 increases with development, signifying its importance in the adult brain (Supp *et al.*, 1996). Most of the attention has been paid to Sp1 and less so to Sp3. However, the function of Sp4 is largely undetermined and poorly understood. Recently, we found that Sp1 regulates the expression of all COX subunit genes in N2a cells (Dhar *et al.*, 2013). However, in primary neurons, it is Sp4 and not Sp1 that plays a more important role in regulating all 13 COX subunit genes (Johar *et al.*, 2013). Moreover, it is Sp4, and not Sp1 or Sp3, that regulates the expression of key glutamatergic receptor subunit genes in neurons (Priya *et al.*, 2013b). Thus, Sp4 mediates the coupling of neuronal activity and energy generation in neurons. In the present study, we hypothesize that Sp4 also regulates genes of the energy-consuming arm of neuronal activity, i.e. Na⁺/K⁺-ATPase, in neurons.

By means of multiple approaches, we tested our hypothesis that Sp4 plays a more important role than Sp1 or Sp3 in functionally regulating the expression of α 1 (*Atp1a1*), α 3 (*Atp1a3*) and β 1 (*Atp1b1*) subunit genes of Na⁺/K⁺-ATPase in murine neurons.

Materials and methods

All experiments and animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996), and all protocols were approved by the Medical College of Wisconsin

Animal Care and Use Committee. All efforts were made to minimize the number of animals used.

Cell culture

Murine Neuro-2a neuroblastoma (N2a) cells were obtained from the American type Culture Collection (ATCC, CCL-131) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO₂.

Mouse primary visual cortical neurons were cultured as described previously for rats (Ongwijitwat & Wong-Riley, 2004). Briefly, 1-day-old mouse pups (*Mus musculus*, C57, both genders, Jackson Laboratory, Bar Harbor, ME, USA; total number of animals: 25) were killed by decapitation. Brains were isolated from the skull and the meninges were removed. The visual cortex was dissected, trypsinized, triturated and plated in six-well poly-L-lysine-coated dishes at a density of 200 000 cells per well. Cells were maintained in Neurobasal-A media supplemented with B27 (Invitrogen). Cytosine β-D-arabinofuranoside (Ara-C) (Sigma, St Louis, MO, USA) was added to the media to suppress the proliferation of glial cells.

In silico analysis of promoters of Na⁺/K⁺-ATPase subunit genes

DNA sequences encompassing 1 kb upstream and 1 kb downstream of the transcription start point (TSP) of murine, rat and human Na⁺/K⁺-ATPase subunit genes (*Atp1a1*, *Atp1a3* and *Atp1b1*) were obtained from the Genome Database in GenBank and aligned using Megalign, DNASTar Lasergene software. Putative specificity (Sp) factor core binding sequence (GGGCGG or CCCGCC) or sites with variation of one or two bases were searched using DNASTar Lasergene software. Regions of high homology were selected for experimental analyses.

Electrophoretic mobility shift and supershift assays

Electrophoretic mobility shift assays (EMSAs) for Sp1, Sp3 and Sp4 interactions with putative *cis*-elements on Na⁺/K⁺-ATPase subunit promoters were carried out as previously described (Johar *et al.*, 2012; Priya *et al.*, 2013a) with minor modifications. Briefly, oligonucleotide probes with putative Sp-factor binding sites (Table 1, based on *in silico* analysis) were synthesized, annealed and labeled by a Klenow fragment fill-in reaction with [α -³²P]dATP (50 µCi/200 ng). Each labeled probe was incubated with 2 µg calf thymus DNA and 5 µg nuclear extract obtained from 1-day-old mouse cerebral cortex or HeLa nuclear extract (Promega, Madison, WI, USA) and processed for EMSA. Supershift assays were also performed and, in each reaction, 2 µg Sp factor-specific polyclonal antibody [Sp1, H-225, SC-14027, Santa Cruz Biotechnology (SCBT), Santa Cruz, CA, USA; Sp3, H-225, SC-13018, SCBT; Sp4, V-20, SC-645, SCBT] was added to the probe/nuclear extract mixture and incubated for 20 min at room temperature. For competition, a 100-fold excess of unlabeled oligonucleotide was incubated with nuclear extract before adding labeled or non-specific oligonucleotide. Reaction mixtures were loaded onto 4% polyacrylamide gel and run at 200 V for 2.5 h in 0.25× TBE buffer. Results were visualized by autoradiography.

Mouse *GM3 synthase* with a known Sp1 binding site was designed as previously described (Xia *et al.*, 2005) and used as a positive control. Sp1 mutants with mutated sequences, as shown in Table 1, were used as negative controls.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed similar to those described previously (Johar *et al.*, 2012; Priya *et al.*, 2013a) with visual cortical tissue obtained from 1-day-old mouse pups. Immunoprecipitation was done with either 2 µg of Sp1, Sp3 or Sp4, or 2 µg anti-nerve growth factor receptor (NGFR) p75 polyclonal goat antibody (SC-6188, SCBT). Semi-quantitative PCR was performed using 1/20th of the precipitated chromatin. Primers targeting promoter sequences near the TSP of Na⁺/K⁺-ATPase subunit genes were designed (Table 2) using methods as previously described (Dhar *et al.*, 2008). GM3 Synthase and neurotrophin 3 (*Ntf3*) promoters with known Sp1 and Sp4 binding sites, respectively, were used as positive controls (Xia *et al.*, 2005; Ishimaru *et al.*, 2007), and β-actin (*Actb*) promoter was used as a negative control (Table 2). As the proximal promoters of Na⁺/K⁺-ATPase subunit genes are very GC-rich, reaction conditions for some amplicons were optimized by adding betaine (Sigma) and/or DMSO (Sigma). PCR products were visualized on 2% agarose gels stained with ethidium bromide.

Promoter mutagenesis studies

Luciferase reporter constructs of Na⁺/K⁺-ATPase subunit promoters were made by PCR cloning of the proximal promoter sequences using genomic DNA prepared from mouse N2a cells as template, digesting with *KpnI* and *HindIII*, and ligating the product directionally into pGL3 basic vector (Promega). Sequences of primers used for PCR cloning and mutagenesis primers are provided in Tables 3 and 4. Site-directed mutagenesis of putative Sp-factor binding sites on each promoter were generated using specific primers. All constructs were verified by sequencing. Each promoter construct was transfected into N2a cells in a 24-well plate using Lipofectamine 2000. Each well received 0.6 µg of reporter construct and 0.06 µg of pGL4.70 promoter-less Renilla luciferase vector (Promega). Some wells also received 1 µg of either of Sp1, Sp3 or Sp4 over-expressing constructs.

To further investigate the effect of KCl stimulation after mutating the Sp-factor binding site, transfected neurons were stimulated with 20 mM KCl in the culture media for 5 h as previously described (Dhar & Wong-Riley, 2009). Cell lysates were then harvested and measured for luciferase activity using the Dual-Luciferase Reporter System (Promega) according to the manufacturer's suggestions. For each sample, luciferase activity was normalized with Renilla luciferase activity.

Sp1, Sp3 and Sp4 silencing using shRNA vectors

Sp1 silencing was carried out using a combination of three to five Sp1-specific 19–25-nt short hairpin RNA (shRNA) sequences against Sp1 (sc-29488, SCBT). Sp3 and Sp4 silencing was carried out using specific shRNA sequences against murine *Sp3* and *Sp4* that were cloned into the pLKO.1 TRC cloning vector (Plasmid 10878; Addgene, Cambridge, MA, USA). Target *Sp3* and *Sp4* shRNA sequences were chosen from the RNAi Consortium's Public TRC Cloning Database at the Broad Institute and are listed in Table 5.

