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Modulation Of CNS Neurotransmitter Levels And Associated Behaviors In Organic Anion Transporter 1 (Slc22a6) And Organic Anion Transporter 3 (Slc22a8) Knockout Mice

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MODULATION OF CNS NEUROTRANSMITTER LEVELS AND ASSOCIATED BEHAVIORS IN ORGANIC ANION TRANSPORTER 1 (Slc22a6) AND ORGANIC ANION TRANSPORTER 3 (Slc22a8) KNOCKOUT MICE

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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LIST OF ABBREVIATIONS

5-HT: Serotonin

5-HIAA: 5-Hydroxy-3-Indoleacetic Acid

ARBEC: Adult Rat Brain Endothelial Cells

BBB: Blood Brain Barrier

BCSFB: Blood Cerebrospinal Fluid Barrier

cDNA: Complementary Deoxyribonucleic Acid

CHO: Chinese Hamster Ovary

CNS: Central Nervous System

COS-7: Green Monkey Kidney Cell Line

CP: Choroid Plexus

CSF: Cerebrospinal Fluid

DA: Dopamine

DAT: Dopamine Transporter

DOPAC: 3,4-dihydroxyphenylacetic Acid

ECD: Electrochemical Detection

GA: L-glutamic Acid

GABA: γ-Aminobutyric Acid

GC: Gas Chromatography

GC-MS/MS: Gas Chromatography Tandem Mass Spectrometry

GFP: Green Fluorescent Protein

HEK293: Human Embryonic Kidney-293

HPLC: High Performance Liquid Chromatography

HPLC-ECD: High Performance Liquid Chromatography with Electrochemical Detection

HRPE: Human Retinal Pigment Epithelium

HVA: Homovanillic Acid

IS: Internal Standard

KYNA: Kynurenic Acid

LD: Light Dark

LLOQ: Lower Limit of Quantitation

LOD: Limit of Detection

LTM: Low Thermal Mass

MATE: Multidrug and toxin extrusion transporter

MB: Marble Burying

MeOH: Methanol

MPP^+: 1-Methyl-4-Phenylpyridinium

MRM: Multiple Reaction Monitoring

mRNA: Messenger Ribonucleic Acid

MS: Mass Spectrometry

NA: Nicotinic Acid

NE: Norepinephrine

NET: Norepinephrine Transporter

NT: Neurotransmitter(s)

OA: Organic Anion

OAT: Organic Anion Transporter

OATP: Organic Anion Transporting Polypeptide

OCT: Organic Cation Transporter

OCTN: Novel Organic Cation Transporter

OFA: Open Field Acitivity

PCR: Polymerase Chain Reaction

QA: Quinolinic Acid

QC: Quality Controls

S2: Second Segment of Proximal Tubule Cells

SD: Standard Deviation

SERT: Serotonin Transporter

SLC: Solute Carrier

SNRI: Serotonin-Norepinephrine Reuptake Inhibitors

SSRI: Selective Serotonin Reuptake Inhibitors

TEA: Triethylamine

TST: Tail Suspension Test

URAT: Urate Transporter

UV: Ultraviolet Detection

WT: Wild-Type

XA: Xanthurenic Acid

ABSTRACT

MODULATION OF CNS NEUROTRANSMITTER LEVELS AND ASSOCIATED BEHAVIORS IN ORGANIC ANION TRANSPORTER 1 (SLC22A6) AND ORGANIC ANION TRANSPORTER 3 (SLC22A8) KNOCKOUT MICE

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According to the World Health Organization, mental disorders represent the leading cause of disability in the US generating ~58 billion dollars in medical costs annually. Additionally, among the US population, ~40 million adults suffer from an anxiety disorder and ~14 million suffer from a major depressive disorder. The association between the persistence of these neurobehavioral conditions and central nervous system (CNS) levels of biogenic amines and metabolites has been studied for half a century. Further, a number of drugs interfering with neurotransmission/metabolism are used clinically for treatment of these disorders. Recently,

some members of the solute carrier (SLC) superfamily, the SLC22 transporter family, which includes organic anion transporters (Oat1, Oat3), were found to be expressed and functional on the apical membrane of the choroid plexus, a component of the blood-cerebrospinal fluid barrier. The cells of this epithelia form tight junctions, which slows penetration of solutes into the brain and limits passive efflux of endogenous solutes from the brain. Therefore, Oat1 and Oat3 are poised to play an active role in the removal of NTs and metabolites from the CSF. Thus, a better understanding of the underlying roles of OATs in regulating CNS neurotransmitters and connecting their activity to complex behaviors may result in improved understanding of the processes governing CNS homeostasis.

Basal locomotor, anxiety-like and depressive-like behaviors in mice of three genotypes (WT, Oat1$^{-/-}$, and Oat3$^{-/-}$) across ages (3-18 mo.) were evaluated using behavioral paradigms (e.g. open field activity (OFA), light-dark (LD), marble burying (MB), and tail suspension test (TST)). Secondly, a simple high performance liquid chromatography-ultraviolet/electrochemical detection (HPLC-UV/ECD) method was developed for quantitation of monoamines and metabolites in mouse whole brain. Following completion of behavioral assessments, whole brain concentrations of monoamines and metabolites were determined using the developed method. Lastly, a novel gas chromatography tandem mass spectrometry (GC-MS/MS) method was developed for quantitation of amino acid neurotransmitters, L-glutamic acid (GA) and γ-aminobutyric acid (GABA), in mouse whole brain. The developed method was used for measurement of whole brain concentrations of GA and GABA in a small subset of WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ mice at 3 and 18 mo.

CHAPTER 1

EXPRESSION AND FUNCTION OF ORGANIC CATION AND ANION TRANSPORTERS (SLC22) IN THE CNS

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1. A. INTRODUCTION

The central nervous system is well insulated from changes in the systemic circulation. This compartmental isolation is achieved by anatomical (*e.g.*, tight junctions) and biochemical (*e.g.*, transporter proteins) mechanisms that exist in the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The endothelial cells of the brain capillaries comprise the BBB, while the BCSFB is comprised of the choroid plexus (CP) epithelia along with the arachnoid villi and circumventricular organs. These tissues regulate the brain penetration and clearance of endogenous (*e.g.*, neurotransmitters or metabolic waste-products) and xenobiotic (*e.g.*, drugs or toxins/toxicants) substances, effectively controlling the composition of the extracellular fluid of the brain (interstitial fluid and CSF) and preserving the CNS milieu. Thus, identification of the transporters present in these barriers, and the properties that govern their function, is fundamental to understanding the maintenance of brain homeostasis and the basis for some disorders involving its perturbation.

The physiological processes governing the movement of charged organic substances across epithelial and endothelial cell sheets have been studied for hundreds of years. By the late

1980's and early 1990's detailed models emerged summarizing the complex interplay between cellular energy, sodium, potassium and proton (H^+) gradients, membrane potential, and dicarboxylate coupled transport that characterize the 'classical' renal organic cation and anion transport systems. Subsequently, the latter half of the 1990's saw the cloning of the first 'renal' organic cation transporters (OCTs) and organic anion transporters (OATs). Detailed investigations using a variety of comparative organ models including renal slices, renal basolateral and brush border membrane vesicle preparations, isolated mammalian and killifish renal tubules, and intact rodent CP and brain capillaries, demonstrated that the physiological characteristics of organic cation and anion transport across each of these tissues was similar (Pritchard and Miller, 1993). It is now confirmed that 'renal' OCTs and OATs are expressed and function in barrier tissues throughout the body including small intestine, liver, placenta, CP and brain capillaries. The OCTs and OATs are classified within the Solute Carrier (SLC) superfamily of transporters, which currently contains over 450 identified transporter proteins divided into ~52 transporter families. OCTs and OATs belong to the same SLC family, family 22 (SLC22), the organic cation/anion/zwitterion transporters.

The purpose of this review is to present an overview of the current knowledge regarding OCT and OAT expression and function within the CNS. A brief introduction of the SLC22 family transporters presently thought to be relevant to brain capillary (BBB) and CP (BCSFB) function is provided, however, for an in-depth accounting of the original cloning, identification and characterization of members of the SLC22 transporter family the reader is referred to several recent reviews covering this aspect (Wright and Dantzler, 2004; Koepsell et al., 2007; VanWert et al., 2010). Perspective is given on how detailed investigation of active

renal organic solute transport fed our current understanding of CNS handling of organic cations and anions. The biochemical and functional evidence supporting OCT and OAT expression in the CNS compartment is summarized.

1. B. ORGANIC CATION AND ANION TRANSPORTERS (SLC22) RELEVANT TO THE CNS

Efficient neurotransmission requires the rapid removal of released neurotransmitters from the synaptic cleft in order to terminate neuronal signaling and prepare for the next event. For monoaminergic signaling, it is well established that members of the SLC6 transporter family expressed in the brain, namely the norepinephrine (SLC6A2), dopamine (SLC6A3) and serotonin (SLC6A4) transporters, represent Na^+-dependent, high affinity (low capacity) neuronal uptake pathways to clear released monoamine neurotransmitters (Pacholczyk et al., 1991; Giros and Caron, 1993; Lesch et al., 1993; Kristensen et al., 2011). However, Na^+ independent transport of choline and catecholamines has been reported in brain/glial cells (Yamamura and Snyder, 1973; Friedgen et al., 1996; Russ et al., 1996; Streich et al., 1996). Now, a growing body of literature strongly supports the involvement of OCTs and OATs (SLC22) expressed in the CNS in the extraneuronal Na^+-independent, low affinity (high capacity) clearance of neurotransmitters and their metabolites from the interstitial fluid/CSF and, thus, contributing to the termination of neurotransmission and the maintenance of CNS homeostasis.

In vitro, cloned OCTs have been demonstrated to interact with an array of structurally diverse compounds including endogenous amines that function in the CNS (*i.e.*, choline, dopamine, epinephrine, histamine, norepinephrine, and serotonin) and therapeutic

agents that target the CNS (*i.e.*, amphetamine, desipramine, fluoxetine, morphine, sertraline), raising the possibility that these transporters might function in the CNS (Table 1.1 and Table 1.2) (Busch et al., 1996a; Busch et al., 1996b; Martel et al., 1996; Gorboulev et al., 1997; Zhang et al., 1997; Breidert et al., 1998; Grundemann et al., 1998; Kekuda et al., 1998; Wu et al., 1998a; Wu et al., 1998b; Murakami et al., 2000; Sweet et al., 2001; Amphoux et al., 2006; Zhu et al., 2012). In addition, some OATs were shown to interact with anionic catecholamine metabolites *in vitro*, including 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-methoxy-3-indoleacetic acid, 3-methoxy-4-hydroxymandelic acid, 4-hydroxy-3-methoxymandelic acid and 3,4- dihydroxymandelic acid (Table 1.3) (Kusuhara et al., 1999; Sekine et al., 2000; Alebouyeh et al., 2003; Ohtsuki et al., 2003; Mori et al., 2004). Expression of OCTs and OATs has been detected in mouse, rat and human brain, consistent with their having a role in the disposition of these compounds in the CNS (Sweet et al., 2001; Sweet et al., 2002). Furthermore, blockade of their transport function correlates with increased monoamines and/or their metabolites in the CNS compartment, *e.g.*, peripheral administration of probenecid (a potent inhibitor of OATs) resulted in increased HVA and 5-HIAA levels in the CSF of mice, rats and humans (Roffler-Tarlov et al., 1971; Emanuelsson et al., 1987a; Emanuelsson et al., 1987b).

Table 1.1. Interaction of monoamine neurotransmitters with organic cation transporters (SLC22).

Compound	Transporter	Interaction	System	Kinetics (mM)			Reference
				Km	Ki	IC50	
Dopamine	rOct1	S	HEK293	1.60±0.30			Amphoux *et al* 2006
		S	*X. laevis*	0.05			Koepsell 1998
		S	HEK293		1.10		Breidert *et al* 1998
		S	*X. laevis*	0.05±0.01			Busch *et al* 1996
	rOct2	S	HEK293	2.10±0.20			Amphoux *et al* 2006
		S	HEK293	2.10			Grundemann *et al* 1998
		S	*X. laevis*	0.65			Koepsell 1998
		I	HRPE			2.3±0.2	Wu *et al* 1998
	hOCT2	S	HEK293	1.40±0.20			Amphoux *et al* 2006
		S	HEK293	0.3±0.16			Busch *et al* 1998
	rOct3	S	HEK293	1.50±0.20			Amphoux *et al* 2006
		I	HRPE			0.62±0.04	Wu *et al* 1998
	hOCT3	S	HEK293	0.8			Zhu *et al* 2012
		S	HEK293	1.03±0.13			Duan & Wang 2010
Histamine	rOct1	I	HEK293		1.4		Grundemann *et al* 1999
	rOct2	S	HEK293	0.89±0.14			Amphoux *et al* 2006
		S	HEK293	0.54	0.39		Grundemann *et al* 1999
	rbOct2	I	CHO			0.43±0.01	Suhre *et al* 2005
	hOCT2	S	HEK293	0.94±0.15			Amphoux *et al* 2006
		S	HEK293	1.3±0.3			Busch *et al* 1998
		I	CHO			3.3±0.05	Suhre *et al* 2005
	rOct3	S	HEK293	0.54±0.09			Amphoux *et al* 2006
	hOCT3	S	HEK293	0.22±0.06			Amphoux *et al* 2006
		S	HEK293	0.18	0.14		Grundemann *et al* 1999
		S	HEK293	0.64±0.24			Duan & Wang 2010
Norepinephrine	rOct1	S	HEK293	0.80±0.25			Amphoux *et al* 2006
		S	HEK293		2.80		Breidert *et al* 1998
	rOct2	S	HEK293	2.10±0.40			Amphoux *et al* 2006

Compound	Transporter	Interaction	System	Kinetics (mM)			Reference
				Km	Ki	IC50	
Norepinephrine		I	HRPE			1.1±0.3	Wu *et al* 1998
	hOCT2	S	HEK293	1.50±0.10			Amphoux *et al* 2006
		S	HEK293	1.9±0.6			Busch *et al* 1998
	rOct3	S	HEK293	1.90±0.38			Amphoux *et al* 2006
		S	HRPE	0.4			Wu *et al* 1998
	hOCT3	S	HEK293	2.63±0.08			Amphoux *et al* 2006
		S	HEK293	1.03			Zhu *et al* 2012
		S	HEK293	0.92±0.17			Duan & Wang 2010
		S	HEK293	0.51			Grundemann *et al* 1998
Serotonin	rOct1	S	HEK293	0.90±0.50			Amphoux *et al* 2006
		S	HEK293		0.65		Breidert *et al* 1998
	rOct2	S	HEK293	0.76±0.05			Amphoux *et al* 2006
	rbOct2	I	CHO			0.66±0.04	Suhre *et al* 2005
	hOCT2	S	HEK293	0.29±0.06			Amphoux *et al* 2006
		S	HEK293	0.08±0.02			Busch *et al* 1998
		I	CHO			0.31±0.02	Suhre *et al* 2005
	rOct3	S	HEK293	00.50±0.10			Amphoux *et al* 2006
		I	HRPE			0.97±0.18	Wu *et al* 1998
	hOCT3	S	HEK293	0.9			Zhu *et al* 2012
		S	HEK293	0.99±0.26			Duan & Wang 2010

[a]h, human; I, inhibitor; m, mouse; r, rat; S, substrate

Table 1.2. Inhibition of organic cation transporters by selective serotonin reuptake inhibitors (SSRIs).

Compound	Transporter	Interaction	System	Kinetics (mM)			Reference
				Km	Ki	IC50	
Desipramine	rOct1	I	*X. laevis*		0.003		Grundemann *et al* 1994
	hOCT1	I	HEK293			0.002	Haenisch *et al* 2012
		I	HeLa		0.005		Zhang *et al* 1998
	hOCT2	I	HEK293			0.075	Haenisch *et al* 2012
		I	*X. laevis*		0.016		Gorboulev *et al* 1997
	rOct3	I	HRPE			0.06	Kekuda *et al* 1998; Wu *et al* 1998
	hOCT3	I	HEK293			0.072	Haenisch *et al* 2012
		I	HEK293			0.005	Zhu *et al* 2012
		I	HRPE		0.014		Wu *et al* 2000
Fluoxetine	hOCT1	I	HEK293			0.003	Haenisch *et al* 2012
	hOCT2	I	HEK293			0.029	Haenisch *et al* 2012
	hOCT3	I	HEK293			0.038	Haenisch *et al* 2012
		I	HEK293			0.022	Zhu *et al* 2012
Imipramine	hOCT1	I	HEK293			0.018	Haenisch *et al* 2012
	hOCT2	I	HEK293			0.015	Haenisch *et al* 2012
	hOCT3	I	HEK293			0.054	Haenisch *et al* 2012
		I	HEK293			0.011	Zhu *et al* 2012
		I	HRPE		0.042		Wu *et al* 2000
Sertraline	hOCT1	I	HEK293			0.009	Haenisch *et al* 2012
	hOCT2	I	HEK293			0.026	Haenisch *et al* 2012

Compound	Transporter	Interaction	System	Kinetics (mM)			Reference
				Km	Ki	IC50	
Sertraline	hOCT3	I	HEK293			0.026	Haenisch *et al* 2012
		I	HEK293			0.007	Zhu *et al* 2012

[a]h, human; m, mouse; r, rat

Table 1.3. Inhibition of organic anion transporters by neurotransmitter metabolites.

Substrate/Inhibitor	Transporter	Interaction	System	Kinetics (mM)			Reference
				Km	Ki	IC50	
DOPAC	hOAT1	I	S2			0.56±0.09	Alebouyeh *et al* 2003
	hOAT3	I	S2			0.99±0.10	Alebouyeh *et al* 2003
5-HIAA	hOAT1	I	S2			0.11±0.02	Alebouyeh *et al* 2003
	hOAT3	I	S2			0.91±0.03	Alebouyeh *et al* 2003
HVA	hOAT1	I	S2			0.07±0.01	Alebouyeh *et al* 2003
	rOat3	S	*X. laevis*	0.27±0.1			Mori *et al* 2003
	hOAT3	I	S2			0.76±0.09	Alebouyeh *et al* 2003
Kynurenic Acid	mOat1	I	COS-7			0.034	Bahn *et al* 2005
	hOAT1	S	*X. laevis*	0.005			Uwai *et al* 2012
	mOat3	I	COS-7			0.008	Bahn *et al* 2005
	hOAT3	S	*X. laevis*	0.005			Uwai *et al* 2012
Quinolinic Acid	mOat1	I	COS-7				Bahn *et al* 2005
	mOat3	I	COS-7				Bahn *et al* 2005
Xanthurenic Acid	mOat1	I	COS-7			0.015	Bahn *et al* 2005
	mOat3	I	COS-7			0.011	Bahn *et al* 2005

[a]h, human; m, mouse; r, rat

Following is a brief summary of the isolation, tissue expression profile and functional characterization of OCTs and OATs known to be expressed in the CNS:

Organic cation transporter 1 (Oct1; *Slc22a1*). Cloned from rat kidney in 1994 (Grundemann et al., 1994). Known orthologs in human, mouse and rabbit (Schweifer and Barlow, 1996; Zhang et al., 1997; Terashita et al., 1998; Green et al., 1999). Oct1 mRNA was detected in intestine, kidney and liver in mouse, rabbit and rat; also in CP, skin and spleen of rat and olfactory mucosa of mouse (Grundemann et al., 1994; Gorboulev et al., 1997; Terashita et al., 1998; Green et al., 1999; Sweet et al., 2001; Slitt et al., 2002; Monte et al., 2004). In humans OCT1 expression was observed in heart, liver and skeletal muscle; lack of detection in human kidney may represent a true species difference (Gorboulev et al., 1997; Zhang et al., 1997). Membrane depolarization, but not the presence of a H^+ gradient, decreased rOct1 transport activity (Grundemann et al., 1994). Thus, mechanistically, rOct1 functions as a facilitated diffusion carrier, characteristic of the basolateral uptake step in renal proximal tubules. Subsequently, immunohistochemistry detected rOct1 signal in the basolateral membrane of renal proximal tubules and in the sinusoidal (basolateral) membrane of hepatocytes (Meyer-Wentrup et al., 1998; Urakami et al., 1998).

Oct2 (*Slc22a2*). Cloned in 1996 from rat kidney (Okuda et al., 1996; Walsh et al., 1996). Orthologs identified in mouse, man, pig and rabbit (Gorboulev et al., 1997; Grundemann et al., 1997; Mooslehner and Allen, 1999; Zhang et al., 2002). Oct2 mRNA expression was mainly observed in kidney in human, mouse and rat (Okuda et al., 1996; Gorboulev et al., 1997; Slitt et al., 2002; Alnouti et al., 2006). Oct2/OCT2 mRNA was also

detected in CP of rats and murine olfactory mucosa (Sweet et al., 2001; Monte et al., 2004). Like rOct1, rOct2-mediated transport was significantly reduced by membrane depolarization and not stimulated by a *trans*-H^+ gradient, again characteristic of the facilitated diffusion basolateral uptake step in renal proximal tubules (Walsh et al., 1996; Sweet and Pritchard, 1999). Subsequently, basolateral targeting of a rOct2-green fluorescent protein fusion construct in intact killifish renal proximal tubules and immunohistochemical staining of proximal tubule basolateral membranes in rat renal slices was observed (Karbach et al., 2000; Sugawara-Yokoo et al., 2000; Sweet et al., 2000).

Oct3 (*Slc22a3*). Isolated from rat placenta in 1998 (Kekuda et al., 1998). Orthologs identified in human and mouse (Grundemann et al., 1998; Verhaagh et al., 1999). Oct3/OCT3 mRNA expression exhibited a wide tissue distribution including adrenal gland, blood vessels, brain, CP, heart/aorta, intestine, kidney, lung, placenta, skeletal muscle and thymus (Kekuda et al., 1998; Verhaagh et al., 1999; Sweet et al., 2001; Slitt et al., 2002; Alnouti et al., 2006) . Hepatic Oct3/OCT3 message expression was strong in humans, but showed little to no expression in mouse and rat (Kekuda et al., 1998; Verhaagh et al., 1999; Slitt et al., 2002; Alnouti et al., 2006). Rat Oct3-mediated transport was reduced when the potential-difference across the cell membrane was decreased and electrophysiology confirmed electrogenic transport of substrate organic cations (Kekuda et al., 1998). Thus, rOct3 also functions as a basolateral facilitated diffusion carrier in kidney.

Organic cation/carnitine transporter (Octn1; *Slc22a4*). Cloned in 1997 from human liver (Tamai et al., 1997). Orthologs identified in mouse and rat (Tamai et al., 1997; Tamai et al., 2000; Wu et al., 2000). Octn1/OCTN1 mRNA was detected in a number of tissues

including brain, CP, heart, intestine, kidney, liver, olfactory mucosa and spleen (Tamai et al., 1997; Tamai et al., 2000; Wu et al., 2000; Sweet et al., 2001; Slitt et al., 2002; Monte et al., 2004; Alnouti et al., 2006). Transport activity of all three Octn1/OCTN1 orthologs was stimulated by a *trans*-applied H^+ gradient, with little to no effect of membrane potential, suggesting it was an apical H^+/organic cation exchanger in kidney (Tamai et al., 1997; Yabuuchi et al., 1999; Tamai et al., 2000; Wu et al., 2000; Tamai et al., 2004). Subsequently, mOctn1 was immunolocalized to the apical membrane of renal proximal tubule (Tamai et al., 2004).

Octn2 (*Slc22a5*). Cloned from a human placental trophoblast cell line in 1998 (Wu et al., 1998b). Mouse and rat orthologs have been isolated (Tamai et al., 1998; Wu et al., 1998b; Nezu et al., 1999; Wu et al., 1999). Octn2/OCTN2 mRNA was reported in a variety of tissues including brain (cerebellum, cortex, and hippocampus), CP, heart, intestine, kidney, liver, placenta, and testis common to all three species (Tamai et al., 1998; Wu et al., 1998b; Wu et al., 1999; Tamai et al., 2000; Sweet et al., 2001; Slitt et al., 2002; Alnouti et al., 2006). Expression also was seen in murine olfactory mucosa (Monte et al., 2004). Initial characterization showed Octn2/OCTN2- mediated transport was pH-dependent, but Na^+-independent, suggesting an apical membrane localization (Wu et al., 1998b; Tamai et al., 2000). Immunohistochemistry/immunoblotting confirmed mOctn2 protein expression in astrocytes and in renal brush border membrane vesicles and rOctn2 protein in astrocytes and neurons from adult rat cerebral cortex (Januszewicz et al., 2009; Cano et al., 2010; Januszewicz et al., 2010).

Octn3 (*Slc22a21*). Identified in mouse in 2000 (Tamai et al., 2000). Murine Octn3 message was found in kidney, olfactory mucosa, small intestine and testis (Tamai et al., 2000; Monte et al., 2004; Duran et al., 2005; Alnouti et al., 2006). Na^+-independent carnitine transport was observed for mOctn3 (Tamai et al., 2000) . Expression of mOctn3 protein in kidney, peroxisomes and testis was detected by Western blot and visualized directly in the apical membrane of proximal tubule cells in tissues sections (Tamai et al., 2000; Lamhonwah et al., 2003; Lamhonwah et al., 2005; Cano et al., 2010). Immunohistochemistry confirmed rOctn3 protein was expressed in astrocytes and neurons from adult rat cerebral cortex (Januszewicz et al., 2010). Immunoblotting detected putative hOCTN3 protein in HepG2 cells and peroxisomes (Lamhonwah et al., 2003; Lamhonwah et al., 2005).

