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Characterization of Basigin and the Interaction Between Embigin and Monocarboxylate Transporter-1, -2, and -4 (MCT1, MCT2, MCT4) in the Mouse Brain

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Characterization of Basigin and the interaction between Embigin and monocarboxylate transporter-1, -2, and -4 (MCT1, MCT2, MCT4) in the mouse brain

By

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Abstract

Basigin and Embigin are members of the immunoglobulin superfamily that function as cell adhesion molecules. Studies of Basigin null mice revealed reproductive sterility, increased pain sensitivity, and blindness. It is thought that the mechanism causing blindness involves misexpression of monocarboxylate transporter 1 (MCT1) in the absence of Basigin. It is known that the transmembrane domain of Basigin interacts with MCT1. In the absence of Basigin, MCT1 does not localize to the plasma membrane of expressing cells and photoreceptor function is disrupted. Studies of the Basigin null mouse brain suggest that MCT1 is properly expressed, which suggests a separate mechanism causes the increased pain sensitivity in these animals, and also that a different protein directs MCT1 to the plasma membrane of expressing cells in mouse brain. Embigin is known to interact with MCT2 in neurons and with MCT1 in erythrocytes. It is not known, however, if Embigin normally interacts with MCT1 in the mouse brain or if Embigin acts to compensate for the lack of Basigin in the Basigin null animals. Therefore, the purpose of this study was to determine if Embigin normally interacts with MCT1, 2, or 4 in the mouse brain and if so, whether the interaction is similar to that between Basigin and MCT1. Expression of Basigin, Embigin, MCT1, MCT2, and MCT4 in mouse brain was assessed via immunoblotting and immunohistochemical analyses. In addition, recombinant protein probes corresponding to the Embigin transmembrane domain were generated for ELISA binding assays using endogenous mouse brain MCTs. It was determined that the proteins in question are rather ubiquitously expressed throughout the mouse brain, and that the cell adhesion molecules Basigin and Embigin may be co-expressed in the same cells as the MCT2 and MCT4 transporter proteins. In addition, it was determined that the Embigin transmembrane domain does not interact with the MCTs. The data therefore suggest that MCTs do not require Basigin or Embigin for plasma membrane expression in mouse brain.

Introduction

The brain is known to be "the most complex piece of matter in the universe" (Bear et al., 2007). Therefore, understanding this organ has created quite a challenge. Neuroscientists have broken down descriptive and functional aspects of the brain into several levels including molecular, cellular, systematic, behavioral and cognitive. The molecular view-point of the brain examines molecules that allow neuron growth and communication, and can monitor what enters and leaves the neurons. Cellular studies of the brain show that various neurons interact with one another and can influence each other. An examination of the systems or complex circuits of neurons reveals that there is an increase in complexity as each circuit varies in how sensory information is analyzed and movements are executed. In addition to basic senses and movements, the brain is able to regulate mood and behavior through normal contributions from these systems. Finally, the cognitive abilities of the brain allow for mental imagery, the use of language, and self-awareness, indicating how extensive the brain's functions are and why it remains an area of enormous interest and exploration (Bear et al., 2007).

The cognitive component of brain function, in addition to discriminative and affective-motivational aspects, unites to produce the sensation of pain that is consciously experienced (Casey, 1999). Pain is the feeling or perception arising from the body, caused by nociception or the sensory process through which painful stimuli are signaled (Bear et al., 2007). This feeling varies among individuals as mood and emotional state can alter the perception of pain. In patients suffering from chronic diseases, the presence of pleasant music, photographs and humorous films reduced pain perception while elements negatively affecting emotions appeared to increase pain (Villemure and

Bushnell, 2002). The stimuli initiating nociception is perceived as aching, throbbing, irritating, sore, stinging, and miserable or unbearable sensations, that are relayed from various tissues throughout the body. Although receptors for nociception appear in most body tissues including skin, bone, muscle, blood vessels, heart, and the majority of internal organs, they are absent from the brain (Bear et al., 2007).

The physiological aspect of pain requires the stimulation of receptors, referred to as nociceptors, which are free, branching, unmyelinated nerve endings that signal when body tissue is being damaged or is undergoing threat of damage (Bear et al., 2007). A variety of nociceptors are present, each responding to different stimuli including mechanical nociceptors conveying strong pressure, thermal nociceptors recognizing extreme temperatures, chemical nociceptors relaying reaction to histamine or other chemicals and polymodal nociceptors which are the most abundant and respond to all stimuli (Bear et al., 2007).

In nociception, fibers carry information to the central nervous system (CNS) for interpretation at different rates due to differences in action potential, conduction, and velocities. This signaling produces two distinct pain perceptions, Aδ fibers relay the first pain, which is described as fast or sharp, and the C fibers are responsible for the duller, longer lasting pain (Bear et al., 2007). The fibers follow either the spinothalamic pathway, traveling from the spinal cord to the brain, or the trigeminal pain pathway, starting at the head or face and relaying back to the brain (Figure 1.1). Activation of cell bodies in the dorsal root ganglia initiates the spinothalamic pathway as signals relay into the dorsal horn of the spinal cord. The fibers enter the spinal cord, branching up and

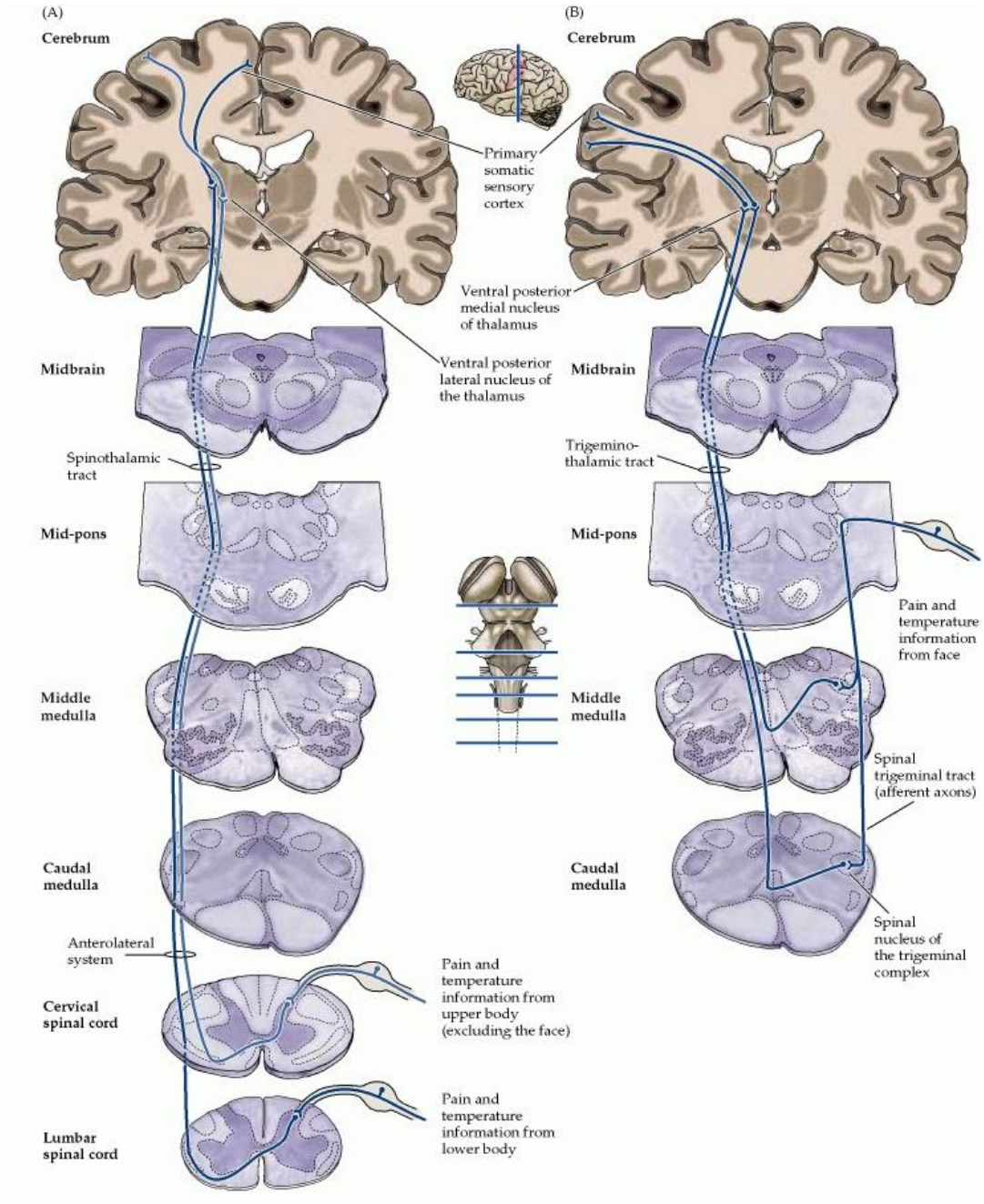


Figure 1.1: The spinothalamic (A) and trigeminal (B) pain pathways. The spinothalamic sensory travels from the spinal cord to the brain whereas the trigeminal signaling is initiated in the face or head and relays back to the brain. (http://www.ncbi.nlm.nih.gov/books/NBK10967/)

down, traveling one or two segments within the zone of Lissauer. From this point they synapse on cells in the substantia gelatinosa, a region in the outer part of the dorsal horn. The signal continues through axons of second order neurons, which receive sensory input from primary afferents (Bear et al., 2007). The axons immediately decussate and follow the spinothalamic track running along the ventral surface of the spinal cord. Spinothalamic fibers extend through the spinal cord, medulla, pons, and midbrain, finally synapsing at the thalamus. The trigeminal pain pathway follows a similar process, using second order axons to synapse at the thalamus. From here, information from both pathways is projected to various areas of the cerebral cortex (Bear et al., 2007).

The Brain

The brain can be divided into four main regions, including the cortex, midbrain, hindbrain and cerebellum (Figure 1.2). The main features of each region of the brain, and how they relate to nociception, are described below.

Cortex: The forebrain, also referred to as the cortex, is made up of two critical structures that further differentiate, the diencephalon and the telencephalon (Bear et al., 2007). The telencephalon is comprised of the olfactory bulbs and two cerebral hemispheres. The gray matter in the telencephalon comprises the basal telencephalon and the cerebral cortex, which is one of the most important structures in the forebrain. The prefrontal cortex is responsible for perception, conscious awareness, cognition and voluntary action (Bear et al., 2007). The diencephalon differentiates into two important structures as well, the

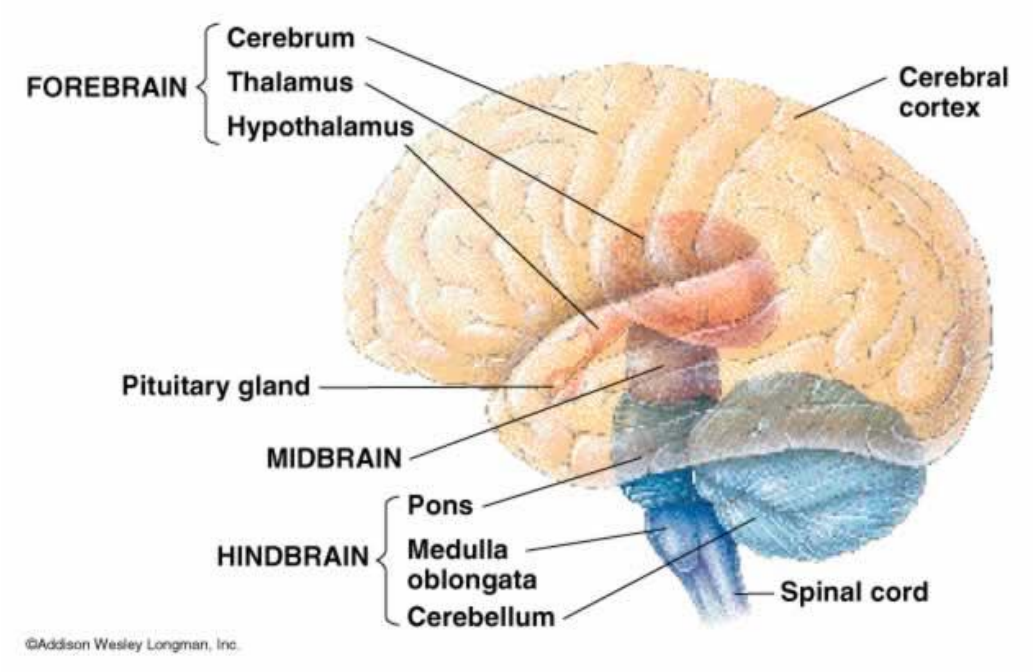


Figure 1.2: The human brain. Four main regions of the brain: Cortex, Midbrain, Hindbrain and Cerebellum are indicated. The overall structure and placement of the different regions is shown (http://linsenbardt.net/?p=2497).

thalamus and hypothalamus. The hypothalamus controls basic bodily functions, while the thalamus is part of the forebrain that receives sensory information regarding vision, audition, and somatic sensation, which it then relays onto the cerebral cortex (Bear et al., 2007). The cortex is then able to distinguish sensory inputs, thoughts, and actions that are constantly changing or weakly established. The cortex is essential in cognitive control, as it keeps the patterns of activity representing goals and the means to achieve them in order. It is not, however, necessary for performing behaviors occurring automatically, such as orientation to an unexpected sound or movement (Miller and Cohen, 2001).

The cortex is also an extremely important structure for perceiving, analyzing, and suppressing pain stimuli. It has been found that stimulation of the thalamus or cerebral cortex can suppress the responses of spinothalamic or trigeminothalamic tract neurons leading to the inhibition of nociception (Casey, 1999). One specific part of the cortex, the anterior cingulate cortex (ACC), is crucial for pain processing. It is involved in working memory and attentional processing, meaning it is able to shift focus toward or away from the pain. The ACC also plays a role in learning associations between neutral and aversive stimuli in classical conditioning, which is important for avoidance of future damage in reoccurring situations. Finally, containing higher motor areas allows the ACC to control defense preparation (Buchel et al., 2002). The structures responsible for the brain defense system include the hypothalamus, periaqueductal gray (PAG), and amygdala. The circuits of this system integrate the myriad endocrine and autonomic responses that are associated with behaviors that are aggressive-defensive (Hsieh et al., 1995). The cortex is therefore an important part of pain regulation and sensation (Figure 1.1).

Midbrain: The midbrain is an intermediate for information passing between the spinal cord and forebrain. Its neurons are contributors to sensory systems and control of movement (Bear et al., 1997). This region of the brain can be divided into three parts including the tectum, tegmentum, and cerebral aqueduct. The tegmentum is specifically involved in the control of voluntary movement. The tectum is further differentiated into two structures, the superior and inferior colliculi, both important for sensory signaling (Bear et al., 2007).

The midbrain is essential for somatic pain perception and suppression. The periaqueducal gray matter (PAG) is a zone of neurons in the midbrain that receives signals from many brain structures, particularly those for emotions. In turn, the PAG has neurons with axons descending into regions of the medulla, specifically the raphe nuclei, which is located in the hindbrain. These neurons extend to the dorsal horn of the spinal cord and are able to depress activity of the nociceptive neurons. This explains the correlation between extreme emotions, stress or determination, and the ability so suppress feelings of pain (Bear et al., 2007). Clinically the PAG has been targeted with electrical stimulation in order to create a state of analgesia and reduce pain for patients (Bear et al., 2007).

