Tissue- and Development-specific Expression of Proton-mediated Peptide Transporters in the Developing Chicken

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ABSTRACT

PepT1, PepT2 and PHT1 are all members of the proton-coupled oligopeptide transporter family, which are important in the transport of amino acids in peptide form. PepT1 acts as a low affinity/high capacity transporter and PepT2 as a high affinity/low capacity transporter for di- and tri-peptides. PHT1 transports di- and tri-peptides as well as histidine. The objective of this study was to profile PepT1, PepT2 and PHT1 mRNA expression in the proventriculus, duodenum, jejunum, ileum, ceca, large intestine, brain, heart, bursa of Fabricius, lung, kidney, and liver in layer chicks on embryonic days 18 and 20 and days 1, 3, 7, 10, and 14 post-hatch. Absolute quantification real-time PCR was used to measure gene expression. PepT1 expression was greatest in the duodenum, jejunum and ileum. Over time, PepT1 expression increased in the duodenum, jejunum, ileum and large intestine and decreased in the ceca. PepT2 expression was greatest in the brain, aiding in neuropeptide homeostasis, and the kidney, aiding in the reabsorption of substrates. Over time, PepT2 expression increased in the bursa of Fabricius and decreased in the proventriculus, duodenum, jejunum and liver. In the small intestine during embryogenesis, PepT2 may function to transport di- and tri-peptides prior to the induction of PepT1. PHT1 expression was expressed in all tissues analyzed. Over time, PHT1 expression increased in the jejunum, large intestine, brain and liver and decreased in the proventriculus. The uptake of peptides in the developing chick is regulated by peptide transporters that are expressed in a tissue- and development-specific manner.

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CHAPTER I. REVIEW OF LITERATURE

Peptide Transport

In 1973, Adibi and Mercer observed that after a human ingested a protein-rich meal, the luminal concentrations of amino acids in both free and peptide form increased. More amino acids were found to be in peptide form within the jejunum in the small intestine. Not surprisingly, no free or peptide amino acids were seen in subjects given a protein-free meal. The absorption of an amino acid was found to be more rapid when it was linked to another amino acid (Adibi and Mercer, 1973). Thus, peptide absorption was further investigated.

In bacteria, the main peptide transporter system includes proteins from the ATP-binding cassette family, consisting of four membrane-associated domains and requiring a substrate-binding protein (Steiner et al., 1995). In eukaryotes, transporter proteins similar in function but differing in distinguishing characteristics were identified and grouped as the "Peptide Transport" family (Steiner et al., 1995) or the "Proton-Coupled Oligopeptide Transporter" superfamily (Botka et al., 2000). Members of this family range from 450 to 790 amino acids in length and contain 12 predicted transmembrane α–helical spans with both the N- and C-termini located within the cell (Herrera-Ruiz and Knipp, 2003). All members also contain three conserved amino acid sequences at the end of transmembrane domain 2, within the intracellular loop, and within transmembrane domain 3 (Herrera-Ruiz and Knipp, 2003). Established peptide transporters in this family are peptide transporter 1 (PepT1), peptide transporter 2 (PepT2), peptide/histidine transporter 1 (PHT1), and peptide/histidine transporter 2 (PHT2) (Herrera-Ruiz and Knipp, 2003). PepT1 has been studied in the greatest detail as it is highly expressed on the apical

membrane of intestinal cells to aid in peptide absorption into the body, indicating a potential pathway for orally ingested pharmaceuticals. PepT2, which in mammals has a 50% amino acid identity to PepT1, has also been investigated as it is expressed in the apical membrane of the kidney, aiding in renal reabsorption of peptides. More recently, PHT1, which transports free histidine as well as di- and tri-peptides, has been observed on the lysosomal membrane and not on the apical membrane of cells. Tissue distribution is very wide among tissues and species (Yamashita et al., 1997; Herrera-Ruiz and Knipp, 2003). PHT2 has been most recently found with high expression levels in cells of the lymphatic system (Sakata et al., 2001). Characteristics of the four proton-coupled oligopeptide transporter family members are summarized in Table 1-1. PHT2 has not been found in the chicken genome and therefore will not be discussed in detail.

Peptide transport has been a highly researched topic with respect to drug transport. PepT1 and PepT2 have been observed to transport beta-lactam antibiotics such as penicillin and cephalosporin, angiotensin-converting enzyme (ACE) inhibitors, aminopeptidase inhibitors, and ester prodrugs (Rubio-Aliaga and Daniel, 2002; Daniel and Kottra, 2004). With an oral dose of these pharmaceuticals, PepT1 transports between 40% and 90% of a dose. PepT2 may also contribute to renal reabsorption of substrate, ultimately affecting pharmacokinetics (Daniel and Kottra, 2004).

Table 1-1. Characteristics of the Proton-coupled Oligopeptide Transporter Family Members

Gene Name	Protein Name	Substrates	Mammalian Tissue Distribution
SLC15A1	PepT1	Di- and Tri-Peptides and Peptide-like Drugs	Intestine, Kidney
SLC15A2	PepT2	Di- and Tri-Peptides and Peptide-like Drugs	Kidney, Brain, Lung, Mammary Gland
SLC15A3	PHT2	Di- and Tri-Peptides and Histidine	Lung, Spleen, Adrenal gland, Thymus
SLC15A4	PHT1	Di- and Tri-Peptides and Histidine	Abundant throughout many tissues

Peptide Transporter 1: PepT1 (SLC15A1)

Structure

The PepT1 transporter was first cloned from the rabbit (Fei et al., 1994), and has been subsequently cloned from the human (Liang et al., 1995), mouse (Fei et al., 2000), sheep (Pan et al., 2001), rat (Hussain et al., 2002), pig (Klang et al., 2005), chicken (Chen et al., 2002), zebrafish (Verri et al., 2003), turkey (Van et al., 2005), and rhesus and cynomolgous monkeys (Zhang et al., 2004).

Like mammalian PepT1, chicken PepT1 contains 23 exons; however, the gene is 149 Megabase pairs (Mbp), which is considerably shorter than the mammalian PepT1 genes, which contain larger introns, particularly at introns 1 and 18 (Frazier et al., 2008). The amino acid sequence of chicken PepT1 is approximately 60% identical to mammalian PepT1 sequences, with the first 15 amino acids being completely different (Chen et al., 2002). This is most likely due to the small amount of sequence identity between exons 1 and 2 of chickens and mammals, even though high sequence identity is exhibited among exons 3-23 (Frazier et al., 2008). Turkey PepT1 is identical to 14 out of

the first 15 amino acids and is overall 94.3% identical to chicken PepT1 (Van et al., 2005). Since the first exon is not conserved between chickens and mammals, there likely are different promoter regions and regulatory elements. The chicken PepT1 promoter is expressed in a tissue-specific manner and is not influenced by the addition of various amino acids and di-peptides, as is mammalian PepT1 (Shiraga et al., 1999; Frazier et al., 2008).

Chicken PepT1 protein was predicted to have 714 amino acids and a molecular mass of 79.3 kiloDaltons (kDa) (Chen et al., 2002). Chicken PepT1, like mammalian PepT1, has a 12-transmembrane domain structure with a large extracellular loop located between transmembrane domains 9 and 10 and the amino and carboxyl termini on the cytoplasmic side of the membrane (Fei et al., 1994; Ogihara et al., 1999; Chen et al., 2002). The N-terminal transmembrane domains form a pore-like structure and the C-terminal domains 7-9 form the substrate binding pocket (Rubio-Aliaga and Daniel, 2002). Particular histidine residues have been observed to be essential for membrane transport function. The His-57 residue found near the extracellular surface in transmembrane domain 2 plays a major role in the H+ binding mechanism, and the His-121 residue found in transmembrane domain 4 is involved in substrate recognition and assists in neutralizing acidic peptides prior to translocation (Terada et al., 1996; Fei et al., 1997; Chen et al., 2000).

Substrates

PepT1 is a low-affinity, high-capacity transporter. Transport via PepT1 is the main mechanism by which di- and tri-peptides produced by luminal digestion are absorbed in the small intestine (Thwaites and Anderson, 2007; Ma et al., 2011). Not all

di- and tri-peptides activate PepT1, suggesting affinity for PepT1 does not necessarily translate to transport via PepT1 (Vig et al., 2006). Neutral and single charged (either positive or negative) dipeptides exhibit the highest PepT1 activation, with lesser activation by dipeptides with two charged residues. PepT1 has no significant contribution for transport of dipeptides with Trp in position two or with two basic amino acid residues (Vig et al., 2006).

Structurally, PepT1 requires its substrate to contain an N-terminal amino group and a C-terminal carboxyl group, as removal of these groups result in the loss of PepT1 activation. There is a size-limitation for amino acids at these termini (Vig et al., 2006). It is important to note that a peptide bond is not needed for PepT1 transport, as ω-amino fatty acids can be transported by PepT1, and the minimal requirements for transport are two ionized groups separated by at least 4 methyl units (Döring et al., 1998). These minimal requirements allow the uptake of δ-aminolevulinic acid for the treatment of tumors as well as many pharmaceutical compounds including β-lactam antibiotics, ACE inhibitors, bestatin and valacyclovir (Ganapathy et al., 1998; Daniel and Kottra, 2004; Thwaites and Anderson, 2007).

PepT1 transports substrates that are larger than one amino acid but smaller than tetrapeptides (Vig et al., 2006). Likewise, chicken PepT1 transports neutral and charged di- and tri-peptides but not amino acids or tetrapeptides, indicating the optimal length of peptides preferred by chicken PepT1 to be 2-3 (Chen et al., 2002).

Transport Mechanism

PepT1 net transport is electrogenic and driven by the inside-negative membrane potential of the cell (Fei et al., 1994; Meredith and Boyd, 2000). This proton driving

force means that substrate uptake relies heavily on extracellular pH and membrane potential (Rubio-Aliaga and Daniel, 2002). Within the rat small intestine, extracellular pH within the villus interspace was observed to be 6.9 and the maximum acidity of 6.5 was observed at 2.13 µm below the villus tip (Freeman et al., 1995). Due to this acidity, neutral substrates are optimal for transport, with anionic and cationic substrates showing lower transport rates (Rubio-Aliaga and Daniel, 2002). For neutral and cationic peptides, the proton:peptide co-transportation ratio is 1:1, for anionic peptides the ratio is 2:1 and cationic peptides depend on membrane potential (Meredith and Boyd, 2000; Daniel and Kottra, 2004).

While PepT1 co-transports H^+ into the cell as substrates are brought in, the acidic pH of the cell is maintained by a Na^+/H^+ exchanger located in the apical membrane which transports one H^+ out of the cell in exchange for one Na^+ into the cell (Adibi, 1997). PepT1 and Na^+/H^+ exchanger protein show coordinate expression in the broiler chicken, with low expression during late embryogenesis rising at day of hatch and continuing to increase post-hatch (Li et al., 2008). In order to maintain the negative potential of the cell cytosol, a Na^+/K^+-ATPase pump exists at the basolateral membrane, transporting 3 Na^+ out of the cell in exchange for 2 K^+ into the cell (Adibi, 1997). Potassium ions taken up leave the cell by potassium channels. After entering the cell, peptides and amino acids are then exported through the basolateral membrane by an unknown transporter (Daniel and Kottra, 2004). PepT1 transport is illustrated in Figure 1-1.

Figure 1-1: Active Transport of Peptides and H+ by PepT1 and PepT2
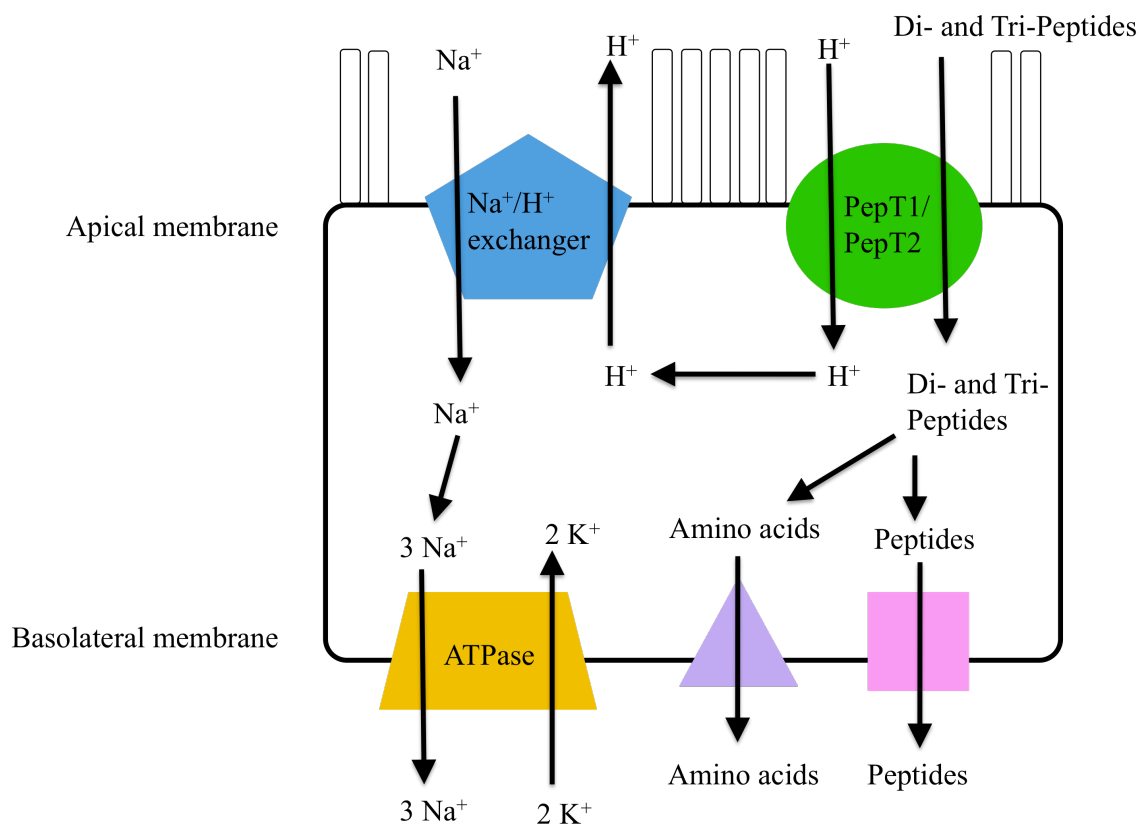
Tissue and Cellular Distribution

PepT1 expression is restricted to the epithelial mucosa throughout the entire rat small intestine, particularly in the villus present on the apical membrane of epithelial cells (Freeman et al., 1995). PepT1 was not found in the goblet cells or less-differentiated epithelial cells located in the crypts of the villi (Ogihara et al., 1996; Hussain et al., 2002). Along the individual villus, PepT1 mRNA was not found in the lower-mid crypt whereas maximal levels were found 100-200 µm from the crypt-villus junction in all intestinal sections (Freeman et al., 1995). Likewise, PepT1 protein expression increased from the base of the villus to the top of the villus (Ogihara et al., 1999). Adult rat PepT1 was localized in the plasma membrane of the apical microvilli on epithelial cells

themselves and not found on the basolateral surface (Ogihara et al., 1999; Hussain et al., 2002). In the mouse, mRNA expression of PepT1 was greatest in the jejunum and ileum, with lesser expression in the duodenum and minimal expression in the colon (Jappar et al., 2010; Ma et al., 2012). Protein expression was strongest in the jejunum, followed by similar expression in the duodenum and ileum, and no expression in the colon (Jappar et al., 2010). Interestingly, the permeability of dipeptides in the intestine was greatest in the duodenum and jejunum, with ileum permeability about 54% that of duodenum and jejunum, and the colon only 6% of duodenum and jejunum (Jappar et al., 2010). Unlike the mouse, human PepT1 mRNA has the greatest expression in the duodenum gradually decreasing to the ileum, consistent with the theory that luminal concentrations of small peptides gradually decrease and concentrations of free amino acids increase as contents move through the intestine (Terada et al., 2005).

In the kidney, PepT1 has only been observed in the apical membrane of S1 and proximal S2 segments of the proximal tubule within the rat nephron. PepT1 was not expressed in any other part of the nephron (Smith et al., 1998). PepT1 mRNA was mainly expressed in the superficial cortex with decreasing expression towards the middle of the kidney and no expression in the deep cortex, inner or outer medulla or papilla (Shen et al., 1999). PepT1 can be detected in the fetal rat kidney as early as embryonic day 17 (Shen et al., 2001).

In the adult broiler chicken, PepT1 was expressed in the small intestine, primarily in the duodenum with lesser expression in the jejunum and ileum (Chen et al., 1999). Kidney and liver showed low expression levels of PepT1, whereas no expression is

detected in the liver, crop, proventriculus, or pectoralis and fibularis longus muscles (Chen et al., 2002).

Regulation

Nutritional status can regulate levels of PepT1 mRNA and protein. In the rat, starvation for both 1 and 4 days showed a marked increase in PepT1 protein, suggesting peptides may be more efficiently absorbed during starvation (Ogihara et al., 1999; Thamotharan et al., 1999). Fasting for 16 hours had little effect on mouse PepT1 mRNA, but PepT1 protein expression was increased more than 2-fold in the duodenum and jejunum, and 1.5-fold in the ileum (Ma et al., 2012). In broiler chickens, PepT1 mRNA expression levels increased 2-fold after a 24-hour feed restriction (Madsen and Wong, 2011). PepT1 expression also was increased in feed-restricted birds when compared to birds fed ad libitum. Feed restriction may increase PepT1 expression in order to maximize amino acid absorption in spite of protein and energy deficiency (Gilbert et al., 2008). Not only was PepT1 upregulated in fasting conditions, peroxisome proliferator-activated receptor α (PPARα) was also upregulated, indicating PepT1 may either be directly or indirectly regulated by PPARα (Madsen and Wong, 2011). Oral administration of WY-14643, a PPARα ligand, caused a significant increase in PepT1 mRNA levels. In PPARα-null mice, starvation did not change PepT1 expression levels, indicating the integral role of PPARα in the PepT1 response (Shimakura et al., 2006).

