EFFECTS OF METHAMPHETAMINE ON THE EXPRESSION OF SULFOTRANSFERASES IN ENDOCRINE GLANDS AND THE LIVER OF RATS

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TENG SUN

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Jilin University

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Thesis Approved:

Guangping Chen

Thesis Adviser

Carey Pope

Michael Davis

Mark E. Payton

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Sulfotransferases

Sulfation

Sulfation (sulfoconjugation or sulfonation) is one of the reactions in phase II drug metabolism targeting a broad range of xenobiotics and endogenous substrates, including drugs, toxic chemicals, steroid hormones, and neurotransmitters (Nimmagadda, Cherala, & Ghatta, 2006). This process is catalyzed by a supergene family of enzymes called sulfotransferases (SULTs), which transfer the sulfuryl group from the ubiquitous donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a hydroxyl-containing or amine-containing group of an acceptor molecule. Two classes of SULTs have been identified: membrane-bound SULTs and cytosolic SULTs. Membrane-bound SULTs are located on the Golgi apparatus of the cell and specifically sulfate peptides, proteins, lipids and glycosaminoglycans (Chapman, Best, Hanson, & Wong, 2004; Falany, 1997; Negishi *et al.*, 2001); cytosolic SULTs are responsible for the sulfation of xenobiotics and small endogenous substrates in the body. The sulfotransferases in this thesis are all referred

to as cytosolic SULTs. In intact cells, the sulfation reaction may be reversed by sulfatases which exist in the endoplasmic reticulum (Nimmagadda *et al.*, 2006; Kauffman, 2004). Sulfation plays a key role in a cell's defensive response against xenobiotics and may have a major function during early development since high SULT expression has been observed in the human fetus (Kauffman, 2004). In most cases, this process is considered to be the detoxification pathway of numerous toxic xenobiotics, because by adding a sulfuryl group on the substrate compound, SULTs increase its water solubility and lead it to be readily excreted from the cell. However, many SULTs are also capable of bioactivating procarcinogens, such as certain dietary and environmental agents, to reactive electrophiles. These electrophiles may covalently bind with DNA and proteins and cause damage to the cell (Glatt *et al.*, 2001). Another major function of SULTs is regulating endocrine status. Sulfation occurs commonly in steroid biosynthesis, the metabolism of catecholamines and iodothyronines as well as the modification of estrogen and androgen receptors (Kauffman, 2004). The transfer reaction of the sulfuryl group of PAPS to the hydroxyl group of a steroid produces a steroid sulfate (Luu-The, Bernier, Dufort, & Labrie, 1996). This commonly-known detoxification reaction (sulfation) may play a role in steroid hormone synthesis since a steroid sulfate is the precursor of most of the steroid hormones (Luu-The *et al.*, 1996). Though the structures and functions of many SULT isoforms were identified years ago, the regulatory mechanism of human SULTs remains as one of the least explored areas in the field (Nimmagadda *et al.*, 2006).

Isoforms, substrate specificity and tissue distribution

SULTs have a wide range of tissue distribution, but isoform expression varies between tissue types. Based on sequence analysis, SULTs have been divided into six families (Pacifici & Coughtrie, 2005). For humans, thirteen SULT isoforms have been identified so far, representing three families: SULT1, SULT2 and SULT4 (Gamage *et al.*, 2006). The SULT1 family, which comprises three SULT subfamilies (SULT1A, SULT1B, and SULT1C) (Freimuth *et al.*, 2000; Fujita, Nagata, Ozawa, Sasano, & Yamazoe, 1997; Weinshilboum *et al.*, 1997), has eight members and typically catalyzes transfer of a sulfuryl group to the hydroxyl group of a phenolic substrate (Pacifici & Coughtrie, 2005). The SULT2 family has two subfamilies (SULT2A and SULT2B) and contains the SULTs specific for the sulfation of hydroxylsteriod. The SULT4 family is least studied family of SULTs so far, and no specific substrate is currently known for this family of enzymes. All that is known about this family is that SULT4A1 is of highly expressed in the brain (Gamage *et al.*, 2006). The gene location, distribution and substrate specificity of human cytosolic SULTs are listed in Table 1. The liver expresses most of the SULT isoforms because it is the main detoxification organ in the body. The SULT1 family is expressed mainly in detoxification organs, indicating that their main function is detoxification, specifically detoxifying phenolic xenobiotics. The SULT2 family is expressed in endocrine glands as well as the target organs of endocrine hormones, such as the adrenal gland and the liver. SULT2 enzymes are reported to be involved in the regulation of cellular hormone systems (Chen, Chen, Head, Mangelsdorf, & Russell, 2007; Glass & Saijo, 2008).

