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Maintenance and regulation of extracellular volume and the ion environment in Drosophila larval nerves

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Abstract

In mammals and insects, paracellular blood barriers isolate the nervous system from the rest of the animal. Glia and accessory cells of the nervous system use pumps, channels, cotransporters, and exchangers collectively to maintain the extracellular ion environment and osmotic balance in the nervous system. At present the molecular mechanisms that regulate this process remain unclear. In humans, loss of extracellular ion and volume regulation in the nervous system poses serious health threats. Drosophila is a model genetic organism with a proven track record for uncovering molecular mechanisms relevant to human health and disease. Here, we review what is known about extracellular ion and volume regulation in larval abdominal nerves, present some new data about the impact of neural activity on the extracellular environment, and relate the findings to mammalian systems. Homologies have been found at the level of morphology, physiology, molecular mechanisms, and mutant phenotypes. The Fray-Ncc69 module regulates extracellular volume in larval nerves. Genetic rescue experiments with the mammalian orthologs prove this module has a direct correlate in humans. This and other molecular homologies, together with the similar physiological needs, suggest that uncovering the molecular mechanisms of ion and volume regulation in larval nerves will likely provide significant insights into this process in mammalian systems.

Keywords

blood-nerve barrier; blood-brain barrier; extracellular fluid; osmoregulation

Introduction

In many insects the hemolymph has an ionic composition that is incompatible with neural function (Bishop et al. 1925; Bone 1944; Brecher 1929; Drilhon 1934; Tobias 1948). For example, the potassium (K) concentration of hemolymph often can range from 35 to 60 mM, levels that would depolarize a neuron and effectively block action potential firing. In a classic experiment nearly 60 years ago, Graham Hoyle found that while bathing locust nerves in 70 mM K saline had little effect on evoked action potential propagation, the injection of a small volume of 70 mM K solution into the nerve blocked firing within seconds (Hoyle 1952). Significantly, within minutes after the injection, action potential activity recovered. This result demonstrated both the presence of an effective barrier isolating the nervous system from the blood, as well as a homeostatic mechanism within the nervous system to maintain low extracellular K concentration around neurons.

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In both mammals and insects, blood-brain barriers play several roles, including: 1) maintaining an extracellular ionic environment within the nervous system that is compatible with electrical activity; 2) controlling the access and levels of neurally active molecules, metabolites, and immune cells; and 3) blocking the entry of harmful substances, such as neurotoxins.

Given the universal need to isolate the nervous system from its environment, it is not surprising that key molecular mechanisms are evolutionarily conserved. Many of the effector molecules responsible for maintaining the extracellular ionic balance have been identified, including proteins that mediate the movement of ions, such as the Na/K ATPase, cotransporters, exchangers, and channels, as well as the proteins that form the cellular junctions that restrict ionic movement. The transfer of solutes across an otherwise impermeable membrane introduces the additional challenge of controlling osmotic pressure and edema, a problem common to all organisms with a blood-nervous system barrier. We do not yet have a full understanding of the physiological and signaling mechanisms that determine how extracellular ion and volume regulation are jointly regulated.

The nervous system of Drosophila is emerging as a valuable model for studying the in vivo mechanisms governing ionic homeostasis and extracellular volume regulation. Important insights have emerged from studies of the peripheral nerves of Drosophila larvae. The larval abdominal nerves have a relatively simple structure, composed of four cells types. The critical paracellular blood-barrier is established by a glial ensheathment located near the surface of the nerve. This arrangement is simpler and experimentally more accessible than that of vertebrates, where the principal paracellular barrier is located at the interface of capillaries that ramify throughout the nervous system (see Table 1). Many of the key molecules are conserved from Drosophila to mammals, making it likely that at least some of the physiological mechanisms are also the same.

Here we review what is known about ion and volume regulation in the larval nerves of Drosophila. Beginning with a morphological description of the nerves and of the cells that form the blood barrier, we turn to the molecular properties of the paracellular junctions, and examine a molecular signaling cassette that regulates extracellular volume, along with the corresponding mutant phenotypes. We next relate the findings in insects to what is known in mammalian systems, and close by describing a path for future studies that will lead to a more complete description of this physiological mechanism.

Morphology of larval nerves

The larval abdomen consists of 8 bilaterally symmetric segments. The first seven, termed A1-A7, have nearly identical arrangements of muscle fibers, sensory cells, and neural connectivity (for review, see Ruiz-Canada and Budnik 2006). The main innervation of each hemisegment is provided by a single abdominal nerve that originates from the lateral edge of each ventral ganglion segment (Figure 1A). There is also a smaller transverse nerve that emerges from the dorsal midline. In the periphery, the abdominal nerves divide into five major branches: the intersegmental nerve branch (ISN), and the four segmental nerve branches (SNa-d). Between the ventral ganglion and the periphery, the nerves float freely in hemolymph. Like the hemolymph of other phytophagous insects, larvae have higher levels of K (circa 40 mM; Begg and Cruickshank 1963; Croghan and Lockwood 1960; Stewart et al. 1994) than is compatible with neural function (Auld et al. 1995; Baumgartner et al. 1996; Schwabe et al. 2005).

