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Localization and characterization of the interactions between Basigin gene products and Monocarboxlate Transporters in the olfactory bulb of the mouse

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Localization and characterization of the interactions between Basigin gene products and Monocarboxylate Transporters in the olfactory bulb of the mouse

By

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CERTIFICATE OF APPROVAL

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Table of Contents

List of Figures and Tables

Abstract

Basigin, the mouse form of the human protein EMMPRIN, is commonly found as a transmembrane homodimer with carboxy termini in the cytosol and extracellular amino-termini. Because of its important role as a cell-to-cell junction molecule, and possible implications for cancer research, a Basigin null mouse was developed in 1996 by Igakura *et al.,* to aid in the study of this protein. Their early research demonstrated that Basigin plays a large role in embryonic development. Mice lacking the Basigin gene are blind from the time of eye opening, and have demonstrated a lack of aversion to offensive odors such as acetic acid and isogine, as well as increased sensitivity to electric foot shock (Naruhashi *et al.,* 1997). Further research demonstrated that Basigin is associated with cell-to-cell communication within the retina of the eye (Ochrietor *et al.,* 2002), and in the olfactory system (Igakura *et al*., 1996). It is thought that Basigin acts as a chaperone for several Monocarboxylate Transporters (MCTs), accompanying them for proper placement in the cell membrane. The focus of this current study is to explore the role and function of Basigin in the olfactory bulb of the mouse. Data from biochemical analysis of tissue samples show that MCTs in the olfactory bulb are unaffected by absence of Basigin. Further study involving immunohistochemistry reveals that MCT2 is the most abundant transporter present in normal olfactory bulbs, and that a metabolic defect does not likely underlie the anosmia exhibited by Basigin null mice.

Chapter 1: Introduction

Unicellular eukaryotes have maintained evolutionary fitness and remained extant via efficient self-containment of necessary adaptations for survival. Many of their interactions with other cells involve defense, either from predatory consumers, or from competing species attempting to occupy their niches, and recognition of self or same to enable exchange of genetic material or to prevent cannibalistic feeding on a potential mate (Dzik, 2010). In multi-cellular organisms, on the other hand, cell specialization precludes some types of cellular activities necessary for independent living in exchange for cohesive tissues that focus exclusively on one or more tasks. Differentiated cells of various tissues use many of the same cellular mechanisms as their unicellular ancestors to achieve specialization (Dzik, 2010). Cells acting as part of a tissue must not only avoid killing each other with secreted wastes, but also communicate identity and needs among each other to survive. This identity is the result of the organization of a cacophony of different and competing signals, all of which are either initiated, mediated, recognized, or acted upon by a group of proteins called cell adhesion molecules (CAMs; Aplin *et al.,* 1999).

Cell adhesion molecules are specialized proteins expressed on the plasma membrane. They participate in myriad functions essential to the life of the cell, and by extension, the life of the organism. These functions include: forming simple connections with other cells or with the extracellular matrix, chaperoning molecules from the endoplasmic reticulum or Golgi body to the plasma membrane, identification as self for immune response, cellular migration or extravasation into tissues from the blood, and signal transduction (Belton *et al.,* 2008); they are an intimate part of nearly every aspect

of cellular life (Dalva *et al.,* 2007). Cell adhesion molecules are also integral to the development of embryonic structures that will eventually develop into the mature tissues of an organism. Cell adhesion proteins fall into four families: the immunoglobulin superfamily (IgSF), integrins, cadherins, and selectins (Lodish*,* 2008). Usually present as transmembrane proteins, CAMs consist of a cytoplasmic region that links to the cytoskeleton, one or more transmembrane domains, and an extracellular region involved in either heterotypic or homotypic binding with other CAMs or similar CAMs, respectively (Goldsby *et al.,* 2000). They are also capable of binding to specific regions on the fibrous molecules of the extracellular matrix (Goldsby *et al.,* 2000). Indeed, CAMs are singularly responsible for the existence of tissues qua tissues in the multi-cellular organism. The IgSF proteins are of particular interest, as they are a group of molecules that are fundamental to the immune system, by which the cells of the organism recognize "self" as distinct from foreign bodies (Goldsby *et al.,* 2000). The burgeoning understanding of the multiple functions and interactions of IgSF CAMs in the life of the cell adds to their desirability as a topic for research.

Members of the Ig superfamily can be soluble proteins (antibodies), but the majority of the functions associated with the IgSF are attributed to the transmembrane members. They are readily identified by their characteristic immunoglobulin domain structures, which are found on the extracellular regions of transmembrane IgSF proteins. The Ig domain is a loop-shaped structure that consists of 70-110 amino acids (Goldsby *et al.,* 2000). These domains consist of variable and conserved regions that allow the individual molecules to be distinguished from each other. Because of this variation, IgSF members have been the targets of much research attempting to identify

specific cell-surface epitopes unique to various pathogens, including, and especially, malignant tumor cells (Pignatelli and Vessey, 1994). It was originally believed that these molecules functioned mainly or exclusively in cell identification and recognition by the immune system, but current trends in research show that IgSF members participate in much more than cell recognition (Ariescu and Jones, 2007). In addition to being important recognizable molecules for the immune system, IgSF members are implicated as attachment points for pathogens and unique indicators of malignancy (in fact, some of them are associated with the facilitation of metastasis; Xue *et al.,* 2005). This distinction makes IgSF members especially important and valuable as objects of study.

The immediate benefit to studying IgSF CAMs resides in their association with disease. Understanding how viruses interact with CAM epitopes could lead to medical treatments that address the main causes of viral infections—the ability of viruses to both express host cell proteins and to use proteins expressed on host cell membranes as a means of attachment and entry to the cytoplasm. Additionally, studying IgSF CAMs will impart greater understanding of the mechanisms involved in malignancy and metastasis. Continuing research could also lead to biotechnological solutions for genetic problems that are not related to cancer or infection. The association of certain CAMs with multi-spanner membrane proteins may provide a means to manufacture designer peptides useful for treating a wide variety of disorders, either acquired or hereditary. Indeed, much work has been done in the study of IgSF CAMs, and that work has uncovered many more questions about these fascinating molecules and their functions within the cell.

The purpose of this research is to add to the body of knowledge of Basigin, a ubiquitous IgSF protein with a growing list of associations and functions. Basigin is a glycosylated cell adhesion molecule located on chromosome 10 of the mouse genome (Mouse genome database, 10/2010). The Basigin gene contains eight known exons, which can be differentially spliced to yield more than one gene product. The gene product named Basigin results from the translation of a mRNA product that has seven exons, while the other known gene product, called Basigin-2, contains eight exons (Mouse genome database, 10/2010). The Basigin protein has two Ig-like domains, and its glycosylated form has a mass of 43-66 kDa (Igakura *et al.,* 1996). The differential masses result from the fact that Basigin is glycosylated differently by cells of various tissues (Fadool and Linser, 1993b.). Basigin-2 has a third Ig-like domain and results from the inclusion of exon 1A in the Basigin gene transcript (Ochrietor *et al.,* 2003). Both gene products are commonly found as transmembrane homodimers with cytosolic carboxy termini and extracellular amino-termini.

The structure of Basigin (Figure 1.1) is highly conserved across species. To date, homologues of Basigin (Basigin itself is the name for the protein as it exists in mice) have been located in humans (Biswas *et al.,* 1995), rats (Kasinrerk *et al.,* 1992), chickens (Seulberger *et al.,* 1990), bovine retina (Yoshimoto *et al.,* 1998), and *Drosophila* (Curtin *et al.,* 2005). In humans, the Basigin homologue is known as Extracellular Matrix Metalloproteinase Inducer (EMMPRIN; Biswas *et al.,* 1995) and as Human leukocyte activation antigen, M6 (Kasinrek *et al*., 1992). In rats, the homologues are called Cluster of Differentiation 147 (CD-147), OX-47, CE9, and PE2 (Finnemann *et al.,* 1997). In the bovine retina, the Basigin homologue is referred to as RPE 7 (Yoshimoto *et al.*, 1998),