Table 1. Gene Location, Distribution and Substrate Specificity of Human Cytosolic SLUTs

SULT Isoform	Chromosomal Location	Substrates	Major Sites of Expression
SULT1A1	16p12.1-11.2	4-Nitrophenol, 1-naphthol, minoxidil,E2, E1, DES, 4-hydroxytamoxifen, naringenin, genistein, dopamine, epinephrine; numerous monocyclic phenols; iodothyronines; 2-naphthol, OH-PhIP	Adult liver, adult gastrointestinal tract, adult platelets, placenta
SULT1A2	16p12.1-11.2	2-Naphthol>minoxidil, 4-nitrophenol; OH-PhIP	No site found
SULT1A3	16p11.2	Dopamine, norepinephrine, isoprenaline, 2-hydroxy-E2 and other catechols, numerous monocyclic phenols; vanillin, 1-naphthol; albuterol and various other β2 agonists; dopamine> tyramine> *p*-nitrocatechol, *m*-nitrocatechol, 4-nitrophenol, *p*-cresol, 4-aminophenol, 4-ethylphenol; E1; minoxidil	Adult gastrointestinal tract, adult platelets, adult brain, placenta, fetal liver
SULT1B1	4q11-13	1-Naphthol> 4-nitrophenol> DES, iodothyronines	Adult liver, adult and fetal gastrointestinal tract
SULT1C2	2q11.2	4-Nitrophenol> OH-AAF	Fetal kidney, lung, and gastrointestinal tract
SULT1C4	2q11.2	No substrate known	Fetal kidney and lung
SULT1E1	4q13	E2, E1, pregnenolone, 17-ethinyl-E2, equilenin; DHEA, 1-naphthol, DES, 4-hydroxytamoxifen, narigenin, genistein; iodothyronines; minoxidil	Fetal liver, lung, and kidney, adult liver and endometrium
SULT2A1	19q13.3	DHEA> epiandrosterone> androsterone> testosterone> E2; cholesterol; lithocholic acid and various other bile acids; pregnenolone> 17-ethinyl-E2> cortisol; minoxidil	Fetal and adult adrenal gland and liver
SULT2B1	19q13.3	DHEA	Adult skin, prostate, and placenta
SULT4A1	22q13.1-13.2	No substrate known	Brain

Abbreviations: DHEA, dehydroepiandrosterone; E2, 17β-estradiol; E1, estrone; OH-PhIP, *N*-hydroxy-2-amino-1-methyl-6-phenylimidazo pyridine; DES, diethylstilbestrol; OH-AAF, *N*-hydoxy-2-acetylaminofluorene.

(Li, Lindsay, Wang, & Zhou, 2008)

Sulfotransferase 1A1

Sulfotransferase 1A1 (SULT1A1) belongs to the SULT1A subfamily, and is also known as the "simple phenol SULT". Human SULT1A1 is the most commonly-studied SULT. It is responsible for the sulfation of phenolic drugs, such as acetaminophen, minoxidil, 17α-ethinylestradiol, etc. (Pacifici & Coughtrie, 2005). SULT1A1 also sulfates endogenous compounds, such as 17β-estradiol and iodothyronines, as well as environmental xenobiotics, such as the isoflavones (Pacifici & Coughtrie, 2005). It is widely distributed in human body, showing high expression in the liver and expression to a lesser degree in brain, breast, intestine, endometrium, kidney, lung, and platelets (Falany & Falany, 1996; Falany, Azziz, & Falany, 1998).

Sulfotransferase 1E1

Sulfotransferase 1E1 (SULT1E1) belongs to the SULT1E subfamily, which is also among the most widely studied of the SULT enzymes. The first SULT protein for which a crystal structure was resolved was mouse SULT1E1 (Kakuta, Pedersen, Carter, Negishi, & Pedersen, 1997; Kakuta, Petrotchenko, Pedersen, & Negishi, 1998). SULT1E family members specifically sulfate 3α-hydroxyl groups of endogenous and xenobiotic estrogens with high affinity (Aksoy, Wood, & Weinshilboum, 1994; Coughtrie *et al.*, 1994; Coughtrie, Sharp, Maxwell, & Innes, 1998; Falany, Wheeler, Oh, & Falany, 1994; Falany, 1997). SULT1E1 is observed to be expressed in some steroid-hormone-responsive tissues, such as the adrenal gland, the testis as well as the placenta, depending on different species (Pacifici & Coughtrie, 2005).

Sulfotransferase 2A1

Sulfotransferase 2A1 (SULT2A1) belongs to the SULT2 family and is also known as the dehydroepiandrosterone (DHEA) SULT. SULT2A1 is the only SULT capable of sulfating DHEA, however, it can also sulfate a variety of other endogenous compounds, such as pregnenolone, cholesterol, cortisol, benzylic alcohols of Polycyclic aromatic hydrocarbons (PAHs), testosterone and the bile salts (Pacifici, 2005). SULT2A1 is the only member of the SULT2 family that is capable of catalyzing the sulfation of the phenolic hydroxy group at the 3 position of estrogens (Parker, Jr., Falany, Stockard, Stankovic, & Grizzle, 1994; Parker, Jr., Stankovic, Falany, Faye-Petersen, & Grizzle, 1995). This DHEA SULT is localized mainly in the fetal zone and neocortex (Parker, Jr. *et al.*, 1994; Parker, Jr. *et al.*, 1995). The crystal structure of human SULT2A1 has been resolved (Pedersen, Petrotchenko, & Negishi, 2000).