Organic anion transporter 1 (Oat1; *Slc22a6*). Isolated in 1997 from rat kidney (Sekine et al., 1997; Sweet et al., 1997). Orthologs known in *C. elegans,* flounder, human, mouse, pig and rabbit (Lopez-Nieto et al., 1997; Wolff et al., 1997; Cihlar et al., 1999; George et al., 1999; Hosoyamada et al., 1999; Race et al., 1999; Bahn et al., 2002; Hagos et al., 2002). Oat1 mRNA was strongly expressed in CP, kidney and olfactory mucosa of mouse and rat (Pacholczyk et al., 1991; Pritchard et al., 1999; Sweet et al., 2002; Monte et al., 2004). Rat Oat1 transport activity was dose-dependently *cis*-inhibited and *trans*-stimulated by the dicarboxylate, glutarate (Sweet et al., 1997). This was consistent with it functioning as an organic anion/dicarboxylate exchanger, the final step in the proposed tertiary active transport system for basolateral renal organic anion uptake (Shimada et al., 1987; Pritchard, 1988). Accordingly, a rOat1-green fluorescent protein fusion construct was targeted to the basolateral membranes of intact renal tubules (Sweet et al., 1999). Subsequently,

basolateral targeting was observed in both human and rat kidneys by immunohistochemistry (Kojima et al., 2002; Motohashi et al., 2002; Cheng et al., 2012).

Oat2 (*Slc22a7*). Originally cloned from rat liver in 1994, however no transport activity was identified (Simonson et al., 1994). In 1998, organic anion transport activity was demonstrated (Sekine et al., 1998). Mouse and human orthologs are known (Enomoto et al., 2002; Kobayashi et al., 2002b). Renal and hepatic mRNA expression was detected in human, mouse and rat; expression in CP was demonstrated for mouse and rat (Sekine et al., 1998; Sun et al., 2001; Buist et al., 2002; Enomoto et al., 2002; Kobayashi et al., 2002a; Kobayashi et al., 2002b; Sweet et al., 2002). Initial membrane localization studies generated species-specific results, with renal rOat2 localized apically in the medullary thick ascending limb and cortical collecting ducts, but hOAT2 targeted to the basolateral proximal tubule membrane (Enomoto et al., 2002; Kojima et al., 2002). Whether or not this represents a true species difference in targeting or unresolved technical issues is unclear. However, a recent report also observed hOAT2 in the basolateral domain of proximal tubules (Cheng et al., 2012).

Oat3 (*Slc22a8*). Isolated in 1999 from rat brain (Kusuhara et al., 1999). Orthologs identified in human, mouse, pig and rabbit (Cha et al., 2001; Sweet et al., 2002; Zhang et al., 2004). Oat3 mRNA was detected in brain capillaries, CP and kidney (Kusuhara et al., 1999; Cha et al., 2001; Sweet et al., 2002; Zhang et al., 2004). Low-level hepatic mRNA expression was reported in rat, but not in mouse (Buist et al., 2002; Kobayashi et al., 2002b; Sweet et al., 2002; Buist and Klaassen, 2004) . Kidneys isolated from Oat3 knockout mice exhibited loss of basolateral uptake of substrate organic anions, suggesting that mOat3-mediated uptake may be driven by organic anion/dicarboxylate exchange (Sweet et al., 2002).

Subsequent evaluation of mouse and rat Oat3 transport energetics, both in intact tissue and *in vitro* with cells co-expressing a Na^+ dicarboxylate cotransporter and Oat3, demonstrated Na^+-dependent *trans*-stimulation by glutarate indicating that it is a basolateral organic anion/dicarboxylate exchanger (Sweet et al., 2003). Hepatic transport of examined organic anions was unaffected in the knockout animals. Basolateral staining for Oat3/OAT3 in rat and human renal proximal tubules has been observed by immunohistochemistry (Kojima et al., 2002; Motohashi et al., 2002; Cheng et al., 2012).

1. C. SLC22 TRANSPORTERS: FROM KIDNEY TO BRAIN

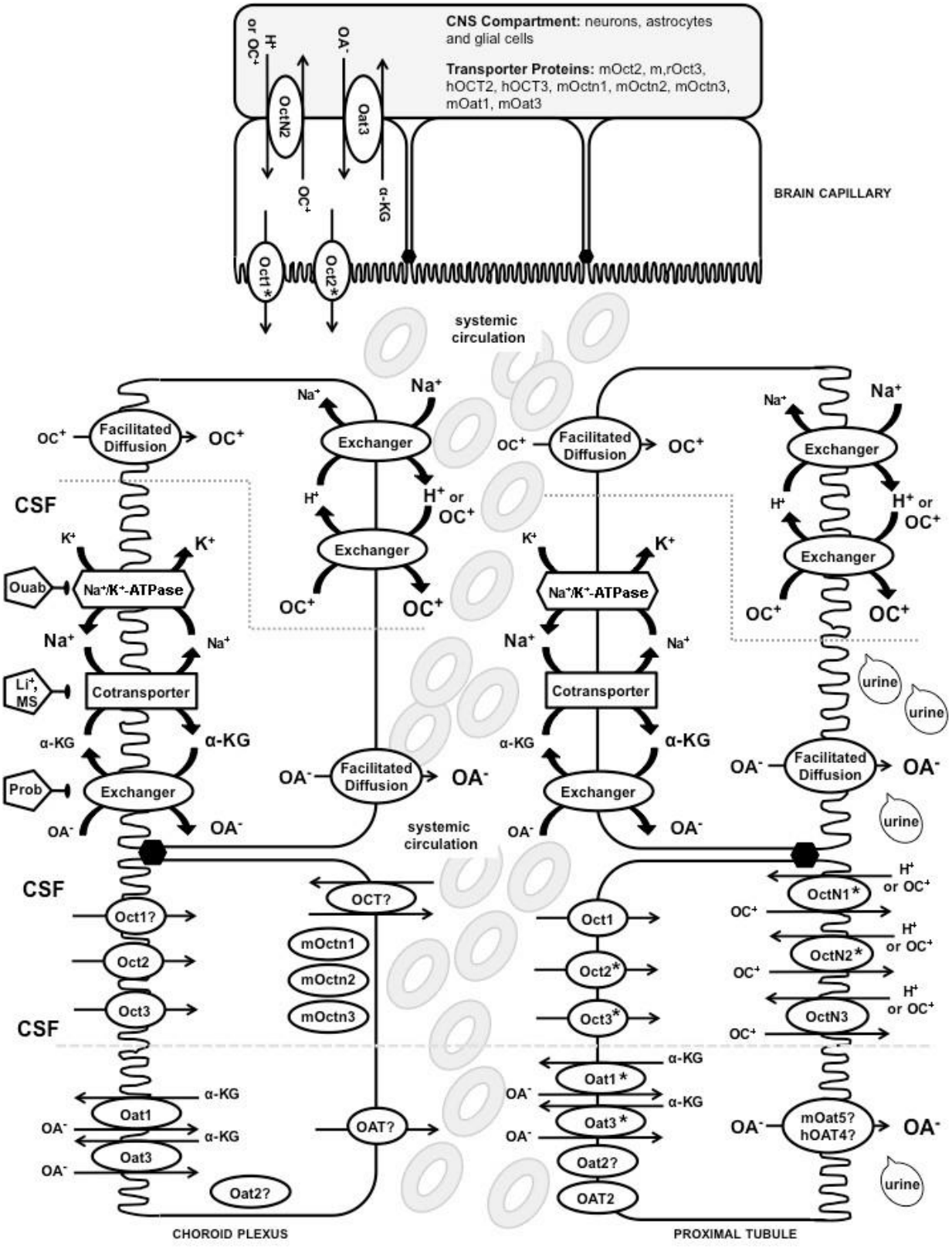
As alluded to above, by the 1980's detailed models describing the physiological characteristics of the pathways for movement of organic cations and anions across renal proximal tubule cells had been developed (Pritchard and Miller, 1993) . These models predicted the existence of proteins to mediate each step in the process, *i.e.*, transporters (Figure 1.1). For example, entry of organic cations into a renal proximal tubule cell from the blood was demonstrated to be associated with the electrochemical gradient, with the membrane potential difference and resulting inside negative resting potential providing the major driving force, which suggested the presence of a facilitated diffusion transporter (uniporter) in the basolateral membrane (Pritchard and Miller, 1993). Exit of organic cations from the cell into the urinary space is energetically uphill and, thus, coupled to an exchange mechanism, *e.g.*, for H^+, suggesting the presence of an exchanger (antiporter) in the apical membrane (Pritchard and Miller, 1993). For organic anions, it is the entry step from the blood that is energetically uphill as the inside negative resting potential must be overcome (Figure 1.1). Cellular energy input is utilized indirectly through a tertiary active process whereby (i)

the Na^+/K^+-ATPase maintains an inwardly directed Na^+ gradient (inhibited by ouabain), (ii) a Na^+/dicarboxylate cotransporter (symporter) uses the inward Na^+ gradient to drive transport of a dicarboxylate into the cell (against its electrochemical gradient; inhibited by lithium or methylsuccinate), and (iii) an exchanger utilizes the established outwardly directed dicarboxylate gradient to drive cellular entry of the substrate organic anion (inhibited by probenecid) (Shimada et al., 1987; Pritchard, 1988; Pritchard, 1990). Similar to organic cation entry, facilitated diffusion is a major mechanism driving the apical efflux of organic anions into the urine (Pritchard and Miller, 1993). Thus, cellular entry and exit of organic cations and anions was known to involve physiologically distinct processes and, presumably, distinct basolateral and apical transporter proteins for each pathway, establishing a tissue polarity of transport (Figure 1.1).

Subsequently, expression assay activity screening of rat renal cDNA libraries led to the cloning of the first OCT, organic cation transporter 1 (rOct1, slc22a1), and the first OAT, organic anion transporter 1 (rOat1, Slc22a6) (Grundemann et al., 1994; Sekine et al., 1997; Sweet et al., 1997). However, since the uptake and efflux steps utilize different physiological driving forces, simply knowing a protein mediated the transport of an organic cation or anion was insufficient to know where (basolateral or apical) to place it within the physiological model describing proximal tubule flux of organic solutes. Further characterization studies demonstrated that rOct1 was sensitive to changes in the membrane potential and, thus, represents the predicted basolateral facilitated diffusion carrier from the physiological proximal tubule model describing the organic cation transport system (Figure 1.1). Work on rOat1 found its activity was linked to an endogenous outward dicarboxylate (α-ketoglutarate) gradient and that it represents the predicted basolateral organic

anion/dicarboxylate exchanger from the physiological proximal tubule model (Figure 1.1). Of course, this was itself an oversimplification, and today there at least three known OCTs that operate via facilitated diffusion, Oct1/OCT1, Oct2/OCT2 and Oct3/OCT3, and at least three that utilize an exchange mechanism, Octn1/OCTN1, Octn2/OCTN2 and Octn3/OCTN3. Similarly, at least two OATs, Oat1/OAT1 and Oat3/OAT3, are conclusively identified as dicarboxylate exchangers, with the mechanisms driving Oat2/OAT2 transport activity still unclear.

Figure 1.1. Organic cation and anion transport systems involving SLC22 transporters in renal proximal tubule, choroid plexus and brain capillary cells. The detailed physiological models describing the energetics of movement of organic cations (OC^+) and organic anions (OA^-) from the systemic circulation to urine through renal proximal tubule cells and from CSF to the systemic circulation through choroid plexus cells are depicted. Upper renal and plexus cells depict OC^+ entry (from blood or CSF, respectively) via a membrane potential difference-sensitive facilitated diffusion mechanism and OA^- entry via a tertiary active process linking Na^+/K^+-ATPase, Na^+/dicarboxylate symport (cotransporter), and dicarboxylate/OA^- antiport (exchanger) activities. Exit of OC^+ is largely accomplished by proton (H^+)/OC^+ antiport (exchanger) linked to a Na^+/H^+ antiport (exchanger) activity. Notably, the same physiological driving forces govern the cellular entry and exit steps of these organic solutes in proximal tubule and choroid plexus, however, the components of each transport system target to the opposite sides of the cell (*i.e.,* basolateral vs. apical) establishing a reversal in the tissue polarity of organic solute transport. For brain capillary cells the basal membrane is in contact with the CNS or brain side and the apical membrane is in contact with the systemic circulation, however, less is known about the mechanisms driving transport in this tissue and the available SLC22 localization data are somewhat unclear in terms of polarity of expression and function in this tissue. Identified transporters associated with each of the physiological steps are identified (in lower cells for choroid plexus and proximal tubule). Currently, Oct1/OCT1, Oct2/OCT2 and Oct3/OCT3 are known facilitated diffusion carriers that support the cellular entry of OC^+ and Octn1/OCTN1, Octn2/OCTN2 and Octn3/OCTN3 transport OC^+ out of the cell in exchange for OC^+ (carnitine) or H^+. Oat1/OAT1 and Oat3/OAT3 are known exchangers that mediate cellular entry of OA^-. Exit of OC^+ and OA^- across the basolateral membrane of choroid plexus cells is less well understood, largely due to the difficulty in experimentally accessing this membrane, however, advances in imaging technology (both instrumentation and probes) are helping to move this area forward. Tissue accessibility has been a major obstacle to the study of brain capillary transport physiology as well. Recently, m,rOct2/hOCT2 protein expression was reported as "mainly" targeted to the apical membrane, however, there was apparent immunopositive signal associated with the basal membrane as well. Therefore, membrane targeting of these transporter orthologs in brain capillary is as yet unclear. Murine Octn1, mOctn2 and mOctn3 protein expression was detected in choroid plexus, however no specific membrane localization was reported. Human OAT2 protein localization in the basolateral membrane of renal proximal tubules has been observed, but Oat2/OAT2's mechanism of transport remains unresolved. In the figure, an asterisk (*) denotes a transporter for which protein expression in human tissue has been reported. OC^+, organic cation; OA^-, organic anion; H^+, proton; K^+, potassium; Na^+, sodium; α-KG, α-ketoglutarate; Ouab, ouabain-sensitive; Li^+, MS, Li^+- and MS-sensitive; Prob, probenecid-sensitive.



In addition to kidney, other barrier tissues, *e.g.*, intestine, liver, placenta, brain capillaries and CP, actively transport organic cations and anions. In the CNS compartment, the choroid plexuses not only produce CSF, but also actively remove organic cationic and anionic compounds from the CSF and secrete them into the systemic circulation for eventual removal by the liver and/or kidneys. Thus, two fundamental questions were, (1) are the physiological mechanisms governing organic cation and anion transport across the brain capillaries and CP the same as those in kidney and (2) if so, are the same (or related) transporters that mediate this function in kidney also responsible for this activity in the brain capillaries and CP?

1. D. CHOROID PLEXUS EPITHELIUM

Similar to kidney, *in vivo* (intraventricular administration or ventriculocisternal perfusion) and *in vitro* (isolated plexus) techniques provided physiological evidence that the CP (from cat, dog, goat, guinea pig, rabbit and rat) actively removes endogenous and xenobiotic organic cations and anions from the CSF (Pappenheimer et al., 1961; Davson and Pollay, 1963; Tochino and Schanker, 1965b; Tochino and Schanker, 1965a; Hug, 1967; Neff et al., 1967). For example, benzylpenicillin, 2,4- dichlorophenoxyacetic acid, hexamethonium, HVA (from dopamine), 5-HIAA (from serotonin), norepinephrine, *para*-aminohippurate, phenolsulfonphthalein, morphine, methotrexate, N^1- methylnicotinamide and salicylate, were all observed to accumulate in CP against their concentration gradients via saturable processes that were temperature sensitive and subject to inhibition (Pappenheimer et al., 1961; Davson and Pollay, 1963; Tochino and Schanker, 1965b; Tochino and Schanker, 1965a; Hug, 1967; Neff et al., 1967; Rubin et al., 1968; Cserr and VanDyke, 1971; Forn, 1972; Lorenzo and Spector, 1973; Pritchard, 1980). Furthermore, the peripheral administration of probenecid (a

known inhibitor of the renal organic anion transport system) blocked active efflux of 5-HIAA and HVA from rat and human brain (Neff et al., 1967; Roffler-Tarlov et al., 1971; Emanuelsson et al., 1987a; Emanuelsson et al., 1987b). Overall, it was concluded that the basic physiological characteristics of the clearance of ionized organic compounds from the CSF/brain were analogous to those identified in renal proximal tubules for clearance from blood.

Subsequently, targeted mechanistic studies were performed in CP primary cell culture, membrane vesicle and intact tissue systems to determine if the molecular energetics of CP organic cation and anion transport were the same as those identified in kidney, *e.g.*, dependence upon ion gradients or membrane potential difference (Figure 1.1). Mediated movement of the prototypical organic cation, tetraethylammonium (TEA), across the apical (ventricular) membrane of cultured rat primary CP epithelial cells was inhibited organic cations, demonstrated to be saturable, and energetically coupled to cellular metabolism (Villalobos et al., 1997; Villalobos et al., 1999). The estimated apparent K_m for apical CP TEA transport in this system was 315 µM, a value similar to that obtained for TEA transport (160-280 µM) in renal basolateral membrane vesicles (Ullrich et al., 1991; Braandle et al., 1992; Villalobos et al., 1997). When choline accumulation was investigated in this same system, movement across the apical membrane was found to be insensitive to manipulations that block tertiary active organic anion transport, namely the removal of external Na^+ or the addition of lithium chloride (Villalobos et al., 1999). However, transport was significantly reduced by depolarization of the membrane potential (characteristic of facilitated diffusion), but marginally affected by adjustments in pH, indicating a H^+ exchange mechanism was not involved (Villalobos et al., 1999).

Experiments conducted with intact rat CP *in vitro* also found that choline accumulation was insensitive to an imposed H^+ gradient, but significantly reduced by membrane depolarization (Sweet et al., 2001). Thus, movement of organic cations across the ventricular (apical) membrane of CP (CSF to blood direction) was mechanistically consistent with basolateral organic cation uptake in renal proximal tubule cells (blood to urine direction).

The mechanisms driving apical organic anion transport were investigated in bovine and rat CP (Pritchard et al., 1999). Accumulation of the organic anion, 2,4-dichlorophenoxyacetic acid, by intact bovine CP and bovine CP brush-border membrane vesicles, was stimulated in the presence of dicarboxylate (glutarate). However, this *cis*-stimulation by dicarboxylate was only observed in the presence of Na^+ and was blocked by lithium or methylsuccinate (Pritchard et al., 1999). Further, when the membrane vesicles were preloaded with glutarate, a significant *trans*-stimulation of 2,4- dichlorophenoxyacetic acid uptake, which was insensitive to Na^+ depletion or inhibition by lithium or methylsuccinate, but sensitive to probenecid (OAT) inhibition, was observed (Figure 1.1). Thus, when dicarboxylate (glutarate) was applied externally, inhibition of the Na^+/dicarboxylate cotransporter by either removal of the Na^+ gradient driving force or application of the inhibitors lithium or methylsuccinate, prevented transport of dicarboxylate into the vesicles for subsequent use in organic anion/dicarboxylate exchange (Pritchard et al., 1999). If the need for Na^+/dicarboxylate cotransporter activity was bypassed, *i.e.*, by preloading the vesicles with glutarate, stimulation of organic anion uptake that was insensitive to inhibition by lithium or methylsuccinate occurred. Virtually identical results were obtained in intact rat CP (Pritchard et al., 1999). These findings paralleled those reported for *p*-

aminohippurate uptake by renal basolateral membrane vesicles (Shimada et al., 1987; Pritchard, 1988). Thus, movement of organic anions across the apical membrane of CP (CSF to blood direction) was mechanistically consistent with basolateral tertiary active organic anion uptake in renal proximal tubule cells (blood to urine direction).

The first evidence that the 'renal' SLC22 transporters might also function in the CNS was obtained when PCR reaction products for Oct2, Oct3, Octn1 and Octn2, but not Oct1, were detected in rat CP (Sweet et al., 2001; Sweet et al., 2002) . PCR also confirmed message expression of Oat1, Oat2 and Oat3 in rat and mouse CP, indicating mRNA expression of all three organic anion transporter paralogs in both species (Sweet et al., 2001). To investigate membrane targeting of OCT and OAT proteins directly, fusion constructs of rOct2 and rOat1 with green fluorescent protein (GFP) were made (Sweet et al., 1999; Sweet et al., 2000). Mechanistic analysis of rOat1-mediated transport indicated that it was an organic anion/dicarboxylate exchanger and should be targeted to the basolateral membrane of renal proximal tubule cells (Sweet et al., 1997). Similar analysis indicated that rOct2 operated as a membrane potential-driven facilitated diffusion carrier, an activity also associated with the basolateral membrane of renal proximal tubule cells (Walsh et al., 1996; Sweet et al., 1999). Accordingly, when the rOat1-GFP or rOct2- GFP fusion proteins were expressed in intact, functional renal proximal tubules *in vitro,* marked basal and lateral membrane fluorescence was observed (Sweet et al., 1999; Sweet et al., 2000). In direct contrast to these results, when these constructs were transfected into intact rat CP *in vitro,* fluorescent signal was exclusively associated with the apical plexus membrane (Pritchard et al., 1999; Sweet et al., 2001). Further, CP isolated from Oat3 knockout mice exhibited significantly impaired accumulation of organic anions *in vitro* (Sweet et al., 2002; Sykes et al.,

2 0 0 4) . These studies exhaustively compared apical organic anion transport in CP from wild type and Oat3 knockout mice and conclusively demonstrated that mOat3 protein is expressed and functional, as well as targeted to the apical membrane, in CP (Sweet et al., 2002; Sykes et al., 2004). Functional and immunocytochemical investigations in rat CP confirmed apical membrane targeting of rOat3 (Nagata et al., 2002; Lowes et al., 2005). A recent study examining antiviral uptake in CP isolated from wild type, Oat1 knockout and Oat3 knockout mice also detected functional activity of mOat1 and mOat3 in the apical membrane (Nagle et al., 2013). This switch in 'targeting polarity' of these transporters between kidney and CP corresponds directly with the fundamental difference between systemic elimination via renal proximal tubule (transcellular movement of substrates from blood to urine) and CP mediated clearance from the CSF (transcellular movement of substrates from CSF to blood) is the order in which the basolateral and apical membrane domains are encountered by solutes. In kidney, cellular entry of substrates from the systemic circulation occurs across the basolateral membrane followed by efflux into the urine across the apical membrane, whereas, in CP cellular entry of substrates from the CSF occurs across the apical membrane followed by efflux into the blood across the basolateral membrane, *i.e.*, functionally there is a difference in tissue polarity (Figure 1.1). Thus, if the same transporters that were identified to mediate this process in the renal proximal tubule, *i.e.*, OCTs and OATs, were also responsible for this transport activity in CP, they would have to target to the opposite membrane in each of these cell types. Therefore, the observed reversal of membrane targeting for OCTs and OATs in CP (as compared to kidney) is in complete agreement with the functional data obtained for the cloned transporters examined in heterologous expression systems and for intact CP (Sweet et al., 1997; Pritchard et al., 1999; Sweet et al., 1999; Sweet et al., 2000; Sweet et al., 2001).

1. E. BRAIN CAPILLARY ENDOLTHELIUM

Investigations into the physiological mechanisms controlling the transport of small organic cations (*e.g.*, choline) and anions (*e.g.*, benzylpenicillin) across the brain capillary endothelium, using isolated brain capillaries and a variety of primary and immortalized cell culture models, indicated that they were similar to those utilized by the kidney (Galea and Estrada, 1992; Sawada et al., 1999; Murakami et al., 2000; Allen and Smith, 2001; Kikuchi et al., 2003). Saturable, energy- dependent, Na^+- and pH-independent, and potential-sensitive mechanisms were demonstrated (Galea and Estrada, 1992; Sawada et al., 1999; Murakami et al., 2000; Allen and Smith, 2001). Messenger RNA expression of mOct1, rOct1, hOCT1, mOct2, rOct2 and hOCT2 was detected in isolated primary brain capillary endothelial cells (Lin et al., 2010). Rat Oct1 and rOct2 were also seen in immortalized adult rat brain endothelial cells (ARBEC) (Lin et al., 2010). In marked contrast, a recent study failed to detect mOct1, mOct2 or mOct3 mRNA expression by quantitative PCR analysis in freshly isolated mouse brain capillaries (Andre et al., 2012). Western blotting and immunocytochemistry detected Oct1/OCT1 and Oct2/OCT2 protein expression in all three species (Lin et al., 2010). While it was concluded that these transporters were "mainly" expressed in the luminal membrane, each technique yielded Oct1/OCT1 and Oct2/OCT2 immunopositive signal associated with the basal membrane as well (Lin et al., 2010). In support of luminal targeting, siRNA knockdown of Oct1/OCT1 or Oct2/OCT2 in primary brain capillary endothelial cells resulted in significantly decreased apical organic cation transport (Lin et al., 2010). Moreover, simultaneous knockdown of both transporters yielded an even greater reduction in apical transport activity than the single knockdown of either transporter (Lin et al., 2010). In agreement with the lack of mOct3 mRNA expression in brain capillaries, no capillary associated fluorescence was observed by immunocytochemistry in brain sections (Andre

et al., 2012). Together, these data strongly support an active role for Oct1/OCT1 and Oct2/OCT2 in CNS permeation of organic cations, but further work is needed to fully resolve expression and function in this tissue *in vivo*.

Octn2/OCTN2 mRNA expression also was observed in bovine and human primary brain capillary endothelial cells (Kido et al., 2001; Berezowski et al., 2004; Miecz et al., 2008). Immunohistochemistry revealed an Octn2 specific signal in the basal membrane of primary bovine brain capillary endothelial cells (Miecz et al., 2008). Murine Octn1, Octn2 and Octn3 proteins appear to be expressed as a group in the cerebellum, CP, hippocampus, hypothalamus, motor cortex, olfactory bulb and spinal cord (Lamhonwah et al., 2008). In opposition to the reported basal targeting of bovine Octn2, functional analysis of carnitine transport across monolayers of primary bovine brain capillary endothelial cells yielded evidence supporting bovine Octn2 transport activity in both the basal (brain side) and apical (blood side) membranes (Kido et al., 2001; Andre et al., 2012). While there is an obvious need to better clarify the contribution of Octn/OCTN transporter function in the brain capillaries, expression in these brain regions is consistent with OCTN function being critical to cerebral energetics and neuronal support and protection.