Similarly, diet composition can regulate levels of PepT1. In rats fed a high protein diet, PepT1 mRNA levels increased (Erickson et al., 1995). On a restricted diet, broiler chicks fed a diet containing 24% crude protein had increased levels of PepT1 mRNA whereas chicks fed a diet containing 12% crude protein had a decrease of mRNA levels

all when compared to chicks fed an 18% crude protein diet (Chen et al., 2005). In Aviagen Line A and B chicks (Aviagen, Huntsville, AL), soybean meal, a high quality protein source, increased the amount of PepT1 expression when compared to corn gluten meal, a lower quality protein source (Gilbert et al., 2008). Oral administration of amino acids decreased PepT1 protein in the jejunum of the rat, suggesting the satiation of the rat for amino acids with the absorption of free amino acids, creating no need for oligopeptide absorption (Ogihara et al., 1999).

PepT1 expression was developmentally regulated in the broiler chicken where it rapidly increased just before hatch (Chen et al., 2005), with a significant increase at the time of hatch (Li et al., 2008). Such a significant increase in expression before food consumption may prepare the bird for intake of peptide substrates when exogenous food becomes available. In the rat, PepT1 expression has been observed in the subapical cytoplasm, basal cytoplasm and in the basolateral membrane immediately after birth, potentially indicating transport activity across the basolateral membrane; however, PepT1 expression becomes confined to the apical membrane at weaning (Hussain et al., 2002). Maximal PepT1 levels were observed in the rat small intestine within a week after birth, rapidly decrease, then increase to adult levels at weaning at about 24 days (Shen et al., 2001). In the broiler chicken, incubation temperature has an effect on PepT1 expression post-hatch. Developmental stage in the broiler chicken was most likely affected with a change in incubation temperature, potentially changing the importance of PepT1 function at an early age. Incubation temperatures at 39.6°C, when compared to the normal 37.4°C, caused similar levels of increasing PepT1 expression until 6 days after hatch when PepT1 expression significantly decreased instead of remaining steady as in chicks incubated at

normal temperature. PepT1 levels for the birds with normal incubation temperatures significantly decreased at day 10 (Barri et al., 2011).

PepT1 may be regulated by the anticipation of food. Peaks of increased mRNA transcript and transport function have been found in a diurnal pattern in association with nocturnal feeding of rats. An increase in the number of apical membrane transporters participating in peptide uptake was observed in both the duodenum and jejunum. Interestingly, the affinity for substrate remained unchanged, suggesting an increase in the number of transporters expressed in the apical membrane and not a change in the type of transporter (Qandeel et al., 2009).

PepT1 may also be genetically regulated. In White Plymouth Rock chickens, birds genetically selected for low weight had induced PepT1 expression on the day of hatch. Peak PepT1 expression in high weight birds was later, at day 7 in females and day 14 in males, possibly suggesting an adaptation to effectively absorb nutrients. Sex may also regulate PepT1 expression as female birds show peak PepT1 expression approximately 7 days before males. Females may need earlier expression of PepT1 due to improved metabolic efficiency or an earlier need for nutrients (Mott et al., 2008). In chickens selected for 51 generations for high and low body weight at 8 weeks of age, highest PepT1 expression was observed in birds selected for low weights and lowest PepT1 expression was observed in birds selected for high weights. An F1 progeny of crosses between high and low weight lines produced chicks of intermediate weight and PepT1 levels. An F2 generation created from the F1 population had a diverse range in body weights and PepT1, suggesting polygenic inheritance for PepT1 regulation in chicks (Zwarycz, unpublished).

PepT1 Knock-out model

There are no known human problems associated with peptide transporter malfunction (Daniel and Kottra, 2004). PepT1 Knock-out mice were found to be as healthy and physically similar in body weight, development and fertility to wild-type mice (Hu et al., 2008). Contrastingly, *Caenorhabditis elegans* lacking PepT1 had severe developmental and reproductive defects associated with a reduction of food-derived peptides (Meissner et al., 2004). Mice without PepT1 showed no upregulation of other peptide or amino acid transporters as compensation, even though increased plasma amino acid levels were observed in PepT1 null mice (Hu et al., 2008; Nässl et al., 2011; Ma et al., 2012). In vitro intestinal uptake studies have shown that PepT1 accounts for 65-80% of GlySar uptake in fed states. In vivo studies in PepT1 knockout mice support the theory that PepT1 is the major transporter for di- and tri-peptides and without PepT1 in the intestine, mice showed drastically reduced levels of GlySar uptake when compared to wild-type mice (Hu et al., 2008; Ma et al., 2012). The importance of PepT1 was increased when high-protein loads of feed are ingested as opposed to when low amounts of protein are ingested (Nässl et al., 2011).

Summary

PepT1 is the main transporter of di- and tri-peptides into the enterocyte from the digesta within the lumen. PepT1 has the ability to transport many peptide-like drugs. As luminal contents move down the small intestine, the amount of di- and tri-peptides decreases due to absorption, with levels of PepT1 protein expression in the small intestine decreasing in the distal tract; however, expression depends upon many factors including the nutritional and developmental state as well as diet composition and genetics.

Peptide Transporter 2: PepT2 (SCL15A2)

Structure

PepT2 was first cloned from the human (Liu et al., 1995) and has subsequently been cloned from the rat (Saito et al., 1996), rabbit (Boll et al., 1996) mouse (Rubio-Aliaga et al., 2000), and zebrafish (Romano et al., 2006). PepT2 cDNA cloned from human kidney encoded for a protein consisting of 729 amino acids with a molecular weight of 81.94 kDa. The protein contains 12 transmembrane domains with an approximately 165 amino acid extracellular loop between transmembrane domains 9 and 10. The N- and C- termini face the cytosol. There was significant homology when comparing human PepT2 and PepT1 proteins, with the amino acid sequences sharing 50% identity and 70% similarity. The highest homology was located within the transmembrane domains (Liu et al., 1995).

Like PepT1, specific histidine residues are conserved across species and have been considered essential for PepT2 protein function. His-87, located near the extracellular surface in the second transmembrane domain, is essential for transport activity, most likely in the binding and translocation of H^+, similar to the function of His-57 in PepT1. His-147, located in the fourth transmembrane domain, appears to play a significant role in maintenance of catalytic activity (Fei et al., 1997). In addition to histidine residues, the amino acids Ile-720 and Leu-722, within the COOH terminus, are integral in the localization of PepT2 to the apical membrane (Klapper et al., 2006). PepT2 mRNA has not been confirmed in the chicken, but has been predicted (NCBI Reference Sequence: XM_422093.3) to be located on chromosome 7 and code for 726 amino acids.

Substrates

PepT2 only transports di- and tri-peptides, regardless of charge, and requires substrates to have an α-amino group (Boll et al., 1996; Amasheh et al., 1997). PepT2 also transports neuropeptides (Teuscher et al., 2001). PepT2 exhibits a 40 times greater substrate affinity for various peptides, irrespective of charge, side-chain structure and modification of α-amino group than PepT1 (Boll et al., 1996; Terada et al., 2000). Most natural dipeptides are high affinity substrates for PepT2 (Biegel et al., 2006); high affinity is classified as a substrate having an affinity constantly less than 0.1 mM (Luckner and Brandsch, 2005). PepT2 shows the highest affinity for dipeptides containing hydrophobic side chains and the trans conformation of the peptide bond (Biegel et al., 2006). Lower affinities were observed for dipeptides with glycine, proline, or an acidic amino acid in the N-terminus position and with basic amino acids at the C-terminus position (Biegel et al., 2006). Tripeptides also had a higher affinity for PepT2, particularly when it contained an uncharged amino acid residue in position 3 (Terada et al., 2000; Biegel et al., 2006).

PepT2 was also observed to not transport amino acids, as there was no difference in transport of glycine and proline between control and PepT2 cDNA-transfected cells (Liu et al., 1995). PepT2 does not transport ACE inhibitors, particularly captopril and enalapril (Boll et al., 1996). PepT2 also does not transport ϖ-amino fatty acids, including 5-aminopentanoic acid, which is the smallest amino fatty acid recognized and transported by PepT1. However, when a carbonyl group is added to the backbone, PepT2 transports it with normal affinity and transport properties, indicating that the carbonyl is essential for substrate recognition by PepT2. A free terminal amino group is also needed for transport

(Daniel et al., 1992); however, it is important that the terminal nitrogen atom be separated by a distance of 1-2 methylene groups from the carbonyl group for high-affinity interaction and transport (Theis et al., 2002).

Transport Mechanism

Transport of dipeptides by PepT2 occurs in an H^+-dependent manner, as shown in HeLa cells transfected with PepT2 cDNA. A significant increase in transport activity was measured at pH 6.0 as opposed to pH 8.0 (Liu et al., 1995). In *Xenopus laevis* oocytes, maximal transport rates of rat PepT2 were increased almost 4-fold by reducing the pH from 8.0 to 6.0 (Boll et al., 1996). As demonstrated in mice, PepT2 operates in an electrogenic mode and was able to transport dipeptides found in the nervous system (Rubio-Aliaga et al., 2000). Like PepT1, PepT2 transport and substrate affinity are dependent upon pH. For neutral substrates, the proton:substrate cotransportation ratio is 2:1 whereas for charged substrates the ratio is variable (Daniel and Kottra, 2004).

PepT2 is characterized as an oligopeptide transporter, like PepT1. Therefore, the two transporters have identical transport mechanisms. PepT2 transport is illustrated in Figure 1-1.

Tissue and Cellular Distribution

In the human, PepT2 RNA was found in the kidney but not in the small intestine (Liu et al., 1995). Rat kidney expressed both PepT1 and PepT2; however, they were confined to distinctly different locations. Within the nephron, PepT2 was predominantly expressed on the apical membrane in the proximal tubes of the deep cortex/outer stripe of the outer medulla. Faint expression was observed in the middle cortex and no expression was found in the superficial cortex, inner stripe of the outer medulla, inner medulla, or

papilla (Shen et al., 1999). There was no PepT2 expression on the basolateral membrane of the kidney (Shen et al., 1999). Expression of PepT2 was greatest in the latter regions of the proximal tubule, particularly in the distal straight part of S2 and S3 segments (Smith et al., 1998; Shen et al., 1999; Rubio-Aliaga et al., 2003). Except for the regions previously discussed, PepT2 was not expressed anywhere else in the nephron. PepT2 expression was also more abundant within the kidney than PepT1 (Smith et al., 1998).

Within the rat brain, PepT2 mRNA was found in the cerebral cortex, while PepT2 protein was found in both the cerebral cortex and cerebellum (Fujita et al., 2004). Within the cortex, cells expressing PepT2 mRNA were found within various layers (Berger and Hediger, 1999). More specifically, Berger and Hediger (1999) found PepT2 mRNA was expressed in astrocytes found throughout the brain. Astrocytes control extracellular fluid composition and potentially aid in the removal of di- and tri-peptide substrates from extracellular fluid, as they release peptidases that break down neuropeptides on their extracellular membrane. Expression of PepT2 on astrocytes suggests that PepT2 is a mechanism of control for fluid composition. PepT2 was also expressed in subependymal cells, ependymal cells lining the cerebral ventricles and epithelial cells of the choroid plexus (Berger and Hediger, 1999). These cells may express PepT2 in order to uptake peptides for intracellular metabolism or control peptide levels in ventricular cerebrospinal fluid (Berger and Hediger, 1999). At the blood-cerebrospinal fluid barrier, PepT2 operates as an efflux transporter (Ocheltree et al., 2005) and plays a role in neuropeptide homeostasis of cerebrospinal fluid (Teuscher et al., 2001). PepT2 was not expressed by the endothelial cells of blood vessels so PepT2 transport is unlikely to contribute to the transport of peptides across the blood-brain barrier (Berger and Hediger, 1999).

Within the lung, PepT2 was localized to the apical membrane of cells of the respiratory epithelium of the large bronchi, alveolar type II pneumocytes and endothelial cells of some smaller vessels in mice and rats (Groneberg et al., 2001; Groneberg et al., 2002b). Type II pneumocytes and tracheal and bronchial epithelial cells exhibited peptide transport activity (Groneberg et al., 2001).

Rat lactating mammary gland as well as epithelial cells isolated from fresh human milk express PepT2 mRNA (Groneberg et al., 2002a). Within the mammary gland, PepT2 was expressed on the apical membrane of the epithelium of main, segmental, and terminal ducts and glands. Groneberg et al. (2002a) suggested PepT2 contributes to reabsorption of peptides that have been broken down by proteases in the milk back into the epithelial cells.

Regulation

Within the kidney, PepT2 may be regulated by hormonal changes. Reduced levels of circulating thyroid hormone due to impaired thyroid function (hypothyroidism) regulate PepT2 levels (Döring et al., 2005). In rats with low thyroid hormone, PepT2 mRNA and protein were increased in the renal tubules. An increase in protein may be a response to increased peptide-bound amino acids in order to counteract the decreased amino-acid-nitrogen availability due to an imbalance in kidney reabsorption (Döring et al., 2005). Epidermal growth factor influences PepT2 by downregulating mRNA expression within a rat kidney proximal tubule cell line (Bravo et al., 2004). Epidermal growth factor is released into the lumen of the distal part of the kidney tubules and promotes cell growth and division, tissue proliferation and nutrient absorption of the kidney. A decrease in Gly-Sar uptake was observed due to a decrease in PepT2 mRNA

leading to reduced PepT2 protein expression (Bravo et al., 2004). Also within the kidney, PepT2 protein and mRNA expression was upregulated in rats simulating chronic renal failure by reduction of nephron mass by 83.3%. No upregulation of PepT1 mRNA was observed, indicating that PepT2 upregulation may be a tissue-specific mechanism within the kidney to prevent damage (Takahashi et al., 2001).

Within the brain, PepT2 appears to be spatially regulated by region-specific factors with very low expression in the ventral forebrain, even in areas of high peptide concentration. Expression of PepT2 in astrocytes was increased in cells that were stimulated to proliferate, suggesting PepT2 expression within astrocytes themselves is regulated (Berger and Hediger, 1999).

Expression of PepT2 was regulated at the cellular level by changing substrate affinities by protein kinase C and protein kinase A-dependent signaling pathways. Wenzel et al. (1999) hypothesized this adaptation occurs to increase efficiency of peptide reabsorption in response to different loads of di- and tri-peptides. The protein kinase C inhibitor, staurosporine, enhanced influx of dipeptides by PepT2 whereas protein kinase C activator, phorbol-12-myristate-13-acetate, inhibited dipeptide transport in LLC-PK1 cells, which were derived from the porcine proximal tubule. Protein kinase A activation increased glucose uptake but inhibited dipeptide transport (Wenzel et al., 1999).

PepT2 Knock-out model

Homozygous PepT2 null mice were healthy and fertile but display a drastically reduced accumulation of hydrolysis-resistant dipeptides in the kidney, confirming the prominent role of PepT2 in renal reabsorption of peptide-bound amino acids (Rubio-Aliaga et al., 2003). Excretion of amino acids in the urine was only minimally altered by

the lack of PepT2, suggesting further hydrolysis of peptides by peptidases not reabsorbed by PepT2 (Rubio-Aliaga et al., 2003). Within the brain, an accumulation of GlySar was observed in PepT2 homozygous null mice, suggesting PepT2 is responsible for cellular uptake of peptides from the cerebrospinal fluid. There was no phenotype observed, suggesting some other transporter may be upregulated to compensate for a lack of PepT2. However, no proton-coupled oligopeptide transporters have been observed to increase in the kidney or the brain due to a lack of PepT2 (Ocheltree et al., 2005).

Summary

PepT2 is a high affinity/low capacity peptide transporter that primarily transports di- and tri-peptides and peptide-like drugs. PepT2 is structurally similar to PepT1 and has the same transport mechanism. Unlike PepT1, PepT2 is not expressed in the intestine and is expressed downstream of PepT1 in the kidney nephron, serving as the transporter for peptides and proteins hydrolyzed within the nephron tubule (Shen et al., 1999). Both physical and hormonal changes within the kidney regulate PepT2 gene and protein expression. Gene and protein expression of PepT2 is found in the brain and functions to transport across the brain-cerebrospinal fluid barrier. Expression of PepT2 is also found in the lung and mammary gland epithelium.

Peptide/Histidine Transporter 1: PHT1 (SLC15A4)

Structure

Rat brain cDNA library screening by Yamashita et al. (1997) resulted in cloning of PHT1 and revealed a cDNA that encoded for a protein 572 amino acids long with a predicted molecular mass of 64.9 kDa. Subsequently, PHT1 has been cloned from the

human (Herrera-Ruiz et al., 2001) and mouse (Sasawatari et al., 2011). The PHT1 transporter is similar to PepT1 and PepT2 in structural analysis, having a predicted protein sequence containing 12 transmembrane domains with intracellular N- and C-termini (Herrera-Ruiz and Knipp, 2003). The PHT1 protein possesses an N-terminal di-leucine-based motif, which is a signal for sorting to endosomes and lysosomes (Sasawatari et al., 2011). The PHT1 amino acid sequence shows similarity to the peptide transporter NTR1 (45% identity and 67% similarity) from *Arabidopsis thaliana* in the putative fifth and sixth membrane spanning portions but only weak similarity to mammalian PepT1 (17% identity and 32% similarity) and PepT2 (12% identity and 27% similarity) (Yamashita et al., 1997).