Sulfotransferase 2B1

Sulfotransferase 2B1 (SULT2B1) belongs to the SULT2B subfamily and was recently detected in a variety of hormone-responsive tissues. The SULT2B1 gene encodes two SULT isoforms, SULT2B1a and SULT2B1b, resulting from different initiation sites of transcription or alternative mRNA splicing (Geese & Raftogianis, 2001; Kohjitani, Fuda, Hanyu, & Strott, 2006; Meloche & Falany, 2001; Koizumi *et al.*, 2009; Her *et al.*, 1998). According to the information of Gene Bank, SULT2B1a is fifteen amino acids shorter in length than SULT2B1b which has the additional fifteen amino

acid at the amino-terminal (N') end of the protein. The final 344 amino acid of both sequences are the same because they are from the same exons on the original gene (Falany, He, Dumas, Frost, & Falany, 2006; Her *et al.*, 1998; Kohjitani *et al.*, 2006; Shimizu, Fuda, Yanai, & Strott, 2003). The SULT2B1b isoform specifically sulfonates cholesterol and cholesterol derivatives, whereas SULT2B1a is specific to pregnenolone. Recent studies indicate that SULT2B1b is responsible for the sulfation of oxysterols, which are cholesterol derivatives and known to have deleterious effects on biological processes (Fuda, Javitt, Mitamura, Ikegawa, & Strott, 2007). There is selective tissue expression of SULT2B1 isoforms in rats. The SULT2B1a isoform is known to be expressed only in the testis and brain of male rats, whereas the SULT2B1b isoform is expressed in most other tissues (Kohjitani *et al.*, 2006; Kohjitani *et al.*, 2006). Different from the other SULT isoforms, both SULT2B1 isoforms likely have little role in xenobiotic detoxification. Although the regulatory mechanisms of these two isoforms still remain unclear, it has been shown that they are involved in regulating lipid and cholesterol metabolism (Chen *et al.*, 2007; Glass & Saijo, 2008).

Endocrine system

The endocrine system is comprised of endocrine glands, the hormones they secrete, and the reaction these hormones produce. Like all other systems in the body, the endocrine system contains a variety of interacting parts. Endocrine glands are the body's main hormone producers and are located throughout the body, from the brain to the testis

or the ovary. They include the hypothalamus, the pituitary gland, the pineal gland, the thyroid and parathyroid glands, the adrenal gland, the thymus gland, and the testis/ovary. The hormones they secrete are distributed to the rest of the body through the bloodstream and interact with the target cells that receive their messages. Special proteins bind to hormones on their way to the target cells to control the amount of hormone available to interact with the target cells. The target cells receive the hormones by specific receptors on the cell membranes. The hormone levels in the blood are maintained by certain regulatory mechanisms. Excessive or insufficient hormone expression causes dysfunction within the body. The endocrine system controls body processes that happen slowly. Other faster body processes, like breathing or body movement, are controlled by the nervous system. The nervous system and the endocrine system work together to maintain normal body functions. Hormone levels can be influenced by environmental factors such as drugs, food components, stresses, diseases, etc. Hormones control biological processes through nuclear receptors at target tissues.

Thyroid hormones

The thyroid gland is located in the front part of the lower neck and produces the thyroid hormones thyroxin (T4) and triiodothyronine (T_3). These hormones are required for normal brain and somatic development and for the important regulation of physiological condition in the body (Zoeller, 2010). They are also involved in the circadian rhythms that govern sleep (Rieser & Kemp, 2010). T_3 plays an important role in the process of blood coagulation, inflammation, metabolism and cell proliferation

(Huang, Tsai, & Lin, 2008; Sprague, Mallett, Rusyniak, & Mills, 2004). As the level of thyroid hormones increase in the bloodstream, so does the speed at which chemical reactions occur in the body. Thyroid hormones also play a key role in bone growth and the development of the brain and nervous system in children (Rieser & Kemp, 2010). The production and release of thyroid hormones is dynamically controlled within a narrow range under normal conditions by the hypothalamus and the pituitary gland (Zoeller, 2010). Many drugs and medications can alter thyroid hormone levels by affecting the hypothalamus, the thyrotropes (in the anterior pituitary), synthesis and secretion from the thyroid gland, and metabolism of thyroid hormones through deiodination, sulfation and glucuronidation (Surks & Sievert, 1995; Haugen, 2009). Thyroid hormone levels can also be affected by drugs through altered affinity of thyroxin-binding globulin. Moreover, drugs can affect absorption of thyroid hormones or actions of thyroid hormones at the hormone target organs (Haugen, 2009; de Groot, Zonnenberg, Plukker, van Der Graaf, & Links, 2005). Environmental chemicals affecting both thyroid hormone circulating level and thyroid hormone action in target tissues are considered to be thyroid toxicants (Zoeller, 2010).

Adrenal hormones

The body has two triangular adrenal glands, one on the upper pole of each kidney, which are anatomically divided into two parts: a cortex and a medulla (Hardy & Cooper, 2010). The adrenal cortex is the outer part of the adrenal gland that synthesizes adrenal steroid hormones, i.e., corticosteroids. Corticosteroids influence or regulate salt and water

balance in the body, the body's response to stress, metabolism, the immune system, and sexual development and function (Maggio & Segal, 2010). Steroid hormones are derived from the same precursor, cholesterol, through a complex series of biochemical reactions (Hardy & Cooper, 2010). Different steroid hormones have different specificities for steroid hormone receptors though they are all derived from the same precursor and have very similar structures (Hardy & Cooper, 2010). The inner part of the adrenal glands, the medulla, produces catecholamines, such as epinephrine, which is also called adrenaline. Epinephrine increases blood pressure and heart rate when the body experiences stresses, known as "fight or flight" responses (Rieser & Kemp, 2010).