The pLKO.1 non-mammalian shRNA control vector, which contains a scrambled shRNA sequence that targets no known mammalian genes, was used as the negative control (SHC002; Sigma).

For transfection, N2a cells were plated at 60% confluency in six-well dishes. Cells were co-transfected the day after plating with either the *Sp1*, *Sp3* or *Sp4* shRNA constructs (4 µg) and enhanced green fluorescent protein (eGFP; 1 µg) vectors or the pLKO.1 non-mammalian control (4 µg) and the eGFP (1 µg) vector using 5 µL JetPrime transfection reagent (PolyPlus Transfection, Illkirch, France) per well. Puromycin at a final concentration of 5 µg/mL was added to the culture medium 1.5 days after transfection to select for purely transfected cells. Green fluorescence was observed to monitor transfection efficiency. Transfection efficiency for N2a cells was around 75%; however, puromycin selection effectively yielded almost 100% transfected cells. N2a cells transfected with shRNA against *Sp1*, *Sp3* or *Sp4* were further stimulated with KCl at a final concentration of 20 mM in the culture media for 5 h as previously described (Dhar & Wong-Riley, 2009). After 5 h of treatment, cells were harvested for RNA and protein isolation.

For transfection of primary neurons in culture, dissociated neurons were plated at a density of 2×10^5 cells per well in a six-well dish and transfected 5 days post-plating with *Sp1*, *Sp3* or *Sp4* shRNA constructs (2 µg) or the pLKO.1 non-mammalian control (2 µg) using 10 µL Neurofect transfection reagent per well. eGFP (0.5 µg) vector was added to visualize transfection efficiency in each well. Transfection efficiency was around 40–50%; however, puromycin selection effectively yielded almost 100% transfected cells.

Sp1, Sp3 and Sp4 over-expression and TTX treatment

The human *Sp1*, *Sp3* and *Sp4* cDNA clones were obtained from Open Biosystems (Lafayette, CO, USA) and cloned into pcDNA Dest40 vector using a Gateway Multisite Cloning kit (Invitrogen) according to the manufacturer's instructions and as described previously (Dhar *et al.*, 2013).

The transfection procedure for N2a cells and primary neuronal culture was similar to that described above with the modification that either 1.5 µg *Sp1*, *Sp3* or *Sp4* over-expression vector or 1 µg of the pcDNA3.1 empty vector, and 0.5 µg of eGFP vector were used for both N2a cells and primary neuronal cultures. Green fluorescence was used to monitor transfection efficiency. Transfected N2a cells were impulse blocked for 3 days with tetrodotoxin (TTX) at a final concentration of 0.4 µM and starting on the day after plating as previously described (Dhar & Wong-Riley, 2009). N2a cells were harvested for RNA and protein isolation 4 days after transfection, whereas primary neuronal cultures were harvested 2 days after transfection.

Real-time quantitative PCR

Total RNA was isolated using TriZol (Invitrogen), given DNAase treatment (Fermentas, Burlington, ON, Canada), and cDNA (Bio-Rad, Hercules, CA, USA) was synthesized according to the manufacturer's instructions. Real-time quantitative PCRs were carried out in the Cepheid Smart Cycler Detection system (Cepheid, Sunnyvale, CA, USA) and/or

CFX96 Touch System (BioRad) using the IQ Sybr Green SuperMix (170-8880; BioRad) following the manufacturer's instructions. Primer sequences are shown in Table 6. PCR runs comprised: hotstart for 2 min at 95°C, denaturation for 10 s at 95°C, annealing for 15 s according to the melting temperature of each primer, and extension for 10 s at 72°C for 15–30 cycles. Melt curve analyses verified the formation of single desired PCR product. Mouse *Actb* (β -actin) and *Gapdh* were the internal controls, and the 2^{-CT} method (Livak & Schmittgen, 2001) was used to calculate the relative amount of transcripts.

Western blot assays

Control and specific *Sp* shRNA-derived protein samples were loaded onto 10% SDS-PAGE gel and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad). Subsequent to blocking, blots were incubated in primary antibodies against Sp1 (1:1000), Sp3 (1:1000), Sp4 (1:1000), Na⁺/K⁺-ATPase α 1 (1:500; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), Na⁺/K⁺-ATPase α 3 (1:500; Pierce Thermo Scientific, Rockford, IL, USA) and Na⁺/K⁺-ATPase β 1 (1:750; Santa Cruz). β -Actin (1:3000; Sigma) served as loading control. Secondary antibodies used were goat-anti-rabbit and goat-anti-mouse antibodies (Vector Laboratories, Burlingame, CA, USA). Blots were then reacted with ECL reagent (Pierce Thermo Scientific) and exposed to autoradiographic film (RPI, Mount Prospect, IL, USA). Quantitative analyses of relative changes were done with Gel Doc (BioRad, Hercules, CA, USA).

Statistical analysis

Sister cultures were divided into various experimental groups and were processed simultaneously. For each experimental group, cells from at least five different wells were used. All values are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was carried out using SPSS software (Version 17.0; IBM Corporation, New York, USA). Raw data were analysed for the parametric or non-parametric distribution. Paired *t*-tests for parametric distribution and Wilcoxon signed-rank tests for non-parametric distribution were used to determine significance between any two groups. The alpha value was set at $P < 0.05$ (*n*, number of wells analysed). Graphs were produced using Microsoft Office Excel software (Version 2010).

Results

Promoters of the Na⁺/K⁺-ATPase subunit gene have a conserved Sp binding site

The proximal promoters of murine Na⁺/K⁺-ATPase subunit gene isoforms were analysed *in silico* with DNA sequence 1 kb 5' upstream and 1 kb 3' beyond the TSP. Several GC box motifs (GGGCGG or CCCGCC) were found on *Atp1a1* and *Atp1b1* promoters. Along with consensus Sp-factor site, *Atp1b1* also had a slightly modified GC-box motif (GCCGCCG and AGGCGGA) close to the TSP. The *Atp1a3* promoter had a CT box motif (CCTCCCC).

Sp4 is abundant in visual cortex neurons and forms more distinct supershift bands than Sp3 or Sp1 on Na⁺/K⁺-ATPase subunit promoters

To determine the abundance of Sp-factors in murine visual cortex and HeLa cells, equal amounts of whole cell extracts were probed for Sp1, Sp3 and Sp4 proteins. Using paired *t*-

tests, Sp4 was found to be more abundant in visual cortical extract than in the HeLa extract, whereas the reverse was true for Sp1 ($P < 0.001$ for all; Fig. 1A). Sp3 had two different isoforms in the HeLa extract. The longer isoform (110 kb) was almost equal in the HeLa and visual cortical extract, whereas the shorter isoform was virtually absent in the visual cortical extract (Fig. 1A).

EMSAs were carried out *in vitro* using ^{32}P -labeled oligonucleotide probes to determine the specificity of individual Sp-factor binding to murine Na^+/K^+ -ATPase subunit gene promoters (Fig. 1B and C). GM3 synthase promoter with known Sp1 binding site served as a positive control (Xia *et al.*, 2005). When incubated with murine visual cortical nuclear extract (Fig. 1B, lanes 1–5), it formed shift bands with Sp1, Sp3 and Sp4 that migrated together (Fig. 1B, lane 1). However, it formed a more prominent supershift band with Sp4 antibody (Fig. 1B, lane 5) but a relatively light supershift band with Sp1 (Fig. 1B, lane 3) or Sp3 (Fig. 1B, lane 4) antibodies. When incubated with HeLa nuclear extract (Fig. 1B, lanes 6–10), the GM3 synthase promoter formed shift bands with Sp1, Sp3 and Sp4 (Fig. 1B, lane 6) that migrated together and a second shift band for Sp3 that migrated at a lower molecular weight (Fig. 1B, lane 6). It formed a light supershift band with Sp4 (Fig. 1B, lane 10) and Sp3 (Fig. 1B, lane 9) antibodies, but formed two supershift bands with Sp1 antibody. The first Sp1 supershift band was distinct, but the second one was a faint smear that migrated more slowly than that of the first supershift band (Fig. 1B, lane 8). When an excess of unlabeled probe was added as a competitor, no shift band was formed with either extract (Fig. 1B, lanes 2 and 7). The intensity and positions of shift and supershift bands obtained with these two extracts was proportional to the abundance of proteins as shown in Fig. 1A.