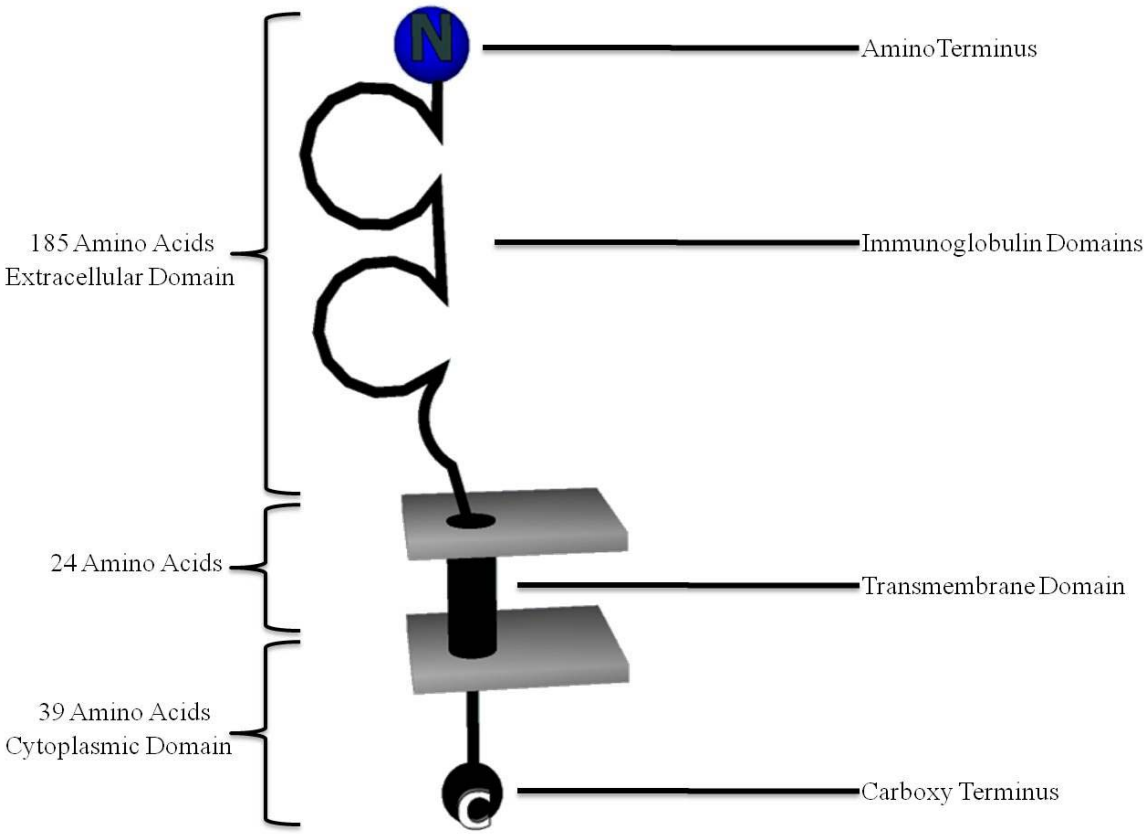

Figure 1.1: A diagram of the Basigin molecule. Basigin consists of ~250 amino acids. The extracellular portion, which contains the Immunoglobulin domains, is ~185 amino acids. Basigin is a single-pass transmembrane protein with a cytosolic carboxy terminus and an extracellular amino terminus (adapted from Wilson *et al.*, 2009).

and in the chicken, HT-7 and 5A11 (Seulberger *et al.,* 1990; Fadool and Linser*,* 1993a.). The high rate of cross-species conservation of these homologues is an indicator that Basigin is of fundamental importance to cellular function.

The early history of Basigin gene products dates back to the 1970s, referencing the fact that Basigin is the antigen for blood group Ok^a, identified on red blood cells (Daniels 2002). It was later noted that the Ok^a blood group antigen is identical to a previously identified marker for M6 leukocyte activation antigen, i.e., Basigin *is* the M6 leukocyte activation antigen marker (Spring *et al.,* 1997). Basigin was later identified as an antigen on the surface of glial cells in the avian retina (Linser *et al.,* 1986). The first monoclonal antibodies for Basigin were derived from embryonic chick retina cells (Linser and Perkins, 1987). It was later transcribed from mRNA isolated from mice (Miyauchi *et al*., 1990). Basigin first emerged into the realm of interest for medical research when it was noted that Basigin is found on the membranes of tumor cells of lung carcinomas and throughout the body on all epithelial layers (Ellis *et al.,* 1989). Later research concluded that Basigin gene products are up-regulated on the plasma membranes of activated T and B lymphocytes (Staffler and Stockinger, 2000). In another study, Basigin was found to be associated with the inducement of extracellular matrix metalloproteinases (MMPs) which are involved in the apoptotic functions of cells and degradation of extracellular matrix proteins (Biswas *et al.*, 1995).

Basigin is putatively associated with cell-to-cell communication in the neural retina of the eye, and in the olfactory system (Igakura *et al.,* 1996). Additionally, Basigin (EMMPRIN) has been implicated in the attempts of tissues to repair and reassemble

subsequent to challenge by injury or cancer (Betsuyaku *et al.,* 2003). This association implicated Basigin as an inducer of the production of interstitial collagenase (Kataoka *et al.,* 1993), which resulted in it first being labeled "tumor cell-derived collagenase stimulatory factor" (Guo *et al.,* 1997). Basigin expression is also known to be up-regulated in tumor cells where it functions to increase the synthesis of matrix metalloproteinases (Zucker *et al.,* 2001). Basigin is also up-regulated in other non-malignant conditions such as rheumatoid arthritis (Konttinen, 2000), chronic liver disease (Shackel *et al.,* 2002), and atherosclerosis (Major *et al.,* 2002). Basigin has become particularly important as a neurological molecule due to research suggesting that human gliomas differentially express Basigin gene products depending on the level of malignancy in the glioma in question (Sameshima *et al.,* 2000). It has also been noted to participate in platelet adhesion to monocytes, stimulating NFκB-driven inflammatory pathways (Schulz *et al.,* 2008).

Basigin was originally scrutinized as a molecule with an important role to play in oncogenesis and cancer progression (Nabeshima *et al.,* 2006), but, in addition to this, research has revealed that it plays a major role in the highly regulated events of embryonic development. In order to better understand this molecule's functions, Takashi Muramatsu and his research team created a Basigin null (-/-) mouse in the laboratory in the 1990s by deleting 0.2 kB from the first exon in the gene, then replacing that section with a neomycin resistance gene and recombining the new construct in cultured pluripotent mouse embryonic stem (ES-D3) cells (Igakura *et al.,* 1998). Male chimeric mice resultant from the ES recombination were then mated with C57BL/6 female mice, producing heterozygotes in the F1 generation. These heterozygotes were interbred to

produce Basigin null mice, whose genotypes were verified via Southern blot analysis (Igakura *et al.,* 1998). Characterization of Basigin null mice revealed multiple embryologic implications resultant from the absence of Basigin, which they concluded to function in intercellular recognition during implantation (Igakura *et al.,* 1998; Muramatsu and Miyauchi 2003). Research using the null mouse has confirmed earlier research in chick retina that concluded that Basigin is integral in neuronal-glial cell interactions in the retina (Fadool and Linser, 1993b.). In fact, Basigin null mice are blind from the time of eye opening (Ochrietor *et al.,* 2002). Additionally, the null mouse has demonstrated difficulty sensing offensive odors, such as acetic acid (Igakura *et al.*, 1996). Of most recent significance is the role of Basigin in the nutrification of neuronal cells.

Nutrient flow restrictions resulting from the presence of the blood-brain barrier have implicated Basigin gene products as necessary molecules for proper transport of nutrients across the plasma membrane (Philp *et al.,* 2003). The tissues of the central nervous system (CNS) utilize neurotransmitter molecules to affect signaling of various sensory and motility data to all areas of the body, and as such, have become adapted to require seclusion from errant molecules that are rather freely distributed by blood flow in other areas of the body. Many of the same molecules found ubiquitously in other tissues are capable of acting as neurotransmitters, and, if allowed to flow into the CNS, could result in a wide array of unintended neurological consequences (Rubin and Staddon, 1999). As such, the capillaries carrying blood to and from the CNS have evolved specialized endothelial cells characterized by a noticeable lack of endocytic vessels and the expression of a large number of tight junctions (Rubin and Staddon, 1999). This combination of tight junctions and reduced endocytic vesicles restricts the flow of many

molecules commonly found in blood to and from the tissues of the CNS, and is the defining constituent of the blood brain barrier (Rubin and Staddon, 1999). The blood-brain barrier effectively restricts the flow of all molecules to and from the brain, excepting those that are very small or lipophillic (Rubin and Staddon, 1999). One difficulty arising from this restriction to the CNS is that some nutrients required by the cells are also hindered or prevented from crossing into the brain. In order to compensate, the cells lining the capillaries in the brain express many proteins associated with active transport or facilitated diffusion of required molecules, such as glucose, amino acids, L-Dopa, and others (Rubin and Staddon, 1999). One group of nutrients that are of great importance for metabolism in the brain are monocarboxylates like pyruvate and lactate, which have limited flow across the blood-brain barrier (Cremer *et al.,* 1979).

Lactate was once thought to be a useless or even harmful dead-end by-product of anaerobic energy metabolism in muscle tissues. Researchers have now come to the conclusion that lactate is the substrate of choice for oxidative metabolism, specifically, as the oxidative source for pyruvate necessary for the mitochondrial tricarboxylic acid cycle in neurons, where the reversible catalytic activity of the enzyme, Lactate Dehydrogenase (Figure 1.2), converts lactate into pyruvate and vice versa on an as-needed basis (reviewed in Schur, 2006). Because of the importance of monocarboxylates such as lactate and pyruvate in cellular metabolism, allowing them to flow freely is essential to the life of the cell. In order to ensure that these monocarboxylates are present in sufficient quantities for metabolism, the CNS expresses a series of molecules that facilitate monocarboxylate diffusion across the plasma membrane. These molecules are known as Monocarboxylate Transporter (MCT) proteins.

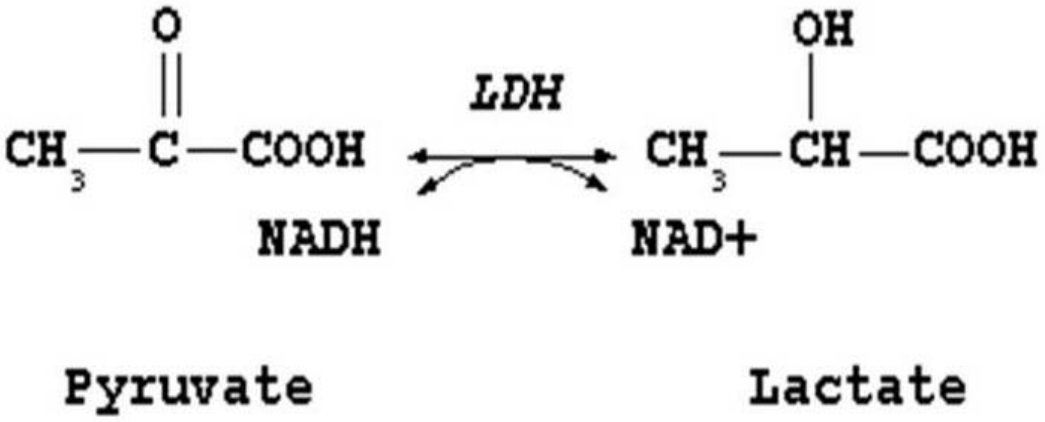

Figure 1.2: A chemical equation depicting the reversible catalytic activity of Lactate Dehydrogenase in the conversion of pyruvate to lactate.