The abdominal nerves of segments A1-A7 each contain about 90 axons, surrounded by three layers of glial cells (Figure 1D). The inner-most layer of glia, termed wrapping glia by Stork et al. (2008), extend processes around both single axons and axon fascicles. The middle

layer consists of subperineurial glia that form the blood-nerve barrier. The perineurial glia form the outermost layer, which underlies the neural lamella, an extracellular matrix.

The three glial cell types are distinguished by their patterns of gene expression and can be identified in enhancer trap lines. The innermost wrapping glia strongly express Nervana2 (Nrv2), a beta subunit of the Na/K ATPase; there are corresponding enhancer trap and gene trap lines. The subperineurial glia form “autocellular” pleated septate junctions—the junctions form between membrane extensions from the same cell—which are the basis for the paracellular blood barrier in the larval CNS and nerves (Stork et al. 2008). They also express proteins required for septate junction formation, including NeurexinIV (NrxIV), Gliotactin (Gli), and Moody (Auld et al. 1995; Bainton et al. 2005; Banerjee et al. 2006a; Baumgartner et al. 1996; Schwabe et al. 2005; Stork et al. 2008). There are GAL4 lines available that use gli and moody enhancer sequences to drive expression in these cells. Unfortunately, no molecular marker has been found that is expressed exclusively in perineurial glia, although the c527 enhancer trap line reportedly labels these cells preferentially (Hummel et al. 2002; Stork et al. 2008). The availability of GAL4 lines that express in subsets of glia allows researchers to test gene function specifically in these cells, in a “mosaic” fashion.

The development of the nerve-associated glia has been the subject of several studies (Edenfeld et al. 2006; Fredieu and Mahowald 1989; Schmid et al. 1999; Schmidt et al. 1997; Sepp et al. 2000; Silies et al. 2007; von Hilchen et al. 2008) and reviews (Klambt 2009; Parker and Auld 2006). For convenience, we provide a brief summary because many of the molecular markers and genetic tools that are used to analyze the larval glia were first characterized in the embryo, when the nerves are formed.

The abdominal nerves are prefigured by motor and sensory axon projections, beginning at about 10 hours post-fertilization (PF) at 25°C (stage 13; Campos-Ortega and Hartenstein 1997). The sensory neuron cell bodies are located in the periphery and the motoneuron cell bodies are located in the developing ventral ganglion. By 16 hours PF (stage 16), most motoneurons have formed synapses with their targets, and the abdominal and transverse nerves (and associated branches) are established. At this stage, twelve glial cells, termed embryonic peripheral glia (ePG), associate with these nerves (Figure 1B and 1C; von Hilchen et al. 2008). Seven of these cells originate in the CNS, having migrated along the nerves. The five others originate from sensory organ precursors in the periphery. The nomenclature for the peripheral glia has led to inconsistencies and confusion. Recently, von Hilchen et al. (2008) proposed a standardized nomenclature, ePG1-12 and provide a table of equivalencies. The correspondence between ePG and larval nerve glia has not been unequivocally established. Nevertheless, several molecular markers and genetic tests provide strong evidence for some of the relationships.

One major difficulty for following peripheral glial development is the tremendous growth of larval abdominal nerves, increasing from a few hundred micrometers at hatching to up to 4 mm in a mature 3rd instar larva (compare inset embryo to 2nd instar larva in Figure 1A). Remarkably, the larval wrapping and subperineurial glia do not divide in larvae (Sepp et al. 2000). Most of their prospective progenitors, the ePG, are located in the periphery, leaving only three to five ePG (#1-5) in a position along the nerve to differentiate into the larval wrapping and subperineurial glia. Stork et al. (2008) report there are two to three wrapping glia per abdominal nerve, which leaves perhaps only three ePG to account for all the subperineurial glia. This fits with published reports and our own observations that even in the longest nerves, there appear to be just a few nuclei of wrapping and subperineurial glia, which can be identified by their large and oblong shape (Auld 1996).

Unlike their internal counterparts, the perineurial glia divide during larval development. In the first instar larvae, nerve perineurial glia are relatively few in number and do not completely envelop the nerves (Stork et al. 2008). Their number subsequently increases through cell division, and they are readily labeled during the 3rd instar with a pulse of BrdU (Leiserson et al. 2000) or identified in MARCM clones (Stork et al. 2008). The origin of the perineurial cells is controversial. Although Edwards et al. (1993) concluded that the CNS perineurial glia were of mesodermal origin, they based their conclusion on the observation that *twist* mutant embryos, which lack mesoderm, also lack perineurial cells. However, the loss of mesoderm causes secondary and non-autonomous effects (e.g., induction of trachea morphogenesis; Sutherland et al. 1996), so other approaches, such as lineage analysis, are needed to establish the origin of perineurial glia.

