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The Distinction of the Interactions Between the Transmembrane Domains of Basigin Gene Products and Monocarboxylate Transporters

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THE DISTINCTION OF THE INTERACTIONS BETWEEN THE TRANSMEMBRANE DOMAINS OF BASIGIN GENE PRODUCTS AND MONOCARBOXYLATE TRANSPORTERS

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Abstract

Although it was once thought that neurons solely rely on glucose as a substrate for cellular energy production, it is now known that small monocarboxylate molecules, like pyruvate, lactate, and ketone bodies, are also utilized. Monocarboxylates are transported across plasma membranes via facilitated diffusion using a family of transport proteins known as monocarboxylate transporters (MCTs). Four MCTs (MCT1, MCT2, MCT3, and MCT4) are expressed within neural tissues. Expression of the MCTs has been tied to co-expression of a cell adhesion molecule belonging to the Basigin subset of the immunoglobulin superfamily (IgSF). Basigin gene products are known to interact with MCT1 and MCT4 in the mammalian neural retina and this association is essential to support the cellular energy needs of photoreceptors. A previous study indicated that Basigin gene products use hydrophobic amino acids within specific regions of the transmembrane domain to interact with MCT1. In the present study, it is hypothesized that the same amino acids within the transmembrane domain are used to interact with MCT4, but that no association exists with MCT2, which typically interacts with a different member of the IgSF subset. Therefore, the purpose of the present study was to assess the association between Basigin gene products and MCT4, and with MCT2. Recombinant proteins corresponding to the transmembrane domain of Basigin gene products were used in in vitro binding assays with endogenous MCT2 and MCT4 from mouse brain protein lysates. Contrary to the hypothesis, it was determined that the transmembrane domain of Basigin gene products binds to both MCT2 and MCT4 *in vitro*. Different amino acids within the transmembrane domain of Basigin gene products are used for each association and the pattern is different from that used in the association with MCT1. The data suggest that Basigin plays multiple roles in the nervous system.

Chapter 1
Introduction

Cell adhesion is an essential process for the maintenance and development of tissues, synaptogenesis, and embryonic development. Connections between cells, mediated by cell-surface proteins, underlie all these processes, and set up the three-dimensional structure of tissues (Gumbiner, 1996). The proteins involved in cell adhesion can be classified into one of four classes of cell adhesion molecules (Figure 1.1), which include the immunoglobulin superfamily (IgSF), cadherins, selectins, and integrins (Alberts et al., 2015). Studies of the expression, structure, and function of cell adhesion molecules are powerful ways to understand the molecular composition of a tissue, and hence its function.

Immunoglobulin Super Family

The IgSF is the largest group of four types of cell adhesion molecules (Beesley et al., 2014; Alberts et al., 2015). Members of the IgSF are typically characterized by a large amino-terminal extracellular domain containing one or more Ig folds, a single transmembrane domain, and a cytoplasmic tail (Aplin et al., 1998). These Ig folds are similar in structure to those found in immunoglobulins, or antibodies, and therefore provide the commonality for this group of proteins (Alberts et al., 2015). IgSF cell adhesion molecules are involved in many processes and found on many cell types, including the development of the nervous system, in which they are

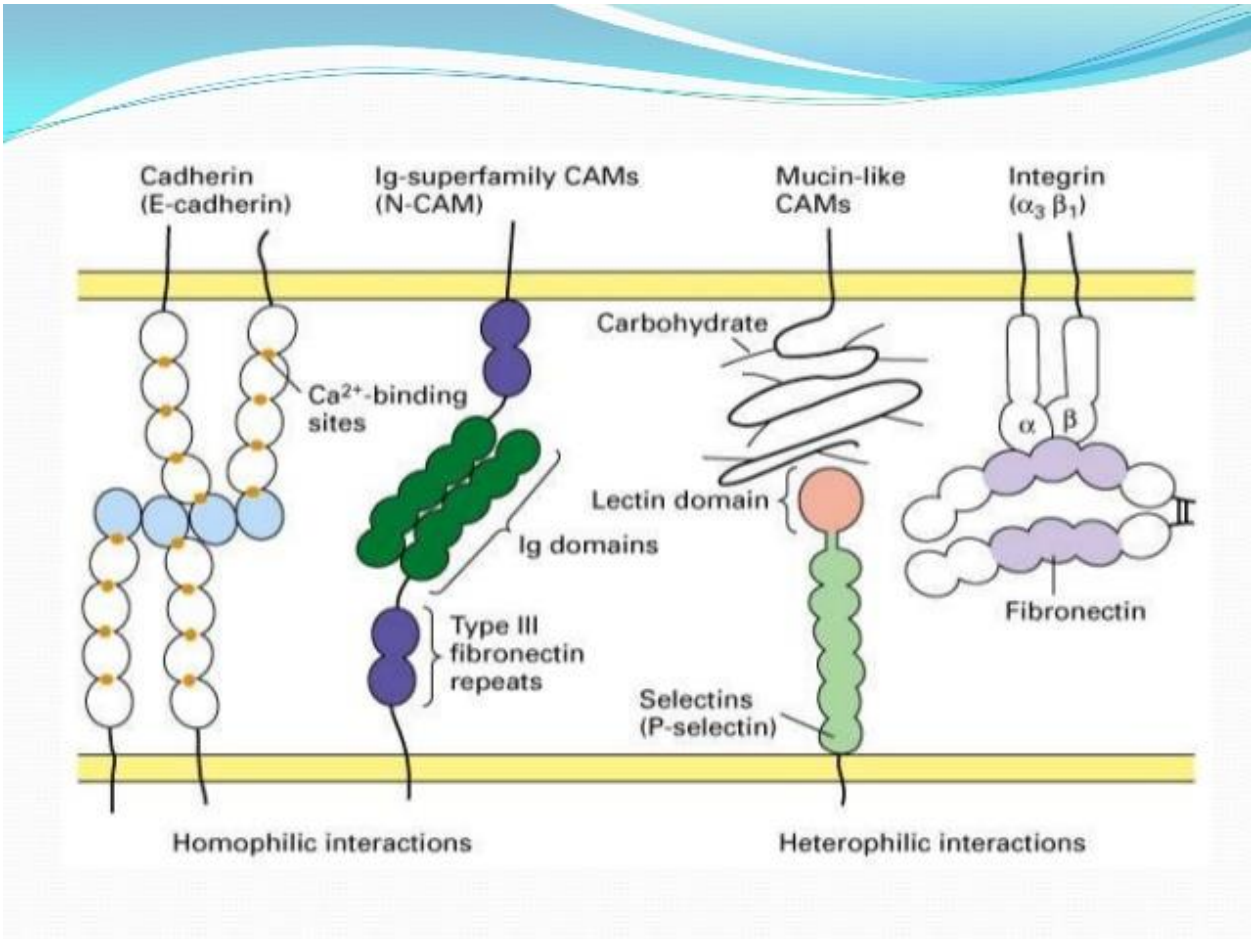

Figure 1.1. The four classes of cell adhesion molecules. These include the immunoglobulin (Ig) superfamily, cadherins, selectins, and integrins. (Alberts et al., 2000).

used in the establishment and maintenance of neuronal connections and axonal guidance (Murase and Schuman, 1999).

Cadherins

Cadherins are a family of transmembrane proteins that possess an extracellular domain with a series of repeats of 100-amino acid cadherin-specific modules (Juliano, 2002). The cadherin family of cell adhesion molecules can be divided into three major subfamilies. The first subfamily is composed of primarily calcium-dependent homotypic cell adhesion molecules referred to as "classic" cadherins. This subset of cadherins specializes in the formation of adherence junctions with actin filaments (Angst et al., 2001). The second subfamily is composed of desmosome-associated cell adhesion molecules. These cadherins form intracellular linkages with intermediate filaments, rather than actin filaments (Hynes, 1999). The final subfamily of cadherins are the proto-cadherins. These are important for the development of the nervous system (Angst et al, 2001).

Selectins

The selectin family of cell adhesion molecules consist of a small family of lectin-like adhesion receptors (Lasky, 1995). Lectins are carbohydrate-binding proteins (Aplin et al., 1998). Selectins possess an amino-terminal domain homologous to calcium-dependent animal lectins, an epidermal growth factor domain, two to nine complement regulatory protein repeats, a transmembrane domain, and a short cytoplasmic tail (Juliano, 2002). Selectins regulate heterotypic cell interactions via calcium-dependent recognition of sialylated glycans, which

plays a role in leukocyte adherence to endothelial cells and platelets during the inflammation process (Springer, 1995).

Integrins

Integrins are cell-surface glycoproteins that serve as receptors for extracellular matrix proteins and connect to the cytoskeleton, hence "integrating" the outside of the cell with the inside of the cell (Juliano, 2002). Integrins exist as heterodimers consisting of two subunits, known as α and β. The α and β subunits each contain an extracellular domain, a membrane spanning region, and cytoplasmic domain (Hynes, 1999; Aplin et al., 1998). Signals originating outside the cell are responsible for cytoskeletal organization, cell motility, and signal transduction, whereas signals originating from inside the cell are responsible for regulation of integrin affinity (Juliano, 2002).

Basigin subset of the IgSF

Within the IgSF exists a subset of proteins that includes two Basigin gene products, two Neuroplastin gene products, and Embigin. These five proteins are classified as a subset of the IgSF based on the similarities of these proteins at the amino acid level, and therefore their structures (Beesley et al., 2014). As members of the IgSF, they share a similar structure, with extracellular Ig-like domains, a transmembrane domain, and a cytoplasmic domain (Figure 1.2; Beesley et al., 2014). A defining feature of this subset of the IgSF is the presence of a glutamate residue within the transmembrane domain (Beesley et al., 2014). The function of this polar amino acid within a hydrophobic domain of the protein has yet to be fully determined. The gene

