ECM-RECEPTOR INTERACTIONS REGULATE
THE DISTRIBUTION OF Kir4.1 and AQP4 CHANNELS
IN RENAL TUBULES AND AT THE BLOOD-BRAIN BARRIER

by

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ABSTRACT

Osmostasis relies on the efficient and selective transport of molecules across barriers, often in a directional manner and this, in turn, is dependent on the asymmetric arrangement of channels within the cells comprising the organs that regulate these processes. The primary aim of this thesis is the identification of the extracellular matrix-receptor interactions important in the generation of polarized channel distribution in two such organs, the kidney and the blood-brain barrier. In the first study, we investigated the involvement of fibronectin and laminin in the basolateral localization of Kir4.1, an inwardly-rectifying potassium channel, and aquaporin-4 (AQP4), a water-permeable channel, in polarized Madin-Darby canine kidney (MDCK) cells. We determined using a variety of approaches that laminin-1 and fibronectin, when present in the culture substrate, significantly stabilize both channel types at the basolateral surfaces of these cells and accordingly induce their increased expression within this membrane domain without requiring a concomitant upregulation of de novo channel synthesis. We also show that the coexpressed laminin receptor dystroglycan (DG) is important for cell surface expression of Kir4.1 but not AQP4, and demonstrate via the use of disintegrin peptides and function-blocking antibodies that their cell-surface expression and stability is also partly reliant on integrin receptors, with αvβ3 being particularly important in the case of the latter. In the second study, we examined the possibility that laminin-dystroglycan binding is involved in the regulation of AQP4 turnover in astrocytes. We first determined that laminin, when applied to primary astrocytes in culture, causes AQP4 amounts at the plasma membrane to increase in a DG-dependent manner while depleting the channel from intracellular sites, brought about by the suppression of channel endocytosis. We then showed that DG binds to inactive dynamin, and that the latter, when freed from this inhibitory influence, functions in cooperation with clathrin to mediate the rapid internalization of AQP4. Finally, we demonstrated that laminin selectively upregulates the cell-surface expression of the M23 isoform of AQP4 only. Our findings therefore reveal that, through their roles in establishing the microscopic architecture of these systems, the extracellular matrix and cell-surface receptors are critical determinants in osmoregulation.
PREFACE

Various authors were involved in the research detailed in chapters 2 and 3 of this thesis. Their contributions are as follows:

Chapter 2

The entirety of this chapter, and the research described therein, was written and carried out by Daniel Tham, who was the primary author of the study.

Chapter 3

The GFP-tagged AQP4 constructs used in this chapter were cloned with the aid of Dr. Bharat Joshi. Otherwise, all other portions of the research were completed solely by the author, Daniel Tham.

All chapters were written under the supervision of Dr. Hakima Moukhles, and all research described within this dissertation has been approved by the animal care committee of the University of British Columbia (application number A06-0319).
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vassopressin</td>
</tr>
<tr>
<td>AQP</td>
<td>aquaporin</td>
</tr>
<tr>
<td>AQP4-M1</td>
<td>M1 isoform of aquaporin-4</td>
</tr>
<tr>
<td>AQP-M23</td>
<td>M23 isoform of aquaporin-4</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BN</td>
<td>Blue-native</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCP</td>
<td>clathrin-coated pit</td>
</tr>
<tr>
<td>Cl</td>
<td>chloride ion</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DAG1</td>
<td>dystroglycan gene locus</td>
</tr>
<tr>
<td>DG</td>
<td>dystroglycan</td>
</tr>
<tr>
<td>DGC</td>
<td>dystroglycan complex</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>Dp71</td>
<td>71-kDa dystrophin isoform</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GLUT1</td>
<td>glucose transporter 1</td>
</tr>
<tr>
<td>K+</td>
<td>potassium ion</td>
</tr>
<tr>
<td>Kir</td>
<td>inwardly-rectifying potassium channel</td>
</tr>
<tr>
<td>Large&lt;sup&gt;myd&lt;/sup&gt;</td>
<td>Large myodystrophic</td>
</tr>
<tr>
<td>LAT1</td>
<td>large neutral amino-acid transporter 1</td>
</tr>
<tr>
<td>LG</td>
<td>globular domain of laminin</td>
</tr>
<tr>
<td>LN</td>
<td>laminin</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cell</td>
</tr>
</tbody>
</table>
MEB  muscle-eye-brain disease
MMP  matrix metalloproteinase
Na+  sodium ion
Na+/K+-ATPase  sodium/potassium antiporter
NCC  sodium/chloride cotransporter
NPA  asparagine-proline-alanine motif
OAP  orthogonal array(s) of particles
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate-buffered saline
PDZ  post-synaptic density protein, discs large, zonula occludens-1
PKA  protein kinase A
PKC  protein kinase C
RGD  arginine-glycine-aspartic acid
SDS  sodium dodecyl sulfate
siCTL  control small interfering RNA
siDAG  small interfering RNA specific for DAG1 gene
siRNA  small interfering RNA
TM  transmembrane
VEGF-A  vascular endothelial growth factor A
WT  wild-type
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CHAPTER 1: INTRODUCTION

Biological systems are exquisitely sensitive to environmental perturbations and, as such, numerous homeostatic mechanisms have evolved to maintain internal conditions within a narrow range of values in complex organisms. Osmostasis – the collective term for the myriad events that stabilize the levels of water and solutes within acceptable bounds – relies on processes that are dependent on the flux of solutes and water molecules across semi-permeable membranes, either via passive diffusion, in which these molecules or ions are motivated either by osmotic and diffusional gradients or by active transport, where the impetus is provided by the expenditure of energy. Because of its inherently directional nature, the systems that participate in these processes are, by necessity, characterized by a certain degree of asymmetry in their architectures. This thesis focuses on identifying the factors that influence the asymmetric distribution of the inwardly-rectifying potassium channel Kir4.1 and aquaporin type-4, otherwise known as AQP4, in the kidney, and of the latter in the blood-brain barrier. Within these two disparate systems, both of which are involved in the maintenance of osmostasis, these channels are crucial in allowing these systems to efficiently regulate the osmotic state of the body and the brain respectively.

This introduction aims to provide the background information pertinent to the discussions contained in the subsequent chapters, beginning with a detailed discussion of the structure, characteristics, and regulation of the inwardly-rectifying potassium channels and aquaporin channels. This is followed by an examination of the mammalian kidney and the blood-brain barrier, with an eye towards the roles that they play, and on the functions of the Kir4.1 and AQP4 channels in these systems. The elements likely to be involved in mediating the polarized localization of the channels will then be addressed, starting with the extracellular matrix (ECM) components laminin and fibronectin, and the cell-surface receptors, the integrins, and dystroglycan, with particular emphasis placed on their roles in generating and maintaining cellular polarity in other systems. This chapter culminates with a section in which the premise and foundational hypotheses of this thesis will be discussed.
1.1 Inwardly-rectifying potassium channels

The identification of potassium currents in muscle exhibiting a strong inward flow, rather than the outward rectification that would be expected of voltage-gated potassium channels, provided the first indication of the existence of the inwardly-rectifying potassium (Kir) channels. The currents carried by these channels were initially considered anomalous, for the reason that they showed a greater dependency on the electrical potential and distribution of potassium ions, than on differences in membrane voltage [1], a characteristic bestowed upon them by the asymmetric fashion in which they are gated by endogenous factors. The Kir channels are rather akin to one-way valves or diodes in the sense that they allow the flow of ions in one direction only. Fifteen Kir subunits are currently known. These are divided into seven subfamilies, and four functional groups. The “ATP-sensitive” Kir channels, comprised of Kir6.1 and 6.2, open in response to decreasing intracellular ATP levels, and so are regulated by metabolic state of the cells. The “G-protein gated” channels, comprised of Kir3.1, 3.2, 3.3, and 3.4 are regulated by the ligation of G-protein coupled receptors, and are sensitive to pertussis toxin. The “classical” Kir channels, whose members include Kir2.1, 2.2, 2.3, and 2.4, display no such reliance, and may be considered to be constitutively active. The “transport” Kir channels, Kir1.1, 4.1, 4.2, and 7.1, participate in the rapid and selective shuttling of potassium ions across cell membranes (reviewed in [2]).

1.1.1 Kir structure and inward-rectification

Kir monomers are comprised of two transmembrane helices linked by an extracellular loop, termed “H5,” that extends into the pore region to form a selectivity filter (Fig. 1-1 A) in an intact channel [3], the formation of which requires the tetramerization of four individual channel subunits (Fig. 1-1B). The considerable homology that exists between the fifteen Kir isoforms permits a certain degree of freedom in this process, and associations between different members of the same subfamily are not uncommon [4] [5]. Heterotetramers containing members from different families are also possible, though rare, with the only currently known example of this being the pairing of Kir4.1 and 5.1 in the CNS and kidney [6-9]. Heteromerization can have important consequences on the characteristics of the resultant channels. For instance, channels assembled from Kir2.1 and 2.2 exhibit properties
distinct from those composed of monomers of either isoform alone [4]. Heteromerization, in some cases, may represent an even more fundamental determinant of channel functionality, as evinced by Kir5.1, whose translocation to the basolateral plasma membrane in MDCK cells following its synthesis is contingent on the coexpression of Kir4.1. Without the latter, it remains locked within intracellular sites and would therefore be unable to mediate a potassium current [10].

The atomic structure of Kir channels has been elucidated in great detail via X-ray crystallography, based on the study of the transmembrane domains of bacterial KirBac1.1, and a chimera possessing sequences derived from KirBac3.1 and mouse Kir3.1 [11, 12], and of the cytoplasmic domains of Kir3.1, 2.1, and 3.2 [13] [14] [15]. Within the lipid bilayer, the transmembrane helices are arranged in such a fashion so that TM1 and TM2 are adjacent, with the former occupying the most external portion of the channel, and the latter positioned inwards, giving rise to the inner limits of the pore region. Additional notable elements of the channel include the H5 extracellular loop, which carries a short pore helix, and the N-terminal amphiphilic “side helix,” which lies at the interface between the membrane and the cytoplasm, and, together with the C-terminal region of the channel, forms an extended pore domain (Fig. 1-1 A and B) [16, 17]. A potassium ion permeating the channel first encounters a selectivity filter comprised by the TXGY(F)G motif contained within the H5 loop, which is highly conserved among all Kir channels. It then passes into a wider central cavity, before encountering a narrow space bordered by the TM2 helices, the movement of which comprises the primary gating mechanism in the Kir types that are G-protein regulated [18]. Beyond this, the ion enters the aforementioned extended pore domain, which doubles the length of the ion conduction pore of the channel.

The determinants that give rise to the inwardly-rectifying properties of the Kir channels are primarily contained within their inner pore region and cytoplasmic domains. While the specifics vary depending on the particular channel subtypes being considered, inward rectification in most cases is based on the directional blockage of potassium ion flow in a competitive fashion by divalent ions, such as Mg$^{2+}$,[19, 20], and by polyamines such as spermine and spermidine [21, 22]. The binding and unbinding of the former leads to the fast inactivation and activation of the channel respectively, while the latter mediates slower
events that result in time-dependent channel gating. Several binding sites for these elements might exist per channel, though it is thought that blockage occurs primarily within the inner pore region. Some speculate that the binding sites in the cytoplasmic region serve mainly to concentrate polyamines close to the pore, thereby increasing their effectiveness [23, 24]. The strength of inward rectification appears to depend in part on the affinity of the pore region for Mg$^{2+}$. Whereas a strongly-rectifying channel such as Kir2.1 contains a negatively-charged aspartic acid residue within its TM2 helix, its place is taken by an uncharged asparagine residue in the weakly rectifying Kir1.1 channel. Introducing aspartic acid into this position, termed the “D/N” site, of Kir1.1 increases Mg$^{2+}$ binding, and consequently increases its rectification strength [25].

1.1.2 Regulation of Kir function and expression

Because they lack the S4 voltage-sensing domain common to all voltage-gated ion channels, Kir channel activity is independent of membrane potential, but is instead regulated primarily by extracellular potassium concentrations. Generally speaking, Kir conductance increases as a function of the square root of [$K^+]_o$ [1, 26], a property that appears to derive from the fact that the two potassium ions can bind concurrently to the selectivity filter of the channel. As well, Kir3.2 and 3.4 channels are activated by increases in intracellular sodium concentrations, while channels of other Kir subfamilies are inhibited by a shift in pH to more acidic levels [27, 28]. The phosphorylation of these channels can play a part as well, with the kinases SGK (serum-glucocorticoid-regulated kinase), PKA, and PKC having been determined to have important roles in regulating Kir cell-surface expression and activity [29-31]. Apart from these mechanisms, another factor of critical importance to Kir regulation is the interaction of these channels with other proteins. While this is most prominently exemplified by the G-protein-regulated Kir3.0 channels, whose activities are modulated by G$\beta\gamma$ binding to their cytoplasmic domains [16, 17, 32-34], and the ATP-regulated Kir6 subfamily, which are inhibited by the sulfonylurea receptors with which they associate [35-38], Kir channels also depend on scaffolding proteins for their intracellular localizations.

This aspect of regulation has been especially well studied in the case of Kir4.1, which is found in a variety of systems, including renal tubular epithelial and glial cells. In the
former, where it is coexpressed, and forms heterotetramers with, Kir5.1, Kir4.1 is restricted to the basolateral domain. In a study published in 2005, Tanemoto et al. [10] showed that the cell-surface expression of Kir4.1 was regulated primarily by its C-terminal PDZ binding motif (SNV), the deletion of which causes a disruption of the normally linear distribution of this channel at the membrane. Using a yeast-two-hybrid approach in a subsequent study, the same group identified a novel membrane-associated guanylate kinase with inverted domain structure 1 (MAGI-1a-long) as being the scaffolding protein involved in the regulation of the proper localization of Kir4.1, which it effects by binding to the PDZ-binding sequence of Kir4.1 via its fifth PDZ domain. This interaction was found to be regulated by salt levels in the diet, and by the phosphorylation state of serine 377 of Kir4.1 [39]. By contrast, Kir4.1 localization in astroglia appears to be dependent on its interactions with syntrophin, a member of the dystroglycan complex, as evidenced by the loss of the channel from perivascular sites in animals deficient for the expression of this scaffolding protein. However, as was the case in renal epithelia, the PDZ domain of this channel remains a crucial determinant here, as the deletion of this motif breaks its link with syntrophin [40] and disrupts the laminin-induced clustering of this channel in cultures of Müller glia [41].

There has been some indication of the possibility that other regulatory mechanisms might exist for Kir4.1. In the study mentioned above [10], it was determined that, in addition to the PDZ domain, a dihydrophobic motif, comprised of two valine residues located at positions 333 and 334, is also required for the proper function of Kir4.1 in epithelial cells. It was seen that the deletion of this motif resulted in the loss of channel expression at the basolateral membrane domain, and causes Kir4.1 to be retained within cytoplasmic sites, and produces a concomitant reduction in activity. As similar motifs in other proteins, and indeed, other Kir channel subtypes, have been identified as being important in mediating interactions with the clathrin complex [42, 43], it is likely that the same is true here. However, as noted by the authors, the dihydrophobic motif in this instance may not be involved in clathrin-mediated endocytosis as it does in these other examples. In this regard, it may serve a role similar to that of the dihydrophobic motif found in CD44, which upregulates the cell-surface expression of this protein, and that of other targets in epithelial cells [44]. Given its potential
implications, it is regrettable that this finding has not been explored more fully in subsequent studies.

1.2 The aquaporins

The aquaporins (AQPs) are a family of small protein channels, of approximately 30 kDa in size, that facilitate the rapid bidirectional transport of water and certain small molecules across lipid membranes. Their function is critical, as their expression greatly increases the water permeability of these membranes to a level far beyond that which would be expected for passive diffusion alone. Currently, 13 mammalian aquaporins have been identified, and these may be divided into three categories based on their particular selectivities. The classical aquaporins, consisting of AQP1, -2, -4, and -5, support the transport of water exclusively, while the aquaglyceroporins, comprised of AQP3, -7, -9, and -10, are permeable to uncharged solutes such as glycerol, as well as water. Unorthodox aquaporins, a class containing the remainder of the aquaporin isoforms, mediate processes yet to be identified at the time of writing (reviewed in [45]). However, the aquaporins participate in a wider array of functions than would be implied by these broad classifications. For instance, in addition to water and glycerol, AQP7 and -10 have also demonstrated a capacity to transport urea when expressed in Xenopus oocytes. AQP6 stands out as the only member of the family capable of carrying several anion species, especially nitrate, possessing a unique asparagine residue within its pore region that allows it to do so. AQP8 is permeable to both ammonia and hydrogen peroxide, the physiological significance of which remains unknown, and the prototypical aquaporin, AQP1, could even potentially play a role in the transmembrane conduction of carbon dioxide and nitric oxide, although these observations have yet to be substantiated by further studies [46-52]. It has been determined that the aquaporins are important factors in a number of biological processes and disease states, including, but not limited to, cataract formation (AQP0), urine concentration (AQP1, -2, and -4), exocrine function, and the production of saliva (AQP5) [53-58].
1.2.1 Aquaporin structure and selectivity

X-ray crystallographical methods have been used to elucidate the structure of the aquaporins, revealing that the channel protein is comprised of six tilted transmembrane α-helices linked by various intra- and extracellular loops (Fig. 1-2 A), possessing an outward appearance resembling that of an “hourglass” (Fig. 1-2 B) [59, 60]. The second intracellular and third extracellular loops (labeled loops B and E in Fig. 1-2 A), which contain short helical segments, protrude inwards from either side of the membrane into the pore region when the channel is in its native configuration (illustrated schematically in Fig. 1-2 C) [61]. These carry the NPA (asparagines-proline-alanine) motif that is common to all aquaporins. Via a series of steric and electrostatic effects, water molecules are compelled to pass through a narrow 20-Å long, 8-Å wide, mostly hydrophobic pore region created by the confluence of the membrane-spanning helices in a single file [62], precluding the formation of stable hydrogen bonds that would otherwise decrease flow velocity and permit the coincident entry of protons [63]. Of note, the arginine residues contained within the conserved NPA motifs mediate the momentary realignment of the dipole of water molecules passing through the pore, a step that is critical in this process. Through this, the rapid and specific conduction of water is ensured.

1.2.2 Aquaporin regulation and sorting

Unlike the majority of channel proteins, the aquaporins possess no discernible endogenous gating mechanisms. However, their activities may still be controlled via other means. AQP0, for example, exhibits increased flow characteristics in the presence of nickel and zinc, or when exposed to environments with an acidic pH [64] [65]. AQP6, is similarly sensitive to pH changes, and is also robustly activated by mercuric compounds, which can cause a 5-to-6-fold rise in water and anion conduction rates [66]. Conversely, divalent cations and low-pH conditions inhibit AQP3 function [67] [68]. Aquaporin regulation may also proceed via cellular events that affect channel localization, with AQP2 being a particularly well-characterized example. AQP2 is expressed at the apical membrane domains of the cells of the collecting tubules of the nephron, as well as within intracellular vesicles, and function synergistically with AQP4 in the reabsorption of water from the post-glomerular filtrate (Fig. 1-4 C). Should conditions arise where an increase in the rate of reabsorption is
desirable, the amount of AQP2 expressed at the cell surface can be upregulated via the secretion of arginine-vasopressin (AVP) from the pituitary. Upon binding, AVP causes the activation of PKA through an increase in cAMP levels, which in turn phosphorylates AQP2 at serine256, thereby causing their translocation to the cell surface [69-72].

AQP4 activity may too be subject to regulation by phosphorylation. The observation that the treatment of defolliculated Xenopus oocytes with the protein kinase C (PKC) activators phorbol 12,13-dibutyrate (PDBu), and phorbol 12-myristate 13-acetate (PMA) causes the phosphorylation and inhibition of exogenously-expressed AQP4 in a dose-dependent manner provided the first indication that this could be the case [73]. It was subsequently established that the reduced activity of AQP4 following the application of PDBu results in a reduction in whole-cell water permeability without the concomitant redistribution of AQP4 channels away from the cell membrane, led to the conclusion that PKC phosphorylation affects the “gating” of this channel, rather than its expression [74], though this view has since been challenged by the results of a more recent study [75]. In the same study, it was demonstrated via the use of a substitution mutant that it was serine180 that acted as the substrate for PKC. Conversely, the phosphorylation of AQP4 by calcium/calmodulin-dependent protein kinase II (CaMKII) increases the apparent permeability of the channel, although the specific mechanism involved remains unresolved [76]. AQP4 may also be a substrate for protein kinase A (PKA), as demonstrated by the fact that the high levels of AQP4 phosphorylation that is normally seen following the histamine-induced internalization of the channel is abolished by micromolar quantities of the PKA inhibitor H89. It is believed that phosphorylation by PKA may represent a crucial step in determining the eventual fate of AQP4 following its internalization from the cell surface, by acting as a signal that prevents the entry of the channel into recycling pathways that would mediate its reinsertion at the plasma membrane [77]. While the precise mechanisms that underlie the phosphorylation-based regulation of AQP4 localization and expression, as well as its physiological significance currently remain unclear, this phenomenon certainly warrants further study, given the potential ramifications of the above findings.

Performing a sequence alignment of several aquaporin types that exhibit divergent expression patterns in kidney epithelia, Deen and van Os, in a study published in 1998 [55],
determined that there existed numerous differences in several locations within their C-termini. Operating on the hypothesis that these might potentially represent sorting determinants that may be important in the regulation of the site-specific localization of AQP4, Madrid et al. [235] therefore synthesized a series of mutant AQP4 constructs bearing deletions within this region of the protein, and expressed them in MDCK cells in order to investigate their involvement in the apico-basal sorting of this channel. It was seen by the authors that the elimination of residues located between Q272 and I302 caused AQP4 to be re-routed to the apical membrane. Upon the further dissection of this region, they saw that this it was in fact comprised of two separate regulatory elements, the first contained between positions 272-281, and the second between residues 282-302, either of which is sufficient to effect proper channel localization. The former contains a tyrosine-based motif resembling those seen in other basolaterally-expressed proteins, and was subsequently determined to be critical in the regulation of AQP4’s interaction with the μ2 subunit of the AP2 clathrin adaptor complex, and the μ3A subunit of the AP3 complex, events which govern the endocytosis of the channel from the plasma membrane, and its transport to late endosomes for degradation. S276, and the residues that border it, was found to conform to a casein kinase II consensus site, the phosphorylation of which strengthens the affinity of AP3 for AQP4. The latter motif contains a cluster of acidic amino acids (VETE) in positions 282-286, followed by a dileucine motif (LIL). The sequential disruption of these led to a stepwise increase in the missorting of AQP4 to the apical membrane domain.