Chicken PHT1 mRNA (NCBI Accession XM_415099.3) has been predicted to be located on chromosome 15 with a protein sequence of 581 amino acids.

Substrates

Histidine, carnosine, a histidine-containing peptide, and other di- and tri-peptides are transported by PHT1 (Yamashita et al., 1997; Bhardwaj et al., 2006; Sasawatari et al., 2011). *Xenopus* oocytes injected with PHT1 sense cRNA showed a 10-fold increase in carnosine transport activity than control water-injected oocytes (Yamashita et al., 1997). Glycylsarcosine was not a substrate for human PHT1, although it is a substrate for PepT1 and PepT2 (Bhardwaj et al., 2006).

Transport Mechanism

In Xenopus oocytes, Yamashita et al. (1997) found a linear 5-fold increase in histidine transport activity in oocytes injected with PHT1 sense cRNA. Transport was found to be saturable, indicating PHT1 is a high affinity histidine transporter (Yamashita

et al., 1997). Similar results were found in COS-7 cells transfected with human PHT1 with an increase in uptake of [^{3}H]histidine and [^{3}H]carnosine in the presence of a proton gradient (Bhardwaj et al., 2006). PHT1 is a proton-coupled transporter and transports histidine and oligopeptides derived from lysosomal degradation across the plasma membrane of lysosomes and into the cytosol (Sasawatari, et al., 2011). PHT1 was not responsible for histidine transport into synaptosomes within the brain, as histidine transport is independent of a transmembrane Na$^+$-gradient (Fujita et al., 2004). The potential transport mechanism for PHT1 is illustrated in Figure 1-2 (Alberts, 2008; Sasawatari et al., 2011).

Figure 1-2: Predicted Active Transport of Peptides, Histidine & H+ by PHT1

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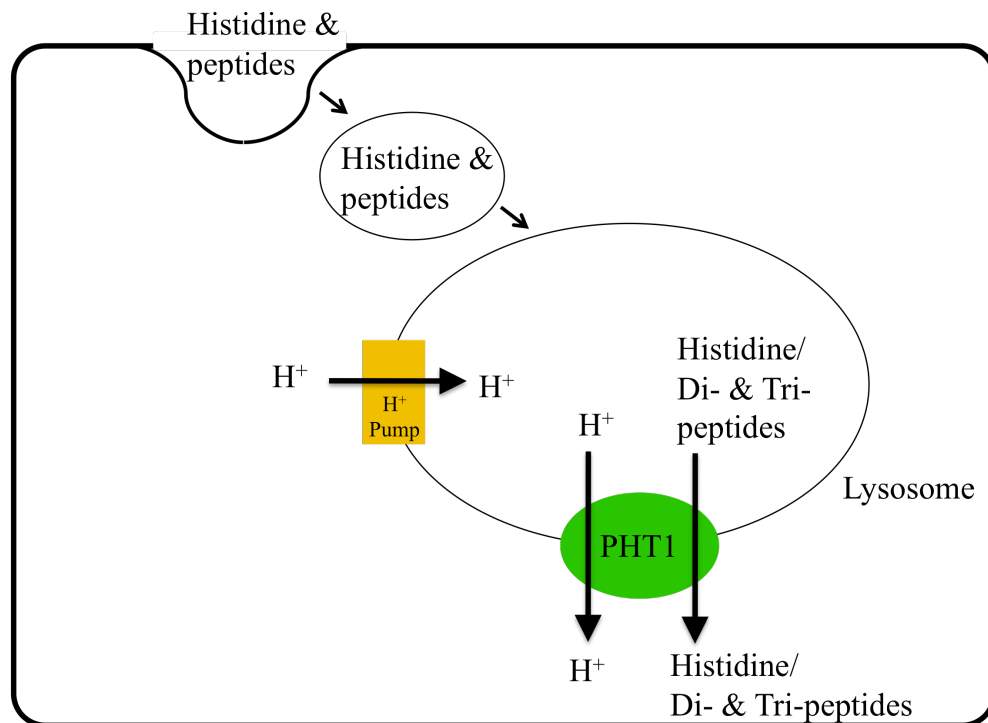
Tissue and Cellular Distribution

In the adult rat, PHT1 DNA expression was widely distributed throughout the gastrointestinal tract of the rat, with expression in the stomach, duodenum, jejunum, ileum, ileocecal junction, cecum and colon (Herrera-Ruiz et al., 2001). Strong PHT1 mRNA expression was also found in the brain and eye with lesser expression in the lung and spleen (Yamashita et al., 1997). Within the brain, strong expression of PHT1 mRNA was detected in the hippocampus, cerebellum, cerebral cortex and pontine nucleus, whereas weak expression was detected in the cerebral cortex, brain stem, thalamus and hypothalamus (Yamashita et al., 1997; Fujita et al., 2004). On a cellular level, PHT1 was detected in both neuronal cells and small nonneuronal cells in the rat brain (Yamashita et al., 1997).

Strong expression of human PHT1, which is 86.5% structurally similar to rat PHT1, was found to be widely expressed in the heart, kidney, leukocytes, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, testis and thymus (Herrera-Ruiz et al., 2001). In the gastrointestinal tract, PHT1 expression was greatest in the colon and jejunum with lesser expression in the duodenum, ileum and stomach. Within the small intestine, PHT1 was expressed in the villus epithelium, particularly in the plasma membrane (Bhardwaj et al., 2006). An immortalized human brain endothelial cell line, hCMEC/D3, also expressed PHT1 mRNA, suggesting a role for PHT1 in function of the human blood brain barrier (Carl et al., 2010).

Strong expression of PHT1 mRNA was found in mouse immune cells, including dendritic cells, activated macrophages and B cells. In a mouse macrophage cell line, RAW264.7, and COS-7 cells, immunohistochemical analysis showed no PHT1 protein

expression at the cell surface, but particular distribution to the late endosomes/lysosomes, which are important in an immune response (Sasawatari et al., 2011).

Regulation

PHT1 was found to be temperature-dependent, as transport activity at 4°C was almost eliminated. Transport by PHT1 was also thought to be proton-dependent as there was a 3-fold greater activity at pH 5.5 when compared to pH 7.5 (Yamashita et al., 1997). Within lysosomes, histidine concentration may be finely regulated to maximize lysosome-dependent signaling events due to the fact that the imidazole ring can function as an acid-base catalyst (Sasawatari et al., 2011).

PHT1 Knock-out Model

Homozygous PHT1 knock-out mice were fertile and appeared healthy. Knock-out mice did not exhibit dextran sodium sulfate induced colitis when compared to normal mice, suggesting PHT1 plays an important role in the immune response. Since lysosome functions within the cell are closely related to immune functions, expression of PHT1 in the control of histidine concentration may impact the efficiency of certain immune responses (Sasawatari et al., 2011).

Summary

Even though PHT1 is structurally similar to PepT1 and PepT2, PHT1 is more widely distributed, plays a different role in peptide transport, and is not located on the apical cell membrane. Widely expressed throughout the body, the peptide and histidine transporter PHT1 allows for products of lysosomal degradation to exit the lysosome into the cytosol. Although observed to be a proton-coupled transporter, further research needs to be conducted to understand the mechanism and regulation of transport through the

lysosomal membrane. Expression of PHT1 is found in the rat, human and mouse but has not been characterized in the chicken.

Peptide/Histidine Transporter 2: PHT2 (SLC15A3)

Sakata et al. (2001) first characterized PHT2 from the rat lung using degenerate PHT1 primers. The PHT2 gene codes for a protein 582 amino acids long and has a core molecular mass of 64.4 kilo Daltons. Like PepT1, PepT2 and PHT1, PHT2 is suggested to have 12 transmembrane domains. PHT2 does not have an N-terminal signal sequence; however, a di-leucine-based lysosome-sorting motif was identified in the putative N-terminal signal sequence. Rat PHT2 mRNA was primarily expressed within the lymphatic system, with abundant expression in the lung, spleen, adrenal gland and thymus. Sakata et al. (2001) suggested PHT2 to play a role in the immune response, with strong expression of PHT2 mRNA found in macrophages, eosinophils and phagocytes.

The amino acid sequence of rat PHT2 is 49% similar in identity to rat PHT1, with the most conserved regions in the putative fifth and tenth transmembrane domains. Rat PHT2 has less similarity to PepT1 and PepT2, with 22% and 24% identity, respectively (Sakata et al., 2001). PHT2 has not been identified in the chicken and will therefore not be further investigated.

Development and function of selected chick organs

The chicken, just as all birds, lays eggs that contain all of the nutrients the embryo needs to survive until hatch, including the protein and lipid-rich yolk. As the embryo grows, it develops those tissues whose function is to transport peptides into the body as well as tissues that need the transported peptides in order to function. It is important to

understand the development of such tissues in order to understand why certain peptide transporters are expressed and why peptide transport is even needed. The incubation period from fertilization until hatch for a chicken is a mere 21 days. During the first two days, rapid growth occurs and cells transform into the basic embryonic body consisting of the endoderm, mesoderm and ectoderm (Bellairs and Osmond, 2005).

Gut Development

A simple, tubular embryonic gut is formed from the endoderm layer by day 3 of incubation, consisting of the foregut, midgut and hindgut. The endoderm layer forms the epithelial lining of the gut and mucosal ducts while the mesoderm forms the muscular wall surrounding the gut. The foregut and hindgut are simple tubes at 50-52 hours of development, whereas the midgut lacks a ventral wall and is open to the yolk sac. By day 5 of development, the foregut and hindgut have extended in length and the midgut is only connected to the yolk sac via the yolk sac stalk. Differences in gene expression differentiate each section of the gut into its eventual specific tissues (Bellairs and Osmond, 2005). From beak to cloaca, the mature gut will consist of the pre-crop esophagus, crop, post-crop esophagus, proventriculus, gizzard, duodenum, jejunum, ileum, ceca and large intestine (Whittow, 2000).

Proventriculus

The region of the foregut which forms the proventriculus swells by the end of day 3 of development and differentiates from the gizzard and begins to form glands by day 7 (Bellairs and Osmond, 2005). In the developed bird, the proventriculus is lined with a mucous membrane, which secretes mucus for digestion. Papillae, containing gastric

glands, project into the lumen. These gastric glands are comprised of oxynticopeptic cells which secrete hydrochloric acid and pepsinogen for digestion (Whittow, 2000).

Small Intestine

The mature small intestine consists of three segments: the duodenum, jejunum and ileum. Divisions between these segments can be seen by day 6 of development, with landmarks being the duodenal-jejunal flexure at the distal end of the duodenal loop and the yolk sac stalk, also known as Meckel's Diverticulum. Starting at day 17 of development, the gut is gradually drawn into the abdomen and is complete at about day 19 when the yolk sac is also withdrawn (Bellairs and Osmond, 2005).

The mature intestine is lined with an absorptive mucosa consisting of chief cells, goblet cells, endocrine cells and epithelial cells, also known as enterocytes (Whittow, 2000; Hall and Guyton, 2011). The absorptive surface area of the intestine is greatly increased by large, circular folds of the mucosal surface, known as folds of Kerckring. The mucosal surface itself is made up of many villi and lined with enterocytes which contain microvilli on the apical membrane (Hall and Guyton, 2011). By day 15 of development, rudimentary villi have formed and by day 18 three waves of developmental stages can be observed. Increasing villus size is observed until hatch, with greatest growth during the day prior to hatch (Uni et al., 2003). After hatch, the small intestine rapidly increases in weight at a faster rate than overall body mass, villus volume increases 3-5 fold and the yolk size decreases approximately 60% within the first 48 hours (Noy and Sklan, 1999). Villus volume increases little within the first 2 days after hatch, then rapidly increases throughout the small intestine until complete by day 7 in the duodenum and by day 14 in the jejunum and ileum (Uni et al., 1998). Villus length also

increases due to a several-fold elevation in enterocyte numbers, which have gained

polarity and developed a defined brush border within hours of hatch (Geyra et al., 2001).

Enterocyte number per villus area remains constant among segments post-hatch ranging

from 200,000 to 280,000 cells (Uni et al., 1998). Crypts begin to form at hatch but

become defined within 2-3 days (Geyra et al., 2001).

Ceca

Unlike mammals, birds have two ceca which begin to form during embryonic day

4. By day 7, they have grown 2-4 mm in length and reach approximately 30 mm at hatch

(Bellairs and Osmond, 2005). In the developed bird, a pair of ceca is found at the juncture

of the small and large intestines. Musculature and villi at the opening to the cecum only

allow liquid to enter the ceca (Whittow, 2000). The ceca are histologically similar to the

small intestine but appear to have the function of microbial fermentation of dietary fiber

and absorption of amino acids and sugars (Sturkie and Benzo, 1986; Whittow, 2000).

Large Intestine

The large intestine, or rectum, begins to elongate after day 4 of development and

is only about 20 mm at hatch (Bellairs and Osmond, 2005). Histologically, the small and

large intestines are similar, with the large intestines having shorter villi and fewer crypts.

The main function of the large intestine is excretion and mineral water balance. The large

intestine is the most caudal portion of the gastrointestinal tract, leading to the cloaca, the

common pathway out of the body for the reproductive and digestive tracts (Whittow,

2000).

Brain

By 29 hours of development, the brain has begun to develop and divide into primary regions. Fibre tracts develop in the brain by 5 days of development. The complete cerebellum forms at about day 10 of development and comes in contact with the cerebral hemispheres by day 16. Gene expression differentiates the pattern of brain and spinal cord tissue throughout development (Bellairs and Osmond, 2005). In the fully developed brain, the forebrain consists of the cerebral hemispheres, thalamus and hypothalamus, which coordinate integration and reflexes. The midbrain consists of the nuclei that respond to sight and sound. The cerebellum consists of the nuclei that maintain equilibrium and coordinate muscle movement (Bellairs and Osmond, 2005).

Heart

The heart begins to form by 30 hours of development and begins beating by 38 hours. Actin potentials in cardiac mesoderm are recorded at 30 hours before heart chambers are formed. Division into left and right sides takes place during day 3-5 and circulation becomes well-established by 56 hours (Bellairs and Osmond, 2005). The mature heart is comprised of 4 chambers, with 2 atria and 2 ventricles and is surrounded by a tough pericardial membrane. The cardiac muscle has no transverse tubules, like those found in the mammalian heart, and has fibers which are much smaller when compared to mammals (Sturkie and Benzo, 1986; Whittow, 2000).

Bursa of Fabricius

The bursa of Fabricius forms on day 4 of development as a diverticulum on the dorsal side of the cloaca from an interaction between the endoderm and mesoderm. It soon becomes highly folded and lymphoid tissue develops at 12 to 13 days of

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development with immunoglobulin synthesis starting soon after (Bellairs and Osmond, 2005). There is rapid bursal growth from hatch to 4 weeks (Whittow, 2000). B cells differentiate within the bursa and are responsible for immunoglobulin and antibody synthesis (Sturkie and Benzo, 1986). Prior to the bursa, B lymphocytes are derived from blood-borne stem cells from the yolk sac (Sturkie and Benzo, 1986).

Lung

The larynx, trachea and lungs are derivatives of the gut and are formed from both mesoderm, which forms the muscles and connective tissue of the lungs, and endoderm, which forms the epithelium of the bronchial tree. As the two lung buds grow, they extend towards the posterior and become nestled deeply between the vertebral ribs. Gene expression directs the characterization of the bronchial tree and air sacs (Bellairs and Osmond, 2005). The lung consists of primary and secondary bronchi. The two primary bronchi have an epithelium containing goblet cells, mucous alveoli and cilia ridges. Four groups of secondary bronchi arise from the primary bronchi and a large number of parabronchi arise from the secondary bronchi (Bellairs and Osmond, 2005). Gas exchange takes place across the surface of the lungs by muscle contraction. The lungs of an adult chicken can hold approximately 300 mL of air (Sturkie and Benzo, 1986).

Kidney

A pair of urogenital ridges forms by 3 days of incubation which give rise to the nonfunctional pronephric kidneys and the more posterior, functional mesonephric kidneys. The mesonephric kidneys are active throughout much of embryonic life and ultimately give rise to the metanephric kidneys, which are fully functional by day 15 of

incubation. The cortex and medulla do not become clearly defined until adulthood (Bellairs and Osmond, 2005). The developed kidney is trilobed.

The functional unit of the kidney is the nephron, which forms urine. Each nephron consists of a glomerulus, where fluid is filtered from the blood, and a tubule where substrates can be reabsorbed into the blood and the fluid is created into urine (Hall and Guyton, 2011). The main function of the kidney is to eliminate wastes and excess water. Within the glomeruli, the kidney filters water, waste and other substances from the blood into the urine. Throughout the rest of the nephron, the kidney subsequently reabsorbs needed water, glucose, sodium and other substances from the urine. After filtration, reabsorption and secretion, waste enters the ureters for excretion as urine (Sturkie and Benzo, 1986; Whittow, 2000; Hall and Guyton, 2011).

Liver

The liver is formed from both the mesoderm and endoderm. By 50-53 hours of development, an endodermal component formed from a diverticulum in the gut invades the mesoderm to form the matrix of the liver. Liver primordium is visible by the end of day 2 and as it grows it comes in contact with the body wall (Bellairs and Osmond, 2005). In the mature chick, the liver is bilobed and contains right and left hepatic ducts, which drain to the duodenum. A branch from the right duct connects to the gallbladder (Whittow, 2000). The liver is involved in metabolism of proteins, fats and carbohydrates as well as detoxification of metabolites. The liver secretes bile, which is stimulated by eating, to emulsify fats for digestion by lipase for absorption. The liver also secretes amylase in the bile for carbohydrate digestion by 4-8 weeks of age (Whittow, 2000).