Hindbrain: The hindbrain also serves as an important intermediate for information being passed between the forebrain and spinal cord (Bear et al., 2007). Morphologically distinct bulges within its walls referred to as rhombomeres characterize it; these organized segments dictate cranial development in vertebrate embryos (Carpenter et al., 1993).

Another characterizing and crucial part of the hindbrain includes the medulla oblongata, responsible for sensory functions. The medulla holds cranial nerve nuclei, which supply somatic sensation from the spinal cord to the thalamus (Bear et al., 1997). In the event that cells from the cranial nerves are destroyed, loss of feeling or anesthesia ensues. An indifference to painful stimuli, even if no other sensory deficits occur, can be detrimental. Individuals with congenital absence of pain, even with early training to avoid damaging situations can develop progressive degeneration of joints and spinal vertebrae. This can lead to skeletal deformation, degeneration, infection and possibly death. Even low levels of nociceptive activity occurring during everyday tasks are vital for indication of strain on one's body from a prolonged posture or particular movement (Bear et al., 2007). Therefore, any analgesia can be potentially harmful to an individual.

The medulla also contains nine nuclei that make up the raphe nucleus. This serotonin-influenced nucleus regulates mood, pain and wakefulness as it fires most when awake and controls sleep/wake cycles. The raphe nucleus has axons projecting into the spinal cord through the dorsal horn. These axons are able to decrease nociceptive neuron activity, alleviating pain (Bear et al., 1997). The medulla is also the site of pyramidal decussation, where axons cross from one side to the other prior to entering the spinal cord. This crossing is the reason one side of the cortex controls movement on the opposite side of the body (Bear et al., 1997).

Cerebellum: This section of the brain is referred to as the "little brain" since it contains as many neurons as the rest of the brain combined, despite being smaller in size (Allen et al., 1997; Bear et al., 2007). The cerebellum is crucial for coordinated and accurate

movements of the ipsilateral (same) side of the body (Bear et al., 2007). It's ability to perform this function is based on taking in all the information as it is physiologically connected with virtually all levels of the central nervous system (CNS) through monosynaptic and multi-synaptic pathways (Allen et al., 1997). The cerebral cortex sends information specifying the goals of intended movement via the pons and once the message reaches the cerebellum, it calculates the precise muscle contractions required to reach the intended goal (Bear et al., 2007).

The traditional view of the cerebellum having only one function, to coordinate movement, is evolving as studies show it may be involved in non-motor functions including complex problem solving, verbal learning and memory, attention and sensory discrimination (Allen et al., 1997). The previous studies showed that when performing an attention task, involvement of the motor system was unnecessary to produce cerebellar activation shown via anatomical magnetic resonance imaging (MRI). The attention to sensory information alone was sufficient to elicit activation of this region (Allen et al., 1997). This impact could then indirectly influence pain perception, as current research indicates individuals whose attention is diverted from pain perceive it as less intense (Villemure and Bushnell, 2002). However, if the individual's attention is not actively directed elsewhere, the painful stimulus dominates over competing non-painful ones. Although it is quite clear that attention can modulate pain, an understanding of the precise cognitive mechanisms behind this is not yet understood (Bantick et al., 2002).

In 1987, Ekerot and colleagues also showed that the cerebellum was involved in nociception, as an increase in nociceptor signaling caused an increase of activity in pathways and neurons of the cerebellum (reviewed in Saab and Willis, 2003). There is

speculation that this increase in activity is simply due to an intention to perform a motor response rather than sensory perception. Further testing, using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) showed perception of acute heat pain, the warm-discrimination task, muscle pain, capsaicin-evoked pain, and allodynia caused increased blood volume or flow in the cerebellar vermis and regions lateral to it (Saab and Willis, 2003). The significance of this lies in the assumption that synaptic activity is the cause of the increase of regional blood volume. The spike in blood flow indicates the presence of the noxious stimuli to the various sensory indicators in the brain, which leads to pain (Saab and Willis, 2003).

Further evidence from Ekerot and colleagues indicated cerebellum influence on pain perception is derived from the postsynaptic dorsal column (PSDC) pathway. This pathway relays visceral nociceptive information to the dorsal column nuclei and relief from pain is acquired by interrupting the path (Saab and Willis, 2003). A region of the cerebellum, the caudal vermis, also holds individual Purkinje cells stimulated by visceral noxious stimulation. Although the same cells could be evoked for a similar response repeatedly, it was unclear what pathway(s) relayed the information to the Purkinje cells (Saab and Willis, 2003).

Despite several studies, the role of the cerebellum in nociception and pain perception is still inconclusive. There is speculation that an underlying intention to perform a motor response is the cause of increased cerebellar activity (Saab and Willis, 2003). Other areas of the brain more traditionally known for pain mechanisms are still credited and clinical evidence to support the cerebellum's role in nociception is required.

Basigin

Cell adhesion molecules can be classified into four groups including cadherins, integrins, the immunoglobulin superfamily, and selectins, based on biochemical structure (Tachikui et al., 1999). The neuronal synapse, or region of contact used in pathways for information transfer, is affected by cell adhesion molecules, which utilize guidance cues to mediate precise connections (Tessier-Labigne, 1994).

The cell adhesion molecule named Basigin is a transmembrane glycoprotein that is a member of the immunoglobulin (Ig) superfamily and possesses two Ig-like extracellular domains (Figure 1.3). Classification within the Ig superfamily is based on conservation of folding of the extracellular domain, in which two β sheets are sandwiched together (Halaby and Mornon, 1998). The function of this class of proteins includes cell adhesion, immunological recognition, and signal reception (Tachikui et al., 1999). The protein portion of Basigin has a mass of 27 kDa and the glycosylated form ranges from 43 to 66 kDa, depending on the tissue in which it is expressed (Igakura et al., 1996; Ochrietor et al., 2003). The name or title of the gene product also varies according to the species and tissue in which it is found. In rat, Basigin is referred to as CE9 (Hubbard et al., 1985) or OX47 (Fossum et al., 1991); while in mouse it is gp42 (Altruda et al., 1989) or CD147. In chicken, Basigin is called HT7 (Seulberger et al., 1990), neurothelin (Schlosshauer and Herzog, 1990) and 5A11 (Fadool and Linser, 1993); but in humans it is leukocyte activation antigen, M6 (Kasinrerk et al., 1992), EMMPRIN or tumor cell-derived collagenase (Biswas et al., 1995). Each of these proteins, despite being found in different species, possesses high amino acid sequence similarity (Toyama et al., 1999).

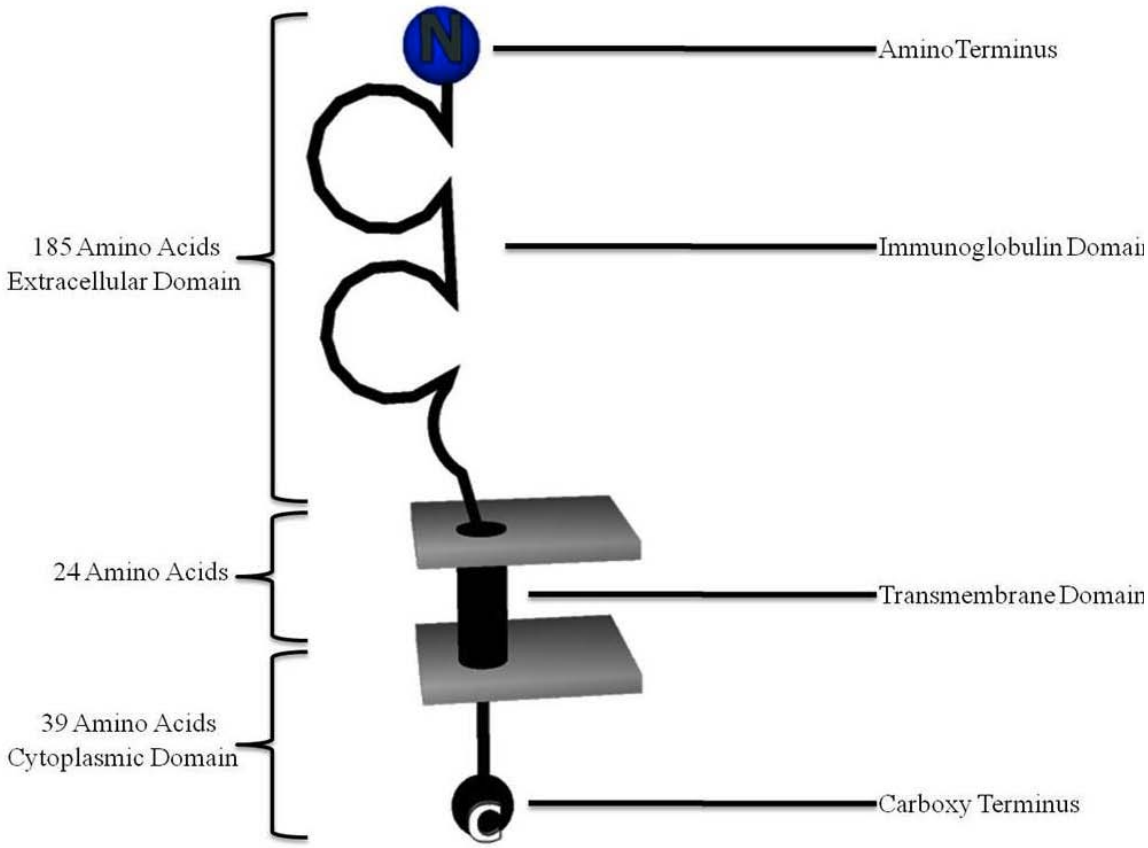


Figure 1.3: A diagram of the Basigin molecule. Basigin is a single-pass transmembrane protein with two extracellular Ig domains at the amino terminus, and a cytosolic carboxy terminus. Basigin consists of ~250 amino acids. The extracellular portion, which contains the Immunoglobulin domains, is ~185 amino acids, the transmembrane domain contains 24 amino acids, and the intracellular domain is ~39 amino acids (Artwork courtesy of Paul Gambon).

The human Basigin gene is located on chromosome 19p13.3, while the mouse Basigin gene is found on chromosome 10 centimorgan (cM) position 42.2. The Basigin gene consists of eight exons, which can be spliced differently to yield multiple gene products (Miyauchi et al., 1991; Mouse genome database, 5/2011). An additional Basigin gene product includes Basigin-2, which contains an identical amino acid sequence to Basigin, with an extra 116 amino acids near the amino-terminus (Ochrietor et al., 2003). Basigin-2 possesses three extracellular Ig domains as a result of inclusion of exon 1A in the transcript. In contrast to Basigin, Basigin-2 is only expressed in the retina, specifically on the surface of photoreceptor cell bodies and inner segments. This limited expression of the glycoprotein Basigin-2 implies a fundamental and specialized role within the retina (Ochrietor et al., 2003).

The biological functions of Basigin are diverse. However, like the majority of the proteins belonging to the immunoglobulin superfamily, it mainly participates in cell adhesion and pattern recognition (Halaby and Moron, 1998). Basigin is widely distributed and is found in mitral cells in the olfactory bulb and Purkinje cells in the cerebellum. Basigin is also found in the thalamus, cerebral cortex, and limbic system, as well as other areas and various organs (Fan et al., 1998b). The protein is involved in immune responses, tumor invasion, and neuronal processes affecting vision, behavior, and olfaction. Other functions of Basigin include physiological events including spermatogenesis, sperm-egg interaction, and embryo implantation (Nakai et al., 2006). The importance of Basigin for these processes is demonstrated by Basigin knockout mice, which have a targeted deletion of the Basigin gene. These animals are blind (Hori

et al., 2000; Ochrietor et al., 2002), reproductively sterile (Igakura et al., 1998), and have decreased pain tolerance (Naruhashi et al., 1997).

Monocarboxylate transporters and Metabolism

Moncarboxylate transporters, as the title implies, assume the responsibility of transporting monocarboxylates such as lactate, pyruvate and ketone bodies, which are vital for intracellular pH regulation and energy supply (Liu et al., 2008). This function is performed using proton or sodium gradients to co-transport the substrates across plasma membranes. The quick movement of monocarboxylates across the plasma membrane is imperative for cellular metabolism and metabolic communication between tissues (Liu et al., 2008). All members of the monocarboxylate transporter (MCT) family have the same structure consisting of 10-12 transmembrane helical domains with both amino and carboxy termini, as well as a large loop region between transmembrane domains 6 and 7, within the cytosol (Wilson et al., 2005).

While there are currently 14 members of the monocarboxylate transporter family that have been isolated and characterized from different tissues (Halestrap and Meridith, 2004), the main focus in this study will be on MCT1, 2 and 4. The focus on these particular MCTs is due to reports that they directly mediate lactate and pyruvate transport, unlike others in this family, which play roles in the transport of thyroid hormone, bumetanide, and L-tyrptophyan (Liu et al., 2008; Manoharan et al., 2006). These three transporters are thought to require ancillary proteins such as Basigin or Embigin (gp70) for appropriate expression and activity in the plasma membrane (Manoharan et al., 2006).

MCT1 (SLC16A) is expressed in a wide variety of tissues including the heart, brain, and retina (Bergersen, 2007; Halestrap and Price, 1999). Expression of MCT1 in the brain is seen in endothelial cells of the capillaries, in both the membrane facing the basal lamina, as well as that facing the lumen of the vessels (Bergersen, 2007). In particular, the neonatal brain expresses high levels of MCT1, indicating the transport of lactate and ketone bodies across the cerebral microvasculature at high rates (Halestrap and Price, 1999). Studies indicate that MCT1 facilitates lactic acid uptake for gluconeogenesis in the liver and kidney of certain species and for oxidation in red skeletal muscle fibers and heart (Ovens et al., 2010). Erythrocytes and cells experiencing hypoxic conditions use MCT1 for lactic acid efflux (Ovens et al. 2010). The intermediate affinity transporter can be inhibited by stilbene disulfonate derivatives such as DIDS and 4,4'-dibenzamido-stilbene-2,2'-disulfonate (DBDS).

As the most widely distributed MCT family member, MCT1 is typically co-expressed with Basigin (Clamp et al., 2004; Wilson et al., 2009). It was proposed that glutamic acid residue positioned as the center of the transmembrane domain of Basigin interacts with MCT1 (Kirk et al., 2000); however it has since been determined that the interactions between Basigin and MCT1 are hydrophobic in nature (Finch et al., 2009). If MCT1 is incubated with DIDS for prolonged periods, it becomes cross-linked to the glycoprotein Embigin, which is a member of the same subset of the Ig superfamily as Basigin (Bergersen, 2007; Wilson et al., 2009).

MCT2 (SLC16A7) is a higher-affinity transporter and is not as widely distributed as MCT 1, despite sharing 60% amino acid sequence identity (Bergersen, 2007; Halestrap and Price, 1999; Ovens et al., 2010). The high binding affinity of MCT2 is important, as

it allows the transporter to take up extracellular lactate for energy when glucose levels decrease below homeostatic range and lactate concentrations are therefore low (Pierre et al., 2000; Pellerin and Magistretti, 1994). MCT2 is found in neurons, specifically in the postysynaptic density at glutamatergic synapses in the cerebellum and hippocampus. Its distribution, along with MCT4, at the synapse supports the proposed glutamate-lactate shuttle between neurons and astrocytes (Bergersen, 2007). The function of MCT2 in the brain is supposedly linked to glutamatergic synaptic transmission (Bergersen, 2007).