Blood barriers of larval nerves

The paracellular barriers to diffusion in larval nerves are provided by pleated septate junctions (pSJs). These are visible as ladder-like structures by transmission electron microscopy. First described in hydra over 50 years ago (Wood 1959), pSJs are found in many epithelial and secretory tissues of invertebrates (Noirot-Timothee and Noirot 1980). There is good evidence that they provide a barrier to diffusion, analogous to tight junctions found in mammals and other vertebrates. Significantly, pSJs are also found in mammals at the nodes of Ranvier of myelinated axons (reviewed in Banerjee et al. 2006b).

The evidence that pSJs provide a barrier to diffusion is abundant. Early investigators proposed a barrier function based on their location near the apical membrane of the cells that form barriers. Lanthanum impregnation studies showed that the ion could not penetrate across the pSJs (Carlson et al. 2000; Juang and Carlson 1994; Lane 1991; Noirot-Timothee and Noirot 1980). The response to treatment with non-isosmotic solutions was also similar to that of tight junctions (Lord and DiBona 1976; Noirot-Timothee and Noirot 1980). More recently, the molecular components of Drosophila pSJs have been identified, along with mutations in the corresponding genes (Banerjee et al. 2006b; Tepass et al. 2001). Mutations in many of these genes disrupt pSJs and/or compromise the paracellular barriers (Banerjee et al. 2006a; Baumgartner et al. 1996; Behr et al. 2003; Faivre-Sarrailh et al. 2004; Genova and Fehon 2003; Lamb et al. 1998; Llimargas et al. 2004; Paul et al. 2003; Schulte et al. 2003; Strigini et al. 2006; Wu et al. 2004; Wu et al. 2007).

In Drosophila, pSJs are found in a wide variety of tissues of ectodermal origin, including epidermis, salivary glands, foregut, hindgut, trachea, and subperineurial glia of the nervous system (Tepass and Hartenstein 1994; Tepass et al. 2001). Consequently, mutations that affect pSJ formation or structure often affect many tissues. Although it is tempting to extrapolate findings about pSJs from one tissue to another, we should do so with caution because pSJs from different tissues may have different proteins and functions. One such difference involves the beta subunit of the Na/K ATPase, Nrv2, a component of ventral ganglion, tracheal, and epidermal pSJs, which is absent in the nerve SPG. Interestingly, Nrv2 is strongly expressed in the wrapping glia which are not thought to form pSJs (Stork et al. 2008).

Regulation of the ion environment and extracellular volume

The presence of a blood barrier requires a physiological system to maintain ionic balance and volume homeostasis (Treherne and Schofield 1981). Such a mechanism is particularly important in the nervous system because electrical activity leads to a rise in extracellular K, which if left unchecked, would block action potential firing. The idea that neural activity would lead to increased extracellular K dates back to the first descriptions of the ionic basis

of the action potential. In squid, tonic excitation of the nerve results in a reduction in the hyperpolarizing after-potential and reduced amplitudes of the action potential, both of which result from increased extracellular K and a more positive equilibrium potential for K (Frankenhaeuser and Hodgkin 1956). Recordings from glia (Schwann cells) in the mud puppy optic nerve indicated that there was a corresponding depolarization of the glial membrane (Orkand et al. 1966). Recordings from Muller glia of the Salamander optic nerve indicated the presence of a "K siphon," where K is ejected from the glial endfeet in response to increased K concentrations at distal processes (Newman et al. 1984). These observations provide strong evidence that one role of glia is to remove excess K from the extracellular environment of axons, maintaining K homeostasis.

The mechanism often invoked to explain how glia accomplish this task can be broken down into two steps: K uptake and spatial buffering. It has been shown that glia take up K via mechanisms that involve K channels, Na/K ATPase, and NKCC cotransporters (Coles and Dietmer 2005; Kofuji and Newman 2004; Walz 2000). Because glia are often large cells, they could provide a reservoir for excess K, which the glial cell would slowly release into the extracellular space. By contrast, the concept of spatial buffering or "K siphoning," pioneered by Newman (1984), is a dynamic process, in which K flows out of the glia away from the site of absorption (Kamasawa et al. 2005; Kofuji and Newman 2004; Menichella et al. 2006; Orkand 1986).

In systems as diverse as cats, rats, and bees, it has been found that increases in extracellular K (e.g., as the result of neural activity) not only causes a flux of K into the glia, but also glial cell swelling, and a decrease in extracellular volume (Coles et al. 1986; Dietzel et al. 1980; Dietzel et al. 1982; Ransom et al. 1985; Svoboda and Sykova 1991; Walz and Hinks 1985). The volume changes are thought to result from osmotic forces arising from the influx of ions across the cell membrane. Inhibition of the NKCC1 cotransporter in optic nerves and primary cultures of astrocytes reduces the glial swelling that arises in response to increased extracellular K, suggesting that NKCC1 mediates the change in volume (MacVicar et al. 2002; Su et al. 2002a; Su et al. 2002b; Walz and Hinks 1986). Analysis of the kinetics of extracellular volume change in response to neural activity suggests that during high frequency firing (100 Hz), the extracellular volume increases by about 20%, and only decreases once the activity has stopped (Svoboda and Sykova 1991).

