HHS Public Access

Author manuscript

Peptides. Author manuscript; available in PMC 2016 October 01.

Published in final edited form as:

Peptides. 2015 October ; 72: 50–56. doi:10.1016/j.peptides.2015.04.020.

Peptides at the Blood Brain Barrier: Knowing Me Knowing You

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Abstract

When the Davis Lab was first asked to contribute to this special edition of *Peptides* to celebrate the career and influence of Abba Kastin on peptide research, it felt like a daunting task. It is difficult to really understand and appreciate the influence that Abba has had, not only on a generation of peptide researchers, but also on the field of blood brain barrier (BBB) research, unless you lived it as we did. When we look back at our careers and those of our former students, one can truly see that several of Abba's papers played an influential role in the development of our personal research programs.

During the mid to late-eighties the Davis Lab became aware of the robust discussions regarding the ability of peptides to cross the BBB (Kastin et al., 1990). As someone who researched central and peripheral neuropeptide processing and degradation throughout the 1980's (Davis et al., 1986; Davis et al., 1984; Davis et al., 1983; Davis et al., 1987; Schoemaker et al., 1982), and had previously investigated a different barrier in the skin (Dill et al., 1983), the debate regarding the delivery of peptides to the brain was interesting, but we did not feel that it was applicable to the Davis Lab of the early to mid 1980's. However, the interest in this area was sparked when we observed, in collaboration with Dr. Terry Moody of the NIH, that some neuropeptides we were studying could be produced by and also regulate the function of cells associated with small cell lung cancer (autocrine growth factors) (Davis et al., 1991).

At this same time period, various groups in our medical pharmacology department at The University of Arizona were interested in the ability of synthetic opioid peptides to act as analgesics (Qi et al., 1990) with the ultimate goal to offset the negative side effects of morphine (dependence, tolerance, and constipation). This is when our interest in Abba's studies, coupled to our ongoing research program in neuropeptides, led to a change in the direction of our research focus.

In this manuscript, we will discuss some of the pivotal research and publications that contributed to this challenging and successful direction for our research program and also formed the bedrock of our early studies on the BBB that actively continues today (Ronaldson and Davis, 2013b; Ronaldson and Davis, 2015; Tome et al., 2015; Yang et al., 2015).

"OUCH There Goes MY Blood Brain Barrier"

Perhaps the early studies for which our research group may be best known are the novel and paradigm shifting studies spearheaded by Dr.'s Jason Huber, Tracy Brooks, Chris Campos and Melissa Seelbach (Brooks et al., 2005; Campos et al., 2008; Huber et al., 2001c; Seelbach et al., 2007). Over a 10 year period these excellent young researchers produced a series of well-designed and carefully executed studies demonstrating that peripheral pain could have a profound impact on the molecular, structural and functional properties of the BBB, and that these alterations can directly affect drug delivery to the brain. However, what has been under appreciated is the role that Abba's early studies on peptide delivery across the BBB played and the influence these foundational studies had on our lab at this time.

There had been considerable interest in the development of peptide analogs of the endogenous opioids to replace morphine, as analgesics, and much of this initial work was carried out by Abba Kastin's group (Coy and Kastin, 1980; Coy et al., 1976; Coy et al., 1978; Kastin et al., 1976), in collaboration with brilliant synthetic chemistry by Dr. David Coy. However, a major stumbling block exists towards development of any analgesic peptide analog, and that is the ability of the BBB to prevent or limit the entry of peptides and their analogs into the brain. This is not however the only issue with peptide drug delivery. Perhaps a more significant problem is the lack of robust metabolic stability in blood, tissue, and at the BBB, due to the presence of numerous, soluble and membrane associated, peptidases (Brownson et al., 1994). This is particularly true for the endogenous, opioid peptide Met-enkephalin, which, has a plasma half-life of around five seconds (Dupont et al., 1977). However, this did not stop Abba and his team who carried out several studies on Met-enkephalin analogs showing brain entry via what was termed a Brain Uptake Index (BUI) (Kastin et al., 1976). They also demonstrated both analgesia and behavioral responses upon peripheral administration (Kastin et al., 1979; Plotnikoff et al., 1976), providing evidence that synthetic peptides could be made metabolically stable, or could be given intra carotid, and could cross the BBB and elicit a biological effect. An obvious and initial challenge was to improve the enzymatic stability of peptide analogs using various chemical strategies to assure that if a peptide was shown to cross the BBB, it did so chemically intact. This point provided the initial entry of the Davis Lab into the area of peptide structure function research at the BBB, where they first demonstrated that several synthetic peptide opioid analogs could be developed that showed enhanced metabolic stability (Abbruscato et al., 1996; Egleton et al., 2000; Greene et al., 1996). Many of these analogs also showed good pain relieving analgesia when given via central (icv) administration, and a few via peripheral administration in pre-clinical pain models. Biodistribution (ADME) studies also showed measurable, intact entry into the brain for several of these peptide analogs (Weber et al., 1992; Weber et al., 1991). Though these early distribution and analgesia studies were interesting and told us that these peptides could enter the brain, the mechanism of transport