MCT4 expression typically occurs in skeletal muscles and white muscle fibers or highly glycolytic cells in which it facilitates lactic acid efflux from the tissue (Juel and Halestrap, 1999; Ovens et al., 2010). The expression of this lower affinity transporter can be up-regulated by cells via Hypoxia-inducible factor 1 (HIF-1α) under hypoxic conditions (Ovens et al., 2010). The low affinity of MCT4 does not hinder its transport of lactate since skeletal muscle and glycolytic cells produce significant concentrations of lactate. This concentration gradient allows MCT4 to adequately export, rather than import lactate (Halestrap and Price, 1999; Bonen et al., 2000).

In the brain, substrates other than glucose can be used as a source of energy metabolism. There is evidence of large amounts of ketone body utilization in newborn babies shortly after birth and newborn rats during the suckling period (Daniel et al., 1977; Vannucci and Duffy, 1974). As animals mature, the brain becomes more glucose dependent upon entering adulthood and ketone body utilization decreases. Contradictions of this occur during periods of exercise or when glucose is being highly utilized while lactate concentration is high, then the brain uses lactate as an energy source (Pellerin, 2005; Dalsgaard et al., 2004). There is also evidence from *in vitro* studies that lactate,

rather than glucose, is the major neuronal energy substrate for tissue surviving an ischemic insult. It's been determined that lactate has neuroprotective properties in the hippocampus and cultured neurons after deprivation of glucose or oxygen, which simulates the conditions encountered during a stroke (Bergersen, 2007). At times when lactate concentrations are low, lactate can still be utilized if a high affinity transporter, such as MCT2, is present (Bergersen et al., 2005). Other *in vitro* studies have shown that neurons, even when both lactate and glucose substrates are available, will select lactate as an energy source (Bouzier-Sore et al., 2003).

Further evidence portrays neurons' preference for extracellular lactate over intracellular lactate, which could be released from nearby astrocytes (Itoh et al., 2003; Aubert et al., 2005). Astroglial cells utilize glucose in *in vitro* studies, suggesting that astrocytes produce the lactate and export it for use by the neurons (Itoh et al., 2003). Therefore, the presence of lactate is important for proper neuron function.

Basigin Null Mice

The importance of Basigin for functions previously discussed is demonstrated by Basigin knockout mice, which were developed by Takashi Muramatsu and his research team in the 1990s (Igakura et al., 1998). These animals have a targeted deletion of the Basigin gene, created by extracting 0.2 kB from the first exon in the gene and replacing it with a neomycin resistance gene. This new construct was then recombined in cultured pluripotent mouse embryonic stem (ES-D3) cells, resulting in male chimeric mice that were mated with C57BL/6 female mice. The herterozygotes produced in the F1 generation were then interbred to produce the Basigin null mice whose genotypes were

verified with Southern blot analyses (Igakura et al., 1998). These animals are blind (Hori et al., 2000, Ochrietor et al., 2002), reproductively sterile (Igakura et al., 1998), and have increased pain sensitivity (Naruhashi et al., 1997).

Behavioral studies conducted on normal and Basigin null mice revealed several abnormalities in sensory and memory functions in the knockout animals. In terms of deficiencies within the nervous system, the mice have an altered sense of pain perception. Although it was originally thought that Basigin null mice have learning and memory deficiencies, it has since been determined that the null mice do not likely have learning or memory deficiencies, as the behavioral tests for these deficiencies rely on visual cues and the mice are blind (Hori et al., 2000). A different behavioral test conducted by Naruhashi et al., (1997) to examine pain tolerance, an electric sensitivity or foot shock test, delivered shock to individual mice placed in a dark compartment through a grid using an isolated stimulator. The electric current was raised in intervals of 0.03 mA given in 0.5 sec intervals until the mouse flinched, vocalized, or jumped, and the current was recorded. In comparison with the wild-type and heterozygous mice, the mutant mice required significantly less current for vocalization or jumping (Naruhashi et al., 1997). This was explained by two factors. Signals from the peripheral sensory neurons are relayed through nerve cells in the thalamus. Basigin in the thalamus, which was found intensely expressed in the anterodorsal and reticular nuclei via in situ hybridization, could then be involved in output from sensory neurons (Fan et al, 1998b; Naruhashi et al., 1997). Alternatively, Basigin in the cerebral cortex could be involved in the sensory signal network (Naruhashi et al., 1997). Expression of Basigin was ubiquitous in the cerebral neocortex, with elevated levels in pyramidal neurons of the Vth layer (Fan et al.,

1998b). Therefore, signaling from the cerebral cortex and thalamus could be affected by the lack of Basigin in null mice causing the increased pain sensitivity.

The blindness of Basigin null mice is thought to be caused by the absence of MCT 1 and MCT4 expression in the retina, resulting in a metabolic deficiency due to inadequate lactate transport between Müller glial cells and photoreceptors (Philip et al., 2003). This transport is thought to be conducted by a shuttle system consisting of Basigin-2 and MCT1 and/or 4 in the membrane of photoreceptor cells and Basigin and MCT1and/or 4 in the membrane of Müller glial cells. The Müller cells use glucose as the main source of metabolic energy. Since glycolysis in Müller cells often out-paces aerobic respiration, lactate is produced and released to serve as a substantial source of metabolic energy for neurons (Poitry-Yamate et al., 1995). In the absence of the lactate metabolon in the retina, which occurs in the absence of Basigin gene products, photoreceptors are not supplied with lactate as an energy source, and they do not function (Philp et al., 2003). Hence the mice are blind from the time of eye opening (Ochrietor et al., 2002). The lactate metabolon that likely exists in the mouse retina is comparable to metabolic shuttling in muscle, in which lactate is transported out of fast glycolytic skeletal muscle fibers by MCT4 to oxidative skeletal muscle fibers expressing MCT1. These systems could also potentially explain the astrocyte-neuron coupling hypothesis of Vanucci and Duffy (1974), as neurons are found to consume lactate produced by astroglia.

Studies performed by the Ochrietor laboratory at the University of North Florida sought to assess MCT expression in the Basigin null mouse brain to determine whether a metabolic defect similar to that occurring in the retina was the reason for sensory pain

deficiencies of the Basigin null mice. This was completed by comparing cerebral membrane-associated expression of MCTs in normal and Basigin null mice. Results from that study indicate that MCT expression (MCT1, MCT2, and MCT4) is not down regulated at the membrane (Ochrietor et al., 2010b), which suggests that the cause of sensory pain deficiencies in Basigin null mice is not faulty neural metabolism. In addition, the data suggest that a different molecule associates with MCTs in the brain for their expression at the membrane. This group hypothesized that Embigin, a transmembrane glycoprotein that belongs to the Basigin subset of Ig superfamily molecules, interacts with MCTs for their proper insertion into the plasma membrane. It was also determined that the concentration of Embigin in Basigin null mouse brains was similar to that in normal mouse brain (Ochrietor, personal observation). This suggests that Embigin is the normal accessory protein for MCT expression and is not compensating for the absence of Basigin in the null animals. Since Basigin and Embigin share significant amino acid sequence homology, it is reasonable to propose that the interaction between Embigin and MCTs is similar to that of Basigin and MCTs.

Embigin

Embigin is a transmembrane glycoprotein and another member of the same subset of the Ig superfamily as Basigin. Like its sister molecule, Embigin contains an N-terminus with two extracellular Ig domains, a single transmembrane sequence containing a glutamic acid residue, and a small intracellular C terminus domain. The mouse Embigin gene is found on mouse chromosome 13 (Muramatsu and Miyauchi., 2003). Formerly referred to as gp 70, it is structurally related to Ig molecules and shares 28%

overall amino acid sequence identity with Basigin and 50% amino acid sequence identity with Basigin in the transmembrane domains (Fan et al., 1998a; Tachikui et al., 1999; Figure 1.4). The Embigin protein is 30 kDa in mass, while the glycosylated form appears as a 66-90 kDa molecule (Fan et al., 1998a). Previously published data suggest that Embigin regulates cellular activity during cell differentiation and tissue remodeling by mediating intercellular recognition (Tachikui et al., 1999). Integrin activity is involved when Embigin is over-expressed, leading to cell-substratum adhesion (Tachikui et al., 1999).

Previous studies indicate the expression of Embigin is more restricted than Basigin, and although strongly expressed in mouse embryos between days 5.0 and 9.0 of gestation, it is only weakly expressed in adult organs (Fan et al., 1998a). The high expression of Embigin in mice is evident during preimplantation and appears localized in the endoderm for early postimplantation embryogenesis (Huang et al., 1990; Fan et al. 1998a). Embigin is also intensely expressed in the visceral yolk sac and embryonic gut around day 8.5 of embryogenesis. In the embryonic ectoderm and mesoderm, on the other hand, there is only weak to moderate expression (Fan et al., 1998a). This trend carries to the brain where Embigin levels appear much lower (Fan et al., 1998a). Adult rats show further variation of Embigin mRNA expression, as seminal vesicles portray the lowest detectable levels, while the heart, lungs, and liver are slightly higher. The kidney, testes, and brain show detectable expression levels of Embigin mRNA as well (Guenette et al., 1997).

Other studies of Embigin allow further understanding of this protein and its various influences. The expression of Embigin suggests that it could be involved in

Figure 1.4: Amino acid sequences of the transmembrane domains of Basigin and Embigin. The two sequences were aligned via BLAST software (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). Significant amino acid identity (50%) was observed between the two sequences within their transmembrane domains. Amino acids that are identical in both Basigin and Embigin are indicated by asterisks (*). The amino acids used by Basigin in the interaction with MCT1 are shown in blue.

tumorigenesis, contributing to the malignant state by interacting with the extracellular matrix (ECM). Embigin regulates cell growth and differentiation in response to components of the extracellular environment (Guenette et al., 1997). The mRNA levels of Embigin are elevated in embryonal carcinoma cells with different differentiation potentials, supporting its involvement in cancer development (Huang et al., 1990). Studies have also proposed that cell adhesion mediated by Embigin requires an integrin accessory molecule (Guenette et al., 1997). This idea is based on enhanced cell-substratum adhesion that was calcium-dependent and inhibited by antibodies specific for integrins and ligands with an exposed arginine-glycine-aspartate (RGD) sequence (Guenette et al., 1997).

An important similarity exists between Basigin and Embigin, as the transmembrane regions of both proteins are highly conserved among species (Tachikui et al., 1999; Figure 1.4). This structural feature suggests Embigin and Basigin can form protein complexes in the plasma membrane (Tachikui et al., 1999). Both glycoproteins are thought to be accessory proteins for MCT expression at the plasma membrane as MCT1 and 4 are found to interact with Basigin whereas MCT2 interacts with Embigin (Wilson et al., 2005). Further studies have also indicated that lysine residues within the extracellular domain of Embigin can be crosslinked to MCT1 if erythrocytes are incubated with DIDS, strengthening the concept of an interaction between Embigin and MCTs (Poole and Halestrap, 1997; Wilson et al., 2009).

The purpose of the present study was to characterize the expression of membrane associated Basigin, Embigin, and MCTs 1, 2, and 4 in the normal mouse brain as a

whole, as well as its dissected parts. The study is aimed at determining whether Embigin normally interacts with MCT1, MCT2, and MCT4 in the mouse brain, and if so, whether the interaction is similar to that between Basigin and MCT1. A model of a lactate shuttle system for the brain, which contains Embigin, MCT2, and MCT4, is shown in Figure 1.5.

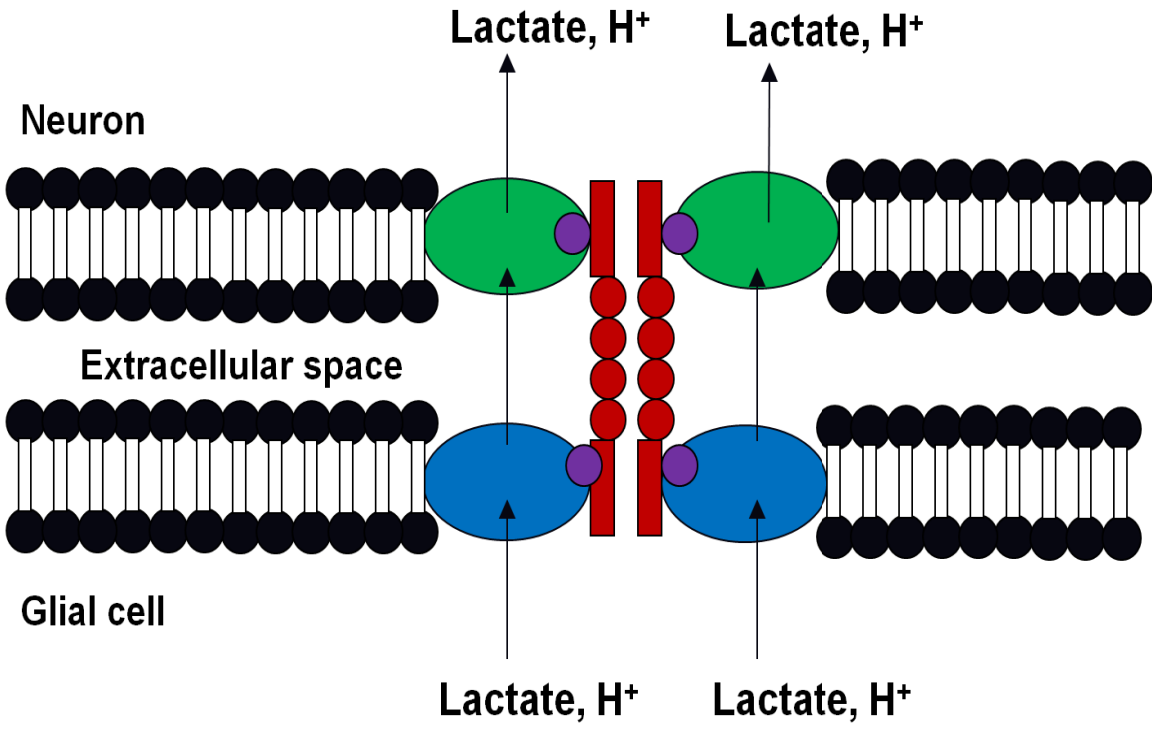


Figure 1.5: The proposed lactate shuttle in the brain. A shuttle complex similar to that thought to exist within the retina was proposed for the mouse brain. The shuttle consists of a complex of Embigin (red structures) and MCT4 (blue ovals) on glial cells and a complex of Embigin and MCT2 (green ovals) on neurons. The complex is thought to transport lactate from glial cells to neurons.

Material and Methods

Generation of mouse brain membrane-associated protein extracts

Normal mice were sacrificed according to an accepted protocol (UNF IACUC #10-009) and the brains were removed immediately and dissected into four parts: cortex, midbrain, hindbrain and cerebellum. The brain sections were homogenized in detergent lysate buffer (150 µL hindbrain and cerebellum; 265 µL midbrain; 500 µL cortex; Invitrogen Corporation, Carlsbad, CA; 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS 0.5% deoxycholate and supplemented with 1mM PMSF [Pierce/ThermoScientific, Rockford, IL]), incubated on ice for 30 minutes, and cleared by centrifugation at 12,000 rpm for 15 minutes. After centrifugation, the supernatants were transferred to 1.5 ml microcentrifuge tubes (USA Scientific, Orlando, FL) and stored at -80°C.

The protein concentrations of the tissue samples were determined using a Coomassie (Bradford) Protein Assay (Pierce/Thermo Scientific). 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 mouse brain extracts were analyzed both undiluted (5µL) or prepared at either a dilution of 1:5 or 1:2 in distilled water (5 µL total volume) and transferred to wells of the same 96-well plate. All wells received 250 µl Coomassie (Bradford) reagent. 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 and the protein concentrations of brain extracts were determined from the graph.