Sex hormones

Sex hormones are also known as sex steroids. They are steroid hormones that control the development and maintenance of sex characteristics by binding to vertebrate androgen or estrogen receptors (Guerriero, 2009). Their functions and effects are controlled either by slow, genomic mechanisms through nuclear receptors or by fast, non-genomic mechanisms through signaling cascades (Thakur & Paramanik, 2009). The two main classes of sex hormones are androgens and estrogens, for male and female vertebrates, respectively. Androgens, also called testoids, include anabolic steroids, androstenedione, dehydroepiandrosterone, dihydrotestosterone, and testosterone, stimulate the activity of accessory male sex organs and development of male secondary

sex characteristics. The female sex hormones, estrogens, are all derived from androgens and include estradiol, estriol, and estrone.

Methamphetamine

The psychostimulant methamphetamine (METH) increases alertness and energy, and in high doses, can induce euphoria, enhance self-esteem, and increase sexual pleasure (Mack, France, & Miller, 2005; Logan 2002). It was approved by the FDA to treat attention deficit hyperactive disorder (ADHD), narcolepsy and exogenous obesity, under the trademark name Desoxyn®^{TM} (Rxlist-Desoxyn, 2010). However, METH also has high potential for abuse and addiction by increasing the levels of dopamine, norepinephrine and serotonin in the brain's psychological reward system (Riddle, Fleckenstein, & Hanson, 2006). METH blocks dopamine reuptake by antagonizing the dopamine transporters on the membrane of the neuron cells and increases the dopamine level in the synapse, resulting in abnormally high levels of dopamine in the brain (Riddle *et al.*, 2006). METH increases dopamine in the brain ten times more than the normal level, and this is four times more than elevation of dopamine by cocaine (Rawson, 2006). METH abuse has many critical consequences, such as anxiety, insomnia, mood disturbances and violent behavior. Long term abuse could even lead to death. A great deal of research has been conducted on METH during the past two decades. METH has been shown to not only affect central nervous system, but also affect the endocrine system in the body. Sprague *et al.* reported that T_4 was strongly induced after high dose MAMA (3,4-methylenedioxymethamphetamine, a METH derivative) treatment (Sprague,

Banks, Cook, & Mills, 2003). METH injection also increases instant and chronic release of adrenal hormones, such as epinephrine (Grace, Schaefer, Gudelsky, Williams, & Vorhees, 2010; Williams, Schaefer, Furay, Ehrman, & Vorhees, 2006; Williams *et al.*, 2000; Schaefer *et al.*, 2008).

Hypotheses and Specific Aims

As stated previously, endocrine glands respond to METH by changing the level of secreted hormones. SULTs are involved in biosynthesis, regulation and metabolism of endocrine hormones. Both METH and SULTs are related to endocrine hormones, however, relatively little research has been conducted looking at the relationship between METH and SULTs. Only one report about METH's effect on SULTs was found in the literature. Dr. Kelsoe's group used microarray to identify a series of candidate genes whose expression is induced by METH treatment. They showed that the mRNA transcription of SULT1A1 was induced 4.3 times more than normal in the amygdala of rats 24 hours after METH treatment (Niculescu, III *et al.*, 2000). The relationship between METH and body cytosolic SULTs has not been reported. The mechanisms of METH effects on SULTs also remain unclear.

Based on the above background information, we hypothesize that the expression of SULTs will be affected by METH in the adrenal gland, the thyroid gland, and the ovary/testis of rats. We are interested in studying the relationship between METH and the expression of different SULT isoforms. We also propose to investigate the effects of

METH on mRNA transcription of different SULT isoforms in those three endocrine glands. To test our hypotheses, we developed the following specific aims:

1. To investigate the effects of METH on protein expression of different SULT isoforms in the adrenal gland, the thyroid gland, and the ovary/testis of rats.
2. To investigate the effects of METH on the mRNA transcriptions of different SULT isoforms in the adrenal gland, the thyroid gland, and the ovary/testis of rats.
3. To investigate the effects of METH on protein expression and mRNA transcription of SULTs in the liver of rats (the liver is the main detoxification organ and one of the targeting tissue of endocrine hormones).

CHAPTER II

METHODOLOGY

Materials

Methamphetamine was purchased from Sigma-Aldrich (St. Louis, MO). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents were purchased from Bio-Rad (Hercules, CA). Fisher BioReagents* EZ-Run* Prestained Rec Protein Ladder was purchased from Fisher Scientific (Pittsburgh, PA). Western blotting chemiluminescence reagent kits (Super Signal West Pico Stable Peroxide and Super Signal West Pico Luminol/Enhancer solutions) were purchased from Pierce Chemical (Rockford, IL). Nitrocellulose membranes (Immobilon-P; Millipore Corporation, Bedford, MA) used for Western blots were purchased from Fisher Scientific (Fair Lawn, NJ). Protein assay reagent was obtained from Bio-Rad (Hercules, CA). Total RNA extraction kit (TRIZOL reagent) was purchased from Molecular Research Center, Inc. (Cincinnati, OH). GoScript™ Reverse Transcription System was purchased from Progema Corporation (Madison, WI). Real-time PCR Kits (qPCR MasterMix Plus for SYBR Green I dNTP) were purchased from EUROGENTEC (San Diego, CA). All other reagents and chemicals were of the highest analytical grade available.

