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Drosophila glia use a conserved cotransporter mechanism to regulate extracellular volume

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Abstract

The nervous system is protected by blood barriers that use multiple systems to control extracellular solute composition, osmotic pressure, and fluid volume. In the human nervous system, misregulation of the extracellular volume poses serious health threats. Here we show that the glial cells that form the *Drosophila* blood-nerve barrier have a conserved molecular mechanism that regulates extracellular volume: the Serine/Threonine kinase Fray, which we previously showed is an ortholog of mammalian PASK/SPAK; and the Na-K-Cl cotransporter NCC69, which we show is an ortholog of human NKCC1. In mammals, PASK/SPAK binds to NKCC1 and regulates its activity. In *Drosophila*, larvae mutant for *NCC69* develop a peripheral neuropathy, where fluid accumulates between glia and axons. The accumulation of fluid has no detectable impact on action potential conduction, suggesting that the role of NCC69 is to maintain volume or osmotic homeostasis. *Drosophila* NCC69 has kinetics similar to human NKCC1, and *NKCC1* can rescue *NCC69*, suggesting that they function in a conserved physiological mechanism. We show that *fray* and *NCC69* are coexpressed in nerve glia, interact in a yeast-two-hybrid assay, and have an essentially identical bulging nerve phenotype. We propose that normally functioning nerves generate extracellular solutes that are removed by NCC69 under the control of Fray. This mechanism may perform a similar role in humans, given that NKCC1 is expressed at the blood-brain barrier.

Keywords

Na-K-Cl transporter; osmoregulation; blood-nerve barrier; blood-brain barrier

Introduction

In animals as diverse as humans and *Drosophila*, the nervous system is isolated from the blood or hemolymph by cellular junctions that restrict the passage of solutes (reviewed in Carlson et al. 2000; Rubin and Staddon 1999). This paracellular barrier permits a tight regulation of the extracellular environment of the nervous system, with ion pumps, cotransporters, exchangers, and channels maintaining ionic homeostasis. In humans, improper ionic homeostasis in the nervous system has serious clinical consequences, including seizures, coma, and death (Somjen 2002). Following brain injury, elevated extracellular solutes cause water to accumulate, resulting in edema and the pathological rise

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of intracranial pressure (Iadecola 1999). Understanding the mechanisms controlling volume and ionic homeostasis is thus critical for developing rational treatments for brain injury.

The Na-K-Cl cotransporter, NKCC1, has been shown to mediate the early swelling response in a mouse model of focal ischemia in the brain (Chen et al. 2005; O'Donnell et al. 2004; Yan et al. 2003). NKCC1 is widely expressed in the nervous system, where it has been proposed to regulate the intracellular level of Cl in neurons and the swelling of glia in response to neuronal activity (Chen and Sun 2005; Delpire 2000; Simard and Nedergaard 2004). Knockdown of NKCC1 reduces the swelling response to ischemia, but its role in vivo under normal conditions is incompletely understood. Analysis of mouse *NKCC1* mutant phenotypes is hampered by the complex anatomy of the nervous system and the masking of behavioral changes caused by the disruption of the vestibular system (Delpire et al. 1999; Dixon et al. 1999; Flagella et al. 1999).

Glial cells have an important role in regulating extracellular ionic composition and volume (Newman 2005; Somjen 2002; Wang and Bordey 2008), and may also be critical for blood-brain barrier function. Oligodendrocytes associated with myelinated axons are connected to astrocytes by gap junctions (Orthmann-Murphy et al. 2008), and may function as a syncytial unit to move K from the axons to blood vessels by a process known as “K siphoning” (Newman 1986). Despite this attractive hypothesis, the full range of glial activities at the blood-brain barrier remain incompletely understood.

Drosophila is a useful model system to elucidate the molecular, cellular and physiological mechanisms in the nervous system. The larval nervous system is far simpler than the mammalian. In larvae a single glial cell layer surrounding the nervous system forms the paracellular barrier that restricts the flow of ions (Baumgartner et al. 1996; Stork et al. 2008). This anatomy allows easy analysis of phenotypes associated with ion homeostasis and volume regulation. By contrast, in humans and mice the analogous barrier is associated with the blood microvessels that penetrate deep within the central and peripheral nervous systems (Zlokovic 2008). Despite these differences, the nervous systems of these organisms face similar physiological challenges. Many of the ion channels, exchangers, and transporters of the *Drosophila* nervous system are evolutionarily conserved, and have orthologs in humans (Rubin 2001). Furthermore, molecules that form paracellular barriers are conserved (Banerjee et al. 2006), suggesting that many molecular mechanisms that mediate ionic and volume homeostasis are also conserved. We show here that fly glia have a molecular mechanism to regulate extracellular volume in larval nerves that is directly related to NKCC1 function in mammals.

One of us (B. F.) has demonstrated that NKCC1 activity is regulated by rat PASK, known as SPAK in humans and mice (Dowd and Forbush 2003). We previously demonstrated that PASK is the ortholog of *Drosophila* Fray, and that loss of function of *fray* in nerve glia results in the accumulation of extracellular fluid between axons and glia (Leiserson et al. 2000). Here, we show that reducing the activity of the cation chloride cotransporter, NCC69, in larval nerve glia causes the same phenotype. NCC69 and Fray function in the subperineurial glia (SPG), the cells that provide the paracellular blood-nerve barrier. The fluid in *NCC69* mutant nerves supports normal action potential activity, suggesting that NCC69 normally regulates extracellular volume by removing solutes from the extracellular space, with little effect on the extracellular ion composition. Measurement of the kinetic parameters of NCC69 in vitro and rescue experiments in vivo demonstrate that NCC69 is the ortholog of human NKCC1. These results suggest that under normal conditions, nerve glia remove excess extracellular solutes to maintain volume and osmotic homeostasis, using Na-K-Cl cotransport mediated by the highly conserved Fray-NCC69 pathway.