It is therefore clear from these examples that aquaporin function may be modulated in a number of ways, and can occur on the level of channel conductivity, localization, or expression at the plasma membrane. The multiplicity of routes involved is perhaps unsurprising, given the importance of the osmoregulatory events that these channels participate in.

1.2.3 Aquaporin-4 isoforms and organization

While individual AQP4 subunits can function as channels, they are more frequently found as tetramers, giving rise to the characteristic intramembrane particles (IMPs) observed via freeze-fracture electron micrography. Of the various AQP4 isoforms known to be
expressed in biological systems, which include AQP4a, c, and e (otherwise known as AQP4-M1, -M23, and -Mz) and their respective spliceoforms AQP4b, d, and f, AQP4-M1 and AQP4-M23 are the most abundant [78], and so were the first to be cloned. For this reason also, they remain, to date, the most thoroughly-characterized AQP4 isoforms. The two differ primarily in their N-terminal sequences, where the former possesses a 22-amino acid extension absent in the latter [79], a consequence of protein translation being initiated at the first methionine (M1), rather than the second (M23) (Fig. 1-3 A) [80].

These two isoforms appear to have antagonistic functions with regards to the organization of AQP4. When present alone in the membrane, AQP4-M1 is diffusely distributed, existing mostly as singlets at one extreme, and forming groups comprised of no more than 12 AQP4 tetramers at the other. M23, however, can spontaneously self-assemble into orthogonal arrays of particles (OAPs) containing upwards of 100 IMPs. When expressed together, OAPs of intermediate sizes form, much like those that are observed in vivo [81], with the final dimensions determined by the relative numbers of each isoform (Fig. 1-3 B) [82], which varies from 3:1 in favor of AQP4-M23 in the brain [81] to 8:1 in favor of AQP4-M1 when the channels are heterologously expressed in epithelial cells [83]. According to data obtained via a live-imaging approach, Crane and Verkman [84] have concluded that OAP formation is dependent on the hydrophobic interactions occurring between adjacent channel tetramers mediated by a valine-alanine-phenylalanine sequence located at the N-terminus of AQP4-M23, which are prevented by the N-terminal extension of AQP4-M1 [84]. As well, the palmitoylation of cysteine residues located at positions 13 and 17 of the N-terminus of M1 may also play a role [85], as the substitution of these residues with alanines causes the resultant mutant M1 channels to exhibit a much increased propensity for self association. But as the effects of this manifest themselves only at relatively low temperatures, it is questionable as to whether this latter mode of regulation might be relevant under more physiological conditions [86].

It has recently emerged that OAP formation may be subject to regulation by a number of external factors: the neuronal isoform of the heparan sulfate proteoglycan, agrin, a component of the extracellular matrix, induces an increase in the expression of AQP4-M23, and decreases the amounts of AQP4-M1 [87], while vasopressin, when applied to LLC-
PK(1) cells expressing AQP4, initially causes an increase in AQP4-M1, but ultimately results in the preferential upregulation of AQP4-M23 [88]. It is likely that the latter acts via the modulation of channel internalization and trafficking, given the apparent importance of serine 111, a PKA phosphorylation consensus site, in mediating the effects of vasopressin [88]. Also, although OAP assembly does not seem to require the presence of an intact PDZ domain [89], the observed association of dystrophin and β-dystroglycan with high-molecular weight aggregates of AQP4 [90] suggests that the DGC may play a part in the accretion of smaller OAPs into higher-ordered structures.

1.3 The role of Kir4.1 and AQP4 in mammalian kidney function

Life in a terrestrial environment poses a unique challenge where systemic osmostasis is concerned. While the excretion of the potentially toxic byproducts of metabolism and excess ions and water is key to ensuring the continued well-being of the organism, the conservation of the latter is necessary also, for the reason that neither are readily abundant, but are absolutely required for various biological processes. In mammals, the renal system has evolved to perform this exact function. In the following section, the various adaptations and features that permit the kidney to exert fine control over systemic osmolarity will be discussed.

The nephron is the most basic unit of the mammalian kidney – the primary biological function of the renal system is entirely based on the collective action of these microscopic structures, which act as highly-selective filters that remove unwanted chemical compounds from the blood and mediate their expulsion via the urinary tract, while allowing for the retention of other essential elements. The first step of this filtration process occurs at what is known as the blood-urine barrier, the interface consisting of the glomerulus, a branching network of capillaries, and the Bowman’s capsule, a portion of which comprises the foremost segment of the nephron. At these sites, the pressure created by differences in the effective cross-sectional areas of the afferent and efferent blood vessels causes water and several ion species to exit the blood and enter the capsule as a filtrate that is roughly isotonic. From thence, the filtrate is then conveyed into the nephron proper, where additional processing steps convert it to a form suitable for excretion as urine. The filtrate first encounters the
proximal convoluted tubule, where water, and ions such as sodium, chloride, potassium, bicarbonate, phosphate, and sulfate are reabsorbed into the interstitium, and eventually into the bloodstream, together with various other solutes, including glucose, amino acids, and certain organic acids. This occurs partly through the paracellular route, which is greatly aided by the comparatively “leaky” nature of the epithelium that comprises this section of the nephron, and partly via a transepithelial route, through apically-expressed specialized transport proteins. At the same time, yet more organic waste products, in the form of various acids and bases, are secreted into the filtrate. The filtrate then enters the loop of Henle, oransa nephroni, which may be subdivided into five discrete sections (namely, the thin descending, thin ascending, thick ascending, and cortical thick ascending limbs) that each possesses distinct properties. Through these segments, the reabsorption of water and certain ions continue, driven by a combination of an increase in interstitial osmolarity and by active transport processes, causing the filtrate to be concentrated further (Fig. 1-4 A; reviewed in [91], [92] and [93]).

The next section of the nephron, the distal convoluted tubule, serves mainly to scavenge the final remnants of NaCl from the increasingly impoverished filtrate. The entry of NaCl into the epithelial cells of this segment is facilitated by NCC, a Na+/Cl- cotransporter (otherwise known as NCCT, or SLC12A3), and the amiloride-sensitive sodium channel ENaC [94-96], while its exit is mediated by a combination of the activities of the antiporter pump Na+/K+-ATPase and the chloride channel CLCKB/A, both of which are localized basolaterally together with the intermediately-inward rectifying potassium channel Kir4.1, one of the subjects of the first study included in this thesis. While the exact purpose of Kir4.1 here is not known, it has recently been speculated that this channel may, in combination with Kir5.1, be involved in the extrusion of the excess intracellular potassium concentrations that would otherwise build up as a consequence of the extended operation of the NCC/ENaC- Na+/K+-ATPase/ CLCKB/A transepithelial transport system described above (Fig. 1-4 B), by acting as a conduit through which potassium ions may pass back into the interstitium or bloodstream [97]. Hereditary mutations that disrupt the function of this channel in the kidney lead to defects that appear to support its hypothesized role in osmoregulation. Patients thusly
affected exhibit symptoms such as hypokalemia, alkalosis, hypomagnesemia, and hypocalciuria [97, 98].

The renal collecting duct represents the primary site of AQP4 expression in the kidney. Within this segment of the nephron, this channel, found exclusively within the basolateral membrane domain of the renal epithelium, functions in tandem with apically-expressed vasopressin-regulated AQP2 to further concentrate the filtrate by establishing a transcellular route through which water can be returned into the bloodstream (Fig. 1-4 C). As such, the dysfunction of AQP2 or AQP4 causes a reduction in the amount of water that the collecting duct is able to recover [56, 99], resulting in symptoms resembling that of nephrogenic diabetes insipidus, a condition characterized by the production of excess volumes of urine.

1.4 The blood-brain barrier

Due to the energy-intensive nature of neural activity, the central nervous system requires a steady supply of metabolites in order to sustain its function. At the same time, the brain is highly-sensitive to disturbances, and so must be isolated from any microbes that might be present in the bloodstream, and even from the activities of the body’s immune system. The blood-brain barrier, comprised of the endothelial cells of the permeating microvasculature of the brain and the endfoot domains of perivascular astroglia, as well as the intervening basal lamina, has evolved to perform these two seemingly conflicting functions by acting as a selective screen, separating the central nervous system from the systemic circulation while permitting the entry of oxygen, hydrophobic solutes and necessary nutrients and the exchange of metabolic waste products. Additionally, the BBB also plays a role in cerebral osmoregulation, and is a central player in a number of pathophysiological states.

The endothelial cells of the cerebral microvasculature are joined together by an abundance of tight junctions composed of occludins and claudins. The cohesiveness that is generated as a result of this arrangement virtually eliminates all gaps in the endothelial cell layer that would otherwise exist, thus rendering the blood vessel impassable to most polar
solute via this route [100]. The relative scarcity of pinocytotic processes and the absence of fenestrations further contribute to the barrier function of the endothelium [101, 102]. This impermeability is offset by the presence of specialized transporters such as LAT1 and GLUT1, expressed on the luminal and abluminal surfaces, which mediate the transendothelial exchange of amino acids and glucose respectively [103, 104]. Astrocytes adjacent to blood vessels project extended membranous structures, known as endfeet, that completely envelop the microvasculature. Within the brain parenchyma, these perivascular astrocytes network to yet other astrocytes via gap junctions, forming a contiguous entity that has been dubbed the “panglial syncitium” [105], a structure crucial to the regulation of cerebral ionic balance.

As the generation of an action potential by a neuron is invariably accompanied by the release of potassium from its internal stores, extended periods of neural activity, in the absence of intervention, would quickly result in the accumulation of excessive amounts of this ion within the extracellular fluid, potentially creating conditions unfavorable for continued nervous transmission. The brain, however, possesses the ability to maintain an extracellular potassium concentration of approximately 3 mM even when subjected to severe and rapid changes in the external environment [106, 107], owing to the various strategies that it employs. Astrocytes are critical players in two such potassium-handling mechanisms, termed potassium buffering and potassium siphoning. In the first, potassium is taken, often together with an accompanying anion type, from “source” regions into the astroglial syncitium, thereby sequestering them from the extracellular space and minimizing their influence on nervous function. In the latter, this excess potassium is conveyed into “sink” regions, such as the bloodstream, driven by the waves of depolarization that propagate through this system of interconnected astrocytes [108]. In these processes, potassium entry is believed to occur through the perisynaptic parenchymal domains of astrocytes through the strongly-rectifying Kir2.1 channel, supplemented by Na⁺/K⁺-ATPase [6], while its exit at the perivascular endfoot is facilitated by Kir4.1 (Fig. 1-5), which is expressed in particularly high amounts at these sites, and possesses intermediate rectifying properties [109]. The function of the latter appears to be especially important, as its elimination results in decreased potassium conduction and the depolarization of resting membrane potential in astrocytes, and leads to
the abrogation of potassium buffering [110, 111]. As well, its disruption has been shown to be associated with an increased susceptibility to epileptic seizure in certain cases [112], further highlighting its role in cerebral osmoregulation.

The water-permeable channel AQP4 is coexpressed with Kir4.1 in astrocytes [113], and may participate in regulating the bulk flow of water required to accommodate changes in the volume of the extracellular space accompanying the rapid ion flux that occurs as a result of the siphoning process [7, 114]. AQP4 may also play a supporting role in neural conduction, as knockout mice deficient for this channel exhibit numerous defects with regards to synaptic plasticity, spatial learning, and the consolidation of fear-based memories [115-117]. As well, pain reception and auditory brainstem response are significantly impaired in these mice [118, 119]. A recent study has determined that the loss of AQP4 expression is accompanied by changes in the activity of Na⁺/K⁺-ATPase and an increase in the degree of gap junction coupling in astrocytes, and significantly affects the potassium-handling properties of these cells [120].

Apart from its role in supporting potassium siphoning, AQP4 may also be involved in the development and resolution of cerebral edema, the aberrant and pernicious accumulation of excess water that can result as a secondary complication of trauma or certain disease states. Depending on etiology, cerebral edema may be broadly classified into two categories. The first, termed cytotoxic edema, can arise when the brain is subjected to the hypoxic conditions that might be experienced following an ischemic episode [121], and is characterized by the swelling of perivascular astrocytes. Edema in these instances is often triggered by the collapse of oxidative phosphorylation, which starves the cell of the ATP on which Na⁺/K⁺-ATPase depends in order to maintain osmotic conditions within these cells [122, 123]. It is believed that it is the disruption of this process leads to the uncontrolled influx of water across the BBB, which in turn causes the swelling that is observed. Alternatively, high concentrations of water within the plasma may drive a similar bulk flow of water into astrocytes, as in the case of water intoxication (Fig. 1-6 A) [121]. The swelling of astrocytic endfeet can cause the microvasculature to become constricted [124], aggravating the damage caused by the initial insult. Vasogenic edema, in contrast to the above, is characterized by the incursion of blood plasma into the brain parenchyma, caused
primarily by the breakdown of the BBB and an increase in vascular permeability (Fig. 1-6 B). The metalloproteinases MMP-2, -3, and -9 have been implicated in the former [125], while the latter appears to be caused by factors such as VEGF-A, which downregulate occludin expression in the endothelial layer [126, 127]. The expression of AQP4 appears to be beneficial where vasogenic edema is concerned: AQP4-null mice, in studies involving cortical freeze injury or tumor implantation exhibited worse neurological outcomes and increased water accumulation compared to WT mice, indicating that this channel is likely required for the clearance of water in these instances [128]. The recent finding that AQP4 serves a similar role in regulating the removal of water from the brain during post-natal development [129] greatly supports this hypothesis. However, it appears that this channel also mediates the water influx that causes cytotoxic edema, as evinced by the fact that AQP4-null mice are protected from the adverse effects of water intoxication and medial cerebral artery occlusion [121]. Interestingly, dystrophin-null mice exhibit a similar resistance to water intoxication, indicating that the asymmetric localization of AQP4 in astrocytes, which is lost in these mice, greatly affects its ability to mediate this movement of water [130].

1.5 The basal lamina

In vivo, epithelial cells, such as those involved in the formation of renal tubules, exist in close association with the basal lamina, and, as already described above, it is also an integral part of the blood-brain barrier. The basal lamina plays several critical roles in the regulation of the morphology and function of these systems. Structurally, the basal lamina is a reticular network composed mostly of the extracellular matrix (ECM) components collagen, laminin and fibronectin, and proteoglycans such as perlecan and agrin, linked together via homotypic and heterotypic interactions. In this thesis, we investigate the contribution of laminin and fibronectin to the organization and expression of Kir4.1 and AQP4 in epithelial Madin-Darby canine kidney cells and primary astrocytes in culture. The following is a brief overview of these proteins.
1.5.1 Laminin

The laminins are secreted heterotrimeric glycoproteins comprised of α, β, and γ subunits. To date, five distinct isoforms of the first, four of the second, and three of the last have been identified in the mouse and human [131], and in various combinations, these give rise to the fifteen laminin types that are currently known to exist (reviewed in [132]). When assembled, most laminin types take on a cruciate appearance, with a patibulum composed of the free N-terminal ends of the β and γ chains, and a central member consisting of, at one end, the N-terminus of the α chain and a coiled-coil domain formed through the intertwining of all three subunits on the other, but, depending on their particular subunit compositions, and on modifications by posttranslational processes, variations possessing either truncated arms, or that are missing a “head” portion, may be possible as well (reviewed in [133]). Laminin is an absolute requirement for the assembly of functional basal lamina, as evidenced by the embryonic lethality induced by the genetic knockout of laminin α1 expression. Animals deficient for this subunit fail to form a complete Reichert’s membrane, and die in utero at E5.5 [134]. The loss of other laminin subunits, similarly, leads to disruptions in basal lamina structure [135-137]. The laminins are capable of both self-association, binding to each other via the globular laminin N-terminal (LN) domains on each of the chains, and of supporting multivalent associations with numerous other proteins, including collagen, and cell-surface receptors such as the integrins and dystroglycan, which interact either with the C-terminal laminin globular (LG) domains or the LN domains of the α subunit [138-141].

The dominant laminin isoforms at the blood-brain barrier are laminin-111 (i.e., α1β1γ1), -211, -411, and -511. The first two are products of astrocytes, while the latter are synthesized primarily by endothelial cells [142-144]. These laminins contribute to the barrier function of the BBB, serving to modulate the cohesion of the endothelial cell layer, and restricting the infiltration of inflammatory cells [143, 145]. Laminin α1 is also found in close association with several epithelial tissues, including those of the adult kidney, where it is initially the most abundant isoform, but is gradually replaced over the course of development with laminin-511, and eventually, laminin-521 [146-148]. It has been demonstrated that mutations in laminin β2 leads to the development of a severe nephritic phenotype [149], suggesting the importance of the laminins in the establishment of the morphology of this
organ. Based on studies performed in other systems, it is likely that the laminins play crucial roles in the processes that regulate tubulogenesis, and cellular migration and polarization within the kidney [150-153][154].

1.5.2 Fibronectin

The fibronectins are an essential and ubiquitous constituent of all basal lamina. They possess a dimeric structure, composed of a pair of segments linked by disulfide bonds. Contained within these segments are various modules that permit their interaction with other components of the ECM, including previously-secreted fibronectins, as well as cell-surface receptors such as the integrins (reviewed in [155]). Their association with the last, in particular, is mediated by the canonical integrin peptide sequence Arginine-Glycine-Aspartic acid (RGD) carried by one of the type-III modules [156]. Upon their initial release from the cell, fibronectins are tightly coiled and non-functional, and it is only following their activation by integrin receptors that the relevant molecular epitopes are exposed which allows fibronectin to induce the clustering of additional integrins at the cell surface. This in turn leads to the recruitment of yet more fibronectins, thereby setting up a positive feedback loop that eventually results in the assembly of the characteristic fibrils after which this ECM molecule takes its name.

As is true for most components of the ECM, fibronectins are involved in the regulation of cell adhesion and migration [157, 158]. With regards to the kidney, fibronectin appears to be localized in especially high concentrations in the regions surrounding the cells of the tubular epithelium, and may play important roles in governing cell survival [159, 160]. It is also possible that they are important in the recovery of the kidney from ischemic states, though the etiologies in this instance currently remain obscure [161].

1.6 The integrins

The integrins are a class of transmembrane cell-surface receptors characterized by their heterodimeric structure. Functionally, the mammalian integrins may be divided into two subgroups based on their affinities. The first, comprised of α3, α6, and α7, pair predominantly with β1, and is involved in laminin binding, while the second, which include
the α subunits αIb, αv, α5, and α8, form dimers with β1 or β3, and is primarily involved in mediating interactions with ECM components possessing the RGD motif, such as fibronectin. One of the chief functions of the integrins is the regulation of cell adhesion, accomplished via their ability to vary their affinity to various ECM molecules on a moment-to-moment basis. This process, known as “inside out” signaling, is a property unique to this receptor class. As well, the integrins are also able to participate in “outside in” events, relaying cues from the external environment, and modifying the behavior of the cell in response (reviewed in [162]). These are mediated through the multitude of secondary effector molecules with which these receptors associate.

The integrins, especially those that contain β1, are critical determinants in renal development, as reflected by their relative abundance in this organ, and their shifting localization within the kidney primordia over time: while β1 integrins are initially found in all cell domains, they redistribute as the kidney matures, and eventually become restricted to the basolateral membranes in tubular epithelia [163, 164], in close apposition with the underlying basal lamina. The genetic ablation of α8 integrin, which is activated by nephronectin, an ECM component expressed exclusively by the kidney, leads to renal agenesis, owing to the dysregulation of the epithelialization process normally required for the formation of functional tubules [165]. The loss of α3, similarly, results in a decrease in the branching complexity of the collecting duct, and causes a malformation of the basal lamina of the glomerulus [166], implicating this isoform in the process that govern tubular morphology and ECM assembly. Finally, if proper α6 function is disrupted via the use of specific blocking antibodies, kidney tubules are allowed to form, but do not polarize [167]. It is worth noting, however, that β1 integrin appears to be required only during nephrogenesis, as its later removal results in no overt harm to kidney structure or function [168].

It was observed by Yu et al. in their 2005 study that apically-expressed β1 integrins were involved in facilitating formation of tubulocysts in two-dimensional MDCK cell cultures subjected to collagen overlay. As the expression of constitutively activated Rac1 or the addition of laminin to the matrix was both sufficient to circumvent the effects of β1 blocking antibodies, it was concluded that β1 and Rac1 constitutes a portion of a signaling
pathway that participates in mediating the reorganization of the ECM and of epithelial monolayers in response to external cues [154]. It is thus likely that the defects observed in the absence of these integrins seen in the above examples are a reflection of the importance of this receptor class in the regulation of epithelial polarity.

1.7 The dystroglycan complex

DG is a highly-conserved cell-surface receptor expressed in numerous organisms, including human, mouse and zebrafish [169-171]. It is encoded by the DAG1 gene, which, in humans maps to chromosome 3p21 [170]. Though initially synthesized as a contiguous 895-amino acid precursor, DG is subsequently proteolytically cleaved into two distinct subunits, termed α- and β-DG, which are then linked to one-another via non-covalent associations [172]. The former is heavily-glycosylated, and forms the extracellular subunit of the protein, interacting with a variety of extracellular matrix (ECM) components, including laminin, agrin, perlecan, and agrin [173-176]. Via β-DG, which mostly comprises the transmembrane and intracellular portions of the receptor, DG interacts and complexes with intracellular components such as syntrophin, dystrobrevin, and dystrophin, collectively forming what is known as the dystroglycan complex (DGC). The DGC was initially isolated from skeletal muscles [177], where it is thought to play a role in maintaining the integrity of the sarcolemma through repeated muscle contractions. Dysfunction of DG, or any of the components with which it is complexed often results in muscular dystrophies. The DGC has subsequently been determined to be expressed in several systems, including the brain.