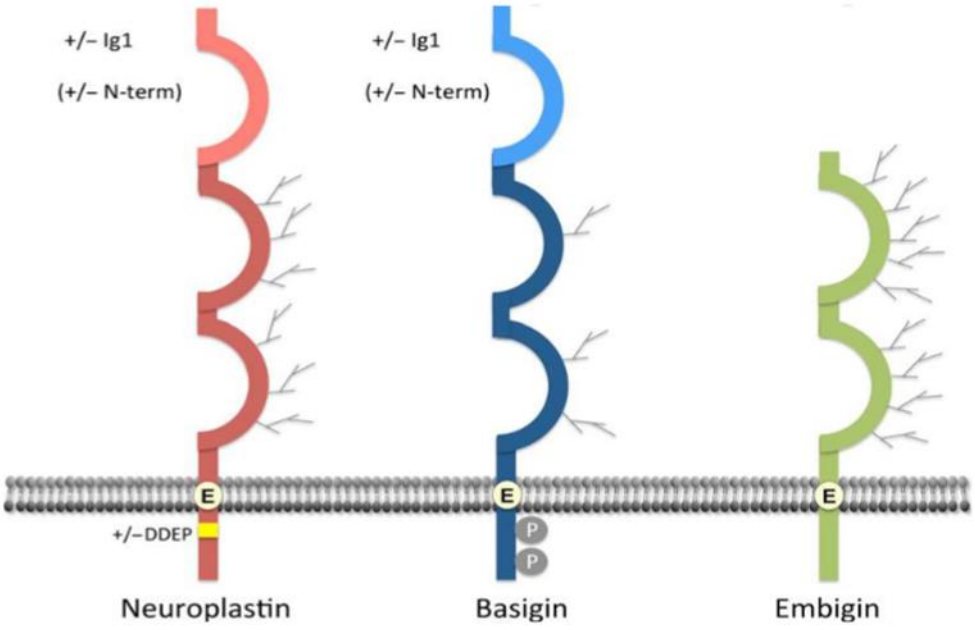

Figure 1.2. Comparison of the structures of Neuroplastin, Basigin, and Embigin. Each protein family possesses extracellular Ig domains, a transmembrane domain, and a cytoplasmic domain. All members of this subset of the IgSF possess a glutamate (E) within the transmembrane domain. There are two Basigin and Neuroplastin gene products. The longer product is depicted by the additional lighter colored domain at the region most distal from the membrane. (Beesley et al., 2014)

for Basigin and the gene for Neuroplastin each produce two main protein products that differ in overall size. The shorter forms of Basigin (Basigin variant-2) and Neuroplastin (Neuroplastin gp55) are ubiquitously expressed and each have two extracellular Ig domains (Beesley et al., 2014). The longer forms of Basigin (Basigin variant-1) and Neuroplastin (Neuroplastin gp65) are expressed in the retina and the brain, respectively, and have three extracellular Ig domains each (Figure 1.2; Beesley et al., 2014). The Embigin gene is unique to this subset, in that it codes for a single polypeptide that has two extracellular Ig domains (Figure 1.2; Beesley et al., 2014). Embigin is known as the "embryonic Ig," based on early studies that indicated that expression is highest during embryonic development (Fan et al., 1998). More recently, it has been determined to be expressed during tissue regression of rat prostate and in mammary glands following hormonal ablation (Guenette et al., 1997), as well as in adult rodent muscle (Lain et al., 2009).

As cell adhesion molecules, members of the Basigin subset of the IgSF play many roles in many different tissues. Basigin variant-2 is known to be involved in glial cell maturation, oocyte maturation, thymic development, and HIV-1 infection (Ding et al., 2002; Renno et al., 2002; Pushkarsky et al., 2001). Conversely, Basigin variant-1 is expressed only in the neural retina and is thought to play a role in regulation of glucose metabolism (Ochrietor et al., 2003; Ait-Ali et al., 2015). The Neuroplastin gene products are specific to the brain and nervous system and are important for mediating neurite outgrowth and plasticity, especially activity-dependent synaptic plasticity (Beesley et al., 2014). Whereas Neuroplastin gp55 is expressed throughout the mammalian brain, Neuroplastin gp65 expression is predominantly found in the hippocampus, cortex, and striatum, with lower concentrations in the brainstem (Hill et al., 1988; Mlinac et al., 2012.; Smalla et al., 2000; Marzban et al., 2003). Embigin regulates cell growth and differentiation during embryonic development (Guenette et al., 1997). Embigin mRNA

levels are elevated in embryonic carcinoma cells, which supports the hypothesis that it is involved in tumorigenesis and cancer development as well (Huang et al., 1990). The common feature of the Basigin subset of the IgSF is their ability to associate with, and direct the expression of, monocarboxylate transporters (Beesley et al., 2014).

Monocarboxylate Transporters

Monocarboxylate transporters (MCTs) are members of the MCT solute carrier family of proteins. All family members possess a similar characteristic structure, consisting of twelve transmembrane helices with intracellular carboxy- and amino-termini, as well as a large cytosolic loop between transmembrane domains six and seven (Figure 1.3; Halestrap and Price, 1999). There are nine different MCT isoforms, but only four of these (MCT1, MCT2, MCT3 and MCT4) are known to transport pyruvate, lactate, and ketone bodies (monocarboxylates) via facilitated diffusion, in a proton-dependent manner (Halestrap and Price, 1999). These isoforms are known to interact with members of the Basigin subset of the IgSF and will therefore be the focus of this discussion. However, because MCT3 is retina-specific (Philp et al., 2001), for this discussion the focus will be on MCT1, MCT2, and MCT4. A comparison of the amino acid sequences of MCT1, MCT2, and MCT4 is shown in Figure 1.4. The area designated by the box shows transmembrane domain 3, which is thought to be the region within MCT1 that interacts with Basigin (Manoharan et al., 2006).

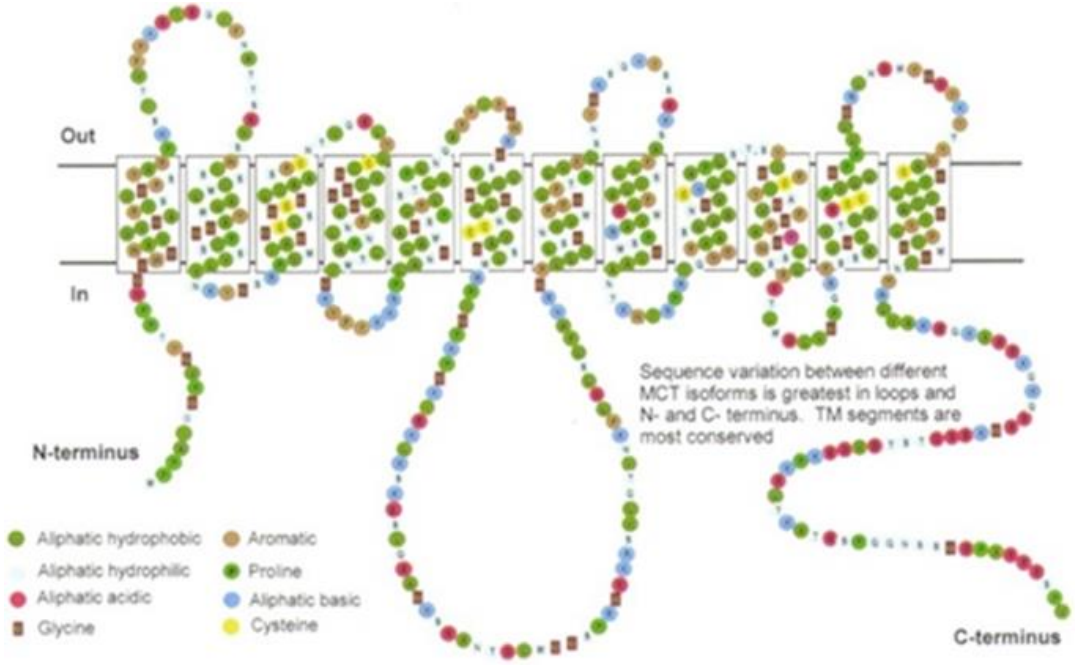

Figure 1.3. Structure of monocarboxylate transporters. The overall structure is depicted using circles that represent each amino acid in the protein. The circles are color-coded to indicate the class of amino acid present at each position. MCTs possess ten to twelve transmembrane domains, with a large cytoplasmic domain between transmembrane domains six and seven. Both the amino- and carboxy-termini are positioned within the cell. (Halestrap and Price, 1999)

Figure 1.4. Amino acid sequence comparison of MCT1, MCT2, and MCT4. The one-letter amino acid code is shown for each transporter. Positions that share identical amino acids are noted with an asterisk (*), whereas positions that have conserved substitutions are noted with a colon (:) or a period (.), depending on the strength of the conservation. The boxed sequence shows transmembrane domain 3, the region of MCT1 thought to interact with Basigin.

The MCT family members are expressed throughout the body. MCT1 is expressed in many tissues and is thought to be responsible for basal monocarboxylate transport across epithelial membranes (Halestrap, 2012). Within the brain, MCT1 and MCT2 expression has been localized to the neocortex, hippocampus, cerebellum, and striatum (Pellerin et al., 1998). In areas where MCT1 and MCT2 are both expressed, they are differentially localized, which may be due to unique functional roles for each transporter (Halestrap, 2012). Expression of MCT2 is largely found in the postsynaptic density of neurons and may therefore facilitate the uptake of monocarboxylates for those cells (Halestrap, 2012). Conversely, expression of MCT1 is largely found in the heart, brain, and retina (Bergersen, 2007; Halestrap and Price, 1999). MCT1 is particularly expressed in the endothelial cells of capillaries in the brain (Bergersen, 2007). MCT4 is widely expressed in glycolytic tissues, such as white skeletal muscle fibers, astrocytes, white blood cells, chondrocytes, and some mammalian cell lines. It is thought that MCT4 may be involved in the export of lactic acid derived from glycolysis (Halestrap, 2012). While highly glycolytic cells are shown to predominantly express and utilize MCT4, cells that have a net influx of lactic acid express and utilize predominantly MCT1 (Kirk et al., 2000). MCT3 is unique to this group in that its expression is restricted to the retinal pigmented epithelium (RPE) of the eye (Philp et al., 2001). Excess lactate from the neural retina is moved into the RPE via apically-expressed MCT1 and out of the RPE toward the choroid via basally-expressed MCT3 (Philp et al., 2001).

The mechanism by which MCTs transport monocarboxylates has been studied in detail using inhibitors, transport kinetics, and site-directed mutagenesis (Halestrap, 2012). The MCT1 transporter has been the prototype molecule used for many of these studies. The mechanism MCT1 utilizes is one in which a substrate binding site, open to one side of the membrane, binds a

proton and lactate anion, causing a conformational change in the protein which brings the two to the opposite surface of the membrane (Halestrap, 2012). The MCT1 transporter specifically uses a lysine residue in the hydrophobic pocket of the substrate-binding channel, that when coupled with the binding of a proton, allows for the binding of the monocarboxylate anion (Halestrap, 2012).

While glucose is considered the main energy source for the brain, lactate and ketone bodies can be utilized by neurons within the brain (Koehler-Stec et al., 1998). It has been proposed that neurons obtain these metabolites to fuel oxidative phosphorylation using an astrocyte-neuron coupling mechanism (Figure 1.5; Magistretti, 2006). It is thought that glucose is taken up by astrocytes (glial cells), using the GLUT-1 transport protein (Magistretti, 2006), and converted to pyruvate via glycolysis. The pyruvate is then reduced to lactate and shuttled to neurons in close association with the astrocyte, which oxidize lactate to pyruvate and continue the process of aerobic respiration (Magistretti, 2006). Lactate converted to ketone bodies within the liver and released into the bloodstream can also be used by neurons, upon conversion of the ketone bodies to pyruvate (Koehler-Stec et al., 1998). In addition, glucose directly enters the neurons, via a separate mechanism, for glycolysis and aerobic respiration in those cells (Magistretti, 2006). Not shown in the diagram are the MCTs that contribute to lactate transport across the membranes of cells.