Materials and Methods

Genetics

The genotypes of the stocks used in this study are listed in Table S1. The two *NCC69* alleles referred to in this study were made by mobilizing the P-element line EY168 (Bellen et al. 2004), in which the P-element had inserted 107 bp 5' of the transcription start site. We screened 112 independent lines by PCR to map excision events. For the *r1* and *r2* alleles, a segment that spanned the breakpoints was amplified by PCR (primer pairs NCC69-U1..L1 and U8..L1, respectively), sequenced, and the location of the breakpoints determined. The *r1* allele has a 537 bp deletion (3L:12,384,465..12,385,007; release r5.22) and the *r2* allele, a 8,743 bp deletion (3L:12,375,902..12,384,644).

Molecular Biology

A list of primers is given in Table S2. A cDNA clone of *NCC69*, GH27027, was obtained from BACPAC Resources and was used for in situ hybridization, yeast-two-hybrid experiments, cell transformation, and transgenics. The human *NKCC1* clone was previously reported (Payne et al. 1995). Sequencing was performed by the Keck Sequence Facility and the DNA Analysis Facility on Science Hill (Yale University). Nucleotide queries were performed on the NCBI BLAST server. Protein sequences alignments and distance relationships were performed using ClustalW (Larkin et al. 2007).

Expression Constructs

A list of expression constructs is given in Table S3. We used a three way ligation strategy to clone HA:*NCC69* into pIZT/V5-His (Invitrogen) for expression in cell culture (pIHANCC69) and pP{UAST} (Brand and Perrimon 1993) for expression in flies (pUHANCC69). First, the insert from GH27027 was amplified with primers HANCC69-EcoRI and 4357RA-703, then digested with EcoRI and XhoI. This fragment was ligated to the appropriate vector, which had been digested with EcoRI and XbaI, along with a 3.3kb XhoI-XbaI fragment from GH27027. For the human *NKCC1* rescue construct, we used the Gateway cloning system (Invitrogen) following the manufacturer's instructions, to clone the human *NKCC1* cDNA into the pTHW vector (Drosophila Gateway Collection, <http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html>). Human *NKCC1* was amplified using primers TEF-TOPO1 and TEF-L1, then cloned into pENTR/D to make an entry clone, which was used in the topoisomerase reaction to make pTH-TEF, a plasmid that contains P{UAS-HA:*NKCC1*}.

Yeast-two-hybrid

A list of bait and prey constructs is shown in Tables S4 and S5. PCR-amplified fragments of Fray (p4624R6, Leiserson et al. 2000) and *NCC69* were cloned into pGBT9 (Bartel et al. 1993) and pGADGH (Clontech). The PJ69-4A yeast strain (James et al. 1996) was transformed using the LiAc method as described by (Gietz and Woods 2001), with minor modifications (see Supplemental Material). Clones that received both bait and prey plasmids were streaked onto plates lacking Trp, Leu, and His. Growth was assessed after two days at 30C.

P-element-mediated transformation and rescue

For the *NCC69* construct (pUHANCC69), we made transgenic lines as described (Leiserson et al. 2000) and obtained six independent insertions. Eight transgenic lines with the human *NKCC1* construct (pUHANKCC1) were obtained from BestGene (Chino Hills, CA).

Histology

Immunostaining was performed as described (Johansen et al. 1989) and analyzed by digital optical microscopy (Halpern et al. 1991) or confocal microscopy (Bio-Rad 1024 and Zeiss LSM510). Image processing was performed with Adobe Photoshop, Zeiss Zen, Bitplane Imaris, and NIH ImageJ software. Primary antibodies used were: rabbit-anti-HRP (1:250; Cappel), goat-anti-HRP (1:200; Cappel), 8D12 mouse-anti-Repo (1:10; Alfonso and Jones 2002) and 16B12 mouse-anti-HA.11 (1:2,000; Covance). The 8D12 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Secondary antibodies used were HRP-donkey-anti-goat (1:200, Jackson ImmunoResearch Laboratories); and the following Alexa antibodies (1:1,000; Molecular Probes): 488-goat-anti-mouse, 568-goat anti-mouse, 488-goat-anti-rabbit (1:500 for the in situ double-labeling), 568-goat-anti-rabbit, 568-donkey anti-goat, and 488-donkey-anti-mouse.

Transmission electron microscopy

Larval fillets were fixed at RT for 1 hour in 4% glutaraldehyde in 0.1M phosphate buffer, pH7.4. The fillets were washed in PBS, stained for 1 hour in 1% osmium tetroxide, dehydrated and embedded in Epon, then silver sections were cut, stained 10 min in 3% uranyl acetate followed by 3 min in 0.3% lead citrate.

In situ hybridization

This was performed with digoxigenin-labeled probes as described in Tautz and Pfeifle (1989), with modifications described in Doe et al. (1991) and the Supplemental Material. Single stranded probes were synthesized using a single primer in a PCR reaction. Prior to hybridization, the probes were boiled for 30 min. Following hybridization, the embryos were stained for at least one hour with alkaline-phosphatase-conjugated sheep-anti-DIG antibodies (Roche), which had been preabsorbed with embryos, at a final dilution of 1:2000. The staining was visualized with NBT/BCIP or Fast Red (Biomedica).

Cell culture and flux assays

The flux experiments were done with *Drosophila* SL2 cells (kindly provided by the Artavanis-Tsakonas lab, Harvard Medical School), an adherent derivative of S2. They were grown at RT in SFM (Gibco) supplemented with 2% fetal bovine serum (FBS, heat-inactivated, Gibco) or Schneider's medium (Gibco) supplemented with 10% FBS (SS). In a 12-well plate, 2.5×10^5 cells were transfected with 1 μ g plasmid (pIHANCC69 or pIZT, the vector control) using Cellfectin (Invitrogen) according to the manufacturer's guidelines. The cells were expanded for ca. 2 weeks, then selected in SFM containing 100 to 800 μ g/ml Zeocin (Invitrogen). After three months, the cells were adapted to SSZ400 (SS supplemented with 400 μ g/ml Zeocin).