Nutrient Digestion

Peptide transport from ingested food within the intestine is arguably the most important form of peptide transport. Without this transport, amino acids as peptides would not enter the blood and be able to nourish the rest of the body. Major physiological changes occur within the chick in regards to protein digestion prior to hatch, immediately after hatch, and as the newly hatched chick grows and intakes exogenous food sources.

Prior to hatch

Prior to hatch, the chick receives nutrients from only the yolk, which is high in fat and protein, through the yolk sac membrane (Uni and Ferket, 2004). The pancreas secretes carboxypeptidase A, trypsin and chymotrypsin by embryonic day 16, pancreatic α-amylase by day 18 and lipase prior to hatch (Uni and Ferket, 2004). In the small intestine, brush border enzymes and major sodium-glucose and ATPase transporters begin expression at embryonic day 15 and begin to increase on embryonic day 19 in preparation for hatch (Uni et al., 2003; Uni and Ferket, 2004).

Immediately Post-Hatch

Birds hatch with an immature gastrointestinal system and are required to quickly undergo a large metabolic and physical change in that system when changing feed sources from the yolk, which is mostly fat, to an exogenous feed, comprised of mostly carbohydrates and proteins (Uni and Ferket, 2004). During the first few days, the chick still utilizes the yolk for growth of the small intestine and energy for maintenance through both the yolk sac membrane and the yolk stalk (Uni and Ferket, 2004). However, the yolk stalk is the primary route of yolk utilization when feed is present in the intestine (Noy and Sklan, 2001). The early utilization of the yolk provides substrate for mucosal

development, as removal of the yolk causes an initial decrease in villus volume and crypt depth (Uni et al., 1998). At hatch, the yolk contains 1.6 g of protein, almost all of which disappears by day 4 post hatch, potentially as the source of amino acids for gastrointestinal growth (Noy and Sklan, 1999).

In addition to morphological changes, brush border enzymes and transporters and pancreatic enzymes are increasing in expression (Uni and Ferket, 2004). Early access to feed enhances body weight, size of pectoralis muscles and the development of the intestine, resulting in an 8-10% heavier body weight at market (Uni and Ferket, 2004). Feed intake triggers enhanced secretions of trypsin and amylase, which are then secreted at a steady state based on feed intake as the chick grows (Sklan and Noy, 2000). Immediately post-hatch, chicks have the ability to degrade disaccharides in the mucosa by the sucrase-maltase complex (Uni et al., 1998). The uptakes of exogenous protein and carbohydrates are low immediately post-hatch but increase as the chick ages, particularly when enzymatic activity and adequate sodium levels are present (Noy and Sklan, 1999).

Post-hatch

Material presented in this section can be found in "Scott's Nutrition of the Chicken" (Leeson et al., 2001). In the proventriculus, hydrochloric acid (HCl) and pepsin are secreted due to vagal stimulation of gastric nerves from feed intake. Hydrochloric acid denatures proteins. The digestive material, or chyme, then moves into the gizzard where mechanical breakdown helps minimize surface area of food particles for degradation. In the small intestine, pepsin activates proteolytic enzymes such as trypsin, chymotrypsin and elastase to further break down polypeptides. These smaller polypeptides are then broken down further into oligopeptides (2-6 amino acids long) and

free amino acids by aminopeptidases and carboxypeptidases that are present in the lumen and mucosa of the small intestine. The jejunum secretes peptidase, which breaks down small peptides into amino acids and dipeptides for absorption. Final hydrolysis of oligopeptides occurs through the action of peptidases present at the surface of the microvilli of enterocytes. Peptides are absorbed into the enterocyte by active transport involving H^+. Once inside the enterocyte, further hydrolysis of peptides occurs and then products, mostly free amino acids, are transported into the bloodstream. Peptide uptake is most rapid in the jejunum whereas amino acid uptake is most rapid in the ileum.

Objectives

In the chicken, PepT1 has been extensively studied in intestine but not in other tissues. Much less is known for the chicken about PepT2 and PHT1, as they are not characterized in the chicken. PHT2 is not found in the chicken genome and therefore was not analyzed here. The objective of this thesis was to perform a comprehensive analysis of developmental and tissue regulation of gene expression of PepT1, PepT2 and PHT1. Gene expression was evaluated through development in both pre- and post-hatch chicks as nutrients are utilized within the developing egg before the consumption of exogenous feed. Gene expression was also evaluated among a wide array of tissues in order to characterize expression throughout the whole chick.

CHAPTER II. MATERIALS AND METHODS

Animals and Tissue Collection

Fertile eggs were obtained from the S37 generation of Dr. Paul Siegel's high antibody selected line of Leghorn chickens at Virginia Tech (Zhao et al., 2012) and were incubated at the Virginia Tech Poultry Farm. After hatch, chicks were moved to Litton-Reaves Hall and caged in groups of 5-6 chicks per cage. On sampling days, chicks were randomly selected from the whole group of remaining chicks. Samples were collected from 5 chicks killed by cervical dislocation on embryonic days 18 (e18) and 20 (e20) and days 1, 3, 7, 10 and 14 post-hatch. Whole brain, heart, proventriculus, duodenum, jejunum, ileum, ceca, large intestine, bursa, kidney, liver, and lung samples were collected from each bird. Bursa of Fabricus and ceca were not collected on e18, whole intestine (as opposed to duodenum, jejunum and ileum segments) was collected at e18, and lungs were not collected on e18 and e20. Intestinal segments were all washed with ice cold phosphate buffered saline (PBS). All samples were collected in two 2 mL microcentrifuge tubes, one containing an aliquot of 20-30 mg of tissue for RNA extraction and one containing the rest of the tissue, and stored at -80°C until use.

Total RNA extraction

RNA was extracted from aliquoted tissue samples using Tri Reagent according to the Isolation of RNA protocol (Molecular Research Center, Inc., Cincinnati, OH). Tissues were homogenized using an IKA ULTRA-TURRAX T25 basic homogenizer and chloroform was used in the phase separation step. RNA was suspended in 100 uL DEPC water (1 mL Diethylpyrocarbonate (Sigma-Aldrich, St. Louis, MO) per liter water, autoclaved) and incubated for 10 minutes at 58°C. Initial concentration was determined

using the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Concentration was confirmed and purity analyzed using Agilent RNA 6000 Nano Chips

on an Agilent 2100 Bioanalyzer (Agilent, Foster City, CA). The manufacturer's protocol

was followed for RNA analysis and 12 samples were run on each Agilent RNA 6000

Nano Chip. Each sample had a RNA Integrity Number greater than 6.5. All extracted

RNA samples were stored at -80°C.

Standard Curve Generation

Standard curves for chicken PepT1 and PepT2 were previously generated;

however, a standard curve for PHT1 needed to be generated. A fragment of chicken

PHT1 gene was cloned into pGEM®-T Easy Vector (Promega), similar to chicken PepT1

and PepT2. Primers for cloning gene fragments, shown in Table 2-1, were designed using

Primer 3 software (Whitehead Institute, 2007) and synthesized by MWG-BIOTECH, Inc.

(Huntsville, AL). Both PepT1 and PHT1 were inserted in the antisense (SP6) orientation

and the plasmid was linearized with the SacII restriction enzyme (20,000 U/mL) and NE

Buffer 4 (New England Biolabs, Ipswich, MA). PepT2 was inserted in the sense (T7)

orientation and the plasmid was linearized with the PstI restriction enzyme (3,000 U/mL)

and NE Buffer 3 (New England Biolabs, Ipswich, MA). A reaction consisting of 5 μg of

plasmid, 1uL of BSA (10 mg/mL), 4 μL of restriction enzyme, 10 μL of restriction

enzyme buffer and DEPC water to a total volume of 100 μL was incubated at 37°C for 4

h followed by 65°C for 20 min. The digest was confirmed by running 4 μL of digest on a

1% agarose (Fisher Scientific) gel. Cut plasmid was purified using the Qiagen PCR

Purification Kit (Qiagen, Valencia, CA). Product concentration and purity were assessed

using the NanoDrop Spectrophotometer. Plasmid stock and cut plasmid were stored at 4°C.

RNA was transcribed from cut DNA plasmid using a MEGAscript® SP6 or T7 in vitro transcription kit (Ambion, Austin, TX), based on the orientation of the insert. A reaction was created using 2 μL of ATP (50 mM), 2 μL of CTP (50 mM), 2 μL of GTP (50 mM), 2 μL of UTP (50 mM), 2 μL of 10X buffer, 2 μL 10X enzyme, and 1 μg template DNA. The reaction was incubated in a 37°C water bath for 16 hours, then treated with DNAse I. RNA was precipitated by adding 30 μL lithium chloride and 15 μL DEPC water and chilling for 2 hours at -20°C. RNA was collected by centrifugation in Thermo Electron Corp IEC Micromax RF centrifuge for 12 minutes at max speed (21,000 rcf), washed with 70% ethanol and resuspended in 20 μL DEPC water. RNA concentration was determined using a Quant-iT Ribogreen RNA Assay Kit (Invitrogen, Molecular Probes, Eugene, OR) and a FLUOstar OPTIMA microplate reader (BMG LABTECH, Germany).

Number of molecules per microliter (N) was calculated using the following equation:

$$N = \frac{\text{Concentration of cRNA (μg/μL)} \times (183.5 \times 10^{13})}{\text{cRNA size (bases)}}$$

Standard cRNA was diluted in a series of 10^{11} to 10^4 molecules per microliter using 10 μg/mL tRNA.

Reverse Transcription

Tissue RNA samples and standard curve RNA dilutions were used to create complementary DNA (cDNA) using the cDNA Archive Kit (Applied Biosystems, Foster

City, CA) for real-time PCR analysis. Each reaction contained 2 μL 10X reverse transcription buffer, 0.8 μL 25x dNTPs, 2 μL 10X random primers, 1 μL multiscribe reverse transcriptase (50 U/μL), 4.2 μL DEPC water, and 10 μL of 0.2 μg/μL sample RNA placed in a thin-walled PCR tube. Tubes were placed in a PTC-200 Peltier Thermal Cycler (MJ Research) for 10 minutes at 25°C followed by 120 minutes at 37°C. The cDNA was diluted 1:30 with DEPC water and stored at -20°C.

Absolute Quantification Real-Time PCR

With a 96-well plate on ice, 2 μL of diluted cDNA were added to each well as well as 23 μL of Real-Time PCR master mix which contained 12.5 μL 2X SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 0.5 μL forward primer (5 μM), 0.5 μL reverse primer (5 μM), and 9.5 μL DEPC water. The plate was sealed with a MicroAmp Optical Adhesive Film (Applied Biosystems) and spun down in a centrifuge to remove bubbles and loaded into an Applied Biosystems 7300 Real-Time PCR machine. The following PCR reaction was run: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The PepT1, PepT2 and PHT1 genes were analyzed and the respective forward and reverse primer sequences are shown in Table 2-1. All primers were created using the Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by MWG-BIOTECH, Inc. Each plate contained samples and a standard curve run in duplicate. The Applied Biosystems computer software, using the standard curve, determined the quantity of RNA for each sample. Since samples were run in duplicate, a mean quantity was generated. The molecules of transporter mRNA per nanogram of total RNA were determined from each sample by dividing the Mean Quantity for each sample by 200.

TABLE 2-1. Forward and Reverse Primers of Genes for Real Time PCR.

Gene	Primer Orientation	Real Time Primer Sequence
PepT1[a]	Forward Real Time Primer	CCCCTGAGGAGGATCACTGTT
	Reverse Real Time Primer	CAAAAGAGCAGCAGCAACGA
	Forward Cloning Primer	ACTGTCAATCCAATCCTGAT
	Reverse Cloning Primer	GACAGTCACGTTCTGAAGAT
PepT2 [b]	Forward Real Time Primer	TGACTGGGCATCGGAACAA
	Reverse Real Time Primer	ACCCGTGTCACCATTTTAACCT
	Forward Cloning Primer	GCTCCCATCAGATTCCAAAG
	Reverse Cloning Primer	TGGATCCCTGCTGATCAAAC
PHT1	Forward Real Time Primer	AAGGCCAGGGAGTCCTTCA
	Reverse Real Time Primer	TGACAGCTTAGCCATCTCAAACA
	Forward Cloning Primer	AAGCCACCTGATGGTAGTGC
	Reverse Cloning Primer	AGGCTGCTGGAAACGTATGA

[a] = designed and cloned by Gilbert, et al. (2007)
[b] = designed and cloned by Wysinski, unpublished

Statistical Analysis

All data were analyzed by ANOVA using JMP® Statistical Discovery Software

from SAS (SAS Institute, Cary, NC). Each gene was analyzed separately. For each gene,

the model included the main effects of tissue and age, the interaction of age by tissue, and

bird number nested within age. Significant effects and interactions ($P < 0.05$) were

further evaluated with Tukey's test for pairwise comparisons.

CHAPTER III. RESULTS

Peptide uptake in the Gastrointestinal Tract

Proventriculus

As shown in Figure 3-1, PepT1 expression in the proventriculus showed no significant developmental change over time. Expression was barely detectable with an average of 5 molecules of PepT1 per ng total RNA over all time points. Expression of PepT2 decreased over time. Greatest expression of PepT2 was observed at e18 with an average of 60 molecules of PepT2 per ng total RNA when compared to expression at d14 with an average of 5 molecules of PepT2 per ng total RNA ($P = 0.015$). Expression of PHT1 also declined from e20 to d7 ($P = 0.010$). Lowest expression of PHT1 was observed at d14 when compared to e18 ($P = 0.0094$) and e20 ($P = 0.0014$).

Small Intestine-Duodenum

As shown in Figure 3-2, expression of PepT1 increased in the duodenum from e18 to d1 with 14,5000 molecules of PepT1 per ng total RNA ($P = 0.021$). Greatest expression of PepT1 among tissues was observed in the duodenum, as well as in the jejunum and ileum (Table 3-1). Expression of PepT2 was greatest in whole small intestine at e18 with an average of 200 molecules PepT2 per ng total RNA, declined in the duodenum at e20 ($P = 0.001$) and remained barely detectable over time. Expression of PHT1 declined from 4,450 molecules of PHT1 per ng total RNA in whole small intestine at e18 to 1,300 molecules of PHT1 per ng total RNA at d7 ($P = 0.046$).

Small Intestine-Jejunum

As shown in Figure 3-3, peak expression of PepT1 was observed within the jejunum at d1 with 33,000 molecules of PepT1 per ng total RNA and was greater than all

other time points ($P < 0.0005$). Other than the d1 peak, PepT1 expression remained constant. Greatest expression of PepT1 among tissues was observed in the jejunum, as well as the duodenum and ileum (Table 3-1). PepT2 expression was highest in whole intestine at e18 with 60 molecules of PepT2 per ng total RNA. Expression of PepT2 decreased from e18 to d3 with 20 molecules of PepT2 per ng total RNA ($P = 0.0086$) and then remained constant with minimal expression. Throughout development, PHT1 expression remained relatively constant, with increasing expression from d3 to d14 ($P = 0.010$).

Small Intestine-Ileum

As shown in Figure 3-4, expression of PepT1 within the ileum dramatically increased ($P = 0.026$) from 820 molecules of PepT1 per ng total RNA at e20 to 15,400 molecules of PepT1 per ng total RNA at d1. Expression was not different from d1 through d14. Highest expression of PepT1 among tissues was observed in the ileum, as well as in the duodenum and jejunum (Table 3-1). Expression of PepT2 was barely detectable throughout development; however, PepT2 expression peaked at d1 with 480 molecules of PepT2 per ng total RNA when compared to all time points except d14 ($P < 0.04$). Expression of PHT1 remained constant throughout development. Average expression was 4,400 molecules of PHT1 per ng total RNA.

Ceca

As shown in Figure 3-5, ceca PepT1 expression increased from 500 molecules of PepT2 per ng total RNA at e20 to d1 with 1,700 molecules of PepT1 per ng total RNA ($P = 0.032$). After d1, PepT1 expression declined to d7 ($P = 0.023$) and then remained constant. Expression of PepT2 remained constant throughout development with an

average expression of 40 molecules of PepT2 per ng total RNA. Likewise, PHT1 expression showed no significant developmental change over time. Average expression of PHT1 was 8,300 molecules of PHT1 per ng total RNA over all time points.

Large Intestine

As shown in Figure 3-6, expression of PepT1 in the large intestine increased from 660 molecules of PepT1 per ng total RNA at d3 to 3,400 molecules of PepT1 per ng total RNA on d10 ($P = 0.020$). Expression of PepT2 remained constant throughout development, with an average expression of 50 molecules of PepT2 per ng total RNA over all time points. Likewise, expression of PHT1 was relatively constant throughout expression with one exception. Expression of PHT1 increased from 3,000 molecules of PHT1 per ng total RNA at d3 to 9,000 molecules of PHT1 per ng total RNA at d10 ($P = 0.030$).

Peptide uptake in other tissues

Brain

As shown in Figure 3-7, expression of PepT1 within the brain was barely detectable from e18 to d10 with an average of 20 molecules of PepT1 per ng total RNA. Expression increased ($P = 0.001$) to 170 molecules of PepT1 per ng total RNA on d14, when compared to all other time points. Expression of PepT2 remained constant over time. Average expression was 3,700 molecules of PepT2 per ng total RNA over all time points. Greatest expression of PepT2 was observed in the brain, when compared to other tissues sampled (Table 3-1). PHT1 expression was constant from e18 to d10 with an

average of 5,100 molecules of PHT1 per ng total RNA. Expression then increased on d14 to 21,000 molecules of PHT1 per ng total RNA ($P = 0.0013$).