When examined *in vivo* using the brain efflux method, movement of OAT substrates such as benzylpenicillin, HVA, indoxyl sulfate, *para*-aminohippurate and thiopurines is mediated by an organic anion transport system physiologically similar to that in kidney (Ohtsuki et al., 2002; Kikuchi et al., 2003; Mori et al., 2003; Mori et al., 2004). For example, the efflux of HVA from rat brain *in vivo* across brain capillaries was blocked by indoxyl sulfate, *para*-aminohippurate or benzylpenicillin, suggesting rOat3 expression and function in this

endothelium (Mori et al., 2003). Similarly, efflux from rat brain of the uremic toxin and OAT substrate, indoxyl sulfate, was inhibited by benzylpenicillin, *para*-aminohippurate or probenecid (Ohtsuki et al., 2002). PCR confirmed mRNA expression of Oat3 in brain capillaries of both mice and rats (Ohtsuki et al., 2002; Kikuchi et al., 2003; Ohtsuki et al., 2004). In agreement with the functional and gene expression data, both mouse and rat Oat3 proteins have been immunolocalized to the basolateral membrane (brain side) of brain capillaries *in vivo* (Kikuchi et al., 2003; Mori et al., 2003; Ohtsuki et al., 2004). Therefore, it is possible that Oat3/OAT3 dysfunction may contribute an as yet unrecognized component to various neurological conditions involving CNS imbalance.

1. F. BEHAVIORAL CONSEQUENCES OF SLC DYSFUNCTION

Oct3 knockout mouse. Both rOct3 and hOCT3 were simultaneously demonstrated to possess properties consistent with the extraneuronal low affinity (high capacity) monoamine transport activity characterized in the CNS (Grundemann et al., 1998; Wu et al., 1998a). In support of this, Oct3 mRNA expression in the CNS compartment was reported in a number of brain regions in rats and mice including the area postrema, CP, dorsal raphe, medial hypothalamus, neurons of the cerebellum, hippocampus, substantia nigra and ventral tegmental area, subfornical organ and thalamus (Sweet et al., 2001; Schmitt et al., 2003; Vialou et al., 2004; Amphoux et al., 2006; Gasser et al., 2006; Vialou et al., 2008). Neuronal and glial cell (astrocyte) expression of hOCT3 mRNA also has been reported (Inazu et al., 2006; Koepsell et al., 2007). More conclusively, mOct3 and hOCT3 protein expression was detected in astrocytes and nondopaminergic neurons within the nigrostriatal dopaminergic pathway and rOct3 protein immunoreactivity was observed in ependymal and glial-like cells in the dorsomedial hypothalamus, as well as in a number of

circumventricular organs, including the area postrema and subfornical organ (Inazu et al., 2003; Vialou et al., 2004; Gasser et al., 2006). Murine Oct3 protein expression was also seen in the subfornical organ (Vialou et al., 2004). In agreement with immunodetection of mOct3 and hOCT3 in astrocytes, primary astrocytes isolated from Oct3 knockout mice exhibited about three-fold lower accumulation of substrate as compared to primary astrocytes isolated from wild type mice (Cui et al., 2009). Further, loss of mOct3 function, either by gene deletion or pharmacological blockade, was demonstrated to be neuroprotective (Cui et al., 2009). Thus, Oct3/OCT3 substrate specificity and protein expression are consistent with this transporter playing an important role in neurophysiology and neuropharmacology, particularly for CNS monoamine signaling.

Initial investigation in Oct3 knockout mice detected a marked (~50%), albeit not significant, drop in steady-state dopamine and norepinephrine levels, but no overt physiological or neural dysfunction suggesting monoamine imbalance (Zwart et al., 2001). Recently, it was confirmed that dopamine transporter (DAT) protein levels are unchanged in these animals (Cui et al., 2009). When examined in the context of water and salt regulation, it was demonstrated that Oct3 knockout mice exhibit altered neural and behavioral responses to changes in blood osmolarity and regulation of salt ingestion, providing evidence that mOct3 plays a functional role in the CNS (Vialou et al., 2004). A subsequent comprehensive survey of dopamine and serotonin (and their metabolites) levels in brains of Oct3 knockout mice revealed significant region-specific alterations (Vialou et al., 2008). For example, dopamine levels were markedly reduced (by ~46%) in the substantia nigra and ventral tegmental areas, and serotonin showed an ~12% drop in the pons. Decreases in other monoamines were also detected including an ~25% reduction in norepinephrine in the substantia nigra and ventral

tegmental areas and an ~20% drop in histamine level in the hypothalamus and thalamus (Vialou et al., 2008).

Despite these alterations in neurotransmitter levels, behavioral assessment with untreated animals found no differences between wild type and Oct3 knockout mice in basal locomotor activity, balance or coordination (Vialou et al., 2008). However, Oct3 knockout mice did show an elevated hyperlocomotor response to administered stimulants, *e.g.*, amphetamine or cocaine (Kitaichi et al., 2005; Nakayama et al., 2007; Vialou et al., 2008). Recent microdialysis studies revealed that dopamine levels in Oct3 knockout mice treated with methamphetamine were increased 2-3-fold compared to similarly treated wild type mice indicating impaired dopamine clearance (Cui et al., 2009). In addition, Oct3 knockout mice exhibited less activity in the center zone (vs. wild type) during an open field activity test, suggestive of increased anxiety-like behavior (Vialou et al., 2008). This observation is in direct contrast to a separate study wherein it was concluded that Oct3 knockout mice display a significantly decreased anxiety-like phenotype based upon performance in the elevated plus maze and open field tests (Wultsch et al., 2009). Oct3 knockout mice roughly doubled the amount of time spent in the open arms of the elevated plus maze and displayed an almost four-fold increase in time spent in the center zone of an open field activity chamber. Resident-intruder (aggression) and Morris water maze (spatial memory) test results were similar between knockout and wild type mice (Wultsch et al., 2009). Clearly, further work is needed with this model to fully elucidate any connections between Oct3/OCT3 dysfunction and deviant behaviors.

Using a knockdown approach, adult wild type mice given a seven-day infusion of antisense mOct3 oligonucleotides directly into the third ventricle exhibited reduced (by ~30%) expression of mOct3 protein in brain (Kitaichi et al., 2005). This downregulation of mOct3 was associated with an enhanced locomotor response to methamphetamine (similar to Oct3 knockout mice), decreased immobility during the forced swim test (*i.e.*, decreased depression-like behavior), and a potentiated antidepressant response to imipramine (Kitaichi et al., 2005). All of these endpoints are consistent with the interpretation that mOct3 functions to remove released dopamine from the interstitial milieu, and when mOct3 activity is reduced/absent in the CNS, dopamine clearance is attenuated resulting in elevated levels of dopamine, enhancing dopaminergic neurotransmission and the associated behavioral response(s). When a three-day ventricular perfusion of antisense Oct3 oligonucleotides was performed in rats, reduction of rOct3 protein expression in the CP and ependymal cells was observed (Nakayama et al., 2007). As with the mice, decreased CNS expression of rOct3 resulted in significantly increased extracellular dopamine levels and hyperlocomotion response induced by methamphetamine administration (Nakayama et al., 2007). Furthermore, methamphetamine levels were significantly elevated in the CSF of rOct3 antisense treated animals (vs. wild type), possibly the result of decreased rOct3 activity in the CP. Together, these data strongly support a role for Oct3/OCT3 expressed in the CNS (neurons, glia, CP) in dopamine clearance and proper termination of catecholaminergic neurotransmission, and possibly in the efficacy of certain antidepressant medications.

Pharmacological manipulation of OCT activity resulted in changes in other neurotransmitters as well. For example, the OCT inhibitor, decynium-22, produced a dose-dependent increase in extracellular serotonin concentration when applied to the medial

hypothalamus of rats (F e n g e t a l . , 2 0 0 5) . This increase in serotonin correlated with altered specific behaviors such as increased grooming, suggesting that OCTs, and rOct3 in particular, function in the CNS and might play a role in adaptive neurophysiological and behavioral responses (Feng et al., 2005). Perfusion of the Oct3/OCT3 inhibitor, corticosterone, into the medial hypothalamus potentiated the response to fenfluramine administration in rats as measured by increased serotonin levels (Feng et al., 2009). Similarly, the norepinephrine metabolite, normetanephrine (Oct3 inhibitor), potentiated the response to venlafaxine in mice as evidenced by increased cortical extracellular norepinephrine (Rahman and Bardo, 2008). Behavioral results in a tail suspension test indicated this enhanced antidepressant-like effects (Rahman and Bardo, 2008). These results provide *in vivo* functional evidence not only in support of the "uptake 2 hypothesis," wherein it was proposed that the concomitant inhibition of both uptake 1 (by SSRIs) and uptake 2 (by normetanephrine or other pharmacological agents) would result in a more rapid onset of antidepressant action (Schildkraut and Mooney, 2004), but also of the concept that OCTs comprise the uptake 2 pathway component and, thus, represent critical targets for future development of drugs for mood disorder therapy.

Investigations of OCT expression in SERT knockout mice found a significant increase in Oct3 mRNA expression in the hippocampus, but not in cortex, striatum, cerebellum or brainstem (Schmitt et al., 2003; Baganz et al., 2008). In the same animals, mOct1 mRNA expression remained unchanged (Schmitt et al., 2003; Baganz et al., 2008). Immunohistochemistry and immunoblotting confirmed mOct3 protein expression in the hippocampus, which was increased by 28% in SERT knockout mice (Baganz et al., 2008). Similar to the earlier studies performed in rats, quantification of serotonin clearance demonstrated that hippocampal OCT inhibition (via application of the inhibitors decynium-22

or corticosterone) was without effect in wild type mice, but produced significant reduction in serotonin clearance in SERT knockout mice (Baganz et al., 2008). Complementary to this evidence, clearance of the Oct3/OCT3 substrate, histamine, was significantly increased in the hippocampi of SERT knockout mice as compared to wild type (Baganz et al., 2008). Finally, it was shown that decynium-22 inhibition of serotonin clearance produced antidepressant-like effects in SERT knockout, but not in wild type mice (Baganz et al., 2008). Together, these data indicate that increased Oct3/OCT3 protein expression may represent compensatory mechanism in response to dysfunctional serotonin clearance.

Oct2 knockout mouse. Rat Oct2 mRNA expression in the CNS compartment was reported to be associated with regions along the brain-CSF barrier, cerebellum, CP, hippocampus and leptomeninges (Sweet et al., 2001; Amphoux et al., 2006). Murine Oct2 protein expression was observed in the amygdala, dorsal raphe, frontal cortex and hippocampus, specifically associated with noradrenergic and serotonergic neurons (Bacq et al., 2012). Thus, some overlap with mOct3 expression might occur, however, no mOct2 protein was detected in the substantia nigra or ventral tegmental area (Bacq et al., 2012). Neuronal expression of hOCT2 mRNA and protein was observed in the cerebral cortex and subcortical nuclei (Busch et al., 1998). In addition, hOCT2 transport activity, but not that of DAT, was inhibited by amantadine at clinical concentrations used in the treatment of Parkinson's disease (Busch et al., 1998). Thus, hOCT2 expression, as well as substrate specificity and affinities (particularly for monoamines), is also consistent with it playing a key role in CNS physiology, monoamine signaling, and drug efficacy. For example, it was suggested that the therapeutic action of the antiparkinsonian drug, amantadine, might involve inhibition of hOCT2-mediated dopamine uptake (Busch et al., 1998).

Cell suspensions derived from cortex and hippocampus of Oct2 knockout mice exhibited markedly decreased norepinephrine and serotonin uptake as compared to suspensions from wild type mice, while dopamine uptake was unaffected (Bacq et al., 2012). Notably, protein expression levels of the norepinephrine transporter, serotonin transporter, and Oct3 were comparable between Oct2 knockout and wild type animals. In contrast to Oct3 knockout mice, dopamine levels between wild type and Oct2 knockout mice were similar. Like Oct3 knockout mice, a significant reduction in norepinephrine was found, however, the reduction in Oct2 knockout animals was associated with the cortex (~38%), hypothalamus (~22%), brain stem, cerebellum, hippocampus and striatum, not the substantia nigra and ventral tegmental areas (Bacq et al., 2012). Serotonin levels were reduced in the hippocampus (~19%), hypothalamus (~27%) and striatum (~20%). These changes did not lead to any overt differences in basal locomotor activity in Oct2 knockout mice (Bacq et al., 2012).

When examined in behavioral models used to assess anxiety, Oct2 knockout mice appeared to exhibit a decreased anxiety phenotype, as measured by a 2-3-fold increase in time spent, and activity in, the center zone (open field and elevated O maze) and decreased (by 40-50%) latency period to feed (novelty suppressed feeding) (Bacq et al., 2012). Significantly increased immobility time was observed in both forced swim and tail suspension tests, suggestive of increased despair and, hence, an increased depressive-like phenotype. These data are consistent with Oct2/OCT2 playing an active role in norepinephrine and serotonin clearance from the brain interstitial fluid and, perhaps, from the CSF via the CP. Similar to the Oct3 knockout mice, Oct2 knockout animals showed an increased sensitivity (vs. wild type) to the acute effects of antidepressants. Venlafaxine and reboxetine were each observed to induce significant dose-dependent decreases in immobility time in the forced swim test (Bacq et al.,

2012). Moreover, in the corticosterone induced depression model, unlike wild type animals, Oct2 knockout mice did not respond to long-term antidepressant (venlafaxine) treatment, indicating Oct2/OCT2 function was required for this effect (Bacq et al., 2012). Identification of these interactions might be the first steps toward new clinical insight to the interindividual variation in patient response to antidepressant and anxiolytic drugs.

1. G. FUTURE DIRECTIONS

Normal brain function can be readily perturbed by improper clearance of metabolic waste products, neurotransmitters and xenobiotic toxins/toxicants. The extrarenal expression and function of the 'renal' OCTs and OATs in the CP and brain capillaries underscores their potential to impact CNS homeostasis and neurotoxicity through active clearance mechanisms. Further studies are needed to fully elucidate the role of SLC22 transporters in these processes. Currently, virtually nothing is known about transporters in the arachnoid villi and circumventricular organs, and the preliminary SLC22 transporter expression profiles reviewed herein are clearly suggestive of a role for these transporters in mediating organic solute flux in these (and other) CNS tissues. In addition, the pia mater is situated between the interstitial fluid of the brain and the CSF in the ventricles and what effect(s) this membrane has on the movement of OCT and OAT substrates between these compartments remains relatively unexplored.

Intriguing data regarding the potential involvement of SLC22 transporters in the etiology of psychostimulant abuse has come to light. Cocaine, 3,4-methylenedioxy-N-methamphetamine (ecstasy), methamphetamine and phencyclidine (PCP) all have been demonstrated to inhibit Oct1/OCT1, Oct2/OCT2, Oct3/OCT3 and Octn2/OCTN2 (Wu et al.,

1998a; Rytting and Audus, 2005; Amphoux et al., 2006). Recently, both the genotype and allele frequency of two independent single nucleotide polymorphisms identified in hOCT3 were found to have significant associations with polysubstance use in subjects with methamphetamine dependence (Aoyama et al., 2006). A separate study identified two different novel independent mutations in hOCT3 exclusively in patients suffering from obsessive-compulsive disorder (Lazar et al., 2008). One was in the gene promoter and significantly increased expression (by ~36%) *in vitro* and the second was in the coding region (non-synonymous Met370Ile mutation) that conferred an ~40% loss in transporter function (Lazar et al., 2008). Thus, the development of selective hOCT2 and/or hOCT3 ligands might be efficacious in the treatment of disorders related to drug abuse and schizophrenia (Nakayama et al., 2007; Taubert et al., 2007; Cui et al., 2009). Further exploration of the nature and magnitude of hOCT2 and/or hOCT3 interactions with anxiolytics and antidepressants are needed to determine whether or not these transporters represent a previously unrecognized component of their pharmacological and therapeutic effects and/or novel pharmacological targets for new drugs. Indeed, recently the "uptake 2 hypothesis" was proposed wherein it was suggested that giving a drug that inhibited the extraneuronal uptake of monoamines (either directly or indirectly) in combination with standard reuptake inhibitors, or a single drug that targets both uptake systems, would 'speed up' the onset of their clinical effects (Schildkraut and Mooney, 2004). Preclinical findings with normetanephrine (metabolite of norepinephrine that inhibits OCTs) administered in combination with venlafaxine support this hypothesis (Rahman and Bardo, 2008)

The endogenous CNS compounds salsolinol and cyclo(his-pro) have been identified as substrates of hOCT2 (Taubert et al., 2007). Salsolinol, and its derivative N-methyl-(R)-

salsolinol, are thought to be integral to the pathogenesis of Parkinson's disease, as their concentrations were elevated in the CSF of Parkinson's patients (Antkiewicz-Michaluk et al., 1997; Naoi et al., 2002). Salsolinol cytotoxicity was significantly increased in cells expressing hOCT2 (vs. mock transfected or hOCT1- or hOCT3-expressing cells) and pretreatment with cyclo(his-pro) was protective (Taubert et al., 2007). Thus, hOCT2 expressed in the CNS, particularly in neurons, might be charged with maintaining the proper balance between intracellular concentrations of salsolinol and cyclo(his-pro), which in turn might be integral to dopaminergic neuronal health (Taubert et al., 2007). Any disruption of this balance could then trigger nigral neural degeneration and the development of Parkinson's-like symptoms. Similarly, the xenobiotic neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+), induced severe Parkinson-like symptoms in humans and non-human primates (Langston et al., 1983; Langston and Ballard, 1984; Langston et al., 1984) and has been identified as a substrate of human, mouse and rat Oct1/OCT1, Oct2/OCT2 and Oct3/OCT3 (Martel et al., 1996; Gorboulev et al., 1997; Zhang et al., 1997; Wu et al., 1998a; Wu et al., 2000; Hayer-Zillgen et al., 2002). Application of OCT inhibitors prevented MPP^+-mediated cytotoxicity in human neurons and astrocytes, as well as in adult rat brain endothelial cells (Shang et al., 2003; Inazu et al., 2006; Liou et al., 2007). Clearly, tissue expression and functional properties of the SLC22 transporters are consistent with their playing a role in modulating neuroprotection/neurodegeneration and, thus, their continued study might provide new insight to CNS pathologies including seizures, Parkinson's, and Reye's syndrome.

Overall, it is intriguing to speculate that OCT- and OAT-mediated clearance of catecholamines and their metabolites from brain interstitial fluid by capillaries, neurons and

astrocytes, and from the CSF by the CP, plays a vital role in the termination of catecholamine signaling in the CNS. Several other tryptophan metabolites, *i.e.*, anthranilic acid, kynurenic acid, picolinic acid, quinolinic acid and xanthurenic acid, have been identified as inhibitors of mOat1 and mOat3 as well (Bahn et al., 2005). In addition to expression and function of rodent Oat1 and Oat3 orthologs in CP and brain capillaries, immunostaining of mOat1 was observed in neurons in the cerebral cortex and hippocampus (Bahn et al., 2005). Thus, further exploration of the interactions between these tryptophan metabolites and the OATs will undoubtedly confer broader scope to the role of OAT expression and function in CNS homeostasis. Whether or not these observations are indicative of the SLC22 transporters being novel targets for the development of CNS-acting anxiolytics, antidepressants or other therapeutics for behavioral disorders is just beginning to be explored. What impact, if any, age, environmental conditions, pathological conditions, nutritional status, reproductive status, etc. have on SLC22 expression and/or function are additional areas that remain to be examined in greater detail.

CHAPTER 2

RESEARCH HYPOTHESES AND SPECIFIC AIMS

A. HYPOTHESIS

2.A.1. The loss of Oat1 and/or Oat3 transporters in the murine CP results in altered concentrations of catecholamines (i.e. dopamine (DA), norepinephrine (NE), serotonin (5-HT)) and relevant metabolites (i.e. 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), 5-hydroxyindole acetic acid (5-HIAA), quinolinic acid (QA), nicotinic acid (NA), and xanthurenic acid (XA)).

2.A.2. The loss of Oat1 and/or Oat3 transporters in the murine CP results in altered anxiety-like and depressive-like behaviors known to associated with monoaminergic neurotransmission.

2.A.3. The loss of Oat1 and/or Oat3 transporters in the murine CP results in altered concentrations of amino acid neurotransmitters GA and GABA.

2.B SPECIFC AIMS TO ADDRESS THE ABOVE HYPOTHESIS

2.B.1. SPECIFIC AIM 1

1. To develop a simple and rapid high performance liquid chromatography-ultraviolet/electrochemical detection method for the quantitation of monoamine neurotransmitters, dopamine (DA), norepinephrine (NE), and serotonin (5-HT), and relative metabolites (3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxy-3-indoleacetic acid (5-HIAA), quinolinic acid (QA), nicotinic acid (NA), and xanthurenic acid (XA)) in mouse whole brain tissue. A method validation will be conducted to evaluate linearity, limits of detection (LOD), lower limits of quantitation (LLOQ), sensitivity, selectivity, intra-day and inter-day accuracy and precision, recovery, and stability.

2. To apply the developed method for the measurement of whole brain concentrations of the monoamine neurotransmitters and relative metabolites in WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ mice at 3, 6, 9, 12, 15 and 18 mo. of age.

2.B.2. SPECIFIC AIM 2

1. To characterize the basal locomotor behavior, anxiety-like behavior, and depressive-like behavior of mice of the three genotypes (WT, Oat1$^{-/-}$, and Oat3$^{-/-}$) across age (3, 6, 9, 12, 15 and 18 mo. of age) using commonly employed behavioral paradigms (e.g. open field activity (OFA), light-dark (LD), marble burying (MB), and tail suspension test (TST)).

2.B.3 SPECIFIC AIM 3

1. To develop a simple and sensitive novel gas chromatography tandem mass spectrometry (GC-MS/MS) method for the quantitation of amino acid neurotransmitters, L-glutamic acid (GA) and γ-aminobutyric acid (GABA), in mouse whole brain tissue. A complete method validation will be conducted to evaluate linearity, limits of detection (LOD), lower limits of quantitation (LLOQ), sensitivity, selectivity, intra-day and inter-day accuracy and precision, instrument injector precision, derivatization efficiency, and stability.

2. To apply the developed method for the measurement of whole brain concentrations of the amino acid neurotransmitters, GA and GABA, in a small subset of WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ mice at 3 mo. and 18 mo. of age.

CHAPTER 3

A SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF MONOAMINE NEUROTRANSMITTERS AND RELATIVE METABOLITES WITH APPLICATION IN MOUSE BRAIN TISSUE

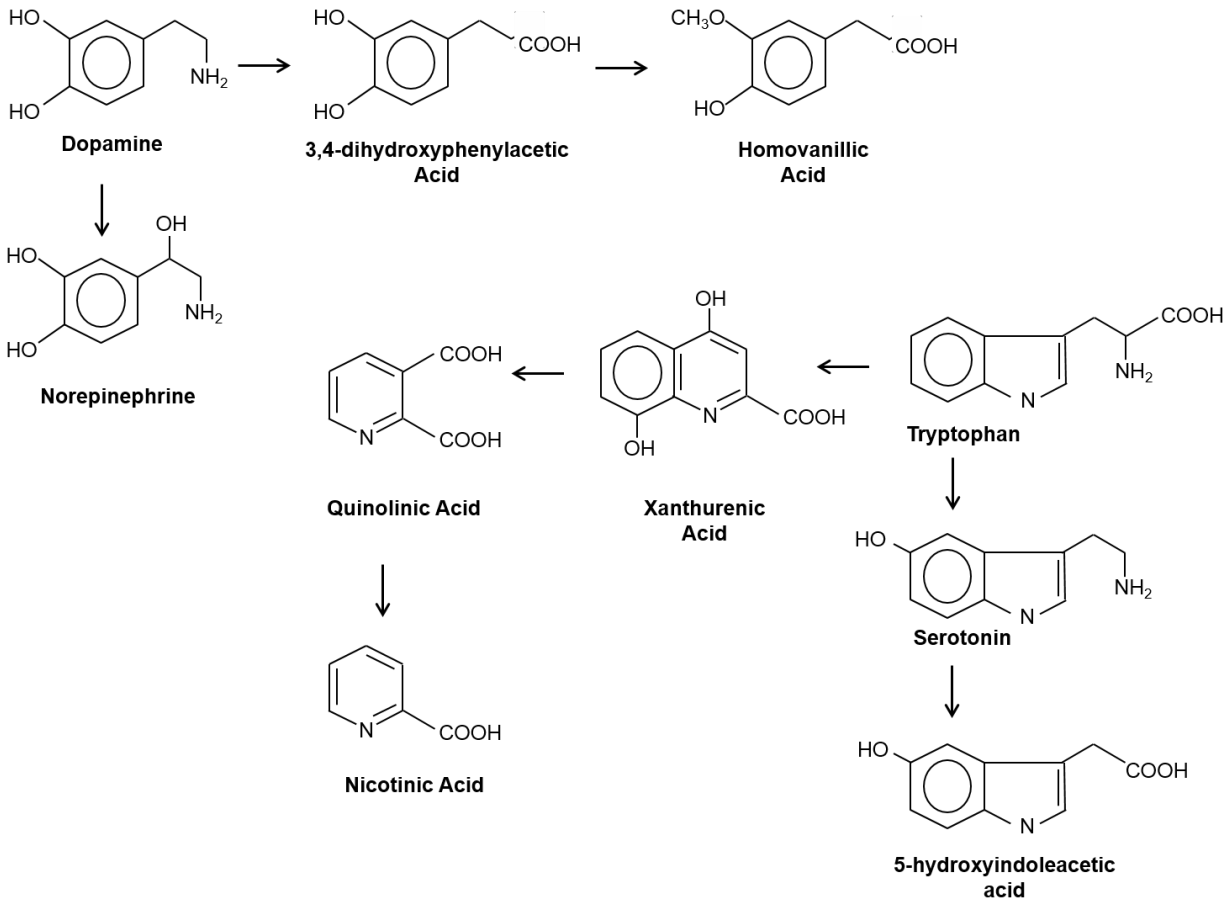
Drawn from manuscript published in *Journal of Liquid Chromatography and Related Technologies*, 2014

3.A. INTRODUCTION

Catecholamines (e.g. dopamine, norepinephrine) and indoleamines (e.g. serotonin (5-hydroxytryptamine)) are widely distributed neurotransmitters in the central nervous system (CNS) that play an important role in physiological and behavioral processes (i.e. sleep, reward and/or reinforcement, motor and cognitive functions, and neurobehavioral disorders) (Carlsson, 1987; Greengard, 2001). Imbalance in CNS levels of these neurotransmitters and their metabolites is associated with a multitude of human neuropsychiatric conditions including anxiety, depression, panic disorder, schizophrenia and Parkinson's disease (Carlsson and Carlsson, 1990; Boyer, 2000). The structure and simplified metabolic pathways are shown in Figure 3.1. The association between CNS concentrations of biogenic amines and the persistence of these neuropsychiatric conditions has been studied for almost 50 years with little improvement in diagnosis, treatment and clinical outcome, let alone the basic understanding of the underlying biochemical mechanisms involved. Recent advances in the knowledge of organic solute transporter tissue distribution and function have suggested a previously unrecognized role for Solute Carrier family 22 (SLC22) members in the maintenance of CNS monoamine

homeostasis (VanWert et al., 2010; Wang and Sweet, 2013; Farthing and Sweet, 2014). Various SLC22 transporters are expressed and functional in brain capillaries (blood-brain barrier), choroid plexus (blood-cerebrospinal fluid barrier), astrocytes and neurons (Farthing and Sweet, 2014). Therefore, accurate quantitation of these monoamine neurotransmitters and their metabolites is essential, not only to advance our understanding of disease states regulated by aminergic neurotransmission and evaluation of novel pharmacotherapies for treating these disorders, but also for the assessment of potential involvement of SLC22 family members in maintaining CNS monoamine homeostasis.