For the flux experiments, cells were grown in shaking cultures, then plated in 96 well plates at 10^5 cells/well, and grown overnight. The ^{86}Rb flux was assayed robotically as described (Darman and Forbush 2002), with the modifications described in the Supplemental Material. The K_m and K_i values were determined for each experiment by fitting the data to single- or double-site binding using a non-linear least-squares approximation (Solver plug-in of Excel).

Electrophysiology

Third instar larvae were filleted and mounted on slides in physiological saline (150 mM Na, 5 mM K, 4 mM HCO₃, 6 mM Mg, 1 mM Ca; 5mM TES; 50mM sucrose, pH 7.2) as previously described (Halpern et al. 1991). Peripheral nerves were stimulated with suction electrodes (6–8 μm wide opening) placed at least 500 micrometers from the bodywall musculature. Evoked excitatory junctional currents (EJCs) were recorded using two electrode voltage clamp (Dagan 8500) with pClamp 9.0 software, as previously described (White et al. 2001). Spontaneous neuromuscular activity was monitored both by two electrode voltage clamp and by single electrode current clamp.

Quantitation of nerve phenotype

Five nerve widths were taken from ca. 220 μm nerve segments of each nerve overlying the middle of bodywall segment A2 to divide the nerve segment into four equal lengths. For the analysis shown in Figures 6A and 6B, the nerve measurements were binned in 0.5 μm intervals, and a normalized cumulative distribution determined. Statistics of the Kolmogorov-Smirnov (K-S) type were used to test whether the difference between the distributions was statistically significant. The critical values were calculated using the large sample approximation for quantiles of the Smirnov test statistic for two samples of different size (Conover 1999). To test for significant differences between the quantiles shown in Figure 6B, we used the “quantile test,” a variation of the median test (Conover 1999). For this analysis, the quantiles were calculated without binning.

For Figure 6C, the width measurements from each nerve segment were treated as an ordered quintuplet (d₁, d₂, d₃, d₄, d₅), that was used to estimate the average cross-sectional area:

$$A = (\pi/48) (d_1^2 + d_1d_2 + 2d_2^2 + d_2d_3 + 2d_3^2 + d_3d_4 + 2d_4^2 + d_4d_5 + d_5^2) \quad (\text{Eq 1})$$

The value A gives the cross-sectional area of a cylinder that has the same volume as the estimated volume of the nerve segment (See Supplemental Material). To arrive at the mean area values shown in Figure 6C and Table 3, we averaged all the A values for each animal, then took the mean of those averages. Standard Student's t-tests were used to determine the p-values.

Results

Cation chloride cotransporters (CCCs) mediate the electroneutral cotransport across the plasma membrane of Cl, along with K (KCCs), Na (NCCs) or Na and K (NKCCs). The CCC family (also known as SLC12) has four major branches (Fig. 1A; reviewed in Hebert et al. 2004; Pullikuth et al. 2003). Two branches contain proteins that transport Na, K, and Cl: the sodium-dependent potassium chloride cotransporter group, N(K)CC; and the sodium-independent potassium chloride cotransporter group, KCC. Humans have four KCC genes with multiple alternatively spliced isoforms, while flies have a single KCC gene (Hekmat-Scafe et al. 2006).

In humans, the N(K)CC group comprises two NKCCs, NKCC1 and NKCC2, and one Na-Cl cotransporter, NCC. Both NKCC2 and NCC are associated with the kidney. NKCC1 has widespread expression, including the brain and peripheral nerves (Hebert et al. 2004). Flies have two members of the N(K)CC group, NCC69 and NCC83 (Fig. 1A), that diverged too early in the N(K)CC lineage to indicate whether they are K-dependent transporters. Here we focus on NCC69, which has extensive primary and secondary amino acid sequence homology to human NKCC1 (Fig. 1B), and which we show below is expressed in nerve glia.

NCC69 is expressed and required in nerve glia

To look for a possible role of *NCC69* in larval nerves, we examined whether the gene is expressed in subperineurial glia, which form pleated septate junctions that provide a paracellular barrier (Stork et al. 2008). These cells are evident in the embryo by their expression of the glial transcription factor Repo, and by their close association with nerves (Fig. 2A, B, and C). Tissue in situ hybridization experiments revealed that these glia express *NCC69* transcripts (Fig 2D and E). The only other prominent expression of *NCC69* was in the dorsal median cells of the ventral ganglion (not shown).

We generated two mutant alleles of *NCC69* and examined the larval abdominal nerves in homozygotes. The alleles have different deletions, but both span the 5' exon of the *NCC69*-RA transcript (Fig. 1C). The abdominal nerves of these mutants have localized swellings or bulges (Fig. 2G). The phenotype is completely penetrant, with bulges appearing along the lengths of the nerves in all the animals examined ($n > 50$), and which could occur in any of the abdominal nerves. *NCC69* mutants survive to adulthood with no other apparent abnormality. Placing either allele in combination with a deficiency that spans *NCC69* does not make the phenotype more severe, suggesting that these alleles are genetic nulls, at least with respect to the nerve phenotype.

Both the expression pattern and the mutant phenotype of *NCC69* suggest that it is required in subperineurial glia. To test this we expressed two independent *NCC69*-RNAi constructs in separate experiments in subperineurial glia. Knockdown of *NCC69* in these glia caused bulges similar to those seen in the *NCC69* mutants (Fig. 2I). By contrast, knockdown of *NCC69* in adjacent neurons had no effect (Fig. 2H). Expressing the *NCC69* cDNA in subperineurial cells of *NCC69* mutants rescues the bulging phenotype (see below and Fig. 5B), while expression in neurons has no effect (data not shown). These experiments demonstrate that the action of *NCC69* is required in subperineurial nerve glia—the same cells in which we observe *NCC69* expression.