Analysis of protein extracts via SDS-PAGE and electroblotting

Aliquots (100 μg each, 20 μl total volume) of mouse brain tissue were transferred to 0.5 ml microcentrifuge tubes (USA Scientific). To each sample, 6.6 μl of LDS buffer (Invitrogen Corporation) and 1.5 μl of β-mercaptoethanol (Fisher, Fairlawn, NJ) 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.

The polyacrylamide gel was placed in a pan with protein transfer buffer (25 mM 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.

The blots were incubated in Blotto (2% instant powdered milk in TBS—137 mM Sodium Chloride, 20 mM Tris, 0.1% Tween-20) for one hour at room temperature or overnight at 4°C, and then the lanes were cut into strips to allow for simultaneous incubation of the various sections of brain (cortex, midbrain, hindbrain, and cerebellum) with antibodies specific for Basigin (Ochrietor et al., 2003), MCT1 (Millipore/Chemicon, Billerica, MA), MCT2 (Millipore/Chemicon), or MCT4 (Millipore/Chemicon). To each of 4 Petri dishes were added strips of nitrocellulose containing normal mouse brain proteins and 10 ml Blotto. Next, the rabbit anti-mouse polyclonal antibodies named above were each added to a dish containing a blot (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 and 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 ~10 ml 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 highest mean intensity signal was set to 100% and the other samples were compared.

Histological analysis of mouse brain

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 Scientific) treated with a drop of poly-L-lysine to facilitate adhesion. Slides containing tissue sections were stored at room temperature.

For histochemistry, tissue sections were rehydrated by incubation in Citrisolv (Fisher Scientific) 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 MasterTech staining protocol (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 Scientific) was added to each slide, and a 22 x 40 mm cover slip (Fisher Scientific) 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, which 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, MCT4, tubulin (diluted 1:100 in pre-incubation buffer; Millipore), and Embigin (diluted 1:100 in pre-incubation buffer; Pierce Scientific). 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). 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 nucleic acid stain was used in the final wash step, the 1,5-bis{[2-(di-methylamino) ethyl]amino}-4, 8-dihydroxyanthracene-9, 10-dione (DRAQ-5) was incubated on the slides for 5 minutes are room temperature in a 1:1000 dilution (Ochrietor et al., 2010a). The slides were then washed a final time as described and mounted. A drop of a solution made by dissolving a small flake of p-phenylenediamine in 30% glycerol was added to each tissue sample and coverslips 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.

A colormetric detection method was also utilized for immunohistochemistry in which the sections were again rehydrated as described and incubated in the pre-incubation solution at 4°C overnight. Pre-incubation medium was removed and 200µl of primary antibody solution was added to the slides, which were incubated at 37°C for 60 minutes in a humid chamber then at 4°C overnight. The antibodies used include those specific for Basigin (diluted 1:200 in the pre-incubation buffer; Ochrietor et al., 2003), MCT1, MCT2, MCT4 (diluted 1:100 in pre-incubation buffer; Millipore) and Embigin (diluted 1:100 in pre-incubation buffer; Pierce Scientific). Slides were washed 10 times each by adding TBS solution to each slide then pouring if off after a minute. Secondary antibody solution, ImmPRESS Reagent Kit, Anti-Rabbit Ig (Vector Laboratories Inc.; Burlingame, CA) was then incubated at room temperature for 60 minutes. The slides were then rinsed with 3 washes of PBS at 5 minutes per wash and incubated with ImmPACT DAB (Vector Laboratories Inc.) for 5 minutes at room temperature. This incubation was followed by a 5 minute running tap water rinse, then incubation in Methyl Green (Vector Laboratories Inc.) at 37°C for 60 minutes. The slides were then rinsed with tap water for two minutes followed by several washes of increasing concentrations (95% to 100%) of ethanol for 5 seconds each. The sections were then incubated in Citrosolv (Fisher Scientific) for three rounds of 5 minutes each. The cover slips were then mounted with Cytoseal 60 (American Mastertech Scientific Inc., Lodi, CA) and imaged using a

Fisher Scientific Micromaster microscope with digital camera and Micron software (Fisher Scientific).

Recombinant Embigin transmembrane domain plasmid production

Expression plasmids containing the cDNA for the entire Embigin transmembrane domain, as well as truncated versions of the domain were produced by PCR and annealing of oligonucleotides respectively. Primer sequences are shown in Table 2.1. The PCR setup included 1μL mouse cerebellum cDNA; 1μL of the forward primer (EmbTMF), 1μL of the reverse primer (EmbTMR), 25μL of the Ex Taq mix (TAKARA Corporation, Madison, WI), for a total volume of 50 μL in a 500 μL microcentrifuge tube (USA Scientific). A three step cycling protocol was used: 95°C for 30 seconds, 55°C for 30 seconds, 68°C for two minutes; for a total of 30 cycles.

All PCR products were analyzed by gel electrophoresis using a 1% agarose gel with 1X Tris-boric acid-EDTA (TBE) buffer. Once the gel was documented, the PCR products were extracted from the gel using a sharp scalpel and placed in a 1.5 centrifuge tube (USA Scientific). DNA was purified from gel fragments via the QIAquick gel extraction kit (QIAGEN, Valencia, CA), following the protocol of the manufacturer. To the excised product was added 180 μL QG buffer. The mixture was incubated at 42°C for 10 minutes to dissolve the gel. Then 60 μL of isopropanol was added and the entire volume was transferred to a QIAquick spin column and centrifuged at 13,000 rpm for 1 minute. After discarding the flow through, 500 μL of QG Buffer and 750 μL Buffer PE were used separately to wash the column, followed by centrifugation at 13,000 rpm for one minute, with the flow through discarded after each wash. A final spin was conducted

Table 2.1: Primers used for amplification of the Embigin transmembrane domain.

Name	Sequence
pETEmbXF	5' CACCATGCGCTCGCACACTGGC 3'
pETEmbXRV	5' GAAGCTCAGCACAACCAGCTCATT 3'
pETEmbTMF	5' CACCCTGGTGCCCCTCAAGCCA 3'
pETEmbTMRV	5' ACAAAGCAGAATGATGGCCACCA 3'
pETEmbTM6RV	5' TGGCTTGAGGGGCACCAG 3'
pETEmbTM19F	5' CACCGCCATCATTCTGCTTTGT 3'
pETEmbTM24RV	5' ACAAAGCAGAATCATGGC 3'
pETEmbTM7F	5' CACCTTTCTGGCCATACTTGCC 3'
pETEmbTM12RV	5' GGCAAGTATGGCCAGAAA 3'
pETEmbTM13F	5' CACCGAAGTCATCCTCTTGGTG 3'
pETEmb18RV	5' CACCAAGAGGATGACTTC 3'
pETBasXFwd	5' CACCATGGCGGCGGCGCTGCTG 3'
pETBasXRV	5' GCGGCTCCGCACACGCAGTGAG 3'

at 13,000 rpm for 1 minute to clear any residual ethanol from Buffer PE. The column was then placed in a clean 1.5 mL microcentrifuge tube (USA Scientific) and 30 μL of elution buffer was applied to the center of the membrane and incubated for 1 minute. The column was centrifuged for one minute at 13,000 rpm to recovers the PCR product.

For annealing of oligonucleotides, appropriate forward and reverse primers, (100 pmol/μL of each) and 1μL of a 20x annealing buffer (0.2 M Tris-HCL pH 7.9, 40 mM $MgCl_2$, 1M NaCl and 20 mM EDTA), in a total volume of 20 μL, were combined and heated to 90°C for 5 min. The mixture was slowly cooled to room temperature.

For cloning into the pET102 (D/TOPO) vector (Invitrogen Corporation), 1.0 μL salt solution, 1.0 μL pET102 vector, and 4.0 μL of annealed plasmid or gel-purified PCR product were combined in a 500 μL centrifuge tube (USA Scientific) and incubated for 5 minutes at room temperature. A negative control plasmid was also generated by incubating 1.0 μL pET102 vector with 1.0 μL salt solution and 4.0 μL of water for 5 minutes at room temperature. These plasmids (2 μL) were then each transformed into 50 μL of chemically competent Top 10 cells (Invitrogen Corporation) following the protocol of the manufacturer. The cells were mixed via stirring with the pipette and then incubated on ice for 30 minutes. The cells were heat-shocked by incubating at 42°C for 30 seconds. The tube was immediately placed on ice for one minute and then 250 μL of Super Optimal Catobolite repression broth (S.O.C.) medium (Invitrogen Corporation) was added. The transformed cells were incubated at 37°C with shaking (220 rpm) for one hour. The entire mixture was then spread onto Luria-Bertani (LB) agar (BD/Fisher Scientific, Woodlawn, NJ) plates containing carbenicillin (Fisher Scientific; 50 μg/mL). The plates were inverted and incubated at 37°C overnight. Multiple colonies were then

picked with a toothpick from the LB-agar plate the following day and added to 25 μL of water in a 500 μL tube (USA Scientific) to generate water cultures.

The colonies were screened using M13R and PD13 primers (Table 2.2). The reaction included 25 μL EX-Taq mix (TAKARA Corporation), 5 μL of the water culture, and 0.5 μM of each primer in a total volume of 50 μL. The reaction was subjected to PCR amplification using a protocol as follows: 95°C for 2 minutes, and 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for two minutes. The amplified PCR products were then analyzed on a 1% agarose gel in 1X TBE buffer. Colonies producing PCR products of the approximate number of base pairs were thought to contain the recombinant DNA insert. These positive colonies (5 μL of water culture) were grown overnight at 37°C with shaking (220 rpm) in 3 mL of Luria Bertani broth containing carbenicillin (50 μg/mL, Fisher Scientific).

The plasmids contained in positive colonies were purified using the QIA prep spin Miniprep Kit (QIAGEN). An aliquot of overnight culture (1.5 mL) was placed into a 1.5 mL microcentrifuge tube (USA Scientific) and centrifuged at 13,000 rpm for one minute. The supernatant was removed and the pellet was resuspended in P1 buffer (250 μL). Buffer P2 (250 μL) was added to the cells and mixed by gently inverting the tube five times. Buffer N3 (350 μL) was added to the solution and also mixed gently by inverting the tube five times. The mixture was centrifuged at 13,000 rpm for 10 minutes. The supernatant was then placed into a QIAprep spin column in a 1.5 mL collection tube using a fine transfer pipette (SAMCO, Morrisville, NC). The column was centrifuged at 13,000 rpm for one minute and the flow-through was discarded. The plasmid DNA was washed with 500 μL of PB buffer and the column was centrifuged for one minute at

Table 2.2: Vector primers used for sequencing

Name	Sequence
PD13	5' CTGGCCGTCGTTTTAC 3'
M13 Rev	5' CAGGAAACAGCTATGA 3'

13,000 rpm. The flow-through was discarded. The spin column was washed with 750 µL of buffer PE and centrifuged for one minute at 13,000 rpm. The flow-through was discarded. The column was centrifuged for one minute at 13,000 rpm to remove residual ethanol. Then buffer EB (50 µL) was added to the center of the membrane. The column was incubated for 1 minute and then centrifuged at 13,000 rpm for 1 minute to recover the plasmid DNA.

The sequence of each construct was verified via automated DNA sequencing. The sequencing was conducted using the Beckman-Coulter Quick Start kit (Fullerton, CA). Six µL of the purified plasmid was placed into a 500 µL centrifuge tube and incubated for 5 minutes at (96^{o}C). Cycle sequencing was initiated by adding 4 µL Quickstart mix (Beckman-Coulter) and 2 µL T7 Reverse primer (1 pmol/µL; Invitrogen Corporation) to the denatured plasmid. The following cycling parameters were used: 30 cycles of 96°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 minutes. The reaction mixture was placed into a clean 1.5 mL microcentrifuge tube (USA Scientific) containing 5 µL of stop solution (2 µL of 3M sodium acetate, pH 5.2; 2 µL of 100 mM Na^{2}-EDTA, pH 8.0; and 1µL of 20 mg/ml glycogen). Sixty µL of cold 100% ethanol was added and the solution was thoroughly mixed and immediately centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed with a fine transfer pipette (SAMCO). The pellet was washed twice with 200 µL of 70% ethanol and centrifuged at 14,000 rpm for two minutes at each wash. Again, a fine transfer pipette was used to remove the supernatant. The precipitated DNA was then dried in Savant Speed Vac SC 100 for 10 minutes. Sample loading solution (25 µL; Beckman-Coulter) was used to resuspend the dried

pellet, which was then analyzed using a Beckman-Coulter CEQ 8000 genetic analyzer (Fullerton, CA).

Expression and purification of Histidine-tagged recombinant proteins

One of each of the expression vectors generated as well as the control probe were individually transformed into BL21 star (DE3) cells (Invitrogen Corporation) following the protocol of the manufacturer. Two μL of plasmid was added to 50 μL of cells in a 3 mL tube and the mixture was incubated on ice for 30 minutes. The cells were heat shocked by incubation at 42°C for 30 seconds and then immediately transferred to ice for two minutes. Room temperature S.O.C. medium (250 μL; Invitrogen Corporation) was added to the cells and they were incubated at 37°C with shaking (220 rpm) for one hour. The entire reaction mixture was transferred to 10 mL LB containing carbenicillin (50 μg/mL; Fisher Scientific, Waltham, MA) and incubated at 37°C with shaking (220 rpm) overnight.

The entire 10 mL of overnight inoculation was transferred to an Erlenmeyer flask containing 250 mL of LB with carbenicillin (Fisher Scientific; 50 μg/mL). The culture was incubated at 37°C with shaking (220 rpm) for three hours. The cultures were then induced in midlog growth with 250 μL of 1mM Isopropyl β-D-thiogalactopyranoside (IPTG, Fisher Scientific,) and allowed to grow overnight at 37°C with shaking (220 rpm).

The induced overnight culture was divided into 6 centrifuge tubes. Cultures were pelleted by centrifugation at 3000×g for 15 min at 4°C and the bacterial cells were resuspended in 10 mL total volume of TALON Xtractor Buffer (Clontech, Mountain View, CA). The entire resuspension was then incubated with lysozyme (100 μL;

Clontech) and DNase I (1 U/μL; Fisher Scientific) for 10 minutes at room temperature with shaking. A protein lysate was formed via centrifugation at 10,000×g for 20 min at 4°C which was then applied to a TALON metal affinity resin (Clontech) for purification.

The TALON metal resin (Clontech) was prepared by resuspending the TALON metal affinity resin through vortexing. Two milliliters of the resin was transferred to a 50 mL centrifuge tube and centrifuged at 700×g for two minutes at 4°C. The supernatant was discarded. The resin was washed with 10 mL of 1X equilibration/wash buffer (Clontech), mixed briefly and centrifuged at 700×g for two minutes at 4°C. This equilibration step was repeated.

The bacterial supernatant was added to the resin and allowed to incubate at room temperature with shaking (100 rpm) for 20 minutes. The mixture was centrifuged at 700×g for five minutes at 4°C. The supernatant was discarded and the pellet was washed with 10 mL of 1X equilibration/wash buffer. The solution was then centrifuged at 700×g for five minutes and the supernatant was discarded again. This wash step was repeated once. One milliliter of the 1X equilibration/wash buffer was added to the pellet and the mixture was vortexed briefly. This solution was then transferred to a 2 mL disposable column that was included in the TALON purification kit (Clontech). The column was held vertically until the resin settled. The buffer was then drained from the column into a waste beaker. The column was washed with 5 mL of 1X equilibration/wash buffer. The purified protein was eluted with 5 mL of 1X elution buffer. Fractions of 0.5 mL were collected and analyzed at 280 nm. Fractions showing peak absorbances were stored in -80°C until further use.