Atp1a1, *Atp1a3* and *Atp1b1* each formed a specific DNA-protein shift complex when incubated with murine visual cortical nuclear extract (Fig. 1C, lanes 1, 8 and 15). They each formed a DNA–protein–antibody supershift complex that was prominent with the Sp4 antibody (Fig. 1C, lanes 5, 12 and 19), very faint with the Sp1 antibody (Fig. 1C, lanes 3, 10 and 17), and moderate with the Sp3 antibody (Fig. 1C, lanes 4, 11 and 18). Competition with excess unlabeled probes eliminated the shift complexes for *Atp1a1*, *Atp1a3* and almost completely for *Atp1b1* (Fig. 1C, lanes 2, 9 and 16). For *Atp1a1* and *Atp1a3*, an excess of unlabeled oligos with mutated Sp sites was not able to out-compete the specific shift bands (Fig. 1C, lanes 6 and 13). The *Atp1b1* probe had two separate Sp binding sites. An excess of unlabeled mutated site 1 oligos caused a reduction in the intensity of the shift band, presumably because site 2 was still intact and was able to compete partially (Fig. 1C, lane 20). An excess of unlabeled mutated site 2 oligos was not able to compete (Fig. 1C, lane 21), and nor was an excess of mutated site 1 plus site 2 (Fig. 1C, lane 22). This indicates that site 2 was a much stronger, functional site than site 1. Labeled oligos with mutated Sp sites on *Atp1a1* and *Atp1a3* served as negative controls, and no shift bands were detected (Fig. 1C, lanes 7 and 14). Labeled oligos of *Atp1b1* with mutated site 1 showed a shift band (Fig. 1C, lane 23), but mutated site 2 did not (Fig. 1C, lane 24).

In vivo interaction of Sp factors with Na^+/K^+ -ATPase subunit promoters

To verify *in vivo* interactions of Sp factors with promoters of Na^+/K^+ -ATPase subunit genes, ChIP assays were performed on cells obtained from murine visual cortex. PCR was carried

out with primers encompassing putative Sp binding sites on chromatin immunoprecipitated with various antibodies. A 0.5% dilution of input chromatin was used as a standard to indicate the efficiency of the PCR. The promoters of GM3 synthase and neuron-specific *Ntf3* gene were used as positive controls, whereas β -actin exon 5 (*Actb*) served as a negative control. Promoters of GM3 synthase, *Ntf3*, *Atp1a1*, *Atp1a3* and *Atp1b1*, each produced a PCR product from DNA immunoprecipitated with anti-Sp1, anti-Sp3 and anti-Sp4 antibodies at positions identical to those of the genomic DNA controls (input) (Fig. 2). The bands obtained with anti-Sp4 antibody are more distinct than that of anti-Sp3 or anti-Sp1 for the Na⁺/K⁺-ATPase isoforms and *Ntf3*. On the other hand, *Actb* (β -actin) yielded no product (Fig. 2). In all cases, immunoprecipitation with NGFR antibodies, an additional negative control, did not yield any PCR product, confirming the specificity of the ChIP reaction. The no-antibody controls (Blank) also gave no PCR product.

Investigation of mutated Sp binding sites on promoter activity

Based on the results obtained with the ChIP primers and EMSA probes that formed Sp-specific complexes, site-directed mutations of these putative Sp binding sites on *Atp1a1*, *Atp1a3* and *Atp1b1* promoters were constructed. Mutating the Sp binding sites on *Atp1a1* and *Atp1a3* promoters led to a significant decrease in their activity as compared with wild type controls ($P < 0.001$ for *Atp1a1*, paired *t*-test; and $P < 0.05$ for *Atp1a3*, Wilcoxon signed-rank test) (Fig. 3). Mutating site 1 on the *Atp1b1* promoter caused a small but significant decrease ($P < 0.05$, paired *t*-test), but mutating site 2 resulted in a much greater decrease in promoter activity ($P < 0.001$, paired *t*-test) (Fig. 3).

To detect the presence of any other Sp binding sites, wild-type and mutant constructs were transfected with specific Sp factor over-expressing vectors. All three promoters (*Atp1a1*, *Atp1a3* and *Atp1b1*) responded to an over-expression of Sp1, Sp3 or Sp4 with a significant increase in luciferase activity as compared to wild-type controls ($P < 0.001$ for all, paired *t*-tests) (Fig. 3). Mutating the Sp binding sites on *Atp1a1* and *Atp1a3* promoters eliminated their responsiveness to the over-expressing vectors (Fig. 3). For *Atp1b1*, site 1 mutants still responded to the over-expression vectors, although to a lesser degree ($P < 0.05$ for all as compared with mutants, paired *t*-tests), but site 2 mutants did not respond to the over-expression vectors (Fig. 3). This indicates that there were no additional Sp binding sites for promoters of *Atp1a1*, *Atp1a3* and *Atp1b1*.

Sp-factor silencing by RNA interference

The effects of shRNA-mediated silencing of Sp1, Sp3 and Sp4 on the expression of Na⁺/K⁺-ATPase genes in N2a cells and in cultured murine visual cortical neurons were then determined. *Cox2* (mitochondria-encoded COX subunit), *Cox7c* (nucleus-encoded COX subunit) and *Ntf3* served as positive controls. In N2a cells, silencing of *Sp1* led to a significant decrease in the expressions of Sp1, *Atp1a1*, *Atp1a3* and *Atp1b1* proteins ($P < 0.001$ for all, paired *t*-tests) (Fig. 4A) and those of *Sp1*, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* mRNAs as compared with control vectors with a scrambled shRNA sequence ($P < 0.001$ for all except *Atp1a3*, paired *t*-tests; $P < 0.05$ for *Atp1a3*, Wilcoxon signed-rank test) (Fig. 4B). Silencing of *Sp3* led to a significant decrease in the expressions of Sp3, *Atp1a1*, *Atp1a3* and *Atp1b1* proteins ($P < 0.001$ for the first three, and $P < 0.01$ for *Atp1b1*, paired *t*-

tests) (Fig. 4A) and those of *Sp3*, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* transcripts as compared with controls ($P < 0.001$ for all except *Atp1a3*, paired *t*-tests; $P < 0.05$ for *Atp1a3*, Wilcoxon signed-rank test) (Fig. 4B). *Atp1b1* protein is heavily glycosylated and formed two or more bands in SDS-PAGE. Silencing of *Sp4* led to a significant decrease in the expressions of *Sp4*, *Atp1a1*, *Atp1a3* and *Atp1b1* proteins ($P < 0.001$ for all, paired *t*-tests) (Fig. 4A) and those of *Sp4*, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* mRNAs as compared with controls ($P < 0.001$ for the first four and $P < 0.01$ for *Atp1b1*, paired *t*-tests; $P < 0.05$ for *Atp1a3*, Wilcoxon signed-rank test) (Fig. 4B).