through the BBB remained elusive. After re-reading several studies by Abba and Bill Banks the issue of intact peptide brain delivery soon became a focus of the Davis Lab, and this challenge was answered with very careful and detailed analytical method development and further development of BBB models. Two approaches were taken. The initial approach was to investigate peptide delivery via a modification of the classical *in vitro* BBB model (Banks et al., 1992). This model was very effective at investigating chemical/biological rank order of peptide uptake as effected by the endothelium and other BBB cell types such as glia (Abbruscato et al., 1996; Gentry et al., 1999; Greene et al., 1996; Witt et al., 2000). It also demonstrated that if BBB soluble and membrane bound peptidases were inhibited by specific protease inhibitors, then endogenous Met-enkephalin could actually have a robust brain uptake (Brownson et al., 1994). But this was not necessarily the best method for looking at endogenous, *in vivo* peptide transport kinetics and mechanisms, though the use of isolated cultures of brain endothelial cells did help cement the concept of an “enzymatic blood-brain barrier” (Brownson et al., 1994). This enzymatic model described a barrier that most believed was present but difficult to describe and study. The answer for us was supported in some of Abba’s earlier work in which he carried out intra carotid injections of radiolabeled peptides and measured brain uptake (Banks et al., 1984; Barrera et al., 1992; Barrera et al., 1991; Kastin et al., 1976). Further investigation by the Davis Lab revealed a number of research groups used variations of this technique to study a range of radiolabeled substance entry into the brain (Cornford et al., 1982; Michaelson and Bradbury, 1982; Pardridge and Oldendorf, 1975; Smith and Takasato, 1986; Spector, 1988; Takasato et al., 1984; Zlokovic et al., 1988; Zlokovic et al., 1990; Zlokovic et al., 1989). These observations of radiolabeled analogs and drug delivery resulted in the recruitment of Dr. Sarah Williams in 1994 from Dr. Malcolm Segal’s lab in St. Thomas’ Hospital, Kings College, and London. Sarah was familiar with Dr. Betza Zlokovic’s dual carotid perfusion technique in guinea-pigs (Zlokovic et al., 1988) and also the Dr. Jane Preston adaption for rats (Preston et al., 1995). This technique was shown to be ideal for investigating the uptake kinetics of intact radiolabeled substances that have a slow brain entry and may be enzymatically unstable to cross the BBB intact. Further the technique allowed a rigorous analysis of the tracers when coupled with state of the art HPLC procedures developed within the Davis Lab (Abbruscato et al., 1996; Williams et al., 1996), to ensure that the brain uptake was indeed intact peptide and not enzymatically degraded products. A number of studies followed in the Davis Lab with several families of opioid peptides that demonstrated many different mechanisms, both saturable and diffusive, that could be targeted to cross the BBB (Abbruscato et al., 1997a; Abbruscato et al., 1997b; Abbruscato et al., 1996; Egleton and Davis, 1999; Egleton et al., 2000; Egleton et al., 2001; Huber et al., 2003; Thomas et al., 1997; Williams et al., 1996; Witt et al., 2001). Coupled with each of these published transport studies were various analgesia assays, which showed that not only could the peptides cross the BBB, but they were CNS and biologically active when they finally did gain entry into the brain and it was not only radiolabel that was quantified, but intact peptide. Regional CNS distribution was even analyzed in some of our early investigations and linked to brain and spinal cord sites that were known to be rich in delta- and mu-opioid receptors (Abbruscato et al., 1997b).