Animals and Drug Treatment

All procedures within this study were carried out with protocols approved by Oklahoma State University Animal Care and Use Committee.

Male and Female Sprague-Dawley rats (Harlan, Indianapolis, IN), 10 to 11 weeks old and 200-300 g body weight were used in this investigation. Rats were housed in a temperature- and humidity-controlled room and supplied with rodent chow and water for at least 1 week before use. Rats were divided randomly into groups of four. Methamphetamine was dissolved in saline and administrated by intraperitoneal injection at 1, 5 and 20 mg/kg to 3 separate groups of rats. The control rats received only the saline vehicle. The treatment was single dose treatment. The rats were euthanized 24 h after the drug treatment. The livers, the thyroid glands, the adrenal glands, and the testes/ovaries were collected from each rat and were snap-frozen in liquid nitrogen. Samples were stored at -80 ℃ until use.

Cytosol Preparation

The liver, the thyroid gland, the adrenal gland, and the testis/ovary tissues were homogenized in 50 mM Tris buffer containing 0.25 M sucrose, 3 mM β-mercaptoethanol and 0.02% (v/v) Tween-20, pH 7.4. All homogenates were centrifuged at 100,000 x g for 1h at 4 ℃. Cytosol aliquots were collected and the concentrations of protein were tested using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA) on Beckman Coulter DU530 Life Science UV/Vis Spectrophotometer (Beckman Coulter,

Brea, CA) according to manufacturer's protocol. The cytosol aliquots were then preserved at -80 ℃ for Western blotting analysis.

Western Blot Analysis

The primary antibodies against rat sulfotransferases rSULT2A1 and rSULT1A1 were provided by Dr. Michael W. Duffel (Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa city, IA). The primary antibody against rSULT1E1 was purchased from Biovision, Inc. (Boston, MA). The primary antibody against rSULT2B1a and rSULT2B1b (sc-t7103) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Briefly, 15 μg cytosol protein from tissue of the liver, the thyroid gland, the adrenal gland or the testis/ovary was separated by electrophoresis (Bio-Rad, Hercules, CA) using 12% SDS polyacrylamide gel. After running 20 minutes at 90V (stacking gel) and 1 hour at 140V (separating gel), the protein bands were transferred overnight at 35 V onto a Polyvinylidene Fluoride (PVDF) nitrocellulose membrane at 4 ℃. Membranes were blocked for 1h with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBST). For all the above-mentioned cytosol proteins, membranes were incubated with one of the following primary antibodies: goat anti-rat AST-IV (rSULT1A1), rabbit anti-human SULT2B1b, rabbit anti-rat STa (rSULT2A1), or mouse anti-rat STe (rSULT1E1). The primary antibodies were diluted to 1:1000 in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20) containing 5% (w/v) nonfat dry milk. The membranes were incubated with the primary antibodies overnight on a shaker at 4 ℃. After incubation, all

membranes were washed 3 times with TBST for 15 min each wash and incubated in secondary antibody (horseradish peroxidase-conjugated Immuno-Pure goat anti-rabbit , rabbit anti-goat and rabbit anti-mouse IgG; H + L) at 1:5000 dilutions in TBST for a minimum of 1 h at room temperature. The membranes were then washed 3 times with TBST for 15 min each wash. Fluorescent bands were developed with 2 ml of substrate containing the same volume of each Super Signal West Pico Luminol Enhancer solution and Super Signal West Pico Stable Peroxidase solution at room temperature for 5 min. The fluorescence image was obtained from VersaDoc Imaging System 5000 (Bio-Rad, Hercules, CA). The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software included with the VersaDoc imaging system.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the liver, the thyroid gland, the adrenal gland and the testis/ovary cells using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. The concentration and purity of the extracted RNA were checked spectrophotometrically by measuring 260/280 absorption ratios. M-MLV Reverse Transcriptase (Promega, Madison, WI) with 1 μg of total RNA was used to synthesize cDNA, and 5 μl of reverse-transcribed product served as the template in polymerase chain reactions.

Real-time PCR was performed on a fluorescence temperature cycler (Applied Biosystems 7500 Real-Time PCR System) using the qPCR MasterMix Plus with SYBR® Green I kit (EUROGENTEC, Fremont, CA) following the manufacturer’s instructions

(Applied Biosystems, Foster City, CA). This system uses fluorescence-based PCR chemistries to provide quantitative detection of nucleic acid sequence using real-time analysis. Amplifications were carried out using 12.5 μl of 2×SYBR® Green PCR Master Mix, which contains SYBR® Green I dye, AmpliTaq® Gold DNA Polymerase, deoxynucleoside triphosphates with deoxyuricil triphosphate, Passive Reference and optimized buffer components (Applied Sysytems), 0.5 μl of each primer, 2 μl of cDNA in a total volumn of 25 μl . Real-time PCR conditions were: activation of DNA polymerase at 95 ℃ for 10 min, followed by 40 cycles of melting at 95 ℃ for 15 sec and annealing and extension at 60 ℃ for 1 min. SYBR® Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during the cycle. A dissociation curve analysis of the amplification products was performed at the end of the PCR run by rapidly increasing the temperature to 95 ℃ followed by immediate cooling to 60 ℃ for 1 min, after which the temperature was gradually increased to 95 ℃ with continuous measurement of fluorescence to confirm amplification of specific transcripts. The overall results obtained were analyzed by Applied Biosystems' 7500 System Sequence Detection Software Version 1.2.3 (Applied Biosystems, Foster City, CA).