Heart

As shown in Figure 3-8, expression of PepT1 in the heart was barely detectable from e18 until d10, with an average of 5 molecules of PepT1 per ng total RNA. Expression of PepT1 increased at d14 to 30 molecules of PepT1 per ng total RNA and was greater ($P = 0.049$) than all other time points. Expression of PepT2 was barely detectable and remained constant over time, with an average expression of 10 molecules of PepT2 per ng total RNA. Expression of PHT1 remained constant over time, with an average expression of 1,500 molecules of PHT1 per ng total RNA.

Bursa of Fabricius

As shown in Figure 3-9, expression of PepT1 within the bursa of Fabricius was barely detectable from e18 until d10, averaging 5 molecules of PepT1 per ng total RNA. Expression of PepT1 increased ($P = 0.047$) from e18 to d14 with 10 molecules of PepT1 per ng total RNA. Expression of PepT2 was constant and barely detectable from e18 until d10 with an average of 15 molecules of PepT2 per ng total RNA. Expression of PepT2 increased ($P = 0.0008$) from d10 to d14 with 85 molecules of PepT2 per ng total RNA. Expression of PHT1 remained constant over time, with an average expression of 8,000 molecules of PHT1 per ng total RNA.

Lung

As shown in Figure 3-10, expression of PepT1 was barely detectable and showed no developmental change over time in the lung. Average expression was 10 molecules of PepT1 per ng total RNA over all time points. Similarly, PepT2 expression showed no

developmental change over time. Average expression was 120 molecules of PepT2 per ng total RNA over all time points. Expression of PHT1 also showed no significant developmental change over time. Average expression was 4,000 molecules of PHT1 per ng total RNA over all time points.

Kidney

As shown in Figure 3-11, expression of PepT1 within the kidney was constant over time, with an average expression of 60 molecules of PepT1 per ng total RNA over all time points. Expression of PepT2 increased ($P = 0.012$) from 1,200 molecules of PepT2 per ng total RNA at e20 to d10, with 5,600 molecules of PepT2 per ng total RNA. Expression then decreased from d10 to d14, with 1,300 molecules of PepT2 per ng total RNA ($P = 0.014$). Expression of PepT2 in the kidney was less than in the brain, but still greater than the rest of the tissues sampled (Table 3-1). Expression of PHT1 remained constant over time, with an average expression of 8,900 molecules of PHT1 per ng total RNA over all time points.

Liver

As shown in Figure 3-12, expression of PepT1 within the liver showed no developmental change over time, with barely detectable expression levels. Average expression was 5 molecules of PepT1 per ng total RNA over all time points. Expression of PepT2 decreased ($P = 0.0038$) from e18, with 630 molecules of PepT2 per ng total RNA, to d1 with 15 molecules of PepT2 per ng total RNA. Expression of PepT2 remained constant and barely detectable through d14. Expression of PHT1 remained relatively constant throughout development. Expression increased from 1,500 molecules

of PHT1 per ng total RNA at d1 to 5,000 molecules of PHT1 per ng total RNA on d10 (P = 0.011).

Overall Gene Expression

To facilitate comparison of tissue-specific gene expression, all tissues at each of the developmental ages are shown for PepT1 (Figure 3-13), PepT2 (Figure 3-14) and PHT1 (Figure 3-15). A significant tissue by age interaction was observed for all genes (Table 3-1). PepT1 expression is greatest within the small intestinal segments when compared to all other tissues and is also greatest at d1 when compared to all other ages (Figure 3-13). PepT2 expression is highly expressed in the brain and kidney when compared to all other tissues (Figure 3-14). PHT1 expression is constitutively expressed among tissues and throughout development (Figure 3-15).
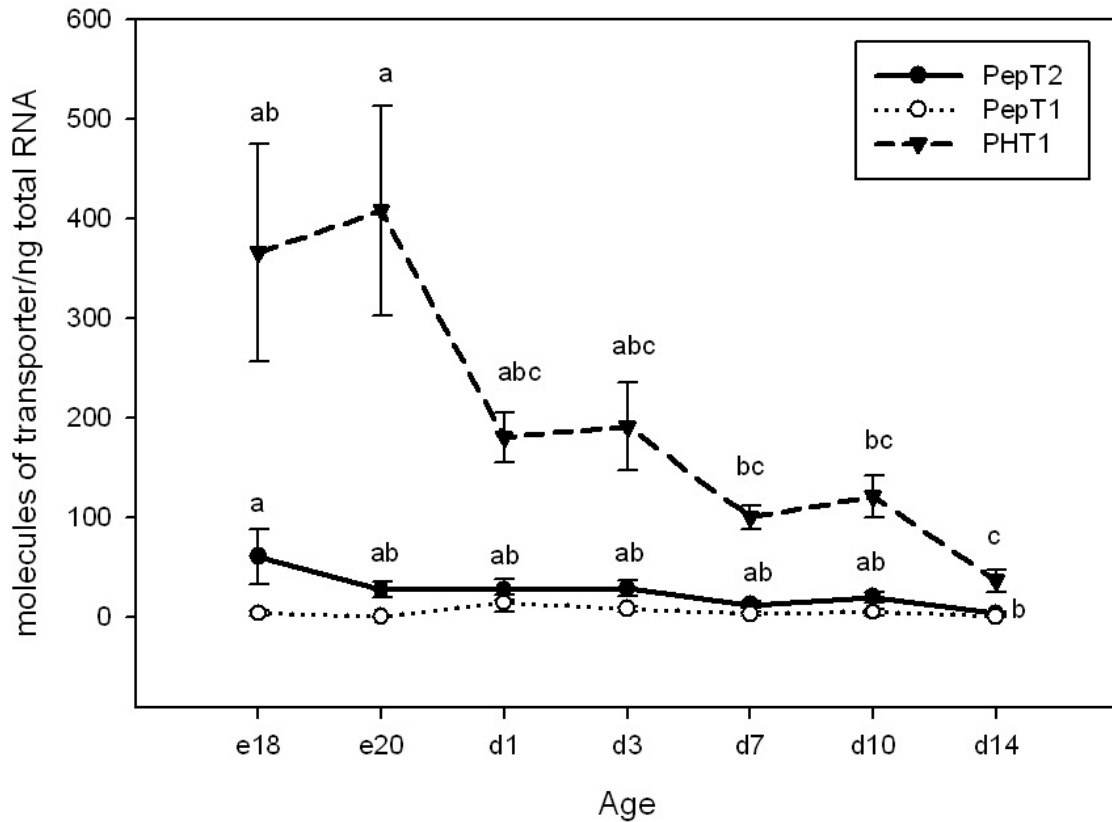
Figure 3-1. Peptide Transporter Gene Expression in the Developing Proventriculus.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the proventriculus. Proventriculus tissue was
excised from 4 birds at embryonic day 18 (e18) and 5 birds from embryonic day 20 (e20),
day 1 (d1), day 3 (d3), day 7 (d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was
extracted from each sample and analyzed for gene expression by Absolute Quantification
Real Time PCR. Each gene was analyzed separately and points within a line without a
common letter differ ($P < 0.05$).
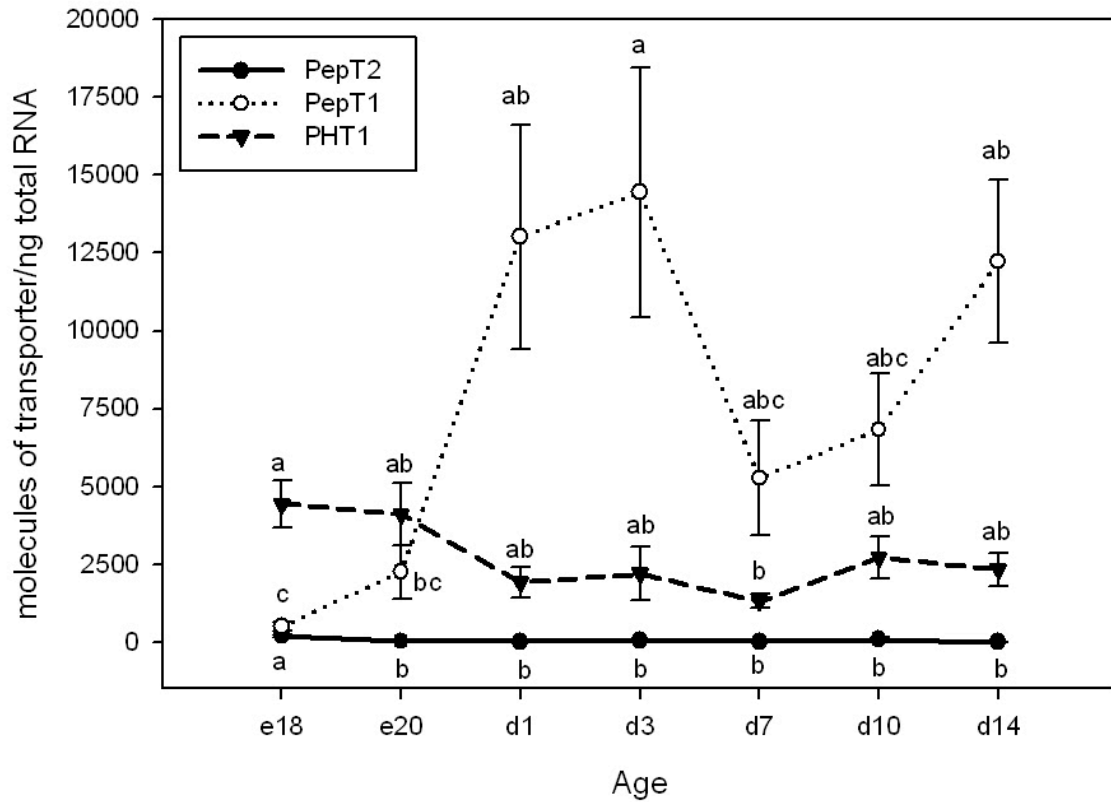
Figure 3-2. Peptide Transporter Gene Expression in the Developing Duodenum.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the small intestine and duodenum. Whole
small intestine tissue was excised from 5 birds at embryonic day 18 (e18). Duodenum
tissue was excised from 5 birds at embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7
(d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was extracted from each sample
and analyzed for gene expression by Absolute Quantification Real Time PCR. Each gene
was analyzed separately and points within a line without a common letter differ ($P <$
0.05).
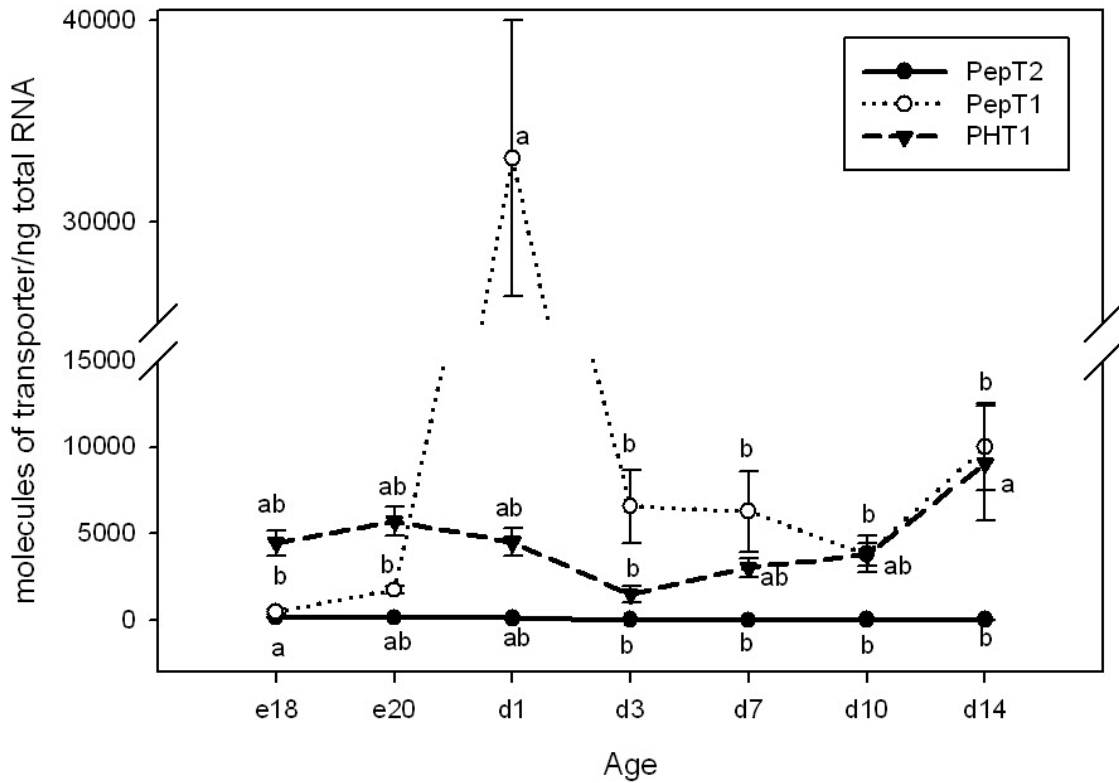
Figure 3-3. Peptide Transporter Gene Expression in the Developing Jejunum.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the small intestine and jejunum. Whole small
intestine tissue was excised from 5 birds at embryonic day 18 (e18). Jejunum tissue was
excised from 5 birds at embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7 (d7), day
10 (d10), and day 14 (d14) post-hatch. RNA was extracted from each sample and
analyzed for gene expression by Absolute Quantification Real Time PCR. Each gene was
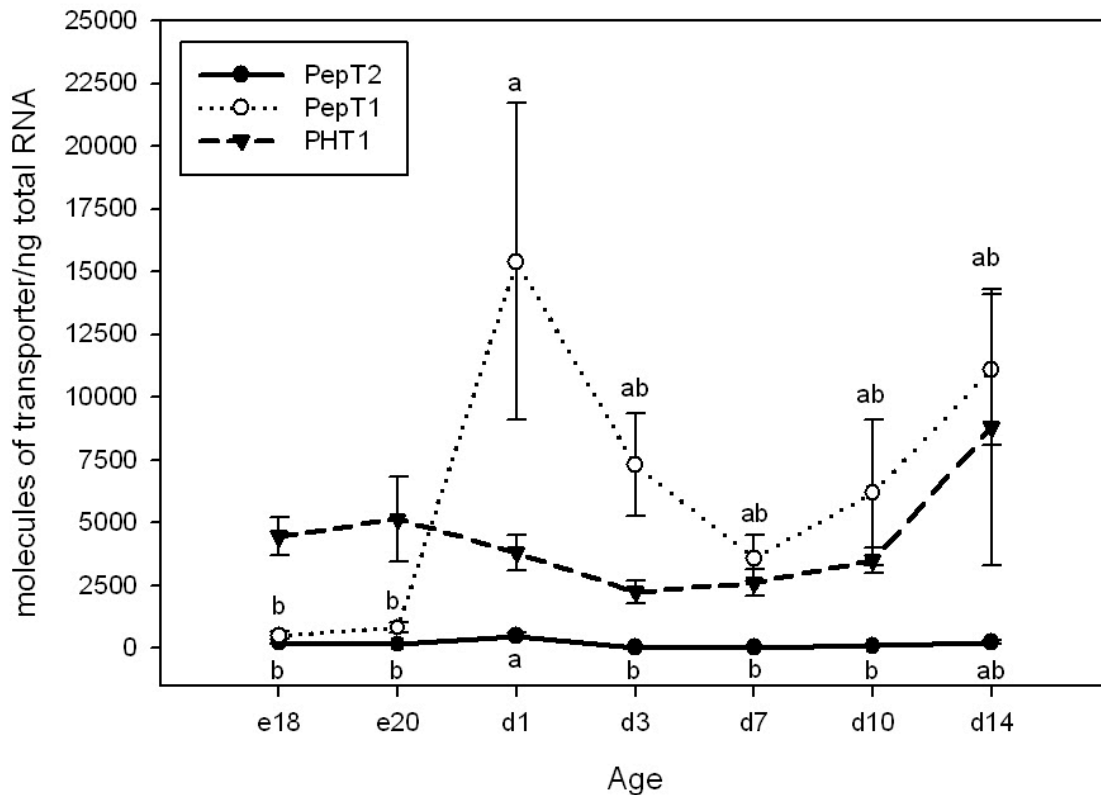analyzed separately and points within a line without a common letter differ ($P < 0.05$).