The evidence to date suggests that some of the same mechanisms of extracellular ion and volume regulation that have been found in vertebrates also apply to Drosophila. We have shown that cotransport by an NKCC1 ortholog regulates the extracellular space of larval nerves (Leiserson et al. 2010). Recently, we obtained evidence that mutants that have chronically active nerves throughout larval life show a corresponding increase in the extracellular volume of their nerves.

The *ether-a-gogo* (*eag*) and *Shaker* (*Sh*) mutations each reduce the expression of specific K channels, and double mutants have enhanced neuronal activity throughout development (Budnik et al. 1990; Ganetzky and Wu 1983; Haugland and Wu 1990). The larval nerves of these mutants are swollen, owing to the accumulation of extracellular fluid between axons and glia (Figure 2B and E). This effect is likely the result of neural activity, as mle^{napts}, a mutation that reduces the hyperactivity of *eag Sh*, also reduces nerve widths back to wild type levels (Figure 2E).

The ultrastructure of *eag Sh* larval nerves resembles that of *fray* and *Ncc69*, mutations that reduce Na-K-Cl cotransport through Ncc69, the ortholog of human NKCC1 (Figure 2). Based on a large body of work on its mammalian homologs as well as genetic data in flies, Fray is a Ser/Thr kinase that activates Ncc69 cotransport (Leiserson et al. 2010;Leiserson et

al. 2000). Nerves of 3rd instar larvae mutant for *fray* or *Ncc69* have fluid between axons and glia. Mosaic knockdown (RNAi) and rescue experiments show that the *fray* and *Ncc69* genes are genetically required in SPG, the cells that form the blood barrier. Mammalian orthologs of Fray and Ncc69, rat PASK and human NKCC1 respectively, can rescue the corresponding fly mutants, demonstrating a strong conservation of the molecular function of these molecules.

In mammalian systems, PASK (also known as SPAK) has been shown to bind, phosphorylate, and activate NKCC1 transport (Dowd and Forbush 2003; Gagnon et al. 2006; Gagnon et al. 2007; Piechotta et al. 2003; Vitari et al. 2006). Given that the fly orthologs are expressed and required in the same nerve glia, and cause a near identical phenotype in the nerves, it is likely the mammalian results also apply to Drosophila. In support of this view, Fray and Ncc69 have been shown to interact in a yeast-two-hybrid assay (Leiserson et al. 2010).

Although nerve bulges in *Ncc69* and *fray* mutants can exceed 70 μm in diameter, 10 times the normal nerve width, the impact on neural transmission is minimal, as judged by behavioral, genetic, and electrophysiological criteria (Leiserson et al. 2010). Mutant larvae crawl normally, are not temperature-sensitive paralytic, and respond normally to nociceptive stimuli. Even when combined with mutations that affect neural activity, *Ncc69* loss of function causes no detectable change in activity. This suggests that the K concentration within the bulges is low, and that the primary abnormality is the accumulation of extracellular fluid with otherwise normal ionic composition.

A model that incorporates these results is shown in Figure 3. Neural activity causes K to flow out of the axon through voltage-gated K channels, resulting in increased concentrations of K. The Na/K ATPase acts to restore the Na and K ion gradients, moving 3 Na ions out of the cell for every 2 K ions into the cell. The net effect is to accumulate solutes in the extracellular space. We propose that Ncc69 functions to remove excess solutes from the extracellular space, importing them into the SPG. Ncc69 would also reduce extracellular K, because it moves 1 K ion along with 1 Na and 2 Cl. In this model, for every two cycles of the Na/K ATPase, Ncc69 cycles once to maintain osmotic balance, exchanging 5 Na for 5 K.

Relationship of Drosophila nerves to mammalian systems

The genetic rescue of *fray* and *Ncc69* mutants by expression of the mammalian orthologs demonstrates the conservation of molecular function. The homologies extend beyond the molecular, however, and can be classified at the level of physiology, morphology, molecular mechanisms, and mutant phenotypes.

Physiology

The nervous systems of flies and mammals are isolated by blood barriers that pose similar physiological problems of ion and volume homeostasis. All organisms use Na and K gradients and fluxes to generate action potentials. There is thus a universal need to regulate extracellular K and maintain ion and volume homeostasis.

Morphology

Mammalian and larval nerves share the common feature of glia that wrap axons. At the ultrastructural level, larval nerves closely resemble immature unmyelinated mammalian fibers, in which a single Schwann cell wraps many axons. While unmyelinated fibers predominate during early development, they remain common in adult mammals, involving nearly 50% of motor axons and 75% of cutaneous axons (Berthold and Rydmark 1995).

Molecular mechanisms

The molecules known to mediate extracellular volume regulation, including Fray, Ncc69, and the pSJ proteins, have human orthologs. Human NKCC1 can substitute for Ncc69 in flies, and rat PASK for Fray, demonstrating the functional conservation of these molecules. Such experiments demonstrate not only that the effector function of each molecule is conserved, but that the regulatory elements are also conserved. Otherwise the alien molecule would not be able to respond appropriately to the regulatory signals in the glial cell. In most cases when a human protein can substitute for the fly homolog, entire pathways have been found to be conserved. Thus, we are optimistic that as further glial molecules are identified that function in the fly Fray-Ncc69 pathway, many will have human orthologs.