Primers were designed with Primer Express Software (Applied Biosystems, Foster City, CA) as follows: rSULT2A1-F537: 5’-TCATGGTTTGAGCACATCCG-3’, rSULT2A1-R661: 5’-CTTGAGGGCCAAATCCAGCTCA-3’; rSULT2B1a-F214: 5'-AGGAT CATCG TGGAA TGGAG-3', rSULT2B1a-R437: 5'-GGTCC CCATC TTTCA GGATT-3'; rSULT2B1b-F75: 5'-CTATG GGGCT CATTG GAGAA-3', rSULT2B1b-R248: 5'-GGGGT AGGTG ACGAT GAAG-3'.

Rat β-actin was used to normalize rSULT2A1, rSULT2B1a, and rSULT2B1b mRNA levels, and the data was analyzed by relative quantification method. Primer sequences for β-actin were: rβ-actin-F320: 5'-AGGCC CCTCT GAACC CTAAG-3', r β-actin-R435: 5'-AGAGG CATAC AGGGA CAACA CA-3'. Each sample was run in duplicate within a reverse transcription qRT-PCR experiment, and each experiment was repeated a minumun of three times.

Data Analysis

One-way ANOVA followed by the Dunnett's test was used to calculate the statistical significance of the difference between the control group means and methamphetamine treatment group means. In all cases, *, $P<0.05$ was considered significant; **, $P<0.01$ was considered very significant. Data presented in the figures are means ± SD (standard deviation) of the data collected separately from at least three individual animals.

CHAPTER III

RESULTS AND FINDINGS

Distribution of rSULT2A1, rSULT1A1, and rSULT2B1b in the liver, the thyroid gland, the adrenal gland and the ovary of female rats

All of the rats used for distribution experiments were from the control group. Figure 1a shows the distribution of SULT2A1 in the liver, the thyroid gland, the adrenal gland and the ovary of female rats. The results show that SULT2A1 is expressed in all four organs, but the expression levels vary varied among these four organs. The highest expression of rSULT2A1 occurs in the liver, which is 50 times more than the lowest expression, found in the thyroid gland. In Figure 1b, one can see that SULT1E1 is expressed in each of the four organs, with highest expression in the liver. Figure 1c shows that SULT2B1b is expressed by all four of these organs with the highest expression occurring in the ovary.

Effects of methamphetamine treatment on rSULT2B1b in the thyroid gland of female and male rats

Figure 2 shows the effect of methamphetamine on the expression of rSULT2B1b in the thyroid gland of female and male rats. In female rats, the highest dose of methamphetamine (20 mg/kg) increased the expression of rSULT2B1b significantly (2.5 fold, $P< 0.05$). In male rats, the highest dose of methamphetamine (20 mg/kg) significantly induced the expression of rSULT2B1b (4 fold, $p< 0.01$). For both male and female rats, the two lower dose treatments (1 mg/kg and 5 mg/kg) didn't show significant effect on the expression of rSULT2B1b ($p> 0.05$).

Effects of methamphetamine treatment on rSULT2B1b in the adrenal gland of female and male rats

Figure 3a shows the effect of methamphetamine on the expression of rSULT2B1b in the adrenal gland of female and male rats. In female rats, the expression of rSULT2B1b was significantly induced by methamphetamine treatment in all three experimental groups (1.6 fold in 1 mg/kg group, $p< 0.01$; 1.7 fold in 5 mg/kg group, $p< 0.01$; 1.4 fold in 20 mg/kg, $p< 0.05$). In male rats , only the lowest dose treatment (1 mg/kg) induced the expression of rSULT2B1b significantly (1.7 fold, $p< 0.05$). The changes in rSULT2B1b expression in the other two highest-dose experimental groups were not statistically significant in males ($p> 0.05$). Reverse transcription qRT-PCR result in Figure 3b shows that in the adrenal gland of male rats, mRNA transcription was

induced significantly ($p < 0.05$) by methamphetamine only in the lowest dose group (1 mg/kg), while the other experimental groups were not significantly induced($p > 0.05$).

Effects of methamphetamine treatment on rSULT2B1b in the ovary of female rats

Figure 4 shows the effects of methamphetamine on rSULT2B1b in the ovary of female rats. Higher doses of methamphetamine significantly induced the expression of rSULT2B1b (2.7 fold in 5 mg/kg group, $p < 0.05$; 2.6 fold in 20 mg/kg group, $p < 0.01$), while rSULT2B1b expression in the lowest dose group (1 mg/kg) was very similar to the expression in the control group.

Effects of methamphetamine treatment on rSULT2B1a in the testis of male rats

Figure 5 shows the effects of methamphetamine on rSULT2B1a in the testis of male rats. As showed in Figure 5a, methamphetamine treatment significantly induced the expression of rSULT2B1a in all three experimental groups (1.8 fold in 1 mg/kg group, $p < 0.05$; 3 fold in 5 mg/kg group, $p < 0.01$; 3.5 fold in 20 mg/kg group $p < 0.05$). The result of reverse transcription qRT-PCR on the testis of rats (Figure 5b) shows that the lower doses of the drug treatment significantly induced mRNA transcription of rSULT2B1a in the testis of rats, while the highest dose group (20 mg/kg) did not change significantly after the drug treatment.