In primary cultures of murine visual cortical neurons, silencing of *Sp1* led to a significant decrease in the mRNA levels of *Sp1* ($P < 0.001$), *Cox2* ($P < 0.01$), *Cox7c* ($P < 0.01$), *Atp1a1* ($P < 0.05$), *Atp1a3* ($P < 0.01$) and *Atp1b1* ($P < 0.01$). All paired *t*-test comparisons were made with controls transfected with pLKO.1 non-mammalian vectors (Fig. 4C). On the other hand, *Ntf3* transcripts showed an increase ($P < 0.05$, paired *t*-test) (Fig. 4C). Silencing of *Sp3* led to a significant decrease in the mRNA expressions of *Sp3*, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* ($P < 0.001$ for all except for *Atp1a3*, which is $P < 0.01$, paired *t*-tests) (Fig. 4C). Silencing of *Sp4* led to a significant decrease in the gene expressions of *Sp4*, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* ($P < 0.001$ for all, paired *t*-tests) (Fig. 4C). *Ntf3* message, however, showed an increase ($P < 0.001$, paired *t*-test) (Fig. 4C), confirming that *Ntf3* is negatively regulated by Sp factors (Ishimaru *et al.*, 2007).

Sp-factor over-expression

The effects of over-expressing *Sp1*, *Sp3* and *Sp4* on Na^+/K^+ -ATPase subunit genes in N2a cells and cultured murine visual cortical neurons were also determined. In N2a cells, over-expressing *Sp1* led to a six-fold increase in its protein level ($P < 0.001$, paired *t*-test) (Fig. 5A) and a 30-fold increase in its mRNA level as compared with empty vector controls ($P < 0.001$, paired *t*-test) (Fig. 5B). It also led to a significant increase in *Atp1a1*, *Atp1a3* and *Atp1b1* protein levels ($P < 0.001$ for all, paired *t*-tests) (Fig. 5A) and *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* mRNA levels ($P < 0.001$ for the first four, paired *t*-tests; $P < 0.05$ for *Atp1b1*, Wilcoxon signed-rank test) (Fig. 5B). Over-expressing *Sp3* led to a 9.5-fold increase in its protein level ($P < 0.001$, paired *t*-test) (Fig. 5A) and a 30-fold increase in its mRNA level ($P < 0.001$, paired *t*-test) (Fig. 5B). It also led to a significant increase in *Atp1a1*, *Atp1a3* and *Atp1b1* protein levels ($P < 0.001$ for all, paired *t*-tests) (Fig. 5A) and *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* mRNA levels ($P < 0.001$ for the first four, paired *t*-tests; $P < 0.05$ for *Atp1b1*, Wilcoxon signed-rank test) (Fig. 5B). Over-expressing *Sp4* led to an eight-fold increase in its protein level ($P < 0.001$, paired *t*-test) (Fig. 5A) and a 29-fold increase in its mRNA level ($P < 0.001$, paired *t*-test) (Fig. 5B). It also led to a significant increase in *Atp1a1*, *Atp1a3* and *Atp1b1* protein levels ($P < 0.001$ for all, paired *t*-test) (Fig. 5A) and *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* mRNA levels as compared with empty vector controls ($P < 0.001$ for the first four, paired *t*-tests; $P < 0.05$ for *Atp1b1*, Wilcoxon signed-rank test) (Fig. 5B).

In cultures of murine visual cortical neurons, over-expressing *Sp1* led to a 60-fold increase in its mRNA level ($P < 0.001$, paired *t*-test) (Fig. 5C). It also led to a significant increase in the mRNA levels of *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* as compared with empty

vector controls ($P < 0.001$ for all except *Atp1a1*, which was $P < 0.01$, paired t -tests) (Fig. 5C). Over-expressing Sp3 led to a 30-fold increase in its mRNA level ($P < 0.001$, paired t -test) (Fig. 5C). It also led to a significant increase in the levels of *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* transcripts ($P < 0.001$ for all except *Cox7c*, which was $P < 0.01$, paired t -tests) (Fig. 5C). Over-expressing Sp4 led to a 29-fold increase in its mRNA level ($P < 0.001$, paired t -test) (Fig. 5C). It also led to a significant increase in the levels of *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* transcripts as compared with empty vector controls ($P < 0.001$ for all except *Cox2*, which was $P < 0.01$, paired t -tests) (Fig. 5C). On the other hand, *Ntf3* mRNAs showed a significant decrease with Sp4 over-expression ($P < 0.01$, paired t -test) but no change with Sp1 or Sp3 over-expression (Fig. 5C). This indicates that *Ntf3* is negatively regulated by Sp4, consistent with previous reports (Ishimaru *et al.*, 2007).

Effect of KCl-induced depolarization and TTX-induced impulse blockade

To determine if the Sp factors themselves are affected by changes in neuronal activity, N2a cells were depolarized by 20 mM KCl for 5 h (Liang & Wong-Riley, 2006; Yang *et al.*, 2006) or impulse blocked by 0.4 μ M TTX for 3 days (Liang & Wong-Riley, 2006; Dhar & Wong-Riley, 2009). Depolarizing stimulation up-regulated mRNAs of *Sp1* ($P < 0.05$, Wilcoxon signed-rank test) and *Sp4* ($P < 0.001$, paired t -test) as compared with unstimulated controls, but *Sp3* was not affected (Fig. 6A). Impulse blockade led to a down-regulation of *Sp1* ($P < 0.05$, Wilcoxon signed-rank test) and *Sp4* ($P < 0.001$, paired t -test), but *Sp3* was not affected (Fig. 6A). Thus, Sp3 is not regulated by neuronal activity.

The effect of depolarizing stimulus was also evaluated after incorporating either wild-type promoter or promoters with mutated Sp site cloned upstream of the luciferase gene in pGL3 vectors. As shown in Fig. 6B, depolarizing stimulation resulted in a significant increase in the activity of *Atp1a1*, *Atp1a3* and *Atp1b1* promoters as compared with unstimulated wild-type controls ($P < 0.001$ for all, paired t -tests). To determine if the up-regulation of Na^+/K^+ -ATPase subunit gene promoters by KCl was dependent on the binding of Sp factors, promoters with mutated Sp binding sites were subjected to KCl treatment. Results indicated that mutant *Atp1a1* and *Atp1a3* promoters failed to respond to depolarization stimulation, and their activity remained significantly lower than those of wild-type controls ($P < 0.001$ for all, paired t -tests) (Fig. 6B). The *Atp1b1* promoter with mutated site 1 was still able to respond to depolarizing stimulation, whereas mutating site 2 significantly reduced its response to depolarization as compared with that of wild-type controls ($P < 0.001$, paired t -test) (Fig. 6B).

To determine if knocking down various Sp factors would have a detrimental effect on the up-regulation of Na^+/K^+ -ATPase subunit genes by KCl, N2a cells transfected with shRNA against Sp1, Sp3 or Sp4 were subjected to 20 mM KCl for 5 h. N2a cells transfected with scrambled vectors served as controls, and *Cox7c* as the positive control gene. As shown in Fig. 6C, depolarizing stimulation resulted in a significant increase in the transcript levels of *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* as compared with unstimulated controls ($P < 0.001$ for all, paired t -tests). When *Sp1* or *Sp4* was silenced, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* failed to respond to KCl. On the other hand, silencing of *Sp3* still permitted some up-regulation of

these four transcripts by KCl ($P < 0.05$ for all, paired t -tests), indicating that Sp3 levels and hence its effect on the expression of target genes are not regulated by neuronal activity.