Figure 3-4. Peptide Transporter Gene Expression in the Developing Ileum.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the small intestine and ileum. Whole small
intestine tissue was excised from 5 birds at embryonic day 18 (e18). Ileum tissue was
excised from 5 birds at embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7 (d7), day
10 (d10), and day 14 (d14) post-hatch. RNA was extracted from each sample and
analyzed for gene expression by Absolute Quantification Real Time PCR. Each gene was
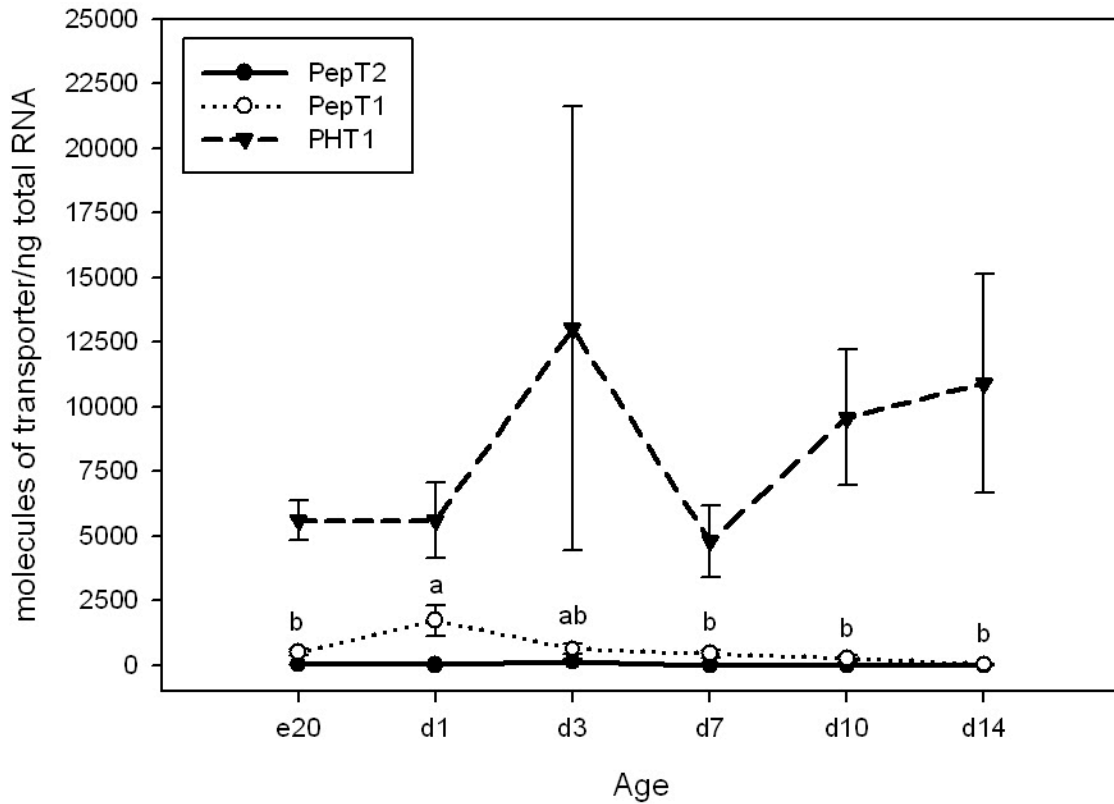analyzed separately and points within a line without a common letter differ ($P < 0.05$).

Ceca

Figure 3-5. Peptide Transporter Gene Expression in the Developing Ceca.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the Ceca. Ceca tissue was excised from only 1
bird at embryonic day 18 (e18) and not included in this graph. Ceca tissue was excised
from 5 birds at embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7 (d7), day 10 (d10),
and day 14 (d14) post-hatch. RNA was extracted from each sample and analyzed for gene
expression by Absolute Quantification Real Time PCR. Each gene was analyzed
separately and points within a line without a common letter differ ($P < 0.05$).

Large Intestine

Figure 3-6. Peptide Transporter Gene Expression in the Developing Large Intestine.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the large intestine. Large intestine tissue was
excised from 5 birds at embryonic day 18 (e18), embryonic day 20 (e20), day 1 (d1), day
3 (d3), day 7 (d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was extracted from
each sample and analyzed for gene expression by Absolute Quantification Real Time
PCR. Each gene was analyzed separately and points within a line without a common
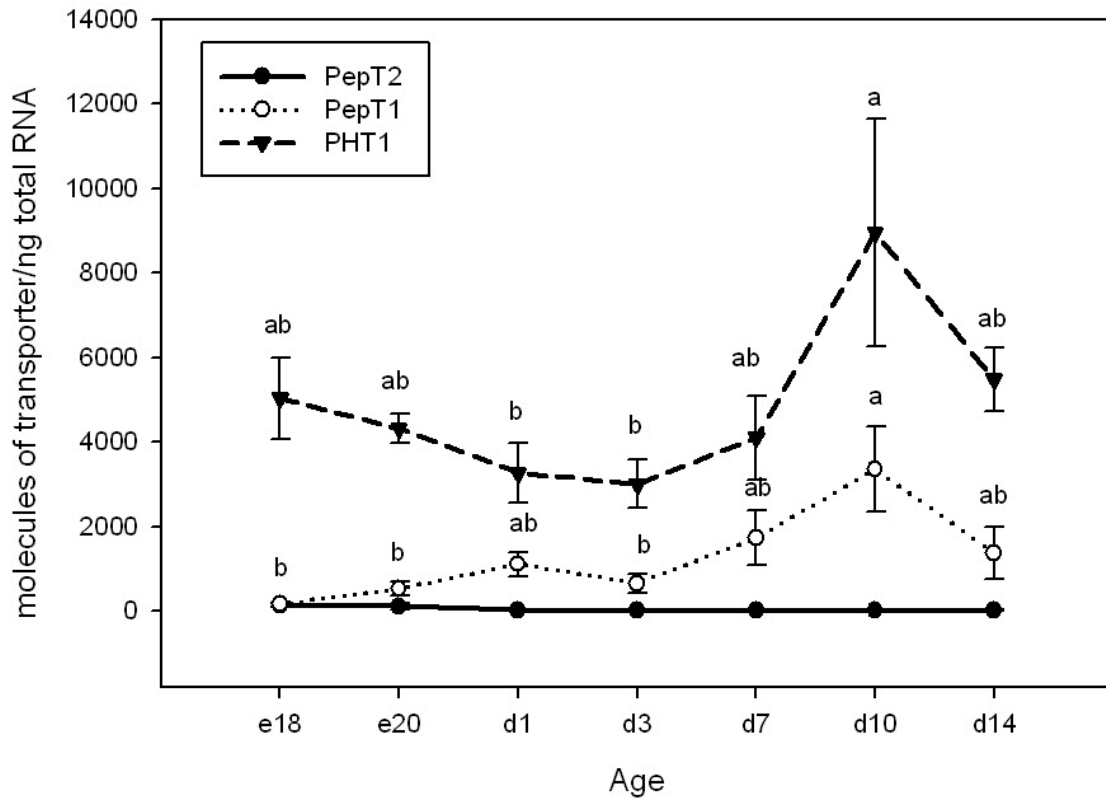letter differ ($P < 0.05$).

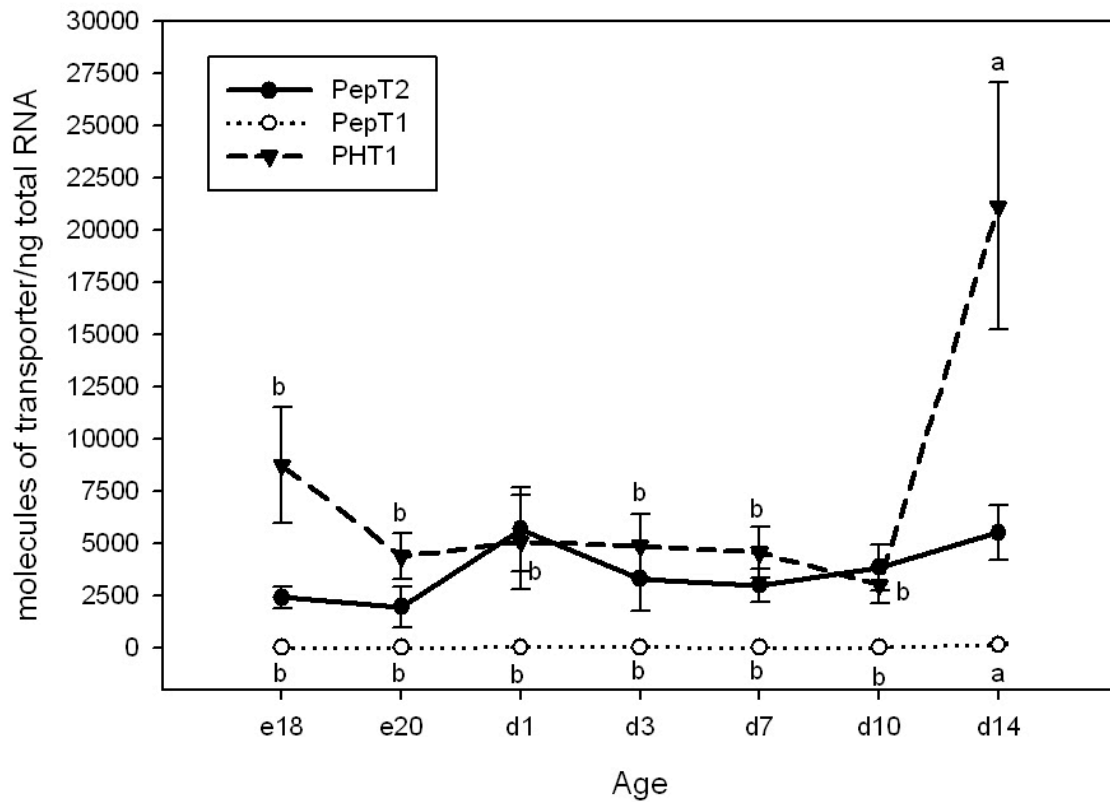**Figure 3-7. Peptide Transporter Gene Expression in the Developing Brain.**
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the brain. Whole brain tissue was excised
from 5 birds at embryonic day 18 (e18), embryonic day 20 (e20), day 1 (d1), day 3 (d3),
day 7 (d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was extracted from each
sample and analyzed for gene expression by Absolute Quantification Real Time PCR.
Each gene was analyzed separately and points within a line without a common letter
differ ($P < 0.05$).

Figure 3-8. Peptide Transporter Gene Expression in the Developing Heart.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the heart. Whole heart tissue was excised from
5 birds at embryonic day 18 (e18), embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7
(d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was extracted from each sample
and analyzed for gene expression by Absolute Quantification Real Time PCR. Each gene
was analyzed separately and points within a line without a common letter differ ($P <$
0.05).

Figure 3-9. Peptide Transporter Gene Expression in the Developing Bursa of Fabricius. Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and Peptide-Histidine Transporter 1 (PHT1) in the Bursa of Fabricius. Bursa of Fabricius tissue was excised from 4 birds at embryonic day 18 (e18) and 5 birds at embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7 (d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was extracted from each sample and analyzed for gene expression by Absolute Quantification Real Time PCR. Each gene was analyzed separately and points within a line without a common letter differ ($P < 0.05$).
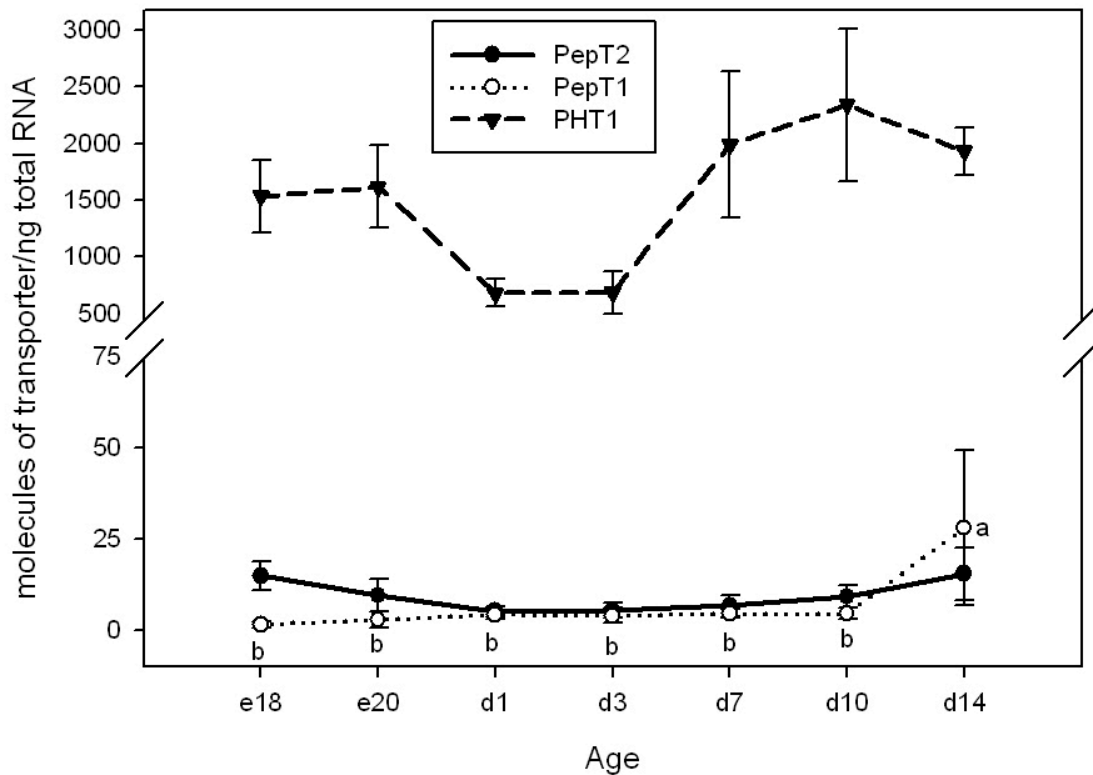
Lung

Figure 3-10. Peptide Transporter Gene Expression in the Developing Lung.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the lung. Lung tissue not was excised from
birds at embryonic day 18 or 20.Lung tissue was excised from 5 birds at day 1 (d1), day 3
(d3), day 7 (d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was extracted from
each sample and analyzed for gene expression by Absolute Quantification Real Time
PCR. Each gene was analyzed separately and points within a line without a common
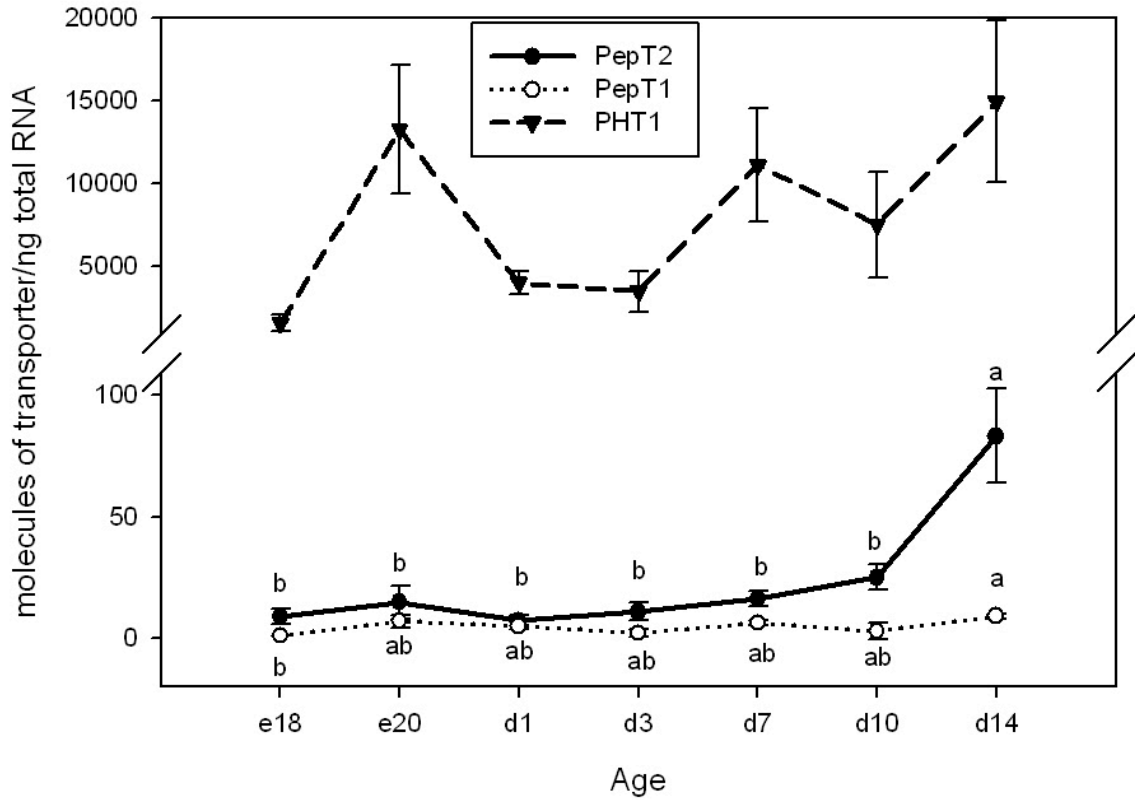letter differ ($P < 0.05$).