Studies have indicated that Basigin interacts with Monocarboxylate Transporter 1 (MCT1) in such a way as to chaperone the lactate transporter from the Golgi body to the plasma membrane, and that Basigin and MCTs are required to be co-localized for proper insertion of either molecule into the plasma membrane, forming a metabolon (Figure 1.3). These metabolons have the potential to interact between neural and supportive cells (Figure 1.4), creating a dedicated lactate shuttle for rapid transport of the monocarboxylates into the neuron (reviewed in Ochrietor and Linser, 2004). This research indicates that the failure of vision (Hori *et al.,* 2000; Ochrietor *et al.,* 2001) and olfaction (Naruhashi *et al.,* 1997) in the null mouse created by Igakura *et al.,* (1996) may be resultant from metabolic deficiencies due to the absence of the required Basigin-MCT metabolon.

Furthermore, Philp *et al.,* (2003) demonstrated a reduction in the membrane expression of MCTs 1, 3, and 4 in the Basigin null mouse retina, indicating that Basigin is very important in targeting MCT molecules to the plasma membrane. Other studies by Ochrietor *et al.,* (2002) showed that the Basigin null mice exhibit apparently normal retinal architecture, but electroretinographs demonstrate a depression in potentials from the time of eye opening, suggesting that the retina is biochemically malformed, i.e., that Basigin plays a role in the formation of the metabolic machinery that is required for photoreceptor cells to function properly (Ochrietor *et al.,* 2002).

This understanding of how Basigin functions in the retina brings other questions to the forefront, among them being the oddity that null mice demonstrate far less

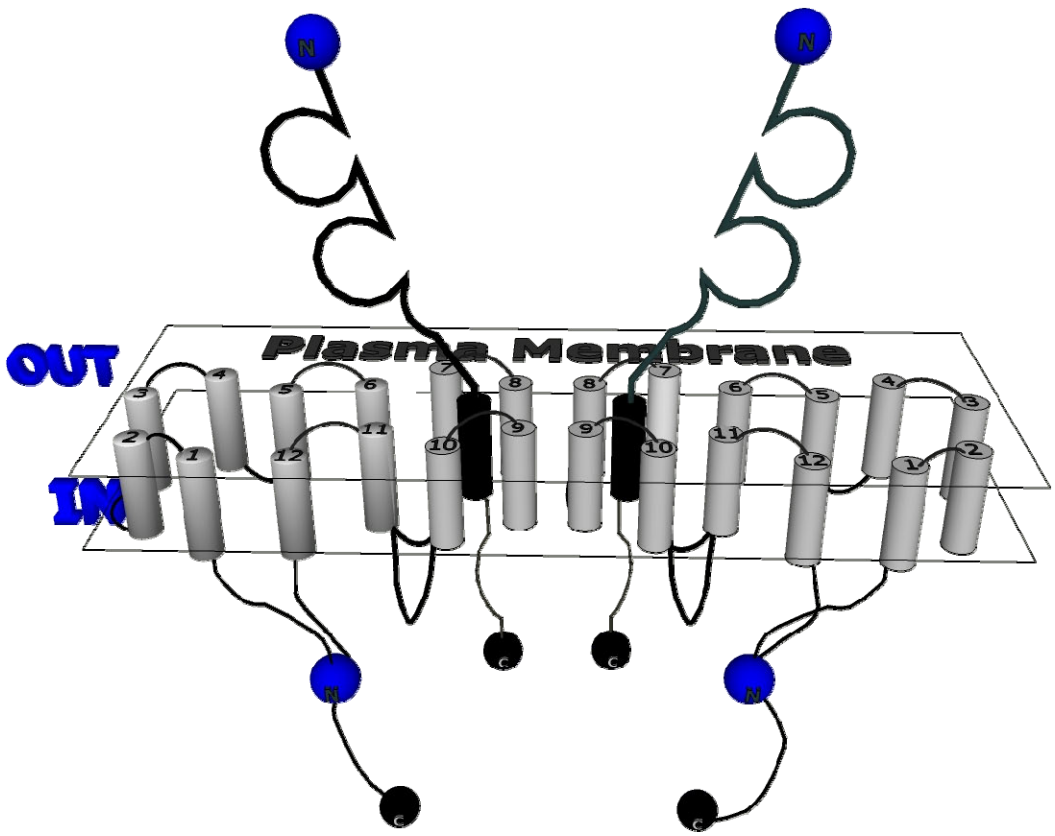

Figure 1.3— A proposed model of the interaction between the Basigin (black) and Monocarboxylate Transporter 1 (MCT1, in gray) 1. The Transmembrane domains of the Basigin homodimer exhibit affinity for those of the MCT1 homodimer (adapted from Wilson *et al.,* 2009).

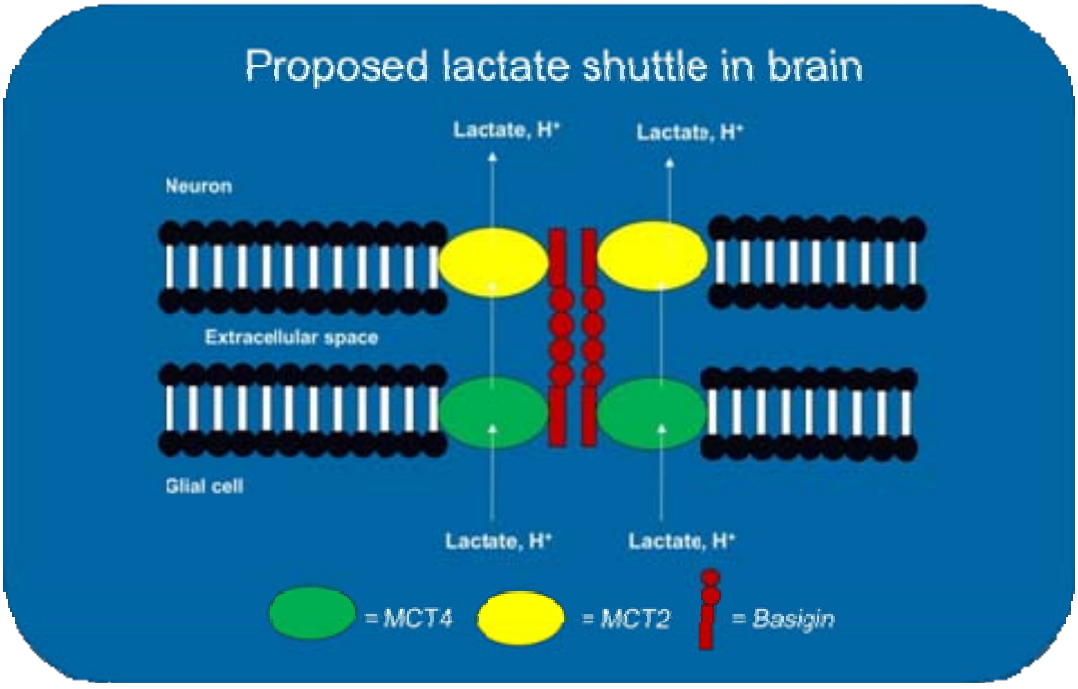

Figure 1.4: Diagram of a proposed lactate shuttle in the brain. Of note are the homophilic interactions between the Basigin homodimers of each cell's metabolon. In this way, lactate can move rapidly from the supportive cell to the neuron.

sensitivity to offensive odors such as acetic acid and isogine than their homozygous normal litter-mates (Igakura *et al.,* 1996). Little to no research has been done on the activity of Basigin in the olfactory system, apart from the discovery of Basigin gene expression in olfactory system tissues (Fan *et al*., 1998). Having identified the gene products there, the question remains as to its functions in the olfactory system of the mouse.

The olfactory system in mice, as shown in Figure 1.5, consists of a group of tissues located in the brain and in the nasal cavity. Olfactory epithelial tissue located in the nasal cavity is separated from the olfactory bulb in the brain by a layer of bone called the cribiform plate (Firestein, 2001). Olfactory cells, in this case, the primary neurons of the olfactory system, extend dendritic cilia into a mucosal layer of the olfactory epithelium (Firestein, 2001).

The cilia of each cell express a particular type of odorant receptor molecule, all of which are G-protein-coupled signal transducers (Buck, 1996). Odorants binding to the receptor proteins trigger the opening and closing of a series of ion channels, which ultimately lead to the depolarization of the cell. Action potentials are transmitted along axonal extensions through the cribiform plate into the olfactory bulb. Multiple cells are stimulated in varying degrees by distinct odorants, and these signals arrive simultaneously (Firestein, 2001). Olfactory receptor neurons terminate and connect with the dendrites of approximately 100 second-order olfactory neurons in the glomeruli of the olfactory bulb (Mackay-Sim and Royet, 2006).