The other molecules demonstrated to have a role in extracellular ion and volume regulation in larval nerves are the components of the pSJs. In the absence of pSJs, the paracellular blood barrier fails allowing ions and solutes to exchange with the hemolymph. Although pSJs have not been observed at the blood barriers in mammals, they are found in the paranodal regions of the nodes of Ranvier, where they form “axo-glial” junctions between the axons and the terminal loops of myelin. Components of the axo-glial pSJs have fly homologs that are found in fly pSJs (Table 2). Knocking down the mammalian pSJ components causes a corresponding reduction in axo-glial pSJs (Bhat et al. 2001;Boyle et al. 2001).

The other presumed components of the apparatus that regulates ion and volume homeostasis in larval nerves have not been tested in vivo. These include the Na/K ATPase, K and Cl channels, transporters, and exchangers, all of which have potential orthologs in Drosophila (Table 3). The Na/K ATPase is of central importance, because it not only generates the Na and K ion gradients, but is also a structural component of pSJs in fly epithelia, trachea, and salivary glands.

Ion/volume misregulation phenotypes

In mice and humans, there is a growing number of ion regulation phenotypes that have been observed in the nervous system and inner ear. Knockdowns of Connexins Cx32 and Cx47 (Anzini et al. 1997; Menichella et al. 2003; Menichella et al. 2006; Odermatt et al. 2003; Oh et al. 1997; Scherer et al. 1998), Kir4.1 (Djukic et al. 2007; Kofuji et al. 2000; Neusch et al. 2001), and KCC3 (Byun and Delpire 2007) result in “extracellular vacuoles” in and around myelinated fibers of the nervous system. Genetic analysis and expression studies have provided a model in which these molecules function together to remove excess K from paranodal regions (Kofuji and Newman 2004; Somjen 2002; Walz 2000).

The gene encoding Cx32 is of particular interest because it is associated with a human inherited neuropathy, Charcot-Marie-Tooth disorder (CMT1X). In mice mutant for this gene, the primary nerve phenotype is a progressive demyelinating neuropathy, characterized by proliferating Schwann cells (“onion bulbs”) and abnormal myelinated fibers (Anzini et al. 1997; Oh et al. 1997; Scherer et al. 1998). Mutants of *Cx47* or *Kir4.1* develop extracellular vacuoles in white matter of the CNS (Neusch et al. 2001; Odermatt et al. 2003), and interact genetically with *Cx32* (Menichella et al. 2006). In the optic nerves of *Cx32 Cx47* double mutants, the size of the vacuoles varies with neural activity (Menichella et al. 2006). Mutation of *KCC3*, one isoform of the K-Cl cotransporter, causes a peripheral neuropathy in mice, with periaxonal swelling (Byun and Delpire 2007; Sun et al. 2010). These results suggest that the vacuoles and swelling in these various mutant combinations are the result of the accumulation of extracellular solutes, similar to the phenotype seen in *fray* and *Ncc69* larval nerves.

Mouse SPAK and NKCC1, orthologs of Fray and Ncc69, are expressed in peripheral nerves, but their function there has not been demonstrated (Alvarez-Leefmans et al. 2001; Piechotta et al. 2003). By contrast, NKCC1 plays a demonstrably essential role in the inner ear, where it is required for a process known as K recycling (Kikuchi et al. 2000; Wangemann 2002). Indeed, the most prominent phenotype of *NKCC1* mouse mutants is deafness and vestibular malfunction (Delpire et al. 1999; Dixon et al. 1999; Flagella et al. 1999), similar to a deafness syndrome in humans caused by loop diuretics that target NKCC1 (Arnold et al. 1981). NKCC1 is expressed in the basolateral membrane of marginal cells of the stria vascularis of the inner ear (Crouch et al. 1997). These cells secrete K into the endolymph through an apical channel, Kv7.1 (isK; Vetter et al. 1996). In response to mechanical stimuli, K from the endolymph passes through apical channels into hair cells, after which it is expelled from the basolateral regions of the hair cells into the extracellular space. From there, it takes a route through and between cells back to the stria vascularis.

Pharmacological or genetic knockdowns of the molecules that mediate K recycling have ultrastructural defects that look remarkably similar to fly *fray* and *Ncc69* mutants. Mice defective for NKCC1 or Kv7.1 accumulate extracellular fluid in the stria vasculari (Santi and Lakhani 1983; Vetter et al. 1996; Wangemann 2002). Similarly, the Kv7.3 and Kv7.4 K channels; ClC-K Cl channels; a variety of Connexins; and KCC3 and KCC4; all participate in this recycling, and give mutant phenotypes that include the accumulation of extracellular fluid (Boettger et al. 2002; Boettger et al. 2003; Wangemann 2002; Zdebik et al. 2009; Zhao et al. 2006).