We next examined whether the peripheral nerve edema of *NCC69* null mutants affects action potential activity and nerve conduction. In *NCC69* mutant larvae, we did not observe phenotypes associated with mutations that affect neuronal activity, such as lack of coordination when crawling, loss of nociception, or temperature-sensitive paralysis. Mutant larvae developed at a normal pace. Thus, although axons in *NCC69* mutants traversed abnormal fluid-filled extracellular environments, the effect on nerve conduction appeared to be minor.

It remained possible, however, that a phenotype might be revealed under conditions of elevated action potential activity. This was examined both electrophysiologically and through genetic tests. Both mutant and wild type nerves of 3rd instar larvae were subjected to trains of tetanic stimuli of increasing frequency (50 pulses at 10, 20, 50, and 100 Hz), and the ability of the motoneuron axons to conduct the pulses was monitored by recording evoked excitatory junction currents (EJCs) from the innervated muscle fibers (Fig. 3 and Table 1). The peripheral nerves were stimulated at least 500 microns from where they branch in the periphery. This approach ensured that action potentials evoked by the stimulus would encounter nerve bulges of *NCC69* mutants as they traveled toward the nerve terminal. Both the *wild type* and mutant nerves showed little evidence of action potential failure, even at the highest stimulation frequencies (at 100 Hz, $p \approx 0.99$; Table 1).

Because an *NCC69* nerve conduction phenotype might not be evident with the acute excitation described above, we used the *eag Sh* (*ether-a-go-go Shaker*) mutation combination to examine the effects of chronic hyperexcitation. In *eag Sh* mutants, multiple voltage-gated K channels are affected, resulting in elevated action potential activity

throughout larval development (Budnik et al. 1990; Ganetzky and Wu 1983; Zhong et al. 1992; Zhong and Wu 2004). As measured by the frequency of spontaneous EJs, the endogenous activity of *eag Sh*; *NCC69* larvae (4.2 to 12.2 Hz, n=5 muscles) was comparable to *eag Sh*; + (0.3 to 12.1 Hz, n=22). We found no evidence that *NCC69* nerve bulges have any effect on action potential function and nerve conduction, when challenged by either acute or chronic hyperstimulation.

Finally, we used a sensitive genetic test to detect reduced action potential function. *NCC69* mutants crawl normally at 37° C. By contrast, mutations that reduce voltage-gated Na channels typically have a temperature-sensitive paralysis phenotype. For example, the *para^{ts1}* mutation, that affects the alpha subunit of the Na(v)1 voltage gated Na channel, results in a characteristic 37° C paralysis in larvae (Ganetzky 1984). The mutation provides a sensitized genetic background useful for identifying mutations that affect action potential function. When combined with a second mutation that reduces membrane excitability (such as *mle^{nap-ts1}*), the double mutant paralyzes at either a lower temperature, or dies (Ganetzky 1984). We found that the *NCC69* mutation did not enhance the severity of the *para^{ts1}* phenotype: *para^{ts1}*; *NCC69^{r2}* double mutant larvae paralyzed at the same temperature as *para^{ts1}* alone (37° C, n>10 animals). At 29° C, a temperature at which *para^{ts1}* larvae are somewhat uncoordinated, larvae mutant for both *para^{ts1}* and *NCC69^{r2}* appear similar to larvae mutant for *para^{ts1}* alone (n=7 animals, each). Thus, the *NCC69* bulges did not further reduce neural activity. In all these assays of nerve function— electrophysiology, genetic and behavioral—*NCC69* bulges had no detectable impact.

NCC69 is a Na-K-Cl cotransporter

We used in vitro cotransporter flux assays to address whether *NCC69* functions as a Na-K-Cl cotransporter analogous to mammalian NKCC1. Our assay was modeled from studies of human and shark cotransporters expressed in a human embryonic kidney cell line (Darman et al. 2001; Payne et al. 1995; Xu et al. 1994). We used a *Drosophila* cell line, SL2, expressing an HA-tagged *NCC69* construct to assay ion transport (Fig. 4). A stable line transfected with HA-tagged *NCC69* shows membrane expression (Fig. 4A).

SL2 cotransporter assays indicate that *NCC69* is a Na-K-Cl cotransporter. To assay for functional *NCC69*, we treated cells with a variety of “activation” conditions, then returned the cells to normal isotonic saline containing ⁸⁶Rb, and assayed how much ⁸⁶Rb entered the cell (⁸⁶Rb is a convenient radioisotope congener of K). Transport is activated when SL2 cells experience a change in osmolarity or a change in Cl concentration. In all conditions tested, the cells transfected with HA-*NCC69* yielded a higher ⁸⁶Rb flux than vector controls (Fig. 4B). The flux is largely abolished by the loop diuretic, bumetanide (Fig. 4B, rightmost category), an inhibitor of mammalian and shark CCCs (Haas and Forbush 2000). The flux varies inversely with increasing concentrations of bumetanide (Fig 4C), consistent with bumetanide acting as a non-competitive inhibitor.

A key feature of cotransporters is the dependence of the transport (flux) of one ion species on the concentrations of other ions. For *NCC69*, the transport of Rb depends on the concentrations of Na (Fig. 4D), Rb (Fig. 4E), and Cl (Fig. 4F). *NCC69* displays kinetics consistent with the cotransport of one Na, one K, and two Cl ions. From the dependence relationship between the flux and the ion concentrations, we calculated the apparent affinity constants (Km) of the cotransporter for the different ion species. Similarly, from the varying bumetanide concentrations, we calculated its inhibition affinity Ki. These values are presented in Table 2, along with published values for human NKCC1. The Km values for the three ions are remarkably similar to the corresponding Km values of human NKCC1. The Ki values show that *NCC69* has a ten-fold greater sensitivity to bumetanide over NKCC1.