Figure 3.1. Structures and simplified metabolic pathways for monoamine and indoleamine neurotransmitters in the CNS



Various analytical methods have been investigated for determining the level of catecholamine neurotransmitters and their metabolites in biological samples such as plasma, urine, and rodent brain (mouse, rat) (Mashige et al., 1994; Fauler et al., 1997; Lakshmana and Raju, 1997; Wang et al., 1999; Chan and Ho, 2000; Tornkvist et al., 2004). These include high performance liquid chromatography (HPLC) coupled with fluorescence, electrochemical (ECD), or mass spectrometry (MS) detection, as well as gas chromatography coupled with MS (Mashige et al., 1994; Fauler et al., 1997; Lakshmana and Raju, 1997; Wang et al., 1999; Chan and Ho, 2000; Tornkvist et al., 2004). HPLC fluorescence detection required cumbersome sample treatments, including a harsh two-step reaction using benzylamine and 1,2-diphenylethylenediamine in weakly alkaline media in the presence of potassium hexacyanoferrate (III) with heating to 50°C for 20 min, in order to generate fluorescent monoamine derivatives (Lakshmana and Raju, 1997). Previous HPLC methods coupled to ECD included reductive electrochemical detection to evaluate various monoamine neurotransmitters and metabolites in cerebrospinal fluid and brain tissue (Schmidt et al., 1990), however, when we evaluated reductive electrochemical detection using our current method, we found that not all of our components of interest were reducible. While methodology yielding very sensitive (LOD = fmol range) detection of catecholamines was reported, the analytical run time was approximately 45 min and we found that we did not need such sensitivity at the whole brain level (Sarre et al., 1992). There are several reported methods utilizing capillary electrophoresis with ECD for such determinations, but they also suffer from extended analytical run time (~40 min) or inability to quantitate all of the components simultaneously, as achieved by our currently developed method (Jin et al., 1999; Parrot et al., 2007). A recent method utilized ECD to determine the concentration of catecholamines, kynurenine, and indole derivatives of

tryptophan in rat brain tissue homogenate with a simple sample preparation, but this method also required an extensive analytical run time (~20 min) and the use of high electrode potentials for some of the analytes, which can be damaging to the electrochemical cells over time (Vaarmann et al., 2002).

One method describing analysis of the kynurenine metabolites from the tryptophan pathway (i.e. quinolinic acid, nicotinic acid, and xanthurenic acid) was conducted with the use of simultaneous ultraviolet and fluorimetric detection requiring a derivitization step, as compared to the proposed simple sample preparation of the current method, and was only described in aqueous clean solutions (Presits and Molnar-Perl, 2003). Additional methods to quantify these metabolites utilizing HPLC with simultaneous ultraviolet-fluorimetric detection have been described in human and rat sera, but none to our knowledge in whole brain tissue homogenate (Badawy and Morgan, 2010; Zhao et al., 2011). HPLC coupled with MS, which can be used to identify analytes on the basis of their mass-to-charge (m/z) ratios, is a popular and powerful technique for analyzing neurotransmitters and their metabolites in several biological fluids (Chan and Ho, 2000; Tornkvist et al., 2004; de Jong et al., 2009; Liao et al., 2010; Zhu et al., 2011; Moller et al., 2012). However, MS methods are highly complex, expensive to perform and require ready access to specialized (and expensive) instrumentation.

Here we report the development of a simple and cost effective method for the sensitive and reproducible determination of three monoaminergic neurotransmitters (dopamine, norepinephrine, serotonin) and relevant metabolites (3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindoleacetic acid, quinolinic acid, xanthurenic acid, nicotinic acid) in mouse brain homogenates with HPLC-UV/ECD. This HPLC-UV/ECD method introduces

several advantages over previous methods in that it uses relatively inexpensive mobile phase constituents, requires minimal sample preparation and small injection volume while preserving good chromatographic separation.

3.B. EXPERIMENTAL

3.B.1. Chemicals and Reagents

ACS reagent grade or higher purity dopamine, norepinephrine, serotonin (5-hydroxytryptophan), 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, homovanillic acid, 3,4-dihydroxybenzylamine, quinolinic acid and nicotinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xanthurenic acid was purchased from Acros Organics (Fair Lawn, NJ, USA). For mobile phase preparation, sodium dihydrogen phosphate (monohydrate), acetonitrile (HPLC grade), phosphoric acid (99%), and triethylamine (HPLC grade) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). EDTA was purchased from EM Science (Gibbstown, NJ, USA) and 1-octanesulfonic acid was purchased from JT Baker (Center Valley, PA, USA). An elution solution used to prepare standards and controls, and for tissue homogenation, consisted of phosphoric acid (0.5%) and acetic acid (1%) (Thermo Fisher Scientific, Fair Lawn, NJ, USA) and deionized water. Ultrapure deionized water (18 megohm-cm) was prepared daily using a Milli-Q Integral Water Purification System from EMD Millipore (Billerica, MA, USA).

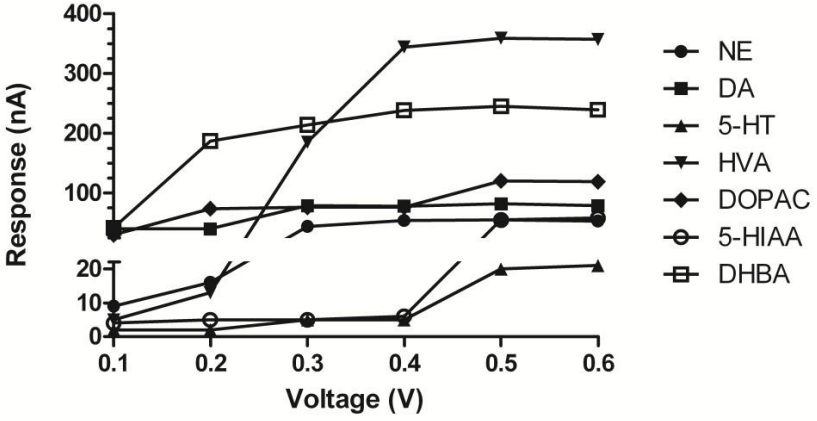
3.B.2 HPLC Equipment and Mobile Phase

The Agilent 1200 series HPLC equipment consisted of a degasser (Model G1322A), solvent delivery system (quaternary) (Model G1311A), autosampler (Model G1329A),

thermostatted column compartment (Model G1316A), variable wavelength detetctor (Model G1314D) (Agilent Technologies, Santa Clara, CA, USA), and a Coulochem III electrochemical detector (Thermo Fisher Scientific (ESA Inc), Fair Lawn, NJ, USA). The analytical column used was an HR-80 C_{18} (80 mm x 4.6 mm I.D) (Thermo Fisher Scientific (ESA Inc)) coupled to a C_{18} guard column (12.5 mm x 4.6 mm I.D.) (Phenomenex®, Torrance, CA, USA). The guard column was replaced after each analytical run of approximately 50 brain tissue homogenate samples. The mobile phase was prepared following ESA, Inc. MD-TM mobile phase technical note and consisted of 75 mM sodium dihydrogen phosphate (monohydrate), 1.7 mM 1-octanesulfonic acid sodium salt, 25 µM EDTA, 10% acetonitrile and 1% triethylamine with a final pH of 3.0 adjusted using concentrated phosphoric acid (H_3PO_4). The mobile phase is commercially available by ESA, Inc.; however, to reduce expenses, it was prepared in house following the technical note for all experiments. A flow rate of 1 mL/min was used with typical HPLC operating pressure of approximately 145 bar and column temperature held at 25°C within the thermostatted column compartment. A 20 µL injection volume of the prepared tissue homogenate sample was accomplished using the Agilent 1200 series Model G1329A autosampler. Component detection was achieved using Agilent Variable Wavelength Detector set to 260 nm coupled to a Coulochem III Electrochemical Detector with the guard cell set to +0.05 V, an E1 voltage of 0 V and an E2 voltage of +0.6 V. This was chosen to be the potential at which the maximum response was given with the lowest amount of background noise for the majority of analytes detected using ECD (Figure 3.2). The detector was operated at high sensitivity setting with a 1 s response time. Peak height was used for computations. Concentrations of analytes were calculated by interpolation of their respective standard curves.

Data acquisition and component computations were performed using ChemStation™ software (Agilent Technologies, Santa Clara, CA, USA).

Figure 3.2. Voltammograms for norepinephrine, dopamine, serotonin, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, and homovanillic acid at various oxidation potentials (+0.1,+0.2, +0.3, +0.4, +0.5, +0.6 V)



3.B.3. Standard and Control Preparation

Stock standards of serotonin, dopamine, norepinephrine, 3,4-dihydroxyphenylacetic acid, nicotinic acid, quinolinic acid, and the internal standard 3,4-dihydroxybenzylamine (1.0 mg/mL) were prepared in an "elution solution" consisting of phosphoric acid (0.5%), acetic acid (1%) and deionized water. Xanthurenic acid was prepared in the elution solution and required a few drops of 1N NaOH to go into solution. Homovanillic acid and 5-hydroxyindoleacetic acid were prepared in 50:50 methanol:deionized water. All stock standards were stored at -80°C. Working standards of dopamine, norepinephrine, serotonin (5-hydroxytryptophan), 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, and homovanillic acid were prepared at 20, 50, 100, 250, 500, 750, and 1000 ng/mL in elution solution. The internal standard, 3,4-dihydroxybenzylamine, was prepared at 100 ng/mL in elution solution. Working quality controls (QC) were prepared at 75, 300, and 600 ng/mL in elution solution. After preparation, the QC samples were stored and maintained at -80°C along with the study samples. Working standards of quinolinic acid, xanthurenic acid and nicotinic acid were prepared at 10, 25, 50, 100, and 200 µg/mL in elution solution. Working quality controls (QC) were prepared at 30, 75, and 150 µg/mL in elution solution. After preparation, the QC samples were stored and maintained at -80°C along with the study samples.

3.B.4. Sample Acquisition and Preparation

Male wild-type C57BL/6J mice (10-13 weeks) were sacrificed and whole brains were removed and snap frozen in liquid nitrogen (average processing time ≤ 60 sec). Samples were stored at -80°C until subsequent analysis. For HPLC analysis, whole brains were weighed and then homogenized with 4 mL of elution solution in 10 mL polypropylene tubes (Eppendorf,

Westbury, NY, USA) using a PowerGen 125 homogenizer (Thermo Fisher Scientific, Fair Lawn, NJ, USA). Prior to homogenization, the internal standard, 3,4-dihydroxybenzylamine, was added to each tube of tissue sample. One mL of homogenate was transferred to a 1.5 mL conical plastic tube and centrifuged at 14,000 x g for 10 min in a refrigerated (4°C) centrifuge (Eppendorf 5810R, Westbury, NY, USA). The cleared supernatants (100 µL) were transferred to plastic autosampler microvials (Waters, Millford, MA, USA) and 5 µL was injected for HPLC analysis.

3.C. RESULTS AND DISCUSSION

3.C.1. Linearity, limits of detection and quantitation, computations

The calibration curve was linear for dopamine, norepinephrine, serotonin, 3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindoleacetic acid throughout the concentration range 20–1000 ng/mL with a mean correlation coefficient of $\geq$ 0.9993 (unweighted normal linear regression, n = 10 analytical runs). The calibration curve was linear for quinolinic acid, xanthurenic acid, and nicotinic acid throughout the concentration range of 10-200 µg/mL with a mean correlation coefficient of $\geq$ 0.9996. The LOD for dopamine, norepinephrine, 3,4-dihydroxyphenylacetic acid, and homovanillic acid was approximately 5 ng/mL and for serotonin and 5-hydroxyindoleacetic acid was approximately 20 ng/mL (n = 3). The LOD for quinolinic acid, xanthurenic acid, and nicotinic acid was approximately 1 µg/mL and determined by evaluation of each analyte in elution solution (n = 3). The LOD was determined using dilutions of standard material and calculated (extrapolated) from each component's standard curve (peak heights were greater than three times the standard deviation of the chromatographic baseline noise). The lowest standard calibrator (i.e. 20 ng/mL and 10

μg/mL) was used as the lower limit of quantitation (LLOQ) and is administratively defined as a combined accuracy and precision within 30% of the nominal amount. The LOD and LLOQ were sufficient for measurement of endogenous concentrations of the neurotransmitters and metabolites in the majority of matrix samples. However, if a compound was found to be less than the LLOQ, it was reported as less than the LLOQ. For HPLC analysis, internal standardization and peak height response were used for all sample computations.

Quantitation of kynurenic acid, an additional metabolite of the kynurenine pathway, also was attempted; however, using the current method, the UV detector did not provide sufficient sensitivity to detect kynurenic acid in the tissue matrix. This was confirmed by detectability of kynurenic acid in standard material and fortified tissue samples (data not shown). In addition, a voltammogram (+0.5 – +1.0 V) was constructed to evaluate electrochemical detection of kynurenic acid, but it remained undetectable in the tissue matrix (data not shown).

3.C.2. Accuracy, precision and recovery

The accuracy and precision for the method was determined by evaluation of replicate tissue samples at fortified analyte concentrations (Table 3.1). Brain tissue samples were divided into two post-homogenization aliquots, the first aliquot was treated as a blank while the second aliquot was fortified with 75, 300 or 600 ng/mL (n = 9 at each level) of dopamine, serotonin, norepinephrine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindoleacetic acid and 30, 75, 150 μg/mL (n = 9 at each level) for quinolinic acid, nicotinic acid, and xanthurenic acid. The "blank" was then subtracted from the fortified sample to normalize for endogenous levels. The combined intra-day (within day) and inter-day (between day) accuracy of the method was reported as percent error of the measured concentration relative to the nominal concentration. The combined intra-day and inter-day precision of the method was reported as

the percent relative standard deviation (% RSD). Using the sample preparation procedure described above, the method demonstrated sufficient accuracy and precision for all analytes (Table 3.1).

Table 3.1. Combined intra-day and inter-day accuracy and precision for all components

	Dopamine			Norepinephrine			Serotonin		
Conc. (ng/mL)	Mean	% Error	% CV	Mean	% Error	% CV	Mean	% Error	% CV
75	73	-3	4.8	64	-14	6.3	66	-12	4.6
300	287	-4	1.3	288	-4	5.2	283	-6	2.3
600	588	2	0.3	590	-2	0.4	583	-3	0.9

	3,4-Dihydroxyphenylacetic Acid			Homovanillic Acid			5-Hydroxyindoleacetic Acid		
Conc. (ng/mL)	Mean	% Error	% CV	Mean	% Error	% CV	Mean	% Error	% CV
75	72	-4	8.4	70	-7	3.3	73	-3	4.9
300	289	-4	1.6	285	-5	1.8	306	2	1.9
600	594	1	0.9	587	-2	1.4	605	1	1.6

	Quinolinic Acid			Nicotinic Acid			Xanthurenic Acid		
Conc. (µg/mL)	Mean	% Error	% CV	Mean	% Error	% CV	Mean	% Error	% CV
30	29	-4	4.0	30	-1	5.1	29	-2	11
75	74	-1	5.4	77	2	2.7	77	-2	1.5
150	157	5	1.6	152	2	2.0	154	3	3.2

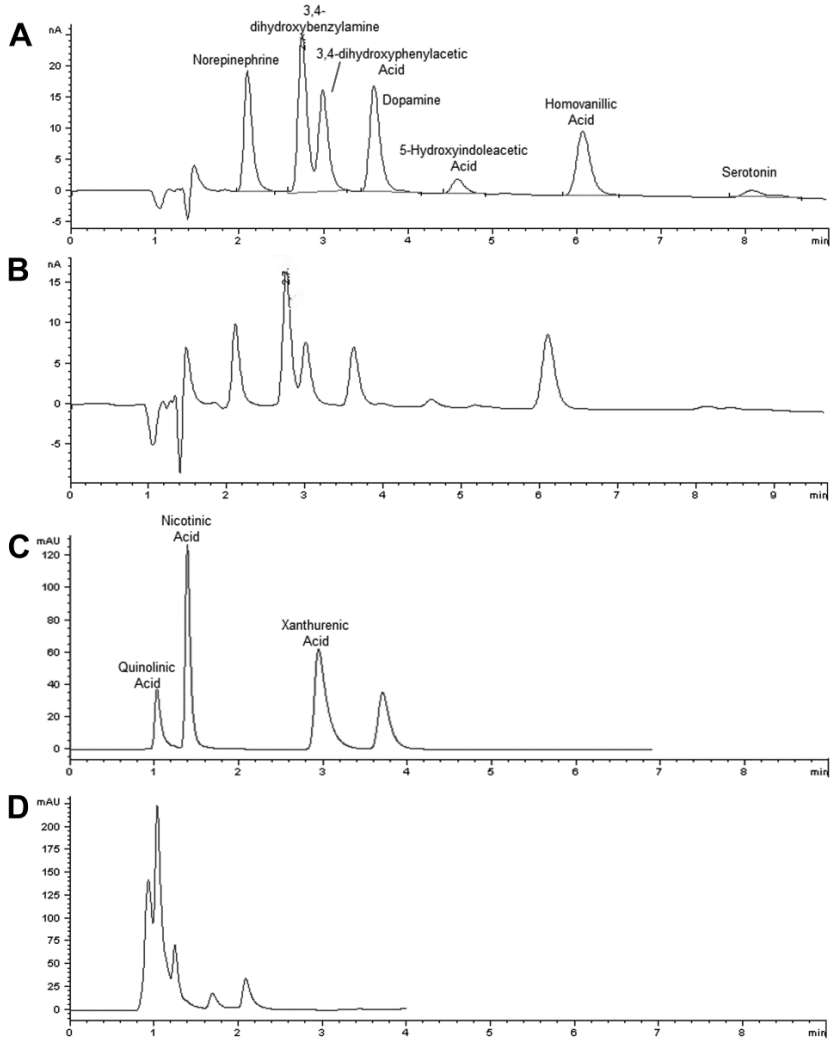
3.C.3. Stability

The stability of all components in standard solution and brain tissue homogenate were evaluated at room temperature and at 4°C for 6 hours (short term stability-representing maximum run time). The tryptophan metabolites quinolinic acid, xanthurenic acid, and nicotinic acid standards were stable for 6 hours at room temperature and 4°C; however, these compounds were not stable at room temperature in brain homogenate over 6 hours possibly indicating metabolic processes. We found that using a 1:10 dilution of each sample with elution solution eliminated the matrix effect and provided stability of the tryptophan metabolites for 48 hours at room temperature and 4°C. Short-term stability of monoamine neurotransmitters and metabolites in standard solution and brain tissue homogenate revealed that all analytes were stable at room temperature and 4°C for 6 hours. All stock standards and working standards were stable when stored frozen at -20°C for 30 days. This time span (30 days) frames the length of time samples were stored frozen during this study. Components were considered stable if the measured concentrations were within the inter-day QC acceptance range.

3.C.4. Chromatography

The method demonstrated good chromatographic selectivity without interferences from other constituents in the matrix at the retention times of each analyte (Figure 3.3). A guard column (changed after ~50 injections) was utilized to extend the analytical column lifetime as the method did not employ a formal sample cleanup step. The analytical column was flushed after each analytical run using acetonitrile:deionized water (70:30, v/v).

Figure 3.3. Representative chromatograms demonstrating electrochemical detection of neurotransmitters and relative metabolites (norepinephrine, dopamine, serotonin, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, and homovanillic acid) in (A) standard solution and (B) brain homogenate. Chromatograms (C) and (D) represent ultraviolet detection of tryptophan metabolites, quinolinic acid, nicotinic acid, and xanthurenic acid, in standard solution and brain homogenate, respectively.



3.D. CONCLUSIONS

Recent *in vitro* evidence suggests a novel role for organic anion and cation transporters from the SLC22 family in the maintenance of CNS monoamine homeostasis [5-7]. In order to explore this possibility, particularly in SLC22 transporter knockout mouse models, a simple and efficient analytical assay capable of simultaneous detection of multiple monoamine NTs and their metabolites was required. Here, we reported a reliable HPLC-UV/ECD method for the evaluation of several neurotransmitters (dopamine, norepinephrine, serotonin (5-hydroxytryptophan)) and their relative metabolites (3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindoleacetic acid, quinolinic acid, xanthurenic acid, and nicotinic acid) in mouse brain homogenate. This method provided sufficient selectivity and sensitivity for quantitation of these endogenous components in the matrix. The method employed a simple sample preparation for tissue with the use of an internal standard (3,4-dihydroxybenzylamine) to provide information regarding analyte recovery. In the future, this assay will be applied to assess changes in CNS monoamine balance in transporter knockout mouse models.

CHAPTER 4

ORGANIC ANION TRANSPORTERS 1 (OAT1) AND OAT3 IN THE MURINE CHOROID PLEXUS: A POTENTIAL MECHANISM FOR MAINTAINING ENDOGENOUS MONOAMINE HOMEOSTASIS IN THE CENTRAL NERVOUS SYSTEM

Drawn from manuscript submitted for publication in *Physiology and Behavior*, 2014

4.A. INTRODUCTION

According to a recent assessment by the World Health Organization, mental disorders represent the leading cause of disability in the US and generate ~58 billion dollars in medical costs each year (NIMH; WHO, 2008). Annually, among the US population 18 years and older, ~40 million individuals suffer from an anxiety disorder, of which ~2 million exhibit obsessive-compulsive disorder (OCD), and ~14 million experience a major depressive disorder (Kessler et al., 2005; WHO, 2008). The association between CNS levels of biogenic amines (i.e. dopamine, serotonin, norepinephrine) and the persistence of these neurobehavioral conditions has been studied for almost 50 years with little improvement in diagnosis and clinical outcomes. Thus, a more complete understanding of the underlying mechanisms regulating CNS monoamine levels may provide new insight to the pathways impacting normal CNS monoamine homeostasis and/or pharmacological action of current medications, potentially leading to new therapeutic approaches to normalizing monoaminergic neurotransmission, resulting in improved treatment and reduced health care costs.

One aspect of CNS regulation of monoaminergic neurotransmission involves pathways targeted toward the efficient removal of released neurotransmitters (NTs), so-called uptake 1 and uptake 2 transport systems. Neuronal uptake 1 activity is comprised of the high affinity, low capacity monoamine transporters for dopamine (DAT), serotonin (SERT), and norepinephrine (NET), which function to ensure rapid reuptake of released NTs by the pre-synaptic neuron. A second, non-neuronal low affinity, high capacity transport system, uptake 2, functions to clear NTs, as well as their degradative metabolites that have diffused away from the synaptic terminal region. The precise nature and role of the uptake 2 pathway in monoaminergic signaling and homeostasis is poorly understood. However, a variety of recent studies comprising pharmacological profiles, mRNA expression, immunohistochemistry, and gene knockdown and knockout have provided evidence suggesting that members of the organic cation/anion/zwitterion transporter family (SLC22) represent a previously unrecognized component of the uptake 2 clearance pathway (Pritchard et al., 1999; Sweet et al., 2001; Ohtsuki et al., 2002; Sweet et al., 2002; Sykes et al., 2004; Kitaichi et al., 2005; Vialou et al., 2008; Wultsch et al., 2009).

The Solute Carrier (SLC) superfamily of transporters contains approximately 370 identified transport proteins divided into 48 families. Neuronal uptake 1 transporters, DAT, SERT, and NET, are members of SLC6, the sodium and chloride dependent neurotransmitter transporter family. The SLC22 family contains 25 members subdivided into three subgroups: organic cation transporters (OCTs), organic anion transporters (OATs), and organic cation/carnitine transporters (OCTNs) (Sweet, 2005; Sweet, 2010; VanWert et al., 2010). The first member of the OAT subfamily, Oat1, was isolated from rat kidney (Sweet et al., 1997). Subsequently, ten additional OATs have been cloned and partially characterized: Oat2-10 and

Urat1 (VanWert et al., 2010). Active renal excretory transport mediated by OATs is a major determinant of the effects of many therapeutics and toxic chemicals (Cihlar et al., 1999; Mulato et al., 2000). In addition to the kidney, active OA transport is an important function of other barrier epithelia. For example, the composition of the extracellular fluid of the brain is tightly regulated and highly insulated from changes in the circulation. This regulation is mediated by the blood-brain and the blood-cerebrospinal fluid barriers, the latter of which is comprised of the choroid plexus (CP), arachnoid membrane, and circumventricular organs (Segal, 1998; Pritchard et al., 1999). The cells of these epithelia form tight junctions, which slow the penetration of solutes, drugs, and other foreign chemicals into the brain and limit the passive efflux of endogenous compounds (i.e. monoaminergic NTs and their metabolites) from the brain(Segal, 1998; Pritchard et al., 1999). Thus, the CP protects the CNS against toxicity by effectively removing environmental pollutants, therapeutic drugs, and waste products of CNS metabolism from the cerebrospinal fluid (CSF) (Forn, 1972; Pritchard, 1980; Suzuki et al., 1987). Because the characteristics of OA transport by the CP are similar to that in kidney, it was hypothesized that the same transporters identified in the kidney could be responsible for mediating OA uptake in CP (Pritchard et al., 1999). Subsequently, Oat1 and Oat3 mRNA expression was detected in adult rat and murine CP (Sweet et al., 2002). For Oat1 and Oat3 expressed in CP to participate in OA removal from CSF to blood, they would have to be targeted to the apical membrane of the CP. In support of this, localization of an Oat1/green fluorescent protein fusion construct (Oat1-GFP) was observed in the apical membrane in intact rat CP transfected *in vitro* (Pritchard et al., 1999). Further, substantial accumulation of the OA fluorescein was observed in the cells and underlying capillaries of CP from wildtype (WT) mice, whereas, CP of Oat3 knockout ($Oat3^{-/-}$) mice exhibited a significant decrease in fluorescein uptake (Sweet et al., 2002). This

suggests that, like Oat1, Oat3 is targeted to the apical membrane in CP and involved in OA transport from CSF to blood. Therefore, Oat1 and Oat3 are poised to play an active role in the removal of NTs and their metabolites from CSF and in protection of the CNS from toxic injury.