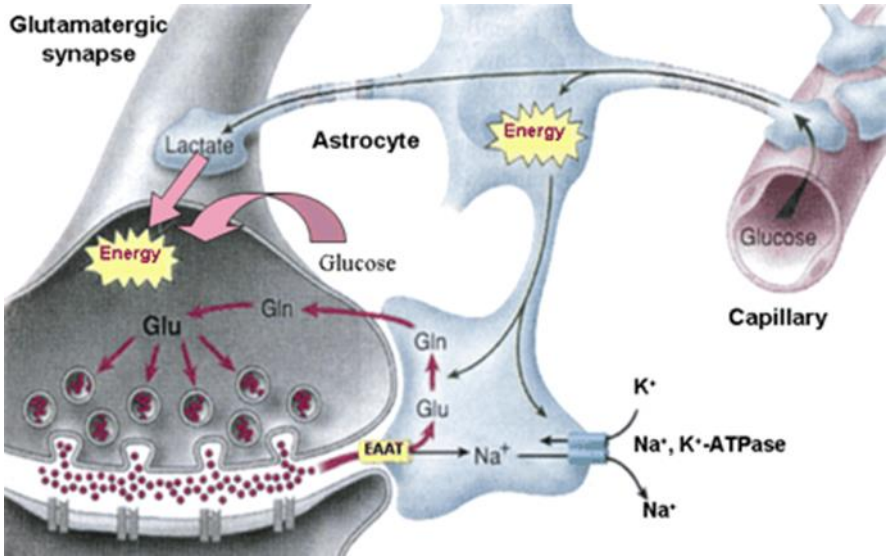

Figure 1.5. The astrocyte-neuron coupling mechanism proposed to exist in the mammalian brain. Glucose is taken up by astrocytes and metabolized to lactate, which is then delivered to neurons and used as a substrate for cellular energy production. (Magistretti, 2006)

The relationship between members of the Basigin subset of the IgSF and MCTs

Numerous studies conducted over the past two decades indicate that MCTs associate with members of the Basigin subset of the IgSF. Basigin gene products are known to associate with MCT1 and MCT4, as demonstrated via *in vitro* and *in vivo* analyses (Kirk et al., 2000; Wilson et al., 2002; Philp et al., 2003; Finch et al., 2009). It has been proposed that expression of MCT1 is dependent of its association with Basigin gene products, because absence of Basigin gene expression in a mouse model system results in the absence of MCT1 protein at the plasma membrane of the cell (Philp et al., 2003). The co-expression of Basigin and MCT1 correlates with a substantially enhanced rate of lactate transport, as compared to cells not expressing both proteins (Kirk et al., 2000). Similarly, a recent study suggests that Neuroplastin gene products associate with MCT2 on neurons, although a direct relationship was not established (Wilson et al., 2013). In another study, Embigin was co-expressed with MCT1 in erythrocytes (Wilson et al., 2009). Although the study was limited by heterologous expression, rather than endogenous expression, it did demonstrate that the two proteins interact at the plasma membrane (Wilson et al., 2009).

The interaction between Basigin gene products and MCT1 has been studied at the molecular level. Binding studies utilizing recombinant forms of the transmembrane domain of Basigin gene products and endogenous mouse neural retina MCT1 demonstrated that this domain of Basigin gene products does bind to MCT1 (Finch et al., 2009). Although it was hypothesized that the central glutamate within the domain would play a significant role in binding to MCT1 (Wilson et al., 2002), it did not appear to play any role in the interaction (Finch et al., 2009). In contrast, hydrophobic interactions were found to be responsible for the interaction with MCT1 (Figure 1.6; Finch et al., 2009).

Figure 1.6. The entire transmembrane domain of Basigin gene products (BasTM-All) is shown using the amino acid one-letter code. It was determined by Finch et al., (2009), that the transmembrane domain of Basigin gene products (BasTM) binds to MCT1 using the hydrophobic amino acids shown in blue. One letter code: A = alanine; E = glutamate; F = phenylalanine; G = glycine; I = isoleucine; L = leucine; M = methionine; P = proline; T = threonine; V = valine; W = tryptophan; Y = tyrosine

In a study using a mouse model system, it was determined that Basigin gene expression affects the expression of MCT1 and MCT4, but not MCT2 (Figure 1.7; Philp et al., 2003). Immunoblotting analyses using detergent-solubilized protein lysates of the neural retinas from Basigin null and control animals indicated that expression of MCT1 and MCT4 proteins was significantly reduced at the plasma membrane of Basigin null animals, as compared to the control animals (Figure 1.7; Philp et al., 2003). In contrast, the expression of MCT2 protein was not different between the two groups (Figure 1.7; Philp et al., 2003). It is known that Basigin gene products interact with MCT1 via the transmembrane domain (Finch et al., 2009). Therefore, the purpose of the present study was to determine if Basigin gene products interact with other members of the MCT family, specifically MCT2 and MCT4, using the same mechanism. Based on previous data, it was hypothesized that Basigin gene products interact with MCT4 in a similar manner to that of MCT1, whereas Basigin gene products do not interact with MCT2 and thus it will serve as a negative control. This hypothesis was tested using a series of biochemical analyses.

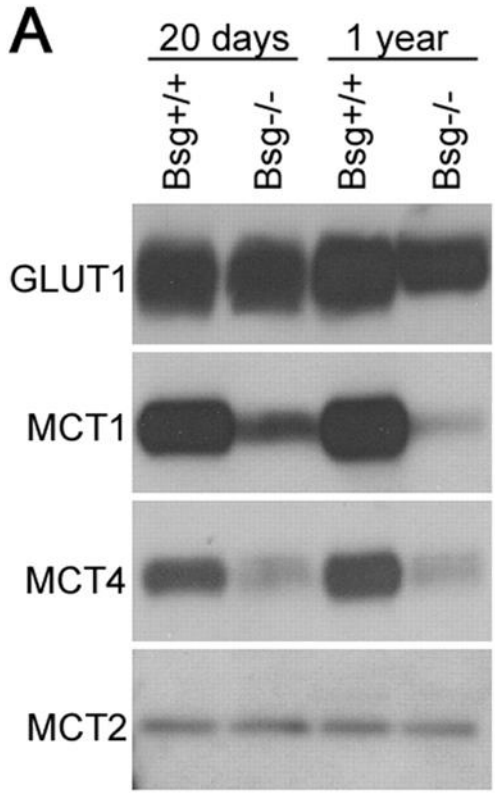

Figure 1.7. Basigin gene products affect the expression of MCT1 and MCT4, but not MCT2 in the mouse neural retina. An immunoblot of neural retina membrane-associated protein expression is shown. Samples from Basigin normal (Bsg +/+) and null (Bsg -/-) were tested from animals at 20-days of age and one year of age. In the top panels, the proteins were probed with an antibody specific for the glutamate transporter GLUT1, which served as a loading control. The remaining panels show the results of probing with antibodies specific for MCT1, MCT4, and MCT2. Expression of MCT1 and MCT4 is reduced in the null animals, as compared to the age-matched normal animals, whereas the expression of MCT2 is similar for all samples. (Philp et al., 2003)

Chapter 2
Materials and Methods

Recombinant protein expression and isolation

Expression plasmids containing the cDNA for the entire transmembrane domain of Basigin gene products, as well as those containing truncated and mutated sequences were previously generated (Finch et al., 2009; Brown, 2016). A plasmid containing no insert was also previously generated and served as the control for binding assays (Finch et al., 2009). The expression plasmid used was pET102 (Invitrogen Corporation, Carlsbad, CA), which allows recombinant proteins containing an epitope of six histidine residues at the carboxy-terminus to be expressed in bacteria. The plasmid codes for 157 amino acids, which mask the hydrophobic nature of the Basigin-specific amino acids and allows a soluble protein to be expressed.

Recombinant protein expression was carried out by transforming BL21 cells (Invitrogen Corporation). The cells were grown to mid-log phase, induced with 1 mM isopropylthiogalactoside (IPTG; Thermo Fisher Scientific, Hampton, NH), and grown overnight at 37^{o}C with shaking.

Recombinant proteins were isolated using the His-TALON system (Clontech, Mountain View, CA). Cells were pelleted by centrifugation at 3000 ×g for 15 minutes and resuspended in X-tractor buffer (20 mL per 1 g of cells). The cells were lysed by incubation for 10 minutes at room temperature in the presence of 100 μg/mL lysozyme (Clontech) and 5 U/mL DNase I (Clontech). A protein lysate was formed by centrifugation at 10,000 ×g for 20 minutes. The

lysate was mixed with TALON purification resin (Clontech) for 10 minutes at room temperature. The resin was washed by centrifugation and the via column gravity filtration. Proteins were eluted from the column using elution buffer (Clontech) in 0.5 mL fractions and the presence of protein was analyzed at 280 nm.

Mouse brain protein lysates

Three adult male mouse brains were obtained using an approved protocol and immediately washed in phosphate buffered saline (PBS). Each brain was homogenized in detergent lysis buffer (0.5% SDS, 0.05 M Tris·HCl, pH to 8.0, plus 1 mM fresh dithiothreitol (DTT)) and incubated on ice for 10 minutes. A protein lysate was generated by centrifugation at 16,000 ×g for 10 minutes.

Bradford-Coomassie protein assay

Protein concentration of lysates was determined using the Bradford-Coomassie protein assay method (Pierce/Thermo Scientific). Concentrations of bovine serum albumin (BSA, Pierce/Thermo Scientific) ranging from 2.0 mg/mL to 0.1 mg/mL were generated and mixed with Bradford-Coomassie binding reagent. The absorbance at 595 nm was measured and a standard curve (absorbance versus protein concentration) was created. The equation from the best-fit trendline (usually logarithmic) was used to determine the concentration of recombinant and mouse brain protein samples.