Hypoxia/Aglycemia/Stroke/Neuroprotection at the BBB

Though the peptide transport studies proved to be quite fruitful in control, healthy, preclinical models, they were not fully instructive as to the mechanism of peptide drug delivery that is involved in models of disease states where drug delivery is critical. During this same time period in the Davis Lab, Abba Kastin and Bill Banks were publishing important and seminal papers indicating that BBB transport of various substances to the brain could be altered via specific disease processes (Banks et al., 1997; Banks and Kastin, 1985a; Banks and Kastin, 1985b; Banks et al., 1999; Banks et al., 2001; Pan et al., 1996). At this same time, Dr. Tom Abbruscato had started working on a NIH fellowship in the Davis Lab studying the cerebrovascular effects of stroke on the *in vitro* BBB phenotype and transport characteristics. Increased cerebrovascular permeability is a critical factor in the development of vasogenic brain edema, a leading cause of death in ischemic stroke. Once again, there was a need for a disease based approach to characterize and understand ischemic brain drug delivery, especially for novel, peptide based neurotherapeutics. Early *in vitro* experiments suggested that astrocytes provide a protective role to the BBB endothelium during hypoxia/aglycemic conditions through the association with E-cadherin (a calcium dependent adherence protein) (Abbruscato and Davis, 1999b). Additional foundational experiments deciphered the cerebrovascular effects of hypoxia and/or aglycemia. Using the membrane-impermeant marker, [^{14}C] sucrose, we found that with hypoxia alone, long exposures (48 h) were needed to result in measurable increases in BBB permeability. Hypoxia/aglycemia exposure resulted in a much shorter time (1–3 h) required for changes in paracellular permeability (Abbruscato and Davis, 1999a). These *in vitro*, pathophysiologic experiments helped to refine *in vitro* conditions that provide the basis for future mechanistic, neurovascular stroke studies. Additional pharmacologic experiments verified that altered endothelial cell calcium flux was responsible for the permeability change observed after both hypoxic and hypoxic/aglycemic exposures. These pathophysiologic experiments sparked interest in understanding the contributions of the NVU to cellular and vasogenic brain edema associated with stroke, but also generated interest into the delivery of stable, peptide based, opioid receptor agonists to the ischemic brain for neuroprotection. This work continues to be developed in the Abbruscato Lab at Texas Tech University Health Sciences Center where further experiments have tested the delivery of both selective and non-selective peptide based analgesics to the ischemic brain. Biphalin, a well characterized, dimeric enkephalin based analgesic that was originally synthesized by Lipkowski et al. (Lipkowski et al., 1982) at Arizona. Biphalin has a unique pharmacologic profile; it has high affinity to MOR and DOR and low affinity to KOR, it crosses the BBB well for a peptide, and has a good serum and brain half-life of 87 and 193 min, respectively (Horan et al., 1993). Still to this date, biphalin has been shown to be one of the most potent, peptide based analgesics synthesized (Feliciani et al., 2013). Recent experiments in Dr. Tom Abbruscato's Lab have shown that biphalin plays a significant role in reducing cellular edema in neurons subjected to oxygen glucose deprivation by modulating the expression and function of the ion transporter Na,K,2Cl-cotransporter (Yang et al., 2011a). Further experiments also validated that these neuroprotective effects of biphalin are also seen in hippocampal slices subjected to oxygen glucose deprivation conditions (Yang et al., 2011b). Biphalin was also shown to significantly decrease edema