Human NKCC1 can substitute for *Drosophila* NCC69 in vivo

Expression of either *Drosophila* NCC69 or human NKCC1 in subperineurial glia rescued the NCC69 bulging nerve phenotype (Fig. 5A–C). The expression of the HA-tagged constructs of either fly NCC69 (Fig. 5E) or human NKCC1 (Fig. 5F) was detected with anti-HA antibody. The human construct expressed less well than the fly. This difference in expression may account for the fact that HA:NCC69 produced a greater degree of rescue than HA:NKCC1 (see below).

A quantitative examination of the degree of nerve bulging confirms the rescue of the NCC69 mutant phenotype. The nerve width distribution for NCC69 is shifted toward larger values compared to the *wild type* control (Fig. 6A). The graph of the fly NCC69 rescue coincides with the *wild type* control, indicating full rescue, while the human NKCC1 rescue is in the middle, indicating partial rescue. Comparisons of selected percentiles (Fig. 6B) and average cross-sectional areas (Fig. 6C) for the four genotypes show a similar trend. The average cross-section area of NCC69 nerves measured over twice normal ($p < 0.005$ to reject the hypothesis H0 that NCC69 is the same as *wild type* or either rescue), while the human rescue gave a value about one quarter above normal (Table 3; $p = 0.02$ to reject the hypothesis H0 that the NKCC1 rescue and *wild type* are the same). Kolmogorov-Smirnov tests on the distributions shown in Fig. 6A (see Table S6 for p-values) and quantile tests for the data shown in Fig. 6B (see Table S7 for p-values) gave similar results. While the NKCC1 rescue deviates significantly from *wild type*, it nevertheless shows a statistically significant rescue of the NCC69 mutant phenotype, demonstrating that the two molecules perform homologous functions in vivo.

NCC69 and Fray function to regulate extracellular volume in larval nerves

Loss of function of the Ser/Thr kinase Fray causes bulging nerves, similar to the NCC69 phenotype. Like NCC69, we previously showed that *fray* is expressed in subperineurial glia, and is required in these cells for normal nerve morphology. We further showed that rat PASK (known in mouse and humans as SPAK) can rescue the *fray* mutant phenotype, demonstrating that Fray and PASK/SPAK are orthologs (Leiserson et al. 2000). There is evidence that the mammalian orthologs of NCC69 and Fray, NKCC1 and PASK/SPAK, bind to one another, and that NKCC1 cotransport is activated by PASK/SPAK. (Dowd and Forbush 2003; Piechotta et al. 2003; Piechotta et al. 2002). Given the strong molecular and functional homologies of these mammalian molecules in flies, we suspected analogous regulation could be occurring in *Drosophila*. To look for an interaction between NCC69 and Fray, we used the yeast-two-hybrid assay. Portions of the Fray protein were assayed against the N-termini of the three putative *Drosophila* CCCs: KCC, NCC69, and NCC83. Of the three, NCC69 showed an interaction (Fig. 7A). This was specific to the C-terminal PF2 domain of Fray, a conserved region among Fray orthologs that was found to interact with NKCC1 in mice (Piechotta et al. 2002). Neither the N-terminal catalytic domain nor the PF1 domains of Fray displayed any interactions with NCC69.

As a test of whether Fray positively regulates NCC69 activity in vivo, we made a *fray* NCC69 double mutant chromosome to test for any additive effects in the nerve phenotype. If Fray is the only kinase that upregulates NCC69, double mutants would have a nerve phenotype that is comparable to that of either mutant alone. Double mutant larvae die before the 3rd instar precluding quantitation of the nerve phenotype comparable to that of the single mutants, presented above. However, the shift in lethal phase shows an additive effect of the lethal phenotype. This effect likely results from a function outside the nerves, as *fray* has vital functions, and bulging nerves in and of themselves have little effect on viability (this work; and Leiserson et al. 2000).

Another approach to test whether Fray positively regulates NCC69 activity in vivo is to compare the mutant nerve phenotypes of the two genes—they would be expected to be very similar. Indeed, by light microscopy, the nerve phenotype of *fray* and *NCC69* look very similar. Bulging nerves, however, can result from the accumulation of fluid in different compartments. In *fray* mutants, the accumulation occurs in the extracellular space, between axons and glia (Leiserson et al. 2000). There are other mutants, such as *inebriated*, in which perineurial glia become abnormally thick (Yager et al. 2001). To see which compartment is abnormally large in *NCC69* larval nerves, we examined thin sections by TEM (Fig. 7C). From this analysis, it is clear that fluid accumulates in the extracellular space between glia and axons. The ultrastructure of this phenotype is strikingly similar to *fray* (compare Fig. 7C with D), suggesting these two genes function in the same cellular process.

Discussion

NCC69 mutant larvae have a peripheral neuropathy similar to *fray*, with swollen nerves in which fluid accumulates between axons and surrounding glia. Our data show that the nerve bulges in *NCC69* mutants arise from the loss of ion cotransport. The morphological and ultrastructural similarity of the *fray* and *NCC69* phenotypes suggests that these genes share similar cellular functions. Both genes are expressed in subperineurial glia, and expression in these glia is necessary and sufficient to rescue the mutant phenotype (Figs. 2 and 5; Leiserson et al. 2000). In flux assays, NCC69 functions as a Na-K-Cl cotransporter, with biochemical characteristics remarkably similar to human NKCC1. Despite several million years in which to diverge, human NKCC1 has kinetic behavior similar to NCC69 and can substitute for NCC69 function in larval glia, showing they perform the same molecular function and are orthologs.

We previously demonstrated that rat PASK and Fray are orthologs. In mammals, it has been shown that PASK can bind and regulate NKCC1 (Dowd and Forbush 2003; Piechotta et al. 2003). Our data shows that the C-terminus of Fray can physically interact with the N-terminus of NCC69 in a yeast-two-hybrid assay, similar to the interaction seen between mammalian homologs of Fray and CCCs (Piechotta et al. 2002). We extend the findings from mammals by showing that in flies, the two molecules function in the same cellular process in vivo: they are both required in subperineurial glia, and loss of function of either molecule in these glia causes the accumulation of fluid in the extracellular compartment of the nerves.