ELISA Binding Assay

Enzyme-linked immunosorbent assays (ELISA) were used as binding assays, as described in Finch et al., (2009). Capture antibody (specific for MCT1, MCT2, or MCT4; 50 ng/mL in phosphate buffered saline [PBS]; Millipore Corporation, Billerica, MA) was plated and incubated overnight at 4°C. The solution was removed, and the wells were washed with PBS containing 0.25% Tween-20 (PBS-T). Bovine serum albumin (BSA; Pierce/Thermo Scientific), diluted to 100 μg/mL in PBS, was added to all wells and incubated at 37°C for 30 minutes. The solution was removed, and wells were washed with PBS-T. Mouse brain protein lysates (100 μg/mL) were added to all wells and incubated at 37°C for 30 minutes. The solution was removed, and wells were washed with PBS-T. Recombinant proteins (diluted to 100 μg/mL in PBS) were added to individual wells in triplicate and incubated at 37°C for 30 minutes. The solution was removed, and wells were washed with PBS-T. Primary antibody specific for the carboxy-terminal six-histidine tag (diluted 1:1000 in PBS; BD Biosciences) was added to all wells and incubated at 37°C for 30 minutes. The solution was removed, and wells were washed with PBS-T. Alkaline phosphatase-conjugated goat anti-mouse secondary antibody (diluted 1:1000 in PBS) was added to all wells and incubated at 37°C for 30 minutes. The solution was removed, and wells were washed with PBS-T. Alkaline phosphatase substrate (PNPP, Pierce/Thermo Scientific) was added to all wells for color development. The reaction was stopped with the addition of 2N NaOH. The absorbance at 405 nm was measured. All runs were performed in triplicate, using different protein samples, and the average absorbance was plotted. Binding was compared for Basigin-containing recombinant proteins with that of the control recombinant protein via a paired, one-tailed T-test. For the binding assays that compared the mutated sequence to the normal sequence, the average absorbance for the protein with the

normal sequence was set to 100% binding and all mutant proteins were compared. Binding was compared for mutant recombinant proteins with that of the normal recombinant protein via a paired, one-tailed T-test.

Affinity binding assays were performed like the simple binding assays described, with the exception that varying concentrations of recombinant protein (10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM) were used. All runs were performed in triplicate, using different brain protein samples. The average absorbance for the protein at 10 μM was set to 100% binding and the absorbances for the other concentrations was compared. A binding curve was generated and the equation for the logarithmic trendline was used to determine the concentration equal to 50% binding. This was determined to be the binding affinity. The similarity of binding of Basigin transmembrane domain recombinant protein for MCT1, MCT2, and MCT4 was compared via a single factor ANOVA.

Immunohistochemistry

Many previous studies by this laboratory have involved the investigation of Basigin gene expression in the retina. It was noted in a recent study in the lab that Basigin and MCT2 expression appears to overlap in tongue, thus providing a biologically relevant application for *in vitro* data. Therefore, mouse tongues were isolated from adult male mice, according to an accepted animal use protocol. The organs were washed in PBS and fixed by incubation for 24 hours in 4% paraformaldehyde in PBS at room temperature. The tissues were embedded in paraffin wax and 5-μm sections were cut and applied to poly-L-lysine-coated glass microscope slides. The retina sections were previously generated (Ochrietor et al., 2003; Tokar et al., 2017).

The tissue sections were rehydrated by incubating in CitriSolve (Fisher Scientific) twice, for 10 minutes each time, followed by 5 minutes in 100% ethanol (Fisher Scientific), then 95% ethanol, then 70% ethanol, and finally tris-buffered saline (TBS). The rehydrated sections were solubilized in a buffer consisting of TBS with 0.1% Triton X-100 (Fisher Scientific) and 2% normal goat serum (Pierce/Thermo Scientific) by incubation at 4ºC overnight. The sections were then incubated in the presence of an antibody specific for Basigin (Ochrietor et al., 2003) or MCT2 (Millipore Corporation, Billerica, MA), diluted to 5 μg/mL in the solubilization buffer for 1 hour at 37ºC, followed by continued incubation at 4ºC overnight. The solution was removed, and the sections were washed several times with TBS. The sections were then incubated in the presence of Alexa488-conjugated or Alexa594-conjugated goat anti-rabbit secondary antibody (Pierce/Thermo Scientific) diluted 1:1000 in solubilization buffer and incubated at 37ºC for 30 minutes. The solution was removed, and the sections were washed several times with TBS. DRAQ5 (Pierce/Thermo Scientific) was added to the first TBS wash at 1:1000 dilution. Coverslips were applied with 30% glycerol containing p-phenylenediamine (Sigma Chemical Company, St. Louis, MO) and the tissues were viewed with an Olympus Fluoview F1000 confocal microscope (Pittsburgh, PA).

Chapter 3
Results

Assessing the ability of Basigin to bind to MCT2 and MCT4

The purpose of the present study was to assess the ability of the transmembrane domain of Basigin gene products to interact with MCT2 and MCT4. Previous studies indicate that Basigin gene products interact with MCT1 using hydrophobic amino acids within the domain (Finch et al., 2009). Other studies suggest that Basigin may interact with MCT4 using a similar mechanism but does not likely interact with MCT2 (Philp et al., 2003). Therefore, the ability of a recombinant form of the Basigin transmembrane domain to bind to endogenous mouse brain MCT2 and MCT4 was tested.

Initially, simple ELISA binding assays were employed to test the ability of the recombinant Basigin transmembrane domain protein to bind to endogenous mouse brain MCT2 and MCT4. The ability of the protein to bind to endogenous mouse brain MCT1 served as the positive control. As observed previously, binding of the Basigin transmembrane domain protein (BasTM-all) to MCT1 was significantly greater than the binding of the vector control protein (control) to MCT1 (Figure 3.1A; $p = 0.0009$). Similarly, the ability of BasTM-all to bind to MCT2 (Figure 3.1B; $p = 0.009$) and MCT4 (Figure 3.1C; $p=0.001$) was significantly greater than binding of the control protein.

Next, the affinity of the transmembrane domain of Basigin gene products for MCT1, MCT2, and MCT4 was assessed. Standard curves in which the recombinant BasTM-all protein

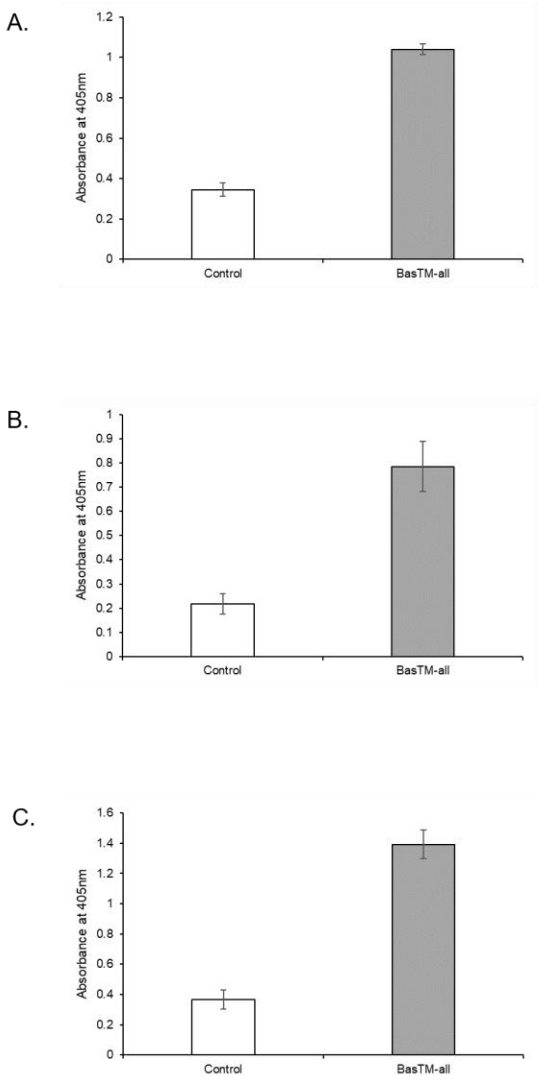

Figure 3.1 Binding of Bas-TM-all to MCTs. The ability of Bas-TM-all to bind to MCT1 (A), MCT2 (B), and MCT4 (C) was assessed via an *in vitro* binding assay. In all cases, the binding of BasTM-all was significantly greater than that of the vector control protein (Control).

was tested over a range of concentrations were used to determine the concentration at 50% binding, which is considered the affinity. The affinity binding curves for BasTM-all against MCT1, MCT2, and MCT4 are shown in Figure 3.2. Table 3.1 shows that the affinity of the BasTM-all protein for each of the transporters. The affinities of BasTM-all for MCT1, MCT2, and MCT4 were not significantly different from each other ($p = 0.086$).

Assessing the ability of the Basigin transmembrane domain to bind MCT2

To determine which amino acids within the transmembrane domain of Basigin gene products are used in the interactions with MCT2, deletion mutants of the domain were used. The domain was fractioned into six amino acid sections and the resulting recombinant proteins were used for simple binding assays. It was determined that the region containing amino acids 1-6 had significantly greater binding to MCT2 than the control protein ($p = 0.004$) and similar to that of the BasTM-all protein ($p = 0.068$; Figure 3.3). Conversely, the regions containing amino acids 7-12 ($p = 0.421$) and 19-24 ($p = 0.310$) were not significantly greater than the control protein (Figure 3.3). The ability of the region containing amino acids 13-18 was significantly lower than that of the control protein ($p = 0.003$; Figure 3.3). To confirm the data obtained through the simple binding assays, affinity assays were performed for each section of the Basigin transmembrane domain (Table 3.2). The affinity for MCT2 of the region containing BasTM 1-6 (0.714 ± 0.391 μM) was significantly greater than that of the entire domain (BasTM-all; 1.70 ± 0.24 μM; $p = 0.041$). The affinities for MCT2 of the regions contain BasTM 13-18 (2.82 ± 0.22 μM) and BasTM 19-24 (2.66 ± 0.30 μM) were significantly lower than that of BasTM-all ($p =$

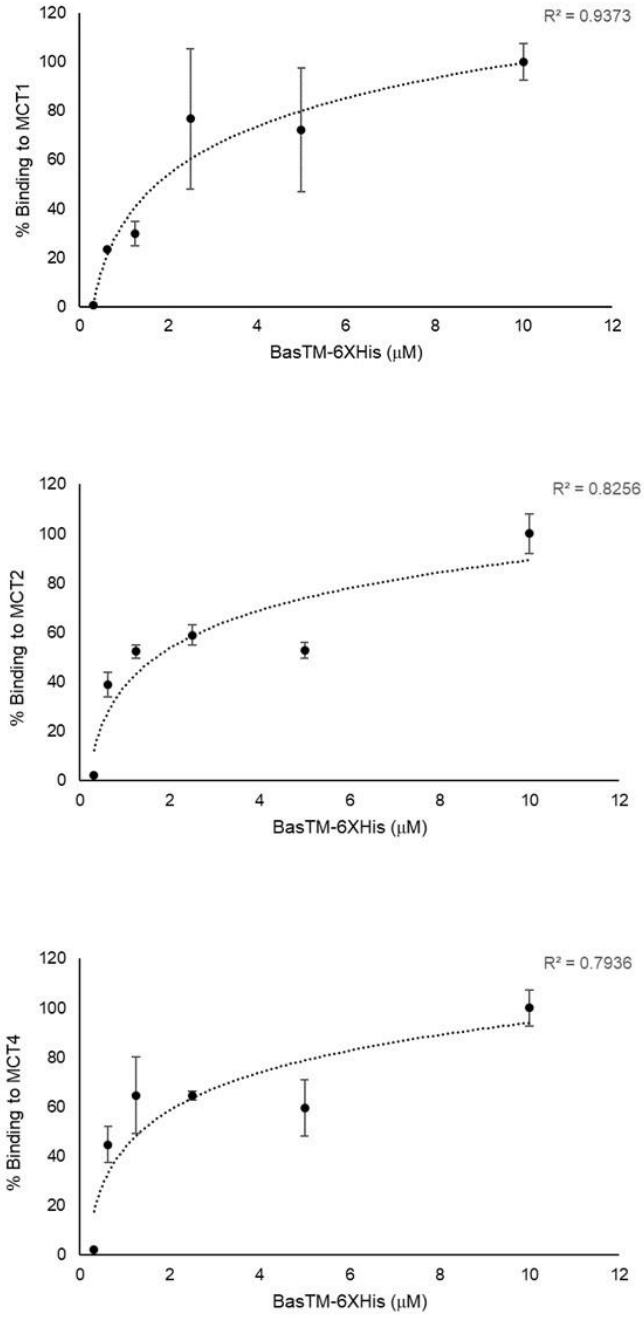

Figure 3.2 Binding curves for BasTM-6xHis with MCT1, MCT2, and MCT4. For each transporter, the absorbance obtained for 10 μM was set to 100% binding, and the other absorbances were compared to it. A logarithmic trendline was applied and the equation was used to determine the affinity. The R^2 value for each trendline is shown.