The protein concentration of each recombinant protein was determined using the Coomassie (Bradford) Protein Assay as previously described.

ELISA assays

Sandwich ELISA analyses served as binding assays (Finch et al., 2009) in which antibodies specific for MCT1, MCT2 or MCT4 (all from Millipore) coated onto wells of a 96 well plate at 0.05 μg/mL in phosphate buffered saline (PBS; 100 μL total volume) and incubated overnight at 4°C. Unbound capture antibody was removed and the wells washed with PBS-T (PBS plus 0.25% Tween-20). Bovine serum albumin (BSA; 100 μg/mL) was added to each well (100 μL total volume) and incubated at 37°C for 30 minutes. Unbound BSA was removed by a PBS-T wash. Mouse brain proteins (100 μg/mL) were added to the wells (100 μL total volume) and incubated at 37°C for 30 minutes. Unbound mouse brain proteins were removed by a PBS-T wash. Embigin transmembrane domain or vector control probes (100 μg/mL; ~5 moles) were added to appropriate wells (100 μL total volume) and incubated for 30 min at 37°C. Unbound probes were removed by a PBS-T wash. An antibody specific for C-terminal 6XHis (Invitrogen Corporation) was then applied to appropriate wells (1:1000 dilution, 100 μL total volume) and incubated for 30 min at 37°C. Unbound C-terminal 6XHis was removed by a PBS-T wash. Alkaline Phosphatase (AP)-conjugated goat anti-mouse IgG (AP-GAM; Pierce/Thermo Scientific) was added to appropriate wells (1:1000 dilution, 100 μL total volume) and incubated at 37°C for 30 min. Unbound AP-GAM was removed by a PBS-T wash. Alkaline phosphatase substrate, (PNPP; Pierce/Thermo

Scientific) was then added to each well and allowed to incubate at room temperature for 30 min or until color development was observed. Reactions were stopped by adding 2 N NaOH (50 μL) and the absorbance at 405 nm was recorded. All runs were performed in duplicate. The average absorbance for each probe was calculated and plotted using Microsoft Excel software.

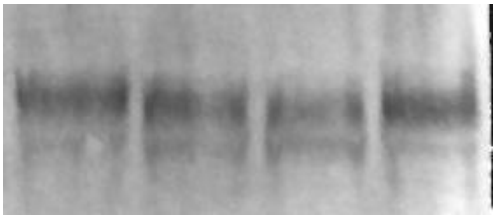
A slightly altered ELISA protocol was used to analyze relative Embigin concentrations in the various parts of the brain examined (cortex, midbrain, hindbrain, and cerebellum). Mouse brain proteins from each section were coated onto wells of a 96 well plate at 100 mg/mL in PBS (100 μL total volume). The plate was incubated at 37°C for 1 hour. Then the protein was removed and without washing, an antibody specific for Embigin (Pierce Scientific, Rockford, IL) was applied to appropriate wells (1:100 dilution, 100 μL total volume) and incubated for 30 min at 37°C. Unbound Embigin antibody was removed by a PBS-T wash. Alkaline Phosphatase (AP)-conjugated goat anti-mouse IgG (AP-GAM; Pierce/Thermo Scientific) was added to appropriate wells (1:100 dilution, 100 μL total volume) and incubated at 37°C for 30 min. Unbound AP-GAM was removed by a PBS-T wash. Alkaline phosphatase substrate, (PNPP; Pierce /Thermo Scientific) was added to each well and allowed to incubate at room temperature until color development was observed. Reactions were stopped by adding 2 N NaOH (50 μL) and the absorbance at 405 nm was recorded. All runs were performed in duplicate. The results were then averaged and plotted in a bar graph using Microsoft Excel software.

Results

Studies have shown that amino acids within the transmembrane domain of Basigin are required for the interaction with MCT1 (Finch et al., 2009). In the absence of Basigin (in the Basigin null mouse neural retina), MCT1 and MCT4 do not localize to the plasma membranes of expressing cells and photoreceptor function is disrupted (Philp et al., 2003). Increased pain sensitivity is another abnormality evident in Basigin null mice (Naruhashi et al., 1997). A recent study conducted by the Ochrietor laboratory at the University of North Florida indicated that MCT expression is not altered in the Basigin null mouse brain (Ochrietor et al., 2010b). It was hypothesized by this laboratory that Embigin acts as the accessory protein for MCT expression in that tissue. Therefore, a primary objective of this study was to characterize the expression of Basigin, Embigin, and MCTs 1, 2, and 4 in mouse brain, to determine if Embigin is the normal accessory protein for MCT1, MCT2, and MCT4 in that tissue. Another primary objective of this study was to determine if the interaction between Embigin and MCT2 is similar to that of Basigin and MCT1 in the neural retina.

Initially, the relative concentrations of Basigin, Embigin, and MCT1, MCT2, and MCT4 were determined via immunoblotting and ELISA analyses. Mouse brains were divided into four sections (cortex, midbrain, hindbrain, and cerebellum) and detergent-soluble (membrane fraction) protein lysates were prepared from each section. Immuoblotting analyses indicated that Basigin is expressed throughout the brain, with the cerebellum having the greatest relative expression (Figure 3.1). Only a single signal at ~50 kDa was observed on all brain blots when the Basigin-specific antibody was used, which indicates that the Basigin-2 gene product is not present in the brain. Embigin

A.



CTX M H CBL

B.

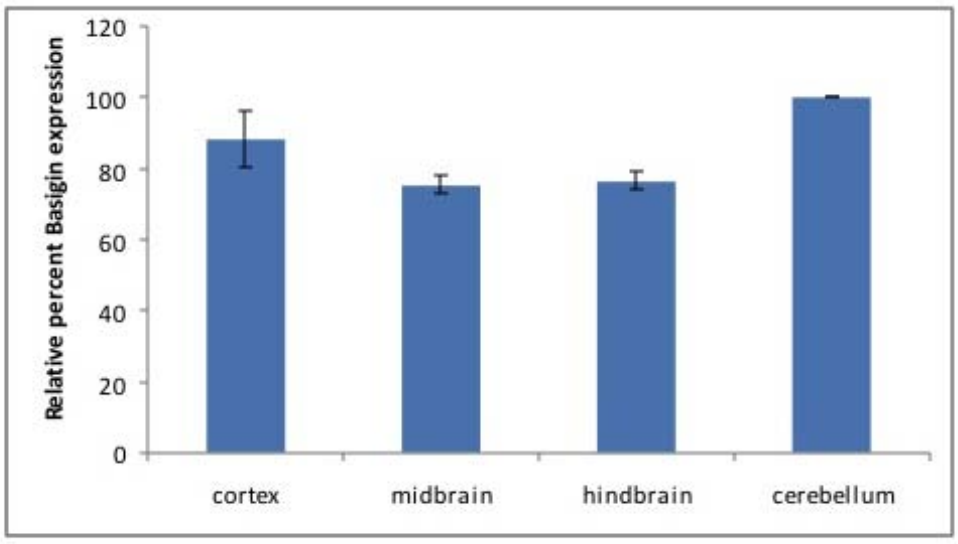


Figure 3.1: A) Basigin expression in mouse brain. Mouse brains were sectioned into four regions: Cortex (CTX), midbrain (M), hindbrain (H), and cerebellum (CBL), and protein lysates were generated for each region. The protein lysates were subjected to SDS-PAGE and subsequent immunoblotting analyses using an antibody that recognizes both Basigin gene products (Ochrietor et al., 2003). A representative blot is shown in panel A. Densitometry analyses were performed and are shown in panel B. Greatest expression was observed in the cerebellum, which was set at 100%. Expression in the other regions was determined relative to that found in the cerebellum. Error bars represent the standard deviation among duplicate trials.

expression throughout the mouse brain was measured via ELISA analysis. It was determined via densitometry analyses that hindbrain and cerebellum have the greatest relative expression (Figure 3.2).

The analyses also revealed that MCT1 (Figure 3.3) and MCT4 (Figure 3.4) expression is apparent in all four examined sections of mouse brain. It was determined via densitometry that little variance of MCT1 or MCT4 expression is found in the four regions, all showing similar levels of expression. Immunoblotting results for MCT2 expression revealed a low concentration of the protein among the various sections of brain, as the MCT2 signals were visibly lighter than those for the other MCTs examined. The highest MCT2 expression was noted in the midbrain (Figure 3.5).

The results of the immunoblotting analyses, in which it was determined that all proteins in question are found rather ubiquitously throughout the brain, led to questions about whether they are expressed on the same cells within the mouse brain. Therefore, paraffin-embedded sections of normal mouse brain were prepared for histological and immunohistochemical analyses. Because all of the antibodies for the proteins of interest were prepared in rabbits, each antibody was used separately in the immunohistochemical analyses. When one compares the images captured using each antibody on successive sections of tissue, it appears that Basigin, Embigin, MCT2, and MCT4 are expressed by cell bodies, which were identified by DRAQ5 staining of nuclear DNA (Figure 3.6). This is further supported by slides prepared via an alternative protocol in which MCT2 and MCT4 again appear expressed in cell bodies (Figure 3.7). There may be some overlap of signal for each antibody used, which suggests that these proteins are co-expressed within

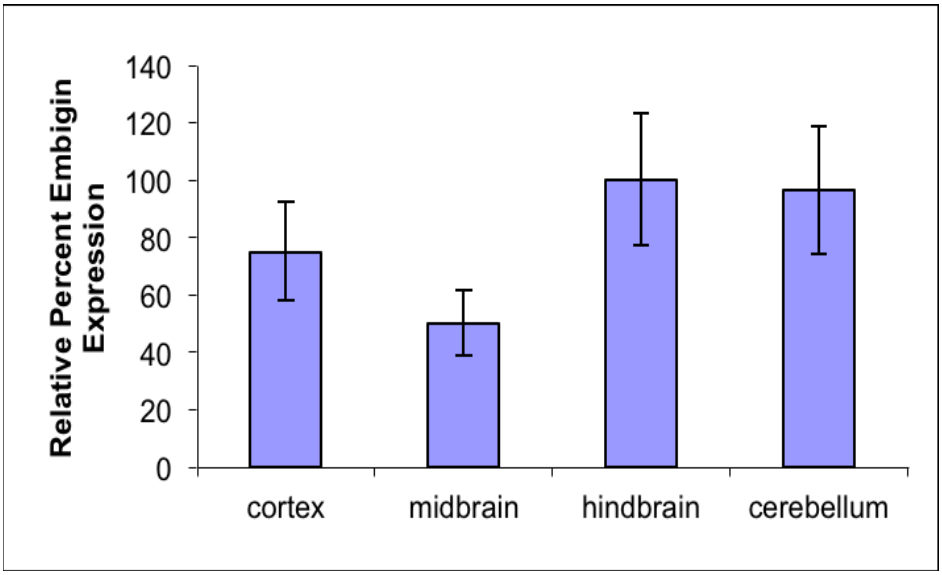
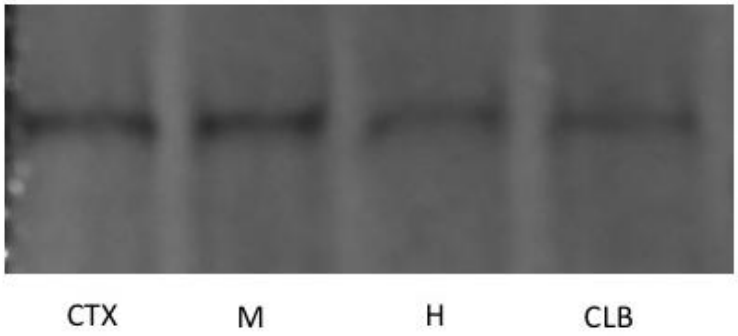


Figure 3.2: Embigin expression in mouse brain. Mouse brains were sectioned into four regions: Cortex, midbrain, hindbrain, and cerebellum, and protein lysates were generated for each region. Mouse brain proteins from each section were subjected to an ELISA assay using an antibody specific for Embigin followed by an Alkaline Phosphatase (AP)-conjugated goat anti-rabbit IgG. Alkaline phosphatase substrate was added to each well and allowed to incubate until color development was observed. Reactions were stopped with 2 N NaOH and the absorbance at 405 nm was recorded. All runs were performed in duplicate. The results were then averaged and plotted. Standard deviations are represented by the error bars.

A.



B.

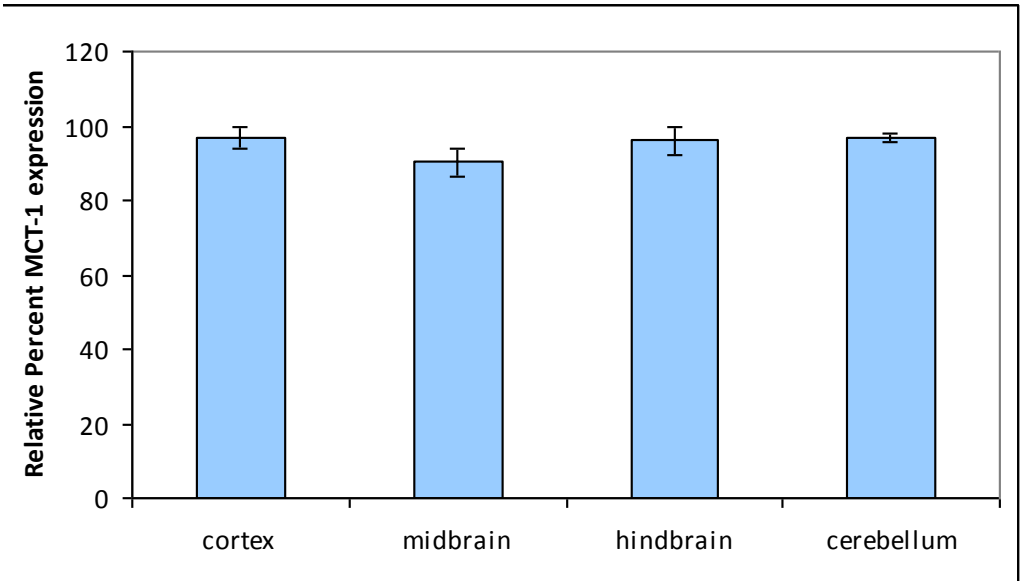
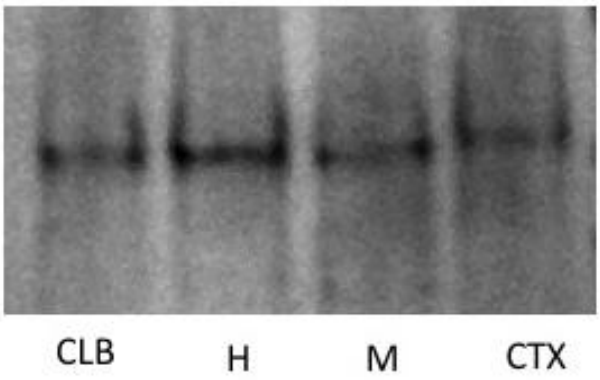


Figure 3.3: MCT1 expression in mouse brain. Mouse brains were sectioned into four regions: cortex (CTX), midbrain (M), hindbrain (H), and cerebellum (CBL), and protein lysates were generated for each region. The protein lysates were subjected to SDS-PAGE and subsequent immunoblotting analyses using an antibody that recognizes MCT1 (Millipore/Chemicon). A representative blot is shown in panel A. Densitometry analyses were performed and are shown in panel B. The largest mean intensity was set as 100% and the other mean intensities were compared. Error bars represent the standard deviation among duplicate trials.

A.



B.