In *NCC69* mutants, we did not observe any of the changes associated with altered neural activity, suggesting that the extracellular fluid that accumulates in *NCC69* mutants nevertheless supports normal action potential activity. In *Drosophila*, mutations that reduce electrical activity commonly causes temperature-sensitive paralysis, and mutation combinations often display an enhanced phenotype, such as a lowered temperature of paralysis or lethality (Budnik et al. 1990; Ganetzky and Wu 1986). The *NCC69* mutation did not show these properties. We found that *NCC69* motoneurons functioned normally in electrophysiology assays and the muscle was able to respond faithfully to trains of stimuli of up to 100 Hz. Even when *NCC69* mutant nerves experienced hyperactivity throughout larval life, as occurs in the mutant combination *eag Sh; NCC69*, there was no evidence of any failure of motoneurons to reliably transmit action potentials. Similarly, *fray* mutant larvae failed to show any evidence of reduced neural activity either in their behavior or in recordings from *eag Sh; fray* mutants (our unpublished observations).

Recently, Sun et al. (2010) reported a survey of the expression and function of *Drosophila* SLC12 gene family members, including *NCC69* (referred to as *CG4357*). Although they were unable to ascribe a cellular function to *NCC69* from their expression and mutation

analysis, they did show that it functioned as a Na-K-Cl cotransporter in a heterologous cell line.

A model for NCC69 function in *Drosophila* nerve glia

Figure 8 shows a working model of ion fluxes in *Drosophila* nerves that accounts for the bulging nerve phenotype seen in *NCC69* mutants. Action potentials leave increased K in the extracellular compartment. The Na/K ATPase moves three Na ions out of the cell for every two K ions it moves into the cell. It is thus both electrogenic (creating net negative charge inside) and osmogenic (increasing the osmotic strength outside). The septate junctions prevent free diffusion of ions to relieve the osmotic stress. We propose that the role of NCC69 is to move ions (and water) into the SPG, which then secretes ions toward the hemolymph. The paracellular barrier in these cells is consistent with a secretory function. The model predicts a stoichiometry of one cycle of Na-K-Cl cotransport for every two cycles of the Na/K ATPase to maintain osmotic equilibrium, resulting in the movement of 5 K into, and 5 Na out of the glial cell.

The normal nerve electrophysiology in *NCC69* mutants is consistent with this model, because it strongly suggests that the bulging phenotype neither results from altered neuronal excitability, nor significantly impacts neuromuscular function. Rather, NCC69 functions to regulate extracellular volume in larval nerves by removing solutes from the extracellular space without dramatically affecting ion composition. With the movement of ions, water naturally follows by osmotic forces. Failure to transport these ions out of the extracellular space causes a build-up of ions in the extracellular space, which in turn draws water into the space by osmosis causing the nerves to bulge.

The model is consistent with data on glia functioning to remove K and restore the normal extracellular ionic environment in the wake of action potentials in axons (Menichella et al. 2006; Newman et al. 1984; Orkand et al. 1966; Walz 2000). In support of the idea that K efflux promotes nerve swelling in *Drosophila*, we find that nerves in hyperactive mutants (*eag Sh*) have larger widths, relative to controls (our unpublished observations). Consequently, the only place for the ions to go is into glia or back into the axons. In this view, neuronal activity causes an accumulation of extracellular solutes which are normally removed by the Fray-NCC69 pathway.

Drosophila nerves as a model for volume regulation of the human nervous system

This mechanism of volume regulation may well function in other systems including humans. *Drosophila* nerves share common traits with human nerves at the level of morphology, phenotypes, and molecules. *Drosophila* nerves resemble non-myelinated fibers of mammals, in which a single Schwann cell can associate with multiple axons. Disruption of ion flow in mammalian systems gives rise to the accumulation of extracellular fluid, with phenotypes that have striking similarities to *fray* and *NCC69* bulging nerves. For example, loss of function of *Cx32* (Scherer et al. 1998), *Kir4.1* (Neusch et al. 2001), *Cx47* (Menichella et al. 2003; Menichella et al. 2006; Odermatt et al. 2003), or *KCC3* (Byun and Delpire 2007) all lead to glial misregulation of ion homeostasis, resulting in the accumulation of extracellular fluid in nerves, nerve tracts, or white matter. Similarly, loss of function of *NKCC1* (Arnold et al. 1981; Santi and Lakhani 1983), the *isk* K channel (Vetter et al. 1996), and *Cx30* (Cohen-Salmon et al. 2007) all result in the accumulation of extracellular fluid in the inner ear. All these mutations result in the misregulation of ion homeostasis, and at the ultrastructural level look remarkably similar to *NCC69* and *fray* bulging nerves.

Finally, this work demonstrates a conserved molecular mechanism, the Fray-NCC69 pathway, that functions in *Drosophila* nerve glia, the cells that form the blood-nerve barrier.

In humans, NKCC1 is also expressed in glia and in the cells that form the blood-brain barrier (Alvarez-Leefmans et al. 2001; O'Donnell et al. 2004; Su et al. 2002; Wang et al. 2003). While a full characterization of its function in these cell types has yet to be done, there is mounting evidence that NKCC1 plays an important role in mediating the swelling response to focal ischemia in glia and the blood-brain barrier (Chen and Sun 2005; O'Donnell et al. 2004; Yan et al. 2003). Our study suggests that in flies, the NKCC1 and SPAK orthologs normally function in the nervous system to regulate extracellular volume, presumably in response to neuronal activity. Given the advantages of *Drosophila*, a proven system for uncovering molecular mechanisms, the relative simplicity of the blood-nerve barrier, and the multiple levels of homology between the fly and human nervous systems, we are confident further work in flies will provide further insights into extracellular volume regulation of the human nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

NCC69 is a member of the *Drosophila* N(K)CC family. **A:** A cladogram showing the relationship between the *Drosophila* (red) and human (black) cation chloride cotransporters (CCCs). Humans have three Na-(K)-Cl cotransporters: one Na-Cl and two Na-K-Cl cotransporters, denoted NCC and NKCC, respectively. *Drosophila* has two related cotransporters, denoted NCC69 and NCC83, but the phylogenetic analysis fails to reveal to which class (NCC or NKCC) these proteins belong. **B:** A model of human NKCC1 showing homology to NCC69. The color-coding shows the similarity to NCC69. The residues annotated with P's are conserved phosphorylation sites that are important for NKCC1 activation in the shark (Darman and Forbush 2002; Vitari et al. 2006). **C:** Map of the *NCC69* genomic region. Two transcripts are predicted, which encode the same protein of 1,171 amino acid residues. The deleted regions are shown of *r1* and *r2*, two alleles that were used in this study, which span 537 and 8,743 bp, respectively.