Effects of methamphetamine on rSULT2B1b in the liver of female and male rats

Figure 6a shows that higher doses of one day methamphetamine treatment significantly induced the expression of rSULT2B1b in the liver of female rats (3.7 fold in 5 mg/kg group, $p< 0.01$; 2.4 fold in 20 mg/kg group, $p< 0.05$), while the lowest dose group (1 mg/kg) showed no significant induction. In the liver of male rats , there was no significant change of the expression of rSULT2B1b after the drug treatment between each experimental group and the control group. However, the reverse transcription qRT-PCR results in the liver of male rats (Figure 6b) showed a marked inhibition of rSULT2B1b mRNA transcription in 1 mg/kg group (40%, $p< 0.01$) and 20 mg/kg group (60%, $p< 0.01$).

Effects of methamphetamine on rSULT2A1 in the liver of male and female rats

Figure 7a shows that the expression of rSULT2A1 in each of the three experimental groups did not change significantly ($p> 0.05$) in the liver of female rats after one day methamphetamine treatment. In the mean time, reverse transcription qRT-PCR result in Figure 7b shows that the mRNA transcription of rSULT2A1 in the liver of female rats was not affected by one day methamphetamine treatment as well. In male rats (Figure 7a), higher doses of methamphetamine treatment significantly inhibited the expression of rSULT2A1 (50% in 5 mg/kg group, $p< 0.05$; 60% in 20 mg/kg group, $p< 0.05$), while the lowest dose treatment of methamphetamine (1 mg/kg) did not significantly affect the expression of rSULT2A1 in the liver of male rats.

Effects of methamphetamine on rSULT2A1 in the ovary of female rats and the testis of male rats

Figure 8a shows that higher doses of one day methamphetamine treatment significantly induced the expression of rSULT2A1 in the ovary of female rats (2 fold in 5 mg/kg group, $p< 0.05$; 3.1 fold in 20 mg/kg group, $p< 0.01$), while the lowest dose group of drug treatment (1 mg/kg) did not change the expression of rSULT2A1 significantly. Figure 8b shows that the expression of rSULT2A1 in the testis of male rats was not affected by one day treatment of methamphetamine.

Effects of methamphetamine on rSULT1A1 in the liver, the thyroid gland, and the ovary of female rats

In Figure 9a, western blotting results demonstrate that higher doses of one day methamphetamine treatment significantly increased the expression of rSULT1A1 in the thyroid gland of female rats (6.5 fold in 5 mg/kg, $p< 0.05$; 5 fold in 20 mg/kg, $p< 0.05$). Figure 9b shows that the expression of rSULT1A1 in the ovary of female rats did not change significantly after the drug treatments. Surprisingly, the expression of rSULT1A1 in the liver of female rats (Figure 9c) was decreased significantly due to methamphetamine treatment (80% in 1 mg/kg group, $p< 0.01$; 80% in 20 mg/kg, $p< 0.01$), while the expression of rSULT1A1 in the 5 mg/kg group was not changed significantly.

Effects of methamphetamine on the expression of rSULT1E1 in the ovary of female rats

The expression of rSULT1E1 in the ovary of female rats was not significantly changed after one day methamphetamine treatment as shown in Figure 10.

FIGURE LEGENDS

Figure 1. Distribution of sulfotransferase isoform 2A1 (Figure 1a), 1E1 (Figure 1b), and 2B1b (Figure 1c) in the liver, the adrenal gland, the ovary, and the thyroid gland of female rats.

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals.

Figure 2. Expression change of sulfotransferase 2B1b 24 hours after a single dose treatment of methamphetamine in the thyroid gland of female and male rats.

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ compared to control group; ** $P<0.01$ compared to control group.

Figure 3. Expression change of sulfotransferase 2B1b 24 hours after a single dose treatment of methamphetamine in the adrenal gland of female and male rats (Figure 3a). mRNA transcription change of sulfotransferase 2B1b after 1 day treatment of methamphetamine in the adrenal gland of male rats (Figure 3b).

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ compared to control group; ** $P<0.01$ compared to control group. Real time RT-PCR results were analyzed by the 7500 System Sequence Detection Software Version 1.2.3 (Applied Biosystems, Foster City, CA). Each sample was run in duplicate within a reverse transcription qRT-PCR experiment, and each experiment was repeated a minumun of three times. The data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ comparing with the control group; ** $P<0.01$ comparing with the control group.

Figure 4. Expression change of sulfotransferase 2B1b 24 hours after a single dose treatment of methamphetamine in the ovary of female rats.

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ compared to control group; ** $P<0.01$ compared to control group.

Figure 5. Expression change of sulfotransferase 2B1a 24 hours after a single dose treatment of methamphetamine in the testis of male rats (Figure 5a). mRNA transcription change of sulfotransferase 2B1a 24 hours after a single dose treatment of methamphetamine in the testis of male rats (Figure 5b).

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ compared to control group; ** $P<0.01$ compared to control group. reverse transcription qRT-PCR results were analyzed by the 7500 System Sequence Detection Software Version 1.2.3 (Applied Biosystems, Foster City, CA). Each sample were run at least three duplications and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ comparing with the control group; ** $P<0.01$ comparing with the control group.