(53%) and infarction (48%) ratios in an in vivo focal model of brain ischemia (Yang et al., 2011b). A recent set of experiments in the Abbruscato Lab have also compared subtype selective peptide agonist to biphalin with respect to stroke neuroprotection and have even confirmed that in all cases, biphalin enhanced stroke immunohistochemical and behavioral neuroprotection in comparison to other subtype-selective opioid receptor agonist that are peptide based (DPDPE and DAMGO) (Yang et al., 2015). Interestingly, the bivalent nature of biphalin, along with the double tyramine moiety, help reduce both glutamate toxicity and oxidative stress associated with ischemia-reperfusion injury. Without the seminal work of Abba with respect to peptide based brain drug delivery our work would not continue with respect to deciphering ischemic brain drug delivery of this promising, peptide based neuroprotectant. Future elucidation of saturable and diffusive mechanisms of ischemic brain drug delivery will need to be deciphered for utilization of peptide based therapies during the stroke neuroprotective time window. It is an exciting possibility that a peripherally delivered, stable, opioid receptor agonist could modulate vital functions needed for brain stroke recovery (reduced vasogenic and cellular brain edema, ROS production and glutamate toxicity). A recent review has focused on the role of blood-brain barrier transporters in the pathophysiology and pharmacotherapy of stroke (Shah and Abbruscato, 2014). This work highlights the recognition of the pivotal role of the neurovascular unit in the pathophysiology of stroke the opportunity for testing of novel therapeutic targets. These studies, that first started in the Davis Lab and then expanded to novel, exciting research directions at Texas Tech University, were noteworthy because they helped answer a constant and prevailing controversy that “peptides do not cross the BBB”. The feasibility of peptide brain delivery during pathophysiologic states that was initiated by the Kastin and Banks team so many years ago continues today.

Inflammatory Pain and BBB Function

Another very interesting observation is that many disease processes often involve inflammatory processes. Since our group in Arizona knew that inflammatory processes could be seen in pain states, this lead to several discussions about how pain could possibly affect BBB function, and possibly effect delivery of pain relieving analgesics to the brain. These discussions, however, were not developed into a full research program until Dr. Jason Huber joined the Davis Lab research group for his post-doctoral training. Jason was a very gifted post-doctoral fellow who had first studied neonatal cocaine exposure and NMDA receptors (Huber et al., 2001a) as a graduate student, but had no previous experience with the BBB field, peptide drug delivery or pain. The initial studies he carried out on peptide transport and analgesia (Bilsky et al., 2000) convinced him that a more disease oriented approach would provide not only more interesting data, but would probably be more relevant (Huber et al., 2001b). What followed was a very carefully designed series of experiments, using in situ brain perfusion, that laid the foundation for the next 15 years of research in the Davis Laboratory, much of it influenced by early observations of Abba and his team in New Orleans.

Peripheral pain leads to a number of changes in both the levels of circulating factors, and also in the activation of various neural pathways (Garland, 2012; Kulmatycki and Jamali, 2007). This can lead to a significant change in not only neural plasticity (Heinricher et al.,