Patients suffering from Duchenne muscular dystrophy, Fukuyama congenital muscular dystrophy, Walker-Warburg syndrome, and muscle eye brain disease, all of which are linked to components of the DGC, often present with mental deficits and ocular defects as well, demonstrating that this complex serves important roles in the central nervous system, with the localization of AQP4 at the perivascular endfeet of astrocytes being one of these (Fig. 1-7 B). Indeed, in these cell domains, AQP4 colocalizes with α1-syntrophin, α-DG, dystrophin, and dystrobrevin [178-180]. Furthermore, AQP4 perivascular localization is lost in the α-syntrophin knockout mouse [181]. The same is true of dystrophin mutants [182, 183]. The interaction of DG with the ECM is also crucial for mediating proper AQP4
localization, as the channel is also mistargeted in Large\textsuperscript{myd} mice, in which the laminin-binding activity of \(\alpha\)-DG is ablated due to its defective O-glycosylation [180]. The localization of AQP4 at the perivascular endfeet of glial cells is crucially dependent on the laminin-binding properties of DG. It has been demonstrated that laminin, when applied to Müller glia and cortical astrocytes in culture, results in the formation of dense clusters of \(\alpha\)-DG and AQP4 on the surface of these cells that are analogous to endfoot domains [41, 184].

As well, the DGC may play a role in epithelial cells, with its importance in facilitating tubulogenesis in numerous systems having been known for quite some time (reviewed in [185]). Additionally, dystroglycan dysfunction is thought to be a primary factor in driving epithelial-mesenchymal transitions in certain carcinoma types (reviewed in [186]). Its importance in this regard likely stems from its involvement in the events that establish cellular polarity: it has been observed, for instance, that the loss of DG in the \textit{Drosophila} oocyte folliculum results in the apicalization of the epithelial cells contained within that structure, and the disruption of the basal cytoskeleton [187]. This, in turn, appears to depend upon the laminin-binding properties of DG, as evinced by the finding of Weir \textit{et al.} that the deletion of the structural determinants that permit this interaction abolishes cellular polarity in mammary epithelial cells [188].

1.8 Project outline

The main thrust of this thesis centers on the elucidation of the cellular events that generate asymmetry in the mammalian kidney and the BBB. As previously discussed, within the kidney, Kir4.1 and AQP4 are found exclusively in the basolateral membrane domains of the epithelial cells that comprise the renal tubules, while in the BBB, AQP4 is heavily concentrated at the perivascular endfoot domains of astrocytes bordering the cerebral vasculature, but is not as concentrated elsewhere in these cells. Given the close association between these cell types and the extracellular matrix, and the well-established role of ECM receptors as determinants of epithelial polarity [189] [190] [154] and AQP4 expression in astrocytes [180, 184], we hypothesize that these elements may too be involved in the regulation of Kir4.1 and AQP4 expression and polarity.
The next chapter of this thesis constitutes an investigation of the factors important in determining the distribution of Kir4.1 and AQP4 in the mammalian nephron, concentrating on three major themes. Using Madin-Darby canine kidney (MDCK) cells as a model system, we first address the heterogenous manner in which these channels are expressed in this system. The nephron is composed of several morphologically distinct segments, all of which possess properties that are strikingly different from those of the others, conferred upon them by the specific protein channels that they express. Kir4.1 and AQP4, despite sharing certain similarities with respect to their expression in other systems [41, 191], are not expressed in the same regions of the nephron: Kir4.1 is found in the distal convoluted tubule, while AQP4 is expressed primarily in the collecting duct, and the separate localization of these channels in these adjacent regions is central to the ability of the kidney to maintain osmostasis, as they mediate processes that are quite different. In order to gain an understanding of how this pattern is established, we investigate how these channels differ in terms of what is required for their proper expression, vis-à-vis the specific ECM molecules and receptors that are involved, focusing primarily on fibronectin and laminin in the case of the former, and on dytroglycan and the integrins for the latter, testing for their role in the regulation of channel expression. We then attempt to determine the specific mechanisms that underlie the actions of fibronectin and laminin, by examining their impact on the diffusional rates of these channels within the basolateral plasma membrane, so as to determine if the ECM affects their lateral stability. Finally, given the associative properties of the molecules that comprise the basal lamina, it is unlikely that either fibronectin or laminin or their receptors function in isolation. We therefore also test for the possibility of crosstalk between these elements, and the impact of this in mediating channel expression and stabilization. Our data reveal that, beyond merely establishing cellular polarity, the interaction of DG and integrins with the ECM also plays a crucial role in the distribution and stabilization of Kir4.1 and AQP4 at the basolateral domain of kidney epithelial cells.

In the third chapter, we investigate the role played by laminin-DG interactions in the establishment of AQP4 polarization at the BBB. Although past studies have described the importance of the binding of these two components in the proper expression of AQP4 at the endfoot structures, no explanation for how this occurs has yet been provided. Based on the
recent finding that DG is involved in the modulation of dynamin activity, we hypothesized that the DG complex, in addition to serving as a scaffolding structure that stabilizes AQP4, also acts to suppress its turnover in perivascular regions in the brain, thereby enriching these for the channel. To test the validity of this theory, we investigated the contribution of laminin and DG to the expression of AQP4 at the cell surface of primary culture astrocytes, and asked the question of whether laminin plays a role in the regulation of AQP4 endocytosis. Following this, we looked at the association between DG and components of the endocytic machinery, and assessed for the involvement of the latter in the cell-surface localization of AQP4. Finally, the divergent effects of this mode of regulation with regards to the two major isoforms of AQP4 were investigated. Our findings in this study demonstrate that laminin, DG and dynamin comprise a complex that regulates AQP4 turnover in astrocytes, and suggest the possibility that the association of these components at the perivascular endfoot may be the cause for the extreme asymmetry in AQP4 distribution in this membrane domain.
Figure 1-1. **Kir4.1 structure.** (A) Diagram detailing the topology of Kir4.1, illustrating the relationship of the major features of the channel, including the two transmembrane regions, the pore helix, and the extensive intracellular domain comprised of the N-terminal side helix and rather sizable C-terminal region. (B) Schematic of Kir4.1 tetramerization (only the rearmost subunits are shown here; others have been omitted for clarity. Kir4.1 only functions as a channel as a tetramer, in which the pore helices and loops of adjacent subunits combine to form a selectivity filter that ensures specificity. The side helices and cytoplasmic tails of the monomers assemble to extend the pore on the cytoplasmic face of the plasma membrane.
A

- Pore helix
- Side helix
- Cytoplasmic domain
- Selectivity filter
- Extended pore
- Pore helices

B

- $K^+$
- Selectivity filter
- Pore helices
- Side helix
- Extended pore
Figure 1-2. AQP4 structure. (A) AQP4 topology. Transmembrane domains and the loops that connect them, as well as the pore helices (labeled “B” and “E”) are shown. (B) A representation of the approximate arrangement of the helices of AQP4. When incorporated into a lipid bilayer, the transmembrane helices of AQP4 are tilted at an angle, and pore helices B and E, which bear a “NPA” motif, protrude into the channel, forming the selectivity filter. Only the pore loops have been included. (C) Schematic of AQP4, highlighting the juxtaposition of the pore helices with the rest of the channel. Through the formation of transient hydrogen bonds with the R-groups of the “NPA” motif, water molecules are propelled through the channel in a single file with great efficiency (see text for further details).
Figure 1-3. AQP4 isoforms and their effects on the assembly of orthogonal arrays of particles (OAP). (A) Three known isoforms of AQP4 exist. The longest, Mz, possesses a 42 a.a. N-terminal region absent in the M1 isoform. Mz is not found in humans and mice, owing to a premature in-frame stop codon that is encountered when translation is initiated at the upstream (−42) methionine. Relative to M1, the N-terminus of M23 bears a 22-amino acid truncation. The molecular masses of Mz, M1, and M23 have been experimentally determined to be 39-, 34-, and 32-kDa respectively. (B) When present in isolation, AQP4-M1 form aggregates containing no more than 12 individual tetramers, whereas M23 will accrete into arrays containing upwards of a hundred tetramers. In combination, the self-associative tendencies of the latter are moderated by the former, and OAP of an intermediate size are seen.
Figure 1-4. The roles of Kir4.1 and AQP4 in the mammalian kidney. The kidneys are central to the maintenance of electrolyte and water levels in terrestrial animals. The nephron (A) is the most basic unit of the kidney. The glomerulus receives blood, which it then filters, removing from it ions present in excess, such as sodium, and waste products such as urea, along with water. The resultant filtrate is passed through the proximal convoluted tubule, the descending limb of the nephron, the loop of Henle, the ascending limb, the distal convoluted tubule, and finally, the collecting duct, from which it is then diverted into the urinary tract for expulsion. Along this route, the content of the filtrate is further modified by specialized mechanisms that alter its salt and water content in various ways depending on the current osmotic state of the animal. In particular, channels expressed by the distal convoluted tubule and collecting duct allow for these sections of the nephron to return sodium ions and water to the bloodstream. Within the former (B), the sodium and chloride cotransporter NCC, localized at the apical domain of the epithelial cells that comprise the renal tubule at that region, mediates the import of these ions. The sodium ions then pass back into the blood via the action of the Na+/K+-ATPase antiporter. Excess intracellular potassium is then removed via the weakly inwardly-rectifying Kir4.1 channel. In the collecting duct (C), apical AQP2 channels function in combination with AQP4 expressed within the basolateral domain to scavenge water from the filtrate, exerting a diuretic effect.
Figure 1-5. Potassium-handling strategies. Action potential generation and synaptic vesicle release are accompanied by the concomitant influx and efflux of sodium and potassium ions. The buildup of excess levels of potassium in the interstitial fluid, which would impede further neural activity if allowed to occur, is prevented in the brain by astrocytes, which rapidly absorb the potassium ions into parenchymal processes. The excess potassium, once absorbed, is either held within the astrocytic syncitium together with an anion such as chloride so as to ensure electroneutrality, as in the case of potassium spatial buffering (left), or is shuttled through these astrocytes into “sink” regions such as the bloodstream via Kir4.1 channels located in their endfoot domains in a process known as potassium siphoning. The bulk movement of water molecules that results as a consequence of this potassium flux is conducted by coexpressed AQP4 channels.
Figure 1-6. The development of edema. Traumatic insults to the brain or certain disease states can trigger the development of edema, of which two major categories are known. (A) Cytotoxic edema results from the rapid influx of water from the bloodstream into the endfeet of perivascular astrocytes via AQP4 channels, and can occur due to metabolic failure in these cells. (B) The development of vasogenic edema is caused by the disruption of the blood-brain barrier via the breakdown of the perivascular basal lamina and an increase in vascular permeability brought about by the combined activities of metalloproteinases and inflammatory factors. If unchecked, this and allow the uncontrolled entry and accumulation of water in the brain parenchyma.
Figure 1-7. The role of the dystroglycan complex in the localization of Kir4.1 and AQP4. Kir4.1 and AQP4 are localized to the basolateral domain of renal tubular cells epithelial cells (A), and the perivascular endfoot domains of astrocytes (B) via their interactions with the dystroglycan complex (C). Dystroglycan binds to laminin extracellularly, and to dystrophin/utrophin intracellularly, which in turn complexes with dystrobrevin and syntrophin. Through the last, the complex interacts with the PDZ-binding motifs found in the C-termini of both Kir4.1 and AQP4. In so doing, the dystroglycan complex forms a bridge connecting the basal lamina and these channels, thereby targeting them to the relevant membrane domains.
CHAPTER 2: REGULATION OF KIR4.1 AND AQP4 EXPRESSION AND STABILITY AT THE BASOLATERAL DOMAIN OF EPITHELIAL MDCK CELLS BY THE EXTRACELLULAR MATRIX

2.1 Introduction

The dystrophin-associated protein (DAP) complex is a multiprotein complex expressed in neuronal, astrocytic, epithelial, and muscle cells [192-198] [199, 200]. Central to the DAP complex is dystroglycan (DG), a molecule composed of a transmembrane protein, β-DG, and an extracellular protein, α-DG. α-DG interacts with the extracellular matrix (ECM) components laminin, agrin, perlecan, and neurexin [201-203], whereas β-DG interacts with the cytoplasmic protein dystrophin, which in turn binds to the actin cytoskeleton. In muscle, the DAP complex and DG have a role in maintaining the structural integrity of the sarcolemma [204, 205]. In the brain, alterations in DG cause a discontinuity in the glia limitans and abnormal neuronal migration [206, 207]. DG is also expressed in a variety of epithelia, including those of the kidney, lung, and testis [208-212]. In the kidney, inhibition of laminin binding to α-DG causes the disruption of epithelial cell differentiation, indicating that the DAP complex plays an important role in epithelial morphogenesis [213]. Furthermore, numerous studies have demonstrated a role for DG in the orientation of polarity. Indeed, DG deletion in Drosophila results in the loss of the apicobasal polarity of epithelial cells as well as the anteroposterior polarity of the oocyte [187, 214]. In mammary cells, DG deletion results in the disruption of laminin assembly in the ECM and a subsequent loss of cell polarity [188]. The large family of heteromeric transmembrane proteins, integrins, has extensively been studied for its role in epithelial cell polarity via their interaction with the ECM. To date, 18 α- and 8 β-subunits have been identified, and these can form 24 distinct integrin dimers [215]. Epithelial cells express several integrins including α1β1, α2β1, α6β1, α3β1, α6β4, α5β1, and αvβ3 [216]. It has been shown that epithelial Madin-Darby canine kidney (MDCK) cells adhere to the ECM via the laminin and collagen receptor α2β1, the laminin, collagen, and fibronectin receptor α3β1, and the vitronectin and fibronectin receptor αvβ3 [217]. In addition, MDCK cells also express α6β4-integrin, but, unlike in other cell types, this receptor does not appear to possess affinity for laminin.
Nonetheless, it has been demonstrated that β1-containing integrins orient the apical domain of polarized MDCK cysts through Rac1 activation and laminin assembly in the basement membrane [154, 190].

In astrocytes of the mammalian brain and Muller cells of the retina, both the inward rectifying potassium channel 4.1 (Kir4.1) and aquaporin-4 (AQP4) aggregate at discrete membrane domains that abut blood vessels and the vitreous body [191, 218]. This polarized distribution is critically dependent on the interaction between laminin and the DAP complex via DG [41, 180, 182-184, 206] and plays a major role in astrocyte- and Muller cell-mediated potassium buffering [219].

In vivo, epithelial cells possess well-defined basolateral and apical membrane domains, each with a distinct complement of proteins. In the case of the epithelial cells of the tubules of the kidney, the basolateral enrichment of Kir4.1 and AQP4 is critical as it allows the renal system to maintain systemic osmostasis [220]. AQP4, which functions in tandem with the vasopressin-regulated AQP2 channel to concentrate the urine and therefore minimize the amounts of water lost via this route, is expressed basolaterally in the cells of the collecting duct [221]. Kir4.1 is found in the distal convoluted tubules of the kidney, where it serves to regulate K⁺ excretion [8, 205]. The basolateral localization of these channels is thus clearly of great importance given the asymmetric transepithelial transport processes that they mediate. However, the specific factors that influence their localization remain to be elucidated. In the present study, we hypothesize that proper distribution of Kir4.1 and AQP4 is dependent on receptor-ECM interactions. To address this question, we used MDCK epithelial cells as a model of renal epithelia to investigate the impact of the ECM on the cell surface localization and stability of Kir4.1 and AQP4 channels. Via immunofluorescence, we first determined that these channels are localized basolaterally in both the mammalian kidney and MDCK cells and that they codistribute with the laminin receptor DG, the DAP component syntrophin, and E-cadherin. We also found that the ECM components fibronectin and laminin-1 both induce an increase in the amounts of either channel at the basolateral domain of MDCK cells. Using fluorescence recovery after photobleaching (FRAP), we demonstrated that fibronectin and laminin-1 stabilize these channels by reducing their diffusion rates within the plasma membrane. Finally, we show that while DG is required to
maintain proper expression of Kir4.1, integrin receptors may play a compensatory role in cells deficient for DG. This is further evidenced by our data showing that RGD-containing peptides known to interact with integrins reverse the reduction of the Kir4.1 diffusion rate induced by laminin, indicating that in epithelial cells, laminin can stabilize Kir4.1 not only via DG, but also via the integrin family of cell surface receptors. Moreover, both RGD peptides and the αvβ3-integrin function-blocking antibody inhibit the reduction of AQP4 diffusion rates, suggesting that αvβ3-integrin plays a role in the cell surface stabilization of AQP4 in epithelial cells.

2.2 Materials and methods

2.2.1 Antibodies

Antibodies used in this study include mouse anti-β-DG, 43DAG1/8D5, against 15 of the last 16 amino acids at the C terminus of the human DG sequence (Novocastra Laboratories, Newcastle-upon-tyne, UK); mouse anti-syntrophin, SYN1351, against Torpedo syntrophin (ABR-Affinity Bioreagents, Golden, CO); mouse anti-E-cadherin against amino acids 735–883 of the human E-cadherin (BD Transduction Laboratories); mouse anti-αvβ3 integrin, CD51/61, against purified adhesion receptor from the human melanoma cell line M21 (Millipore); rabbit anti-AQP4 against rat GST AQP4 corresponding to residues 249–323 (Alomone Laboratories, Jerusalem, Israel); and rabbit anti-Kir4.1 against amino acids 356–375 of the intracellular C-terminal region of rat Kir4.1 (Alomone Laboratories).

2.2.2 Cell culture

Type II MDCK cells were transfected with the cDNA coding for AQP4 (25) and Kir4.1 (48) inserted into the enhanced green fluorescent protein expression plasmids EGFP-C3 (Clontech Laboratories) and EGFP-C1, respectively. Cells were grown in high-glucose DMEM containing 10% fetal bovine serum (Medicorp, Montreal, QC), 1% penicillin/streptomycin, and 1% L-glutamine (GIBCO-BRL) in a humidified incubator at 37°C with 5% CO2-95% air and transfected 1 day after plating using the Effectene reagent following the supplier's instructions (Qiagen, Mississauga, ON). Individual colonies of
MDCK cells stably expressing either GFP-AQP4 or GFP-Kir4.1 were selected using 500 μg/ml geneticin-containing DMEM and grown at a density of 3 × 10^3 cells/ml for 72 h. For confocal microscopic analysis, FRAP, and cell surface biotinylation, the cells were grown on 12-mm coverslips, eight-well Ibidi μ-slides (Ibidi, Martinsried, Munich, Germany), and 25-mm PET track-etched filters with 0.4-μm pores (BD Biosciences, Mississauga, ON), respectively. These were coated for 18 h with 100 μg/ml collagen IV, 5.0 or 50.0 μg/ml fibronectin, or 9.6 or 96.0 μg/ml mouse Engelbreth-Holm-Swarm (EHS) Sarcoma laminin-1 (Sigma-Aldrich) prepared in DMEM. For the fibronectin-competition experiment, the specific fibronectin-competing peptides RGD (17.5 μg/ml) and GRGDSPK (35 μg/ml; Sigma-Aldrich) were applied to the cells in suspension for 5–10 min before plating. To ensure saturation of all potential binding sites, GFP-Kir4.1- and GFP-AQP4-expressing MDCK cells in suspension were exposed to these peptides in 900-fold molar excess (compared with fibronectin) before their plating on 50 μg/ml fibronectin-coated filters, as well as for the entire duration of the culture period. The FRAP experiments pertaining to blocking the interaction between αvβ3-integrin and the ECM were performed first by incubating the cells for 30 min at room temperature with 10 μg/ml of the αvβ3-integrin function-blocking antibody and then by plating them on substrates containing 50 μg/ml exogenous fibronectin or 96 μg/ml laminin-1.

2.2.3 Cell surface biotinylation

MDCK cells grown on 25-mm PET track-etched filters were placed on ice and washed with cold PBS containing 1.0 mM CaCl₂ and 0.5 mM MgCl₂ (DPBS) three times for 5 min each, and then their basolateral membrane was labeled for 30 min at 4°C using 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL). The biotin solution was removed, and the reaction was quenched with 50 mM NH₄Cl in DPBS for 10 min. The cells were extensively washed and incubated for 1 h with extraction buffer (25 mM Tris, pH 7.4, 25 mM glycine, 150 mM NaCl, and 5 mM EDTA) containing 1% Triton X-100 and 1× complete protease inhibitor cocktail (Roche, Laval, QC, Canada). The biotinylated proteins were precipitated using streptavidin covalently attached to agarose beads (Pierce Biotechnology).
The apicobasal expression of Kir4.1 and AQP4 was determined by cell surface biotinylation on confluent MDCK cells grown on PET track-etched filters. The biotin was applied on the apical or basolateral domains, and subsequent sample collection and analysis steps were carried out using the method described above.

2.2.4 Immunoblotting

The biotinylated proteins were denatured by boiling at 95°C for 9 min in reducing sample buffer, then separated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON). The blots were first blocked with a solution of 5% nonfat milk dissolved in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and then probed using antibodies against AQP4 (1:1,000), Kir4.1 (1:750), β-DG (1:500), syntrophin (1:500), and E-cadherin (1:500). The bound antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:2,000; Pierce), and signals were visualized on Bioflex Econofilm (Interscience, Markham, ON) using chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

2.2.5 Immunofluorescence

For immunofluorescence, rat cryostat kidney sections and cells grown on 12-mm glass coverslips were washed three times with PBS for 5 min each. The sections were fixed using 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, and the cells were fixed using either 2% PFA or methanol-acetone (80–20%) for 20 min. After three additional washes with PBS, the sections and cells were blocked and permeabilized at room temperature (20–22°C) with a solution containing 2% BSA and 0.3% Triton X-100 in PBS. They were then incubated at room temperature for 1 h with primary antibodies against β-DG (1:50), E-cadherin (1:100), or syntrophin (1:100) and rinsed with PBS (3 × 15 min). Subsequently, they were incubated with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-rabbit IgG for 1 h (1:700; Molecular Probes). After several washes with PBS, kidney sections and cells were mounted on glass coverslips and slides, respectively, using Prolong Gold Antifade reagent (Invitrogen, Burlington, ON). To confirm the specificity of the labeling, control sections and cells were treated equivalently in the absence of primary
antibodies. Fluorescent labeling was visualized using a confocal microscope (Fluoview 1,000; Olympus) and an Uplan Apochromat 1.35 NA ×60 objective (Olympus).