Table 3.1 Binding affinity of BasTM-6xHis for MCT1, MCT2, and MCT4 in μM.

Transporter	Binding affinity (μM)
MCT-1	1.74 +/- 0.12
MCT-2	1.70 +/- 0.24
MCT-4	1.36 +/- 0.19

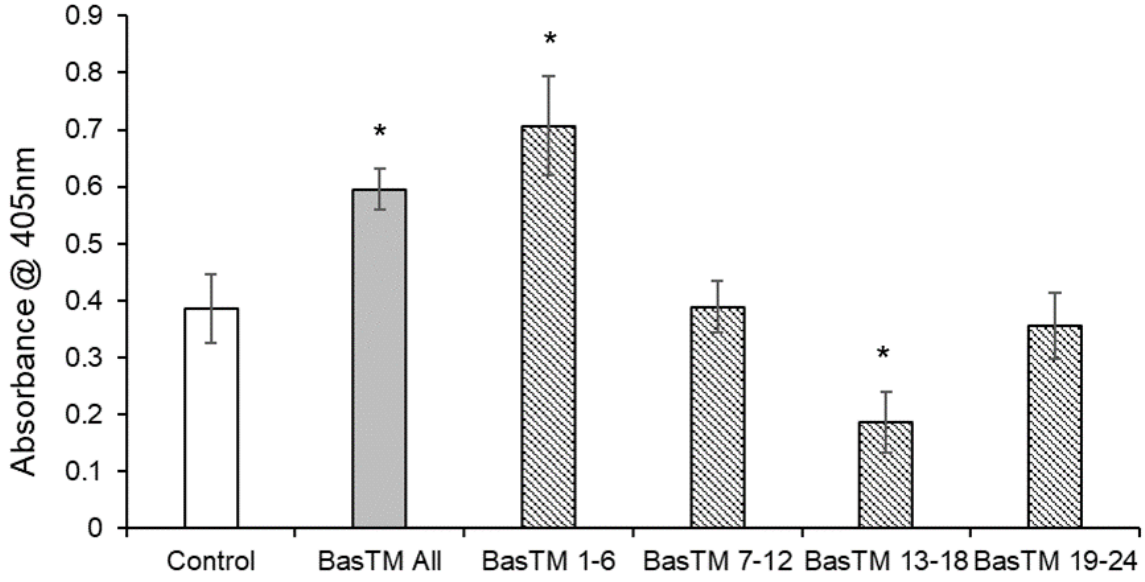

Figure 3.3 Binding of BasTM truncation mutants to MCT2. The ability of each section of the transmembrane domain of Basigin (BasTM 1-6, BasTM 7-12, BasTM 13-18, BasTM 19-24) to bind to MCT2 was assessed through an *in vitro* binding assay. The binding of each BasTM protein was individually compared to that of the control protein using a paired, one-tailed T-test. The asterisk (*) represents p=<0.01.

Table 3.2 Binding affinity of BasTM-all and the Bas-TM truncation mutants for MCT2 in μM. The p-value was obtained via a paired, one-tailed T-test comparing each mutant to the BasTM-all protein.

Protein	Affinity	p-value
BasTM-all	1.70 ±0.24 μM	
BasTM 1-6	0.714±0.391 μM	0.041
BasTM 7-12	1.70±0.88 μM	0.492
BasTM 13-18	2.82±0.22 μM	0.019
BasTM 19-24	2.66±0.30 μM	0.045
BasTM-all-E13G	1.43±0.266 μM	0.187

0.019, p = 0.045, respectively). Contrary to the data obtained from the simple binding assay, the affinity for MCT2 of the region containing BasTM 7-12 (1.70±0.88 μM) was the same as that for BasTM-all. These data suggest that binding of the transmembrane domain of Basigin to MCT2 employs amino acids within the first twelve residues of the domain.

The glutamate residue found in the center of the transmembrane domain of Basigin, at position 13, has been hypothesized by others to be essential for the interaction with MCTs (Kirk et al., 2000; Wilson et al., 2002). Although the affinity data generated through this study suggest otherwise, a direct assessment of the residue was performed using a recombinant version of the entire Basigin transmembrane domain, in which the amino acid glutamate was mutated to glycine (BasTM-all-E13G), and in an affinity binding assay (Figure 3.4). The affinity of the BasTM-all-E13G protein for MCT2 was determined to be 1.429 ± 0.266 μM, which is not statistically different from the affinity of the wild-type sequence for the transporter when compared using a paired, one-tailed T-test (p = 0.187; Table 3.2). These data suggest that the glutamate plays no role in the interaction between Basigin and MCT2.

To determine which amino acids in the segment containing amino acids 1-6 are used in the interaction with MCT2, deletion mutants in which individual amino acids were mutated to glycine were used. Binding of each mutant was compared to that of the BasTM 1-6 protein (Figure 3.5). It was determined that the proteins containing an alanine-to-glycine mutation at position 3 (p = 0.001) and a leucine-to-glycine at position 4 (p = 0.01) had significantly lower binding to MCT2 than the BasTM 1-6 protein. These amino acids are likely used in the association with MCT2. The proteins containing a methionine-to-glycine mutation at position 1 (p = 0.001), an alanine-to-glycine mutation at position 2 (p = 0.048), and a tryptophan-to-glycine

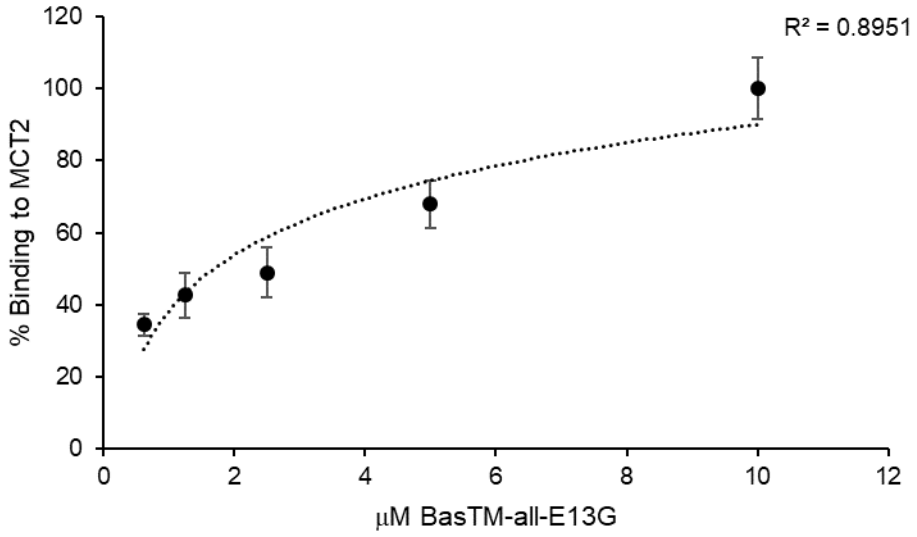

Figure 3.4 Binding curve of BasTM-all-E13G for MCT2. The absorbance obtained for 10 μM was set to 100% binding, and the other absorbances were compared to it. A logarithmic trendline was applied and the equation was used to determine the affinity. The R^2 value for the trendline is shown.

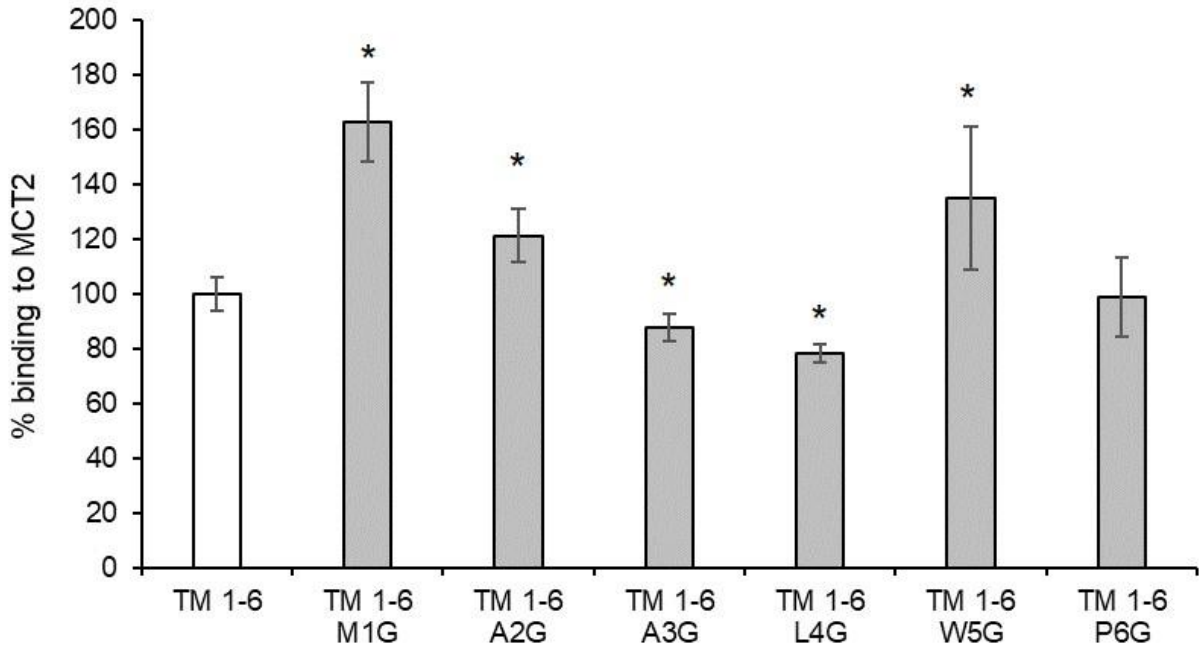

Figure 3.5 Binding of BasTM 1-6 mutants to MCT2. The ability of each amino acid to contribute to binding to MCT2 was evaluated through an *in vitro* binding assay, and compared to that of the BasTM 1-6 sequence, which was set to 100% binding. Mutants with binding greater than 100% are thought to inhibit the interaction with MCT2, whereas those with binding less than 100% are thought to be involved in the interaction. *=$p<0.05$ when individually compared to BasTM 1-6 via a paired, one-tailed T-test.

mutation at position 5 (p = 0.05) had significantly greater binding to MCT2 than the BasTM 1-6 protein. These amino acids likely inhibit the interaction with MCT2.