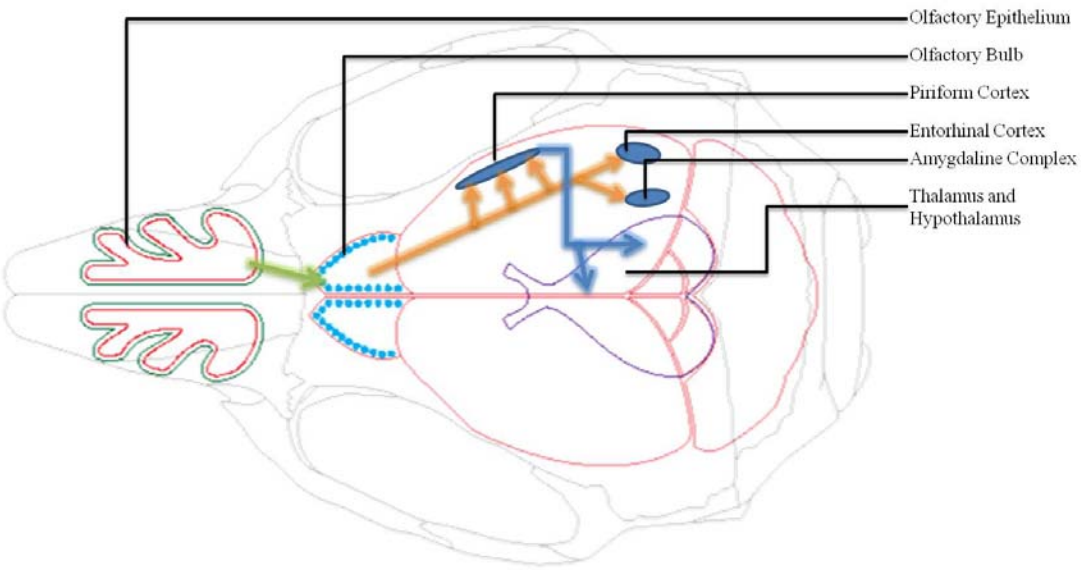

Figure 1.5 – Overview of the olfactory system in mice. Sensory stimuli enter the nose, and bind to G-protein coupled receptors on the dendrites of olfactory sensory neurons (OSNs) in the mucosal layer of the olfactory epithelium in the nasal cavity. Action potentials send signals through the axons of the OSNs, which exit the nose and enter the skull via the cribiform plate, where they terminate and synapse with mitral cells and tufted cells, forming the glomeruli of the olfactory bulb. Signals continue along the mitral axons, which terminate in the internal plexiform and granular cell layer, where they synapse with other neurons headed to other areas of the brain, including the piriform cortex, the entorhinal cortex, and amygdaline complexes. These structures are collectively referred to as the olfactory cortex, as they function in responding to signals from the anterior olfactory neurons. Further signaling occurs from the olfactory cortex areas to the thalamus and hypothalamus. Other neurons feed signals back to the olfactory bulb (not shown). Diagram adapted from deCastro, 2009

There are an estimated 25,000 primary axons converging into each of approximately 2000 glomeruli in the human olfactory bulb (Bear *et al*., 2007). Olfactory receptor neurons are mapped to glomeruli in the olfactory bulb that are specific to the type of odorant to which they are sensitive (Bear *et al*., 2007). The summation of signals from various cell-types in the olfactory epithelium allows the olfactory bulb to differentiate odors and signal their identity to the olfactory cortex in the cerebral cortex. From the cerebral cortex, odor information can be transmitted to the thalamus and other areas of the brain associated with memory and emotions (Bear *et al*., 2007). Olfactory signals are thus represented in the brain both spatially and temporally, as there is great redundancy in the types of odorants to which a particular receptor is sensitive. Each receptor, however, responds with a varying frequency of action potentials to each stimulant, allowing for further fine-tuning of odor recognition by the olfactory bulb (Bear *et al*., 2007).

Many cellular systems are either completely conserved or slightly modified across tissues and organs. Given the nature of neurological functioning from one sensory organ to the next, it is reasonable to assume that the metabolic requirements and structures present in the retina are also present in the olfactory bulb. As such, the hypothesis fueling this study is that in the Basigin null mouse, MCT1 and MCT4 are down-regulated in the plasma membranes of the olfactory bulb, since there is no Basigin present to shuttle the transporters to the membrane. Additionally, these proteins would appear unhindered in the normal mouse.

The primary focus of this work was to localize the expression of Basigin gene products and MCT molecules on the membranes of cells of the olfactory bulbs of mice, and to determine if MCT molecules are mis-expressed in the olfactory bulbs of Basigin null mice. This was accomplished via biochemical analysis of homogenized samples of olfactory tissue from normal and Basigin null mice. Immunoblotting and subsequent densitometry analyses of the samples indicated the presence and relative concentrations of Basigin, MCT1, MCT2, and MCT4 in olfactory tissue. Immunoblotting analyses also revealed that MCTs are not mis-expressed in the absence of Basigin. Immunohistochemistry of the normal mouse olfactory bulb showed that of the three MCT proteins studied, only MCT2 seems to be present in significant quantities, and is expressed by neuronal processes throughout the olfactory bulb. Expression of Basigin was observed in the granule cell layer, as well as on blood vessel endothelial cells, which also express MCT1 and MCT4. This indicates that the anosmia demonstrated by behavior tests in null mice is not resultant from a metabolic defect of the olfactory bulb.

Chapter 2: Materials & Methods

—Generation of Mouse Olfactory Bulb membrane-associated protein extracts—

Normal and Basigin null mice were sacrificed via an approved protocol (UNF IACUC #10-009). Olfactory bulbs were dissected from the brains. The tissue was placed it into a 1.5 ml microcentrifuge tube with 500 µl of detergent lysis buffer—10 mM imidazole, 100mM KCl, 1% Triton X-100, 1mM EDTA, 5mM MgCl (Philp *et al.,* 2003)— and homogenized by drawing the tissue up into a micropipette tip repeatedly. Detergent-solubilized homogenates were incubated on ice for 30 minutes and then centrifuged at 12,000 rpm for 15 minutes. After centrifugation, the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and stored at -80° C.

—Bradford Protein Assay—

The protein concentration of the tissue samples was determined using a Coomassie (Bradford) Protein Assay (Pierce/Thermo Scientific, Rockford IL). Dilutions of bovine serum albumin (BSA, Pierce/Thermo Scientific) were prepared as standards ranging from 0.2 mg/ml to 1.0 mg/ml. Aliquots (5 µl) of each standard were transferred to individual wells of a 96-well polystyrene EIA/RIA microplate (Corning, Corning, NY). Water (5 µl) was used as a blank. Samples of normal and Basigin null mouse olfactory bulb extracts were prepared at either a dilution of 1:4 or 1:1 in distilled water and transferred to wells of the same 96-well plate. Coomassie (Bradford) reagent (250µl) was added to all wells. The absorbance of each standard and sample was measured at 595 nm using a BioTek Powerwave XS plate reader (Biotek Instruments, Winooski,

VT). The average absorbance for each standard was calculated and used to generate a standard curve for BSA using Microsoft Excel Software (Redmond, WA). The standard curve was used to perform a linear regression to derive a formula used to calculate the protein concentrations of the olfactory bulb extracts.

—Analysis of protein extracts—

Aliquots (100 μg each, 20 μl total volume) of normal and Basigin null mouse olfactory bulb tissue were transferred to a 0.5 ml microcentrifuge tube. To each sample 6.6 μl of LDS buffer (Invitrogen Corporation, Carlsbad, CA) and 1.5 μl of β-mercapto ethanol (Fisher, Fairlawn, NJ) each were added per 50 μg of sample protein. Samples were heated to 95 °C for 5 minutes. Proteins (50 μg per lane) were separated within individual wells of a 4-12% gradient polyacrylamide gel using BupH Tris-HEPES SDS running buffer (Pierce/Thermo Scientific). Other wells contained Prosieve® marker protein (10 μl, Lonza, Rockland, ME). The proteins were electrophoresed at 120 volts until the dye front fully migrated through the gel.

—Electro-blotting of protein extracts—

The polyacrylamide gel was placed in a pan with protein transfer buffer (25mM Tris base, 190mM glycine, 0.1% SDS, 20% methanol). A transfer chamber was prepared by placing pre-cut pieces of sponge pad (Invitrogen Corporation**)** into the chamber, followed by a pre-cut piece of Whatman #1 filter paper (Whatman, Piscataway, NJ). Next, the gel was placed in the chamber, and covered with a pre-cut piece of nitrocellulose transfer membrane (Osmonics/GE, Minnetonka, MN) followed by a second

piece of Whatman #1 filter paper. The remaining space in the chamber was filled with more pre-cut pieces of sponge pad. The chamber was sealed and placed in the SureLock apparatus (Invitrogen Corporation), and then subjected to 30 volts of current for 60 minutes. The apparatus was disassembled and the nitrocellulose paper was incubated in 20 ml Fast Green stain (0.1 % fast green in a solution of methanol, acetic acid, and H_2O in a 5:1:5 ratio) and destained with three changes of 50 ml de-staining solution (methanol, acetic acid, and H_2O in a 5:1:5 ratio). The blots were stored in water at room temperature until the immunoblotting analyses were performed.

—Immunoblotting Analyses—

The blots were incubated in Blotto (2% Instant powdered milk in TBS-T—137 mM Sodium Chloride, 20 mM Tris, 0.1% Tween-20) for one hour at room temperature, and then the lanes were cut into strips to allow for simultaneous incubation with antibodies specific for Basigin (Ochrietor *et al.,* 2003), MCT1 (Millipore, Billerica, MA), MCT2 (Millipore), or MCT4 (Millipore). To each of 4 Petri dishes were added a strip of nitrocellulose containing normal and Basigin null mouse olfactory bulb proteins and 10 ml Blotto. Next, rabbit anti-mouse polyclonal antibodies for Basigin (Ochrietor *et al.,* 2003), MCT1 (Millipore), MCT2 (Millipore), or MCT4 (Millipore) were each added to a dish (10 µl each). The samples were gently shaken at 70 rpm at 37°C for 60 minutes. Samples were washed with several changes of 20 ml TBS. Then, 10 ml of Blotto containing polyclonal Alkaline Phosphatase-conjugated goat anti-rabbit secondary antibody (5 µl, Pierce/Thermo Scientific) was added to each dish. Blots were shaken at 70 rpm at 37° C for 30 minutes. Blots were washed in TBS as described. An alkaline

phosphatase substrate (Bio-Rad, Hercules, CA) was used to develop each blot, as directed in the manufacturer's instructions. Blots were incubated in ~10ml of substrate at room temperature with shaking until signal developed. Blots were dried and imaged using a Gel Logic 1500 Imaging System (Carestream Health, Inc., Rochester, NY) with Molecular Imaging software (Kodak, Rochester, NY). For each analysis, Microsoft Excel software was used to calculate the mean intensity of the signals. The mean intensity of the signal for the normal sample was set to 100% and the null sample was compared.