2.2.6 FRAP

MDCK cells stably expressing either GFP-Kir4.1 or GFP-AQP4 were grown to confluence for 48 h in 8-well Ibidi μ-slides (Ibidi), and images were acquired on a confocal microscope (FV1000; Olympus) using an Uplan Apochromat 1.35 NA ×60 objective and fully opened pinhole. Areas of interest encompassing the coincident plasma membranes of two adjacent GFP-AQP4 or GFP-Kir4.1 cells were photobleached over 15 frames using a 405-nm simultaneous scanner set to 60% power. All images were captured at a resolution of 512 × 512 using a scan rate of 2 μs/pixel, a frame rate of 1.1 s/frame over a period of ~45 s. Fluorescence recovery was analyzed, and “best-fit” curves derived, using the built-in nonlinear regression function of Prism 4.00 (GraphPad Software, La Jolla, CA). FRAP analysis was also conducted on Kir2.1 in MDCK cells transiently transfected with Kir2.1 cDNA inserted into the pEGFP-C1 expression plasmid (48).

2.2.7 Small interference RNA transfection

Adherent MDCK cells cultured for 1 day to near-confluence were transfected with small interference (si) RNAs specific for canine Dag1 (ON-TARGETplus SMARTpool siRNA reagents, Dharmacon; 100 pmol for cells grown on 12-mm coverslips, 250 pmol for cells grown on 25-mm PET track-etched membranes) using Lipofectamine 2000. Controls for nonspecific siRNA activity were performed using equivalent amounts of ON-TARGETplus Non-targeting siRNA.

2.2.8 Quantitative and statistical analysis

Fluorescence intensity analysis from three different experiments was performed using ImagePro Plus (Media Cybernetics). Six fields containing an average of 100 cells were analyzed for each experiment. Images of individual optical sections at the level of the basolateral surface from the same experiment were acquired and quantified under identical settings. In the case of Western blotting experiments, the quantifications were performed by densitometric analysis. All statistical analyses were performed using Prism 4.00 software.
For the FRAP assay, fluorescence values at each time point were first normalized to prebleach values using Microsoft Excel, then plotted and analyzed with GraphPad Prism.

As previously described in opossum kidney cells [202], Kir4.1 fluorescence was seen at the basolateral plasma membrane as well as in the Golgi complex of MDCK cells (Appendix D). To quantify the basolateral pool of Kir4.1 fluorescence, the total Kir4.1 fluorescence was subtracted from that of basolateral E-cadherin, giving rise to the fluorescence intensity of intracellular Kir4.1. This value was subsequently subtracted from the total Kir4.1 fluorescence intensity, enabling the determination of Kir4.1 fluorescence intensity associated with the basolateral plasma membrane.

In the case of the biotinylation assays, densitometric analyses were performed to quantitate the fibronectin- and laminin-induced changes in the amounts of Kir4.1 and AQP4 at the basolateral membrane domain. These were first normalized against E-cadherin and were then normalized against the signal seen in the control untreated cells.

2.3 Results

2.3.1 Kir4.1 and AQP4 are localized at the basolateral plasma membrane domain in kidney epithelial cells

To examine the distribution of Kir4.1 and AQP4 in the renal cortex and medulla, cryostat sections of rat kidneys were double-immunolabeled for Kir4.1 or AQP4 and E-cadherin. Consistent with previous observations [8, 221], labeling for Kir4.1 and AQP4 was observed at the basolateral domain of epithelial cells where it colocalizes with E-cadherin (Fig. 2-1, A–C and G–I).

We next examined whether Kir4.1 and AQP4 were codistributed with β-DG, syntrophin, and E-cadherin in confluent epithelial MDCK cells stably expressing GFP-Kir4.1 or GFP-AQP4. AQP4 is mainly localized at the plasma membrane whereas Kir4.1 is also associated with the Golgi apparatus as has been previously described in opossum kidney cells [202]. In addition, we found that GFP-Kir4.1 and GFP-AQP4 codistribute with syntrophin (Fig. 2-2, D–F, F’, M–O, and O’) and E-cadherin (Fig. 2-2, G–I, I’, P–R, and R’) at the basolateral domain with a more obvious codistribution at the lateral domain. While the 2%
PFA fixation necessitated by the use of the antibody against β-DG caused substantial flattening of the epithelial monolayer (Fig. 2-2, C' and L'), lateral codistribution of Kir4.1 and AQP4 with β-DG is still evident (Fig. 2-2, A–C, C’, J–L, and L’).

To further demonstrate that both Kir4.1 and AQP4 are expressed predominantly at the basolateral membrane of MDCK cells in culture, we performed biotinylation studies of the apical and basolateral domains of cells expressing these channels (Fig. 2-3). We found that both Kir4.1, detected in its monomeric and tetrameric forms (~67 and ~268 kDa) (Fig. 2-3 A), and AQP4 (Fig. 2-3 B) are predominantly expressed at the basolateral domain. β-DG is also localized to the same domain.

2.3.2 Fibronectin and laminin increase cell-surface expression and reduce lateral mobility of Kir4.1 and AQP4 at the basolateral plasma membrane domain of MDCK cells

We next sought to determine whether the interaction of the DGC with the ECM plays a role in the basolateral localization of Kir4.1 and AQP4. MDCK cells expressing GFP-Kir4.1 and GFP-AQP4 were grown for 72 h on coverslips coated with either fibronectin or laminin-1 (5.0 FN = 5.0 μg/ml fibronectin; 50.0 FN = 50.0 μg/ml fibronectin; 9.6 LN = 9.6 μg/ml laminin; 96.0 LN = 96.0 μg/ml laminin) (Fig. 2-4). The amounts of Kir4.1 and AQP4 localized at the lateral domain of MDCK cells were then determined via ImagePro analysis of confocal micrographs using E-cadherin staining as a marker of the basolateral domain. We found that the cells cultured on 50.0 μg/ml fibronectin, 9.6 μg/ml laminin, or 96.0 μg/ml laminin presented significantly greater fluorescence intensity for both Kir4.1 and AQP4 compared with control cells cultured on uncoated coverslips (Fig. 2-4, A and B). Collagen IV produced no change in the fluorescence intensity of either channel (data not shown). In addition, cell surface biotinylation of the basolateral domain of MDCK cells cultured on permeable supports coated with low and high concentrations of either fibronectin or laminin (both of which are effectively retained by the culture surface; Appendix A) revealed an increase in the cell surface localization of both Kir4.1 and AQP4 (Fig. 2-5, A and C, and Appendix B). It is important to point out that the total expression levels of both channel types remained unchanged when the cells were treated with fibronectin or laminin (Appendix C). Densitometric analysis showed that fibronectin and laminin-1 resulted in a significant 1.3- and 1.9-fold increase in Kir4.1 cell surface amounts, respectively (Fig. 2-5 B). AQP4
amounts at the cell surface also increased approximately twofold in the presence of fibronectin and laminin-1 (Fig. 2-5 D). The greater increase of Kir4.1 fluorescence intensity (Fig. 2-4 A) compared to its cell surface expression (Fig. 2-5 A) may be due to the detection of not only the fluorescence associated with the cell surface but also of a sub-surface pool of GFP-Kir4.1, of which a significant portion is found within the Golgi (Appendix D). To determine the mechanisms underlying the increased channel expression at the cell surface, we next investigated whether fibronectin and laminin alter the lateral mobilities of Kir4.1 and AQP4 within the plasma membrane. The cells were cultured to confluence in eight-well µ-slides coated with either 50.0 µg/ml fibronectin or 96.0 µg/ml laminin, and 2-µm sections of lateral membranes were photobleached so that <50% of initial fluorescence remained. The fluorescence intensity within the bleached areas was then measured every 1.1 s over 45 s. Both fibronectin and laminin caused a dramatic reduction in the rate of recovery and mobile fractions of GFP-Kir4.1 (Fig. 2-6, A and B) and GFP-AQP4 (Fig. 2-6, C and D). These data reveal a role for both fibronectin and laminin in the regulation of Kir4.1 and AQP4 diffusion rates at the plasma membrane of epithelial MDCK cells. To determine whether the effect of fibronectin and laminin is specific to Kir4.1, we tested their effect on the diffusion rates of GFP-Kir2.1 by FRAP analysis using parameters identical to those used for GFP-Kir4.1. We found that fibronectin induces a significant reduction in the fluorescence recovery of Kir2.1, whereas laminin has no effect compared with control untreated cells (Fig. 2-6, E and F).

2.3.3 DG is involved in Kir4.1 and AQP4 basolateral distribution regulated by fibronectin and laminin

Our previous studies in astrocytes and Müller glia showed that laminin-induced redistribution of Kir4.1 and AQP4 is mediated via DG [41, 184, 222]. Here, we used siRNA targeted against canine DG (si-Dag1) to silence its expression and determined whether DG is involved in the laminin-induced increase in basolateral expression of Kir4.1 and AQP4 by both fluorescence and cell surface biotinylation (Figs. 7 and 8). We found that patches of cells were effectively knocked down for DG, and we estimated these patches to constitute ∼50% of the cell population. Cells deficient for DG express reduced levels of Kir4.1 fluorescence (compare Fig. 2-7, D–F to A–C). However, AQP4 fluorescence is comparable
to that seen in control cells (compare Fig. 2-7, J–L to G–I), despite the fact that DG expression was, significantly decreased by the siRNA treatment in both cases, and resulted in severe alterations to cellular polarity (Appendix E). These results are supported by the cell surface biotinylation data, showing that DG deficiency significantly reduces Kir4.1 cell surface expression (Fig. 2-8, A and B) but does not alter that of AQP4 (Fig. 2-8, C and D).

To determine whether the laminin-induced increase in cell surface expression of Kir4.1 and AQP4 is due to the interaction of laminin with cell surface receptors other than α-DG, GFP-Kir4.1- and GFP-AQP4-expressing MDCK cells transfected with si-Dag1 were grown in the presence of 96 μg/ml laminin-1 for 72 h, and the membrane expression of Kir4.1 and AQP4 was determined by cell surface biotinylation. We found that laminin-1 induces an increase in both Kir4.1 and AQP4 cell surface expression (Fig. 2-8). Of particular interest, this laminin-induced increase is more robust in si-Dag1-transfected cells than in control cells (Fig. 2-8, A and C). These results show that, in the presence of exogenous laminin, DG deficiency in MDCK cells does not impair the ability of laminin-1 to alter cell surface expression of these channels and suggest that other laminin receptors may also be involved.

To investigate further the effect of fibronectin on Kir4.1 and AQP4 cell surface expression, we used the two RGD motif-containing disintegrin peptides Arg-Gly-Asp (RGD) and Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) known to block fibronectin binding to integrins. To ensure saturation of all potential binding sites, GFP-Kir4.1- and GFP-AQP4-expressing MDCK cells in suspension were exposed to 900-fold molar excess (compared with fibronectin) of these peptides before plating on fibronectin-coated filters as well as during the entire culture period. Via cell surface biotinylation, we found that GRGDSPK (xRGD) reduces significantly the effect of fibronectin on the cell surface expression of both Kir4.1 (Fig. 2-9, A and B) and AQP4 (Fig. 2-9, C and D).

2.3.4 RGD-containing peptides and αvβ3-integrin antibody alter AQP4 and Kir4.1 mobility on fibronectin and laminin

Having demonstrated that the rate of recovery and mobile fractions of Kir4.1 and AQP4 are significantly decreased in the presence of fibronectin and that integrin-blocking
peptides inhibit the fibronectin-induced increase in Kir4.1 and AQP4 cell surface expression, we next sought to determine the effect of fibronectin and these peptides on the rate of recovery of both channels using FRAP analysis. We found that both RGD and GRGDSPK (xRGD) significantly inhibit the effect of fibronectin, resulting in a faster rate of recovery and higher mobile fractions than those of control cells (Fig. 2-10, A, A’, B, and B’). When applied in conjunction with laminin, both peptides restore significantly the diffusion rate of Kir4.1 (Fig. 2-10, C and C’). However, only GRGDSPK is effective in reversing the effect of laminin on the mobility of AQP4 (Fig. 2-10, D and D’). These data reveal a differential effect of RGD-containing peptides on Kir4.1 and AQP4 stability at the plasma membrane.

Although both Kir4.1 and AQP4 are regulated by laminin-1 and fibronectin, Kir4.1 is more responsive than AQP4 to RGD-containing peptides in the presence of laminin. Conversely, AQP4 is more responsive than Kir4.1 to these peptides in the presence of fibronectin.

Given that αvβ3-integrin interacts with ECM proteins including fibronectin via its RGD binding motif [223] and that RGD-containing peptides significantly reverse the decreased channel diffusion induced by fibronectin (Fig. 2-10, A, A’, B, and B’), and laminin (Fig. 2-10, C, C’, D, and D’), we hypothesized that this reversal occurs via blockage of the interaction between the ECM and αvβ3-integrin. We therefore assessed Kir4.1 and AQP4 diffusion rates in cells treated with either 50 μg/ml fibronectin or 96 μg/ml laminin plus 10 μg/ml of αvβ3-integrin function-blocking antibody by FRAP (Fig. 2-11). Here, the cells were incubated with the αvβ3-integrin antibody for 30 min at room temperature before being plated on a substrate containing either fibronectin or laminin and in culture media containing the αvβ3-integrin antibody. First, we showed that the αvβ3-integrin antibody effectively blocks adhesion of GFP-Kir4.1- and GFP-AQP4-expressing cells to fibronectin (Appendix F). Second, we performed FRAP analysis and the data show that the decreased diffusion rates of AQP4 induced by either laminin or fibronectin are reversed by the αvβ3-integrin antibody (Fig. 2-11, B, B’, D, and D’); however, those of Kir4.1 remain unchanged (Fig. 2-11, A, A’, C, and C’). These data suggest that αvβ3-integrin plays a role in the ECM-induced stabilization of AQP4 at the basolateral domain.
2.4 Discussion

2.4.1 Laminin-DG regulation of basolateral expression of Kir4.1 and AQP4

The ECM is a critical determinant of epithelial morphology and organization. Several studies have demonstrated that laminin assembly plays a crucial role in the polarization of MDCK cells [190, 224] and that interactions between laminin and β1-integrin, relayed via a Rac1-dependent mechanism, are necessary for the proper polarization of three-dimensional MDCK cysts [190]. While previous studies have detailed the importance of DG in mediating epithelial and oocyte polarity in Drosophila [187], the role of this high-affinity laminin receptor [173] in the distribution of polarized proteins in epithelial cells remains to be determined.

Our previous findings have described a role for DG in the laminin-dependent localization of Kir4.1 and AQP4 in glial cells [41, 180, 184]. In astrocyte cultures, we have shown that laminin-1 requires DG for the formation of macrodomains containing laminin, the DG complex, and both Kir4.1 and AQP4 [184, 222]. In the intact mouse brain, α-DG, Kir4.1, and AQP4 are concentrated at perivascular domains of astrocytes; however, this distribution pattern is lost in the Large\textsuperscript{myd} mouse, presenting a hypoglycosylation of α-DG and a subsequent loss of laminin binding to α-DG [180]. These findings strongly suggest that DG-laminin interaction is important in the polarized distribution of Kir4.1 and AQP4 \textit{in vivo}. To determine whether this type of interaction plays also a role in regulating the basolateral distribution of Kir4.1 and AQP4 in epithelial cells, we used MDCK epithelial cells as a model of renal epithelia. In the present study, we show that laminin-1 and fibronectin induce a significant increase in the levels of both Kir4.1 and AQP4 at the basolateral plasma membrane domain of MDCK cells, indicating that they are important determinants of polarized channel expression in the renal system. These data suggest that water permeability and potassium conductance of the plasma membrane of renal epithelial cells may be regulated by the ECM. DG and syntrophin are localized basolaterally in MDCK cells where they codistribute with Kir4.1 and AQP4, supporting a role for the DAP complex in the regulation of the polarized distribution of these channels. Indeed, siRNA-mediated knockdown of DG resulted in a decrease in basolateral Kir4.1 expression in the absence of
exogenous laminin, indicating that DG is crucial for the polarized distribution of this channel in epithelial cells. However, in the presence of exogenous laminin increased basolateral expression of both Kir4.1 and AQP4 occurs in MDCK cells deficient for DG. This suggests that regulation of the polarity of these channels by laminin in epithelial cells involves DG but also other laminin receptors such as those of the integrin family. Indeed, numerous studies have described synergistic interactions between DG and receptors of the integrin family [175, 225, 226].

2.4.2 DG and integrins contribute to the basolateral stabilization of Kir4.1 and AQP4 by laminin and fibronectin

To address the mechanisms underlying the increase in channel expression in the presence of fibronectin and laminin, we performed FRAP analysis on the lateral membrane of cells expressing GFP-tagged Kir4.1 or AQP4 channels cultured on either ECM molecule. We found that both caused significant reductions in the mobilities of the channels in a dose-dependent manner. These results show that the ECM regulates the stabilization of both channels at the membrane, thereby contributing to their polarized distribution at the basolateral domain of epithelial cells. This ECM-mediated stabilization of the channels may result from a direct mechanism whereby fibronectin and laminin have access to the lateral domains due to the partial polarization of the cells grown on glass coverslips. Alternatively, it may be due to an indirect mechanism involving cell signaling resulting from the interaction of the ECM with the basal cell domains. Reduced endocytosis may participate in the increased ECM-induced stabilization of the channels; however, we believe that to be unlikely as the FRAP analysis was conducted over short time periods (45–60 s).

We have also investigated the effect of the ECM on the stability of Kir2.1 at the plasma membrane of MDCK cells by FRAP. Our results show a decrease in Kir2.1 diffusion rate in the presence of fibronectin but not laminin. Although both Kir4.1 and Kir2.1 contain a PDZ-binding motif at the C terminal that enables their interaction with the DAP complex and therefore with laminin in the ECM [227], Kir2.1 stabilization at the membrane does not seem to be regulated by laminin. This is likely due to the fact that Kir2.1 interacts with the DAP complex with a lower affinity than Kir4.1 [227]. These data argue that laminin-induced
stabilization of Kir4.1 is mediated by the DAP complex and that inwardly rectifying potassium channels containing a PDZ-binding motif are differentially regulated by laminin.

The expression of several fibronectin-binding integrins [203] and the stabilization of both Kir4.1 and AQP4 in MDCK cells in the presence of fibronectin, prompted us to investigate the possible implication of integrins in this effect. The application of fibronectin-integrin-blocking peptides Arg-Gly-Asp (RGD) and Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) in tandem with fibronectin to MDCK cells followed by cell surface biotinylation shows that GRGDSPK reduces significantly the effect of fibronectin on Kir4.1 and AQP4 expression at the basolateral domain (Fig. 2-9, B and D). In addition, FRAP analysis shows that RGD peptides inhibit the effect of fibronectin, resulting in a faster rate of recovery and higher mobile fractions of Kir4.1 and AQP4. These data suggest that fibronectin receptors, most likely integrins, play a role in limiting the rate of lateral diffusion within the plasma membrane of both Kir4.1 and AQP4. We then determined the effect of these disintegrin peptides on laminin-induced retardation of channel diffusion and found that GRGDSPK enhances the rate of lateral diffusion of Kir4.1 and AQP4 over cells treated with laminin alone, suggesting that laminin’s effect involves the contribution of fibronectin and fibronectin-binding receptors as well. Addition of exogenous laminin-1 to MDCK cells leads to the accumulation of endogenous fibronectin, suggesting that the laminin-induced effects may be indirect in that they involve fibronectin (Appendix A). Furthermore, the inhibition of fibronectin binding to αvβ3-integrin using a function-blocking antibody (Fig. 2-11) indicates that αvβ3-integrin plays a role in the ECM-mediated stabilization of AQP4 but not Kir4.1 at the basolateral domain of MDCK cells. The reversal of the fibronectin-induced decreases in Kir4.1 diffusion rate by RGD-containing peptides and the inability of the αvβ3-integrin antibody to prevent this effect suggest that the ECM-mediated stabilization of Kir4.1 may be regulated by α5β1-integrin [217].