Normal brain function can be disturbed by improper clearance of monoamine NTs and their metabolites (Sullivan et al., 1978; Giroud et al., 1990). Several acidic metabolites of the monoamine NTs dopamine (DA), serotonin (5-HT), and norepinephrine (NE) are known substrates, or inhibitors, of Oat1 and Oat3; including 5-hydroxyindole-3-acetic acid (5-HIAA), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA), 5-methoxy-3-indoleacetic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxymandelic acid, 4-hydroxy-3-methoxymandelic acid, and 5-methoxytryptophol (Kusuhara et al., 1999; Sekine et al., 2000; Ohtsuki et al., 2002). Furthermore, peripheral administration of probenecid (a potent inhibitor of OATs) results in increased HVA and 5-HIAA levels in the CSF (Forn, 1972; Emanuelsson et al., 1987a; Emanuelsson et al., 1987b). Thus, changes in expression and function of Oat1 and Oat3 in CP may play a previously unrecognized role in CNS monoaminergic homeostasis and they may represent unexplored contributors to behavioral states associated with monoamine imbalance in the CNS.

In the current series of investigations, we studied basal motor behavior, and anxiety-like and depressive-like behaviors in Oat1 and Oat3 knockout mice compared with age-matched WT mice bred in-house. We then assessed monoamine homeostasis by measuring tissue levels of neurotransmitters (DA, NE, 5-HT) and relevant metabolites (DOPAC, HVA, 5-HIAA, QA, NA, and XA) using high performance liquid chromatography methodology. Together, the findings of the current study extend the behavioral and biochemical phenotypes of Oat knockout mice, as these mice displayed a significant age-dependent change in a measure of anxiety-like behavior

without concomitant changes in their basal motor behavior. Further, the current findings include previously unknown correlative changes in neurotransmitter and relevant metabolite concentrations in aged mice.

4.B. METHODS

4.B.1. Subjects

Male and female wild-type (WT), Oat1 knockout ($Oat1^{-/-}$), and Oat3 knockout ($Oat3^{-/-}$) mice of C57Bl6J background were bred and housed in university animal facilities until they reached specific ages of interest: 3, 6, 9, 12, 15, or 18 mo. Sample size and mean weight for each group are listed in Table 4.1. Subjects were housed in groups of 2 to 5 to avoid the possible confounding effects of isolation or over-crowding. Subjects had ad libitum access to food and water, and were maintained under 12/12 hour light/dark cycle. All behavioral assessments were conducted during the light phase of the cycle.

Animal use and care procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

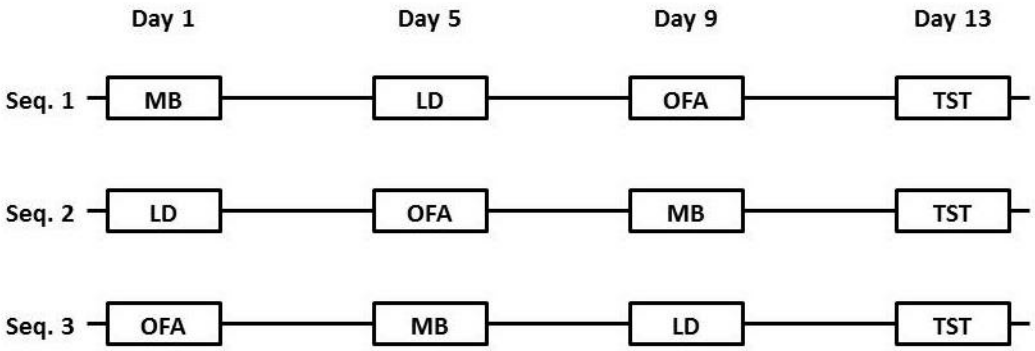
Table 4.1. Strain sample size (n) and mean weight (g).

Gender	Age	n			Mean Weight (g) ± SEM		
	(mo)	WT	Oat1$^{-/-}$	Oat3$^{-/-}$	WT	Oat1$^{-/-}$	Oat3$^{-/-}$
Female	3	9	9	6	20 ± 1	20 ± 1	20 ± 1
	6	7	10	6	21 ± 1	21 ± 2	23 ± 2
	9	8	6	6	22 ± 1	23 ± 1	25 ± 1
	12	7	7	6	22 ± 1	25 ± 1	26 ± 1
	15	2	8	6	25 ± 1	24 ± 1	27 ± 1
	18	7	10	2	26 ± 1	26 ± 1	27 ± 2
Male	3	7	9	6	26 ± 2	26 ± 1	25 ± 1
	6	6	10	6	27 ± 2	29 ± 1	26 ± 1
	9	12	6	10	27 ± 1	30 ± 2	30 ± 1
	12	6	7	6	29 ± 2	31 ± 1	30 ± 2
	15	2	10	6	30 ± 1	35 ± 4	37 ± 1
	18	9	9	2	32 ± 3	33 ± 2	38 ± 2

4.B.2. Behavioral Tests

All subjects were weighed and handled daily for 5 days prior to the start of behavioral assessments to minimize stress-induced handling artifacts. Each animal was assessed in all of the following procedures: open field activity (OFA), light dark exploration (LD), marble burying (MB), and tail suspension test (TST). Animals were randomly assigned to one of the three sequences to eliminate any potential test order bias with a minimum of 3 consecutive days between assessments in the individual paradigms (Figure 4.1). OFA and LD procedures were monitored and recorded with high sensitivity Fire-I firewire cameras (UniBrain Fire-I, San Ramon, CA) suspended above the apparatus and interfaced to AnyMaze software (Stoelting, Wood Dale, IL). The TST was performed at the end of all sequences due to the stress and potentially lasting deficits in motivation induced by this assessment. Prior to the start of any assessment, the mice were allowed to acclimate to the test room for one hour. Following completion of a testing sequence, mice were sacrificed using cervical dislocation and whole brains removed, snapped frozen using liquid nitrogen (<2 min from time of dislocation to snap frozen), and stored at -80°C until further analysis.

Figure 4.1. Schematic depicting the behavioral assay regimen for each sequence. Animals were randomly assigned to one of the three sequences to eliminate any potential test order bias with a minimum of three consecutive days between assessments in the individual paradigms. The TST was performed at the end in all sequences due to the stress and potentially lasting deficits in motivation.



4.B.2.1. Open Field Activity

Animals were tested for a 30 minute trial on 3 consecutive days. The OFA chambers consisted of 41 cm x 41 cm x 46 cm acrylic cubicles. The center zone was defined as a 20 x 20 cm square in the center of the chamber. Mice were then placed in the center zone of the chamber. Total distance traveled and total time mobile were used as indices of basal motor activity. Total time spent in the center zone and the numbers of entries to the center zone were used as indices to determine the degree of thigmotaxis. Mice were evaluated daily for 3 consecutive days in order to determine baseline behavior in a novel environment and habituation to a novel environment. Each subject was tested at the same time each day; however, time of day was randomized across the subjects in each group.

4.B.2.2. Light Dark Exploration

The procedure utilized was based on a previously published study (Bourin and Hascoet, 2003). Animals were tested for a single 30 minute trial. The LD chambers consisted of a single experimental chamber (42 cm x 42 cm x 30 cm) with an enclosed black acrylic box insert (41 cm x 21 cm x 30 cm). There was a small opening (7 cm x 5 cm) present in the base of the black box permitting movement between the light and dark zones. The light zone was comprised of the remainder of the experimental chamber (41 cm x 21 cm x 30 cm) with white walls. Suspended 45 cm above the light zone was a 100 W lamp. Animals were placed in the light zone near the dark zone entrance. Total time spent in the light zone and the numbers of entries into the light zone were scored as indices of anxiety-like behavior using AnyMaze software.

4.B.2.3. Marble Burying

The MB procedure was based on previously published studies (Deacon, 2006; Thomas et al., 2009). Animals were tested for a single 20 minute trial. The testing apparatus consisted of a polycarbonate cage (20 cm x 30 cm x 19 cm) filled to a depth of 5 cm with wood chip bedding (Harlan Sani-Chip, Indianapolis, IN). Marbles (n = 20 blue glass) were evenly spaced in a 4 x 5 grid across the surface of the bedding. Individual mice were placed into the center of the apparatus and left undisturbed in visual and acoustic isolation for 20 min. At the end of the assessment, the mice were removed and the number of marbles buried was manually scored. Marbles buried was defined as ≥ 67% of the marble covered by bedding.

4.B.2.4. Tail Suspension Test

The TST procedure used was based on that described by Steru et al. (1985) with modifications by Yoshikawa et al. (2002) (Steru et al., 1985; Yoshikawa et al., 2002). The apparatus consisted of a 40 cm X 3 cm X 0.5 cm acrylic bar fixed horizontally 40 cm above the laboratory bench providing a smooth flat surface on which to affix the mouse tail. Testing was performed in both acoustic and visual isolation from other subjects. During testing, the mouse was attached to the bar by medical adhesive tape placed approximately 1 cm from the tip of its tail, suspended in an inverted position above a foam pad placed on the bench top. Total time immobile was scored manually over a 6 min assessment. Immobility was defined as the absence of any limb or body movements, except for those caused by respiration.

4.B.3. Analysis of Monoamines and Relative Metabolites

A high performance liquid chromatography (HPLC) method utilizing electrochemical detection (ECD) for the determination of serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA), norepinephrine (NE), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and utilizing ultraviolet (UV) detection for the determination of nicotinic acid (NA), quinolinic acid (QA), and xanthurenic acid (4, 8-dihydroxyquinoline-2-carboxylic acid, XA) in whole brain was developed (Farthing et al., 2014). An internal standard, 3,4-dihydroxybenzylamine (DHBA), was used to account for any loss in analyte due to sample preparation. Brain tissue samples were prepared for HPLC analysis by homogenization in an aqueous solution (deionized water with 1.0% acetic acid and 0.5% phosphoric acid, pH 3.0), followed by centrifugation at 10,000 x g for 10 min (6°C). Resultant supernatants were transferred to plastic HPLC autosampler vials (Waters®, Milford MA, USA) with 5µL injected into the HPLC for analysis. For analysis of QA, NA, and XA, a 1:10 dilution of the supernatants was made to reduce metabolic matrix effects. The HPLC equipment consisted of an Agilent 1200 series degasser (Model G1322A), solvent delivery system (quaternary) (Model G1311A), autosampler (Model G1329A), thermostatted column compartment (Model G1316A), variable wavelength detector (Model G1314D) (Agilent Technologies, Santa Clara, CA, USA), and a Coulochem III electrochemical detector (Thermo Fisher Scientific (ESA, Inc), Fair Lawn, NJ, USA). The analytical column used was an HR-80 C_{18} (80 mm x 4.6 mm I.D; Thermo Fisher Scientific, Fair Lawn, NJ, USA) coupled to a C_{18} guard column (12.5 mm x 4.6 mm I.D.; Phenomenex®, Torrance, CA, USA). The mobile phase consisted of 75 mM sodium dihydrogen phosphate (monohydrate), 1.7 mM 1-octanesulfonic acid sodium salt, 25 µM EDTA, 10% acetonitrile, 1% triethylamine with a final pH of 3.0

adjusted using concentrated H_3PO_4 (prepared using Thermo Fisher Scientific (ESA, Inc.) recommendations for catecholamine analysis), and used at a flow rate of 1.0 mL/min. An injection volume of 5 µL of the prepared tissue homogenate sample was accomplished using the Agilent 1200 series Model G1329A autosampler. Component detection was achieved using ESA, Inc. Coulochem III Electrochemical Detector with a voltage setpoint of 600 mV for DA, 5-HT, NE, DOPAC, HVA, and 5-HIAA. The voltage setpoint was established by conducting a voltammogram for all components and determining the voltage at which responses were optimal. Component detection was achieved for QA, NA, and XA at an UV absorbance wavelength of 260 nm. Data acquisition and component computations were performed using ChemStation™ software (Agilent Technologies, Santa Clara, CA, USA).

4.B.4. Statistical Analyses

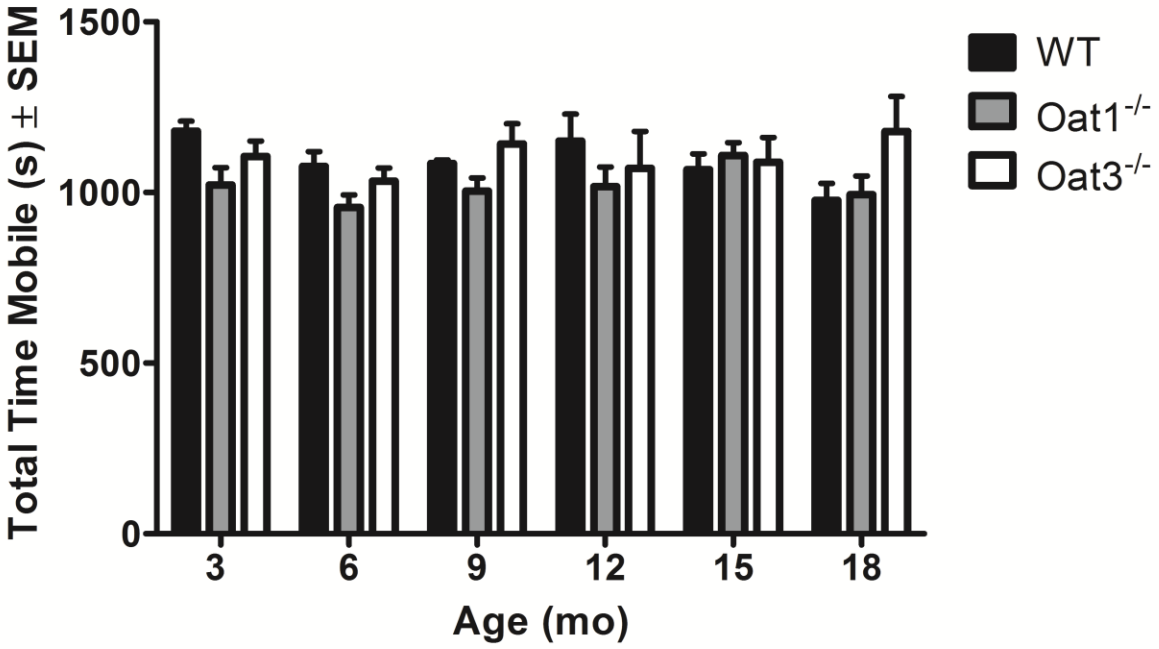
One-way and two-way analyses of variance (ANOVA) were used as appropriate. One-way ANOVA was used to investigate comparisons within genotype where the independent variable was age. Two-way ANOVA was used to investigate comparisons across genotypes where the independent variables were genotype and age. Two-way ANOVA was also used to investigate gender specific comparisons within a genotype where the independent variables were gender and age. Post-hoc comparisons were conducted using Bonferroni pairwise comparisons. The α level for statistical significance was $p < 0.05$.

4.C. RESULTS AND DISCUSSION

4.C.1. Open Field Activity

WT, Oat1$^{-/-}$ and Oat3$^{-/-}$ mice exhibited similar total time mobile (Figure 4.2) and total distance traveled (data not shown) at all age. Additionally, this value remained consistent regardless of age or genotype demonstrating that these parameters had no effect on baseline motor behavior [$F(10,220) = 1.02$, $p = 0.4$]. In fact, there were no significant differences in the total time mobile when comparing Day 2 vs. Day 3 at any given age or within genotype across age (data not shown). When habituation to a novel environment was evaluated by comparing total time mobile on Day 1 vs. total time mobile on Day 2, no significant differences in habituation patterns across genotypes at any given age or within genotype across age were identified (data not shown). There were no significant differences among groups in the time spent in the center zone or the number of center zone entries (data not shown).

Figure 4.2. Total time mobile (mean ± SEM) in an open field for WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ mice across age. Mice of all three genotypes exhibited similar total time mobile indicating basal motor activity was not significantly different across genotype or age. Data are from male and female animals, see Table 4.1 for group details.



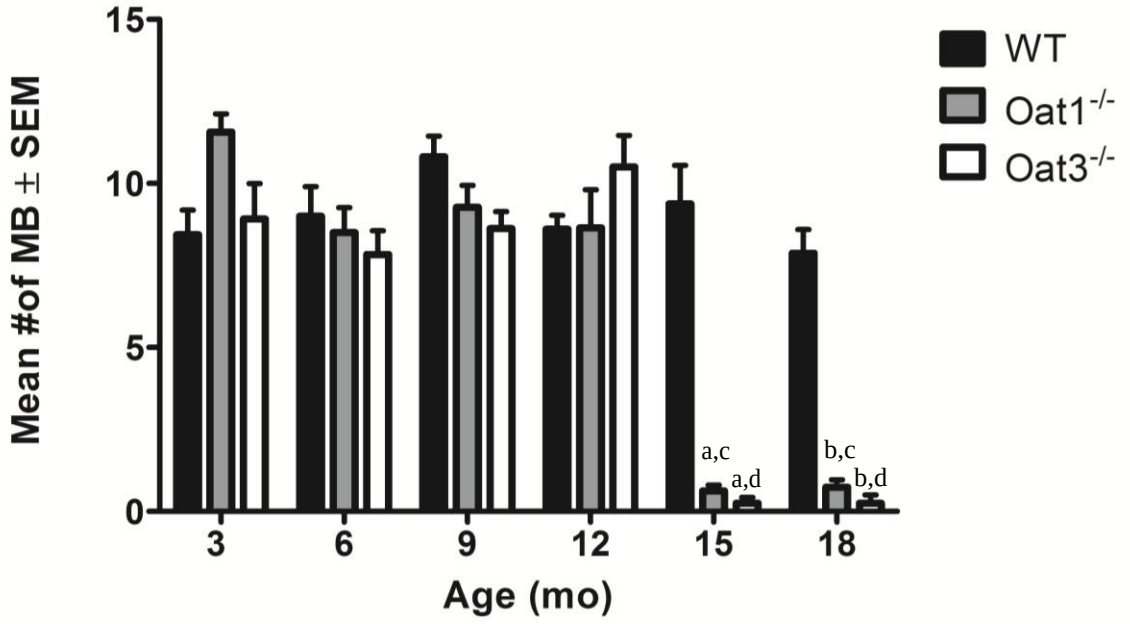
4.C.2. Light Dark Exploration

The number of transitions between the dark and lighted compartments and the time spent in the light zone are measures of anxiety-like behavior. There were no statistically significant differences in the number of transitions or the time spent in the light compartment.

4.C.3. Marble Burying

No significant differences in the number of marbles buried were observed within or across (WT vs. Oat1$^{-/-}$, WT vs. Oat3$^{-/-}$, Oat1$^{-/-}$ vs. Oat3$^{-/-}$) genotypes from 3 to 12 mo of age (Figure 4.3). However, at 15 and 18 mo of age, both Oat1$^{-/-}$ and Oat3$^{-/-}$ animals exhibited a sudden and significant reduction in the number of marbles buried vs age-matched WT control animals [$F(10,220) = 12.56$), $p<0.0001$]. The number of marbles buried by WT mice remained consistent from 3 to 18 mo of age (Figure 4.3). The decrease in burying behavior at 15 and 18 mo observed in Oat1$^{-/-}$ and Oat3$^{-/-}$ animals was significant compared to Oat1$^{-/-}$ and Oat3$^{-/-}$ animals 3 to 12 mo of age, respectively.

Figure 4.3. The number of marbles buried (mean ± SEM) by WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ mice across age. Oat1$^{-/-}$ and Oat3$^{-/-}$ mice almost completely eliminated burying behavior at 15 and 18 mo of age. $^{a}P < 0.001$ compared to WT 15 mo, $^{b}P < 0.001$ compared to WT 18 mo, $^{c}P < 0.001$ compared to Oat1$^{-/-}$ at 3, 6, 9 and 12 mo., $^{d}P < 0.001$ compared to Oat3$^{-/-}$ at 3, 6, 9 and 12 mo.

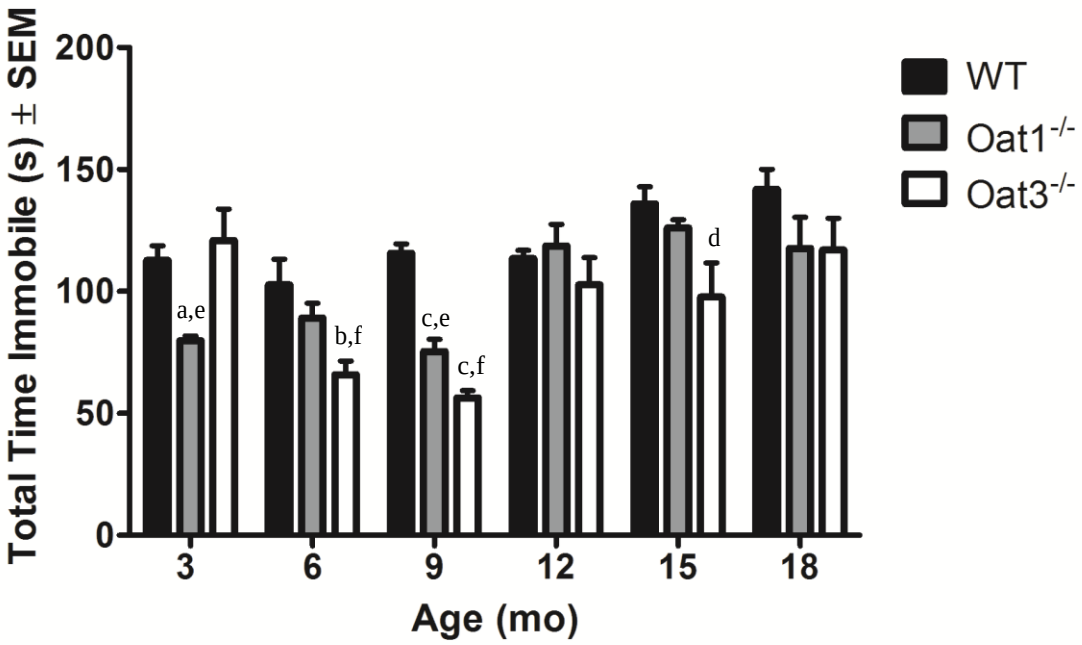


4.C.4. 4. Tail Suspension Test

Three mice (33%) in the 3 mo WT group failed to provide valid data in the tail suspension test because they repeatedly climbed their tails. This behavior has been previously reported as prevalent among mice of C57Bl/6 background between 8-10 weeks of age [Dalvi 1999, Mayorga 2001]. Interestingly, we had no mice successfully climb their tails in either of the Oat deficient genotypes at 3 mo of age.

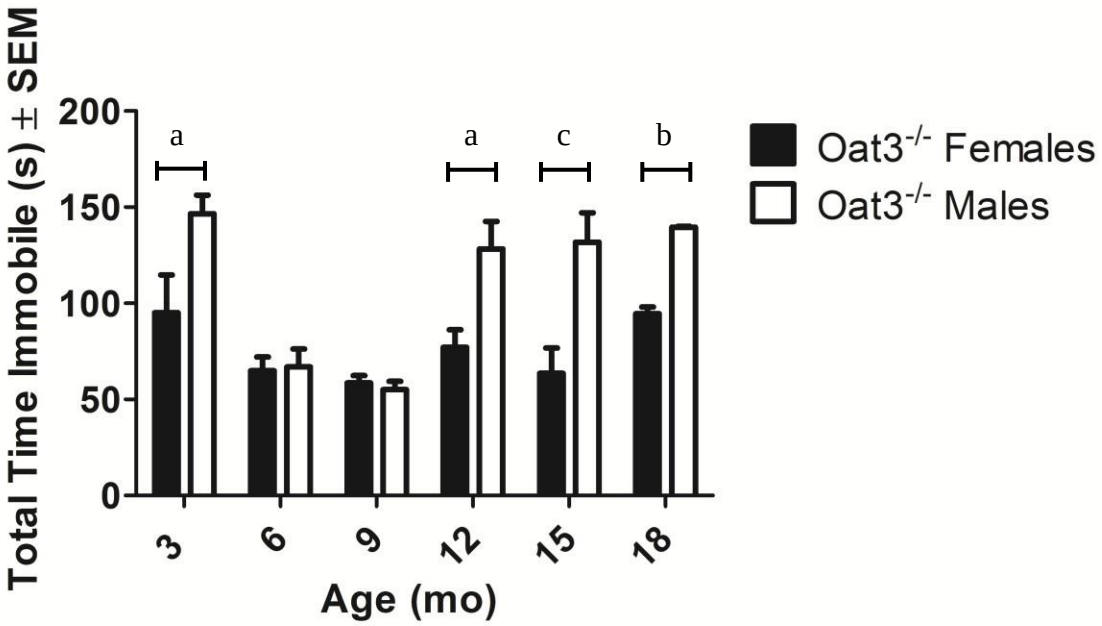
The mean time spent immobile (seconds) for each of the genotypes across age is shown in Figure 4.4. As can be seen, genotype and age had a significant interaction effect on the mean time spent immobile particularly from 3 to 9 mo of age [$F(10,220) = 3.06$), $p = 0.001$]. Post-hoc comparisons revealed that 3 and 9 mo Oat1$^{-/-}$ mice spent significantly less time immobile than similarly aged WT control animals; and a similar trend was observed at 6 mo, but this difference did no reach significance. Oat1$^{-/-}$ mice exhibited a significant increase in total time spent immobiles (vs. 9 mo) from 12 to 18 mo of age, to a level similar to that for WT animals. Oat3$^{-/-}$ animals were similar to WT at 3 mo of age; however, at 6 and 9 mo, they also exhibited a significant decrease in total time immobile vs. WT (Figure 4.4). Immobility time at 12 and 18 mo was similar between WT and Oat3$^{-/-}$ mice; however, unlike Oat1$^{-/-}$ animals, Oat3$^{-/-}$ total time immobile was significantly reduced vs WT at 15 mo. Like Oat1$^{-/-}$ mice, Oat3$^{-/-}$ animals also demonstrated a significant decrease in total time immobile from 12 to 18 mo of age vs. Oat3$^{-/-}$ at 9 mo. Except for the 3 mo Oat3$^{-/-}$ animals, Oat1$^{-/-}$ amd Oat3-/- mice showed similar activity patterns in the TST.