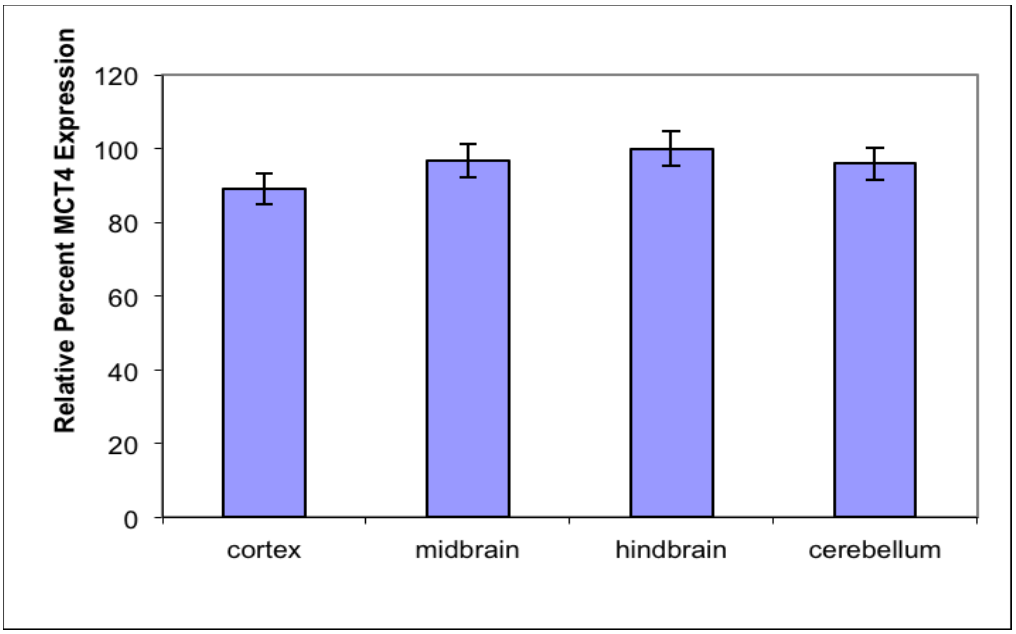
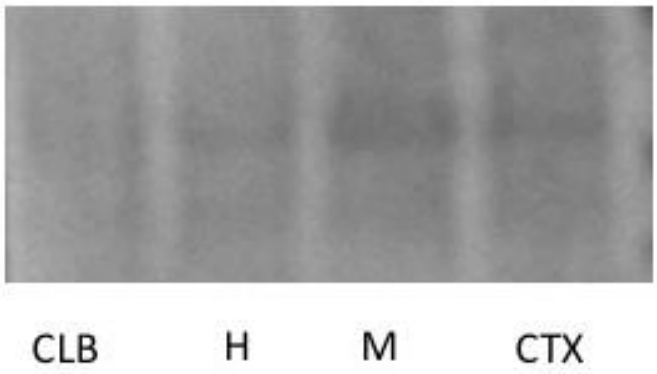


Figure 3.4: A) MCT4 expression in mouse brain. Mouse brains were sectioned into four regions: Cortex (CTX), midbrain (M), hindbrain (H), and cerebellum (CBL), and protein lysates were generated for each region. The protein lysates were subjected to SDS-PAGE and subsequent immunoblotting analyses using an antibody that recognizes MCT4 (Millipore). A representative blot is shown in panel A. Densitometry analyses were performed and are shown in panel B. Greatest expression was observed in the hindbrain, which was set at 100%. Expression in the other regions was determined relative to that found in the hindbrain. Error bars represent the standard deviation among duplicate trials.

A.



B.

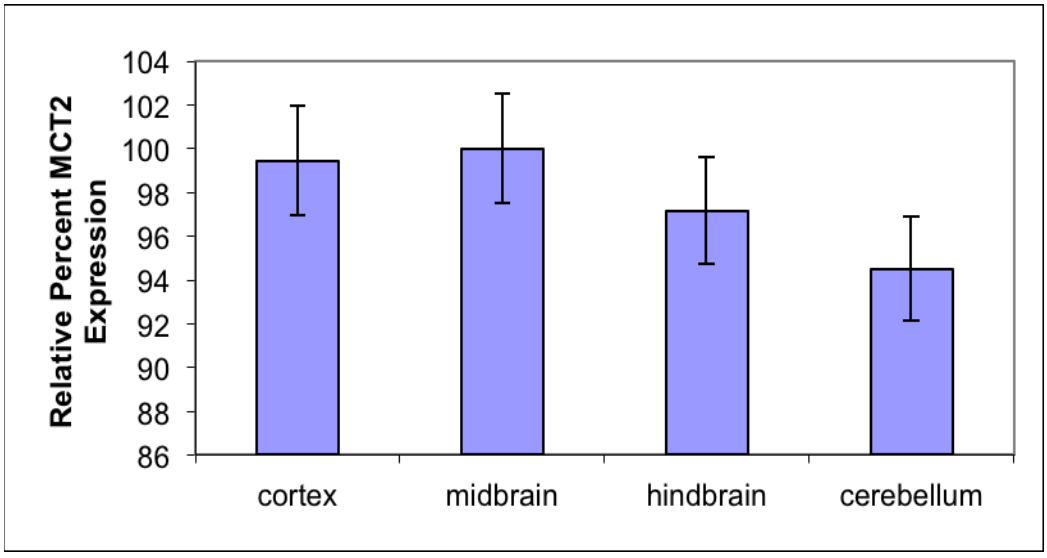


Figure 3.5: A) MCT2 expression in mouse brain. Mouse brains were sectioned into four regions: Cortex (CTX), midbrain (M), hindbrain (H), and cerebellum (CBL), and protein lysates were generated for each region. The protein lysates were subjected to SDS-PAGE and subsequent immunoblotting analyses using an antibody that recognizes MCT2 (Millipore). A representative blot is shown in panel A. Densitometry analyses were performed and are shown in panel B. Greatest expression was observed in the midbrain, which was set at 100%. Expression in the other regions was determined relative to that found in the midbrain. Error bars represent the standard deviation among duplicate trials.

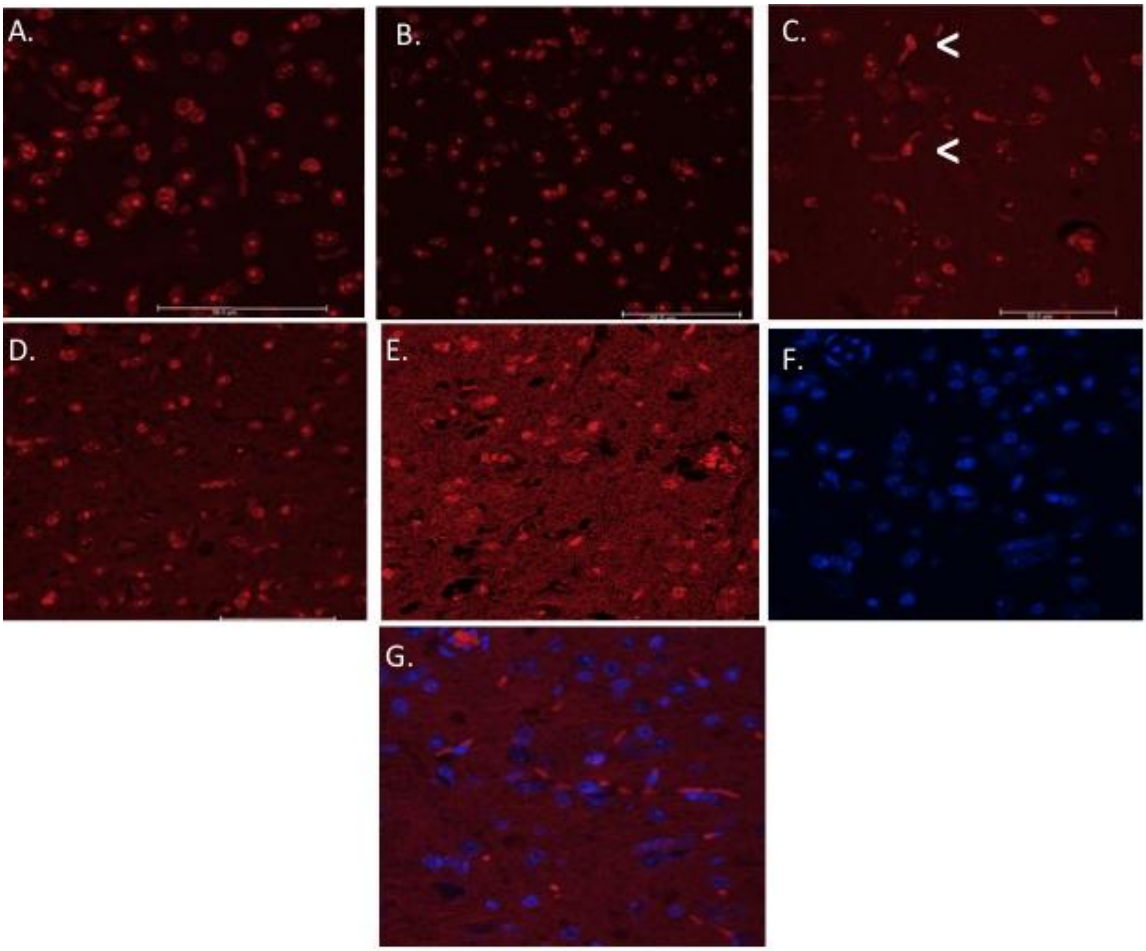


Figure 3.6: Protein expression in the mouse brain. Paraffin-embedded normal mouse brain sections were subjected to immunohistochemical analyses using antibodies specific for Basigin (A, red); Embigin (B, red); MCT1 (C, red); MCT4 (D, red); and MCT2 (E, red). A negative control slide was also generated using the Alexa-Fluor 594 polyclonal goat anti-rabbit secondary antibody (F, red) and the DNA-binding dye DRAQ5 (F and G, blue). Comparison of panels F and G with the others suggests that Basigin, Embigin, MCT2, and MCT4 are expressed by cell bodies within the brain and may be expressed by the same cells. MCT1 appears to be expressed by blood vessel endothelial cells (arrowheads), although a similar pattern of signal was observed using the secondary antibody alone (compare F with C). The magnification bars shown in panels A-E represent 50 μm.

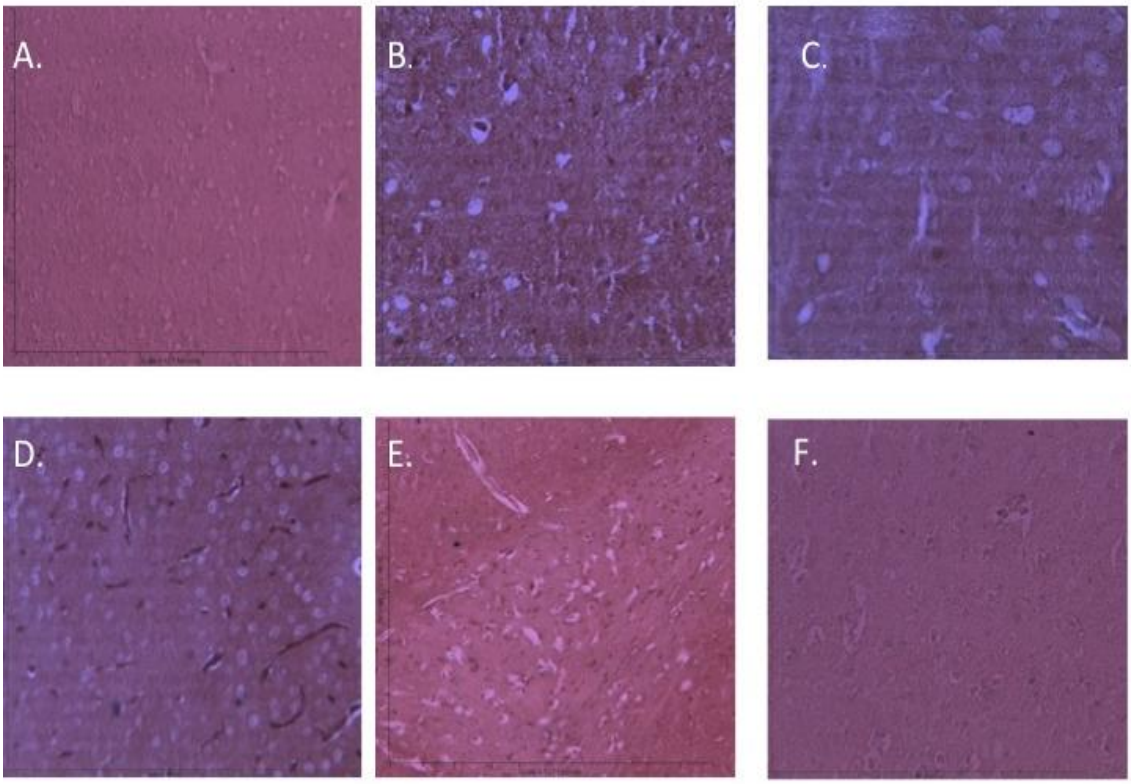


Figure 3.7: Protein expression in mouse brain. Paraffin-embedded normal mouse brain sections were subjected to immunohistochemical analyses using antibodies specific for Basigin (B); Embigin (C); MCT1 (D); MCT2 (E); and MCT4 (F). A negative control slide was also generated using the ImmPRESS Anti-Rabbit Ig Peroxidase antibody (A). Comparison of panels E and F with others suggests that MCT2 and MCT4 are expressed by cell bodies within the brain and may be expressed by the same cells. MCT 1 appears to be expressed by blood vessel endothelial cells. Panel B shows Basigin expression throughout the tissue, in both cell bodies and blood vessel endothelial cells. Panel C shows that Embigin expression is concentrated in cell bodies as well. The magnification bars shown in panels A-D and F represent 127 and that in panel E represents 254 μm.

the tissue. The expression of MCT1 appears to be restricted to blood vessels. Although a similar signal was observed using the secondary antibody alone in the fluorescence slides (Figure 3.6), slides prepared via colormetric detection show MCT1 in blood vessels and no signal using the secondary antibody alone (Figure 3.7). Signals within blood vessel endothelial cells were also observed using the antibodies specific for Basigin and MCT4 (Figures 3.6 and 3.7).

The cresyl violet histochemical analyses of the sections indicate that the region of brain shown is within the midbrain (data not shown). This assessment is based on the presence of the cerebral aqueduct, superficial and deep gray layers of the superior colliculus, and the optic nerve layer of the superior colliculus, when compared to Franklin and Paxinos (2008).

The immunoblotting and immunohistochemical results led to questions concerning the nature of the molecular interaction between Embigin and the MCTs. Since these proteins are expressed rather ubiquitously in the brain and appear to be expressed by the same cells (Embigin, MCT2, and MCT4), the ability of the transmembrane domain of Embigin to bind to MCT2, MCT4, and MCT1 was then examined. Recombinant protein probes corresponding to the entire putative transmembrane domain of Embigin, as well as six-amino acid segments of the domain, were prepared by molecular methods for use in ELISA binding analyses. Initially, the ability of the transmembrane domain of Embigin to bind to MCT2 was assessed. Endogenous MCT2 was captured from mouse brains and probed with the recombinant Embigin transmembrane domain probes. The association between recombinant Embigin transmembrane domain and MCT2 was no different from that of the control protein

probe, which consists of the pET102 vector-specific amino acids, and MCT2 (Figure 3.8). Similar studies were conducted using endogenous MCT4 (Figure 3.9) and MCT1 (Figure 3.10), and again it was determined that the transmembrane domain of Embigin does not interact with either transporter.