Prospects

The homologies between ion and volume regulation in flies and mammals suggest findings in one will provide insights into the other. Drosophila has a rich tool kit for discovering and analyzing molecular mechanisms of biological processes. For most genes, mutations and/or RNAi lines are available that can be used to knock down gene function. Transgenic gain and loss of function experiments are particularly powerful, as it is possible to drive expression of a dominant-acting construct in specific cells or tissues at any time during development. This is done using bipartite expression systems such as GeneSwitch or TARGET (Han et al. 2000; McGuire et al. 2003; Osterwalder et al. 2001; Roman et al. 2001). Other tools, such as the FLP and MARCM allow sophisticated lineage and genetic mosaic analysis (Lee and Luo 2001; Struhl and Basler 1993; Xu and Rubin 1993). Perhaps the most exciting prospect for Drosophila is to use it to screen for mutations that by themselves, or in combination with other mutations, affect ion/volume homeostasis. Given the dramatic phenotypes associated with homeostatic dysfunction in the larval nerves, it should be possible to rapidly extend the relevant signaling cassettes to new molecular players. The genes identified in the fly would then point to the relevant candidate genes to study in mammals.

One major advance from a screen for glial genes would be to identify the molecules upstream of Ncc69 that trigger its activation. In mice, members of the WNK kinase family of Ser/Thr kinases have been shown to work with SPAK and OSR1 to regulate NKCC1 activity in response to changes in osmolarity and intracellular Cl, but the sensing mechanism that activates these kinases remain elusive (Kahle et al. 2008; Richardson and Alessi 2008). What exactly are the molecules that sense the need to activate cotransport, and what exactly are they sensing? Is it low Cl, stress on the cytoskeleton from osmotic forces, a combination of those two, or something else entirely? Answering these questions would be very helpful in understanding how extracellular ion/volume is regulated in larval nerves. In mammals, NKCC1 is expressed in a wide variety of cells and locations, including parts of the nervous system, including neurons (Dzhala et al. 2005; Plotkin et al. 1997b; Wang et al. 2002), glia (Alvarez-Leefmans et al. 2001; Su et al. 2002b; Wang et al. 2003), endothelial cells of brain

capillaries (O'Donnell et al. 2004), and the epithelium of the choroid plexus (Plotkin et al. 1997a). In these different cell types, a variety of cellular functions have been ascribed to NKCC1, including setting the internal Cl concentration in neurons and glia (Hubner and Rust 2006; Wang et al. 2003), K homeostasis and volume regulation in glia (Chen and Sun 2005), and the secretion of cerebrospinal fluid and brain interstitial fluid (Davson et al. 1987; O'Donnell et al. 2004).

NKCC1 has been implicated in the swelling that is associated with stroke and traumatic brain injury (Chen and Sun 2005; Yan et al. 2001). Endothelial cells of the brain capillaries respond to ischemia by an elevated secretion of NaCl and water into the brain parenchyma, resulting in swelling (O'Donnell et al. 2004). This response precedes the breakdown of the blood-brain barrier, and is responsible for the initial swelling following ischemic attacks. NKCC1 also mediates swelling in astrocytes in response to ischemia, which likely restricts blood flow (Chen and Sun 2005).

Yet despite the widespread expression of NKCC1, the knockout phenotype is relatively modest. This is probably due to functional redundancy among the proteins involved in maintaining the blood-brain barrier, complicating the genetic analysis. By contrast, the homologous Ncc69 cotransporter of Drosophila is principally expressed by the glia of the blood-nerve barrier (the SPG), and has a pronounced loss of function phenotype. The genetic analysis of Drosophila orthologs, such as *fray* or *Ncc69*, is a good way to understand the mechanisms that regulate homeostasis in the mammalian nervous system. A better understanding of the molecular basis of ion/volume homeostasis would in turn speed the development of effective clinical treatments for the swelling associated with stroke and brain injury.

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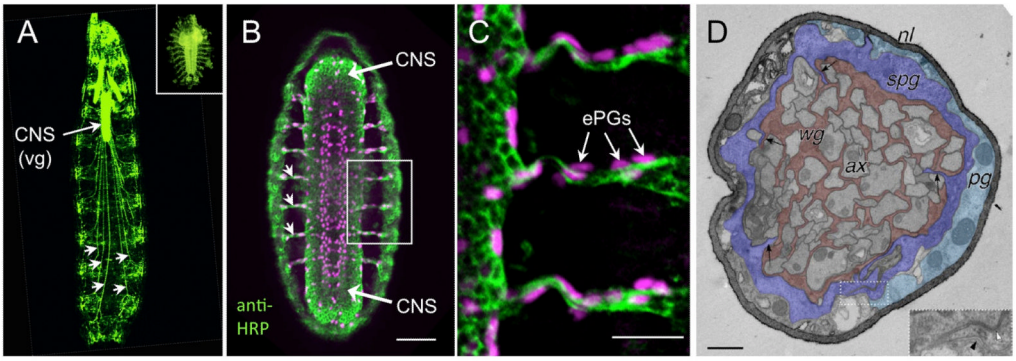

Figure 1.