Figure 2.

NCC69 is expressed and required in subperineurial glia. **A:** Drosophila whole embryo, stage 16, labeled with anti-HRP to reveal neurons and neuronal projections (green) and anti-Repo to label glial nuclei (magenta). At this magnification, the nerves (arrowheads) can be easily identified linking the CNS to the periphery in a segmentally repeated and bilaterally symmetric pattern. Bar: 50 microns. **B, C:** Higher magnification of region boxed in **A** showing the double label **B** and the anti-Repo (magenta) channel **C**. At this magnification, the labeling reveals individual nuclei (arrows) of glial cells that are closely associated with the axons. These glial cells have been shown to express *fray* and Gliotactin, and will give rise to the subperineurial glia in larval nerves. Bar: 20 microns. **D, E:** Drosophila whole embryo, stage 16, labeled with anti-HRP (green) and probed with *NCC69* anti-sense DNA (magenta). The nerve glia express the *NCC69* mRNA (arrowheads), visible in the double-labeled image **D** and the *NCC69* (magenta) channel **E**. Bar: 20 microns. **F, G:** Fillet preps from 3rd instar larvae, stained with DAB anti-HRP to label the nervous system. *Wild type* **F**

nerves appear uniform in width (arrowheads). By contrast, *NCC69* **G** mutant nerves have localized bulges and swellings (arrowheads). Fillet preps from 3rd instar larvae expressing an RNAi construct targeted against *NCC69* in neurons **H** or subperineurial glia **I**. When the RNAi construct is expressed in neurons, the nerves appear wild type (compare with **F**). Expression of the RNAi construct in nerve glia causes bulges in the nerves **G**. Genotypes: **F**, *CS*. **G**, *NCC69[r1]*. **H**, *w*, *C155-GAL4/+; UAS-RNAi(NCC69)/+*. **I**, *w*; *Gli-GAL4/+; UAS-RNAi(NCC69)*. **F–I** are to the same scale (Bar = 20 microns).

Figure 3. Evoked action potential activity is unaffected in *NCC69* mutants

Excitatory junctional currents (EJCs) were recorded from specific ventral longitudinal muscle fibers of a *wild type* (CS) and an *NCC69^{r2}* larva, in response to trains of stimuli at 10 and 50 Hz evoked by shocking the nerve at a distance of at least 500 micrometers from the innervated segment. This ensured that in *NCC69* mutants, the resulting action potentials would encounter bulges as they traveled toward the nerve terminal. The failure of an action potential to arrive at the nerve terminal results in a missing EJC (asterisks above the wild type trace at 50 Hz). The all-or-nothing response indicates that the EJCs are a reliable indicator of action potential propagation. The shock artifacts, which appeared as upward deflections on the traces, have been clipped for easier viewing.

Figure 4.

In vitro analysis of NCC69 cotransporter function. **A:** Anti-HA staining of 1B4, a stable SL2 cell line transfected with a construct to express HA:NCC69. A high proportion (ca. 85%) of the cells express HA:NCC69 at varying levels. The construct is detected at the cell membrane (inset), as well as in intracellular compartments. Bar: 10 microns (inset, 5 microns).

B: Assays of ^{86}Rb flux in SL2 transfected cell lines expressing HA:NCC69 versus IZT (vector alone). Cell lines were subjected to four different “activation” conditions: isotonic (control), hypertonic, hypotonic, and 0 Cl isotonic media. In the three activation conditions, the HA:NCC69-transfected cell line responded with a greater ^{86}Rb flux than the control. The flux was inhibited by bumetanide, a compound that inhibits cotransporters from humans and other species. **C–F:** Dependence of ^{86}Rb flux in HA:NCC69 cells on bumetanide **C**, Na **D**, Rb **E**, and Cl **F** concentrations. The results are shown for a representative experiment with individual data points and a curve fit using a non-linear least squares algorithm. For Na and Rb, the data best fit using a model that assumes a single ligand-binding site; for Cl, the best fit is consistent with two ligand-binding sites. The data points shown as black diamonds with error bars represent the average of four replicates with the standard error about the mean. Where the error bars are not visible, the standard error is smaller than the symbols.

Figure 5.

Human NKCC1 can substitute for NCC69 *in vivo*. 3rd instar fillets stained with anti-HRP, **A–C** or anti-HA **D–F**. **A, D:** *NCC69*. Many nerve bulges are visible (arrowheads in **A**), corresponding to the accumulation of extracellular fluid between glia and axons. Staining of this prep with anti-HA **D** serves as a negative control for the rescue experiments. Genotype: *w; NCC69^{r2}*. **B, E:** Fly rescue. Expressing the *NCC69* cDNA in subperineurial glia in *NCC69* mutants reduces the nerve swelling considerably (arrowheads in **B**). Staining with anti-HA **E** reveals ample expression of the HA:*NCC69* transgene in the nerve. Genotype: *w; Gli-GAL4; NCC69^{r2}, UAS-HA:NCC69*. **C, F:** Human rescue. Expressing the human *NKCC1* cDNA in subperineurial glia in *NCC69* mutants also reduces nerve swelling (arrowheads in **C**), demonstrating that these two genes encode functional orthologs. Genotype: *w; Gli-GAL4; UAS-HA:NKCC1 NCC69^{r1}*. Bar: 50 microns; **A–F** are all to the same scale.