Kidney

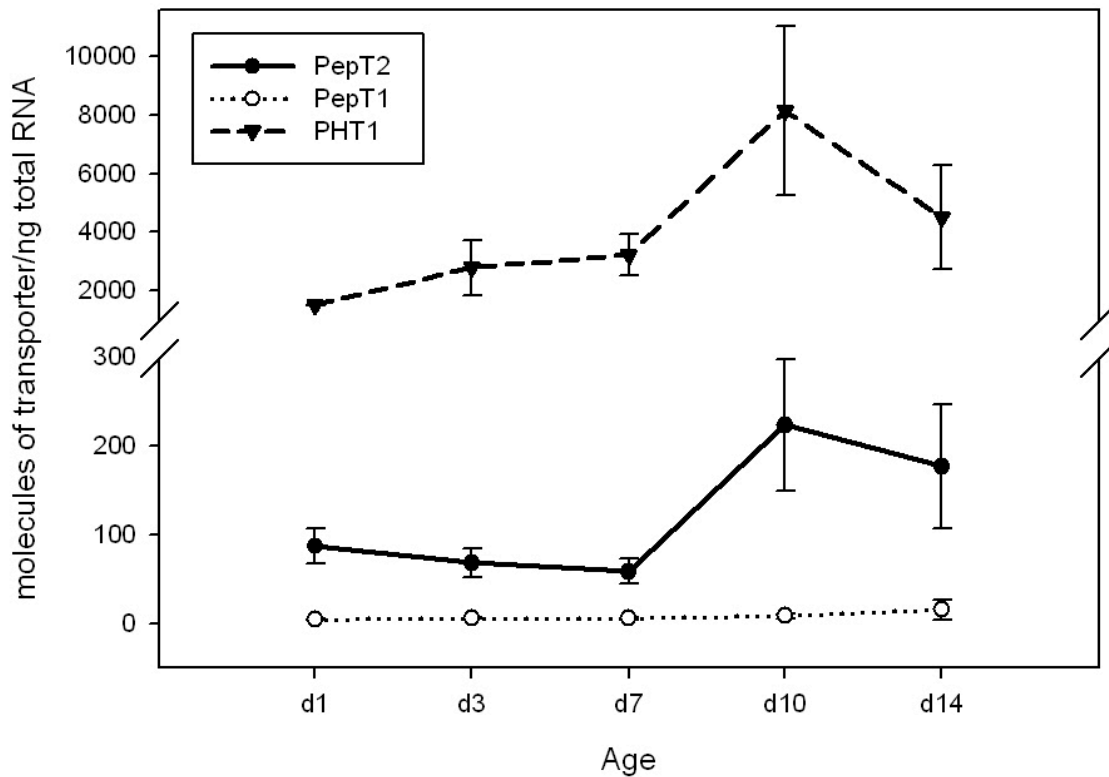**Figure 3-11. Peptide Transporter Gene Expression in the Developing Kidney.**
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the Kidney. Kidney tissue was excised from 5
birds at embryonic day 18 (e18), embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7
(d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was extracted from each sample
and analyzed for gene expression by Absolute Quantification Real Time PCR. Each gene
was analyzed separately and points within a line without a common letter differ ($P <$
0.05).
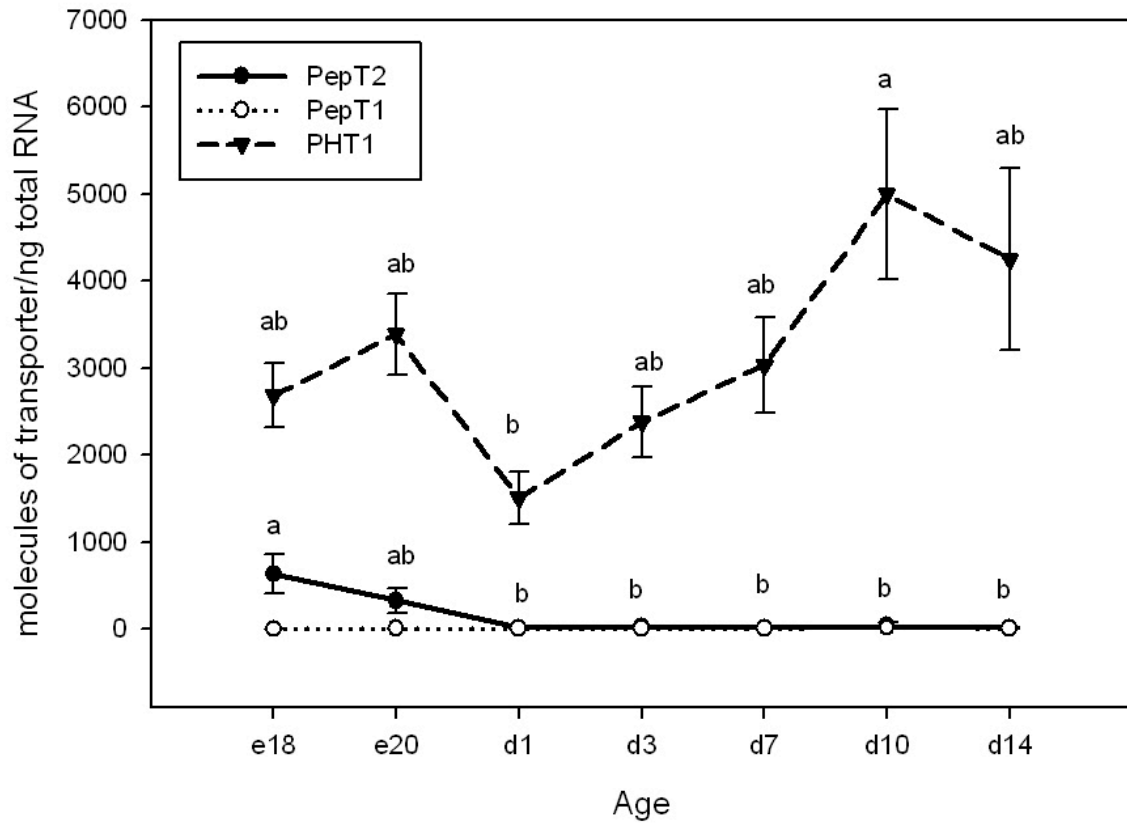
Figure 3-12. Peptide Transporter Gene Expression in the Developing Liver.
Expression of Peptide Transporter 1 (PepT1), Peptide Transporter 2 (PepT2), and
Peptide-Histidine Transporter 1 (PHT1) in the Liver. Liver tissue was excised from 5
birds at embryonic day 18 (e18), embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7
(d7), day 10 (d10), and day 14 (d14) post-hatch. RNA was extracted from each sample
and analyzed for gene expression by Absolute Quantification Real Time PCR. Each gene
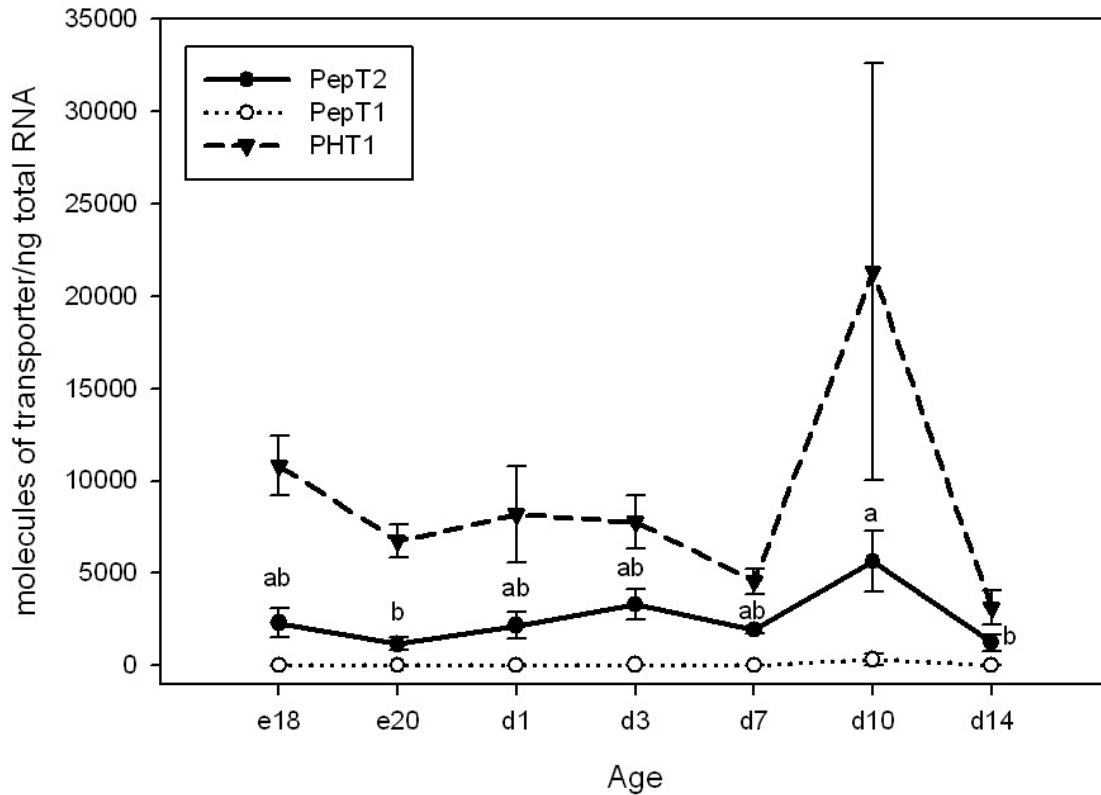was analyzed separately and points within a line without a common letter differ ($P <$
0.05).
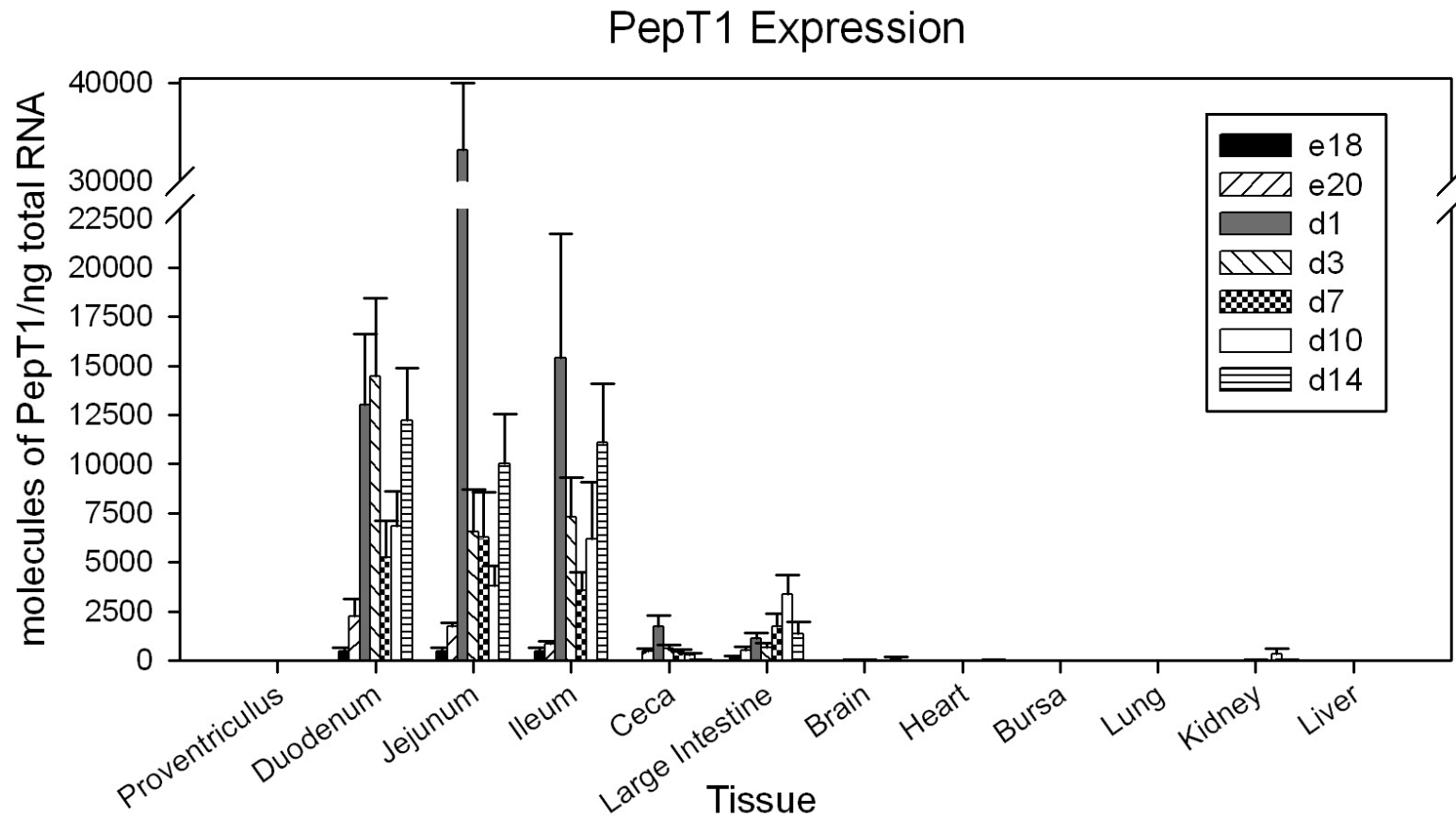
Figure 3-13. Expression of Peptide Transporter 1 (PepT1) in developing chick tissues. Five birds were sampled at embryonic day 18 (e18), embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7 (d7), day 10 (d10), and day 14 (d14) post-hatch. Proventriculus, duodenum, jejunum, ileum, ceca, large intestine, whole brain, heart, bursa of Fabricius (bursa), lung, kidney and liver samples were collected from each bird at every day sampled, except whole intestine (as opposed to duodenum, jejunum and ileum segments) was collected at e18, lungs were not collected on e18 and e20, and only 4 samples of bursa of Fabricius and ceca were collected on e18. RNA was extracted from each sample and analyzed for PepT1 gene expression by Absolute Quantification Real Time PCR.
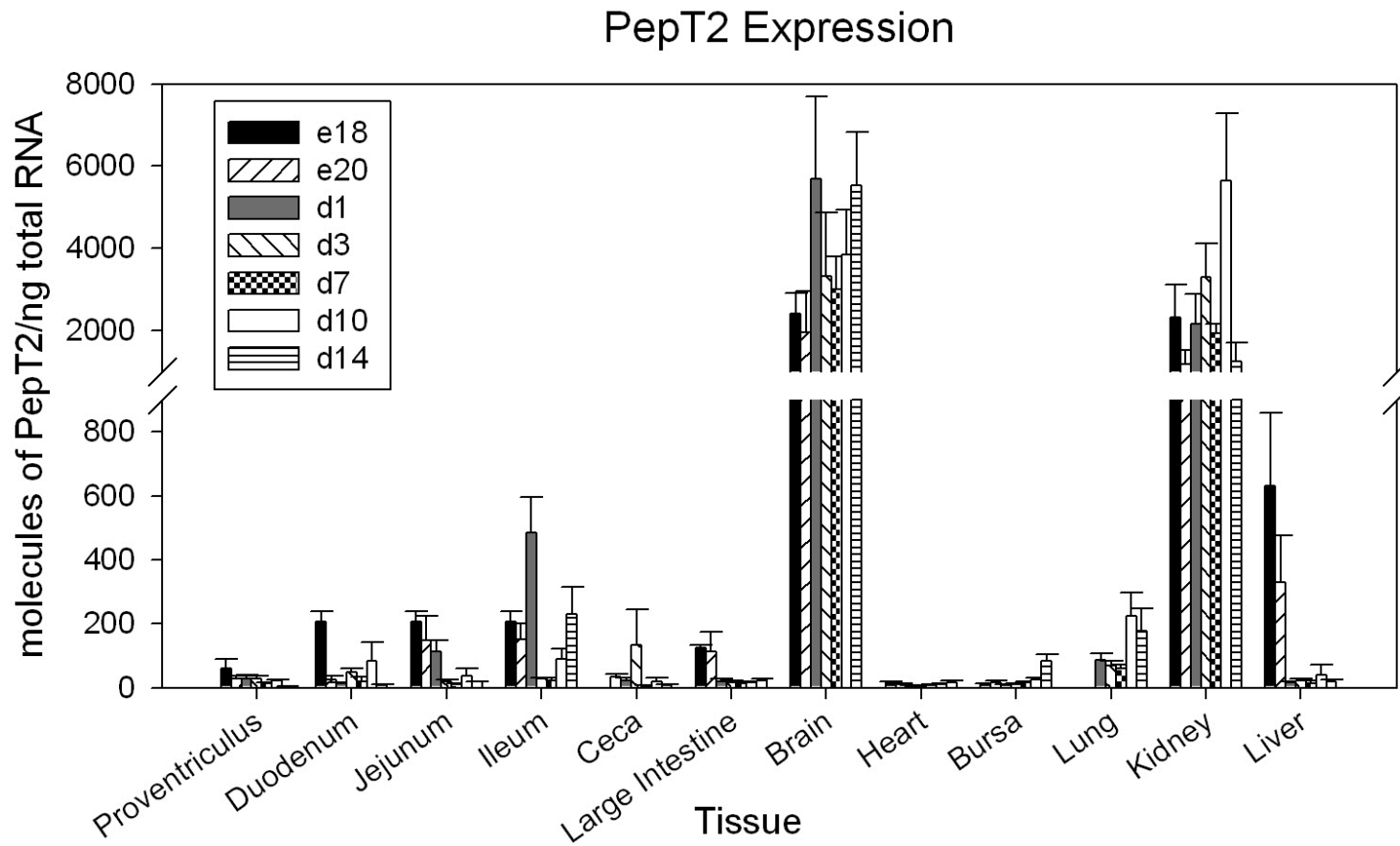
Figure 3-14. Expression of Peptide Transporter 2 (PepT2) in developing chick tissues. Five birds were sampled at embryonic day 18 (e18), embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7 (d7), day 10 (d10), and day 14 (d14) post-hatch. Proventriculus, duodenum, jejunum, ileum, ceca, large intestine, whole brain, heart, bursa of Fabricius (bursa), lung, kidney and liver samples were collected from each bird at every day sampled, except whole intestine (as opposed to duodenum, jejunum and ileum segments) was collected at e18, lungs were not collected on e18 and e20, and only 4 samples of bursa of Fabricius and ceca were collected on e18. RNA was extracted from each sample and analyzed for PepT2 gene expression by Absolute Quantification Real Time PCR.
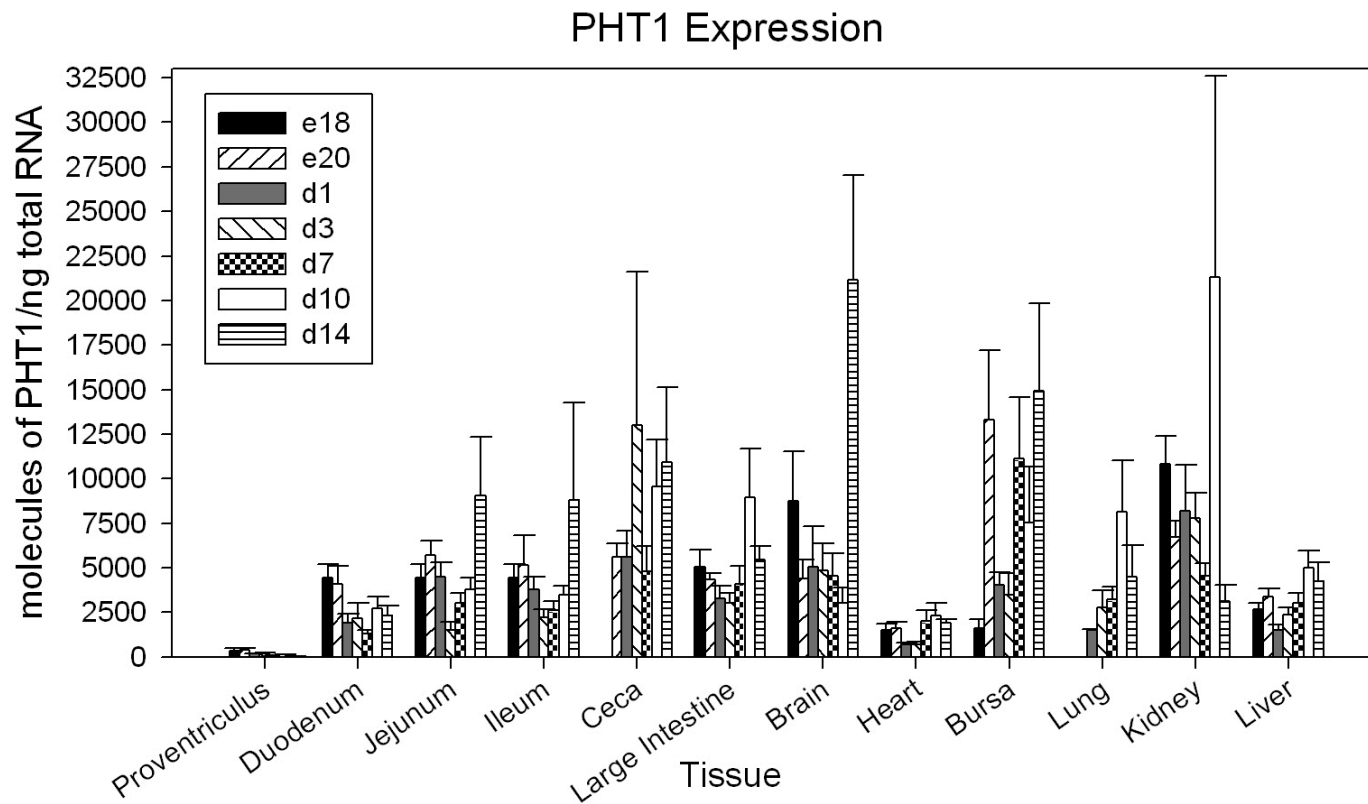
Figure 3-15. Expression of Peptide/Histidine Transporter 1 (PHT1) in developing chick tissues. Five birds were sampled at embryonic day 18 (e18), embryonic day 20 (e20), day 1 (d1), day 3 (d3), day 7 (d7), day 10 (d10), and day 14 (d14) post-hatch. Proventriculus, duodenum, jejunum, ileum, ceca, large intestine, whole brain, heart, bursa of Fabricius (bursa), lung, kidney and liver samples were collected from each bird at every day sampled, except whole intestine (as opposed to duodenum, jejunum and ileum segments) was collected at e18, lungs were not collected on e18 and e20, and only 4 samples of bursa of Fabricius and ceca were collected on e18. RNA was extracted from each sample and analyzed for PHT1 gene expression by Absolute Quantification Real Time PCR.