To evaluate the effect of impulse blockade on Na^+/K^+ -ATPase subunits, N2a cells were treated with TTX for 3 days. N2a cells transfected with empty vectors were taken as controls, and *Cox7c* was the positive control gene. TTX significantly reduced the transcript levels of *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* ($P < 0.001$ for the first three, paired t -tests; $P < 0.05$ for *Atp1b1*, Wilcoxon signed-rank test) (Fig. 6D). This indicates an overall suppressive effect of TTX on Na^+/K^+ -ATPase subunit genes in neurons. To determine if over-expressing Sp factors would rescue the detrimental effect of TTX, N2a cells were transfected with Sp1, Sp3 or Sp4 expression vectors before subjecting them to 3 days of TTX. Results indicated that over-expressing any one of the three Sp factors effectively rescued *Atp1a1*, *Atp1a3* and *Atp1b1* from being down-regulated by TTX (values were all the same or above those of empty vector controls; paired t -tests) (Fig. 6D).

Sp1 and Sp4 were up regulated by KCl-induced depolarization and down-regulated by TTX-induced impulse blockade. In the presence of Sp1 or Sp4 shRNA, KCl-induced up-regulation of Na^+/K^+ -ATPase subunits was prevented. Both Sp1 and Sp4 rescued Na^+/K^+ -ATPase subunits down-regulated by TTX-induced impulse blockade. Hence, both Sp1 and Sp4 are involved in the activity-dependent regulation of Na^+/K^+ -ATPase subunits in neurons.

Homology of Sp binding sites

Functional Sp binding sites on the 5'-untranslated regions of *Atp1a1*, *Atp1a3* and *Atp1b1* genes were conserved among humans, mice and rats (Fig. 7).

Discussion

Using multiple approaches, the present study documents that Sp4 plays a major role in the positive regulation of Na^+/K^+ -ATPase subunits $\alpha 1$, $\alpha 3$ and $\beta 1$ in primary neurons. Silencing Sp4 prevented depolarization-induced up-regulation of these subunits, and over-expressing Sp4 rescued them from TTX-induced down-regulation. The expression of these subunits as well as that of Sp4 itself is activity-dependent.

To date, the known specificity protein/Krüppel-like family (Sp/KLF) consists of 25 members (for a review see Suske *et al.*, 2005). Among them, Sp1, Sp3 and Sp4 are structurally similar and share critical amino acid homology in their DNA binding domains. Thus, they compete for the same GC-boxes and other closely related sequences located on the promoters of many genes (for reviews see Suske, 1999; Suske *et al.*, 2005). Sp1 and Sp3 are ubiquitous, but Sp4 is neuron-specific and its expression increases with development (Supp *et al.*, 1996; Suske, 1999; Safe & Abdelrahim, 2005). An abnormal level of Sp4 is associated with human diseases. For example, a high level of Sp4 is detected in brains of Alzheimer's patients (Villa *et al.*, 2012), whereas a reduced level is associated with bipolar disorders (Pinacho *et al.*, 2011). Polymorphism in the Sp4 gene is linked to schizophrenia and other psychiatric disorders (Zhou *et al.*, 2010). Sp4 hypomorphic mice have various neuronal abnormalities, such as vacuolization in the hippocampus, sensory and motor

deficits, loss of contextual memory, and loss of reproductive capability, despite the presence of a normal-looking reproductive system (Supp *et al.*, 1996; Zhou *et al.*, 2005). Sp4 has both activating and repressing domains for transcription (Suske, 1999; Wierstra, 2008). During neuronal differentiation, Sp1 dissociates from the promoter region of Sp target genes (Milagre *et al.*, 2012), and Sp4 increases its transcriptional repressor function on genes controlled by Sp1, such as those involved in mitosis (Mao *et al.*, 2006, 2007). With development, there is a switch in dominance from Sp1 to Sp4 in neurons (Mao *et al.*, 2006, 2007). Recently, we found that Sp4 regulates the expression of all 13 subunits of *COX* in neurons (Johar *et al.*, 2013). Thus, it regulates genes vital for energy generation (*COX*) as well as genes involved in energy consumption (Na^+/K^+ -ATPase; present study) in neurons.

The present study documents for the first time how the silencing and over-expression of Sp factors affect Na^+/K^+ -ATPase subunit genes in murine visual cortical neurons and N2a cells. Silencing or over-expressing Sp4 had a much greater effect on primary neurons than on N2a cells, whereas silencing or over-expressing Sp1 or Sp3 had a much lower effect on primary neurons than on N2a cells. These findings are consistent with previous reports that Sp1 and Sp3 are present only in low amounts in the brain (Kobayashi & Kawakami, 1997) and that the predominant isoform in adult neurons is Sp4 (Mao *et al.*, 2006, 2007). Among Sp factors, Sp4 is a major regulator of Na^+/K^+ -ATPase subunit gene expression in primary neurons.

Na^+/K^+ -ATPase holozyome is made up of α and β subunits. The larger α subunit is a major determinant of ligand-binding, ATP hydrolysis, and the transport of Na^+ and K^+ ions (Kaplan, 2002; Geering, 2008). The four α -subunits ($\alpha 1$ to $\alpha 4$) are distributed in a cell-specific manner. In the brain, $\alpha 1$ is found in both neurons and glia, $\alpha 2$ in glia, and $\alpha 3$ in neurons, but $\alpha 4$ is absent (McGrail *et al.*, 1991; Morth *et al.*, 2009; Poulsen *et al.*, 2010; Bottger *et al.*, 2011). The smaller β isoform is heavily glycosylated and has three isoforms ($\beta 1$ to $\beta 3$). In the brain, $\beta 1$ is found in neurons and glia, $\beta 2$ in glia, and $\beta 3$ in oligodendrocytes (McGrail *et al.*, 1991; Bottger *et al.*, 2011). Hence, functional enzymes in neurons possess either $\alpha 1$ or $\alpha 3$ and β subunits. The $\alpha 1$ subunit has a greater affinity for Na^+ and lower affinity for K^+ and ATP than $\alpha 3$. Thus, $\alpha 1$ -containing enzymes probably operate under normal physiological conditions, whereas $\alpha 3$ -containing enzymes may participate more in restoring membrane potentials of highly active neurons (Jewell & Lingrel, 1991; Munzer *et al.*, 1994; Habiba *et al.*, 2000; Blanco, 2005). The functional Sp-binding site on $\alpha 1$ promoter (present study) is well conserved in the rat brain, kidney and liver (Yagawa *et al.*, 1992; Nomoto *et al.*, 1995; Kobayashi & Kawakami, 1997). The $\alpha 3$ isoform is expressed mainly in excitable tissues, such as neurons and cardiac myocytes (Kamitani *et al.*, 1992; Bottger *et al.*, 2011). The human $\alpha 3$ promoter has two consensus Sp sites (Benfante *et al.*, 2005). In rats, an atypical Sp binding site is located very close to the upstream site reported in the human $\alpha 3$ promoter (Murakami *et al.*, 1997). The functional Sp site on the murine $\alpha 3$ promoter (present study) is comparable to that of the rat promoter. The $\beta 1$ subunit associates with the α subunit in the endoplasmic reticulum as a prerequisite to the release of the enzyme from this organelle and its insertion into the plasma membrane (for reviews see Blanco, 2005; Geering, 2008). In tissues including the brain, the level of $\beta 1$ -mRNA correlates with the activity of the enzyme (Gick *et al.*, 1993). However, the

abundance of $\beta 1$ mRNA is significantly lower than that of the α subunit in many tissues including the brain (Sweadner, 1989; Geering, 1990; Shao & Ismail-Beigi, 2001), suggesting that β -subunit synthesis is tightly regulated and that it controls the abundance of the holoenzyme. The positive regulation of the $\beta 1$ subunit by Sp factors, especially Sp4, directly affects the regulation of the entire holoenzyme in neurons.