Anatomy of the larval nervous system. **A.** An intact 2nd instar larva expressing GFP in the nervous system. The abdominal nerves (some of which are marked with arrowheads) are hundreds of microns long, linking the ventral ganglion (vg) to the periphery. **Inset:** Nervous system of an embryo (fillet prep) to the same scale as the larva, stained with the neuronal marker anti-HRP. **B.** Whole embryo stained with anti-HRP (green) and anti-repo (magenta). At this stage the precursors of the subperineurial glia, the embryonic peripheral glia (ePG, magenta) are migrating along the nerves (green), which are less than 20 μm long. Bar: 50 microns. **C.** Enlarged view of the boxed area in **B** shows the close association of the ePG (some of which are indicated by arrows) with the nerves Bar: 20 microns. **D.** TEM of a 3rd instar larval abdominal nerve, showing the four cellular components: axons (ax), wrapping glia (wg), subperineurial glia (spg), and perineurial glia (pg). The inset shows an example of an autocellular septate junction between two processes of a SPG (with permission from Stork et al. 2008). Bar: 1 micron.

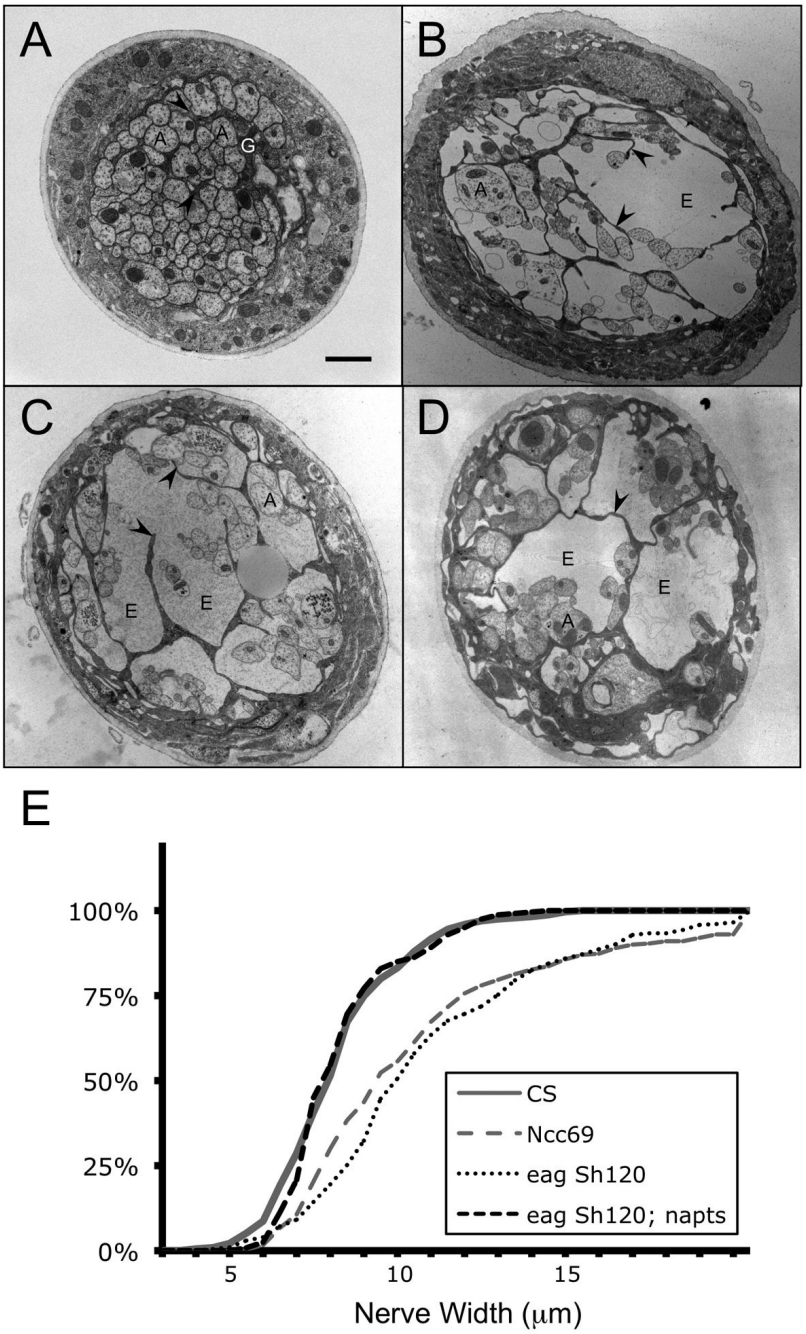

Figure 2.