2009), but also in the efficacy of analgesics to treat the pain (Kulmatycki and Jamali, 2007). A question of major importance within the BBB field is how does this process, known as pain, regulate barrier function? With the current conceptualization of the neurovascular unit (NVU) initially described by the NIH Stroke Program Research Group in 2002, in which all brain cells and also components of the blood have the potential to regulate BBB function (Egleton and Abbruscato, 2014; Hawkins and Davis, 2005), it is highly likely that pain itself can regulate the barrier and directly affect drug delivery. Several studies have investigated how various pain models can regulate barrier function. During a series of studies we first demonstrated using three separate pain models that there was a significant increase in BBB permeability to sucrose (Brooks et al., 2005; Huber et al., 2002a; Huber et al., 2001c). Changes in sucrose indicate that there had been a change in the paracellular permeability (leakiness) of the barrier, probably due to changes in the high transcellular resistant, tight junctions. These types of techniques were commonly used in other barrier systems and were initially utilized by the Davis Lab to characterize changes in paracellular permeability and tight junction expression at the BBB induced by *in vitro* hypoxia-aglycemia to simulate brain ischemic stroke (Abbruscato and Davis 1999a, 1999b) and subsequent studies indicated that this was indeed the case with respect to inflammatory pain as well. Using various models of inflammatory pain we further demonstrated that several tight junction protein - protein interactions were significantly altered. Tight junctions at the BBB consist of a complex interaction of transcellular and cellular proteins that maintain close cell-to-cell contacts between the endothelial cells of the BBB (Hawkins and Davis, 2005). In the lambda-carrageenan plantar injection model of inflammatory pain, an increased permeability to sucrose starts as early as one hour post injection of carrageenan and follows a biphasic pattern (Huber et al., 2002a). The initial BBB alteration response is maintained through six hours and peaks again at 48 hours post carrageenan (Huber et al., 2002a). During this time a number of significant changes are occurring at the tight junctions wherein the tight junctions also demonstrate the biphasic response. Occludin, a transmembrane protein, has a major role, not only in maintaining cell-to-cell BBB tightness, but is also a major scaffolding protein for various cell signaling pathways (Feldman et al., 2005). During carrageenan induced inflammatory pain, occludin follows a similar response pattern to sucrose with a reduction in expression levels which parallels the changes observed in sucrose permeability. In contrast, ZO-1 a cytoplasmic, accessory tight junction protein component is increased in expression during the initial phase of permeability (Huber et al., 2002a). To confirm that the observed response was actually due to the physiological response to the painful stimuli, and not due to circulating carrageenan, critical, *in vitro* studies were performed and demonstrated that carrageenan had no direct effect on endothelial cells (Huber et al., 2002b). These studies indicated that there was a differential controlled response of the BBB to the noxious painful stimuli. Subsequent studies revealed that this was a complex process that involves disruption of occludin-occludin disulfide bond formation, thus preventing formation / maintenance of oligomeric organization and assembly, coupled with an alteration in the cellular compartmentalization/trafficking of both occludin and ZO-1 (McCaffrey et al., 2008). The oligomeric organization of tight junctions is a vital component of maintaining optimal barrier function (McCaffrey et al., 2007). The mechanism that regulates these changes has been extensively studied. It is apparent that the process involves endothelial activation as there is an increase in ICAM-1 expression on the endothelial cells

coupled with activated microglia (Huber et al., 2006). Changes in ICAM-1 RNA are seen as early as 15 minutes post carrageenan injection, with the protein changes occurring at one hour and maintained throughout the next 48 hours (Huber et al., 2006). During this time, there was also a significant change in the levels of various circulating cytokines (Huber et al., 2006), many of which have previously been linked to activation of endothelial cells (Blann, 2000). These studies provide compelling evidence that hyperalgesia could lead to changes in BBB molecular and functional properties. The question remained as to whether this was specific to the carrageenan insult or was true for multiple models of pain. In the original studies, we had seen similar magnitude responses of sucrose permeability in three different models of pain (Huber et al., 2001c). In a subsequent follow up study with the complete freunds adjuvant (CFA), similar changes in permeability were seen with a biphasic response to sucrose permeability (Brooks et al., 2006). However, though there were molecular changes seen in the tight junctions, they were different to those seen in the carrageenan model, perhaps representing the different immune response profile seen in the two distinct pain models. Subsequent studies concentrated on the carrageenan model, as it demonstrated no tissue damage as the CFA model did.

Mechanism Proposed – Protein Trafficking

The next set of studies concentrated on trying to discover a mechanism. The signaling involved in pain processing involves two major pathways. The first is the transmission of the nociceptive signal via various neurons, while the second is via the inflammatory response. There is considerable evidence that both neuronal signaling and inflammatory signals can regulate the neurovascular unit (Egleton and Abbruscato, 2014), but the role of these two pathways in pain and BBB alterations are not clear. Inhibition of the nociceptive input was carried out via a perineural pre-injection of bupivacaine to the saphenous, tibial, and common peroneal nerves, prior to injection of carrageenan (Campos et al., 2008). This injection did not prevent the inflammation of the paw, however it did inhibit the pain processing and also the molecular and functional changes induced by carrageenan injection (Campos et al., 2008). For the first time this showed that a stimulation of a peripheral pain pathway was responsible for changes in BBB integrity. The role of oxidative stress and COX enzymes were also investigated and in vivo inhibition of both oxidative stress and prostaglandin synthesis significantly ameliorated the changes in BBB permeability, indicating that there was also a significant role for inflammation and ROS in the BBB changes (Brooks et al., 2008; Lochhead et al., 2012). Finally an inhibition of TGFβ signaling also prevented the changes in tight junction proteins and the associated sucrose permeability (Ronaldson et al., 2009). These studies all showed that the regulation of tight junction proteins and hence BBB integrity is complicated and can involve input from the brain, and from the peripheral immune response.