Figure 6.

Quantitation of the nerve phenotype. **A:** Cumulative frequency graphs of nerve widths. Curves from animals with a higher proportion of large nerve widths are shifted to the right; those with a lower proportion are shifted to the left. The rightmost curve is for *NCC69*. The fly rescue (expressing HA:*NCC69* in the nerve glia in *NCC69* mutants) shifts the curve leftward to overlap the wild type curve. The human rescue (expressing HA:*NKCC1* in the nerve glia of *NCC69* mutants) shifts the curve leftward as well, but for the most part does not overlap the wild type curve. The *NCC69* distribution is significantly different from the others ($p < 0.005$; see Results and Table S6). **B:** Percentiles from the distributions shown in **A**. The 50th, 75th, 80th, 85th and 90th percentiles are shown. In each case, *NCC69* has a value that shows a statistically significant difference from the values of the other genotypes ($p < 10^{-4}$; see Results and Table S7). **C:** Average cross-section area of the nerves from the genotypes shown in **A**. The average cross-section area from *NCC69* is significantly larger than the other genotypes (one-tail t test $p < 0.005$; Table 3).

Figure 7. NCC69 and Fray interact by yeast-two-hybrid assay and have strikingly similar mutant nerve phenotypes. **A:** Yeast-two-hybrid interactions between NCC69 and Fray. **B–D:** Transmission electron micrographs of nerves from 3rd instar larvae. In *wild type* **B**, axons (“A”) and glial cell processes (“G” and arrowheads) are closely associated, with little extracellular space. By contrast, in *NCC69* **C** and *fray* **D** mutant nerve bulges, axons and glia may be separated by a large amount of extracellular space (“E”). Bar in **D**: 1 micron; **B–D** are to the same scale. Genotypes: **B**, *CS*. **C**, *w*; *NCC69*^{*r2*}. **D**, *ry fray*^{*r1*}.

Figure 8.

A model of NCC69 function in larval nerves, showing how the Na/K ATPase could create the need for solute removal from the extracellular space in larval nerves. The diagram shows a simplified view of the nerve, consisting of an axon and the subperineurial glia, whose septate junctions (SJ) restrict paracellular flow. The model leaves out two classes of glia, the wrapping glia, which are located inside the nerve, and the perineurial cells which are located on the outside, because they do not form septate junctions, and would not pose much of a barrier to paracellular ion flow. The ion flows in the diagram are represented by arrows. Several known ion flows (e.g., I_h and I_{Na}) have been omitted for the sake of simplicity. **1:** The flow of ions is initiated by the action potential which leaves increased extracellular K in its wake through voltage-gated K channels. **2:** In each cycle, the Na/K ATPase removes 2 K ions from the extracellular space, and replaces them with 3 Na ions. **3:** Cl ions move to balance the gain in positive charge. These Cl ions could flow from other parts of the extracellular space and/or from intracellular sources, e.g., through Cl channels or transporters. The net effect of the Na/K ATPase is to accumulate NaCl in the extracellular space. If left unchecked (as in *NCC69* mutants), the accumulation of NaCl draws water into the extracellular space through osmosis, causing swelling. **4:** NCC69 relieves the pressure by transporting solutes into the subperineurial glia, causing it to swell. **5:** The subperineurial cell exports solutes, presumably into the hemolymph, to maintain volume homeostasis. **6:** K flows down the axoplasm to replace the K that is lost.

Table 1Failure rates of EJC's in *wild type* and *NCC69* mutants

Genotype	frequency (Hz)			
	10	20	50	100
<i>CS (wild type)</i>	0 (0)	1.1 (0.9)	9.5 (3.2)	23.1 (7.2)
<i>NCC69²</i>	0 (0)	0 (0)	0 (0)	2.9 (3.2)
p-value (t-test)	NA	0.886	0.995	0.989

Nerves were subjected to trains of 50 shocks at the frequencies shown, and currents recorded from various muscles (MFs 6, 7, 12, or 13) under voltage clamp. Failures of the muscle to respond were tallied. Mean numbers of failures (and standard error in parentheses) are given for the two genotypes. For CS, n=12 muscles except at 100 Hz where n=8. For NCC69, n=7 muscles, except at 100 Hz where n=6. The p-values result from testing the hypothesis that NCC69 nerves have more failures than wild type.

Table 2

Kinetic parameters of NCC69

	K_m (mM)			K_i (μM)
	Na	Rb	Cl	Bu
Dros NCC69	7.44 (0.66)	1.48 (0.16)	30.32 (1.85)	0.049 (0.007)
Human NKCC1	14.57 (1.24)	1.95 (0.09)	31.25 (0.79)	0.69 (0.13)

The kinetic parameters for NCC69 were calculated from data represented in Figure 4. The parameters were calculated using a non-linear, least squares fit algorithm (see Materials and Methods). The mean from a minimum of six experiments is shown with the standard error about the mean shown in parentheses. The parameters for human NKCC1 are from published data (Isenring et al. 1998), and are presented here for comparison.

Table 3Mean cross-sectional areas of *NCC69* and rescues

Genotype	mean area A	std err	n	width
<i>CS (wild type)</i>	52.4	3.7	10	8.2
<i>NCC69²</i>	106.4	8.5	5	11.6
Fly rescue	50.8	1.7	4	8.0
Human rescue	66.9	3.8	4	9.2

For each animal, the average area, A (μm^2), was calculated from 10 to 12 nerve segments as described in Experimental Procedures. These values of A were then averaged for each animal. The mean of those averages is what is presented in column 2, with the corresponding standard error in column 3. The number of animals (n) is shown in column 4. Column 5 gives the corresponding nerve width (μm), derived from the value in column 2.