Figure 4.4. Tail suspension test revealed a significant genotype and age interaction effect on the mean time spent immobile. Oat$^{-/-}$ mice tended to display decreased immobility at younger ages (i.e. 3, 6 and 9 mo) compared to age matched WT and themselves at older ages (i.e. 12, 15 and 18 mo). $^{a}P<0.05$ compared to 3 mo WT, $^{b}P<0.05$ compared to 6 mo WT, $^{c}P<0.01$ compared to 9 mo WT, $^{d}P<0.05$ compared to 15 mo WT, $^{e}P<0.05$ compared to Oat1$^{-/-}$ at 12, 15 and 18 mo, $^{f}P<0.05$ compared to Oat3$^{-/-}$ at 12, 15 and 18 mo.



There were no gender specific differences across ages within the WT or Oat1$^{-/-}$ mice. In stark contrast, Oat3$^{-/-}$ mice displayed a significant gender and age interaction effect on the mean time spent immobile [$F(5,56 = 3.7, p = 0.006$] (Figure 4.5). Post-hoc comparisons revealed that Oat3$^{-/-}$ females spent significantly less time immobile compared to their males counterparts at ages 3, 12, 15 and 18 mo ($p \leq 0.05$).

Figure 4.5. Tail suspension test data for Oat3$^{-/-}$ mice parsed by gender. TST data revealed a significant gender and age interaction effect on the mean time spent immobile. Female Oat3$^{-/-}$ mice spent less time immobile compared to age matched Oat3$^{-/-}$ males at 3, 12, 15 and 18 mo. $^{a}P < 0.05$, $^{b}P<0.01$, and $^{c}P<0.001$ compared to age matched counterpart.



4.C.5. Whole brain concentrations of monoamines and relevant metabolites

Table 4.2 lists the mean concentrations of monoamine neurotransmitters (5-HT, DA, and NE) and relevant metabolites (HVA, 5-HIAA, DOPAC, QA, NA and XA) for each experimental group. Genotype and age had a significant interaction effect on the mean concentrations of several of the monoamine neurotransmitters and their relative metabolites. Post-hoc comparisons revealed that tissue levels of DA in $Oat1^{-/-}$ and $Oat3^{-/-}$ mice at 12, 15 and 18 mo of age were significantly decreased compared to age matched WT ($p<0.05$). Similarly, concentrations of the DA metabolites, DOPAC and HVA were significantly lower in aged $Oat^{-/-}$ mice (12, 15 and 18 mo) compared to age-matched WT mice ($p<0.05$). Tissue levels of NE in $Oat1^{-/-}$ and $Oat3^{-/-}$ mice at any given age were not significantly different compared to age matched WT. Interestingly, post-hoc comparisons also revealed that mean tissue concentrations of 5-HT were significantly decreased in WT at 12, 15 and 18 mo of age compared to age matched $Oat1^{-/-}$ and $Oat3^{-/-}$ mice ($p<0.05$). There was an age related decline in WT mice that was absent across all ages in OAT knockout mice. There was no significant change in mean concentrations of 5-HIAA across genotypes at any given age.

One-way ANOVA indicated that the mean concentrations of DA and NE were not significantly different in WT mice across age; however, there was a trend observed for an age dependent decrease in NE concentrations in WT mice ($p = 0.06$). This analysis also revealed a significant difference within the $Oat1^{-/-}$ and $Oat3^{-/-}$ mice across age ($p<0.01$). The mean concentrations of DA and NE at 12, 15 and 18 mo of age in $Oat1^{-/-}$ were significantly lower than concentrations at 3 through 9 mo of age in $Oat1^{-/-}$ mice ($p<0.01$). A significant decrease in mean DA and NE concentrations in aged mice was also observed within the $Oat3^{-/-}$ genotype

($p<0.01$). Similarly, the mean concentrations of DOPAC at 12, 15 and 18 mo of age in $Oat1^{-/-}$ was significantly lower than concentrations at 3 through 9 mo of age in $Oat1^{-/-}$ mice ($p<0.01$). A significant decrease in mean DOPAC concentrations in aged mice was also observed within the $Oat3^{-/-}$ genotype ($p<0.01$). HVA concentrations at 12, 15 and 18 mo of age was also significantly lower compared to ages 3 through 9 mo within both $Oat1^{-/-}$ and $Oat3^{-/-}$ knockout strains. In stark contrast, mean 5-HT concentrations were relatively unchanged in both $Oat1^{-/-}$ and $Oat3^{-/-}$ mice across age, but were found to be significantly decreased within WT across age ($p<0.01$). Concentrations of 5-HT in 12, 15 and 18 mo WT mice were significantly less than mean concentrations observed in WT mice from 3 through 6 mo of age ($p< 0.05$). There was no significant change in mean concentrations of 5-HIAA within genotypes across age.

Lastly, the kynurenine metabolites, QA, NA, and XA, were evaluated in 3, 12 and 18 mo old mice for all three genotypes (Table 4.3). No differences in the mean concentrations of these metabolites either across genotype at any given age or within genotype across age were detected.

Table 4.2. Monoamine neurotransmitter and metabolite concentrations in whole brain in WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ across age.

		Mean Conc. [ng/mg] ± SEM					
Genotype	**Age (mo)**	**DA**	**NE**	**5HT**	**DOPAC**	**HVA**	**5-HIAA**
WT	3	5.5 ± 1.2	4.7 ± 1.2	4.1 ± 0.8	0.5 ± 0.02	0.3 ± 0.04	0.5 ± 0.06
	6	5.3 ± 0.7	4.6 ± 0.9	4.4 ± 1.2	0.7 ± 0.06	0.5 ± 0.09	0.7 ± 0.l
	9	4.7 ± 1.0	4.2 ± 1.4	4.0 ± 0.5	0.5 ± 0.09	0.4 ± 0.06	0.6 ± 0.04
	12	4.6 ± 0.5	4.3 ± 1.4	2.2 ± 0.4	0.5 ± 0.04	0.8 ± 0.06	0.5 ± 0.04
	15	4.0 ±0.4	3.9 ± 0.3	2.1 ± 0.6	0.5 ± 0.06	0.6 ± 0.02	0.6± 0.06
	18	5.4 ± 1.1	3.7 ± 0.8	2.4 ± 0.2	0.6 ± 0.04	0.6 ± 0.08	0.4 ± 0.1
Oat1KO	3	5.7 ± 1.0	5.2 ± 1.7	4.6 ± 0.8	0.8 ± 0.05	0.8 ± 0.06	0.5 ± 0.05
	6	4.8 ± 0.7	5.1 ± 0.8	4.3 ± 0.8	0.6 ± 0.04	0.8 ± 0.02	0.5 ± 0.03
	9	4.5 ± 0.6	4.6 ± 0.4	3.9 ± 0.8	0.7 ± 0.08	0.6 ± 0.01	0.6 ± 0.02
	12	3.0 ± 0.4	3.0 ± 0.5	3.8 ± 1.1	0.4 ± 0.09	0.2 ± 0.06	0.7 ± 0.08
	15	2.8 ± 0.3	2.7 ± 1.0	4.3 ± 0.8	0.4 ± 0.03	0.4 ± 0.06	0.6 ± 0.05
	18	3.2 ± 0.5	3.0 ± 0.6	3.1 ± 0.5	0.3 ± 0.04	0.4 ± 0.05	0.5 ± 0.05
Oat3KO	3	5.4 ± 0.4	4.8 ± 0.8	3.5 ± 1.0	0.7 ± 0.06	0.5 ± 0.05	0.6 ± 0.05
	6	5.2 ± 0.9	6.3± 1.3	3.9 ± 1.2	0.8 ± 0.05	0.6 ± 0.05	0.6 ± 0.07
	9	4.6 ± 0.5	4.2 ± 0.6	3.7 ± 1.1	0.6 ± 0.02	0.6 ± 0.06	0.5 ± 0.04
	12	2.9 ± 0.8	2.5 ± 0.3	3.7 ± 0.4	0.4 ± 0.07	0.3 ± 0.09	0.7 ± 0.04
	15	2.7 ± 0.5	3.0 ± 0.5	4.0 ± 0.5	0.3 ± 0.02	0.4 ± 0.04	0.5 ± 0.08
	18	3.0 ± 1.0	2.1 ± 0.6	3.6 ± 0.7	0.3 ± 0.05	0.3 ± 0.06	0.6 ± 0.05

Table 4.3. Kynurenine pathway metabolite concentrations in whole brain in WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ at 3, 12, and 18 mo of age.

Genotype	Age (mo)	Mean Conc. [µg/g] ± SEM		
		QA	NA	XA
WT	3	28.8 ± 4.2	26.7 ± 3.5	15.4 ± 2.4
	12	32.4 ± 6.5	23.5 ± 5.9	13.2 ± 3.2
	18	33.2 ± 5.1	23.8 ± 5.2	12.6 ± 4.2
Oat1KO	3	27.6 ± 9.0	25.4 ± 3.4	12.6 ± 4.8
	12	24.9 ± 4.8	26.5 ± 2.8	13.5 ± 3.4
	18	23.9 ± 5.1	25.7 ± 4.1	14.0 ± 2.5
Oat3KO	3	24.6 ± 26	25.8 ± 3.6	16.2 ± 4.5
	12	26.5 ± 6.3	27.3 ± 4.4	14.7 ± 5.5
	18	30.2 ± 10.0	31.9 ± 8.2	13.7 ± 4.4

4.D. DISSCUSION

The aim of the present study was to evaluate neurochemical and behavioral effects associated with loss of Oat1 or Oat3. Four behavioral paradigms were used to provide information on basal motor behavior, and anxiety-like and depressive-like behaviors across 6 age points from 3 mo to 18 mo in WT and OAT knockout mice bred and housed under the same conditions. Following the behavioral assessments, concentrations of neurotransmitters and their respective metabolites were determined using HPLC methodology. Our studies provide the first evidence of the significant effects of OAT transporter deletion on multiple measures of anxiety-like/depressive-like behaviors and concentrations of monoamine neurotransmitters and their metabolites in whole brain.

The OFA paradigm is a classical test of exploratory locomotor behavior that provides a measure of basal activity and thus is an essential component of behavioral phenotyping. In addition, patterns of activity within the field can be determined providing additional information about novel strains. In open fields, animals that spend more time in the periphery of the field (thigmotaxis) are said to display a more anxious-like phenotype than those that freely explore the center of the field (Prut and Belzung, 2003). Additionally, generalized locomotor hyperactivity may reflect environmental neophobia (an increased fear of novel environments). In the current study, the total time spent mobile and the total distance traveled in the OFA paradigm were parameters used to measure basal motor activity across genotype at any given age. There were no significant differences in these measures across age for each of the genotypes or across genotype at any given age (as seen in Figure 1, and data not shown). In addition, when parsed by gender, there were no gender-specific effects on basal motor function. Therefore, any alterations observed in anxiety-like or depressive-like behaviors in the additional

paradigms are not simply reflective of genotype or aged-related differences in basal motor activity. Similarly, there were no significant differences in thigmotactic behavior observed when comparing the distance traveled in the center zone and time in the center zone as a percent of the total distance traveled and total time mobile in the field across strain by age, genotype, or gender. Finally, when we evaluated habituation to a novel environment by comparing total distance traveled and total time mobile in the field on Day 1 vs. Day 2, we found that there were no significant differences across age for any of the genotypes, or across genotype at any given age, in habituation patterns.

Marble burying has been suggested as a model to measure anxiety-like behaviors, specifically compulsive behaviors, and as a possible model of obsessive compulsive disorder in rodents (Thomas et al., 2009; Albelda and Joel, 2012). The effects of OAT deletion were most profound in the MB procedure where $Oat1^{-/-}$ and $Oat3^{-/-}$ mice almost completely ceased burying behavior at 15 and 18 mo of age. These findings may suggest decreased compulsive-like behavior in the aged OAT knockout mice. Because results from the OFA (Figure 1) failed to demonstrate any significant differences in the total time spent mobile or the total distance traveled in the field, the MB results do not simply reflect lower basal activity levels, but appear to demonstrate true differences in burying behavior. The pattern of anxiolytic effect seen in our aged knockout mice is similar to what has been observed in serotonin transporter (SERT) and dopamine transporter (DAT) knockout mice when studied in the MB procedure (Line et al., 2011; Pang et al., 2011; Fox et al., 2013). However, it has been proposed that the decrease in burying behavior observed in DAT knockout mice was a result of hyperactivity and disinterest in the marbles noted during the MB procedure (by personal observation; Fox et al., 2013). In marked contrast to the locomotor observations in DAT KO mice during the MB procedure, no

differences in locomotor activity during the MB procedure was observed in either knockout strain across all ages. It should be noted that throughout the 30 min test, the mice were active, but at 15 and 18 mo the $Oat^{-/-}$ and $Oat3^{-/-}$ animals essentially maneuvered around the cage without disturbing the marbles (personal observation).

It was generally hypothesized that due to similarities in both function and location of the Oat1 and Oat3 transporters, behavioral and neurochemical alterations would be similar between the two genotypes across age. In the paradigms measuring anxiety-like behavior, this hypothesis remains true with the $Oat1^{-/-}$ and $Oat3^{-/-}$ strains providing similar behavioral alterations; however, in the TST paradigm measuring depression-like behavior, the genetic background of OAT deficient mice displayed some differences. When comparing the TST data in the knockout strains to each other at each age, the point where they significantly deviated from each other was at 3 mo of age. As demonstrated in Figure 4.4, genotype and age had a significant impact on the mean time spent immobile with 3 mo $Oat1^{-/-}$ mice spending significantly less time immobile than $Oat3^{-/-}$ and WT mice at 3 mo. When comparing the knockout genotypes relative to WT at each time point, there were differences at varying ages with an overall trend of knockout mice spending less time immobile compared to WT. The $Oat1^{-/-}$ mice spent significantly less time immobile compared to age-matched WT at 3 and 9 mo of age, while the 6 mo $Oat1^{-/-}$ mice tended to also spent less time immobile than WT. $Oat3^{-/-}$ mice spent significantly less time immobile compared to age-matched WT at 6 and 9 mo of age. Perhaps, the inconsistencies in this measure of depressive-like behavior can be partly explained by the observed gender-specific variations in mean time spent immobile found only within the $Oat3^{-/-}$ strain. $Oat3^{-/-}$ females spent significantly less time immobile compared to their male counterparts at ages 3, 12, 15 and 18 mo of age. Nonetheless, the decreased immobility

observed in our OAT knockout animals in the TST is comparable to published observations in the DAT, SERT, and norepinephrine transporter (NET) knockout mice, providing further evidence for involvement of OAT transporters in behaviors known to be regulated by monoaminergic neurotransmission (Xu et al., 2000; Holmes et al., 2002; Perona et al., 2008).

Mean brain concentrations of DA and major metabolites, DOPAC and HVA, were significantly lower in aged (12, 15 and 18 mo) Oat1 and Oat3 deficient mice compared to aged matched WT levels. Interestingly, this decrease in DA did not cause any alterations in locomotion (i.e. hypolocomotion) as evident from the equivalent total distance traveled and total time mobile between all three strains in the OFA paradigm, as one would expect based on previous reports (Haenisch and Bonisch, 2011). It is possible that this alteration in DA concentration was not severe enough to elicit any phenotypical response in locomotor behavior or that the paradigm does not have adequate sensitivity to detect the degree of change. Conversely, mean tissue levels of 5-HT were found to be significantly lower in aged WT mice, but remained relatively unchanged in OAT knockout mice across age providing a possible correlative neurochemical change that could partly explain observed alteration in burying behavior in the $Oat1^{-/-}$ and $Oat3^{-/-}$ knockout mice . However, changes in tryptophan metabolic pathway enzyme levels may also occur and this remains to be explored.

Because tryptophan has two metabolic pathways, the serotonergic pathway leading to production of 5-HT and 5-HIAA, and the kynurenine pathway leading to the production of uremic toxins (i.e. QA, NA, and XA) reportedly associated with inflammatory and non-inflammatory neurological diseases (i.e. schizophrenia) (Heyes et al., 1992; Erhardt et al., 2009; Olsson et al., 2012). In addition, given that these metabolites have been shown to interact *in*

vitro with Oat1 and Oat3 transporters, it was of interest to investigate the possible impact of OAT deletion on concentrations of these metabolites in brain tissue. However, based on our findings, it appears that transporter deletion did not significant impact concentrations of these kynurenine metabolites.

This study provides the first evidence indicating that Oat1$^{-/-}$ and Oat3$^{-/-}$ mice may serve as useful tools to further delineate the mechanisms underlying CNS control of monoamine homeostasis and suggesting a potential role for these transporter proteins in neurobehavioral disorders such as anxiety and depression. Alterations in the glutamatergic neurotransmission system have recently been shown to play a clinical role in compulsivity (Chakrabarty et al., 2005; Egashira et al., 2008). Thus future studies aimed to study the effects of Oat1 and Oat3 deletion on this system are needed as well. In addition, it would be of interest to investigate if pharmacologic antagonism of the Oat1 and Oat3 transporters produces similar behavioral and neurochemical effects as are observed in the OAT knockout models and reported consequences following administration of current pharmacotherapies used clinically for treatment of these affective disorders (i.e. selective serotonin reuptake inhibitors, serotonin/norepinephrine uptake inhibitors (Borsini et al., 2002; Cryan et al., 2005; Li et al., 2006; Castagne et al., 2011). However, comparisons of pharmacological antagonism of Oat1 and Oat3 vs. genetic deletion should be conservative as differences in behavioral phenotypes may be reflective of deletion during development.

Further, it is also of interest to study the effects of Oat1 and Oat3 deletion on other factors shown to be involved in maintaining the CNS environment. As mentioned previously, one aspect of CNS regulation of monoaminergic neurotransmission involves pathways targeted

toward the efficient removal of released neurotransmitters (NTs), so-called uptake 1 and uptake 2 transport systems. Neuronal uptake 1 is comprised of the well-studied DAT, SERT, and NET systems, which function to ensure rapid reuptake of released NTs by the pre-synaptic neuron. Additionally, members of the organic cation/anion/zwitterion transporter family (SLC22) have been suggested as the second, non-neuronal low affinity, high capacity transport system, uptake 2, which functions to clear NTs and their degradative metabolites from the synaptic terminal region. Recent studies have found that NET knockout mice exhibited upregulation of the other reuptake transporters, DAT and SERT, in the brain indicating a compensatory change in the within the uptake 1 system as a result of loss of NET during development (Solich et al., 2011). Similarly, SERT KO mice demonstrated upregulated organic cation transporter 3 levels, providing evidence of compensatory changes in SLC22 transporters (proposed uptake 2 mechanism) in response to loss of one of the uptake-1 transporters during development (Schmitt et al., 2003; Baganz et al., 2008). Lastly, as discussed above, many factors can influence clearance of neurotransmitters and relevant metabolites, including enzymatic breakdown. It was recently reported that DAT knockout mice exhibited down-regulated tyrosine hydroxylase, the enzyme responsible for metabolism of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor for DA and subsequently NE (Haenisch and Bonisch, 2011). In contrast, Daws et al found that SERT knockout mice exhibited no change in the levels of monoamine oxidase enzymes, MAO-A and MAO-B, responsible for catalyzing the oxidation of monoamine NTs to their acidic metabolites (Daws, 2009). Nonetheless, enzyme levels are still a potential factor that should later be investigated in this knockout model. In conclusion, behavioral and neurochemical analyses in OAT knockout mice revealed alterations in both anxiety-like and depressive-like behaviors with correlative changes in clearance of monoaminergic

neurotransmitters and metabolites. Additional work is required to further understand how genetic deletion of these transporters produces decreased susceptibility to anxiogenic and depressive phenotypes.

CHAPTER 5

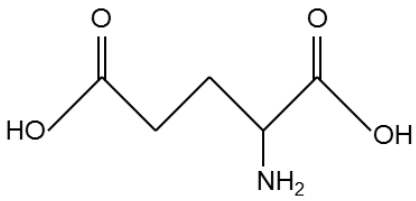
DETERMINATION OF L-GLUTAMIC ACID AND γ-AMINOBUTYRIC ACID IN MOUSE BRAIN TISSUE UTILIZING GC-MS/MS

Drawn from manuscript submitted to *Journal of Chromatography B*, 2014.

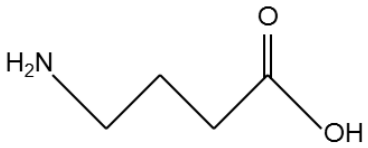
5.A. INTRODUCTION

L-Glutamic acid (GA; MW=147.13 g/mol) and γ-aminobutyric acid (GABA; MW=103.12 g/mol) are neurotransmitters found in the brains of mammals. GABA is synthesized from GA by glutamate decarboxylase (Figure 5.1), yet the functions of these two neurotransmitters have opposing effects. GA is considered a major excitatory neurotransmitter in the central nervous system (CNS), while GABA is considered a major inhibitory neurotransmitter. Quantitation of these two neurotransmitters is important to the field of neuropharmacology, as they have been associated with learning and memory, the sleep cycle, and in clinical conditions including schizophrenia, Alzheimer's disease, Parkinson's disease, depression, anxiety, and obsessive-compulsive disorder (Bjarkam et al., 2001; Reinoso-Suarez et al., 2001; Das, 2003; Lewis et al., 2005; Yang and Shen, 2005; Bhagwagar et al., 2007; Gronli et al., 2007; Egashira et al., 2008; Schaeffer and Gattaz, 2008).

Figure 5.1. Structures of L-Glutamic Acid and γ-Aminobutyric Acid.



L-Glutamic Acid



γ-Aminobutyric Acid

High performance liquid chromatography with electrochemical detection (HPLC-ECD) techniques have previously been used to study amino acid concentrations in the brain (Canevari et al., 1992; Murai et al., 1992; Boyd et al., 2000; Monge-Acuna and Fornaguera-Trias, 2009). Since the amino acids are not naturally electroactive, these methods typically require a derivatization step (usually using o-phthalaldehyde (OPA) in the presence of a thiol (e.g. β-mercaptoethanol) or a sulfite group). However, despite the sensitivity that can be achieved using HPLC-ECD, the electroactive derivatives are often unstable, and it has been reported that resolution may be problematic due to unknown peaks of biological origin that elute closely to GABA (Rowley et al., 1995; Clarke et al., 2007; Buck et al., 2009).

In addition, there have been several publications using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of GA and GABA in biological samples (Piraud et al., 2003; Song et al., 2005; Eckstein et al., 2008). However, to our knowledge, there are no currently published methods utilizing gas chromatography-tandem mass spectrometry (GC-MS/MS) offering quantitation of both GA and GABA in brain tissue. In this article, we describe a rapid and selective GC-MS/MS method for the detection and quantitation of GA and GABA in mouse brain homogenate. Stable isotopes of each compound were used as internal standards. The use of MethElute™ reagent (as described in (Farthing et al.)) rapidly derivatized GA and GABA and their isotopologues in the heated GC injection port, which required less than 20 sec for completion and utilization of the low thermal mass (LTM) technology provided high resolution and fast chromatography. The method demonstrated excellent linearity from 0.5 to 100 μg/mL, with limits of detections of 100 ng/mL and 250 ng/mL for GA and GABA, respectively. The method was successfully used to assess the effects of deletion of the organic

anion transporter 1 (Oat1) and Oat3 in the mouse model on brain tissue concentrations of glutamate and GABA.

5.B. EXPERIMENTAL

5.B.1. Chemicals, Reagents, and Gases

Standard material of L-Glutamic acid and γ-aminobutyric acid were purchased from Sigma Aldrich (St. Louis, MO). The $^{13}C_5$-L-Glutamic acid (M+5 stable isotope, purity 99%) and U-$^{13}C_4$-4-aminobutyric acid (M+4 stable isotope, purity 98%) internal standards were purchased from Cambridge Isotopes (Andover, MA).

MethElute™ derivatization reagent (0.2 M trimethylanilinium hydroxide in methanol, pH ≥ 10) was purchased from Thermo Scientific (Waltham, MA). Methanol (MeOH) (HPLC grade, 99.9% purity) was purchased from Acros Organics (Fair Lawn, NJ). Ultrapure deionized water (18 Ωohm-cm) was prepared daily using a Milli-Q Integral Water Purification system from EMD Millipore (Billerica, MA).

Gases used for GC-MS/MS analyses were helium (grade 5.5 purity), nitrogen (ultra high purity), isobutane (Matheson 99.99% purity) and methane (research grade purity) and were all purchased from Roberts Oxygen Company (Rockville, MD). Gas purifiers used to remove hydrocarbons and moisture from the gases were purchased from Agilent Technologies (Santa Clara, CA).

5.B.2. Equipment and Software

The Agilent 7890 GC, LTM series II fast GC module, 7000A GC-MS triple quadrupole, and 7693 autosampler were purchased from Agilent Technologies (Santa Clara, CA). The Agilent MassHunter software was used for GC-MS/MS data collection and processing including GC-MS/MS Acquisition (version B.05.02.1032), Qualitative Analysis (version B.04.00), and Quantitative Analysis (version B.05.00)

An Eppendorf Model 5417R centrifuge was used to centrifuge down any particulate in the tissue homogenate prior to the derivatization step (Hauppauge, NY). The Savant DNA110 SpeedVac® used for evaporating and concentrating samples was purchased from Thermo Fisher Scientific (Waltham, MA).