These studies also indicate that the tight junctions are opening, in each case, allowing sucrose to cross the BBB. This is interesting mechanistically, but may not be necessarily clinically relevant. The question remains as to what is regulating/interfering with drug delivery of the actual drugs that are used to treat pain? Several studies from Kastin's group indicate that inflammation can regulate transporter function at the BBB (Pan et al., 2011; Pan et al., 2008; Yu et al., 2007). Of particular interest to us were the studies that showed

that inflammation could regulate the activity and expression of efflux transporters, most notably P-glycoprotein (Pgp), which is the major efflux transporter for morphine and opioid analgesics (Yu et al., 2008; Yu et al., 2007). This interest was driven primarily due to the role of Pgp in limiting opioid peptide delivery (Dagenais et al., 2001), thus was a link to our earlier focus on opioid peptide transport. Furthermore, Dr. David Miller's group at the NIEHS described a series of elegant studies that demonstrated the role of Pgp in the efflux transport of a range of substances, and also demonstrated a series of signaling mechanisms that regulated the transport, which included several inflammatory processes (reviewed in (Miller, 2014)). To address these questions we developed a series of complementary studies to investigate the transport of several opioids, including codeine and morphine, and to determine if Pgp was involved. Three hours after carrageenan injection, codeine was found to have a significant increase in delivery to the brain (Hau et al., 2004; Lochhead et al., 2012), in contrast the transport of morphine was decreased (Seelbach et al., 2007). Both codeine and morphine enter the brain largely via diffusion; however morphine is also a substrate for the efflux pump Pgp. When Pgp was inhibited with cyclosporine-A there was a dose dependent increase in morphine uptake by the brain (Seelbach et al., 2007). Subsequently it was demonstrated that the inflammatory pain induced changes in morphine uptake were due to an increased expression of Pgp at the BBB (Seelbach et al., 2007). This was a truly interesting finding that confirmed the role for Pgp and links back to both the studies of Kastin and Miller. Further it indicates that *in vivo* BBB transporter regulation can occur via changes in the periphery, and subsequently this combination of tight junction and efflux transporter regulation has been seen in other peripheral disorders, including models of diabetes (Hawkins et al., 2007a; Hawkins et al., 2007b). So, how is this expression and function of p-glycoprotein regulated? Initial studies indicate that this is in part via regulation of caveolin-1 mediated cellular trafficking (McCaffrey et al., 2012). Recent proteomic analysis of cellular fractions confirms the association of Pgp with caveolae, as demonstrated by the association with both Cavin-1 and Cavin-2 (Tome et al., 2015). The fractionation analysis also indicates two distinct caveolae associated pools of Pgp potentially associated with the dynamic nature of caveolae (Tome et al., 2015). This association may be an essential component for the rapid movement of Pgp to and from the plasma membrane reported in several studies (Miller, 2014). The role of caveolae in regulating Pgp is probably, in part, due to cellular trafficking but also probably involves the numerous signaling components in caveolae. Interestingly, treatment with diclofenac a NSAID, actually promoted expression levels of Pgp resulting in reduced morphine uptake into the brain when given to control animals (Sanchez-Covarrubias et al., 2014). In contrast, when given as a pretreatment prior to carrageenan, it prevented the carrageenan induced reduction in morphine transport (Sanchez-Covarrubias et al., 2014). This implies that the regulation of PgP function at the BBB is complex. When tight junction function was assessed, diclofenac inhibited opening (leak) induced by carrageenan, and also had a trend to reducing basal permeability of the BBB to sucrose (Brooks et al., 2008). How COX enzyme inhibition regulates PgP is unclear, however it is known that some of the other enzymes involved in Prostaglandin synthesis are found within the caveolae, including prostacyclin synthase (Spisni et al., 2001), this has been linked to a role in angiogenesis, other factors involved in angiogenesis are also known Pgp modulators (Hawkins et al., 2010). Thus there appears to be a differential regulation dependent on the underlying stimuli. Within the blood of the