Table 3-1. Main Effects and Interactions of Variables within Proton-Coupled Oligopeptide Transporter Family Gene Expression

Item	PepT1	PepT2	PHT1
	Molecules of mRNA/ng total RNA[1]		
Tissue[2, 3]			
Proventriculus	3[b]	21[c]	1,961[d]
Duodenum	7,793[a]	57[c]	2,717[cd]
Jejunum	8,860[a]	79[c]	4,572[bcd]
Ileum	6,411[a]	173[c]	4,365[bcd]
Ceca	555[b]	62[c]	7,991[abc]
Large Intestine	1,245[b]	47[c]	4,883[abcd]
Brain	39[b]	3,693[a]	7,404[ab]
Heart	7[b]	9[c]	1,540[d]
Bursa of Fabricius	3[b]	34[c]	8,196[ab]
Kidney	62[b]	2,541[b]	8,935[a]
Liver	6[b]	153[c]	3,179[cd]
SEM (n=35)	570	176	919
P-value	<0.0001	<0.0001	<0.0001
Age[4]			
e18	175[c]	586	4,737[ab]
e20	534[c]	363	5,309[ab]
d1	5,864[a]	779	3,670[b]
d3	2,698[b]	638	3,915[b]
d7	1,575[bc]	460	3,833[b]
d10	1,892[bc]	894	6,268[ab]
d14	3,181[b]	653	7,491[a]
SEM (n=60)	455	140	732
P-value	<0.0001	0.1112	0.0006
Interaction			
Tissue x Age (*P*-value)	<0.0001	<0.0001	0.0002

[1] All means expressed as molecules of mRNA per nanogram of total RNA

[2] Means within a column without a common letter differ, $P < 0.05$

[3] Lung was not included in analysis, as tissue samples were not taken at e18 and e20 causing an unbalanced model

[4] Means within a column without a common letter differ, $P < 0.05$

CHAPTER IV. DISCUSSION

The Proton-Coupled Oligopeptide transporter family consists of PepT1, PepT2, PHT1 and PHT2, all of which transport di- and tri-peptides. PHT1 and PHT2 also transport histidine. These transporters are structurally similar, with 12 transmembrane proteins and an intracellular loop, but differ in sequence identity. Distribution of these transporters also differs greatly, as peptide transport is needed in different parts of the body. The proton-coupled oligopeptide transporter family has not been studied as in depth in the chicken as it has been in mammals. PepT1 has been studied the most and has been cloned from the chicken, but PepT2 and PHT1 only have computer predicted sequences. PHT2 has not even been identified in the chicken. This study investigated PepT1, PepT2 and PHT1 mRNA expression in various tissues throughout the developing chicken.

PepT1

PepT1 transports di- and tri-peptides with a low affinity and high capacity and was found in this study to be expressed the greatest within small intestinal tissue. PepT1 was expressed at much lesser quantities in the other tissues observed throughout development, at levels similar to background levels and possibly not biologically significant. Chen et al. (2002) found similar results in broiler chickens with the strongest hybridization to mRNA from the duodenum, jejunum, ileum, and less hybridization from kidney and cecum. No hybridization was observed in the liver, crop, or proventriculus.

Analysis of individual small intestinal segments showed an increase of PepT1 in all segments from e18 to d1. After d1, no difference in expression was observed in the duodenum and ileum; however, jejunum PepT1 expression peaked at d1 and decreased at

d3 to levels similar to expression in the duodenum and jejunum. Quantities of PepT1 mRNA were not different among small intestinal segments. Li et al. (2008) observed similar expression patterns in male Aviagen commercial broilers, with all small intestinal segments increasing in PepT1 mRNA expression from e18 to d14, although an extreme peak in jejunum PepT1 at d3 was not observed. Gilbert et al. (2008) also observed an increase of PepT1 mRNA from e20 to d14 within all segments of Aviagen commercial broilers. Quantities of PepT1 mRNA were greatest in the duodenum when compared to the ileum, with jejunum quantities being intermediate.

Although expression of PepT1 in the large intestine and ceca are not significantly greater than the rest of the tissues sampled, expression in these tissues may be at biologically significant levels. A larger sample size may help clarify mRNA abundance. Expression in the latter portions of the gastrointestinal tract would be advantageous in order to absorb all possible di- and tri-peptides before they are eliminated from the body. Terada et al. (2005) observed slight PepT1 expression in the human colon and rectum, however no PepT1 protein was observed in these segments. Chen et al. (1999) observed PepT1 mRNA in the rumen and omasum of sheep and dairy cows, which contribute to bacterial fermentation (Akers and Denbow, 2008)**,** similar to the ceca in chickens. Further investigation into the expression of PepT1 protein in the large intestine and ceca will provide a better characterization of PepT1 in the latter portions of the gastrointestinal tract.

Although chicken PepT1 has been observed in the small intestine, it has not been characterized on a cellular level. Whole intestine was excised from each chick and homogenized; therefore, it is unknown exactly where PepT1 resides in the chicken

intestine. Freeman et al. (1995) reported PepT1 in the epithelial mucosa, particularly in the villus of epithelial cells, which line the rat small intestine. More specifically, Hussain et al. (2002) and Ogihara et al. (1999) observed PepT1 on the apical membrane of epithelial cells but not on the basolateral membrane. Chicken PepT1 has not been specifically localized to one area of the small intestine, although similar cellular distribution would be expected. Further research needs to be done to confirm cellular distribution in the chicken.

Increase of PepT1 expression from pre- to post-hatch may be explained by the consumption of exogenous food by the chick after hatch. Observing expression of PepT1 immediately post-hatch, on day of hatch, may provide more insight into the increasing pattern of PepT1 expression post-hatch. If PepT1 expression is increased immediately after hatch, in ovo feeding may increase PepT1 expression prior to hatch for more efficient feed utilization once the chick has hatched. After hatch, the chick transitions from utilizing the yolk, which is mostly fat, to a carbohydrate and protein based feed at hatch. At hatch, enterocytes, which line the small intestine, are immature and lack a brush-border membrane, which is where PepT1 presumably resides. After hatch, enterocytes mature and villi elongate to increase surface area for absorption. Greater expression of PepT1 may occur to efficiently utilize the increased peptide load from exogenous feed consumed by the chick. PepT1 expression may also increase simply due to the structural changes in the small intestine from embryonic stages to post-hatch, as enterocyte numbers increase with maturity. PepT1 expression may not change within each enterocyte cell but increase in the overall tissue due to an increased number of enterocytes.

The significant distribution of PepT1 mRNA to only the small intestine suggests an importance, based on magnitude, of PepT1 in the small intestine. Interestingly, homozygous PepT1 null mice were healthy and physically similar in body weight and development when compared to wild-type mice (Hu et al., 2008). This questions the importance of PepT1 in the mouse, as no upregulation of other peptide or amino acid transporters were observed to compensate even though increased plasma amino acid levels were observed. However, Daniel (2004) suggested peptides are absorbed faster and more efficiently than free amino acids in the small intestine. In the chicken, Gilbert et al. (2007) found an abundance of amino acid transporters in the distal segments of the small intestine. If these amino acid transporters function at the same rate, regardless of PepT1 expression, perhaps PepT1 expression in the small intestine is not essential, but when expressed, utilizes protein-containing diets more efficiently. Homozygous PepT1 null chickens would characterize the importance of PepT1 within the chicken.

In the kidney, Smith et al. (1998) observed PepT2 in the rat nephron which functions to reabsorb peptides that have been filtered out of the body into the urine. PepT1 serves as the first, high capacity transporter of peptides to keep them in the body for utilization. Although PepT1 mRNA was minimally observed in the developing chicken, it does not mean that PepT1 protein is not present or that PepT1 expression is not expressed later in development. Overall, protein expression must be observed throughout the chicken to confirm functionality of PepT1 throughout the chick.

PepT2

In this study, greatest expression of PepT2 was observed in the brain, with lesser expression in the kidney and little to no expression in the gastrointestinal tract, lung,

heart, bursa of Fabricius and liver. Low expression in tissues may not be biologically significant as expression levels were barely detectable regardless of age. Expression at such low levels may illustrate the sensitivity of quantitative PCR, as any contamination could amplify and provide a false positive result. In the situation where the change in expression over time is, in fact, real, PepT2 may function as an embryonic transporter in the proventriculus, duodenum, jejunum and liver. Expression of PepT2 in each of these tissues declines post-hatch. Perhaps PepT2 functions as an important peptide transporter in the embryonic gastrointestinal system before PepT1 is expressed. Further research needs to be conducted for PepT2 expression prior to e18 in the developing chick in order to properly assess PepT2 expression prior to hatch. Conversely, expression of PepT2 increased in the bursa of Fabricius at d14. Rapid bursal growth occurs from hatch to 4 weeks of age (Whittow, 2000) and PepT2 may provide peptides to the developing tissue. Many small peptides have been observed that can modulate the immune system and have been used in vaccines and as treatments for immune deficiencies and cancer (St Georgiev, 1990). PepT2 may transport these small peptides in the chicken shortly after hatch. Further investigation into the substrates of PepT2 and spatial distribution of PepT2 mRNA in the bursa will help characterize bursal PepT2 function.

In this study, PepT2 was expressed greatest in the brain. Likewise, PepT2 was observed widely distributed throughout the rat brain by Berger and Hediger (1999), particularly in astrocytes, subependymal cells and ependymal cells. Expression of PepT2 in the mammalian brain aids in the homeostasis of neuropeptides at the blood-cerebrospinal fluid barrier. Similar function would be expected of PepT2 in kidney and brain in the chick; however, function of chicken PepT2 needs to be further investigated.

PepT2 was also expressed at high levels in the kidney, second only to expression in the brain when compared to all other tissues sampled. These high expression levels agree with mammalian distribution of PepT2. Shen et al. (1999) observed PepT2 in the rat kidney, particularly in the apical membrane of the nephron. More specifically, PepT2 is situated on the apical membrane in the S2 and S3 segments of the nephron. It is advantageously situated downstream of PepT1 in order to effectively reabsorb peptides that are in the urine. Although PepT2 would be expected to serve the same functional purpose in the chicken kidney, further research needs to be conducted in order to confirm cellular distribution and function.

PHT1

Expression of PHT1 has been found in immune cells, such as dendritic cells, activated macrophages and B-cells (Sasawatari et al., 2011). This supports the wide range of distribution found in mammals. Herrera-Ruiz et al. (2001) observed PHT1 mRNA by using a Southern blot of RT-PCR products in rat stomach, small intestine, ceca and colon and in human heart, kidney, leukocytes, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, testis and thymus. The wide distribution of PHT1 expression observed in mammals is similar to the wide distribution observed in chickens found in this study. Such wide distribution questions distribution of PHT1 expression within each of these tissues. Expression on the membrane of the lysosome, and not on the apical membrane of the cell, is apparent due to a N-terminal di-leucine-based motif within the protein structure. However, lysosomes are found in more than just immune cells; therefore, further investigation into the cellular localization of PHT1 within tissues is needed to clarify the function and purpose of PHT1.

Expression of PHT1 has not been reported in the chicken; therefore, this study serves as a basis of mRNA distribution of PHT1 in the chick. In this study, PHT1 expression levels in tissues and through development were not as variable as PepT1 and PepT2 expression. PHT1 was observed in all tissues analyzed. PHT1 expression increased in liver, brain, large intestine and jejunum post-hatch, suggesting an increase in expression due to maturation of the tissue. Regulation of PHT1 needs to be further examined as an increase with age may be due to the influence of growth factors on PHT1 expression. Further research needs to focus on the function of PHT1, as tissues may need its transport function as they grow and develop.

PHT1 has not been studied as nearly in depth as PepT1 and PepT2 and therefore less information is available for comparison and characterization, both in the mammal and in the chicken. PHT1 also needs to be further investigated to understand the function and cellular distribution of PHT1, particularly in the chicken. In depth investigation into PHT1 expression in the spleen, bursa of Fabricius, where B cells are produced, and the thymus, where T cells are produced, would lead to an increased understanding of PHT1 in the immune cells in the chick.

Chapter V. EPILOGUE

The results from this study indicate PepT1 and PepT2 are distributed in different tissues throughout the developing chick, with PepT1 highly expressed in the small intestine and PepT2 in the brain and kidney. On the other hand, PHT1 is widely distributed throughout the chick. These results in the chicken follow the same pattern as seen in mammals. The developmental profiles of PepT1, PepT2 and PHT1 vary for different tissues.

It is important to note that only mRNA levels were examined in this study and not protein levels. Further research into the protein distribution among tissues and throughout development would create a more complete picture of peptide transporter function in the chicken, as mRNA expression does not always reflect protein expression or function. This is a comprehensive study and the anticipation would be that the protein distribution and function would be observed in a similar pattern to mammalian expression. Gene expression with very low mRNA levels detected by real-time PCR may not be biologically significant.

It is important to realize that although replicates for each time point were taken, the variation between chicks may be substantial. The rapid growth of chick embryos in the last 2-3 days of incubation is astonishing. Many factors can cause differences when a chick will hatch, and whether a chick will hatch early or late can cause great variations in its embryonic development at e18 and e20. Likewise, when measuring expression immediately post-hatch, differences in hatch time and access to feed and water may play important roles in subsequent gene expression. One solution to this would be to attain a larger sample size in order to compensate for any fast or slow developing chicks. In this

study, a sample size of 5, and in some cases 4, was used and may not have compensated for the variation in development within each day.

Knowledge of the distribution and function of peptide transporters may be key in designing orally ingested pharmaceuticals. Designing a peptide-like drug to effectively utilize initial absorption by PepT1 in the small intestine coupled with the reabsorption in the kidney by PepT2 would be an effective route of treatment.

Although this study shows a comprehensive distribution of PepT1, PepT2 and PHT1 throughout the young chick, future research in the function and cellular distribution of transporters, particularly PepT2 and PHT1 would provide greater insight into peptide utilization in the whole chick. In-situ hybridization should be performed to complement the mRNA quantification of this study and spatially observe mRNA distribution within tissues. Protein expression should be observed by western blot to confirm the presence and quantity of a functional protein in specific tissues and immunohistochemisty should be performed in order to localize proteins within tissues. Comparison of mRNA and protein distribution and expression will create a much more complete picture of these genes. The best indicator of the function of a gene would be to create a knock-out animal lacking that particular gene. Although challenging, a homozygous null PepT1, PepT2 or PHT1 chicken would provide great insight into the true function of the role the gene plays in the chicken as a whole.

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