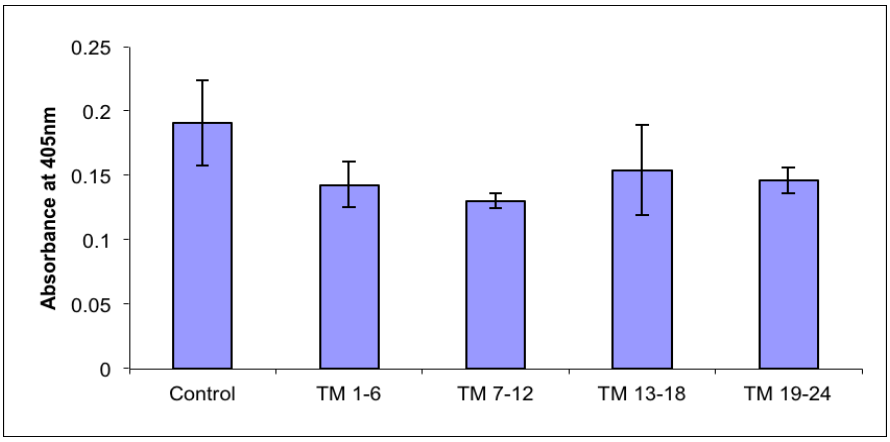


Figure 3.8: A sandwich ELISA was performed in which MCT2 was captured from mouse brain lysates. Probes consisting of six amino acid segments of the transmembrane domain of Embigin (TM 1-6, TM 7-12, TM 13-18, TM 19-24) or the pET102 vector-specific amino acids (control) were incubated with MCT2, followed by an antibody specific for the 6X-His epitope (Invitrogen Corporation) and alkaline phosphatase (AP)-conjugated secondary antibody (Thermo Fisher Scientific). AP substrate was added to wells and the reaction was stopped with the addition of 2N NaOH after color development. The absorbance at 405 nm was measured and recorded. Tests were performed in duplicate. Standard deviations are shown as error bars. No statistically significant binding was observed with any of the probes tested.

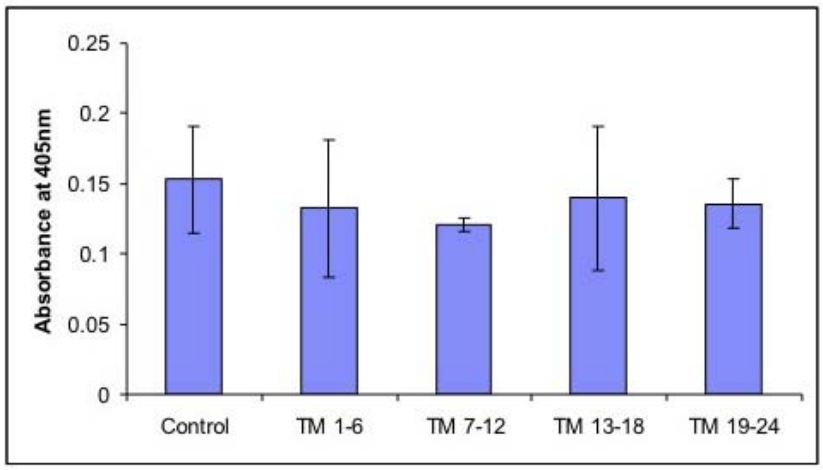


Figure 3.9: A sandwich ELISA was performed in which MCT4 was captured from mouse brain lysates. Probes consisting of six amino acid segments of the transmembrane domain of Embigin (TM 1-6, TM 7-12, TM 13-18, TM 19-24) or the pET102 vector-specific amino acids (control) were incubated with MCT4, followed by an antibody specific for the 6X-His epitope (Invitrogen Corporation) and alkaline phosphatase (AP)-conjugated secondary antibody (Thermo Fisher Scientific). AP substrate was added to wells and the reaction was stopped with the addition of 2N NaOH after color development. The absorbance at 405 nm was measured and recorded. Tests were performed in duplicate. Standard deviations are shown as error bars. No statistically significant binding was observed with any of the probes tested.

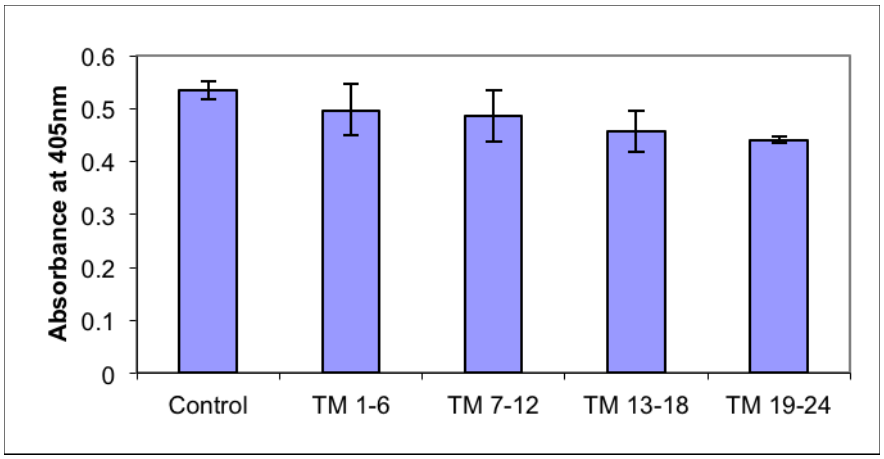


Figure 3.10: A sandwich ELISA was performed in which MCT1 was captured from mouse brain lysates. Probes consisting of six amino acid segments of the transmembrane domain of Embigin (TM 1-6, TM 7-12, TM 13-18, TM 19-24) or the pET102 vector-specific amino acids (control) were incubated with MCT1, followed by an antibody specific for the 6X-His epitope (Invitrogen Corporation) and alkaline phosphatase (AP)-conjugated secondary antibody (Thermo Fisher Scientific). AP substrate was added to wells and the reaction was stopped with the addition of 2N NaOH after color development. The absorbance at 405 nm was measured and recorded. Tests were performed in duplicate. Standard deviations are shown as error bars. No statistically significant binding was observed with any of the probes tested.

Discussion

It has been proposed that the proton-linked monocarboxylate transporters MCT1, MCT2, and MCT4 must interact with a cell adhesion molecule of the IgSF for expression at the plasma membrane. Cell adhesion molecules within the Basigin subset of the IgSF have specifically been implicated in this role of accessory protein (Kirk et al., 2000; Wilson et al., 2005; Bergersen, 2007). Although it has been documented that MCTs do require Basigin for membrane expression (Kirk et al., 2000; Wilson et al., 2002; Philp et al., 2003), other studies indicate that MCTs and Basigin do no always have overlapping expression (Clamp et al., 2004). Studies of the Basigin null mouse, by this laboratory, indicate that although associations between Basigin and MCT1 occur (Philp et al., 2003) via hydrophobic interactions (Finch et al., 2009) in the neural retina, associations between Basigin and MCT1 do not occur in the brain (Ochrietor et al., 2010b). The purpose of this study was to determine if Embigin, a member of the Basigin subset of the IgSF, is the natural accessory protein for MCTs in the brain, and if so, to determine if a hydrophobic association is used.

Immunoblotting and ELISA analyses were first performed to determine the gross expression patterns of Basigin, Embigin, MCT1, MCT2, and MCT4 in the mouse brain. Immunoblotting was performed on isolated cortex, midbrain, hindbrain, and cerebellum from adult mouse brain using antibodies specific for Basigin and the MCTs, whereas an ELISA analysis of the same regions was performed for Embigin, as that antibody does not recognize the protein antigen in a denatured form (J. Ochrietor, personal observation). It was determined that the proteins of interest are rather ubiquitously expressed

throughout the mouse brain, which correlates with the results of previous studies (Fan et al., 1998a, Fan et al., 1998b; Morris and Flemlee, 2008; Halestrap and Price, 1999).

In an effort to better understand the results of the initial biochemical analyses of Basigin, Embigin, and MCT expression, immunohistochemical analyses of paraffin-embedded mouse brain sections were performed. Although it can be inferred that Basigin, Embigin, MCT2, and MCT4 are co-expressed by cells of the midbrain, based on the comparison of signals from successive sections, an overlay of signals within a single section could not be performed since all antibodies for this study were generated in rabbits. It is intriguing that Basigin appears to be expressed by cells that also express MCT2 and/or MCT4, even though it does not direct their expression at the plasma membrane (Ochrietor et al., 2010b). Basigin expression was found throughout the tissue on the surface of cell bodies. Expression of Basigin, MCT1, and MCT4 also appear to colocalize on blood vessel endothelial cells, which is supported by previous studies of mouse neural retina (Ochrietor et al., 2001). In support of the hypothesis that Embigin is the accessory protein for MCT expression in the mouse brain, its expression was determined to be similar to that of MCT2 and MCT4 in that tissue. Based on the pattern of expression, it is likely that Embigin is the natural accessory protein for MCT2 and MCT4 in mouse brain, and not merely compensating for the lack of Basigin in the Basigin null mice, as other recent studies by this laboratory have determined that Embigin expression in the Basigin null mouse brain is similar to that of control brains (J. Ochrietor, personal observation). That is to say that Embigin expression is not upregulated in the Basigin null mouse brain to compensate for the lack of Basigin expression.

It was proposed that a shuttle complex is formed in the brain in which Embigin interacts with MCT2 on neurons and MCT4 on glial cells to direct their expression to the plasma membrane so that transport of metabolites between cells can occur. This is a molecular explanation for the astrocyte-neuron coupling mechanism hypothesized to exist within the brain several decades ago (Magistretti, 2006) and similar to a shuttle complex hypothesized to exist within the neural retina (Ochrietor and Linser, 2004). To determine whether Embigin directly interacts with MCT2 and MCT4 in a manner similar to that of Basigin and MCT1 (Finch et al., 2009), recombinant proteins corresponding to the transmembrane domain of Embigin were generated for ELISA binding assays using endogenous mouse MCTs. Although the proteins appear to be expressed by the same cells within the brain, there does not appear to be a direct interaction between Embigin and MCTs via the transmembrane domain of Embigin. This should not be completely surprising, as although Basigin and Embigin share 50% amino acid similarity in their transmembrane domains, the similarity is not within the regions determined to be involved in the Basigin-MCT1 association (Finch et al., 2009). Studies undertaken by Kathryn Fletcher in her UNF undergraduate honors thesis indicate that the extracellular domain of Embigin also does not interact with MCTs. A future study, in which the Embigin cytoplasmic tail will be assessed, should determine whether any portion of the Embigin protein interacts with MCTs.

Although the data suggest that Embigin and MCTs do not interact, several caveats must be discussed. The studies employed herein only examined the involvement of Embigin amino acids and not the associated carbohydrates. Obviously, no carbohydrates are attached to the Embigin molecule within the transmembrane domain, but the

possibility exists that extracellular carbohydrate moieties direct the interaction. In addition, while conducting immunoblotting and ELISA assays in this study, the same concentrations of recombinant and endogenous proteins were used that had been successful in previous binding assays of Basigin and MCT1 (Finch et al., 2009). It is possible that Embigin does in fact bind to the MCTs but at a much lower affinity than the Basigin-MCT1 interaction, as Embigin probes may have been used at too low of a concentration to generate a visible reaction required in the ELISA.

The results of this study suggest that Embigin does not participate in a shuttle complex with MCTs in the mouse brain, as depicted in Figure 1.5. However, it is not yet known whether another accessory protein acts as the chaperone for MCT expression or if MCTs do not need an accessory protein for expression at the plasma membrane. Future studies will be required to uncover the nature of MCT expression in the mouse brain.

References

Allen, Greg, Richard B. Buxton, Eric C. Wong, and Eric Courchesne. "Attentional Activation of the Cerebellum Independent of Motor Involvement." *Science* 275 (1997): 1940-943.

Altruda, F., P. Cervella, M.L. Gaeta, A. Daniele, F. Giancotti, G. Tarone, G. Stefanuto and L. Silengo. "Cloning of cDNA for a novel mouse membrane glycoprotein (gp42): shared identity to histocompatibility antigens, immunoglobulins and neural-cell adhesion molecules." *Gene* 85 (1989): 445-452.

Aubert, A., R. Costalat, P. Magistretti, and L. Pellerin. "Brain Lactate Kinetics: Modeling Evidence for Neuronal Lactate Uptake upon Activation." *Proceedings of the National Academy of Sciences of the United States of America* 102.45 (2005): 16448-6453.

Bantick, Susanna J., Richard G. Wise, Alexander Phoghaus, Stuart Clare, Stephen M. Smith, and Irene Tracey. "Imaging How Attention Modulates Pain in Humans Using Functional MRI." *Oxford Journal* 125.2 (2002): 310-19.

Bear, Mark F., Barry W. Connors, and Michael A. Paradiso. *Neuroscience: Exploring the Brain*. Philadelphia, PA: Lippincott Williams & Wilkins , 2007.

Bergersen, L.H. "Is Lactate Food for Neurons? Comparison of Monocarboxylate Transporter Subtypes in Brain and Muscle." *Neuroscience* 145.1 (2007): 11-19.

Biswas, C., Y. Zhang, R. DeCastro, H. Guo, T. Nakamura, H. Kataoka and K. Nabeshima. "The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily" *Cancer Research* 55 (1995): 434-439.

"Basic Local Alignment Search Tool (BLAST)." National Center for Biotechnology Information. Web. 7 Feb. 2010. <http://www.ncbi.nlm.nih.gov/>.

Bonen, A., D. Miskovic, M. Tonouchi, K. Lemieux, M. C. Wilson, A. Marette, and A. P. Halestrap. "Abundance and Subcellular Distribution of MCT1 and MCT4 in Heart and Fast Twitch Skeletal Muscles." *American Journal of Physiology-Endocrinology and Metabolism* 278.6 (2000): 1067-077.

Bouzier-Sore, A., P. Canioni, PJ Magistretti, and L. Pellerin. "Lactate Is a Perferetial Oxidateive Energy Substrate over Glucose for Neurons in Culture." *Journal of Cerebral Blood Flow and Metabolism: Offical Journal of the International Society of Cerebral Blood Flow and Metabolism* 23.11 (2003): 1298-1306.

Buchel, Christian, Karin Bornhovd, Markus Quante, Volkmar Glauche, Burkhard Bromm, and Cornelius Weiller. "Dissociable Neural Responses Related to Pain Intensity, Stimulus Intensity, and Stimulus Awareness within the Anterior Cingulate Cortex: A Parametric Single-Trial Laser Functional Magnetic Resonance Imaging Study." *The Journal of Neuroscience* 22.3 (2002): 970-76.

Carpenter, Ellen M., Judy M. Goddard, Osamu Chisaka, Nancy R. Manley, and Mario R. Capecchi. "Loss of Hox-A1 (Hox-1.6) Function Results in the Reorganization of the Murine Hindbrain." *Development* 118 (1993): 1063-075.

Casey, Kenneth L. "Forebrain Mechanisms of Nociception and Pain: Analysis through Imaging." *Proceedings of the National Academy of Sciences* 96 (1999): 7668-674.

"Central Pain Pathways: The Spinothalamic Tract - Neuroscience - NCBI Bookshelf." Web. 01 June 2011. <http://www.ncbi.nlm.nih.gov/books/NBK10967/>.