animals treated with diclofenac and or carrageenan there is a significant increase in the levels of TNF (Sanchez-Covarrubias et al., 2014). TNF has been previously linked to regulating both tight junction and efflux transporter function at the BBB (Bauer et al., 2007; Lv et al., 2010; Pan et al., 2008; Yu et al., 2007). Though if TNF is responsible it is probably not the only factor as the highest TNF levels were reported in the carrageenan rats treated with diclofenac, animals for which the morphine and sucrose transports were similar to control (Sanchez-Covarrubias et al., 2014). This indicates that diclofenac and carrageenan regulate the barrier via different and potentially antagonistic mechanisms. This also raises the question as to how other drugs, that may be co-administered with morphine can regulate morphine brain uptake during pain. Acetaminophen is a common addition to opioid therapy, a recent study revealed that acetaminophen could lead to an increase in Pgp levels at the BBB resulting in a reduced morphine uptake and subsequent reduced analgesia induced by morphine (Slosky et al., 2013). The increase in Pgp was reported to be via an upregulation of constitutive androstane receptor (CAR) mediated transcriptional regulation of Pgp (Slosky et al., 2013). Other studies have also shown transcriptional regulation of efflux transporters including Pgp at the BBB via ahryl hydrocarbon receptor (AHR) mediated transcription (Wang et al., 2011). There is the potential for these interactions to be clinically relevant, and reports in the literature indicate that combination of diclofenac with an opioid, lead to reduced requirements for opioid for similar levels of analgesia. So, is this a BBB transport phenomenon (Ronaldson and Davis, 2013a) ? The differential regulation noted above is also important when considering non-efflux transporters at the BBB that are also regulated in models of inflammatory pain. The organic anion transporting polypeptide Oatp1a4 is expressed at the BBB and is involved in transporting substances into the brain. This transporter is upregulated via a proposed TGFβ mediated mechanism (Ronaldson et al., 2011). This upregulation is inhibited by diclofenac (Ronaldson et al., 2011). What makes this recent observation really interesting is that unlike the Pgp response to diclofenac in control animals (Sanchez-Covarrubias et al., 2014), basal expression of Oatp1a4 is not regulated by treatment, it is only the inflammatory pain stimulated increase in Oatp1a4 that is prevented (Ronaldson et al., 2011).

Summary and Discussion

The above studies clearly demonstrate that there is a truly dynamic and complex regulation of both the physical and transport barrier systems at the BBB. These studies also show that there is most likely a complex interaction of multiple cytokines and growth factors that differentially regulate tight junctions, efflux transporters and uptake transport systems. I trust that we have also demonstrated how Abba Kastin's studies starting in the 70's and beyond have influenced our thinking. Both directly and indirectly Abba's studies on peptides and the BBB have also had a significant impact on the training of multiple Davis Lab graduate students (Tom Abbruscato, Ken Witt, Vincent Hau, Brian Hawkins, Sharon Hom, Melissa Seelbach, Christopher Campos, Jeff Lochead, Lucy Sanchez-Covarrubias), and post doctoral fellows (Sarah Thomas, Richard Egleton, Karen Mark, Rachel Brown, Jason Huber, Tracy Brooks, Anne Wolka, Scott Ocheltree-Hynes, Colin Willis, Gwen McCaffery, Patrick Ronaldson) and many dedicated and enthusiastic undergraduates in our

laboratory. Perhaps more importantly Abba has been a great friend and we will miss reading his insightful papers.

Acknowledgments

Support: NIH R01 grants 5R01 DA011271 and 5R01 NS042652 to TPD, and R01 NS076012 to TJA.

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