2.4.3 Summary

To date, evidence from the literature has focused on the role of the interactions of DG and integrins with laminins in the morphogenesis and wound healing in the kidney, salivary gland, and lung [189, 201, 228, 229]. Our findings reveal a new role for DG and integrins
and show that the interaction of these receptors with the ECM plays an important role in the polarized distribution of Kir4.1 and AQP4 at the basolateral domain of kidney epithelial cells and therefore is critical to the maintenance of osmoregulation in the renal system.
Figure 2-1. Kir4.1 and AQP4 are localized basolaterally in kidney epithelial cells. Coronal Kidney sections were double immunolabeled for Kir4.1 (A) or AQP4 (G) and the basolateral marker E-cadherin (B and H). The merged images show that Kir4.1 and AQP4 codistribute with E-cadherin (C and I) in kidney epithelial cells. Zoomed images of the boxed areas in A-C and G-I are shown in D-F and J-L, respectively. Scale bars, 50 µm for A-C and G-I and 25 mm for D-F and J-L.
Figure 2-2. **Kir4.1 and AQP4 are expressed basolaterally in MDCK cells.** Confluent MDCK cells expressing either GFP-Kir4.1 (A, D, and G) or GFP-AQP4 (J, M, and P) were immunolabeled for β-DG (B and K), syntrophin (E and N), or E-cadherin (H and Q). The merged images of XY (C, F, I, L, O, R) and ZY views (C’, F’, I’, L’, O’, R’) show that Kir4.1 and AQP4 co-distribute with components of the dystrophin associated protein complex as well as with E-cadherin at the basolateral membrane domains of MDCK cells. Scale bar, 50 μm.
Figure 2-3. Kir4.1 and AQP4 are expressed predominantly within the basolateral membrane domain of MDCK cells. Biotinylated cell surface fractions from the apical (AP) and basolateral (BL) domains of confluent MDCK cells expressing either GFP-Kir4.1 or GFP-AQP4 were immunoblotted for Kir4.1 (A; arrow points at the tetramer and monomer, respectively) and AQP4 (B) as well as β-DG (bottom panels in A and B).
Figure 2-4. Fibronectin and laminin increase Kir4.1 and AQP4 levels at the basolateral domains of MDCK cells. MDCK cells expressing GFP-Kir4.1 (A) or GFP-AQP4 (B) were grown on coverslips coated with fibronectin (5.0 μg/ml FN or 50.0 μg/ml FN) or laminin-1 (9.6 μg/ml LN or 96.0 μg/ml LN) for 72 hours. Histograms represent the mean GFP fluorescence intensity normalized to that of the respective untreated controls ±SEM of three experiments. The asterisks represent statistically significant increases in the fluorescence intensity compared with control cells as determined by the two-tailed Student’s t-test (*p<0.05; **p<0.005).
Figure 2-5. Fibronectin and laminin increase cell surface expression of Kir4.1 and AQP4 at the basolateral domains of MDCK cells. Biotinylated cell surface fractions from control cells (CTL) and cells grown on either fibronectin (5.0 μg/ml FN or 50.0 μg/ml FN) or laminin-1 (9.6.0 μg/ml LN or 50.0 μg/ml LN) for 72 hours were immunoblotted for Kir4.1 (A), AQP4 (C), β-DG (A and C), syntrophin (A and C) and E-cadherin (A and C). Histograms of the densitometric analysis represent the mean pixel intensity ±SEM relative to control of three experiments (B and D). The asterisks represent statistically significant increases of Kir4.1 and AQP4 cell surface expression compared with control cells as determined by the two-tailed Student’s t-test (*p<0.05).
Figure 2-6. Fibronectin and laminin reduce the membrane diffusion of Kir4.1 and AQP4 in MDCK cells. MDCK cells expressing GFP-Kir4.1 (A), GFP-AQP4 (C), or GFP-Kir2.1 (D) grown in 8-well m-slides coated with fibronectin (50.0 FN) or laminin-1 (96.0 LN) for 48-72 hours were analyzed by FRAP. Data points represent averaged percent fluorescence intensity ±SEM of GFP-Kir4.1, GFP-AQP4, and GFP-Kir2.1 recovered in the bleached area and normalized to the intensity observed prior to the bleaching in the same cell (n>8 cells/condition). The tables summarize the percent mobile fraction of GFP-Kir4.1 (B), GFP-AQP4 (D) and GFP-Kir2.1 (E) in control cells and cells grown on fibronectin or laminin-1 (**p<0.0001; *p=0.0042; *p=0.0384).
Kir4.1 Mobile Fraction (%)

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<th></th>
<th>UT</th>
<th>50.0 FN</th>
<th>96.0 LN</th>
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<tr>
<td>Mobile Fraction (%)</td>
<td>83.60 ± 2.31</td>
<td>42.94 ± 1.39***</td>
<td>37.29 ± 1.15**</td>
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AQP4 Mobile Fraction (%)

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<th>UT</th>
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<th>96.0 LN</th>
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<tr>
<td>Mobile Fraction (%)</td>
<td>90.53 ± 2.88</td>
<td>28.19 ± 3.24***</td>
<td>28.28 ± 2.56***</td>
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Kir2.1 Mobile Fraction (%)

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<tr>
<td>Mobile Fraction (%)</td>
<td>43.86 ± 8.10</td>
<td>23.55 ± 1.53*</td>
<td>36.40 ± 7.87</td>
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</table>
Figure 2-7. Dystroglycan is essential for the proper localization of Kir4.1 but not AQP4. MDCK cells expressing GFP-Kir4.1 (A and D) and GFP-AQP4 (G and J) were transfected with Dag1 siRNA (siDag1; D-F and J-L) and immunolabeled for β-DG (B, E, H and K). siCTL-transfected cells were used as control (A-C and G-I). The dotted lines show fields containing cells deficient in dystroglycan. Scale bar, 50 μm.
Figure 2-8. Dystroglycan is not the only laminin receptor involved in the laminin-induced increase in cell surface expression of Kir4.1 and AQP4 in MDCK cells. Biotinylated cell surface fractions from control (siCTL) and Dag1 siRNA-transfected (siDag1) GFP-Kir4.1 and GFP-AQP4 expressing cells grown in the absence (-LN) or the presence of 96 μg/ml laminin-1 (+LN; A and B) for 72 hours were immunoblotted for Kir4.1 (A), AQP4 (C), β-DG and E-cadherin (A and C). Histograms of the densitometric analysis represent the mean pixel intensity ±SEM relative to control of three experiments (B and D). The asterisks represent statistically significant increases of Kir4.1 and AQP4 cell surface expression compared with control cells as determined by the two-tailed Student’s t-test (* p<0.05; ***p<0.001).
Figure 2-9. RGD and xRGD peptides inhibit the fibronectin-induced increase in cell surface expression of Kir4.1 and AQP4. Control MDCK cells, and cells incubated with Arg-Gly-Asp (RGD) or Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK = xRGD) peptides, were cultured to confluence over 72 hours on fibronectin-coated cell culture filters, and then subjected to cell surface biotinylation of the basolateral domain. Cell surface fractions were immunoblotted for Kir4.1 (A) or AQP4 (C), and for E-cadherin (A and C). Histograms of the densitometric analysis represent the mean pixel intensity ±SEM relative to fibronectin-treated cells of three experiments (B and D). The asterisks represent statistically significant decreases of Kir4.1 and AQP4 cell surface expression compared with fibronectin-treated cells as determined by the two-tailed Student’s t-test (* p<0.05).
Figure 2-10. RGD peptides reverse the fibronectin- and laminin-induced reduction in channel mobility. Cells expressing either GFP-tagged Kir4.1 or AQP4 were incubated with RGD or GRGDSPK (xRGD) peptides, plated on substrates containing 50 mg/ml exogenous fibronectin (FN) or 96 mg/ml laminin-1 (LN), and then subjected to FRAP analysis. Although fibronectin potently retards the lateral diffusion of Kir4.1, xRGD completely abolished this effect restoring channel mobility level to that seen for untreated cells (A and A'; ***p<0.0001). RGD and xRGD are also effective in significantly increasing AQP4 diffusion rates in the presence of FN (B and B'; **p=0.0015 and 0.0036, respectively). While both RGD and xRGD peptides are capable of significantly reversing the effect of laminin, raising Kir4.1 mobility to levels even beyond those of control cells (C and C'; ***p<0.0001), only xRGD is capable of partially reversing the effect of laminin on AQP4 mobility (D and D'; **p=0.0074).
### Kir4.1 Mobile Fraction (%)

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<td>FN</td>
<td>32.61 ± 3.32</td>
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<tr>
<td>FN + RGD</td>
<td>38.78 ± 3.70</td>
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<tr>
<td>FN + xRGD</td>
<td>54.96 ± 3.59***</td>
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### Kir4.1 Mobile Fraction (%)

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<tr>
<td>LN + RGD</td>
<td>85.62 ± 7.23***</td>
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<tr>
<td>LN + xRGD</td>
<td>102.30 ± 5.10***</td>
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### AQP4 Mobile Fraction (%)

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<tr>
<td>UT</td>
<td>59.94 ± 8.67</td>
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<tr>
<td>FN</td>
<td>57.91 ± 6.63</td>
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<tr>
<td>FN + RGD</td>
<td>78.57 ± 4.90**</td>
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<tr>
<td>FN + xRGD</td>
<td>76.47 ± 5.16**</td>
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### AQP4 Mobile Fraction (%)

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<tr>
<td>UT</td>
<td>76.49 ± 2.22</td>
</tr>
<tr>
<td>LN</td>
<td>31.90 ± 3.32</td>
</tr>
<tr>
<td>LN + RGD</td>
<td>30.69 ± 2.81</td>
</tr>
<tr>
<td>LN + xRGD</td>
<td>48.97 ± 4.33**</td>
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Figure 2-11. αvβ3 integrin plays a role in the regulation of the fibronectin and laminin-induced stability of AQP4 at the plasma membrane. Cells expressing either GFP-tagged Kir4.1 or AQP4 were incubated for 30 min at room temperature with 10 mg/ml of αvβ3 integrin antibody, plated on substrates containing 50 μg/ml exogenous fibronectin (FN) or 96 μg/ml laminin-1 (LN), and then subjected to FRAP analysis. The αvβ3 integrin antibody does not significantly affect the lateral mobility of Kir4.1 in MDCK cells grown in the presence of either fibronectin or laminin (A, A’, C and C’), however it inhibits that of AQP4 in MDCK cells grown on laminin (D and D’; *p=0.04). Note that while the predicted recovery of AQP4 is not significantly different between cells treated with fibronectin and cells treated with fibronectin plus the αvβ3 integrin antibody (B’), the initial recovery (up to 15 seconds) is accelerated in cells treated with fibronectin plus the αvβ3 integrin antibody.
AQP4 Mobile Fraction (%)

FN  19.45  0.59
FN + αvβ3Ab  30.13  0.66

Kir4.1 Mobile Fraction (%)

FN  29.62  0.42
FN + αvβ3Ab  32.04  0.43

Kir4.1 Mobile Fraction (%)

LN  46.70  0.46
LN + αvβ3Ab  65.79  0.67*

Kir4.1 Mobile Fraction (%)

AQP4 Mobile Fraction (%)

FN  19.45  0.59
FN + αvβ3Ab  30.13  0.66

Kir4.1 Mobile Fraction (%)

AQP4 Mobile Fraction (%)

LN  46.70  0.46
LN + αvβ3Ab  65.79  0.67*
CHAPTER 3: ASTROCYTIC AQUAPORIN-4 CELL-SURFACE EXPRESSION AND TURNOVER ARE REGULATED BY DYSTROGLYCAN, DYNAMIN, AND THE EXTRACELLULAR MATRIX

3.1 Introduction

The aquaporins are a family of water-permeable channels that are expressed in a wide variety of tissues. They primarily serve to facilitate rapid water conduction into and out of cells, although certain aquaporins are also known to be permeable to small solutes such as urea and glycerol, and even carbon dioxide [230]. Aquaporin-4 is the most abundant aquaporin isoform in the mammalian central nervous system. It is expressed primarily by astroglial cells at glia limitans externa at the brain surface, the glia limitans interna of the ventricles, structures which define the boundaries between the brain and the cerebrospinal fluid, and those at the blood-brain barrier (BBB; [113, 231]). In the BBB, AQP4 is predominantly localized to the perivascular endfeet of astrocytes [178], which are structures that form where these cells come into contact with the blood vessels, and it is likely that this perivascular pool of AQP4 functions to mediate water flow between the blood and the brain. While animals that lack AQP4 expression exhibit no detectable behavioural defects or gross morphological disruptions in BBB structure [232], they do present deficits in olfaction and audition [182, 233], indicating that the channel may play a central role in brain function. Studies of these animals have also revealed the importance of this channel in pathophysiological states. In cases of severe insults to the brain, such as stroke or injury, in which cytotoxic and vasogenic edema can develop, and often are the main contributors to morbidity or death [234]. The former is caused by the influx of water into the brain through the BBB, which leads to cellular swelling, while the latter is associated with the breakdown of the BBB itself, and the consequent flow of fluid into the extracellular spaces of the brain. AQP4-null mice display improved neurological outcome following water intoxication or ischemic stroke by preventing the accumulation of water in astrocytic endfeet [121], and cytotoxic edema resulting from meningitis is reduced in these animals as well [235]. Paradoxically, the vasogenic edema resulting from intraparenchymal fluid infusion, cortical freeze injury, brain tumor and brain abscess was found to be worse in these animals,
compared to wild-type controls [128; [236], implicating AQP4 in bulk water clearance. The modulation of AQP4 activity or expression in the brain could therefore result in the amelioration of these disease states.

Two major isoforms of AQP4 are expressed in astrocytes: M1, which has been reported to be approximately 34 kDa in size, and the smaller 31-kDa M2 isoform, which differs from the former by a 22 amino acid truncation from its N-terminus [79]. The impact of this seemingly minor difference is profound. Whereas the M1 isoform exists primarily as singular tetramers that can infrequently self-assemble into groups containing no more than 12 similar subunits, tetramers of M2 possess the capacity to further organize themselves into massive orthogonal arrays of particles (OAPs) comprised of more than four hundred linked subunits. OAPs generated from a mixture of these two isoforms appear to be held in balance via their interaction, with an excess of M23 contributing to an increase in aggregate size, and M1 inhibiting accretion [81]. Transcripts corresponding to a third “Mz” isoform [237], have been found in rat brains as well, but as the protein is expressed in comparatively low amounts, its contribution to channel organization can most likely be considered to be minimal.

It appears that the regulation of AQP4 trafficking occurs on a number of fronts. A study published by Madrid et al. in 2001 [238], demonstrated that the expression of AQP4 at the basolateral domain of epithelial MDCK cells falls under the control of two independent sorting signals contained within the C-terminus of the channel. The distal signal, a dileucine motif found between residues 288 and 294, causes the rerouting AQP4 to the apical pole when absent or mutated. The proximal signal, an YxxΦ motif, determines the interaction of AQP4 with the μ2a and μ3 subunits of the AP2 and AP3 complexes, thereby governing the uptake of the channel via clathrin-dependent pathways and its eventual targeting to lysosomes. The rate at which AQP4 was shuttled into the degradative pathway was found to be greatly influenced by the casein kinase II-mediated phosphorylation of a serine residue located N-terminally to the tyrosine motif. A subsequent study [77] showed that, in human gastric cells, phosphorylation is similarly involved in the regulation of AQP4 expression at the cell surface. It was seen, upon the treatment of cells with histamine, that the PKA phosphorylation of AQP4 increased tenfold. This however, failed to occur if the addition of
histamine was preceded phenylarsine oxide, an inhibitor of endocytosis, which indicated that this mode of phosphorylation is conditional on AQP4 being internalized. These lines of evidence, combined with the finding that the washout of histamine resulted in the delayed reemergence of AQP4 from internal stores even in the absence of de novo protein synthesis, led the authors of this study to speculate that PKA-mediated phosphorylation may serve to retain AQP4 in recycling pathways. A more recent study [75], performed in Xenopus oocytes induced to express various AQP4 isoforms, determined that PKC activation resulted in the rapid clearance of the channel from the cell surface, with M23 exhibiting the greatest degree of sensitivity.

DG is a highly-conserved cell-surface laminin receptor expressed in numerous organisms, including human, mouse and zebrafish [169-171]. DG interacts and complexes with intracellular components such as syntrophin, dystrobrevin, and dystrophin, collectively forming what is known as the dystroglycan complex (DGC). At the perivascular regions of the brain, AQP4 is coexpressed with α1-syntrophin and with α-DG [178-180], and the evidence linking the function of the DGC to AQP4 expression is quite ample. Indeed, the perivascular localization of AQP4 is lost in the α-syntrophin knockout mouse [181], and the same appears to be true of dystrophin mutants [182, 183]. The localization of AQP4 at the perivascular endfeet of glial cells is crucially dependent on the laminin-binding properties of DG, as seen by the fact that the channel is also mistargeted in Large<sup>myd</sup> mice, in which the laminin-binding activity of α-DG is ablated due to its defective O-glycosylation [180]. It has been demonstrated that the application of laminin to Müller glia and cortical astrocytes in culture, results in the formation of dense clusters of α-DG and AQP4 on the surface of these cells that are analogous to endfoot domains [41, 184]. Further, AQP4 clustering in astrocytes is significantly reduced in instances where DG expression has been disrupted via siRNA and we observed that laminin-induced clusters of AQP4 and the membrane domains with which these channels are associated exhibit significantly reduced lateral mobility, as measured using the fluorescence recovery after photobleaching assay [222]. A similar reduction of AQP4 mobility was observed in MDCK cells cultured on laminin as well [239]. Based on these data, it has been proposed that the interaction between the DGC and the ECM plays a key role in tethering AQP4 at the perivascular astrocyte endfoot.
However, the highly asymmetric concentration of AQP4 in the endfoot regions of astrocytes observed may be mediated by mechanisms beyond tethering alone. Indeed, in the aforementioned study, we established via cell-surface biotinylation that the diffusional retardation induced by laminin in MDCK cells is accompanied by an increased expression of AQP4 at the basolateral plasma membrane [239], implying that laminin-dystroglycan interaction may regulate the expression levels of AQP4 at the plasma membrane by altering its internalization. A study published by Zhan et al. [240] showing that DG interacts with the GTPase dynamin-1 in the brain, the latter of which is known to be involved in the scission of newly-formed caveolae and clathrin-coated pits from the plasma membrane, supports this hypothesis. We therefore chose in the present study to investigate if DG might, in addition to its role as a scaffolding molecule, also serve to extend AQP4’s longevity at the cell surface by suppressing the dynamin-mediated endocytosis of this channel in astrocytes. Via a variety of approaches, we determined that the cell-surface expression of AQP4 is dependent on DG, and is potentiated by laminin. We demonstrated that laminin exerts its effect not by increasing the synthesis of AQP4, but rather by suppressing its rapid uptake at the plasma membrane that would otherwise take place. We observed that DG interacts with caveolin-1 and dynamin in astrocytes, and showed that the latter, functioning cooperatively with clathrin, is important in the regulation of AQP4 endocytosis. We also provide evidence indicating that dynamin’s interaction with DG may reduce the availability of the former, and serve to modulate its function. Finally, we saw that laminin selectively upregulates the M23, but not the M1 isoform of AQP4. Based on these lines of evidence, we propose that laminin, DG, and dynamin comprise the primary components of a complex involved in the regulation of clathrin-mediated AQP4 endocytosis, and that their interaction serves to promote the enrichment of M23-based orthogonal arrays of particles at astrocytic endfeet.

### 3.2 Materials and methods

#### 3.2.1 Antibodies

The following antibodies were used: rabbit polyclonal IgG raised against a GST-conjugated polypeptide corresponding to residues 249-323 of rat AQP4 (Alomone Laboratories, Jerusalem, IL), rabbit polyclonal IgG targeting residues 244-323 of human
AQP4 (Santa Cruz Biotechnology, California, USA), mouse anti-β-DG, 43DAG1/8D5, specific for 15 of the last 16 amino acids at the C-terminus of the human dystroglycan sequence (Novocastra Laboratories, Newcastle-upon-tyne, UK), mouse anti-hemagglutinin (HA) IgG (Abgent, California, USA), mouse anti-EEA-1 specific for residues 3-281 of the human protein (BD Biosciences, Ontario, Canada), mouse anti-human TfR (targeting residues 3-28 of the tail region of the protein; Zymed Laboratories Inc./Invitrogen, Ontario, Canada), and rabbit anti-caveolin1 made against the N-terminal region of the human protein (Santa Cruz Biotechnology, California, USA).

3.2.2 Plasmids and constructs

The RFP-tagged Rab-5a, -7, and -11a constructs used in this study were provided as a generous gift by Dr. James D. Johnson. The M1 and M23-AQP4 chimeras bearing GFP between valines 141 and 142 of the second extracellular loop of the channel protein were generated via overlap extension PCR using a protocol similar to that described by Tajima et al. [241], and cloned into the expression vector pcDNA3.1 (Invitrogen/Life Technologies, Ontario, Canada).

3.2.3 Cell culture

Primary astrocytes used in this study were cultured from the dissected cortices of 1-day-old Sprague-Dawley rat pups (Charles River) in accordance with protocols (#A06-0319) approved by the animal care committee of the University of British Columbia. Unless stated otherwise, all cell types were maintained and manipulated in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine (all Gibco, Ontario, CA). In the case of experiments investigating the effects of laminin on AQP4 uptake and distribution, cells were incubated with mouse Engelbreth-Holm-Swarm sarcoma laminin-111 (Sigma-Aldrich, Missouri, USA) at a concentration of 30 nM over a period of approximately 18 hours prior to assays. All transfections were performed with cells in suspension, using Lipofectamine 2000 (Invitrogen, Ontario, CA) as the means of delivery. Rat Dag1-specific and control non-targeting siRNA (ON-TARGETplus SMARTpool; Dharmaco, Illinois, USA) were applied at a final concentration of 100 nM, while 1 and 10 μg of GFP-AQP4-pcDNA3.1 and control plasmids were used for cells grown on 12 mm coverslips and 75 cm²
flasks respectively. Assays were performed 48-72 hours following transfection. Viral infections were performed on adherent cells, grown to 80% confluence. As with the transfection experiments, 48-72 hours were allowed to elapse before effects were assessed. Where indicated, cells were treated with 160 µM dynasore monohydrate and 25 µM chlorpromazine hydrochloride (Sigma-Aldrich, Missouri, USA) for a period of 90 and 30 minutes respectively prior to the beginning of any subsequent assays.

3.2.4 Cell surface and pulse-chase biotinylation

In cell-surface biotinylation experiments, primary astrocytes were first washed thrice with ice-cold DPBS, and were then treated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin dissolved in DPBS (Pierce Biotechnology, Illinois, USA) for 30 minutes at 4°C. After this, excess biotin was quenched with 50 mM NH₄Cl in DPBS, then the cells were washed extensively before being incubated for 30 minutes in extraction buffer (25 mM Tris pH 7.4, 25 mM glycine, 150 mM NaCl and 5 mM EDTA) containing 1% Triton X-100 and protease inhibitors (Roche, Quebec, CA). The biotinylated proteins were precipitated using streptavidin covalently attached to agarose beads (Pierce Biotechnology, Illinois, USA). Bound proteins were re-solubilized and denatured using reducing/loading buffer (50mM Tris pH 6.8, 2% SDS, 10% glycerol, and bromophenol blue), and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (see below). For pulse-chase biotinylation, cells were washed in PBS, and then treated with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Illinois, USA) in 125 mM NaCl, 2 mM CaCl₂ and 10 mM TEA for 30 minutes at 4°C, before being washed again and incubated at 37°C with culture medium for 15 and 30 minutes to allow the uptake of biotinylated cell-surface proteins to occur. Biotin bound to proteins not taken into the cell over that time period was cleaved via two successive 15-minute treatments with 50 mM reduced glutathione (Sigma-Aldrich, Missouri, USA) in a buffer consisting of 75 mM NaCl and 75 mM NaOH at 4°C. This was then followed by two more 15-minute incubations with 50 mM iodoacetamide (Bio-Rad, Ontario, CA), dissolved in ice-cold PBS containing 1% BSA. The subsequent cell lysis, protein precipitation, and denaturation steps were carried out using the methods already described.
3.2.5 Electrophoresis and immunoblotting

For SDS-PAGE, samples were first separated on 10% polyacrylamide gels containing SDS, and then electrotransferred to nitrocellulose membranes (Bio-Rad, Ontario, CA). The blots were first blocked with a solution of 5% non-fat milk dissolved in TBST (TBST-M 5%; 20 mM Tris and pH 7.5, 150 mM NaCl and 0.1% Tween-20), and then probed using antibodies against AQP4, β-DG, or HA (diluted 1/1000, 1/500 and 1/250, respectively, in TBST-M 5%) for 2 h. Membranes were washed thoroughly with TBST and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (diluted 1/2000; Pierce, Illinois, USA) for 1 h and after extensive washing, the signals were visualized via chemiluminescence using Immobilon Western HRP substrate (Millipore, Massachusetts, USA). Samples for one-dimensional blue-native electrophoresis were prepared in the manner described by Schägger and von Jagow [242], with sample separation performed on linear 6% polyacrylamide gels. Protein complexes were immobilized on PVDF membranes (Bio-Rad, Ontario, CA), which were then blocked with PBS containing 5% non-fat milk powder, before they were incubated with anti-AQP4 IgG (diluted 1/1000 in 1% milk in PBS). Secondary incubation was performed under similar conditions, and antibody detection was carried out using methods already described for SDS-PAGE.