Drosophila bulging nerve mutants. **A-D:** Transmission electron micrographs of nerves from 3rd instar larvae. In *wild type* **A**, axons ("A") and glial cell processes ("G" and arrowheads) are closely associated, with little extracellular space. By contrast, in *eag Sh* **B**, *Ncc69* **C**, and *fray* **D** mutant nerve bulges, axons and glia may be separated by a large amount of extracellular space ("E"). **E.** 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. Both *Ncc69* and *eag* Sh^{120} mutant larvae have larger nerve widths than *wild type* (CS). Introducing the *mle*^{napts} mutation into *eag* Sh^{120} suppresses neuronal hyperactivity and restores nerve widths to wild type levels. Bar in **A:** 1

micron; **A-D** are to the same scale. Genotypes: **A**, *CS*. **B,** *eag* Sh^{120}. **C,** *w;* $Ncc69^{r2}$. **D**, *ry* $fray^{r1}$. **E,** *eag* Sh^{120}*;* mle^{napts}.

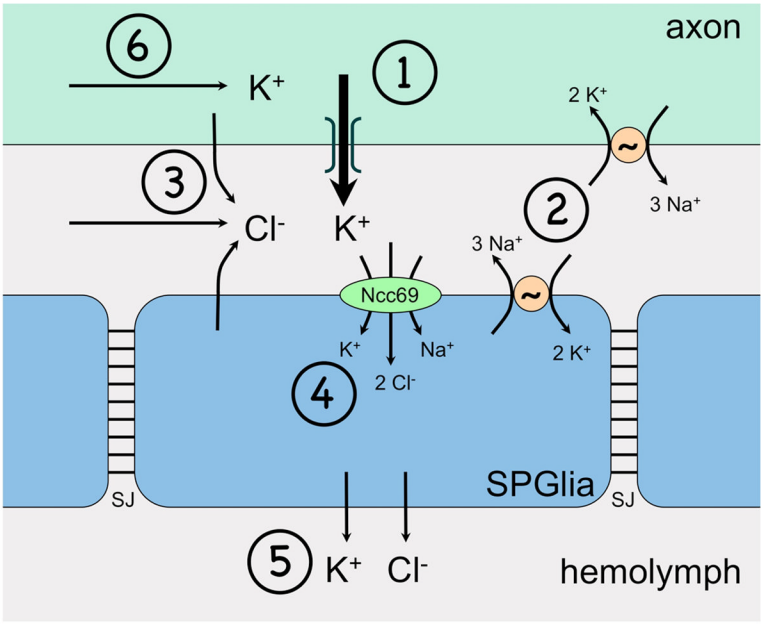

Figure 3.

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 whose roles are unknown: the wrapping glia, which are located inside the nerve, and the perineurial cells which are located on the outside. They are not essential in this model 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. **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 (From Leiserson et al. 2010 with permission.).

Table 1
Properties of fly and human paracellular barriers of the nervous system

Property	Drosophila	Human
location of blood barriers	• subperineurial glia	• perineurium (squamous epithelium) • endothelial cells of brain/nerve microvessels • choroidal epithelium (Blood-CSF Barrier)
paracellular junction	• septate junction	• tight junction (perineurium, endothelial cells, choroidal epithelium) • septate junction (axon-glia)
cells that regulate extracellular volume	• subperineurial glia	• endothelial cells of microvessels • choroid plexus • perineurium? • cerebrospinal fluid drainage pathways

Table 2
Mammalian homologs of Drosophila pleated septate junction molecules

Drosophila	Mammal
NeurexinIV	NCP1 (Nrx/Caspr1/Paranodin)
Coracle	Band4.1
Neuroglian	Neurofascin (NF155)
D-Contactin	Contactin
Sinuous, Megatrachea	Claudins (tight junction proteins)
Gliotactin	Neuroligin3
Discs Large	Dlg1-4
varicose	VAM-1/PALS2
Na/K ATPase subunits	Na/K ATPase subunits
Lachesin	IgLONs

The first four Drosophila pSJ molecules have been found in mammalian pSJs; the others have not. Sinous and Megatrachea are related to Claudins, molecules that have been found at tight junctions, the predominant paracellular barrier in mammals.

Table 3
Drosophila homologs of mammalian ion/volume homeostasis molecules

Function	Drosophila	Mammal
Na-K-Cl cotransport	Ncc69, Ncc83	NKCC1
K-Cl cotransport	Kcc	KCC3, KCC4
Ser/Thr kinase	Fray	OSR1 and SPAK/PASK
K channel (inward rectifier)	Ir, Irk2, Irk3	Kir4.1
K channel (voltage-gated)	KCNQ	Kv7.3, Kv7.4
Cl channel	ClC-a	ClC-Ka, ClC-Kb
Gap junction	(Innexin family)	Connexin family
Aquaporin	Drip, CG4019, CG17662, CG17664, CG7777, Bib*	AQP4

Table shows mammalian molecules, described and referenced in the text, that have been shown to function in ion or volume homeostasis in the nervous system or inner ear with corresponding functions. The only Drosophila orthologs with genetic rescue data are Ncc69 and Fray (Leiserson et al. 2010; Leiserson et al. 2000). The candidate orthologous Drosophila molecules were obtained from OrthoDB (Kriventseva et al. 2008); where no putative orthologs were found, related molecules of similar function are shown in parentheses.

* Evidence suggests that while Bib is homologous to Aquaporins, it may not function as a water channel (Tatsumi et al. 2009).