—Histological preparation of mouse olfactory bulbs—

Normal (for Basigin) mice were sacrificed via an accepted protocol (UNF IACUC #10-009) and the brains were immediately removed and fixed in a 4% paraformaldehyde solution for 48 hours. Tissues were then transferred into 70% ethanol and stored at 4° C. Tissues were dehydrated in increasing concentrations of ethanol (70% to 100%) and then paraffin embedded using a Microm STP 120 Spin Tissue Processor (Microm International / Pierce / Thermo Scientific, San Jose, CA). Sections were cut using a model 820 rotary microtome (American Optical, Southbridge, MA) set at 5 micron thickness. Sections were transferred from the microtome to a warm water bath. After warming, sections were adhered to microscope slides (Fisher) treated with a drop of poly-L-lysine to facilitate adhesion. Slides containing tissue sections were stored at room temperature.

—Histochemistry and immunohistochemistry—

For histochemistry, tissue sections were rehydrated in Citrisolv (Fisher) for three rounds of 10 minutes each, followed by 5 minute washes in decreasing concentrations (100% to 70%) of ethanol. Sections were stained with cresyl violet according to the protocol listed in the MasterTech staining kit (American MasterTech Scientific, Inc., Lodi, CA). Briefly, after dehydration, sections were rinsed in H_2O, and then placed in cresyl violet solution for 10 minutes, followed by a rinse in H_2O. Each section was then dipped in 70% reagent alcohol 10 times. Next, sections were dipped in absolute alcohol 3 times, followed by clarification in Citrisolv for 3 rounds of 5 minutes each. A drop of permount (Fisher) was added to each slide, and a 22 x 40 mm cover slip (Fisher) was applied.

For immunohistochemistry, sections were rehydrated as described and incubated in a pre-incubation solution (TBS containing 2% normal goat serum and 0.0015% Triton X-100) for 60 minutes at 37º C in a humid chamber. Pre-incubation medium was removed and 200 µl of primary antibody solution was added to the slides and the slides were incubated at 37º C for 60 minutes in a humid chamber. The antibodies used include those specific for Basigin (diluted 1:200 in the pre-incubation buffer; Ochrietor *et al.,* 2003), MCT1, MCT2, and MCT4 (diluted 1:100 in pre-incubation buffer; Millipore), and neuron-specific tubulin (diluted 1:100 in pre-incubation buffer; Millipore). Slides were washed 10 times each by adding TBS solution to each slide and then pouring it off after one minute. Secondary antibody solutions were prepared by adding Alexa-Fluor 594 polyclonal goat-anti-rabbit antibodies (diluted 1:100 in pre-incubation buffer; Invitrogen

Corporation) and FITC goat anti mouse (diluted 1:100 in pre-incubation buffer; Invitrogen Corporation). Aliquots of 200 µl of secondary antibody solution were added to each slide as appropriate. After 30 minutes of incubation at 37°C in a humid chamber, the slides were washed as described. A drop of a solution made by dissolving a small flake of p-phenylenediamine in 30% glycerol was added to each tissue sample and cover slips were applied to the slides. All slides were stored at -20º C. The sections were imaged using a TCS SP5 II confocal microscope (Leica, Deerfield, IL) equipped with Leica Confocal Software for image acquisition and adjustment at the University of Florida Whitney Laboratory for Marine Biosciences. Microsoft PowerPoint software was used to generate the figures.

Chapter 3: Results

Mindful of the fact that very few published studies deal with Basigin in the olfactory bulb, and fewer still deal with the relationship between Basigin and MCTs in the same tissue, a primary objective of this study was to localize these proteins in the olfactory bulb, and discuss any implications based upon the data. Membrane-associated olfactory bulb proteins extracted from normal and Basigin null mice were separated using SDS-PAGE, electroblotted, and analyzed via immunoblotting using an alkaline phosphatase detection system. Once the signal had been developed, imaging and densitometry software were used to quantify the relative amounts of proteins present in normal versus Basigin null samples. Immunoblotting data (Figure 3.1) confirmed that Basigin is present in the mouse olfactory bulb. The signal was observed at 45 kDa. Additionally, the absence of an additional signal at 55 kDa. indicates that the Basigin-2 gene product is not present in the olfactory bulb. MCT2 was also present in both normal and Basigin null mouse olfactory bulbs; however, the signals for MCT1 and MCT4 were difficult to detect in either normal or Basigin null samples. The other aim of the biochemical analysis was to determine whether any of the MCTs were mis-expressed at the membrane as a result of the absence of Basigin in the null mouse. The blots (Figure 3.1) show that the signal for MCT2 is present in relatively similar quantities in both normal and Basigin null mice. Additionally, MCT1 and MCT4 are under-represented in both normal and Basigin null mice.

The immunoblotting results led to questions about the expression of these four proteins in the normal mouse olfactory bulb. To visualize the architecture of the tissue, sections of normal mouse olfactory bulbs were stained with cresyl violet (Figure 3.2).

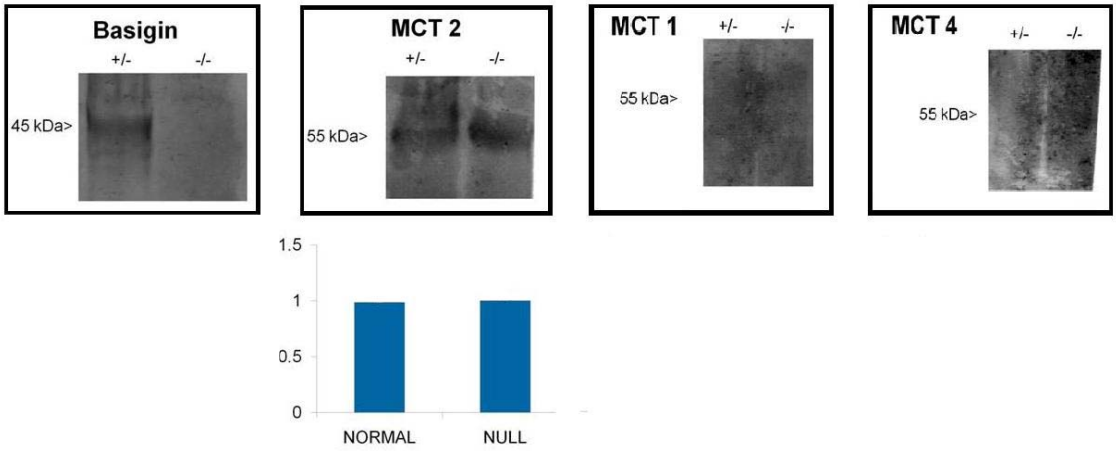

Figure 3.1: Immunoblot images of normal (+/-) and Basigin null (-/-) mouse olfactory bulb protein extracts. Basigin and MCT2 signals are distinct in the +/- samples, whereas MCT2, but not Basigin is present in the -/- samples. The signals for MCT1 and MCT4 are too faint to be detected in either sample.

Cresyl staining identifies Nissl substance—rough endoplasmic reticulum within neuronal cell (Krause, 1996). Tissue sections stained using this method show the cell bodies within the olfactory bulb. In the outer region lies the glomerular cell layer (G; Figure 3.1). Each glomerulus, as shown in Figure 3.3, is made up of processes of olfactory neurons from the nasal epithelium, processes of mitral cells, and periglomerular neurons, and the processes of astrocytes and olfactory ensheathing cells. More centrally positioned within the olfactory bulb is the external plexiform layer (EP), which contains the processes of mitral cells and the mitral cell layer (M), in which the cell bodies of mitral cells are located (Figure 3.2). Mitral cells synapse with granule cells, whose cell bodies are located in the granular cell layer (GC) and whose processes extend into the internal plexiform layer (IP; Figure 3.2). From this area, processes extend caudally to other areas of the brain. The olfactory bulb is a relay that interprets the spacial and temporal values of signals coming from the olfactory mucosal epithelium in the nasal cavity and sends the interpreted signal to other areas of the brain for discrimination and behavior modulation in response to odor stimuli.

Immunohistochemical analyses of the expression of Basigin and MCTs in the mouse were then performed. Normal mouse brains were isolated and prepared. Basigin gene expression was observed throughout the granule cell layer, as well as blood vessel endothelial cells of the mouse olfactory bulb (Figure 3.4)

Immunohistochemical analyses of MCT expression supported the results of the immunoblotting analyses. Significant expression of MCT2 was observed throughout the tissue (Figure 3.5), especially within cells surrounding the glomeruli, the mitral cell layer,