5.B.3. GC-MS/MS Instrument Conditions

The final GC and MS instrumental parameters used for sample analysis are listed in Table 5.1 and Table 5.2, respectively.

Table 5.1. Agilent 7890A GC and Autosampler Parameters

GC Inlet	
Mode	Pulsed Splitless
Temperature (°C)	235
Total Flow (mL/min Helium)	54
Septum Purge Flow (mL/min Helium)	3
Injection Pulse Pressure (psi until 1 min)	25
Purge flow to split vent (mL/min Helium at 1.1 min)	50
Gas saver (mL/min Helium after 3 min)	15
GC Program	
Initial GC Oven Temperature (°C)	30
Initial Oven Hold Time (min)	1
Rate (°C/min)	75
Final GC Oven Temperature (°C)	300
Final Over Hold Time (min)	1.4
Run Time (min)	6
Equilibration Time (min)	0.1
GC Transfer Line (°C)	300
Oven Max Temperature (°C)	340
GC Column	
LTM Column	DB-5ms, 15m x 250µm x 0.25µm
Head Pressure (psi)	2.14
Flow (mL/min Helium)	1
Average Velocity (cm/sec)	47
Holdup Time (min)	0.5
GC Autosampler	
Syringe Size (µL)	10
Injection Volume (µL)	1
Post Washes A	3 with ethanol
Post Washes B	3 with acetone
Samples Washes	1
Sample Wash Volume (µL)	4
Samples Pumps	3
Viscosity Delay (sec)	3
Air Gap (µL)	0.2
GC Autosampler Barcode Mixer	
Mixer	Enabled
Mixer Cycle	1
Mixer Time (sec)	10
Mixer Speed (rpm)	2,000

Table 5.2. Agilent 7000A MS Parameters

Helium Quench Gas (mL/min)	2.25
Nitrogen Collision Gas (mL/min)	1.5
Isobutane Reagent Gas (mL/min)	2
Ion Source Temp (°C)	350
Quad 1 Temperature (°C)	150
Quad 3 Temperature (°C)	150
Ion Source	Chemical Ionization (CI)
Mode	Positive Ion (PCI)
Electron Energy Mode	Use tune settings
Solvent Delay (min)	1.0
Run Time (min)	6.0
Time Filter Enabled	
Peak Width	0.7
MS1 Resolution	Unit
MS2 Resolution	Unit
Dwell Time (msec)	50
Scan Rate (cycles/sec)	5
Collision Energy-GA (V)	15
Collision Energy-GABA (V)	20
Electron Multiplier (V)	1600
Delta EMV (V)	400
HED (kV)	-10
Instrument Tuning (Mass Calibration)	
Tune MS weekly or as necessary	
MRM Transitions	
GA derivative (precursor ion/product ion)	m/z 204/144
d5-GA derivative (precursor ion/product ion)	m/z 209/148
GABA derivative (precursor ion/product ion)	m/z 146/101
D4-GABA derivative (precursor ion/product ion)	m/z 150/105

5.B.4. GC-MS/MS Calibration Standards and Internal Standards

Stock standard solutions of GA and GABA (1 mg/mL) were prepared in deionized water and deionized water:MeOH (50:50 v/v), respectively, and stored at -20°C until analysis. GA was found to be insoluble in aqueous solutions with varying percentages of organic modifiers (e.g. 70/30 v/v; 80/20 v/v; 90/10 v/v deionized water:MeOH). Working calibration standards (0.5, 2.5, 5, 10, 25, 50 and 100 μg/mL) were prepared containing GA and GABA, and their stable isotopes GA M+5 and GABA M+4 (internal standards) and stored at -20°C until analysis. The calibration standards were prepared by adding 50 μL of standard to the microvial insert (Agilent, Santa Clara, CA) and evaporated to dryness (~45 min at 45°C) using the Savant DNA SpeedVAC. For reconstitution, 50 μL of the MethElute™ derivatizing reagent was added to the autosampler microvial containing the sample residue, capped, and vortexed for 15 sec prior to placement on the autosampler. The internal standard (I.S.) working solution was prepared by appropriate dilutions of the stock solution with deionized water. The I.S. working solution contained 10 μg/mL GA M+5 and GABA M+4.

5.B.5. Brain Sample Preparation

Tissue samples were homogenized prior to analysis by GC-MS/MS. For sample analysis, brain homogenates were thawed, vortexed for 30 sec, and centrifuged at 2500 rpm for 5 min. Following centrifugation, 50 μL of brain homogenate and 50 μL of internal standard were added to the microvial insert (Agilent, Santa Clara, CA) and evaporated to dryness (~45 min at 45°C) using the Savant DNA SpeedVAC. For reconstitution, 50 μL of the MethElute™

derivatizing reagent was added to the autosampler microvial containing the sample residue, capped, and vortexed for 15 sec prior to placement on the autosampler.

5.B.6. Method Validation

The method was validated in terms of linearity, sensitivity, selectivity, intra- and inter-day accuracy and precision, and stability. The linearity was evaluated using a weighted (1/x) quadratic analysis of calibration standards from 0.5-100 μg/mL (n = 3). The sensitivity was established by determining the lower limit of quantitation (LLOQ) and the limit of detection (LOD) for each component. The combined inter-day and intra-day accuracy and precision of the method was evaluated using calibrators from the standard curves (n = 3). Instrument injector precision and derivatization reproducibility were evaluated by using replicate injections of the 25 μg/mL calibration standard (n=6). The stability of GA and GABA were evaluated using the mid calibration standard (10 μg/mL) at 0, 3, 5, 7 and 18 hours at room temperature on the autosampler. Freeze/thaw stability was evaluated using wild-type control brains prepared and injected following one freeze thaw cycle.

5.B.7. Computations

Component computations were performed using MS multiple reaction monitoring (MRM) mode and peak height response with MassHunter quantitation software (Agilent Technologies, Santa Clara, CA). The seven point calibration curve was a plot of the standard concentrations versus the ratio of the peak height of the component and its internal standard. Calibration curves were constructed for each analytical run.

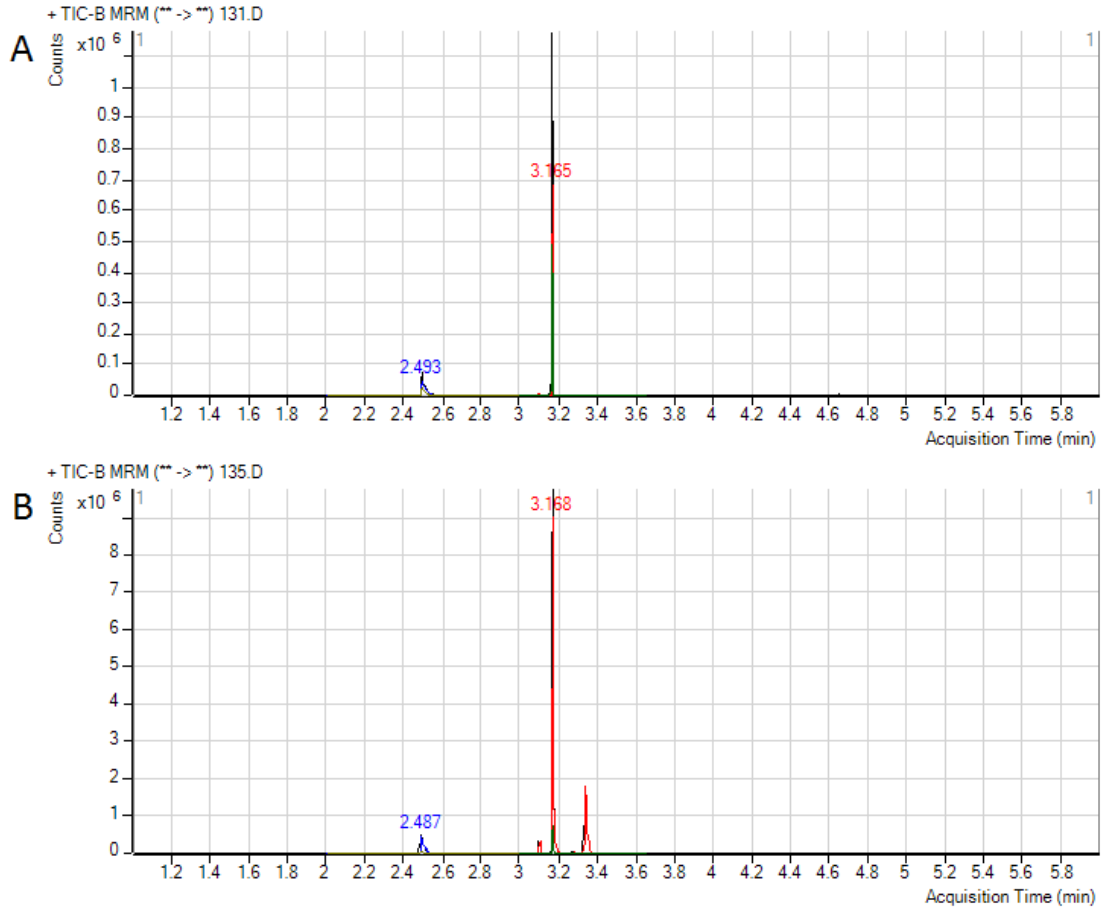
5.C. RESULTS AND DISCUSSION

5.C.1. GC-MS/MS Method Development and Optimization

DB-17ms and DB-5ms columns were evaluated for this method. The DB-5ms column provided better peak shape of GABA, as compared to the moderately polar DB-17ms column, most likely due to the non-polar nature of the GABA derivative.

MS/MS optimizations were performed for both GA and GABA. Samples were injected in chemical ionization mode (positive ion mode) with data collected at unit mass resolution in MRM mode. The precursor ions were isolated in the first quadruple and further fragmented with different collision energies (15, 20 V) with optimal collision energies of 15V for GA and 20V for GABA. The third quadruple then sorts these fragments for detection. The main characteristic ions of GA and GABA produced major product ions at *m/z* 144 and *m/z* 101, respectively. Therefore, the precursor/product ion pairs of *m/z* 204 → *m/z* 144 and *m/z* 146 → *m/z* 101 were chosen as MRM transitions of GA and GABA, respectively. Figure 5.2 shows chromatogram overlays representing MRM of the 25 µg/mL standard and a representative brain sample.

Figure 5.2. Chromatographic overlays representing typical multiple reaction monitoring of the (A) 25 µg/mL standard and (B) a representative brain sample.



The method can be made more sensitive by altering the sample preparation. For the current purpose, a 1:1 ratio (50μL sample: reconstituted in 50 μL derivatizing agent) was used for reconstitution; however, by changing the ratio (v/v) (e.g. 150 μL sample: reconstituted in 25 μL derivatizing agent) sensitivity was increased 5 fold (data not shown).

5.C.2. GC-MS/MS Method Validation

The calibration curve (peak height ratio of analyte to internal standard vs. concentration) for both GA and GABA was evaluated using a 1/x weighted quadratic analysis and was linear over the range of 0.5-100 μg/mL (n = 3) with a correlation coefficient value ≥ 0.998. A typical calibration plot for each component is shown in Figure 5.3. The lower limit of quantitation (LLOQ) for both components was defined as the lowest calibration standard (0.5 μg/ml), which can be accurately and precisely determined with less than 20% total error. The limit of detection (LOD) was determined by conducting dilutions of each component's lowest calibration standard until the MS signal approached background noise. The LOD was determined to be 100 ng/mL and 250 ng/mL for GA and GABA derivatives, respectively (Figure 5.4).

Figure 5.3. A typical calibration curve (peak height ratio of analyte to internal standard vs. concentration) for (A) GA and (B) GABA using a 1/x weighted quadratic analysis. The standards used for calibration were prepared at concentrations of 0.5, 2.5, 5, 10, 25, 50 and 100 µg/mL.

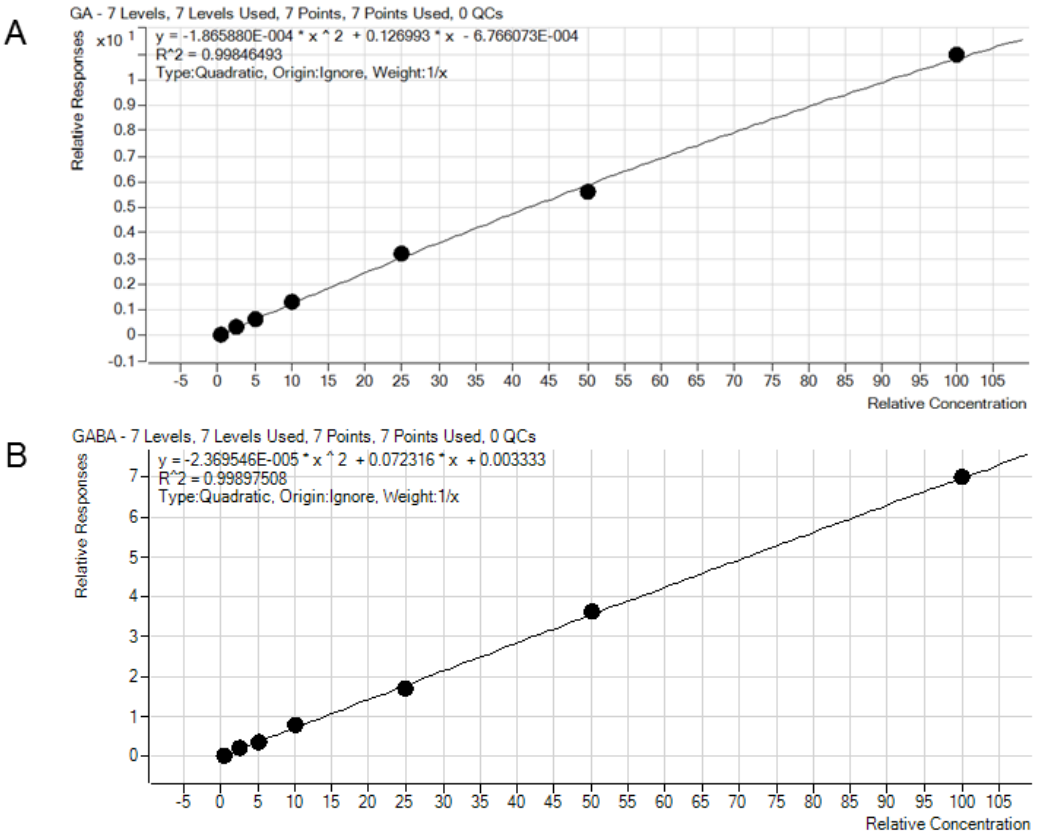
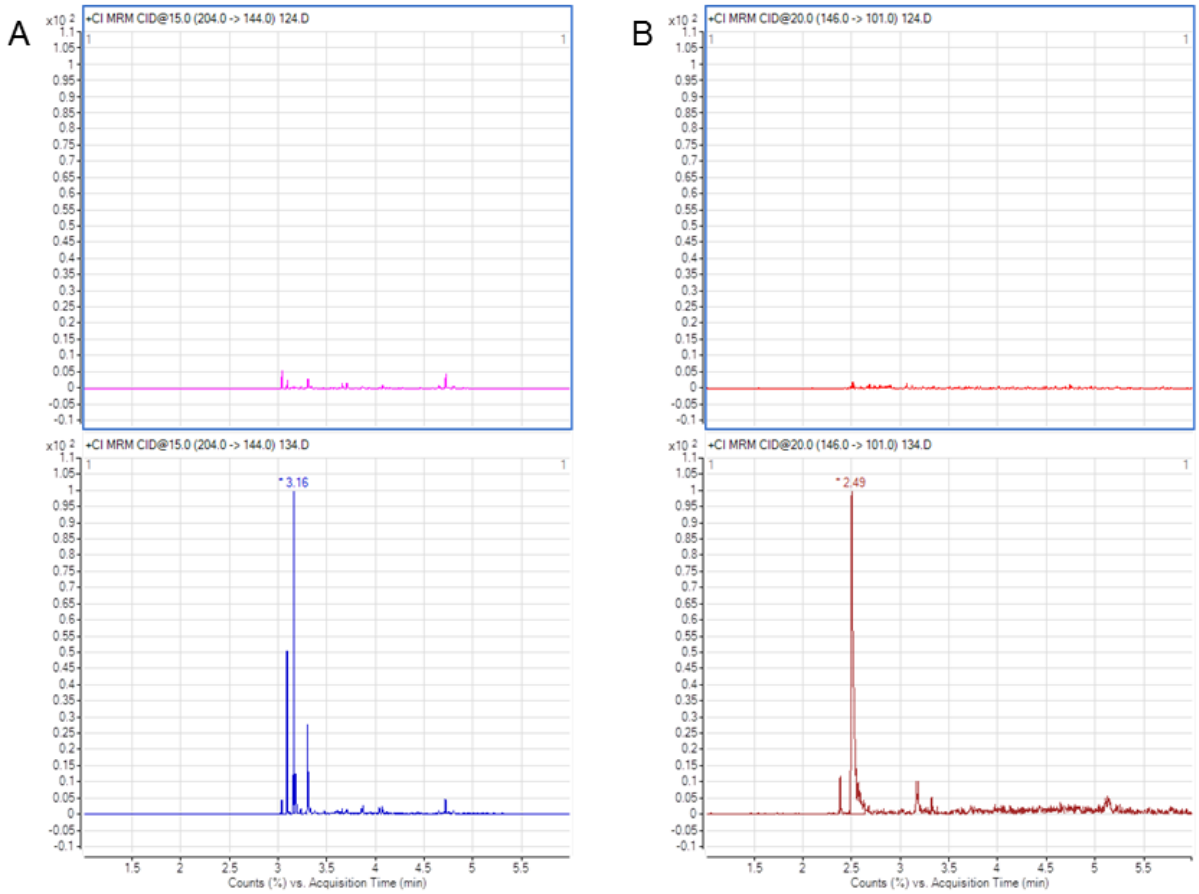


Figure 5.4. Chromatogram overlays representing multiple reaction monitoring of the LOD for GA (panel A bottom; 100 ng/mL at RT = 3.16 min) and GABA (panel B bottom; 250 ng/mL at RT = 2.49 min) relative to MethElute™ derivatizing agent only (top panels).



The combined intra-day and inter-day accuracy and precision were evaluated using standard curves (Table 5.3). Accuracy (% error) was determined by the difference between ((measured concentration-nominal concentration)/(nominal concentration))*100. Precision (%CV) was determined by dividing the standard deviation by the mean (SD/mean)*100. Intra-day instrument injector precision and online heated GC inlet derivatization reproducibility were evaluated by using repetitive injections of the 25 μg/mL calibration standard (Table 5.3). The instrument injector precision was less than 7% C.V. and online heated derivatization procedure was very reproducible.

Table 5.3. Intra-day and Inter-day accuracy and precisions of GA and GABA calibration standards.

Combined Intra-Day and Inter-Day Accuracy and Precision									
GA					**GABA**				
Conc.	Measured Conc.	SD	% Error	% CV	Conc.	Measured Conc.	SD	% Error	% CV
0.5	0.5	0.02	-3.3	3.2	0.5	0.5	0.02	0.0	3.5
2.5	2.5	0.18	-1.6	7.4	2.5	2.5	0.03	1.2	1.2
5	4.8	0.22	-3.3	4.6	5	4.9	0.03	-1.8	0.7
10	10.8	0.21	7.8	2.0	10	10.4	0.33	4.2	3.1
25	25.9	1.07	3.9	4.1	25	23.7	0.20	-5.2	0.8
50	47.4	1.51	-5.2	3.2	50	51.3	0.36	2.7	0.7
100	101.1	0.59	1.1	0.6	100	97.9	2.69	-2.1	2.7

Instrument Precision and Derivatization Reproducibility									
GA					**GABA**				
Conc.	Measured Conc.	SD	% Error	% CV	Conc.	Measured Conc.	SD	% Error	% CV
25	25.4	1.59	1.4	6.3	25	24.1	0.3	-3.7	1.4

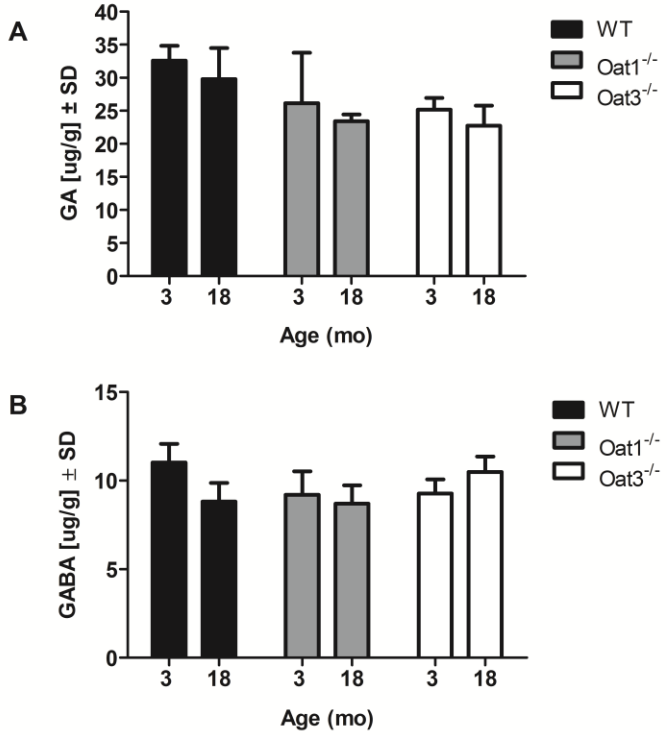
A stability study was performed to evaluate the component's stability in the MethElute™ derivatizing reagent. The stability of GA and GABA were evaluated using the mid calibration standard (10 µg/mL) at 3, 5, 7 and 18 hours at ambient room temperature on the autosampler, as compared to freshly prepared and analyzed 10 µg/mL calibration standard. The results of the stability study indicate that GA and GABA are stable in the MethElute™ derivatizing agent for at least 7 hrs (typical run length), but are not stable at 18 hrs (< 10% degradation of GA, and ~30-40% degradation of GABA). Freeze/thaw stability was also evaluated using wild-type control brains (n =4) prepared and injected following one freeze thaw cycle. Both components were stable through one freeze thaw cycle.

5.C.3. Method Application

The validated method has successfully been applied for the quantitative detection of GA and GABA in mouse brain tissue of organic anion transporter (OAT) knockout mice. Mice (C57Bl/6J background) of three genotypes: wild-type (WT), Oat1 transporter knockout ($Oat1^{-/-}$), and $Oat3^{-/-}$ at two ages (3 mo vs. 18 mo of age) were used for initial assessment (n = 4 in each group). It was hypothesized based on our previous behavioral assessments (specifically, in the marble burying procedure, a model of compulsive-like behavior) that the GA/GABA system may be altered over age from the loss of OAT transporter function in the brain. Brain tissue samples were previously collected following completion of the behavioral assessments, and homogenates were aliquoted and stored at -80°C until analysis. The concentrations of GA and GABA in brain homogenates for each group are shown in Figure 5.5 (panel A). As can be seen from Figure 5.5 (panel B), GABA remained relatively unchanged across and within genotype. GA concentrations also remained unchanged within all three genotypes; however, a trend was

observed with lower concentrations of GA in both of the aged OAT knockout genotypes compared to age matched WT mice.

Figure 5.5. The concentrations of GA and GABA in brain homogenates for each group are shown. As can be seen in panel B, GABA concentrations remained relatively unchanged across and within genotype. GA concentrations also remained unchanged within all three genotypes (panel A); however, a trend was observed with lower concentrations of GA in both of the aged (18 mo) OAT knockout genotypes, as compared to age matched WT mice. There were n = 4 mice in each of the groups (e.g. WT 3 mo, WT 18 mo, Oat1$^{-/-}$ 3 mo, etc.).



5.D. CONCLUSIONS

We developed a rapid and selective GC-MS/MS method for the detection and quantitation of GA and GABA in mouse brain homogenate. The method employed an online hot inlet gas phase derivatization technique, which required less than 20 sec for completion and fast chromatography by utilization of the LTM technology. The method demonstrated excellent linearity from 0.5 to 100 μg/mL, with limits of detections of 100 ng/mL and 250 ng/mL for GA and GABA, respectively. The method was used to investigate the role of organic anion transporter deletion on concentrations of GA and GABA in the mouse brain.

CHAPTER 6

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

According to the World Health Organization, mental disorders represent the leading cause of disability in the US generating ~58 billion dollars in medical costs annually. Additionally, among the US population, ~40 million adults suffer from an anxiety disorder and ~14 million suffer from a major depressive disorder. The association between the persistence of these neurobehavioral conditions and CNS levels of biogenic amines and metabolites has been studied for half a century. Further, a large number of drugs interfering with neurotransmission/metabolism are used clinically for treatment of these disorders. Recently, some members of the SLC superfamily, the SLC22 transporter family, which includes Oat1 and Oat3, were found to be expressed and functional on the apical membrane of the choroid plexus, a component of the blood cerebrospinal fluid barrier. The cells of this epithelium form tight junctions, which slows penetration of solutes into the brain and limits passive efflux of endogenous solutes from the brain. Therefore, Oat1 and Oat3 are poised to play an active role in the removal of NTs and metabolites from the CSF. Thus, this dissertation intended to provide an increased understanding of the physiological roles of OATs in regulating CNS neurotransmitter and relevant metabolite concentrations and connect their activity to complex behaviors known to be associated with alterations in neurotransmission.