Clamp, M. F., J. D. Ochrietor, T. P. Moroz, and P. J. Linser. "Developmental Analyses of 5A11/Basigin, 5A11/Basigin-2 and Their Putative Binding Partner MCT1 in the Mouse Eye." *Experimental Eye Research* 78.4 (2004): 777-89

Dalsgaard, M., B. Quistorff, E. Danielsen, C. Selmer, T. Vogelsang, and N. Secher. "A Reduced Cerebral Metabolic Ratio in Exercise Reflects Metabolism and Not Accumulation of Lactate within the Human Brain." *Journal of Physiology* 554.2 (2004): 571-578.

Daniel, PM, ER Love, and OE Pratt. "The Transport of Ketone Bodies into the Brain of the Rat (in Vivo)." *Journal of Neurological Sciences* 34.1 (1977): 1-13.

Fadool, J.M and P.J. Linser. "5A11 antigen is a cell recognition molecule which is involved in neuronal-glial interactions in avian neural retina" *Develop. Dyn*. 196 (1993): 252-262.

Fan, Qi-Wen, Kenji Kadomatsu, Kenji Uchimura, and Takashi Muramatsu. "Embigin/basigin Subgroup of the Immunoglobulin Superfamily: Different Modes of Expression during Mouse Embryogenesis and Correlated Expression with Carbohydrate Antigenic Markers." *Developmental Growth Differ.* 40 (1998a): 277-86.

Fan, Qi-Wen, Shigeki Yuasa, Naohiko Kuno, Takao Senda, Miya Kobayashi, Takashi Muramatsu, and Kenji Kadomatsu. "Expression of Basigin, a Member of the Immuoglobulin Superfamily, in the Mouse Central Nervous System." *Neuroscience Research* 30 (1998b): 53-63.

Finch, Nicole A., Paul J. Linser, and Judith D. Ochrietor. "Hydrophobic Interactions Stablizie the Basigin-MCT1 Complex." *Springer Science+Business Media* 28 (2009): 362-368.

Fossum, S.S. MAllett, A.N. Barclay. "The MRC OX-47 antigen is a member of the immunoglobulin superfamily with an unusual transmembrane sequence" *European Journal of Immunology* 21 (1991): 671-679.

Franklin, Keith B. J., and George Paxinos. *The Mouse Brain in Stereotaxic Coordinates*. 3rd ed. New York: Elsevier/Academic, 2008.

Gambon, Paul L. "Localization and Characterization of the Interactions between Basigin Gene Products and Monocarboxylate Transporters in the Olfactory Bulb of the Mouse." Thesis. University of North Florida, 2011.

Guenette, R. Sean, Srikala Sridhar, Mark Herley, Marilyn Mooibroek, Paul Wong, and Martin Tenniswood. "Embigin, a Developmentally Expressed Member of the Immunoglobulin Super Family, Is Also Expressed during Regression of Prostate and Mammary Gland." *Developmental Genetics* 21.4 (1997): 268-78.

Halaby, D.M., and J.P.E Mornon. "The Immunoglobulin Superfamily: An Insight on Its Tissular, Species, and Functional Diversity." *Journal of Molecular Evolution* 46 (1998): 389-400.

Halestrap, Andrew P., and Nigel T. Price. "The Proton-linked Monocarboxylate Transporter (MCT) Family: Structure, Function and Regulation." *Biochemistry Journal* 343 (1999): 281-99.

Halestrap, A., and D Meredith. "The SLC16 Gene Family-from Monocarboxylate Transporters (MCTs) to Aromatic Amino Acid Transporters and beyond." *Pflugers Archive European Journal of Physiology* 447.5 (2004): 619-28.

Hori, A., N. Katayama, S. Kachi, M. Kondo, K. Kadomatsu, J. Usukura, T. Muramatsu, S. Mori, Y. Miyake. Retinal Dysfunction in Basigin Deficiency. Invest Opthamol Vis Sci. 41 (2000): 3128-3133.

Hsieh, Jen-Chuen, M. Stahle-Backdahl, O. Hagermark, S. Stone-Elander, G. Rosenquiest, M. Ingvar. "Traumatic nociceptive pain activates the hypothalamus and the periqueductal gray: a positron emission tomography study". *Pain* 64 (1995): 303-314.

Huang, Ruo-Pan, Masayuki Ozawa, Kenji Kadomatsu, and Takashi Muramatsu. "Developmentally Regulated Expression of Embigin, a Member of the Immunoglobulin Superfamily Found in Embryonal Carcinoma Cells." *Differentiation Ontogeny and Neoplasia* (1990): 76-83.

Hubbard, A.L., J. R. Bartles and L.T. Braitermann. "Idenfication of rat hepatocyte plasma membrane proteins using monoclonal antibodies" *Journal o f Cell Biology* 100 (1985): 1115-1125.

Igakura, Tadahiko, Kenji Kadomatsu, Osamu Taguchi, Hisako Muramatsu, Tadashi Kaname, Teruo Miyauchi, Ken-ichi Yamamura, Kimiyoshi Arimura, and Takashi Muramatsu. "Roles of Basigin, a Member of the Immunoglobulin Superfamily, in Behavior as to an Irritating Odor, Lymphocyte Response, and Blood-Brain Barrier." *Biochemical and Biophysical Research Communications* 224 (1996): 33-36.

Igakura, T., K. Kadomatsu, T. Kaname, H. Muramatsu, Q. Fan, and T. Miyauchi. "A Null Mutation in Basigin, an Immunoglobulin Superfamily Member, Indicates Its Important Roles in Peri-implantation Development and Spermatogenesis." *Developmental Biology* 194.2 (1998): 152-65.

Itoh, Y., T. Esaki, K. Shimoji, M. Cook, M. Law, E. Kaufman, and L. Sokoloff. "Dicholoroacetate Effects on Glucose and Lactate Oxidation by Neurons and Astroglia in Vitro and on Glucosse Utilization by Brain in Vivo." *PNAS* 100.8 (2003): 4879-884.

Juel, C., and A. Halestrap. "Lactate Transport in Skeletal Muscle- Role and Regulation of the Monocarboxylate Tranporter." *The Journal of Physiology* 517 (1999): 633-42.

Kasinrerk, W., E. Fiebiger, I. Stefanova, T. Baumruker, W. Knapp and H. Stockinger. "Human leukocyte activation antigen M6, a member of the Ig superfamily, is the species homologue of rat OX-47, mouse basigin, and chicken HT7 molecule" Journal of Immunology 149 (1992): 847-854.

Kirk P, MC Wilson, C Heddle, MH Brown, AN Barclay, AP Halestrap. "CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression" *European Molecular Biology Journal* 19 (2000): 3896-3904.

Liu, Qingpo, Shijuan Dou, Guang'en Wang, Zhimin Li, and Ying Feng. "Evolution and Functional Divergence of Monocarboxylate Transporter Genes in Vertebrates." *Gene* 423 (2008): 14-22.

Magistretti PJ. (2006) Neuron-glia metabolic coupling and plasticity. *J. Exp. Biol.* 209, 2304-2311.

Manoharan, Christine, Marieangela C. Wilson, Richard B. Sessions, and Andrew P. Halestrap. "The Role of Charged Residues in the Transmembrane Helices of Monocarboxylate Transporter 1 and Its Ancillary Protein Basigin in Determining Plasma Membrane Expression and Catalytic Activity." *Molecular Membrane Biology* 23.6 (2006): 486-98.

Miller, Earl K., and Jonathan D. Cohen. "An Integrative Theory of Prefronal Cortex Function." *Annual Review of Neuroscience* 24 (2001): 167-202.

Miyauchi T, Y. Masuzawa and T. Muramatsu. "The Basigin group of the immunoglobulin superfamily: complete conservation of a segment in and around transmembrane domains of human and mouse Basigin and chicken HT7 Antigen." *Journal of Biochemistry* 110 (1991): 770-74.

Morris, M. E. and M. A Felmlee. Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse γ-hydroxybutyric acid. Am. Assoc. Pharm. Sci. 10, (2008): 311-321.

Mouse Genome Database (MGD) at the Mouse Genome Informatics website, The Jackson Laboratory, Bar Harbor, Main. World Wide Web (5/2011). http://www.informatics.jax.org.

Muramatsu, T., and T. Miyauchi. "Basigin (CD147): a Multifunctional Transmembrane Involved Iin Reproduction, Neural Function, Inflammation and Tumor Invasion." *Histology Histopathology* 18 (2003): 981-87.

Nakai, Masaaki, Li Chen, and Romana A. Nowak. "Tissue Distribution of Basigin and Monocarboxylate Transporter 1 in the Adult Male Mouse: A Study Using the Wild-Type and Basigin Gene Knockout Mice." *The Anatomical Record Part A* 288A (2006): 527-35.

Naruhashi K, K. Kadomatsu, T. Igakura, Q. Fan, N. Kuno, H. Muramatsu, T. Miyauchi, T. Hasegawa, A. Itoh, T .Muramatsu and T. Nabeshima. 1997. "Abnormalities of sensory and memory functions in mice lacking Basigin gene." *Biochemical and Biophysical Research Communications*. Vol 236(3) (1997): 733-737.

Ochrietor, Judith D., and Paul J. Linser. "5A11/Basigin Gene Products Are Necessary for Proper Maturation and Function of the Retina." *Developmental Neuroscience* 26.5-6 (2004): 380-87.

Ochrietor, Judith D., Tatiana P. Moroz, Paul J. Linser. "The 2M6 antigen is a Müller cell-specific intracellular membrane-associated protein of the sarcolemmal-membrane-associated protein family and is also Top." *Molecular Vision* 16 (2010a): 961-969.

Ochrietor JD, P. Peterson , L.N. Little, E. Stotz-Potter. (2010b) Learning and memory deficits of Basigin null mice are not likely caused by a metabolic defect. Mol. Biol. Cell 20: Abstract #1624.

Ochrietor, J., T. Moroz, M. Clamp, A. Timmers, T. Muramatsu, and P. Linser. "Inactivation of the Basigin Gene Impairs Normal Retinal Development and Maturation." *Vision Research* 42.4 (2002): 447-53.

Ochrietor, J., T. M. Moroz, K. Kadomatsu, T. Muramatsu, and Paul J. Linser. "Retinal Degeneration Following Failed Photoreceptor Maturation in 5A11/Basigin Null Mice." *Experimental Eye Research* 72.4 (2001): 467-77.

Ochrietor, Judith D., Tatiana P. Moroz, Leslie Van Ekeris, Michael F. Clamp, Stephanie C. Jefferson, Ana C. DeCarvalho, James M. Fadool, Graeme Wistow, Takashi Muramatsu, and Paul J. Linser. "Retina-Specific Expression of 5A11/Basigin-2, a Memeber of the Immunoglobulin Gene Superfamily." *Investigative Ophthalmology & Visual Science* 44.9 (2003): 4086-096..

Ovens, Matthew J., Andrew J. Davies, Marieangela C. Wilson, Clare M. Murray, and Andrew P. Halestrap. "AR-C155858 Is a Potent Inhibitor of Monocarboxylate Transporters MCT1 and MCT2 That Binds to an Intracellular Site Involving Transmembrane Helices 7-10." *Biochemistry Journal* 425 (2010): 523-30.

Pellerin, L. "How Astrocytes Feed Hungry Neurons." *Molecular Neurobiolgy* 32.1 (2005): 59-72.

Pellerin, L., and P. J. Magistretti. "Glutamate Uptake into Astrocytes Stimulates Aerobic Glycolysis: A Mechanism Coupling Neuronal Activity to Glucose Utilization." *Proceedings of the National Academy of Sciences of the United States of America* 91.22 (1994): 10625-0629.

Philp, Nancy J., Judith D. Ochrietor, Carla Rudoy, Takashi Muramatsu, and Paul J. Linser. "Loss of MCT1, MCT3, and MCT4 Expression in the Retinal Pigment Epithelium and Neural Retina of the 5A11/Basigin-Null Mouse." *Investigative Ophthalmology & Visual Science* 44.3 (2003): 1305-311.

Pierre, K., L. Pellerin, R. Debernardi, B. M. Riederer, and P. J. Magistretti. "Cell-specific Localization of Monocarboxylate Transporters, MCT1 and MCT2, in the Adult Mouse Brain Revealed by Double Immunohistochemical Labeling and Confocal Microscopy." *Neuroscience* 100.3 (2000): 617-27.

Poitry-Yamate, C., S. Poitry, and M. Tsacopoulos. "Lactate Released by Muller Glial Cells Is Metabolized by Photoreceptors from Mammalian Retina." *The Journal of Neuroscience* 15 (1995): 5179-191.

Poole, R. C., and A. P. Halestrap. "Interaction of the Erythrocyte Lactate Transporter (monocarboxylate Transporter 1) with an Intergral 70-kDa Membrane Glycoprotein of the Immunoglobulin Superfamily." *The Journal of Biological Chemistry* 272.23 (1997): 14624-4628.

"Primer: Structure of the Brain." *Linsenbardt.net « A Blog for Brooke and Andy*. Web. 01 June 2011. <http://linsenbardt.net/?p=2497>.

Saab, C. and W.D. Willis. "The Cerebellum: Organization, Functions and Its Role in Nociception." *Brain Research Reviews* 42.1 (2003): 85-95.

Schlosshauer, B. and K.-H. Herzog. “Neurothelin: an inducible cell surface glycoprotein of blood-brain barrier-specific endothelial cells and distinct neurons” Journal of Cell Biology 110 (1990): 1261-1274.

Seulberger, H., F. Lottspeich and W. Risau. “The inducible blood-brain barrier-specific endothelial cells and distinct neurons” *EMBO Journal* 9 (1990): 2151-2158.

Tachikui, Hiroshi, Nobuyuki Kurosawa, Kenji Kadomatsu, and Takashi Muramatsu. "Genomic Organization and Promoter Activity of Embigin, a Member of the Immunoglobulin Superfamily." *Gene An International Journal on Genes and Genomes* 240 (1999): 325-32.

Tessier-Lavigne, M. "Axon Guidance by Diffusible Repellants and Attractants." *Current Opinion in Genetics & Development* 4.4 (1994): 596-601.

Toyama, Y., M. Maekawa, K. Kadomatsu, T. Miyauchi, T. Muramatsu, and S. Yuasa. "Histological Characterization of Defective Spermatogensis in Mice Lacking the Basigin Gene." *Anatomy Histology Embryology* 28 (1999): 205-13.

Vannucci, RC, and TE Duffy. "Influence of Birth on Carbohydrate and Energy Metabolism in Rat Brain." *American Journal of Physiology* 226 (1974): 933-40.

Villemure, Chantal, and M. C. Bushnell. "Cognitive Modulation of Pain: How Do Attention and Emotion Influence Pain Processing?" *Pain* 95 (2002): 195-99.

Wilson, Marieangela C., David Meredith, Chotirote Bunnun, Richard B. Sessions, and Andrew P. Halestrap. "Studies on the DIDS-binding Side of Monocarboxylate Transporter 1 Suggest a Homology Model of the Open Conformation and a Plausible Translocation Cycle." *The Journal of Biological Chemistry* 284.30 (2009): 20011-0021.

Wilson, Marieangela C., David Meredith, Jocelyn E. Manning Fox, Christine Manoharan, Andrew J. Davies, and Andrew P. Halestrap. "Basigin (CD147) Is the Target for Organomercurial Inhibition of Monocarboxylate Transporter Isoforms 1 and 4." *The Journal of Biological Chemistry* 280.29 (2005): 27213-7221.

Wilson, M. C., Meredith, D., Halestrap, A. P. Fluorescence resonance energy transfer studies on the interaction between the lactate transporter MCT1 and CD147 provide information on the topology and stoichiometry of the complex in situ. *J Biol Chem* 277 (2002): 3666-3672.

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