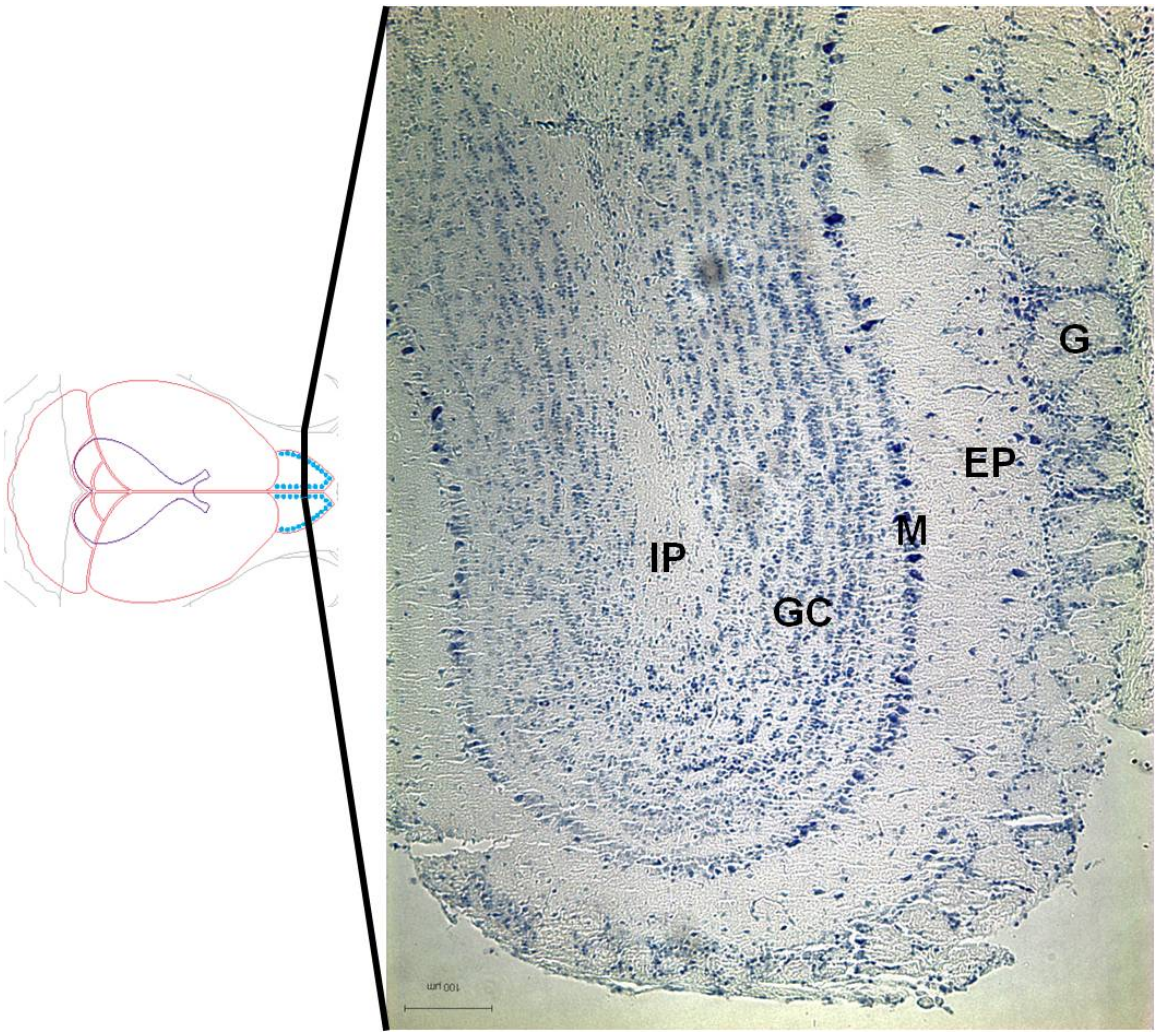

Figure 3.2: Cresyl violet stain of normal mouse olfactory bulb. The cartoon diagram at the left indicates the relative position of the coronal section. Normal mouse olfactory bulbs were fixed, embedded in paraffin, and sectioned at 5 µm thickness. Sections were rehydrated and stained with cresyl violet as described. The magnification bar represents 100 µm. Abbreviations: G = Glomerular cell layer; EP = External Plexiform layer; M = Mitral cell layer; IP = Internal Plexiform layer; GC = Granule cell layer.

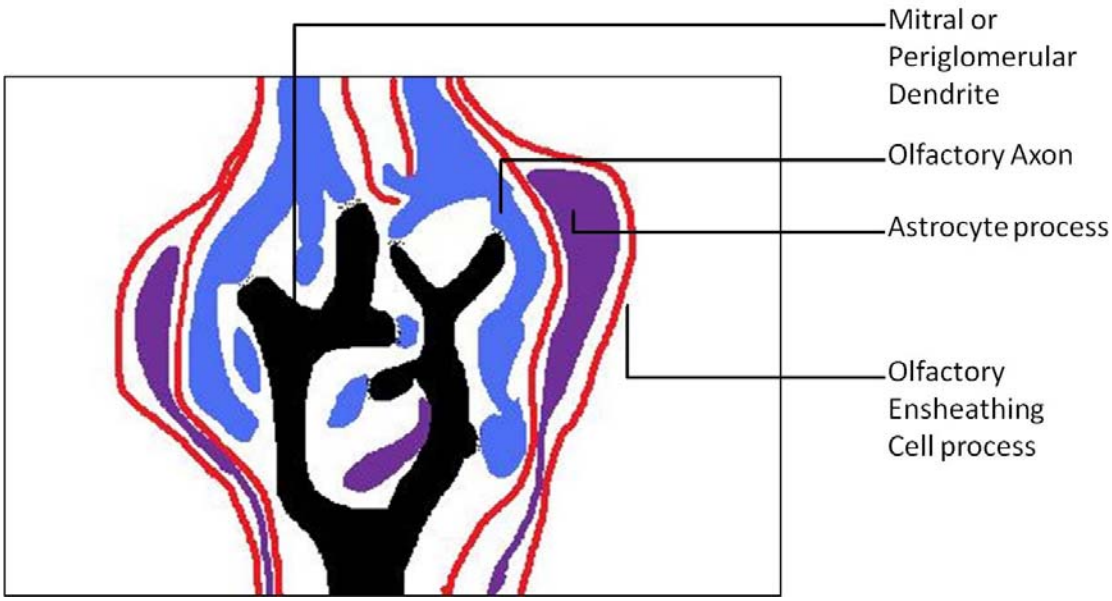

Figure 3.3: Diagram of the cellular architecture of a single glomerulus in the olfactory bulb. Primary olfactory axons (blue) synapse with both mitral and periglomerular cell dendrites (black). Olfactory ensheathing cell (red) and astrocyte processes (purple) envelop each glomerulus (adapted from Raisman 2001).

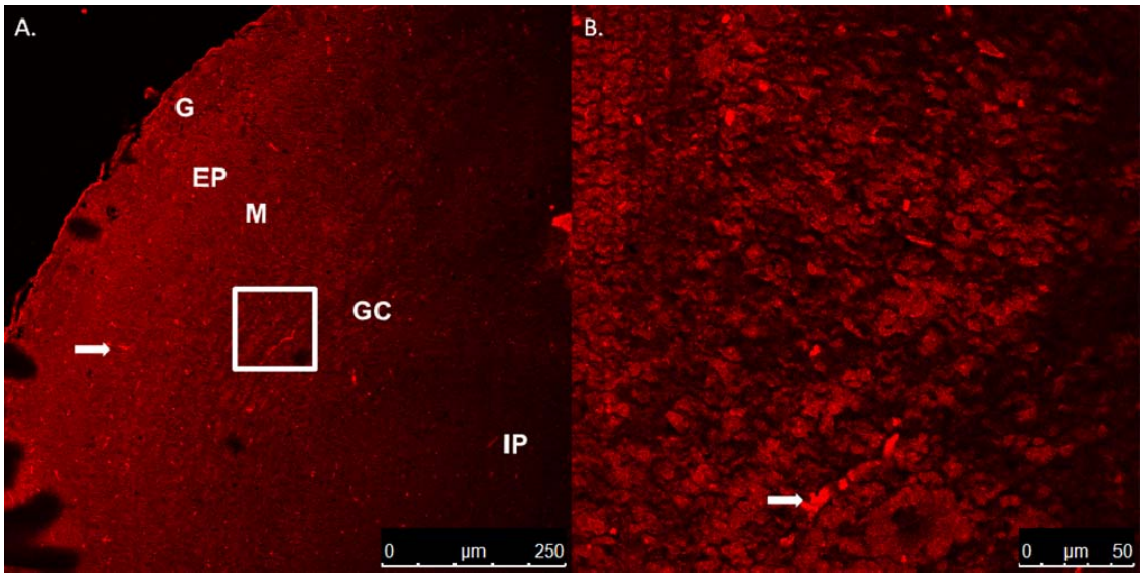

Figure 3.4: Basigin gene expression in the mouse olfactory bulb. Normal mouse olfactory bulbs were fixed, embedded in paraffin, and sectioned at 5µm thickness. Sections were rehydrated and subjected to immunohistochemistry using an antibody specific for Basigin (Ochrieter *et al.,* 2003) and Alexa Fluor 594 secondary antibody (Invitrogen Corporation). Expression was observed within the granule cell layer and blood vessel endothelial cells (arrows). Magnification bar in A = 250 µm; magnification bar in B = 50 µm. Abbreviations: G = Glomerular cell layer; EP = External Plexiform layer; M = Mitral cell layer; IP = Internal Plexiform layer; GC = Granule cell layer.