To determine which amino acids in the segment containing amino acids 7-12 are used in the interaction with MCT2, deletion mutants in which individual amino acids were mutated to glycine were used. Binding of each mutant was compared to that of the BasTM 7-12 protein (Figure 3.6). It was determined that mutation of no amino acids resulted in a significant decrease in binding. On the contrary, mutation of the phenylalanine at position 7 (p=0.003), the leucine at position 8 (p=0.006), the isoleucine at position 10 (p=0.031), and the alanine at position 12 (p=0.009) had significantly greater binding to MCT2 than BasTM 7-12. Mutation of the valine at position 11 had similar binding to MCT2 as BasTM 7-12 (p=0.247). The amino acid at position 9 is glycine and therefore was not mutated.

Assessing the ability of the Basigin transmembrane domain to bind MCT4

To determine which amino acids within the transmembrane domain of Basigin gene products are used in the interactions with MCT4, deletion mutants of the domain were again used. It was determined that the regions containing amino acids 1-6 and 7-12 had significantly greater binding to MCT4 than the control protein (p = 0.018, 0.024, respectively; Figure 3.7) and similar to that of the BasTM-all protein (p = 0.099, 0.250, respectively), whereas the region containing amino acids 19-24 was not greater than the control protein (p = 0.255; Figure 3.7). The ability of the region containing amino acids 13-18 was significantly lower than that of the control protein (p = 0.002; Figure 3.7). To confirm the data obtained through the simple binding assays, affinity assays were performed for each section of the Basigin transmembrane domain

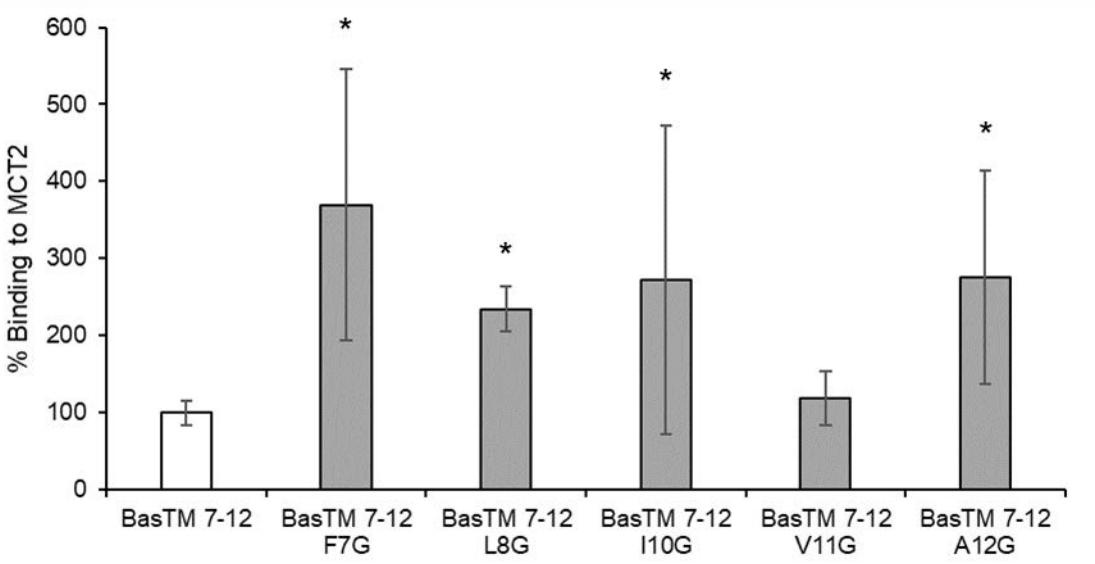

Figure 3.6 Binding of BasTM 7-12 mutants to MCT2. The ability of each amino acid to contribute to binding to MCT2 was evaluated through an *in vitro* binding assay, and compared to that of the BasTM 7-12 sequence, which was set to 100% binding. Mutants with binding greater than 100% are thought to inhibit the interaction with MCT2, whereas those with binding less than 100% are thought to be involved in the interaction. *=$p<0.05$ when individually compared to BasTM 7-12 using a paired, one-tailed T-test.

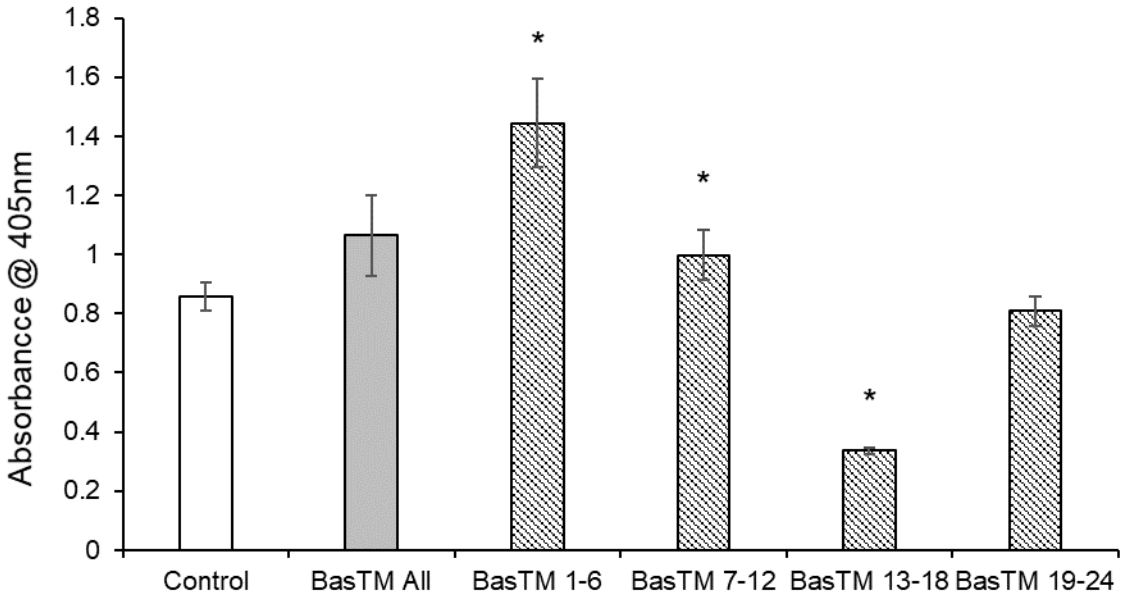

Figure 3.7 Binding of BasTM truncation mutants to MCT4. The ability of each section of the transmembrane domain of Basigin (BasTM 1-6, BasTM 7-12, BasTM 13-18, BasTM 19-24) to bind to MCT4 was assessed through an *in vitro* binding assay. The binding of each BasTM mutant protein was individually compared to that of the BasTM-all protein using a paired, one-tailed T-test. The asterisk (*) represents p=<0.01.

Table 3.3 Binding affinity of BasTM-all and the Bas-TM truncation mutants for MCT4 in μM. The p-value was obtained via a paired, one-tailed T-test comparing each mutant to the BasTM-all protein.

Protein	Affinity	p-value
BasTM-all	1.36±0.19 μM	
BasTM 1-6	1.76±0.433 μM	0.185
BasTM 7-12	2.03±0.14 μM	0.015
BasTM 13-18	0.838±0.280 μM	0.032
BasTM 19-24	2.44±0.12 μM	0.012
BasTM-all-E13G	1.69±0.127 μM	0.043

(Table 3.3). The affinity for MCT4 of the region containing BasTM 1-6 (1.76±0.433 μM) was similar to that of the entire domain (BasTM-all; 1.36 ±0.19 μM). The affinities for MCT4 of the regions containing BasTM 7-12 (2.03±0.14 μM) and BasTM 19-24 (2.44±0.12 μM) were significantly lower than that of BasTM-all. Surprisingly, the affinity for MCT4 of the region containing BasTM 13-18 was significantly greater than that of BasTM-all (0.838±0.280; $p = 0.032$). These data suggest that amino acids within the first six residues of the domain may play a role in binding to MCT4, but amino acids within residues 13-18 play a more significant role in the interaction. The glutamate residue within the transmembrane domain of Basigin gene products is found at position 13. The role of this amino acid was tested using the recombinant version of the entire Basigin transmembrane domain, in which the amino acid glutamate was mutated to glycine (BasTM-all-E13G), in an affinity binding assay (Figure 3.8). The affinity of the BasTM-all-E13G protein for MCT4 was determined to be 1.69 ± 0.127 μM, which is statistically greater than the affinity of the wild-type sequence for the transporter ($p = 0.043$; Table 3.3). These data suggest that the glutamate plays a direct role in the interaction between Basigin and MCT4.

To determine which amino acids in the segment containing amino acids 1-6 are used in the interaction with MCT4, deletion mutants in which individual amino acids were mutated to glycine were used. Binding of each mutant was compared to that of the BasTM 1-6 protein (Figure 3.9). It was determined that the protein containing an alanine-to-glycine mutation at position 3 ($p = 0.046$) had significantly lower binding to MCT4 than the BasTM 1-6 protein. This amino acid likely binds to MCT4. The proteins containing a methionine-to-glycine mutation at position 1 ($p = 0.009$), an alanine-to-glycine mutation at position 2 ($p = 0.041$), a tryptophan-to-glycine mutation at position 5 ($p = 0.023$), and a proline-to-glycine mutation at

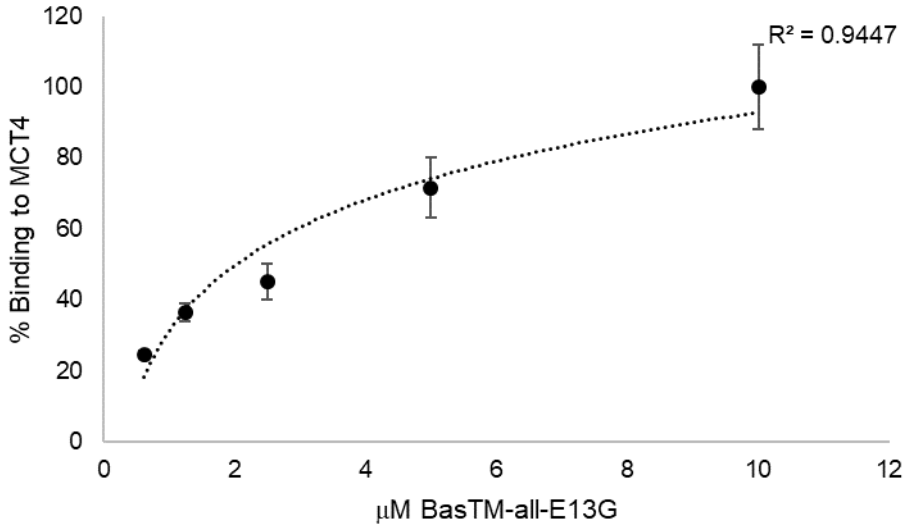

Figure 3.8 Binding curve of BasTM-all-E13G for MCT4. The absorbance obtained for 10 μM was set to 100% binding, and the other absorbances were compared to it. A logarithmic trendline was applied and the equation was used to determine the affinity. The R^2 value for the trendline is shown.