3.2.6 Immunofluorescence

In our immunofluorescence studies, primary astrocytes grown on 12-mm glass coverslips coated with 0.1 mg/ml poly-D-lysine (Sigma-Aldrich, Missouri, USA) were washed three times with DPBS, before fixation with 2% PFA. Following three additional washes with PBS, they were blocked and permeabilized at room temperature (20–22°C) with a solution containing 2% BSA and 0.3% Triton X-100 in PBS. They were then incubated at room temperature for 1 h with primary antibodies against AQP4 and HA (both diluted 1/100 in blocking solution) and rinsed with PBS. Subsequently, they were incubated with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-rabbit IgG for 1 h (1/700; Molecular Probes, USA). The cells were then mounted on glass slides using Prolong Gold Antifade reagent (Invitrogen, Ontario, CA). To control for the non-specific binding of secondary antibodies, control experiments from which primary antibody labeling steps were
omitted were also performed in tandem. Labeling was visualized using an Olympus Fluoview 1000 confocal microscope.

3.2.7 Quantitative and statistical analysis

Densitometric analysis was performed to quantify relative protein amounts across sample sets, and normalizations were performed against the loading controls specified in the text. For the quantitation of colocalization, confocal stacks were collapsed into single images from which the Mander’s coefficients for the overlap of the relevant channels were then calculated via ImageJ. All statistical analyses were performed with a combination of Excel 2007 (Microsoft, Washington, USA) and Prism 4.0 (GraphPad Software, California, USA).

3.3 Results

3.3.1 Laminin increases aquaporin-4 expression at the plasma membrane in a dystroglycan-dependent manner

Our previous finding that exogenous laminin triggers a gain in the expression of AQP4 at the basolateral domain of epithelial MDCK cells when it is present in the culture substrate [239], prompted us to first investigate if laminin might also be capable of exerting a similar effect in astrocytes. Utilizing a biotinylation approach, we determined that primary astrocytes cultured in the presence of exogenous laminin, compared to untreated controls, did indeed exhibit an approximately threefold (2.97 ± 0.37) increase in AQP4 amounts at the plasma membrane (Fig. 3-1 A and C). Consistent with our previous study [239], laminin does not affect total expression levels of AQP4 expression (Fig. 3-1 B), but changes its localization within the cell. The observation that the channel becomes depleted from the intracellular pool (Fig. 3-1 A) strongly supports this conclusion.

Perhaps rather counter-intuitively, given the well-demonstrated spatial and functional association between DG and AQP4 in vivo and in vitro [184], and the former’s function as a laminin receptor [243], this DAP complex component fails to respond to exogenous laminin in the same manner as AQP4 or syntrophin (Fig. 3-1 A). Nonetheless, we decided to pose the question of whether this DAP complex component is necessary for the expression of AQP4 at the plasma membrane. Via biotinylation, we saw that the siRNA-mediated ablation of DG
expression led to a pronounced loss of AQP4 from the cell surface (Fig. 3-1 D). Taken together with the above data, this demonstrates that DG is central in the regulation of AQP4 localization. However, it would seem that the relationship between the two is not bound by direct proportionality.

3.3.2 Laminin causes AQP4 to be depleted from early endosomes

Based on the observation that laminin increases the amounts of AQP4 detected via biotinylation at the cell-surface without causing a concomitant elevation in whole-cell channel levels, we postulated that astrocytic AQP4 is sequestered in intracellular domains when laminin is not present. To investigate this possibility, we first performed an experiment to determine whether AQP4 is colocalized with markers of intracellular vesicles known to be important in mediating the transport of protein cargoes. Primary astrocytes cultured on glass coverslips were stained with a polyclonal antibody against AQP4, and a monoclonal antibody against the early endosome marker EEA-1, the recycling endosomes marker transferrin receptor TfR, and the late endosome marker LAMP-1. In addition, astrocytes expressing the RFP-tagged markers of early, late, and recycling endosomes, Rab-5a, Rab-7, and Rab-11 were also immunolabeled for AQP4 [244-248].

It was seen via confocal microscopy that, among these markers, only EEA-1 and, to a lesser extent, Rab-5a possessed any appreciable degree of colocalization with AQP4, thereby indicating that a portion of the intracellular pool of AQP4 is found within early endosomes. Laminin appeared to be an important factor in the regulation of this fraction of channels: whereas 21.89 ± 3.05 % of AQP4-linked fluorescence was detected in EEA-1-positive vesicles in control cells, this association was virtually halved in cells treated with laminin (Fig. 3-2 A and B), and a similar trend was also seen in the case of Rab-5a (Appendix H). These data suggest that the laminin-induced increase in AQP4 cell surface expression may be due in part to the depletion of AQP4 from an endocytic pathway.

3.3.3 Laminin inhibits AQP4 internalization

We next sought to investigate the mechanism underlying laminin-induced increase of AQP4 cell surface expression. Following our earlier observation that laminin may result in
the reapportioning of AQP4 from the intracellular sites to the cell surface, we hypothesized that laminin might stabilize AQP4 at the plasma membrane, via the reduction of its uptake into the cell. To investigate this hypothesis, we performed pulse-chase biotinylation experiments to assess the effects of laminin on the endocytosis of AQP4. We observed that, in untreated control astrocytes, AQP4 exhibits a high rate of endocytosis at 37°C, with virtually all channels that were initially biotinylated being taken into the cell by the end of the 30-minute assay period (Fig. 3-3 A and B). However, when astrocytes are treated with laminin AQP4 uptake virtually ceases suggesting that laminin suppresses AQP4 endocytosis.

3.3.4 Dystroglycan associates with the endocytic components caveolin1 and dynamins I and II

The effects of laminin on AQP4 endocytosis, together with the fact that DG depletion leads to the loss of channel expression at the cell surface, led us to next ask the question of whether and how DG might be involved in the regulation of AQP4 turnover. As previous studies have demonstrated that the endocytic components caveolin-3 and dynamin interact with DG in smooth muscle and in the brain, respectively [240, 249], we therefore chose to investigate if equivalent links existed in astrocytes. We determined that, via a caveolin-1-specific antibody, β-DG could be co-immunoprecipitated from lysates of astrocyte cultures (Fig. 3-4 A). This, however, was not seen when non-reactive serum was used instead (Fig. 3-4 A). Similarly, dynamin II and β-DG co-immunoprecipitate, whereas control antibodies specific for connexin-43 failed to immunoprecipitate dynamin II or β-DG (Fig. 3-4 B and C).

3.3.5 Dynamin and clathrin regulate AQP4 turnover

Given dynamin’s well-established role in mediating endocytic processes, its observed association with DG and the role of DG in the clustering of AQP4 at the plasma membrane of astrocytes, we chose to determine if dynamin also regulates AQP4 endocytosis in astrocytes. Astrocytes cultured on glass coverslips were treated with 160 μM dynasore monohydrate for 90 minutes and processed for immunofluorescence and confocal imaging. We found that that the level of AQP4-associated fluorescence was increased in dynasore-treated compared to control astrocytes (Fig. 3-5 A). Furthermore, laminin induces more clustering of AQP4 in dynasore-treated astrocytes compared to astrocytes treated with
laminin alone (Fig. 3-5 A). Cell surface biotinylation shows a significant increase of AQP4 cell surface expression in dynasore-treated cells (Fig. 3-5 B) corroborating the results seen by immunofluorescence (160 µM Dyn; Fig. 3-5 A). Taken together, these findings demonstrate that the GTPase activity of dynamin, which is central to its ability to sever nascent endocytic vesicles from the plasma membrane, is indeed crucial for the regulation of surface AQP4 levels. Using a pulse-chase approach, we then observed that the treatment of cells with dynasore monohydrate decreases the amount of AQP4 uptake to 9.63 ± 15.76 % at the 30-minute mark, as opposed to 92.55 ± 22.61 % for control cells.

To more directly test for dynamin’s involvement, we infected astrocytes with an adenovirus carrying a HA-tagged dominant-negative K44A mutant construct of dynamin II. Similarly to dynasore, we found that this GTPase-deficient dynamin halts AQP4 uptake, as reflected by the greater amounts of AQP4 at the cell surface (Fig. 3-5 D, E and F). Also, as verified by pulse chase biotinylation, this increased expression most probably stemmed from the inhibition of the uptake of AQP4, which fell from an average of 104.55 ± 8.35 % after 15 minutes for control cells to near-zero levels in dynamin K44A-expressing cells at the equivalent time point (-11.88 ± 16.47 %; Fig. 3-6 C and D). Taken together, these data strongly indicate that the regulation of surface AQP4 levels in astrocytes is dependent on the GTPase activity of dynamin.

We next investigated if clathrin was also involved in AQP4 endocytosis. To do this, cells were treated with 25 µM chlorpromazine hydrochloride, a pharmacological inhibitor of clathrin-mediated endocytosis [250], for a period of 30 minutes, and then labeled the astrocytes for AQP4 and clathrin. Compared to untreated controls, astrocytes treated with chlorpromazine express higher levels of AQP4 at the cell surface, as reflected by the intense fluorescence for AQP4 delineating the peripheral regions of individual cells (Fig. 3-7 A). Additionally, cell-surface AQP4 levels were assessed via biotinylation (Fig. 3-7 B and C). Here, chlorpromazine upregulates AQP4 cell-surface expression by approximately 2.92 times (± 0.75). These data suggest that AQP4 endocytosis occurs through a dynamin- and clathrin-dependent pathway.
Caveolin1, however, does not play a role in AQP4 uptake, despite having been identified earlier as an interacting partner of DG. Silencing caveolin1 expression in cultured astrocytes via the transfection of a specific siRNA construct did not alter the cell-surface expression of AQP4 or β-DG compared to control cells transfected with a control scrambled siRNA (Fig. 3-8 A and B). Furthermore, the interaction between caveolin1 and β-DG does not appear to be regulated by laminin, as evidenced by our observation that identical amounts of β-DG are co-immunoprecipitated with caveolin1 whether or not cells are cultured in the presence of laminin (Fig. 3-8 C and D).

3.3.6 GTP regulates dystroglycan association with the GTPase dynamin

While we found that dystroglycan interacts with components of the endocytic pathway, the exact nature of this interaction was as yet unclear. Thus, we performed a series of experiments designed to investigate factors important in regulating this interaction. Given laminin’s role in inhibiting AQP4 endocytosis, we tested whether laminin regulates the degree of association between dynamin II and β-DG. To do this, we probed β-DG immunoprecipitates from astrocytes treated with laminin and control untreated astrocytes for dynamin II and found no significant changes in the amount of dynamin II. This suggests that (Fig. 3-9 A and B) laminin’s effects on AQP4 turnover and localization are not mediated directly via the modulation of DG-dynamin binding.

However, the affinity of dynamin for DG does appear to be dependent on the state of dynamin. As assessed via immunoprecipitation assays, cells treated with the drug dynasore monohydrate, a specific inhibitor of the GTPase activity of dynamin [251], exhibited a lower degree of binding between dynamin and DG compared to control cells treated with DMSO only (Fig. 3-9 C and D). As treatment with the drug would likely lead to the eventual accumulation of GTP-bound dynamin within cells, this result strongly suggests that DG preferentially binds to GDP-bound or guanoside-depleted (i.e. ‘inactive’) forms of dynamin, suggesting that DG may inhibit the function of dynamin by sequestering it.
3.3.7 Laminin regulates the cell-surface expression of the M23 isoform of AQP4, but not that of the M1 isoform

As mentioned above, the relative abundance of the M1 and M23 isoforms of AQP4 is a critical determinant of OAP dimension. Because of the potential functional implications of this, particularly with respect to channel distribution and organization at the astrocytic-vascular interface, we decided in our next experiment to investigate if the ECM might be a factor that governs how each of these isoforms is expressed. To do so, we expressed M1 or M23 AQP4 bearing GFP tags in each of their second extracellular loops in two sets of CHO cells, which were then treated with laminin. Via cell-surface biotinylation, we then compared the cell-surface amounts of AQP4 in these cells with that of untreated controls also expressing these tagged AQP4 constructs. We found that, in the absence of laminin, M23-AQP4 is expressed at higher levels at the cell surface compared to the M1 isoform, despite the fact that M1 and M23 AQP4 are detected in equivalent amounts in the input fraction, thereby indicating that M23 possesses a greater tendency to be localized at the plasma membrane, which may in turn explain the observed predominance of that isoform at the surface of astrocytes (Fig. 3-10 A). Further, while M1 poses a near-negligible response to exogenous laminin, cells expressing M23-AQP4 exhibit a doubling of the amounts of this isoform at the plasma membrane (Fig. 3-10 A and B). These data show that laminin regulates cell surface expression of M23-AQP4 but not that of M1-AQP4.

3.4 Discussion

In a previous study, we determined that certain components of the extracellular matrix, via their interactions with cell-surface receptors, regulates the behavior of AQP4 in Madin-Darby canine kidney (MDCK) cells, impacting not only the levels at which the channel was expressed at the plasma membrane, but it’s diffusional stability as well [239]. However, MDCK cells spontaneously polarize in culture once confluent, adopting apical and basolateral domains that each contain distinct complements of proteins (AQP4, for instance is localized virtually exclusively to the latter), and for this reason they proved ill-suited as a model system for addressing questions regarding the means through which AQP4 localization is established ab initio within systems in which it is natively expressed.
In the present study, we have chosen to elucidate these mechanisms using primary rat cortical astrocyte cultures. In the mammalian brain, the localization of AQP4 is highly polarized: whereas it is highly concentrated to the perivascular “endfoot” regions of these cells that form where they contact perivascular basal lamina, which is rich in the extracellular matrix protein laminin, the distal parenchymal domains are largely devoid of this channel. Once isolated, and cultured, however, astrocytes will revert to an unpolarized state in the absence of exogenous factors, and AQP4 becomes expressed in a diffuse manner throughout the plasma membrane.

3.4.1 Laminin-DG interactions are crucial in maintaining the cell-surface expression of AQP4

Prior work conducted in our lab has previously demonstrated that polarity can be restored, after a fashion, via the addition of laminin-111. Cells thusly treated develop dense clusters on their surfaces comprised of AQP4, laminin, and several members of the DGC, including dystroglycan and syntrophin [184], arrayed in an arrangement that is analogous to that which is observed at perivascular sites within the brain. We have now extended our analysis by showing in this study that laminin not only contributes to AQP4 localization, but also the expression levels of this channel at the cell surface. We have further demonstrated that, with the silencing of DG expression, AQP4 ceased to be detected at the cell membrane in any appreciable quantity. It therefore appeared that DG not only regulates the localization of AQP4 within the astrocyte membrane, but it is absolutely required for the maintenance of channel amounts at the membrane as well. The above results, when extrapolated to the in vivo setting of the intact brain, would seem to indicate that the highly asymmetric distribution of AQP4 within at perivascular sites, once supposed to be the product solely of the tethering of AQP4 to the basal lamina via the DGC as an intermediary, might also involve the enrichment of these channels at the plasma membrane.

Based on our analysis, which showed that laminin treatment does not affect global AQP4 amounts, it would appear that the enrichment of AQP4 at the plasma membrane arises not through an increase in the de novo synthesis of channels, but rather from a reapportioning of existing channels within the cell. This hypothesis finds further support in our qualitative observation that the increase in the plasmalemmal AQP4 pool is concomitant with a
corresponding decrease in the amount of channels that remain in the cell. The results of our co-labeling experiments suggest that this fraction of AQP4 may be held largely within intracellular vesicles positive for the early-endosome markers EEA-1 and Rab-5a, the proportions of which appear to be subject to regulation via laminin. This prompted us to further investigate the question of whether laminin might impinge on AQP4 endocytosis. Via pulse-chase biotinylation, we saw that while surface AQP4 is aggressively taken up into untreated control cells, laminin severely suppresses channel endocytosis. The strikingly divergent fates of AQP4 that we observed may provide a possible explanation for the channel’s distribution in perivascular astrocytes as the decreased uptake and selective retention of channels would result in the eventual enrichment of AQP4 in that region of the membrane, even in the absence of events that might increase channel expression via other means, such as increased protein synthesis or the upregulation of export pathways.

3.4.2 DG is part of a complex involved in the regulation of AQP4 turnover

Given the central role that DG plays in the localization of AQP4, we therefore posed the question of whether it might be involved. Via immunoprecipitation assays, we determined that dystroglycan interacts with both caveolin-1 and a variety of dynamin isoforms, the latter of which was then demonstrated to be directly involved in the regulation of AQP4 endocytosis, and, consequently, its cell-surface expression. We subsequently found that the inhibition of the GTPase activity of the latter in astrocytes via the use of the pharmacological compound dynasore monohydrate effectively halved the amount of dynamin co-immunoprecipitated with DG, which in turn pointed to the fact that DG might preferentially associate with the “inactive” GDP-bound form of dynamin, or its guanosine-depleted form. The above result likely indicates that the laminin-dystroglycan interaction may, in part at least, derive the effects that it exerts on AQP4 turnover via the sequestration of a portion of the cell’s complement of dynamin, thereby moderating the rate of endocytosis. These results are in agreement with, and expands upon, that of a study published by Zhan et al. [240], in which it was seen that dystroglycan, when transfected into cells previously lacking it, dramatically reduces the rate at which surface transferring receptor is brought into the cell, from which it was then inferred that dystroglycan might be involved in the regulation of endocytosis.
The treatment of astrocytes with chlorpromazine hydrochloride, a cholestatic compound that inhibits clathrin-mediated endocytosis via the dislocation of AP-2 and their associated clathrin subunits from plasmalemmal sites to endosomes [250], was determined to have very similar effects to dynasore monohydrate, dramatically increasing AQP4 cell-surface expression. This was perhaps unsurprising given the well-characterized cooperative roles of dynamin and clathrin in clathrin-mediated endocytosis (CME; [252]), and the fact that AQP4 contains within its c-terminus a tyrosine-based “YxxΦ” motif that confers upon it a propensity to interact with the adaptor protein 2 complex, also known to be important in the same process [238].

3.4.3 The DG-mediated suppression of endocytosis is specific to the M23 isoform of AQP4

We also saw, via immunoprecipitation assays, that laminin in no way increases the degree to which DG binds to dynamin. This was greatly unexpected, especially in light of the earlier finding that laminin also does not increase the cell-surface amounts of DG, indicating that the action of laminin may be less direct than was first supposed. Our observation that laminin preferentially increases the cell-surface expression of the OAP-forming M23 isoform of the channel over that of M1 provides an alternate avenue through which laminin binding could mediate the accumulation of greater amounts of AQP4 at the cell surface. The robustness of this bias, in combination with our other prior observations and those of other groups, leads us to surmise that it must be the M23 isoform that exerts the greater influence over the organization and function of the perivascular endfoot with regards to the regulation of water permeability. We further believe that this also represents a central link in the chain of events regulating the enrichment of AQP4 at the perivascular endfeet of astrocytes, and it is this piece of data that forms the keystone for the following model, which we have also illustrated in figure 11. We propose that, at endfoot sites, units of DG recruited via their interactions with the laminin-rich perivascular basal lamina bind to dynamin, lowering the effective concentration of the latter to suppress endocytosis within a limited radius. This, in turn, allows for the gradual accumulation of several protein species, including AQP4. Individual units of M23, due to their capacity for self-association [81], would then act as the initial nuclei for the accretion of OAPs. This hypothetical model is substantiated by freeze-fracture studies conducted by others that have confirmed that the endfoot domains of
astrocytes are indeed enriched in AQP4-based OAPs [253]. AQP4 channels inserted into the parenchymal domain of astrocytes, conversely, remain for a short period of time only before they are shuttled into early endosomes through a dynamin- and clathrin-mediated pathway, as a means of maintaining AQP4 amounts at this domain at a comparatively modest level. We believe that it is through these mechanisms that the extreme asymmetry observed in the distribution of AQP4 is generated.