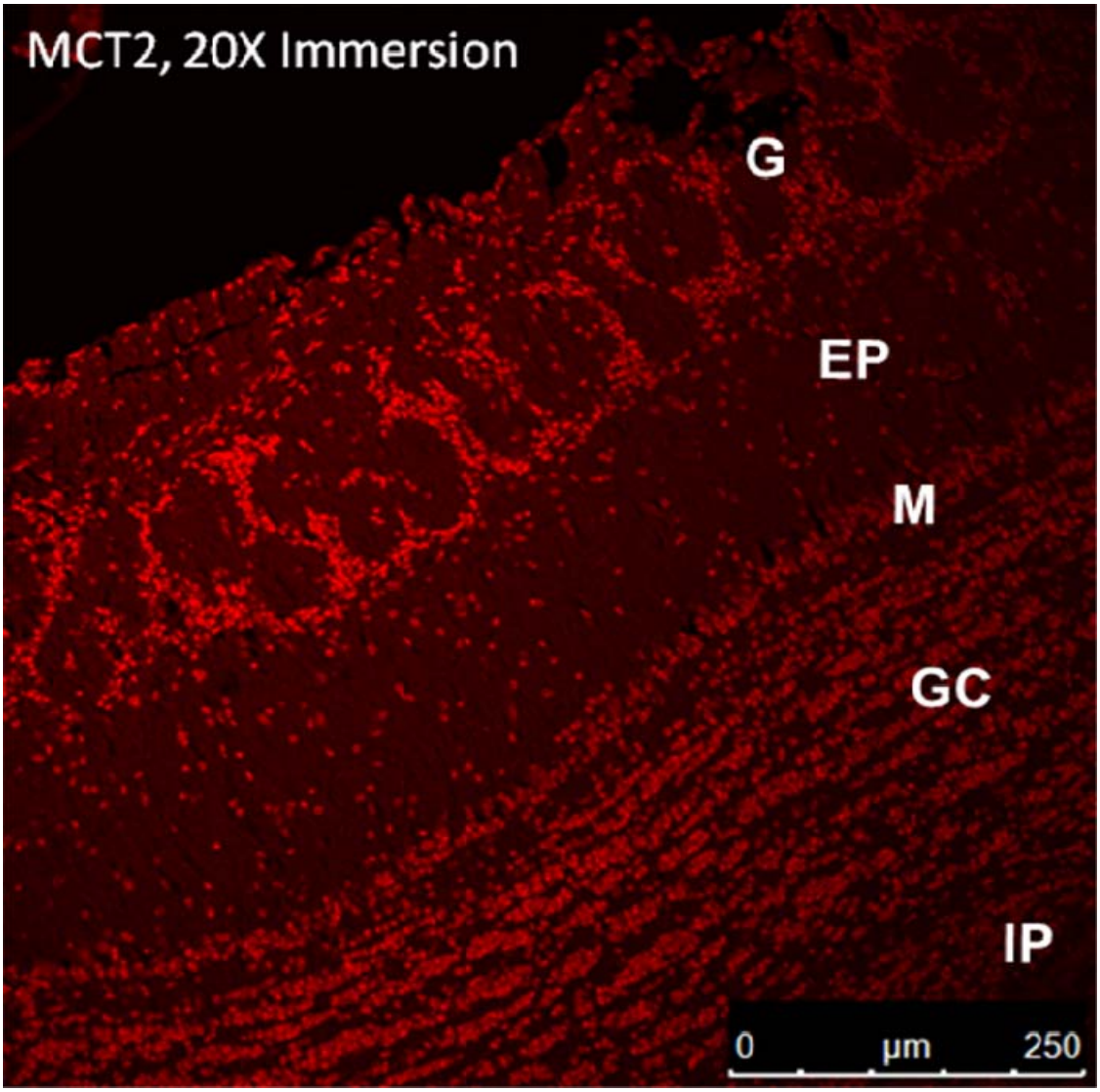

Figure 3.5: MCT2 gene expression in the mouse olfactory bulb. Normal mouse olfactory bulbs were fixed, embedded in paraffin, and sectioned at 5 µm thickness. Sections were rehydrated and subjected to immunohistochemistry using an antibody specific for MCT2 (Millipore) and AlexaFluor 594 secondary antibody (Invitrogen Corporation). Expression was observed throughout the glomerular, mitral, and granule cell layers of the olfactory bulb. Magnification bar = 250 µm. Abbreviations: G = Glomerular cell layer; EP = External Plexiform layer; M = Mitral cell layer; IP = Internal Plexiform layer; GC = Granule cell layer.

and the granule cell layer. Expression of MCT 2 in the glomerular cell layer was determined to be within neurons, as MCT2 expression overlapped that of neuron specific Tubulin (figure 3.6). In contrast, expression of MCT1 and MCT4 was observed only within blood vessel endothelial cells (Figure 3.7)

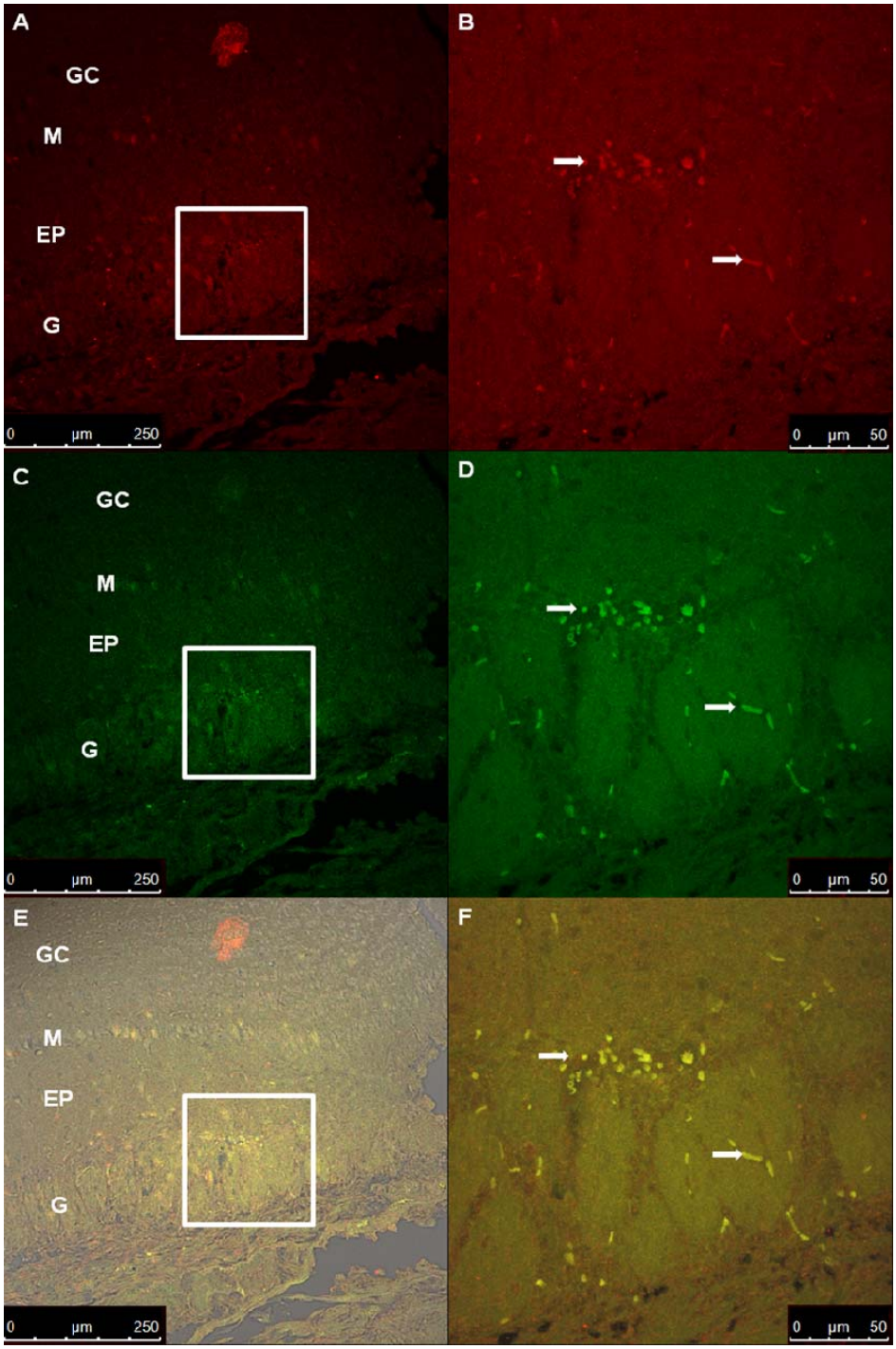

Figure 3.6: Expression of MCT2 and neuron-specific Tubulin within the olfactory bulb of the mouse. Normal mouse olfactory bulbs were fixed, embedded in paraffin, and sectioned at 5µm thickness. Sections were rehydrated and subjected to immunohistochemistry using antibodies specific for MCT2 (Millipore; A, B) and neuron-specific Tubulin (Millipore; C, D). Expression of both proteins was observed in neuronal processes (arrows). Overlay images (E, F) confirm co-localization of MCT2 and Tubulin expression. Magnification bar in A, C, E = 250 µm; magnification bar in B, D, F = 50 µm. Abbreviations: G = Glomerular cell layer; EP = External Plexiform layer; M = Mitral cell layer; GC = Granule cell layer

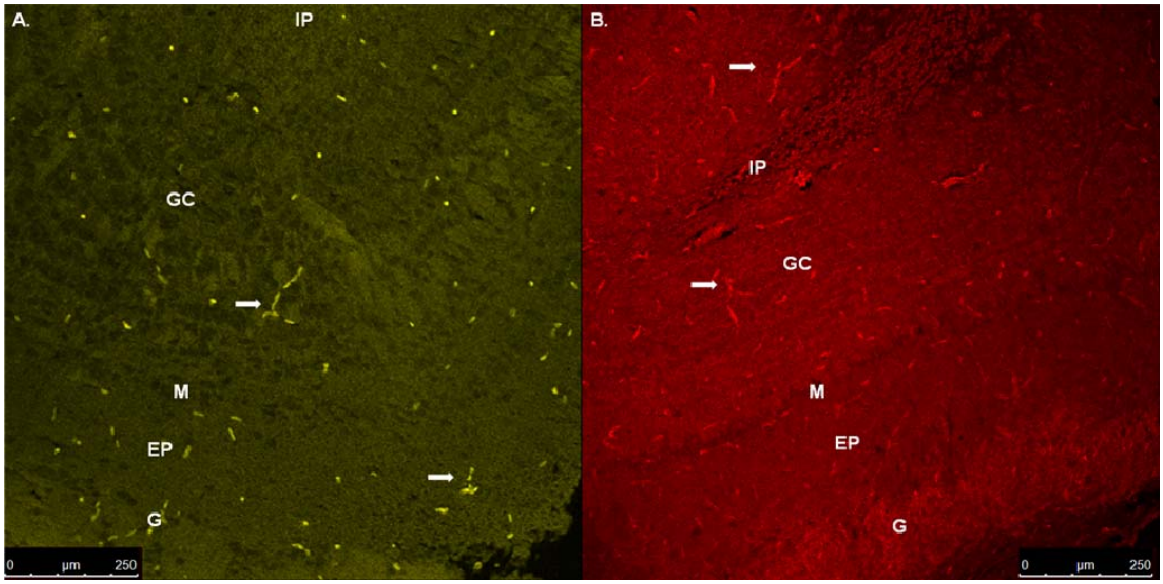

Figure 3.7: Expression of MCT1 and MCT4 within the mouse olfactory bulb. Normal mouse olfactory bulbs were fixed, embedded in paraffin, and sectioned at 5µm thickness. Sections were rehydrated and subjected to immunohistochemistry using antibodies specific for MCT1 (Millipore; A) and MCT4 (Millipore; B), and AlexaFluor 594 Secondary antibody (Invitrogen Corporation). Expression was observed exclusively in the blood vessel endothelial cells (arrows). Abbreviations: G = Glomerular cell layer; EP = External Plexiform layer; M = Mitral cell layer; IP = Internal Plexiform layer; GC = Granule cell layer.