Previously, we found that nuclear respiratory factor 1 (NRF-1) negatively regulates $\alpha 1$, positively regulates $\beta 1$ and does not regulate $\alpha 3$ in neurons (Johar *et al.*, 2012). Thus, NRF-1 and Sp factors, especially Sp4, operate in an opposing, complementary, as well as concurrent and parallel manner with respect to the sodium pump. The mechanism is opposing for the two transcription factors with respect to $\alpha 1$ (positive for Sp and negative for NRF-1). It is complementary for $\alpha 3$ (Sp regulates but NRF-1 does not), and concurrent and parallel for $\beta 1$ (both are positive regulators) (Johar *et al.*, 2012; present study). The negative regulation of $\alpha 1$ by NRF-1 and the positive regulation of $\alpha 3$ by Sp4 may be advantageous to neurons during repeated firing of action potentials, when activity of the $\alpha 3$ -containing enzyme is in greater demand than that of the $\alpha 1$ -containing enzymes. Neuronal activity also stimulates both NRF-1 (Liang & Wong-Riley, 2006; Yang *et al.*, 2006) and Sp4 (present study). Hence, with increased neuronal activity, the level of $\alpha 1$ is kept in check via its negative regulation by NRF-1, whereas that of $\alpha 3$ is enhanced by the positive regulation from Sp4. The level of $\beta 1$, and hence of the whole enzyme, is positively regulated by both NRF-1 and Sp4 under both basal and excited states.

Previously, we reported that NRF-1 mediates the tight coupling between energy metabolism, neuronal activity and energy consumption by regulating the expressions of all 13 COX subunit genes (Dhar *et al.*, 2008), specific glutamatergic neurochemicals (Dhar & Wong-Riley, 2009; Dhar *et al.*, 2009a,b), and $\alpha 1$ and $\beta 1$ subunits of Na^+/K^+ -ATPase (Johar *et al.*, 2012). Our recent studies identified Sp factors in regulating the expression of all 13 subunits of COX in N2a cells (Dhar *et al.*, 2013) and in primary neurons (Johar *et al.*, 2013). In addition, we found that it is Sp4, and not Sp1 or Sp3, that regulates the expression of key glutamatergic receptor subunits in neurons (Priya *et al.*, 2013b). Thus, Sp factors, especially the neuron-specific Sp4, also mediate the coupling of energy generation, neuronal activity and energy consumption at the transcriptional level in neurons.

Acknowledgments

This work was supported by National Institutes of Health Grant EY018441 and Grant T32-EY14537. We thank Shilpa Dhar and Bindu Nair for their professional help and J. Fogerty for providing entry vectors for Sp1, Sp3 and Sp4 over-expression vectors. We also thank Mr Jignashu Yagnik for his professional assistance with statistical analyses.

Abbreviations

ChIP	chromatin immunoprecipitation
COX	cytochrome c oxidase
EMSA	electrophoretic mobility shift assay
N2a	Neuro-2a neuroblastoma

NGFR	growth factor receptor
NRF-1	nuclear respiratory factor 1
shRNA	short hairpin RNA
Sp1	specificity factor 1
Sp3	specificity protein 3
Sp4	specificity protein 4
TSP	transcription start point
TTX	tetrodotoxin

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A Protein levels

B GM3 synthase

Gene promoter	GM3 synthase									
³² P-labeled oligo	+	+	+	+	+	+	+	+	+	+
Unlabelled oligo	-	+	-	-	-	-	+	-	-	-
Visual Cortex NE	+	+	+	+	+	-	-	-	-	-
HeLa NE	-	-	-	-	-	+	+	+	+	+
Anti-Sp1	-	-	+	-	-	-	-	+	-	-
Anti-Sp3	-	-	-	+	-	-	-	-	+	-
Anti-Sp4	-	-	-	-	+	-	-	-	-	+

C Isoforms of Na⁺/K⁺-ATPase

Gene promoter	<i>Atp1a1</i>								<i>Atp1a3</i>								<i>Atp1b1</i>							
³² P-labeled oligo	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-
Unlabelled oligo	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Unlabelled mutant oligo	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-
³² P-labeled mutant oligo	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+
Visual cortex NE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anti-Sp1	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Anti-Sp3	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Anti-Sp4	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-

Fig. 1.

Relative protein levels of Sp factors and *in vitro* EMSA and supershift assays. (A) Relative levels of Sp factors in whole cell extracts of mouse visual cortex and HeLa cells. Sp4 levels were significantly higher in murine visual cortical tissue than in HeLa cells, whereas the reverse was true for Sp1. The shorter isoform of Sp3 was more prevalent in HeLa cells and was virtually absent in murine visual cortex. (B,C) *In vitro* binding of Sp factors to GM3 synthase and *Atp1a1*, *Atp1a3* and *Atp1b1* promoters. ³²P-labeled oligonucleotides (oligos), excess unlabeled oligos as competitors, excess unlabeled mutant oligos as competitors,

HeLa nuclear extract, nuclear extract from murine visual cortex and Sp factor antibodies (against Sp1, Sp3 or Sp4) added to or absent from the reactions are indicated by a + or a – sign, respectively, above each lane. Arrows mark the specific shift, supershift and non-specific bands. GM3 synthase served as a positive control and showed shift (B, lanes 1 and 6) and supershift bands for Sp1 (B, lanes 3 and 8), Sp3 (B, lanes 4 and 9) and Sp4 (B, lanes 5 and 10), whereas the addition of an excess of unlabeled competitors did not yield any band (B, lanes 2 and 7). Note that the HeLa nuclear extract gave a stronger Sp1 supershift band than the murine visual cortical nuclear extract (lane 8 versus lane 3), but the opposite was true for Sp4 supershift (lane 10 versus lane 5). Such differences were proportional to those of protein levels shown in A. For *Atp1a1*, *Atp1a3* and *Atp1b1* oligos, shift bands were found for Sp factors (C, lanes 1, 8 and 15), and supershift bands were relatively faint for Sp1 (C, lanes 3, 10 and 17), moderate for Sp3 (C, lanes 4, 11 and 18) and more prominent for Sp4 (C, lanes 5, 12 and 19). Pre-incubation with unlabeled oligos out-competed interactions for *Atp1a1* and *Atp1a3* (C, lanes 2 and 9), but less completely for *Atp1b1* (C, lane 16). Unlabeled *Atp1a1* and *Atp1a3* oligos with mutated Sp sites failed to compete (C, lanes 6 and 13). For *Atp1b1*, which contained two Sp sites, unlabeled site 1 mutant oligos partially competed (C, lane 20), whereas unlabeled site 2 mutant oligos and unlabeled site 1 plus site 2 mutant oligos failed to compete the shift reactions (C, lanes 21 and 22, respectively). ³²P-labeled *Atp1a1* and *Atp1a3* promoters with mutated Sp sites yielded no shift bands (C, lanes 7 and 14). For *Atp1b1*, ³²P-labeled site 1 mutants showed a relatively strong shift band (C, lane 23), but labeled site 2 mutants failed to show any band (C, lane 24). This indicates that site 2 was a much stronger Sp binding site. Note that Sp4 had much stronger supershift bands than Sp1 or Sp3 for all three Na⁺/K⁺-ATPase promoters.

Fig. 2.

In vivo ChIP assays for the binding of Sp factors to Na⁺/K⁺-ATPase subunit promoters. PCRs were performed on murine visual cortical chromatin precipitated with anti-Sp1 (Sp1 lane), Sp3 (Sp3 lane) or Sp4 (Sp4 lane) antibodies. Anti-NGFR p75 antibody and no antibody served as negative controls (NGFR and Blank lanes, respectively). Input lane represents 0.5% of input chromatin. GM3 synthase and Neurotrophin 3 (*Ntf3*) promoters served as positive controls, and β -actin (*Actb*) as a negative control. Interactions were present for all three Sp factors with *Atp1a1*, *Atp1a3* and *Atp1b1* promoters. However, the

bands obtained with anti-Sp4 are more distinct than those with anti-Sp3 or anti-Sp1 antibodies.