3.4.4 Summary

Previous studies on the involvement of the dystroglycan complex in mediating AQP4 distribution in astrocytes have thus far primarily cast their focus on its function in the localization of these channels to astrocytic endfeet [178-180, 184]. In the present study, we have provided evidence to demonstrate that dystroglycan, via its interactions with both the perivascular ECM and dynamin, also serves to facilitate the enrichment of the channel within these cellular domains. To the best of our knowledge, our effort represents the first description of a mechanism capable of creating and maintaining cellular polarity in this manner.
Figure 3-1. Dystroglycan and laminin are involved in the regulation of AQP4 cell-surface expression in astrocytes. Intracellular fractions (Intracellular) and biotinylated cell surface (Cell surface) fractions from control untreated astrocytes (-LN) and astrocytes treated with 24 nM laminin-1 (+LN) were immunoblotted for AQP4 and β-DG (A). Overall AQP4 proteins levels in untreated and laminin-treated astrocytes (B). Histogram summarizing the differences in protein expression levels of AQP4 at the cell surface of untreated and laminin-treated astrocytes, normalized against β-DG levels (C). Values represent relative normalized mean pixel intensities ±SEM from three different experiments. The asterisk indicates a statistically significant increase of AQP4 expression compared to untreated astrocytes, as determined by a two-tailed Student’s t-test (*p=0.033). Cell-surface proteins isolated via biotinylation assay from siCTL and siDag1-transfected primary hippocampal astrocyte cultures were immunoblotted for AQP4, β-DG, and with streptavidin-HRP to visualize all biotinylated proteins (D).
Figure 3-2. Laminin decreases the amount of AQP4 present in EEA-1-positive intracellular vesicles. Untreated control cells (-LN) and laminin-treated astrocytes (+LN) were double immunolabeled with antibodies specific for AQP4 and EEA-1. Images were acquired across successive focal planes via confocal microscopy and then summed to produce Z-stack images (A). The degree of colocalization between AQP4 and EEA-1-containing endocytic vesicles were quantified from collapsed stacks (B).
Figure 3-3. Laminin inhibits AQP4 endocytosis in astrocytes. Control untreated (-LN) and laminin-treated astrocytes (+LN) were subjected to pulse-chase biotinylation, and fractions isolated at the time-points indicated were separated via SDS-PAGE and immunoblotted for AQP4 and β-DG (A). Histogram showing the effect of laminin on the rates of AQP4 endocytosis (B). Values represent the average change, in percent, in the protein levels of internalized AQP4 at each time-point over that seen at 0' from three different experiments. Symbols indicate statistically significant differences, as determined by a two-tailed Student’s t-test (*p=0.017; †p=0.026).
Figure 3-4. Dystroglycan associates with caveolin 1 and dynamin in astrocytes.
Caveolin 1-containing immunoprecipitates were separated via SDS-PAGE and immunoblotted for β-DG and Cav-1 (IP; A). Non-reactive rabbit IgG (NRIgG) is used to control for non-specific binding. β-DG (top; B) and dynamin-containing immunoprecipitates (C) were immunoblotted for dynamin II and β-DG. Connexin 43 immunoprecipitates immunoblotted for dynamin was used as a negative control against the possible non-specific interaction between the intracellular domains of transmembrane proteins and dynamin (bottom; B). Whole cell protein extracts (Input) and fractions of proteins remaining in the supernatant after the immunoprecipitation (Sup) were also immunoblotted for the indicated proteins.
Figure 3-5. The inhibition of dynamin leads to the accumulation of AQP4 at the cell surface. Primary astrocyte cultures were either treated with DMSO, treated with dynasore monohydrate (Dyn), laminin (+LN), or both, before being double immunolabeled for AQP4 and β-DG (A). Cell-surface biotinylation was performed to assess the impact of dynamin inhibition on AQP4 and β-DG expression at the plasma membrane. Whole cell lysates (Input) and unbound proteins (Intracellular) were also immunoblotted for these proteins. A representative immunoblot of AQP4 and β-DG cell surface expression in dynasore and DMSO-treated astrocytes (B). The histogram represents the mean pixel intensity ±SEM of AQP4 cell surface expression normalized to that of β-DG from three different experiments (C). The asterisk indicates a statistically significant difference compared to DMSO-treated astrocytes, as determined by a two-tailed Student’s t-test (* p=0.0456). Primary astrocyte cultures infected with adenovirus carrying a construct for HA-tagged dominant-negative dynamin (DynK44A) and mock-infected (Mock) control cells were immunolabeled for AQP4 and HA (D). The effects of DynK44A on the total expression of AQP4 and β-DG (Input) as well as the amounts of these proteins at the plasma membrane (Cell surface) and in intracellular sites inaccessible to biotin (Intracellular) were also assessed via cell-surface biotinylation (E). Histogram summarizing the average difference in cell-surface AQP4 expression (following normalization against β-DG), ±SEM, for three separate experiments (F). Asterisk indicates a statistically significant deviation from mock-infected controls, as determined by two-tailed Student’s t-test (*p=0.0472).
AQP4 Cell-surface Expression (Normalized to DG)

A

DMSO

160 mM Dyn

+LN

160 mM Dyn +LN

D

HA/AQP4

-LN

+LN

Mock

DynK44A

B

AQP4

β-DG

Input

Cell surface

Intracellular

E

Mock

DynK44A

Mock

DynK44A

Mock

DynK44A

C

F

AQP4 Cell-surface Expression (Normalized to DG)
Figure 3-6. The endocytosis of AQP4 is regulated by dynamin. DMSO- and Dynasore-treated astrocytes (Dyn) were subjected to pulse-chase biotinylation, and fractions isolated at the time-points indicated were separated via SDS-PAGE and immunoblotted for AQP4 (A). Histogram showing the effect of dynasore on the rates of AQP4 endocytosis (B). Values represent the average percent increases in the levels of internalized AQP4 at each time-point over that seen at 0’ from five separate experiments. Symbols indicate statistically significant differences, as determined by a two-tailed Student’s t-test (*p=0.0150; †p=0.0470). Primary astrocytes infected with adenoviruses carrying a construct expressing dominant-negative dynamin (DynK44A) and control cells (Mock) were subjected to pulse-chase biotinylation assays, and the amount of AQP4 internalized at each of the indicated time points was determined via SDS-PAGE (C). Histogram summarizing the differences seen in AQP4 internalization for each treatment over four such experiments (D), where values shown represent the average increase (in percentage points) over that for the equivalent 0’ control. Symbols indicate statistically significant differences between pairs of values, as determined via two-tailed Student’s t-tests (*p=0.0214; †p=0.0006; #p=0.0003).
Figure 3-7. **Clathrin regulates AQP4 expression at the cell surface.** Untreated control cells (UT) and cells treated with 25 μM chlorpromazine (25 μM CPZ) were double-immunolabeled with antibodies against AQP4 and clathrin heavy chain, and imaged using confocal microscopy (A). Control cells and cells treated with the concentrations of chlorpromazine indicated were subjected to cell-surface biotinylation, following which the cell-surface fraction was analysed using SDS-PAGE, and probed for AQP4 and β-DG (B). Histogram illustrating changes seen in the cell-surface expression of AQP4, as normalized against that of β-DG in the same cells. Symbol indicates that differences observed were statistically significant, as assessed via the Student’s *t*-test (*p=0.0069).
Figure 3-8. Caveolin 1 is not involved in AQP4 turnover. Cell surface proteins were isolated via biotinylation from cells treated with siRNA corresponding to caveolin 1 (siCav) and control siRNA (siCTL), and analysed via SDS-PAGE (A). Histogram (B) shows the relative amounts of AQP4 expressed at the plasma membrane in both groups, normalized against β-DG expression. Caveolin 1-containing immunoprecipitates were isolated from control (-LN) and laminin-treated (+LN) cells, analysed via SDS-PAGE, and the input, immunoprecipitate (IP) and supernatant (Sup.) fractions were probed for caveolin 1 and dystroglycan (C). Histogram (D) compares the amounts of β-DG co-immunoprecipitated with caveolin 1 for each group.
Figure 3-9. The DG-Dynamin II interaction is modulated by dynamin II activity, but not by laminin binding. β-DG-containing immunoprecipitates prepared from astrocytes treated of 160 μM dynasore monohydrate (Dyn), an inhibitor of the GTPase activity of dynamin, or DMSO (used here as a control) were analyzed by SDS-PAGE and then immunoblotted for the dynamin II and β-DG (A). Similar immunoprecipitation experiments were performed on laminin-treated and untreated control astrocytes (C). Whole cell protein extracts (Input) and fractions of proteins left in the supernatant after the immunoprecipitation (Sup) were also immunoblotted for the dynamin II and β-DG (A and C). The histograms represent the mean pixel intensities ±SEM of the dynamin II signal normalized to that of β-DG from five different experiments (B and D). The asterisk indicates a statistically significant difference compared to DMSO-treated astrocytes, as determined by a two-tailed Student’s t-test (B; *p=0.0119).
**Figure 3-10. Laminin affects the cell-surface expression of only the M23 isoform of AQP4.** Laminin-treated (+LN) and untreated control CHO cells expressing GFP-tagged M1 and M23 isoforms of AQP4 were subjected to biotinylation analysis, and the input and biotinylated (Cell surface) fractions were isolated and probed for the proteins indicated (A). Histogram summarizes the results obtained over 4 such experiments (B). Symbols indicate statistically-significant differences between groups, as determined via the Students t-test (*p=0.0408).

**A**

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**B**

Histogram showing AQP4 expression at the plasma membrane (normalized to DG) for M1, M1+LN, M23, and M23+LN treatments. The histogram bars are labeled with error bars indicating statistical significance (*p=0.0408).
Figure 3-11. A schematic detailing the involvement of laminin-DG interactions in mediating the asymmetric distribution of AQP4 in astrocytes. AQP4 amounts in the parenchymal domain of perivascular astrocytes are maintained at relatively low levels (1.) via rapid channel turnover. AQP4 is recruited via its clathrin adaptor protein interaction sites to nascent clathrin-coated pits (2.), which are then ligated from the plasma membrane via the action of dynamin (3.). The contents of the resultant endocytic vesicle (4.) are either shuttled into degradative pathways, or are recycled back to the cell surface to begin the process anew. At the endfoot domain, the laminin (5.) the perivascular basal lamina, largely comprised of laminin, serves to retain the extracellular matrix receptor dystroglycan (6.), which in turn binds to, and sequesters dynamin in its inactive states (7.), which leads to a suppression of AQP4-M23 uptake. AQP4-M23 are immobilized via a combination of their interactions with the dystroglycan complex and their propensity to form OAPs via homomerization (8.), thereby resulting in the maintenance of a comparatively large pool of AQP4 channels at the astrocyte endfoot.
CHAPTER 4 - CONCLUSION

This thesis primarily focuses on the principles that underlie the generation of polarized channel distribution in two osmoregulatory systems – the kidney and the blood-brain barrier. In chapter 2, we described a study explicating the involvement of interactions between the extracellular matrix and cell-surface receptors in the localization of the water channel aquaporin-4 (AQP4) and the inwardly-rectifying potassium channel Kir4.1 to the basolateral membrane domain of renal tubular epithelial cells, and chapter 3 was a characterization of how laminin-dystroglycan interactions mediate the exclusive expression of AQP4 at the perivascular endfoot structures of astrocytes. In the following section we will summarize these studies and discuss them within a broader context with the aim of comparing how they relate to other studies that have previously been conducted in the related fields and synthesizing their salient findings where possible. As well, we will briefly discuss additional data that fall outside the scope of these studies, and speculate on potential routes of investigation for the future.

4.1 Chapter 2

4.1.1 The polarized expression of AQP4 and Kir4.1 within renal tubular epithelia and kidney function

Kir4.1 and AQP4 are expressed in the basolateral membrane domains of tubular epithelial cells of the distal convoluted tubule and collecting duct, respectively. The former functions in concert with the sodium chloride co-transporter NCC and Na⁺/K⁺-ATPase to prevent excessive salt loss, and the latter, together with AQP2, serves to scavenge water from the post-glomerular filtrate. Important as these functions are, the factors important in the localization of Kir4.1 and AQP4 within the kidney have remained, by and large, neglected as a subject of investigation. Chapter 2 of this thesis describes a study in which we investigated the importance of the interactions between laminin and fibronectin and the cell-surface receptors of the integrin class and dystroglycan in determining the expression of these channels at the basolateral domain of renal epithelial cells in culture.
4.1.2 ECM-receptor interactions in the regulation of the cell-surface expression of Kir4.1 and AQP4 in renal epithelial cells

We performed colabeling studies to assess the patterns of distribution of Kir4.1 and AQP4 channels with respect to that of dystroglycan, a high-affinity laminin receptor, and observed that the channels colocalized significantly with the latter, which was highly suggestive of the possibility that the latter might be involved in the regulation of the polarized distribution of these channels. Indeed, the importance of DG in mediating cell-polarity has been well-demonstrated in numerous studies. For instance, Deng et al. [187] observed that Drosophila mosaic mutants lacking DG expression in follicle and disc epithelia exhibit extensive apicalization in these cells, resulting from the aberrant overexpression of markers associated with this membrane domain. As well, DG appeared to be important in the regulation of oocyte antero-posterior polarity. In the same study, a requirement for DG in establishing the proper organization of the basal cytoskeleton was also seen, and it was speculated that this may reflect a role for DG in extracellular matrix reorganization.

Using Madin-Darby canine kidney cells as a model system for renal tubular epithelia, we determined via a combination of immunofluorescence and cell-surface biotinylation approaches that cells cultured on substrates containing fibronectin and laminin expressed Kir4.1 and AQP4 in greater quantities within their basolateral domains. While these data may initially appear to only reinforce the importance of ECM-receptor interactions in establishing overall cell polarity, and imply that the increases in channel expression may only be a secondary effect of the cultures attaining a more polarized state under the influence of exogenous extracellular matrix proteins, our coincident observation that the increase in channel expression far exceeded that of the polarity marker E-cadherin argues that the effects of laminin are in fact quite specific.

4.1.3 The role of fibronectin and laminin in the basolateral stabilization of Kir4.1 and AQP4

It had been demonstrated in several prior studies that the localization of Kir4.1 and AQP4 in perivascular astroglia is greatly dependent on the DG complex, as evinced by the fact that the loss of α-syntrophin and dystrophin both result in the redistribution of AQP4
from the endfoot domain of these cells. As well, the Large$^{\text{myd}}$ mutation, which disrupts the O-glycosylation of the mucin-like domain of DG, has similar effects on Kir4.1 and AQP4 [180-182]. These data have led some to posit that the DG complex essentially acts as a bridge linking the channels to the relatively immobile extracellular matrix via its capacity to interact with both, thereby effecting the stabilization of the former at the membrane. While elegant in its simplicity, direct evidence supporting this proposed mechanism has thus far been scant.

Therefore, we sought to ascertain the validity of this model in this study. Via fluorescence recovery after photobleaching (FRAP), we saw that the macroscopic diffusional rates of Kir4.1 and AQP4 were indeed significantly decreased when cells were cultured in the presence of fibronectin and laminin. Additionally, this effect appeared to result not from a generalized immobilization of the entire population of membrane proteins, but is rather more selective, as demonstrated by our observation that Kir2.1, which does not interact with the DG complex despite possessing the motifs that would allow it to do so, is affected by fibronectin only, and not laminin.

4.1.4 The stabilization of AQP4 via motifs that allow its interactions with the clathrin complex

While the above data may initially appear to substantiate the “molecular tether” model of DG function, our subsequent analysis of several mutant forms of AQP4 using FRAP revealed that this receptor may not be necessary for the stabilization of this channel. We were, for instance, unable to detect any deviation in the fluorescence recovery profiles of MDCK cells expressing mutant AQP4 bearing a deletion eliminating the C-terminal PDZ-binding motif, which is necessary to allow its interaction with the DG complex through syntrophin [254], compared to those of cells expressing the full-length construct, regardless of whether the cells were cultured on laminin-coated surfaces. This largely ran counter to our expectations, especially as the same motif is a crucial determinant of Kir4.1 clustering in Müller glia following laminin treatment [41]. However, as others have also observed that the deletion of this SSV sequence has little bearing on the expression of AQP4 at the basolateral membrane domain of MDCK cells, our result is probably a reflection of the fact that its requirement is variable from system to system, and that AQP4 anchorage is governed
through other means in this cell type [238]. To determine what this mechanism might be, we performed similar analyses of other mutant forms of the channel. Our examination of the mutant in which residues 272-281 had been deleted produced the most striking results. While the diffusional rate of this mutant was comparable to that of wild-type AQP4 in cells grown on untreated glass surfaces, its mobility was much less restricted by the addition of exogenous laminin.

Given the lack of evidence suggesting that the residues deleted in this mutant were at all important in mediating AQP4’s interactions with the DG complex, these data highlighted the possibility that the effect that laminin has on AQP4 expression and localization in MDCK cells might be transmitted via routes less direct than is posited in the accepted model. There is, however, some consistency between our findings and those of a study published by Madrid et al. [238], in which it was demonstrated that the YxxΦ motif contained within this sequence is involved in governing AQP4’s interaction with the μ2A subunit of clathrin adaptor protein complex 2 (AP2). Could associations with clathrin stabilize AQP4? Evidence suggests that this could indeed be the case. E-selectin, a receptor expressed in endothelial cells under inflammatory conditions associates with α-adaptin, a member of the clathrin complex, and is recruited to clathrin-coated pits as a result, where it is seen to form clusters [255]. It is therefore not unreasonable to assume that a similar mechanism may serve to assemble AQP4 in MDCK cells into more ordered states at the membrane, and that the reduced diffusional rates seen for the channels may be a consequence of this organization.

It was also seen in the same study mentioned above that cytokine activated human umbilical vein endothelial cells expressed a certain portion of E-selectin receptors within lipid rafts, and that the disruption of these via methyl-β-cyclodextrin treatment interfered with the interactions between these receptors and their corresponding ligands, resulting in decreased leucocyte rolling [255]. In a previous study, our lab showed using primary astrocyte cultures that AQP4, too, was localized to detergent-resistant domains, and that the laminin-induced clustering of this channel also depended upon the latter being intact [222]. Our data indicate that it is possible that AQP4 localization in MDCK cells may likewise require the cooperation of lipid rafts and clathrin, and it would be quite feasible to perform
assays verifying the importance of either owing to the ready availability of tools that allow for the detection, visualization, and disruption of the activities of these components. A study could, for instance, investigate the colocalization of AQP4 with lipid raft markers such as transferrin receptor, ganglioside marker 1 (GM1), or flotillin, and with clathrin heavy chain in MDCK cells cultured on substrates of various compositions. siRNA or pharmacological compounds (such as statins that inhibit cholesterol synthesis in the case of lipid rafts and chlorpromazine in the case of the clathrin complex, for example) could then be used in conjunction with a FRAP-based approach to determine the importance of these factors in the stabilization of AQP4.

4.1.5 The involvement of receptors other than DG in Kir4.1 localization

Despite sharing certain common features with AQP4, early indications pointed to the possibility that the localization of Kir4.1 may in fact be determined by pathways that are quite different. The ablation of DG synthesis via siRNA transfection, while having no discernible effect on AQP4 expression, eliminated Kir4.1 from the lateral membranes of MDCK cells. Obtaining direct verification of DG’s involvement in the stabilization of Kir4.1 via a FRAP-based approach proved to be quite challenging, however, due to the relative lack of Kir4.1-associated fluorescence in cells affected by the siRNA construct. Determining if the PDZ-binding domain of Kir4.1 is involved in its localization may therefore be a viable substitute, given the motif’s role in facilitating the channel’s interaction with the DG complex via syntrophin in Müller astrocytes [41], and in the regulation of channel expression [10]. Doing so could be particularly useful in elucidating the different ways in which the expression of these channels is governed by providing an explanation for why DG is required in the case of one but not the other.

4.1.6 Synergy between integrin receptors and dystroglycan in the regulation of Kir4.1 and AQP4 expression

It is important to note that DG is not an absolute requirement, as the loss of Kir4.1 that we observed following the siRNA-mediated knockdown of DG expression may be quite effectively compensated for via the addition of exogenous laminin to the culture substrate. This result, together with the fact that fibronectin, for which DG possesses no verifiable
affinity, also causes an upregulation of the expression and stabilization of both Kir4.1 and AQP4, suggests that an alternative ECM receptor must exist.

Indeed, RGD-containing peptides, which competitively inhibit fibronectin binding to certain integrins, prevent the fibronectin-induced increase in Kir4.1 and AQP4 cell surface expression and reverse the fibronectin-induced reduction in both channels’ diffusion rates. In a similar vein, the αvβ3 integrin function blocking antibody alters the reduction of AQP4 diffusion rates induced by fibronectin, suggesting that αvβ3 integrin plays a role in the stabilization of AQP4 at the basolateral domain of epithelial cells. It was, however, also observed that the effects resulting from the disruption of fibronectin-receptor binding extend not just to cells grown on substrates enriched for fibronectin, but also those cultured on laminin-coated surfaces. These particular results are especially interesting, for they reveal that the effects of laminin are, on some level, transmitted via integrins specific for fibronectin only, and that the latter can in some respects act as a substitute for the former under certain conditions.

The above raises a crucial question: how is it that a receptor should have a role in mediating the effects of an ECM molecule to which it does not bind? We believe that the resolution of this apparent contradiction may lie in the fact that there exists a certain level of crosstalk between the ECM, as well as their respective receptors, which occurs across a number of levels, mediated by cellular responses that provide yet further feedback. Support for this hypothesis is provided by our analysis of how ECM composition is influenced by the initial substrate. In this experiment, MDCK cells were grown on semi-permeable polycarbonate cell culture inserts coated with fibronectin and laminin over a period of 72 hours, before being harvested and subjected to analysis by western blotting to assess the relative levels of either of these molecules within the ECM. We saw that cells cultured in the presence of exogenous laminin exhibited greatly increased levels of fibronectin compared to those cultured on uncoated surfaces (compare lanes 1 and 3 of Appendix A, part A). It therefore appears that, when provided with an appropriate substrate, MCDK cells possess the capacity to alter their ECM in rather significant ways.
When viewed in light of this result, our previous finding that exogenous laminin is able to offset the loss of DG with regards to the enrichment of Kir4.1 at the basolateral membrane domain appears less paradoxical. If it is assumed that DG acts in this scenario to organize laminin (Fig. 4.1 A), as it has been shown to do in systems as diverse as astrocytes and mammary epithelial cells [175, 188], then it follows that its loss would also perturb the structure of the ECM at large, thereby resulting in the disruption of integrin-fibronectin interactions and consequently, the proper localization of Kir4.1 (Fig. 4.1 B). However, culture surfaces coated with laminin (which self associates when at sufficiently high concentrations) allow cells to circumvent the loss of DG by providing an already organized substrate to which fibronectin can attach, to the benefit of Kir4.1 expression (Fig. 4.1 C).

While some aspects of this proposed mechanism await direct experimental verification, it is nonetheless clear that Kir4.1 localization requires some degree of cooperation between fibronectin and laminin, and the dystroglycan and integrin complexes.

4.1.7 The different mechanisms regulating Kir4.1 and AQP4 expression and localization in MDCK cells - summary and suggestions for future investigation

This study has highlighted the fact that, despite the superficial similarities between them, such as their coexpression in the perivascular domain of astroglia [218], and their apparently coupled functions and association with the components of the dystroglycan complex in that system [180], the distribution of Kir4.1 and AQP4 in renal epithelia is regulated via pathways that are in certain respects quite distinct. Whereas Kir4.1 requires the participation of both DG and integrins (Fig. 4.2 A), AQP4 localization appears to be solely based on the latter, and likely involves clathrin function, as discussed earlier (Fig. 4.2 B).