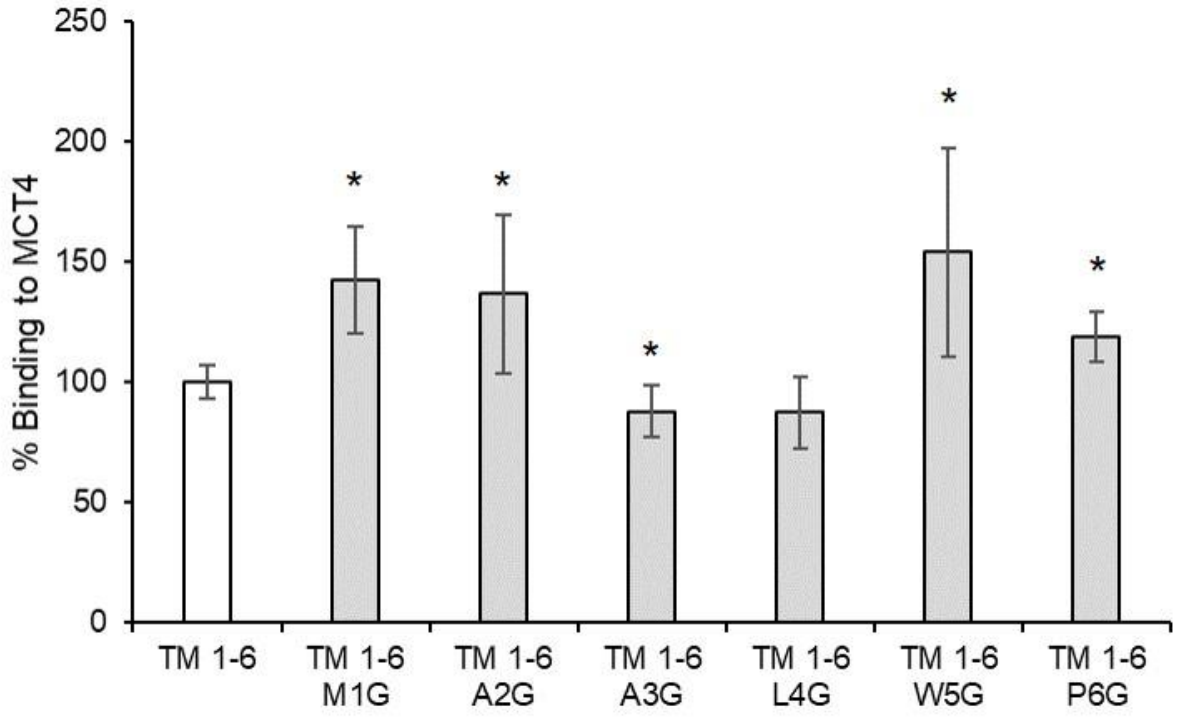

Figure 3.9 Binding of BasTM 1-6 mutants to MCT4. The ability of each amino acid to contribute to binding to MCT4 was evaluated through an *in vitro* binding assay, and compared to that of the BasTM 1-6 sequence, which was set to 100% binding. Mutants with binding greater than 100% are thought to inhibit the interaction with MCT4, whereas those with binding less than 100% are thought to be involved in the interaction. *=$p<0.05$ when individually compared to BasTM 1-6 using a paired, one-tailed T-test.

position 6 ($p = 0.008$) had significantly greater binding to MCT4 than the BasTM 1-6 protein. These amino acids are likely inhibitory in the interaction with MCT4.

To determine which amino acids in the segment containing amino acids 13-18 are used in the interaction with MCT4, deletion mutants in which individual amino acids were mutated to glycine were again used. Binding of each mutant was compared to that of the BasTM 13-18 protein (Figure 3.10). It was determined that the protein containing a glutamate-to-glycine mutation at position 13 ($p = 0.022$) had significantly lower binding to MCT4 than the BasTM 13-18 protein. This amino acid likely binds to MCT4. The proteins containing a leucine-to-glycine mutation at position 15 ($p = 0.010$), a valine-to-glycine mutation at position 16 ($p = 0.002$), a leucine-to-glycine mutation at position 17 ($p = 0.002$), and a valine-to-glycine mutation at position 18 ($p = 0.004$) had significantly greater binding to MCT4 than the BasTM 13-18 protein. These amino acids are likely inhibitory in the interaction with MCT4.

Assessing the expression of Basigin and MCT2 in vivo

To assess the biological relevance of the ability of Basigin to bind to MCT2, immunohistochemical analyses were conducted. Paraffin-embedded sections of mouse retina were subjected to immunohistochemical analyses using antibodies specific for Basigin and MCT2 (Figure 3.11). While Basigin is found on the Muller cells, photoreceptor cells, and blood vessels of the retina, MCT2 expression predominates in the inner and outer plexiform layers, where synapses between neurons form. A more recent study by this laboratory investigated the expression of Basigin gene products within the mouse tongue (Figure 3.12). Basigin and MCT2 overlap in the taste buds on the surface of the tongue epithelium.

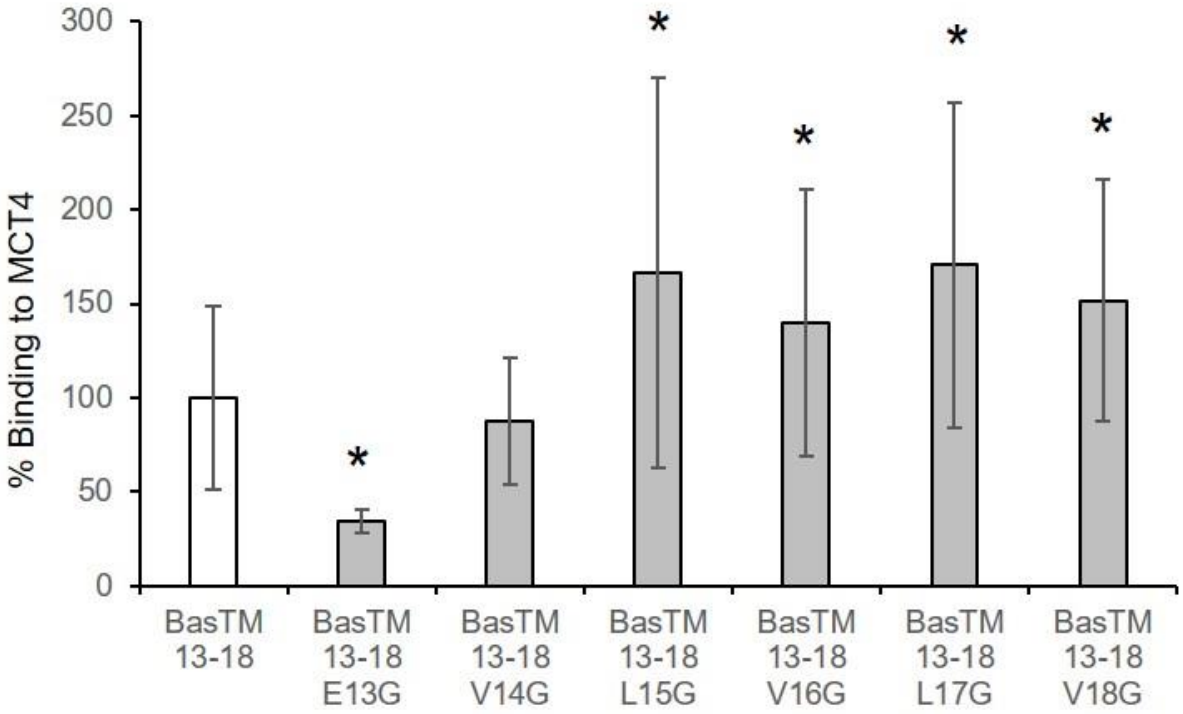

Figure 3.10 Binding of BasTM 13-18 mutants to MCT4. The ability of each amino acid to contribute to binding to MCT4 was evaluated through an in vitro binding assay, and compared to that of the BasTM 13-18 sequence, which was set to 100% binding. Mutants with binding greater than 100% are thought to inhibit the interaction with MCT4, whereas those with binding less than 100% are thought to be involved in the interaction. *=$p<0.05$ when individually compared to BasTM 13-18 using a paired, one-tailed T-test.

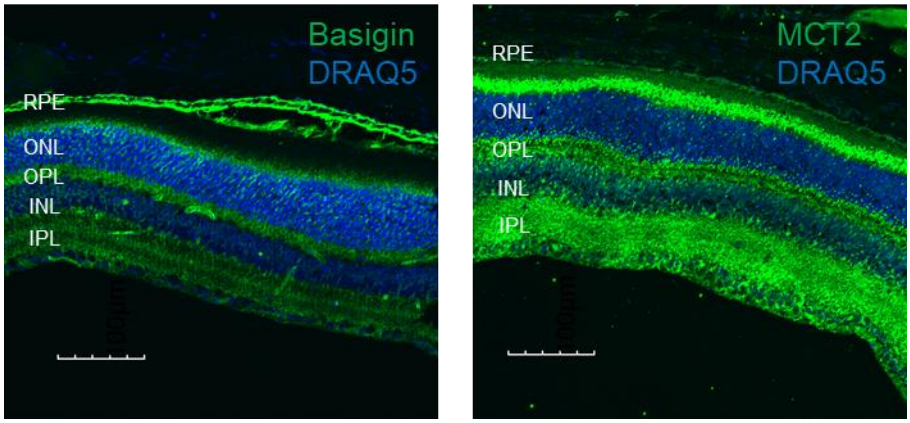

Figure 3.11. Expression of Basigin and MCT2 in the mouse retina. In both panels, the green fluorescence represents protein expression (Basigin or MCT2) and the blue fluorescence represents DRAQ5, which binds DNA. The magnification bar represents 50 μm. Abbreviations: RPE, retinal pigmented epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer.