Fig. 3.

Mutation (*mut*) of Sp-factor binding sites decreased promoter activity of Na⁺/K⁺-ATPase subunit genes compared with that of wild-type (*wt*). Mutating the Sp binding site in *Atp1a1* and *Atp1a3* promoters resulted in a significant decrease in luciferase activity. For *Atp1b1*, two different mutants were generated. Site 1 mutant had a 20% decrease in promoter activity, whereas site 2 mutant had a 60% reduction. Over-expressing Sp1, Sp3 or Sp4 together with *Atp1a1*, *Atp1a3* or *Atp1b1* wild-type constructs resulted in significant increases in luciferase activity. However, over-expressing each of the Sp factors together

with the *Atp1a1* or *Atp1a3* promoter containing mutated Sp sites resulted in no change in luciferase activity. For *Atp1b1*, over-expressing each of the Sp factors together with site 1 mutation did result in some increase in promoter activity, whereas with site 2 mutation it did not. $n = 5$ for each group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $X_{\text{non-significant}}$. All mutants and wild-type with over-expression are compared with respective wild-types. Mutants with over-expression were compared with respective mutant constructs.

Fig. 4.

Silencing of *Sp4* in primary neurons had a greater effect on reducing the expression of Na^+/K^+ -ATPase subunit genes than that of *Sp3* or *Sp1*. Silencing of Sp factors was carried out with shRNA vectors, and *Ntf3* and *COX* subunits (*Cox2*, mitochondria-encoded; *Cox7c*, nucleus-encoded) served as positive controls. (A) Western blots revealed protein levels of Sp factors, Atp1a1, Atp1a3 and Atp1b1 in N2a cells. Both bands of Atp1b1 were included in the measurement. Both isoforms of Sp3 were down-regulated by shRNA against Sp3. β -Actin served as a loading control. $n = 5$ for each data point. (B) Real-time PCR analysis of

mRNA levels in N2a cells revealed a significant down-regulation of respective Sp factors, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* with shRNA against each of the Sp factors. $n = 6$ for each data point. (C) Real-time PCR analysis of mRNA levels in cultured primary neurons of mouse visual cortex revealed a significant down-regulation of Sp factors, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* with shRNA against each of the Sp factors. On the other hand, *Ntf3* showed a significant up-regulation with Sp4 shRNA. This confirms a negative regulation of *Ntf3* by Sp factors (31). $n = 6$ for each data point. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and X non-significant when compared with controls.

Fig. 5.

Over-expressing Sp factors increased protein and mRNA levels of Na^+/K^+ -ATPase subunit genes in mouse visual cortex. *Ntf3* and *COX* subunits (*Cox2*, mitochondria-encoded; *Cox7c*, nucleus-encoded) served as positive controls. (A) Western blots revealed an up-regulation of protein levels of Sp factors, Atp1a1, Atp1a3 and Atp1b1 in N2a cells. β -Actin served as a loading control. $n = 5$ for each data point. (B) Real-time PCR analysis of mRNA levels in N2a cells revealed significant up-regulation of Sp factors, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* with over-expression of each of the Sp factors. $n = 6$ for each data point. (C) Real-

time PCR analysis of mRNA levels in primary neurons of mouse visual cortex revealed a significant up-regulation of *Sp* factors, *Cox2*, *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* with over-expression of each of the *Sp* factors. However, *Ntf3* showed a significant down-regulation with *Sp4* over-expression. $n = 6$ for each data point. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; X non-significant when compared with controls.

Fig. 6.

The regulation of Na^+/K^+ -ATPase subunit genes by Sp factors is associated with neuronal activity. (A) KCl-induced depolarization significantly up-regulated mRNA levels of Sp1 and Sp4 while Sp3 was not affected. TTX-induced impulse blockade significantly down-regulated mRNA levels of Sp1 and Sp4 while Sp3 was not affected. NRF-1 served as a positive control, as it is known to be regulated by neuronal activity. (B) KCl depolarization significantly increased promoter activity in all wild-type promoters. However, mutating the Sp binding sites on *Atp1a1* and *Atp1a3* promoters prevented such an increase in their activity. For *Atp1b1*, mutating the putative Sp binding site 2 prevented the promoter from responding to KCl, whereas mutating site 1 did not. All wild-type + KCl and mutant + KCl were compared with the wild-type. (C) KCl-induced depolarization significantly increased mRNA levels of all three Na^+/K^+ -ATPase subunit genes compared with those of controls.

Knocking down Sp1 or Sp4 with shRNA prevented the increase in *Cox7c*, *Atp1a1*, *Atp1a3* and *Atp1b1* mRNAs normally induced by KCl. On the other hand, knocking down Sp3 led to an increase in RNA levels of these three transcripts as compared with those of controls. (D) TTX-induced impulse blockade led to reduced levels of *Cox7c* and all three Na⁺/K⁺-ATPase subunit transcripts as compared with controls. Over-expression of Sp-factors rescued all three Na⁺/K⁺-ATPase subunit transcripts from TTX-induced down-regulation. *n* = 5 for each data point; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; and ^Xnon-significant when compared with controls.

Atp1a1

H		GTTTCGCGGCGGTGGGCGGGGCCA-GACG	
M	-23	GTG-CACG-CGTGGGCGGAGCCATCACG	+3
R		GTG-CACG-CGTGGGCGGAGCCATCACG	

Atp1a3

H		GGCCAGCGCCGCCCCCTCCGCGGACG	
M	-81	GGCCGGAGCCGCCTCCC-CCGCGGGCG	-55
R		GGCCAGAGCCGCCTCCC-CCGCGGGCG	

Atp1b1

			Site 1		Site 2		
H		ACCGGGCCGCTAGAGGGCGGGGCGGCACG					
M	-73	GCCGTGCCGCCGGTAGGCGGAGCTACGG					-46
R		GCGGTGCCGCCGGTAGGCGGAGCTACGG					

Fig. 7.

Aligned partial sequences of *Atp1a1*, *Atp1a3* and *Atp1b1* promoters from humans (H), mice (M) and rats (R) showed conserved putative Sp binding sites. *Atp1a1* has typical and *Atp1a3* and *Atp1b1* have atypical Sp binding motifs. *Atp1b1* has two consecutive atypical Sp binding sites that are denoted as site 1 and site 2. Solid boxes indicate Sp sites and highlighted areas indicate conserved bases.

Table 1

EMSA probes

Gene	Position	Sequence
<i>Atp1a1</i>	+4/-17	F: 5'-TTTTCGT GGGCGG AGCCATCACGC-3' R: 5'-TTTTCGTGATGGCT CCGCC CACGC-3'
Mutant <i>Atp1a1</i>	+4/-17	F: 5'-TTTTCGT GGAAAGG AGCCATCACGC-3' R: 5'-TTTTCGTGATGGCT CCITC CACGC-3'
<i>Atp1a3</i>	-53/-81	F: 5'-TTTGGCCGGAGCCG CTCCCC CGGGCGCG-3' R: 5'-TTTTCGCGCCCGCGGGGG AGG CGGCTCCGGCC-3
Mutant <i>Atp1a3</i>	-53/-81	F: 5'-TTTGGCCGGAGCCG TTTTT CCCCGCGGGCGCG-3' R: 5'-TTTTCGCGCCCGCGGG AAAAA CGGCTCCGGCC-3'
<i>Atp1b1</i>	-50/-71	F: 5'-TTTTCGTG CCGCCG GTA GGCGG AGCT-3' R: 5'-TTTAGCT CCGCCT AC CGGCGG CACG-3'
Mutant1 <i>Atp1b1</i>	-50/-71	F: 5'-TTTTCGTG CTTTT GGTAG GGCGG AGCT-3' R: 5'-TTTAGCT CCGCCT AC CAAAA GCACG-3'
Mutant2 <i>Atp1b1</i>	-50/-71	F: 5'-TTTTCGTG CCGCCG GTA TTTT AGCT-3' R: 5'-TTTAGCT AAAAA CTAC CGGCGG CACG-3'
Mutant3 <i>Atp1b1</i>	-50/-71	F: 5'-TTTTCGTG CTTTT GGTAG TTTT AGCT-3' R: 5'-TTTAGCT AAAAA CTAC AAAAA GCACG-3'
GM3 synthase	-58/-38	F: 5'-TTTTCGCGA CCCCGCCCCG CCTA-3' R: 3'-TTTTCAGGCGGGGGCGGGGTCGCGC-5'

Positions of probes are given relative to the TSP. Putative Sp-factor binding sites are in bold. Mutated nucleotide sequences are underlined.