Several central questions persist, however. For instance, the nature of DG’s role in the regulation of Kir4.1 expression is currently not exactly known. Does it accomplish this via direct physical interactions with Kir4.1, as in the classic model of DGC function, or does it rely on other pathways? A more precise dissection of the DGC in MDCK cells, either via siRNA-mediated knockdown, or deletion mutants, should be helpful in answering this question. It has also been assumed thus far that the effects of laminin arise solely from its ability to polymerize. The use of the E4laminin fragment in high molar excess to prevent
laminin self-assembly would allow for this issue to be resolved, and would be particularly useful in allowing for dystroglycan’s role in the organization of the ECM and in modulating Kir4.1 expression (see previous section) to be more clearly defined. Synthetic peptides corresponding to domains of laminin important in regulating polymerization would also serve a similar purpose. By the same token, the E3 laminin fragment could also be used in a similar fashion to block DG-laminin interactions to either eliminate or confirm the possibility of the involvement of secondary laminin receptors. In the case of AQP4, which does not require DG but nonetheless responds to exogenous laminin, blocking antibodies could be used to identify the alternate receptor.

Additionally, it is as yet unclear in both cases how integrin-class receptors are able to mediate channel localization, given the lack of data indicating that they possess the ability to interact with cellular components capable of binding to these channels, unlike DG. It is possible that their action is indirect, and is effected via the cytoskeleton. Integrins serve a prominent role in the regulation of cell adhesion and migration, activities that are dependent on the reorganization of cortical actin networks (reviewed in [256]). Given that dystrophin is similarly associated with the cytoskeleton, it could act as an appropriate intermediary, tethering Kir4.1 via syntrophin. Indeed, evidence suggesting that these components of the dystroglycan complex can function independently of dystroglycan already exists, in the form of a study performed by Cote et al. [257], in which it was determined that the cellular distributions of both these components remained unaffected in muscles of myodystrophic mice. As well, it was seen in the same study that integrin expression was upregulated, strongly hinting at the involvement of this receptor type. Further, an association has been identified between Dp71, vinculin, and integrins, based on sedimentation data [258]. Studies designed to elucidate if such a crossover exists could take several forms, one of which would be the assessment of the importance components that are known to function downstream of integrins to organize the cytoskeleton, such as vinculin or talin, or GTPases such as Rho or Rac [259]. The availability of dominant negative mutants and specific pharmacological inhibitors of these should doubtlessly facilitate such an undertaking.

Finally, while this study has revealed much about the regulation of Kir4.1 and AQP4 localization in renal systems, it remains to be seen how our results might be applied to
understanding kidney function and development. One of the foremost considerations that will have to be addressed in future studies is whether the differences seen between these two channel types in this study play a role in dictating their distinct localizations within the nephron in vivo. This question will need to be addressed on two fronts. Firstly, it will have to be determined if channel expression is dependent on the composition of the extracellular milieu in which renal tubules are embedded, and secondly, it will also be important to assess how this is influenced by the various cell-surface receptors types found in renal epithelia. Doing so would necessitate a thorough characterization of the contribution of ECM-receptor interactions across various regions of the developing kidney as it pertains to Kir4.1 and AQP4 distribution, perhaps via immunohistochemical methods. A similar approach could also be applied to the study of mutant animals lacking certain integrin or laminin subunits in order to ascertain the individual value of these components.

4.2 Chapter 3

4.2.1 The importance of the localization of AQP4 to the endfoot regions of astrocytes in the regulation of proper osmolyte and fluid exchange at the blood-brain barrier

AQP4 is highly-expressed by perivascular astrocytes of the mammalian brain, and represents the sole conduit for water across the blood-brain barrier. Functioning in concert with the potassium channel Kir4.1, this channel serves to facilitate neural conduction by allowing for the rapid shuttling of the excess potassium ion generated as a by-product of repeated action potential cycles from the extracellular fluid spaces of the brain into the blood. As well, several studies have ascribed to AQP4 a role in the development and resolution of brain edema, a condition that can arise as a secondary complication in instances of traumatic injury and disease, and that is quite often the leading contributor to morbidity and mortality in these cases. Previous studies have identified interactions between the dystroglycan complex and the perivascular ECM as being an important factor in AQP4 localization, resulting in the development of a “bridge” model for DGC function (as discussed in the introduction and the preceding sections in this chapter). However, such a tethering mechanism alone may be insufficient to account for the high degree of asymmetry seen in terms of the distribution of AQP4. Chapter 3 of this thesis describes a study in which we investigate if this interaction might have additional effects in the regulation of AQP4.
expression at the cell surface. Via a combination of experimental approaches, it was determined that laminin and DG are also critical determinants of AQP4 uptake, and that they might be important in enriching AQP4 expression at the perivascular endfoot by suppressing clathrin-mediated endocytosis in a domain-specific manner.

### 4.2.2 The role of integrins in AQP4 localization in astrocytes in culture

In this study, it was seen that the treatment of primary astrocyte cultures with laminin resulted in the increase of the amounts of AQP4 found at the cell surface, and that the silencing of DG expression effectively abrogates this upregulation. This observation stands in stark contrast to our previous findings, in which it was observed that not only was DG unnecessary for AQP4 localization in MDCK cells, it was rather integrin αvβ3 that was important in mediating the responses of this channel to exogenous laminin. What could account for such a striking difference?

There indeed appears to be much overlap in terms of the ECM receptors that are expressed in both systems. For instance, integrins α3β1, α6β1 and α6β4, all of which exhibit an affinity towards laminin, have been identified in both MDCK cells and in regions proximal to the cerebral microvasculature [203, 260-262], as has, of course, DG. However, previous work performed in our laboratory has ascertained that β1 integrins, unlike DG, or components of its attendant complex, do not co-cluster with AQP4 under the influence of laminin but instead remains diffusely distributed, and that α6β4 is not expressed at all in astrocytes in culture. The additional finding that exogenous fibronectin, added in any amount, has no discernable effects on AQP4 (our unpublished data), further reinforces the notion that DG is, by and large, the primary effector of AQP4 organization, at least in this case.

It is important to note that the above does not preclude the involvement of integrins in vivo. In fact, their particular localization within the intact brain is highly suggestive that they may well be involved in the establishment of astrocytic morphology, if not in determining AQP4 localization directly. It would therefore be interesting to investigate how these functions might be disrupted in knockout animals deficient for the aforementioned
receptors. Further, it is possible that the apparent divergence between the two systems may originate not just from differences in the receptors that they possess, but from differences in the composition of their associated complexes, which may also be variable as a result of other factors, as exemplified by dystrophin, the expression and localization of which changes during cellular differentiation and brain development [263] [264]. It might therefore be productive if these could be characterized with an eye towards identifying their involvement in AQP4 localization in the developing fetal brain. Doing so might yield important information regarding how channel polarity is established in astrocytes, and may reveal a greater degree of commonality between regulatory mechanisms in different systems.

4.2.3 The ECM plays a role in modulating endocytic rates

Via a pulse-chase biotinylation approach, we established that the addition of laminin leads to a drastic decrease in the rate at which AQP4 is taken into the cell via clathrin-mediated endocytosis. This is largely in agreement with those of Batchelder and Yarar [265], who recently determined that clathrin-coated pits (CCP) proximal to sites rich in cell-matrix adhesions exhibited slower dynamics compared to more distal ones. Despite their superficial similarities, it is the differences between these two studies, in terms of the methodology employed and their respective findings, that may be more valuable in providing insight into the mechanism that underlies this phenomenon, and in illuminating possible avenues for future research.

In their study, Batchelder and Yarar saw a significant degree of colocalization between ligand-bound β1 integrin and clathrin-coated pits in BSC1 cells cultured on fibronectin-coated surfaces. The effects that they observed therefore appeared to be mediated primarily via this particular ECM-receptor combination. By contrast, our results imply that AQP4 uptake is regulated chiefly via a mechanism involving laminin and DG. The above may indicate that there exists a general link between cell adhesion and the suppression of endocytosis, a probability that would seem to find support in Batchelder and Yarar’s observation that plant lectins, which bind to glycosylated proteins at the cell-surface in a non-specific manner, also promotes the extension of CCP lifetimes. It is important to
note, however, as was previously discussed, that fibronectin and integrins appear to play a negligible role in the expression or localization of AQP4 in our system. It may be possible, though, that this may stem from the inability of astrocytes to properly organize fibronectin in an in vitro setting when the latter is supplied in isolation in a fashion that permits the manifestation of its effects, perhaps owing to the lack of the appropriate receptors. In such a case, the use of patterned culture substrates or fibronectin-coated beads might allow for the circumvention of this shortfall, and should be considered in subsequent studies.

Batchelder and Yarar propose two candidate mechanisms to explain how cell-matrix interactions could inhibit clathrin-mediated endocytosis. In the first, the cell-surface receptor acts as an intermediary, tethering components of the clathrin-coated pit to the substrate, physically restricting its uptake. The second mechanism involves signalling events triggered through the ligation of ECM components by receptors that result in changes to the functions of endocytic components and/or to cytoskeletal organization. These hypotheses are easily testable using RGD-containing peptides in the case of fibronectin, and the E3 fragment of laminin, as these would bind to and stimulate their respective receptors, but would not facilitate adhesion, at least not to the extent of their full-length counterparts. Our data provides concrete evidence that a third possibility exists, in the form of the dystroglycan-mediated suppression of dynamin function. Regardless of the specific details of how it occurs, however, it now seems clear that the ECM is important in the regulation of endocytosis.

4.2.4 A possible role for clathrin in mediating AQP4 localization in astrocytes

Another salient point emerging from the Batchelder study was the fact that matrix-receptor interactions resulted not in the complete elimination of CCPs, but rather in the increase of the time periods over which they dwell at the cell surface. Indeed, our finding that DG inhibits dynamin function, rather than that of components of the clathrin complex, implies that this mode of regulation acts at the level of vesicle scission, rather than pit formation. Considering the coincident distribution patterns of AQP4 and DG [184], and the relatively short ranges at which endocytic suppression appears to operate ([265]; see above description), it is therefore likely that AQP4-AP2 interactions may also play a role in AQP4
stabilization in astrocytes, as it appears to in MDCK cells (see discussion in section 4.1.4 of this chapter; Appendix G). One possible approach that could be used to determine if this might be the case would be to examine the diffusional stability of the AQP4 273-282 deletion mutant that we used previously, modified with an internal GFP tag, via immunofluorescence and FRAP. Comparing the results obtained for this mutant to the one lacking the terminal SSV sequence should be especially enlightening, as it would reveal the relative contributions of the DG and clathrin complexes in the anchoring of AQP4.

4.2.5 The endocytic itinerary of AQP4

While we have shown that AQP4 is internalized via EEA-1- and Rab-5a-positive endocytic vesicles that probably correspond to early endosomes, its subsequent fate is unclear, due to the absence of any discernable colocalization between the channel and other trafficking markers using an immunofluorescence approach. It is possible that this is due to the fact that this step of the endocytic process is the rate limiting step, and that the channel protein is shuttled through the remainder of its itinerary with such rapidity that it is given little opportunity to accumulate in the latter steps, thereby precluding its detection in the associated vesicles. What happens to AQP4 following its initial uptake nonetheless remains an interesting and relevant question worthy of further investigation.

Two major outcomes exist regarding the path taken by AQP4, and these may be non-mutually exclusive. First, AQP4 taken into early endosomes may, after a certain delay, be recycled back to the plasma membrane. Alternatively, it could be targeted to the degradative pathway, in which case it is likely that lysosomes would be involved. To address these possibilities, dominant negative mutants of either Rab-11 or Rab-7, the wild-type versions of which are known to regulate protein transport to these systems [247, 248] could be transfected into astrocytes, which should cause the eventual accumulation of AQP4 in these respective vesicular bodies.

4.2.6 Implications and possible practical applications

We have demonstrated in this study that laminin-DG interactions are involved in the site-specific suppression of endocytosis, which is required for the persistence of AQP4 at the
cell-surface in astrocytes, and provides a possible mechanism explaining the asymmetric distribution of AQP4 at perivascular endfeet structures at the blood-brain barrier. Our results reveal a previously unknown aspect of dystroglycan complex function, and represent a significant step forward in our understanding of how astrocyte polarity is established and maintained at the blood-brain barrier. Additionally, in shedding light on the mechanisms involved in the regulation of AQP4 cell-surface expression in astrocytes, these results may provide inroads to the development of novel pharmacological strategies for the treatment of brain edema.

As previously discussed, AQP4 is centrally involved in the development and resolution of brain edema, but the requirement and role for this channel is variable depending on the exact phase of the disorder being considered: while AQP4 appears to be detrimental in the case of cytotoxic edema, the resolution of vasogenic edema appears to benefit from it. Thus far, studies focusing on the modulation of AQP4 activity by compounds have mostly turned up candidates that are unlikely to be effective in a clinical setting, such as zinc [266], or that appear to operate via as yet unidentified mechanisms, as with edaravone, a free-radical scavenger [267]. By comparison, our study has resulted in the elucidation of a regulatory pathway that should be highly amenable to exploitation, and a potential means by which this might be accomplished, in the form of chlorpromazine, a compound that has been proven to be effective in the treatment of other brain disorders. Additionally, the involvement of the extracellular matrix provides another potential route of intervention. It is known that metalloproteinases are often activated as part of the response to brain ischemia, and that these are involved in the degradation of laminin. While it has been determined that the inhibition of these enzymes can have a protective effect on the neurons bordering the infarct area by preventing them from undergoing apoptosis [268], our findings show that doing so could also be effective in preserving AQP4 distribution in astrocytes, which could be especially useful in instances of vasogenic edema. Finally, given that the cell-surface expression of AQP4 may be readily assessed for via surface immunolabeling, it is possible that this could form the basis of high-throughput screens designed to identify novel compounds capable of up- or downregulating channel endocytosis.
Figure 4-1. A model describing a possible mechanism through which exogenous laminin compensates for the loss of DG expression in the localization of Kir4.1. In an untreated cell (A), endogenous laminin is organized by dystroglycan, which in turn binds to and presents fibronectin to fibronectin-binding integrin receptors. Through this, the localization of Kir4.1 channels at the basolateral domain is established and maintained. However, when the expression of DG is silenced (B), the ECM becomes disrupted, and Kir4.1 is lost from the plasma membrane. This phenotype is rescued when laminin is present in sufficient amounts (C), at which point it can either spontaneously self-assemble, or be organized via interactions with lower-affinity receptors, such as β1-integrins. Together with endogenously-expressed fibronectin, it can then again form an appropriate substrate for receptor binding, and provide the cues required to restore Kir4.1 polarity.
Figure 4-2. Possible mechanisms involved in the localization of Kir4.1 and AQP4.
The localization of Kir4.1 (A) is dependent on both DG and fibronectin-binding integrins. Through the talin/vinculin/Arp2/3 complex, the latter organizes the actin cytoskeleton, and regulates Kir4.1, perhaps through the recruitment of the DG complex (left). While our data indicates that DG is not absolutely required, and that its absence may be compensated for when sufficiently high amounts of laminin are present, this does not necessarily eliminate the role of this receptor in the regulation of Kir4.1 expression under physiological conditions. It is also quite possible that integrins could regulate the localization of Kir4.1 through dystrophin and syntrophin, as there is some evidence suggesting that these components can be localized to the proper cellular domains independently of DG. In the case of AQP4 localization (B), it is integrin αvβ3 that acts as the primary receptor for the ECM. This perhaps serves to recruit AQP4 into clathrin-coated pits located within detergent-resistant membrane domains. The actin cytoskeleton, given its function in the regulation of clathrin-coated pit morphology, could also be involved.
REFERENCES


APPENDIX A

Figure A.1. Polycarbonate cell culture inserts effectively retain ECM molecules. MDCK cells were grown to confluence over a period of 72 hours on permeable polycarbonate cell culture inserts (pore size 0.4 μm) previously coated with either fibronectin (50.0 μg/ml FN) or laminin-1 (96.0 μg/ml LN) for 18 hours. Lysates prepared from the adherent cells were immunoblotted for fibronectin (A) and laminin-1 (B) to assess the efficacy of the coating regiments used in the present study. Note that exogenous laminin-1 (96.0 μg/ml LN) results in the accumulation of endogenous fibronectin (A).
Figure B.1. Laminin increases cell surface expression of Kir4.1 at the basolateral domains of several MDCK clones expressing GFP-Kir4.1. Densitometric analysis of cell surface expression of β-DG (A) and Kir4.1 (B) in control GFP-Kir4.1 MDCK clones (D4, B5 and G8) grown in the absence of laminin (-LN) and GFP-Kir4.1 clones grown on 96 mg/ml laminin-1 (+LN) for 72 hours.
Figure C.1. Fibronectin and laminin do not change total expression levels of Kir4.1 and AQP4. Total protein extracts from GFP-Kir4.1 (A) and GFP-AQP4 cells (B) grown on either 50.0 μg/ml fibronectin (50.0 FN) or 96.0 μg/ml laminin (96.0 LN) for 72 hours or in the absence of exogenous fibronectin or laminin (UT: untreated) were immunoblotted for Kir4.1 (A) AQP4 (B) as well as for E-cadherin and actin (A and B). Note that total expression levels of either Kir4.1 or AQP4 remain unchanged in the presence of fibronectin and laminin when compared to control untreated cells. Both E-cadherin and actin were used as loading controls.
Figure D.1. The intracellular pool of Kir4.1 is associated primarily with the Golgi apparatus. Merged images of confluent MDCK cells expressing either GFP-AQP4 (A-D) or GFP-Kir4.1 (E-H) and immunolabeled for calnexin (A and E), GM130 (B and F), the transferrin receptor (TfR; C and G) and LAMP-2 (D and H). Scale bar, 5 μm. Note that in addition to its association with the plasma membrane, Kir4.1 is also associated with the Golgi complex.
Figure E.1. Dystroglycan is involved in maintaining polarized epithelial morphology. Whole cell extracts from control (siCTL) and Dag1 siRNA-transfected (siDag1) GFP-Kir4.1 and GFP-AQP4 expressing cells were immunoblotted for β-DG and histograms of the mean pixel intensity ±SEM of four experiments determined by densitometric analysis (A). The asterisks represent statistically significant decreases of β-DG expression compared with control cells as determined by the two-tailed Student’s t-test (* p<0.05; ***p<0.001). Confluent MDCK cells expressing either GFP-Kir4.1 or GFP-AQP4 were transfected either with control siRNA (siCTL) or Dag1 siRNA (siDag1) or left untransfected (UT) and the short axis, long axis and aspect ratio were measured from three experiments where three random fields containing each an average of 10 cells were chosen. The asterisks represent statistically significant changes as determined by the two-tailed Student’s t-test (* p<0.05, *** p<0.0001).

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<tr>
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<th>AQP4</th>
<th>Kir4.1</th>
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<tr>
<td>Short Axis (mm)</td>
<td>UT 18.6±0.6</td>
<td>siCTL 20.2±1.1</td>
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<tr>
<td>Long Axis (mm)</td>
<td>UT 25.6±0.7</td>
<td>siCTL 24.9±0.6</td>
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<tr>
<td>Aspect Ratio</td>
<td>UT 1.4±0.0</td>
<td>siCTL 1.3±0.1</td>
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APPENDIX F

Figure F.1. The $\alpha_v\beta_3$ antibody blocks the adhesion of Kir4.1 and AQP4-expressing cells to fibronectin. Untransfected MDCK cells (A), and the GFP-Kir- and GFP-AQP4-expressing cells (B and C) were grown to confluence, and immunolabeled for $\alpha_v\beta_3$ integrin. Note that untransfected MDCK cells as well as GFP-Kir4.1 and GFP-AQP4 cells express $\alpha_v\beta_3$ integrin at the basolateral membrane domain. GFP-Kir4.1- or GFP-AQP4-expressing MDCK cells were pre-incubated with 10 $\mu$g/ml of $\alpha_v\beta_3$ integrin antibody for 30 min at room temperature, and then plated onto fibronectin-coated coverslips. After 1 hour, non-adherent cells were removed via vigorous agitation. The adherent cells were labeled with DAPI, and imaged via confocal microscopy. Histograms represent the mean cell count ±SEM per field for control cells (FN) and cells treated with the $\alpha_v\beta_3$ integrin antibody (FN+Ab). Ten fields per condition for each cell type were analyzed. ($**p<0.0001$; ††$p=0.0002$).
Figure G.1. AQP4 stabilization is not dependent on the PDZ domain, but rather requires a tyrosine-based motif. MDCK cells expressing a GFP-tagged mutant construct of AQP4 lacking the final three amino acids of the wild-type sequence (321-323DEL) were plated on uncoated glass surfaces (UT), or on substrates containing 96 µg/ml laminin-1 (LN), and then subjected to FRAP analysis (A). Under either condition, the recovery profiles of the mutant closely matched that of the WT channel, represented here by blue and red dashed lines (the SEMs for these have been omitted for clarity). An AQP4 mutant in which residues 272-281 have been deleted (272-281DEL), when subjected to a similar experiment, exhibits greater lateral mobility when the cells were cultured on laminin compared to the WT channel, demonstrating the importance of the tyrosine-based motif contained within this region of the protein in mediating the stabilizing effects of laminin.
Figure H.1. The association of AQP4 with Rab5-positive endocytic vesicles is regulated by laminin. Untreated control cells (-LN) and laminin-treated astrocytes (+LN) expressing RFP-tagged Rab5 were immunolabeled for AQP4, and imaged via confocal microscopy across several focal planes, which were then combined (A). The association between AQP4 and vesicles containing fluorescent Rab5 were quantified from these stacked images (B).
Figure I. Treatment with glutathione effectively eliminates surface-bound biotin. As a demonstration of glutathione’s efficacy in removing biotin bound to cell-surface proteins, a control experiment was conducted in which two pulse-chase biotinylation assays were performed concurrently on untreated primary astrocyte cultures. The first (the results of which are shown on the left) was performed using conditions identical to those employed for similar experiments in this study, whereas in the second (shown right), the glutathione and subsequent iodoacetamide treatment steps were omitted.
Figure J.1. AQP4 aggregation is regulated by dystroglycan and laminin. Cell lysates prepared from astrocytes treated with control siRNA, Dag1 siRNA, a combination of control siRNA and laminin, or Dag1 siRNA and laminin were separated by BN-PAGE (A) and SDS-PAGE (B), and then analysed via Western blotting to assess the involvement of dystroglycan and laminin in the regulation of AQP4 aggregate size.