Chapter 4: Discussion

The Basigin null mouse, originally developed as a means to study the effects of Basigin in the generation and progression of cancer (Igakura *et al.*, 1996), has taken on a new role in the study of cellular interactions. When researchers began analyzing the molecule, it quickly became clear that Basigin has many more functions in the body than simply the inducement of extracellular matrix metalloproteinases. Not only has Basigin been implicated as a player in the functioning of neurological processes (Fadool and Linser, 1993a.), but study of the protein has led to a greater understanding of metabolic functions of neurons (Ochrietor and Linser, 2004). Prior to this work, it was already understood that Basigin and MCTs interact, forming a metabolon that serves as a rapid shuttle for monocarboxylates like pyruvate and lactate (Philp *et al.*, 2003). In the retina, this shuttle is thought to be of utmost importance, as the photoreceptor cells need a constant and rapid supply of lactate to promote neuronal function (Philp *et al.*, 2003). In absence of the metabolon, the photoreceptor neurons never functions (Ochrietor *et al.,* 2002) and eventually degenerate (Ochrietor *et al.,* 2001). The hypothesis underlying the present study is that the apparent anosmia noted in the null mouse by Igakura *et al*. (1996) is likely due to a similar absence of Basigin and MCT. To investigate the hypothesis, membrane-associated proteins from normal and Basigin null mouse olfactory bulbs were subjected to immunoblotting analyses to determine the presence of MCTs within the tissue and also to determine if mis-expression is observed in the absence of Basigin. As expected, Basigin was present in the normal, but not the null mouse. A signal for Basigin, but not Basigin-2 was observed in the samples, which is consistent with the literature (Ochrietor *et al.,* 2003). MCT2 is prominent in both normal and

Basigin null mouse olfactory bulbs; however the concentrations of MCTs 1 and 4, in both normal and Basigin null mice, appeared to be too low to get a conclusive signal on the blot.

The data from the immunoblotting assays fueled an investigation of the localization of expression of the four proteins in a normal mouse olfactory bulb. Basigin is expressed in blood vessel endothelial cells as well as granule cells within the normal mouse olfactory bulb. MCT2 is expressed throughout the olfactory bulb, specifically in the glomerular, mitral, and granule cell layers of the tissue. MCTs 1 and 4 are expressed exclusively in the blood vessel endothelial cells, and not in any of the other layers of the tissue.

Results of the immunohistochemical and biochemical analyses suggest that Basigin functions differently in the olfactory bulb than in the retina. In the retina, Basigin gene products interact with MCT1 and MCT4 at the plasma membrane of Müller glial cells, photoreceptor neurons, and blood vessel endothelial cells (Philp *et al.,* 2003). In the olfactory bulb, such interactions are limited to blood vessel endothelial cells. Expression of Basigin and MCT2 does not overlap within the granule cell layer. In addition, the relative abundance of MCT2 in the Basigin null olfactory bulb is consistent with previous data that concludes MCT2 does not interact with Basigin (Philp *et al.,* 2003; Wilson *et al*., 2005). Therefore, no neuron-glial coupling via a Basigin-MCT metabolon is observed in the mouse olfactory bulb.

These data fail to support the hypothesis stated. Basigin does not appear to be involved in a metabolon relationship with MCT1 or 4 in the olfactory bulb that couples

neurons and glial cells. It is therefore unlikely that a metabolic deficiency within the Basigin null olfactory bulb underlies the anosmia observed in these animals.

One explanation of the different functions of Basigin in the eye and in the olfactory bulb has to do with cellular anatomy. The blood brain barrier is an effective gate against the flow of molecules like lactate into the central nervous system (Rubin and Staddon, 1999). As such, cells within the CNS must either synthesize the chemicals they need or be completely dependent upon active transport or facilitated diffusion from the blood to obtain them. One important cell that helps to offset the deficit of lactate in the CNS is the astrocytes, which has been noted to produce significant quantities of lactate and pyruvate for use within the CNS (Pellerin *et al.*, 1998). These supportive cells can effectively alleviate any deficit of metabolites within the neurons in their vicinity. This suggests that perhaps there is an inverse relationship between the location of astrocytes in the CNS tissue and the necessity for the Basigin-MCT complex. In the olfactory bulb, each glomerulus is intimately associated with one or more astrocytes (de Saint Jan and Westbrook, 2005), whereas in the retina, the location of astrocytes is limited by the location of the vasculature in that they do not intimately associate with the photoreceptor neurons (Schnitzer, 1987). Since the glomeruli are the points of entry for neurological signals from the olfactory epithelium outside the brain, it stands to reason that perhaps the region surrounding these cells is adequately supplied with monocarboxylates from astrocytes that can be transported via the high affinity MCT2 molecule into the neuronal cells.

The co-localization of neuron-specific tubulin with MCT2 on neuronal cell processes indicates that MCT2 is indeed expressed by neurons within the mouse olfactory

bulb. It was determined that MCT1 and 4 are expressed by the blood vessel endothelial cells, which explains the faint signal observed on the immunoblots. It is likely that Basigin interacts with MCT1 and MCT4 on blood vessel endothelial cells, which transport monocarboxylates from the bloodstream into the tissue. It is not yet known whether MCT1 or MCT4 mis-expression occurs on the blood vessel endothelial cells of the Basigin null mouse. Although mis-expression is likely, it probably does not account for the selective anosmia observed in Basigin null mice.

The fact that the olfactory bulb is not the primary source of sensory input is indicative of lowered metabolic requirements. Looking to the model of the retina, high energy requirements necessitated a metabolon shuttle for lactate. The metabolic needs of the retina are high so as to maintain ready sensory cells that can be stimulated rapidly and repeatedly, as opposed to cells of the CNS, which are not involved in such constant and rapid stimulation as is typical of sensory stimulation (Sokoloff, 1999). It is possible that the olfactory mucosal epithelium in the nose has similar metabolic requirements to those of the retina. Since the olfactory bulb is merely interpreting signals from the olfactory mucosa, it stands to reason that it may not have the same requirement for rapid energy replenishment that would be inherent in the place where sensation is occurring.

Basigin null mice respond with lessened sensitivity to the presence of offensive odors like acetic acid and isogine (Igakura *et al.,* 1996). This was determined by placing a filter paper coated with the irritating odorant under the bedding of the mice, and then counting the number of visits to the area with the odorant (Igakura *et al.,* 1996). Basigin null mice were noted to visit the area more often than their normal littermates (Igakura *et al.,* 1996). This led the researchers to conclude that Basigin null mice have lessened

olfactory capability (Igakura *et al.,* 1996). Results from this project indicate that the source of the anosmia may not be in the olfactory bulb. It is possible, however, that absence of basigin has deleterious effects within the nasal epithelium that result in anosmia.

It is also possible that the Basigin null animals are quite capable of detecting the irritating odors. Kobayakawa *et al*., (2007) indicate that there are multiple ways in which odor sensation and behaviors in response to that odor may be limited. They noted that the glomeruli seem to be subdivided into three domains that line up along the dorso-ventral axis in the olfactory bulb, and are associated with interpretation and mediation of different odors (Kobayakawa *et al.*, 2007). Kobayakawa's research group created mutant mice that did not express the normal population maps of the olfactory glomeruli. Changing the mapping of odorants in this way altered the avoidance behavior of the mice (Kobayakawa *et al.*, 2007). Failure to avoid irritating odors involves both sensation of the odor and behavior in response to the sensation. In order to properly identify an odor, it is necessary that the olfactory architecture be properly arranged. If odors are not properly recognized as in the mutant mice above, behavioral responses to them will vary from the norm. It is possible that Basigin null mice have improperly coded glomeruli, resultant from errors in embryological development associated with the absence of Basigin.

In order to determine the nature of the anosmia in Basigin null mice, further study will need to answer these questions:

- What is the full extent of the olfactory insensitivity of Basigin null mice?
- Is the population coding of the olfactory glomeruli intact?

- Is the neurological framework of the nasal epithelium intact?
- Is there Basigin-dependent modulation of behavior in response to odorants that occurs in the brain outside the olfactory bulb?

Basigin is a rather ubiquitous protein whose functions are only beginning to be fully documented. Little is known of the ways that Basigin affects the development and function of the brain in mice. Even less is known of the role of the molecule in olfaction. The purpose of this study was to expand upon what is known. The data resultant from the various assays conducted herein lead to the conclusion that any anosmia in the Basigin null mouse is not resultant from a Basigin-mediated metabolic malfunction in the olfactory bulb. Further study will be required to uncover the nature of the peculiar insensitivity to irritating odors of the Basigin null mouse.

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