Table 2

Primers for ChIP assays

Gene	Sequence	Amplicon length (bp)	Position
<i>Atp1a1</i>	F:5'-AGAGACCCAGACACCCGCGT-3' R:5'-GAGCCCGTGCCTCCTCCTCA-3'	221	-44/+177
<i>Atp1a3</i>	F:5'-GCTCCTGATTGGCCGGAGCC-3' R:5'-GACAGACGCACGCTCCCACC-3'	120	-90/+29
<i>Atp1b1</i>	F:5'-GAGAGGGCCAGCAGAGCTGC-3' R:5'-GGGCGCACGCCCTACCTTTAC-3'	237	-240/-3
GM3 synthase	F:5'-CACCTACTTCTCGGCTGGAG-3' R:5'-AATTCAGCCCCGGACAGT-3'	198	-182/+15
<i>Ntf3</i>	F:5'-GAGCAAACCTCCAAAATGCCAGG-3' R:5'-AAAGTTGCGCCGGGCTATCTC-3'	219	-149/+70
<i>Actb</i>	F:5'-TGAGAGGGAAATCGTGCGTGAC-3' R:5'-GCTCGTTGCCAATAGTGATGACC-3'	149	+2191/+2339

Positions of amplicons are given relative to the TSP.

Table 3

Primers for PCR cloning of Na⁺/K⁺-ATPase subunit promoters

Gene	Sequence
<i>Atp1a1</i>	F: 5'-AAAAGGTACCGATGCTGTGGATGCTGTTC-3' R: 5'-AAAAAAGCTTGGTCCTGTTGCTGGAGAC-3'
<i>Atp1a3</i>	F: 5'-AAAAGGTACCCTGCAAGATGGTGGCACTC-3' R: 5'-AAAAAAGCTTGAAGAGCGGCGTTCAGAC-3'
<i>Atp1b1</i>	F: 5'-AAAAGGTACCGCACAGACCAGGAGTCAGG-3' R: 5'-AAAAAAGCTTGCTGCCTTCCTCCTTGGC-3'
<i>Cox6b</i>	F: 5'-AAGGTACCGCCAGCCCTTAATTGTTTTC-3' R: 5'-AAAAGCTTTCGCAACTAAAAGCTCCACA-3'

Table 4

Mutagenesis primers

Gene	Sequence
<i>Atp1a1</i>	F: 5'-GTGACGTGCACGCGT GAAA AGCCATCACGCAGG-3' R: 5'-CCTGCGTGATGGCT CTTTT CACGCGTGCACGTCAC-3'
<i>Atp1a3</i>	F: 5'-GATTGGCCGAGCC AAAA CCCCCGCGGGCGCGGGCATATG-3' R: 5'-CATATGCCCGCGCCCGGGGGG TTTT GGCTCCGGCCAATC-3'
<i>Atp1b1 Mut1</i>	F: 5'-GATTGGCTTGCCGT GCTTTT GGTAGGCGGAGCTAC-3' R: 5'-GTAGCTCCGCCTAC CAAA GCACGGCAAGCCAATC-3'
<i>Atp1b1 Mut2</i>	F: 5'-GCCGTGCCGCCGTA AGTTTT AGCTACGGATGGTG-3' R: 5'-CACCATCCGTAGCT TAAAA CTACCGGCGGCACGGC-3'
<i>Cox6b</i>	F: 5'-CAGCACTAGTTAGGC AGAGTTT GGCGGATTCTGAGTCTAC-3' R: 5'-GTAGACTCAGAAATCCGCC AAACT CTGCCTAACTAGTGCTGG-3'

Putative Sp-factor binding sites are in bold. Mutated nucleotide sequences are underlined.

Table 5*Sp3* and *Sp4* shRNA sequences (in bold)

Gene		Sequence
<i>Sp3</i>	1	5' CCGG— ATGAGAACTGTTGGTATT A—CTCGAG—TAAATACCAACAGTTTCTCAT—TTTTG 3'
		5' AATTCAAAAA— ATGAGAACTGTTGGTATT A—CTCGAG—TAAATACCAACAGTTTCTCAT 3'
	2	5' CCGG— TTACCTTTGTACCAATCAATA —CTCGAG—TATTGATTGGTACAAAGGTAA—TTTTG 3'
		5' AATTCAAAAA— TTACCTTTGTACCAATCAATA —CTCGAG—TATTGATTGGTACAAAGGTAA 3'
<i>Sp4</i>	1	5' CCGG— CCAGTAACAATCACTAGTGTT —CTCGAG—AACACTAGTGATTGTTACTGG—TTTTG 3'
		5' AATTCAAAAA— CCAGTAACAATCACTAGTGTT —CTCGAG—AACACTAGTGATTGTTACTGG 3'
	2	5' CCGG— CTGGACAACAGCAGATTATTA —CTCGAG—TAATAATCTGCTGTTGTCCAG—TTTTG 3'
		5' AATTCAAAAA— CTGGACAACAGCAGATTATTA —CTCGAG—TAATAATCTGCTGTTGTCCAG 3'

Table 6

Primers for real-time PCR

Gene	Sequence
<i>Atp1a1</i>	F: 5'-ATCTGAGCCCAAACACCTGCTAGT-3' R: 5'-AAGCGTCCTTCAGCTCTTCATCCA-3'
<i>Atp1b1</i>	F: 5'-ACGAGGCCTACGTGCTAAACATCA-3' R: 5'-TTGAACCTGCACACCTTCCTCTCT-3'
<i>Atp1a3</i>	F: 5'-AGCCGCCAAGATGGGGGACAAAA-3' R: 5'-TGTGTCAGACCCTGCACGCAGTC-3'
<i>Sp1</i>	F: 5'-CTCCAGACCATTAACTCAGTG-3' R: 5'-ATCATGTATTCCATCACCACCAG-3'
<i>Sp3</i>	F: 5'-TCAGGCACAGACAGTGACCCCT-3' R: 5'-AGCGTGAGTGTCTGAACAGGCG-3'
<i>Sp4</i>	F: 5'-TTGCAGCAAGGCCAGCAGACC-3' R: 5'-GCTTCTTCTTCTGTTCACTGCT-3'
<i>Cox2</i>	F: 5'-TCTCCCTCTCTACGCATTC-3' R: 5'-CAGGTTTTAGGTCGTTTGTG-3'
<i>Cox7c</i>	F: 5'-ACCCAGATCCAAAGTACACGG-3' R: 5'-ATGTTGGGCCAGAGTATCCG-3'
<i>Actb</i>	F: 5'-GTGACGTTGACATCCGTAAGA-3' R: 5'-GCCGACTCATCGTACTCC-3'
<i>Gapdh</i>	F: 5'-AGGTCGGTGTGAACGGATTG-3' R: 5'-GGGGTCGTTGATGGCAACA-3'