In Chapter 3, a simple HPLC-UV/ECD method was developed and validated for the simultaneous determination of various monoamine neurotransmitters (DA, NE, 5-HT) and

relevant metabolites (DOPAC, HVA, 5-HIAA, QA, NA, XA) in mouse brain tissue. This method introduced several advantages over previous methods in that it uses relatively inexpensive mobile phase constituents, requires minimal sample preparation and small injection volume, while preserving sufficient chromatographic separation of the 9 components of interest, simultaneously. This assay was developed to successfully determine concentrations of the NTs and metabolites at the whole brain level in WT, Oat1$^{-/-}$ and Oat3$^{-/-}$ mice. Metabolic product accumulation occurs over time; therefore, the neurochemical measures were examined at multiple time points (e.g. 3, 6, 9, 12, 15 and 18 mo) in both male and female mice to determine key milestones in the development of neurobehavioral abnormalities. This approach is consistent with the age- and gender-dependent development of different psychopathologies in humans. This design represents a novel methodology as relatively few studies provide a systematic evaluation of neurochemical and behavioral changes over time, particularly in aged animals.

The preliminary studies in Oat1$^{-/-}$ and Oat3$^{-/-}$ mice presented in Chapter 4 of this dissertation provide the first behavioral data indicating that loss of function of Oat1 and Oat3 in the CP results in measurable behavioral consequences. Basal locomotor, anxiety-like and depressive-like behaviors in mice across genotype (WT, Oat1$^{-/-}$ and Oat3$^{-/-}$) and across ages (3-18 mo) were evaluated using behavioral paradigms (OFA, LD, MB and TST). The OFA paradigm is considered a classical test of exploratory locomotor behavior that provides a measure of basal activity and, thus, is an essential component of behavioral phenotyping. In addition, patterns of activity within the field can be determined providing additional information about novel strains. In the current study, the total time spent mobile and the total distance traveled in the OFA paradigm were parameters used to measure basal motor activity across

genotype at any given age. We found that there were no significant differences in these measures across age for each of the genotypes and across genotype at any given age. Therefore, any alterations observed in anxiety-like or depressive-like behaviors in the additional paradigms are not simply reflective of genotype or aged-related differences in basal motor activity. Similarly, there were no significant differences in thigmotactic behavior observed when comparing the distance traveled in the center zone and time in the center zone as a percent of the total distance traveled and total time mobile in the field across strain by age, genotype, and gender. Finally, when we evaluated environmental neophobia by comparing total distance traveled and total time mobile in the field on Day 1 vs. Day 2, we found that there were no significant differences across age for each of the genotypes or across genotype at any given age in habituation patterns.

Perhaps the most interesting finding in Chapter 4 was the behavioral consequence of Oat transporter deletion evident in the MB paradigm. Marble burying has been suggested as a model to measure anxiety-like behaviors, specifically compulsivity, and has been proposed as a possible model of obsessive compulsive disorder in rodents (Albelda and Joel 2012). In the current study, $Oat1^{-/-}$ and $Oat3^{-/-}$ mice nearly eliminated burying behavior at 15 and 18 mo of age. These findings may suggest decreased anxiety-like or compulsive-like behavior in the aged Oat deficient mice and suggest that these knockout mice may be useful as a model of anti-anxiety/compulsivity at older ages (i.e. 15 and 18 mo). Because results from the OFA did not detect any significant differences in the total time spent mobile or the total distance traveled in the field, the marble burying results do not simply reflect lower basal activity levels, but demonstrate true differences in burying behavior. The pattern of anxiolytic effect in our aged knockout mice is similar to what has been observed in the marble burying procedure for SERT

and DAT knockout mice, and when WT mice are administered therapeutic agents used clinically to treat such disorders (e.g. citalopram, fluoxetine) (Kobayashi et al., 2002a; Line et al., 2011; Pang et al., 2011; Fox et al., 2013)

The TST paradigm measuring depressive-like behavior provided the first significant differences between Oat1$^{-/-}$ and Oat3$^{-/-}$ mice. When comparing the knockout strains to each other at each time point, the point where they significantly deviated from each other was at 3 mo of age, with Oat1$^{-/-}$ mice exhibiting significantly decreased immobility compared to Oat3$^{-/-}$ and WT mice at 3 mo. When comparing the knockout genotypes relative to WT at each time point, there were significant differences at varying ages with an overall trend of knockout mice spending less time immobile compared to WT. The Oat1$^{-/-}$ mice spent significantly less time immobile compared to WT at 3 and 9 mo of age, while the 6 mo Oat1$^{-/-}$ mice tended to also spent less time immobile than WT. Oat3$^{-/-}$ mice spent significantly less time immobile compared to age-matched WT at 6, 9 and 15 mo of age. Perhaps, the inconsistencies in this measure of depressive-like behavior can be partly explained by the first and only observed gender-specific variations. Oat3$^{-/-}$ females spent significantly less time immobile compared to their males counterparts at ages 3, 12, 15, and 18 mo of age. This was an important finding because it highlights the importance of not collapsing data across genders without evaluating each gender separately with appropriate sample sizes as most pharmacologic and behavioral studies are conducted at earlier ages (3 mo - 6 mo) and only using one gender. Interestingly, there were no gender-specific differences observed within the Oat1$^{-/-}$ strain. Nonetheless, this observation in our knockout mice of decreased immobility in the TST is comparable to published observations in the DAT, SERT, and norepinephrine transporter (NET) deficient mice providing further evidence for involvement of OAT transporters in behaviors known to be

regulated by monoaminergic neurotransmission (Xu et al., 2000; Holmes et al., 2002; Perona et al., 2008).

Following completion of behavioral assessments, whole brain concentrations of monoamines and metabolites were determined using the developed HPLC-UV/ECD method described in Chapter 3. The biochemical data on brain NT levels presented in Chapter 4 of this dissertation represent the first correlative evidence that the hypothesis of OAT involvement in maintaining endogenous CNS levels of NTs and metabolites is correct. As discussed in Chapter 3, tissue levels of DA in Oat1$^{-/-}$ and Oat3$^{-/-}$ mice at 12, 15 and 18 mo of age were significantly decreased compared to age matched WT. Similarly, concentrations of the DA metabolites, DOPAC and HVA were also significantly lower in aged Oat$^{-/-}$ mice (12, 15 and 18 mo) compared to age-matched WT mice. In contrast, NE concentrations in Oat1$^{-/-}$ and Oat3$^{-/-}$ mice at any given age were not significantly different compared to age matched WT; however, there was a trend for NE concentrations to decrease with age in all genotypes. Interestingly, mean tissue concentrations of 5-HT were significantly decreased in WT at 12, 15 and 18 mo of age compared to age matched Oat1$^{-/-}$ and Oat3$^{-/-}$ mice while there was no significant change in mean concentrations of 5-HIAA. Lastly, we found that there were no differences in the mean concentrations of the kynurenine metabolites, QA, NA, and XA, across genotype at 3, 12 and 18 mo or within genotype across the specified ages.

Based on our extreme findings with aged knockout mice nearly ceasing burying behavior in the MB procedure, which has been suggested in literature to be used as a model of compulsive-like behavior (Ichimaru et al., 1995; Thomas et al., 2009), and given that abnormalities in the glutamate/γ-aminobutyric acid system have been implicated in the

physiology of this disorder (Egashira et al., 2008), we hypothesized that GA and/or GABA concentrations may be altered over age from the loss of OAT transporter function in the CNS. In addition, *in vitro* studies have demonstrated that both of these amino acid NTs interact with OAT transporters (Mori et al., 2003). Therefore, based on this information, investigations of whole brain concentrations of GA and GABA were logical progressions.

Lastly, as described in Chapter 5, a novel GC-MS/MS method was developed for quantitation of amino acid neurotransmitters, GA and GABA, in mouse brain tissue. There were no currently published methods utilizing GC-MS/MS offering quantitation of both GA and GABA in brain tissue. In this dissertation, we describe a rapid and selective GC-MS/MS which utilized stable isotopes of each compound as internal standards. The use of MethElute™ reagent rapidly derivatized GA and GABA and their stable isotopes in the heated GC injection port, which required less than 20 sec for completion and utilization of the low thermal mass (LTM) technology provided high resolution and fast chromatography. The method demonstrated excellent linearity from 0.5 to 100 μg/mL, with limits of detections of 100 ng/mL and 250 ng/mL for GA and GABA, respectively. The developed method was successfully used for measurement of whole brain concentrations of GA and GABA in a small subset of WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ mice at 3 and 18 mo. Brain tissue samples were previously collected following completion of the behavioral assessments, and homogenates were aliquoted and stored at -80°C until analysis. The concentration of GABA remained relatively unchanged across and within genotype. GA concentrations also remained unchanged within all three genotypes; however, a trend was observed with lower concentrations of GA in both of the aged OAT knockout genotypes compared to age matched WT mice.

These studies have only scratched the surface in terms of our understanding of the endogenous physiological roles of these transporters in the CP. Our study provides the first evidence suggesting that the complex behavioral profiles observed in the Oat1$^{-/-}$ and Oat3$^{-/-}$ mice may serve as a tool to better understand the mechanisms underlying neurobehavioral disorders such as anxiety, compulsivity and depression. However, it should be noted that the CNS is a dynamic system that has many additional factors (i.e. other transporters, receptors, enzymes) responsible for maintaining concentration of endogenous substances (i.e. neurotransmitters and their metabolites) and thereby behaviors associated with their concentrations. Thus, immediate studies should include investigations of the impact of Oat1 deletion on the levels of Oat3 and vice versa. It would also be of interest to investigate if pharmacologic antagonism of Oat1 or Oat3 elicits similar behavioral and neurochemical consequences as observed in Oat$^{-/-}$ mice. Additionally, given our understanding of the overlapping specificities for NTs and their metabolites and overlapping tissue expression profiles of Oat1 and Oat3, it would be of interest to develop an Oat1/Oat3 double knockout mouse model to truly delineate the role of OAT's, although this is a difficult challenge due to linkage of the genes and it remains unknown if a double knockout model would be embryonic lethal. Lastly, future studies exploring the redundancy in the CNS should also include investigations of the levels of other transporters known to be involved in neurotransmission (i.e. SLC6 transporters (DAT, NET, SERT) and OCTs (uptake 2)) as a response to delection of OAT transporters. Evidence of compensatory changes and the dynamic interplay between these two transporters families (SCL6 and SLC22) in knockout animals has already been reported with SERT knockout mice exhibiting increased levels of Oct3 (Schmitt et al., 2003; Baganz et al., 2008). Continuing our discussion of the dynamic nature of the CNS environment, other important investigations to be considered also

includes evaluating levels of metabolic enzymes (i.e. MAOs, TH, COMT) responsible for maintain homeostatic concentrations of neurotransmitters, and evaluating receptor levels (i.e. dopamine receptors and serotonin receptors) in these knockout models.

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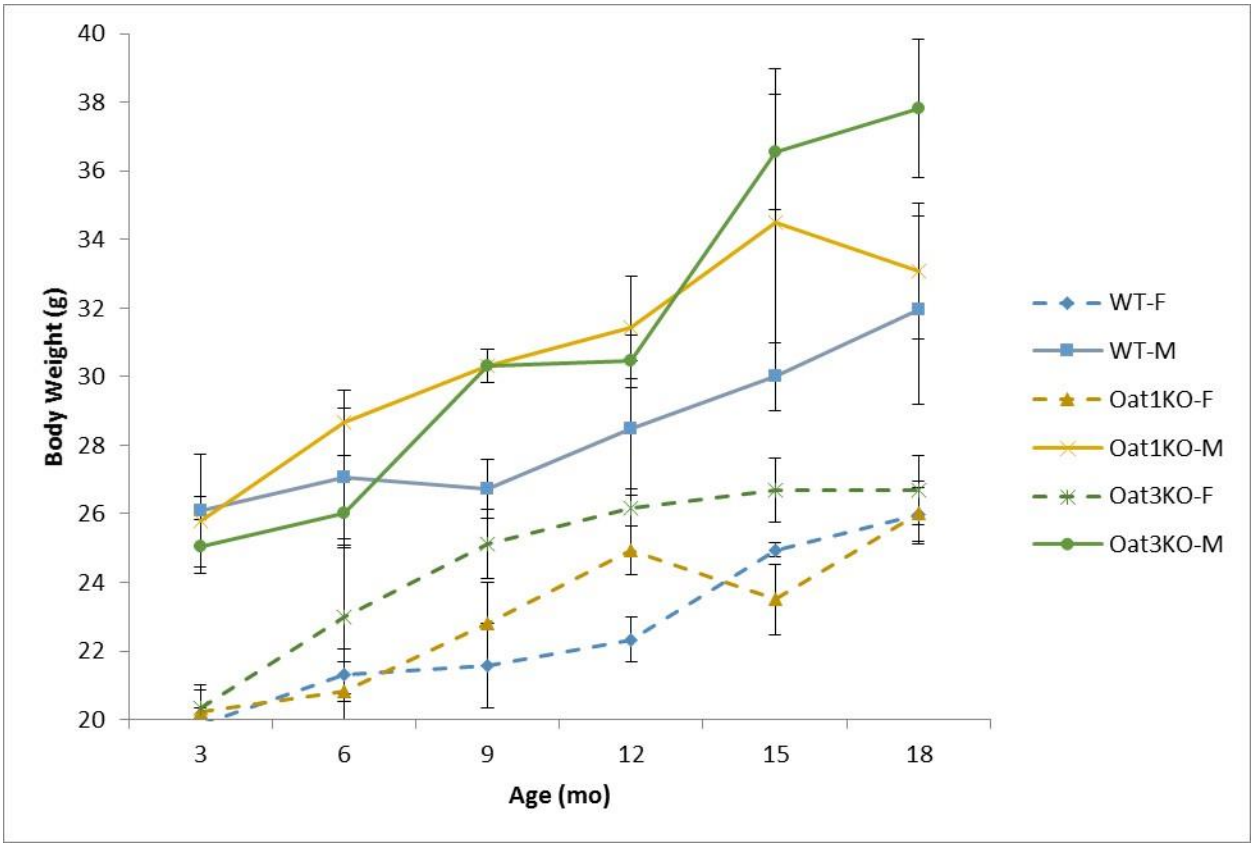
APPENDIX I

COMPARISON OF BODY WEIGHT (G) BY GENOTYPE AND GENDER ACROSS AGE

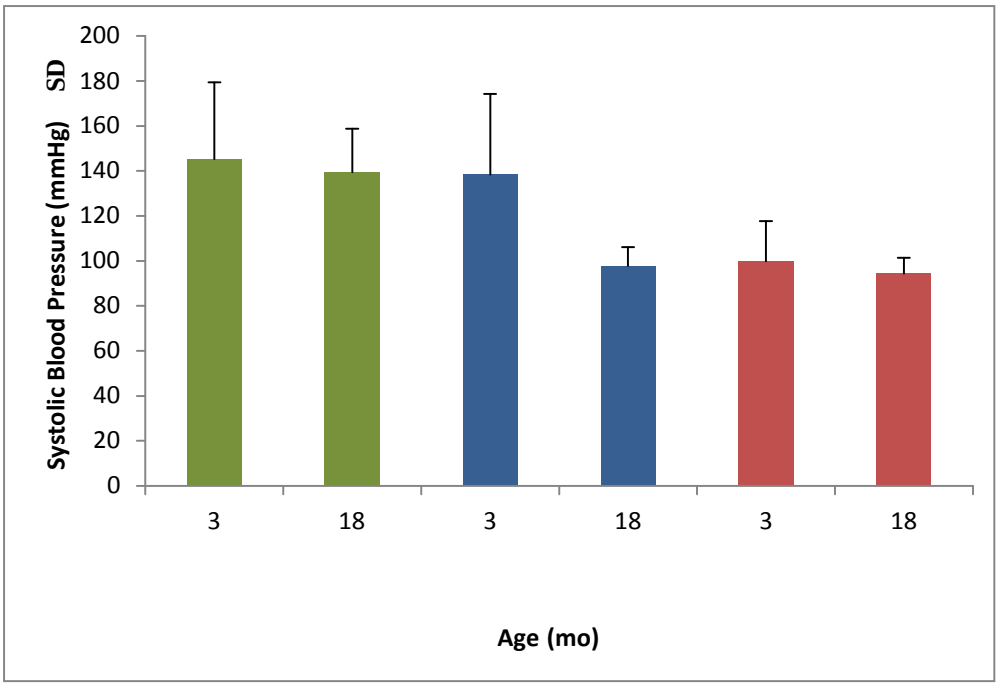
Body weight was collected for all animals used in the behavioral analyses described in Chapter 4. As demonstrated in the graph below, males of all genotypes (solid lines) had higher body weights compared to their age matched female counterparts (dashed lines). Despite this increase in body weight, mice remained similar in their overall baseline motor activity as indicated by the total time mobile and total distance travelled in the open field paragidm as discussed in Chapter 4.

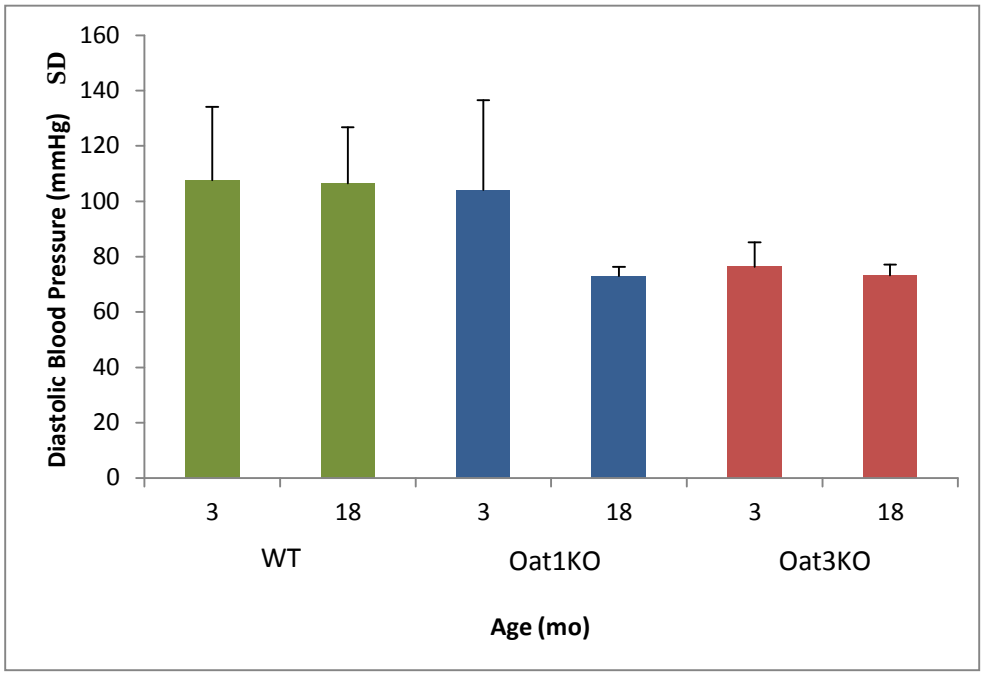


APPENDIX II

ASSESSMENT OF BLOOD PRESSURE BY GENOTYPE AND AGE

Vallon *et al* reported in a 2008 article in the Journal of the American Society of Nephrology that Oat3$^{-/-}$ mice had 10-15% lower systolic, diastolic, and mean arterial blood pressure (MABP) compared to WT and Oat1$^{-/-}$ mice. Systolic blood pressure was measured using he tail-cuff method in awake mice, while MABP was measured in anesthetized mice via intra-arterial catheter. Systolic and diastolic blood pressure was measured in a small subset of WT, Oat1$^{-/-}$, and Oat3$^{-/-}$ mice (n = 4-6 in each group) using the tail-cuff method and correlated with the findings reported by Vallon *et al*. As demonstrated in the graph below, Oat3$^{-/-}$ demonstrated decreased systolic and diastolic blood pressure compared to WT and Oat1$^{-/-}$ at 3 mo of age. Interestingly, we found that at 18 mo of age, both Oat1-/- and Oat3-/- mice demonstrated decreased systolic and diastolic pressure compared to WT mice at 18 mo. These results indicate the potential implication of these transporters in blood pressure regulation.





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- Identifies and consents appropriate study patients
- Receive study samples
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- Designed and implemented behavioral experiments to assess anxiety/depressive-like behaviors in transgenic mice
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- Developed HPLC and GC/MSMS methods to determine neurotransmitter concentrations in mouse brain homogenates
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- Provide technical and organizational leadership in alignment with company objectives
- Provides technical support for the development of new formulations, manufacturing processes, and testing methods by proactively engaging product development teams and scientific leadership

Peer Reviewed Scientific Publications

1. Farthing D., Sica D., Fakhry I., Larus T., Ghosh S., **Farthing C.**, Vranian M., Gehr T. Simple HPLC-UV method for determination of iohexol, iothalamte, p-aminohippuric acid and n-acetyl-p-aminohippuric acid in human plasma and urine with ERPF, GFR and ERPF/GFR ratio determination using colorimetric analysis. J of Chrom B (2005) 826:267-272.

2. Farthing D., Sica D., Gehr T., Wilson B., Fakhry I., Larus T., **Farthing C.**, Karnes H.T. A simple and sensitive HPLC method for determination of inosine and hypoxanthine in human plasma from healthy volunteers and patients presenting with chest pain and potential acute cardiac ischemia. J of Chrom B (2007) 854:158-164.

3. Farthing D., Gehr L., Karnes H.T., Sica D., Gehr T., Larus T., **Farthing C.**, Xi L. Dose-dependent effects of salicylic acid on post-ischemic ventricular function and purine efflux in isolated mouse hearts. Biomarkers (2007) 12(6):623-634.

4. Maynor L, Carl DE, Matzke GR, Gehr TWB, **Farthing C**, Farthing D, Brophy DF. An In Vivo-In Vitro Study of Cefepime and Cefazolin Clearance During High Flux Hemodialysis. Pharmacotherapy (2008) 28(8):977-983.

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9. Fowler A., Syed A., Knowlson, S. Sculthorpe, R., Farthing D., Dewilde, C., **Farthing, C.**, Larus, T., Martin, E., Brophy, D., Gupta, S., Medical Respiratory Intensive Care Unit Nursing, Fisher, B., Natarajan, R. Intravenous Ascorbic Acid Reduces Organ Failure and Injury Biomarkers in Humans with Severe Sepsis. J of Trans Med (2014) 12: 32-42.

10. **Farthing, C.**, Halquist, M., Sweet, DH. A Simple High-Performance Liquid Chromatographic Method for the Simultaneous Determination of Monoamine Neurotransmitters and Relative Metabolites with Application in Mouse Brain Tissue. Manuscript accepted J of Liq Chrom Rel Sci 2014.

11. **Farthing, C.**, Nicholson, K., Sweet, DH. Organic Anion Transporter 1 (Oat1) and Oat3 in the Murine Choroid Plexus: A Potential Mechanism for Maintaining Endogenous Monoamine Homeostasis in the Central Nervous System. Manuscript submitted to Physiology and Behavior, 2014.

12. **Farthing, C.**, Farthing, D., Gress, RE, Sweet, DH. Determination of L-Glutamic Acid and γ-Aminobutyric Acid in Mouse Brain Tissue Utilizing GC-MS/MS. Manuscript submitted to J Chrom B, 2014.

Scientific Posters

1. Farthing D., Sica D., Fakhry I., Larus T., Ghosh S., **Farthing C.**, Vranian M., Gehr TWB. Simple HPLC-UV method for determination of iohexol, iothalamate, p-aminohippuric acid and n-acetyl-p-aminohippuric acid in human plasma and urine with ERPF, GFR, and EPRF/GFR ration determination using colorimetric analysis. Poster Pittcon (Orlando Fl, 2005)

2. Farthing D., Sica D., Gehr T., Larus T., **Farthing C.**, Karnes H.T.. Rapid luminescence method for determination of inosine and hypoxanthine in human plasma. Poster, Pittcon (New Orleans, LA, March 2008).

3. Farthing D., Gehr T., Sica D., **Farthing C.**, Larus T., Fakhry I., Karnes H.T. Rapid and simple luminescence method for detection of acute cardiac ischemia. Poster, Biomarker Assay Development Conference (San Diego, CA, Jan 2009).

4. **Farthing, C**., Nicholson, K., Shelton, K., Sweet D. Evaluation of the loss of Oat1 and Oat3 function in the murine choroid plexus and its influence on behaviors regulated by monoaminergic neurotransmission. Poster, Virginia Commonwealth University Research and Career Day Conference (Richmond, VA, Oct 2011), Virginia Commonwealth University Pharmacology/Toxicology Retreat (Richmond, VA, Oct 2011).

5. Natarajan, R., Fisher, B., DeWilde, C., Priday, A., Syed, A., **Farthing, C.**, Larus, T., Knowlson, S., Fowler, A. Parenteral Vitamin C Attenuates Markers of Organ Injury and Inflammation in Severe Sepsis. Poster, American Thoracic Society International Conference (San Francisco, CA, May 2012).

6. **Farthing, C.**, Nicholson, K., Shelton, K., Sweet, D. Evaluation of the loss of Oat1 and Oat3 function in the murine choroid plexus and its influence on behaviors regulated by monoaminergic neurotransmission. Poster, Virginia Commonwealth University, Graduate Research Symposium (Richmond, VA, April 2013).

7. **Farthing, C.**, Nicholson, K., Sweet, D. Organic Anion Transporters (SLC22) in the Murine Choroid Plexus Impact Monoamine Homeostasis in the Central Nervous System. Poster, American Association of Pharmaceutical Science Annual Meeting (San Antonio, TX, November 2013), Virginia Commonwealth University Research and Career Day (Richmond, VA, Nov 2013).

Honors & Awards

2013 Altria Group Tobacco Products Regulatory Science Fellowship
2014 Virginia Commonwealth University Leadership Award

Leadership & Service Experience

2007-2008	Adult Career Development Center *Volunteer Teacher*
2008-2009	Richmond SPCA *Adoption Specialist*
2011-Present	Kersey Creek Elementary School *Science Fair Judge*
2011-2013	VCU School of Pharmacy Graduate Affairs Committee *Student Representative of Executive Committee*
2011-2013	VCU Pharmaceutics Graduate Student Association *Vice President*
2011-2013	VCU AAPS Student Chapter *Vice-Chair*
2011-2014	VCU Diversity Strategic Planning Committee *Student Representative of Executive Committee*
2013-2014	VCU Women in Science *Member*
2013-2014	American Association of Pharmaceutical Scientists (AAPS) *PPDM Section Student Representative of Executive Committee*

Professional Memberships

2011-Present	American Association of Pharmaceutical Scientists
2012-2014	American Society for Pharmacology and Experimental Therapeutics