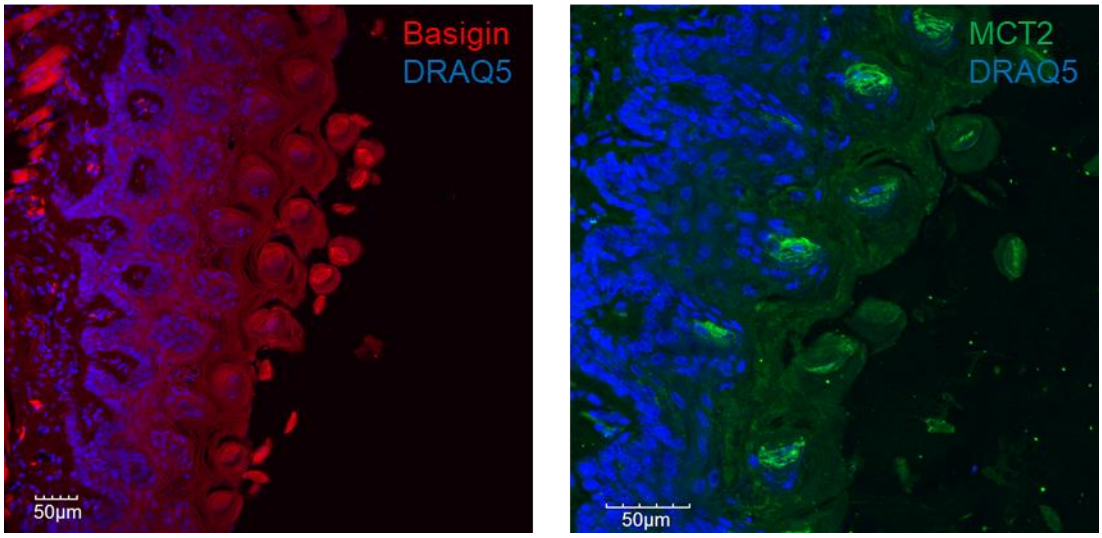

Figure 3.12 Expression of Basigin and MCT2 in mouse tongue. In the left panel, Basigin expression is represented by red fluorescence. In the right panel, MCT2 expression is represented by green fluorescence. In both panels, DRAQ 5 binding to DNA is represented by the blue fluorescence. The magnification bars represent 50 μm.

Chapter 4
Discussion

It is generally accepted that the proton-linked monocarboxylate transporters MCT1, MCT2, and MCT4 require an interaction with a cell adhesion molecule of the Basigin subset of the IgSF for expression at the plasma membrane and transport as an accessory protein to express at the plasma membrane (Kirk et al., 2000; Philp et al., 2003; Wilson et al., 2009). It was thought that Basigin gene products interact with MCT1 and MCT4 (Kirk et al., 2000; Philp et al., 2003), whereas the Neuroplastins interact with MCT2 (Wilson et al., 2013). Little is known of Embigin, but it was shown to interact with MCT1 (Wilson et al., 2009). The purpose of the present study was to characterize the interaction between Basigin gene products and MCT4, to determine if that interaction is like that for MCT1. During the investigation, it was determined that the interactions of Basigin gene products with MCT1 and MCT4 are quite distinct from each other. Additionally, although it was designed to serve as a negative control, an interaction between Basigin gene products and MCT2 was identified. That interaction differs from those of the other two transporters. In all, the data suggest that Basigin gene products, especially Basigin variant-1, which is ubiquitously expressed, are highly versatile proteins.

While simple binding studies are useful in establishing interactions between proteins, affinity binding assays allow the interaction to be quantified and compared. Initially, interactions between the transmembrane domain of Basigin and MCT1 were used as a positive control and to validate the study system used. Affinity data for the Basigin-MCT1 interaction

obtained through this study was similar to those of previous studies and suggest that the interaction is of moderate affinity (Howard et al., 2010). This study demonstrated that the affinity of Basigin for MCT2 and for MCT4 were statistically similar to that of MCT1. This is the first study to indicate that Basigin can interact with MCT2, which was previously thought to interact solely with the Neuroplastins (Beesley et al., 2014). The moderate affinity for MCT4 is not surprising considering a recent study in which an interaction between Basigin and MCT4 was determined to be crucial for development of glioblastoma and disruption of that interaction can reverse that development (Voss et al., 2017).

The interactions between Basigin and monocarboxylate transporters are distinct. A previous study in which the interaction between the transmembrane domain of Basigin gene products and MCT1 was investigated indicated that hydrophobic amino acids on both "ends" of the domain were used (Finch et al., 2009). In the present study, it was determined that the interaction between Basigin and MCT2 utilizes two of the three amino acids at the extracellular-facing portion of the membrane-spanning domain that are used by MCT1. However, no amino acids within the cytoplasmic-facing portion of the membrane-spanning domain are used. In contrast, it was determined that for the interaction with MCT4, Basigin uses only one amino acid within the extracellular-facing portion of the membrane-spanning domain used for the interactions with MCT1 and MCT2, but also uses the glutamate residue positioned at the center of the domain. A summary of the interactions is shown in Figure 4.1.

One exciting component of the present study was the finding that the glutamate residue within the transmembrane domain of Basigin is used in the interaction with MCT4. In previous studies of Basigin and MCT1, it was hypothesized that the polar, charged amino acid glutamate

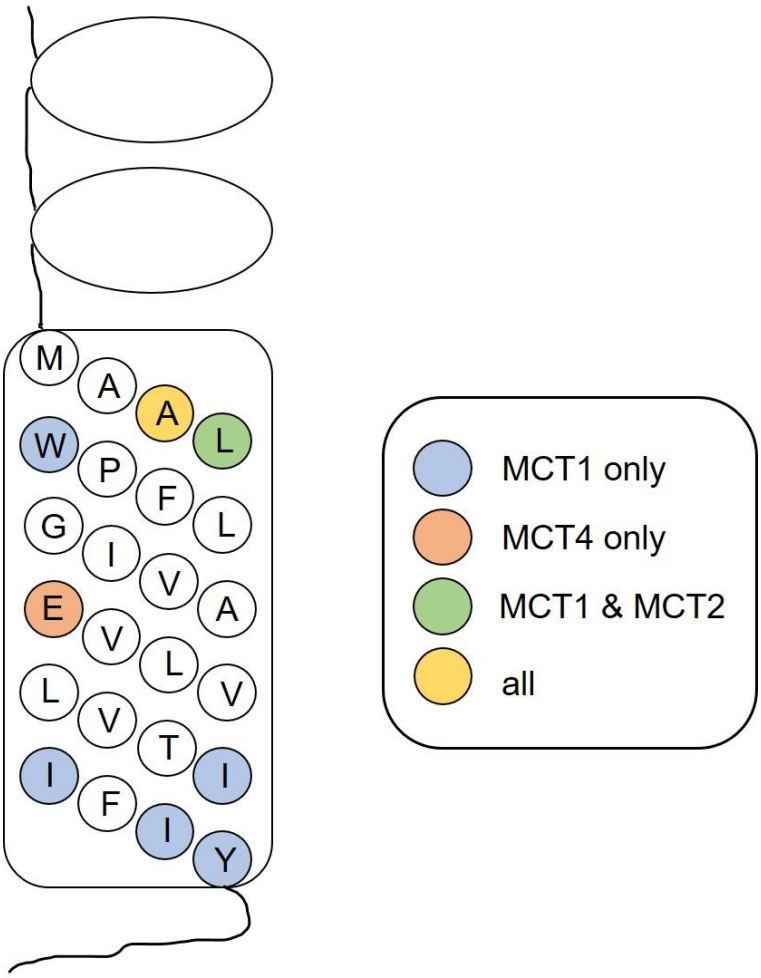

Figure 4.1. Summary of Basigin binding to monocarboxylate transporters. The transmembrane domain is depicted with the one-letter code for the individual amino acids shown in the circles. Amino acids within white circles are not used to bind any MCT. Amino acids within blue circles are used by MCT1 only. Amino acids within red circles are used by MCT4 only. Amino acids within green circles are used by MCT1 and MCT2. Amino acids within yellow circles are used by MCT1, MCT2, and MCT4.

must have a specific function within the hydrophobic domain of Basigin, and that the function was for the purposes of chaperoning MCT1 to the cell surface (Kirk et al., 2000). That hypothesis was not supported by data from a previous study by this laboratory (Finch et al., 2009). However, the role of the glutamate was never determined. In the present study, it was determined through two independent assays that glutamate interacts with MCT4. When the residue was mutated to glycine within a recombinant protein consisting of the entire BasTM domain (BasTM-E13G), the affinity of the protein for MCT4 was statistically lower than that of the original BasTM protein. In addition, the simple binding assay using deletion mutants consisting of BasTM 13-18 showed that mutation of glutamate to glycine produced significantly decreased binding to MCT4 when compared to the original BasTM 13-18 sequence. A recent study using glioblastoma stem cells indicated that the interaction between Basigin and MCT4 can be disrupted by acriflavine (Voss et al., 2017). The acriflavine molecule binds to the extracellular domain of Basigin, as determined by surface plasmon resonance, and can prohibit proliferation of the glioblastoma cells (Voss et al., 2017). Even more impressively, disruption of the Basigin-MCT4 interaction in mice with glioblastoma xenographs, showed significant inhibition of tumor progression in both early and late stages of the disease (Voss et al., 2017). The data presented herein suggest that the interaction between acriflavine and Basigin creates either a conformational change in the Basigin molecule that prevents the glutamate from interacting with MCT4 or changes the charge on the glutamate residue within Basigin and hence prevents an interaction with MCT4.

Another exciting finding from this study is the fact that Basigin binds to MCT2. Previous studies in which the expression of MCTs in the absence of Basigin gene expression was investigated indicated that expression of MCT1 and MCT4 were altered in the absence of

Basigin, whereas the expression of MCT2 was unaffected (Philp et al., 2003). The immunohistochemical analyses presented in this study provide an explanation for that observation. Basigin and MCT2 are not expressed in the same places within the retina. Therefore, Basigin does not affect MCT2 expression in that tissue. The immunohistochemical analyses of mouse tongue suggest that Basigin and MCT2 expression do overlap in that organ and provide biological relevance to the biochemical data obtained. Unfortunately, an analysis of MCT2 expression in the mouse tongue in the absence of Basigin expression is beyond the current abilities of the laboratory and cannot be undertaken. The laboratory has not been able to produce a Basigin-null animal in several years.

In conclusion, it has been demonstrated that the transmembrane domain of Basigin can interact with MCT1, MCT2, and MCT4. The amino acids used in the interactions differs by transporter. The observed preference of Basigin for MCT1 and MCT4 is therefore more likely an effect of overlapping expression, rather than the ability to interact.

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