Figure 6. Expression change of sulfotransferase 2B1b 24 hours after a single dose treatment of methamphetamine in the liver of female and male rats (Figure 6a). mRNA transcription change of sulfotransferase 2B1b 24 hours after a single dose treatment of methamphetamine in the liver of male rats (Figure 6b).

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ compared

to control group; ** P<0.01 compared to control group. reverse transcription qRT-PCR results were analyzed by the 7500 System Sequence Detection Software Version 1.2.3 (Applied Biosystems, Foster City, CA). Each sample were run at least three duplications and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * P<0.05 comparing with the control group; ** P<0.01 comparing with the control group.

Figure 7. Expression change of sulfotransferase 2A1 24 hours after a single dose treatment of methamphetamine in the liver of female and male rats (Figure 7a). mRNA transcription change of sulfotransferase 2A1 24 hours after a single dose treatment of methamphetamine in the liver of female rats (Figure 7b).

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * P<0.05 compared to control group; ** P<0.01 compared to control group. reverse transcription qRT-PCR results were analyzed by the 7500 System Sequence Detection Software Version 1.2.3 (Applied Biosystems, Foster City, CA). Each sample was run in duplicate within a reverse transcription qRT-PCR experiment, and each experiment was repeated a minumun of three times. The data presented are means ± S.D. of the data collected separately from at least three independent animals. * P<0.05 comparing with the control group; ** P<0.01 comparing with the control group.

Figure 8. Expression change of sulfotransferase 2A1 24 hours after a single dose treatment of methamphetamine in the ovary of female rats (Figure 8a) and the testis of male rats (Figure 8b).

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ compared to control group; ** $P<0.01$ compared to control group.

Figure 9. Expression change of sulfotransferase 1A1 24 hours after a single dose treatment of methamphetamine in the thyroid gland (Figure 9a), the ovary (Figure 9b), and the liver (Figure 9c) of female rats.

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of the data collected separately from at least three independent animals. * $P<0.05$ compared to control group; ** $P<0.01$ compared to control group.

Figure 10. Expression change of sulfotransferase 1E1 24 hours after a single dose treatment of methamphetamine in the ovary of female rats.

The densitometric quantification of protein bands was performed with Quantity One 4.6.5 software of the Bio-Rad VersaDoc imaging system. Each western blotting experiment was repeated at least three times and the data presented are means ± S.D. of

the data collected separately from at least three independent animals. * $P<0.05$ compared to control group; ** $P<0.01$ compared to control group.

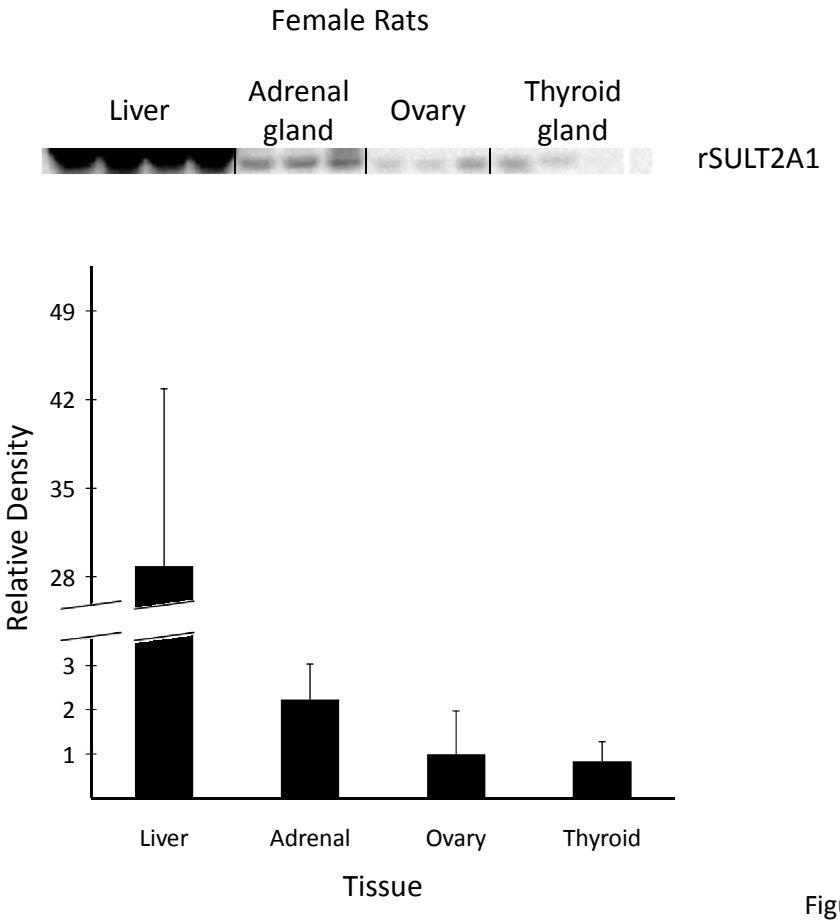
Female Rats
Liver
Adrenal gland
Ovary
Thyroid gland
rSULT2A1
Relative Density
49
42
35
28
3
2
1
Liver
Adrenal
Ovary
Thyroid
Tissue

Figure 1a

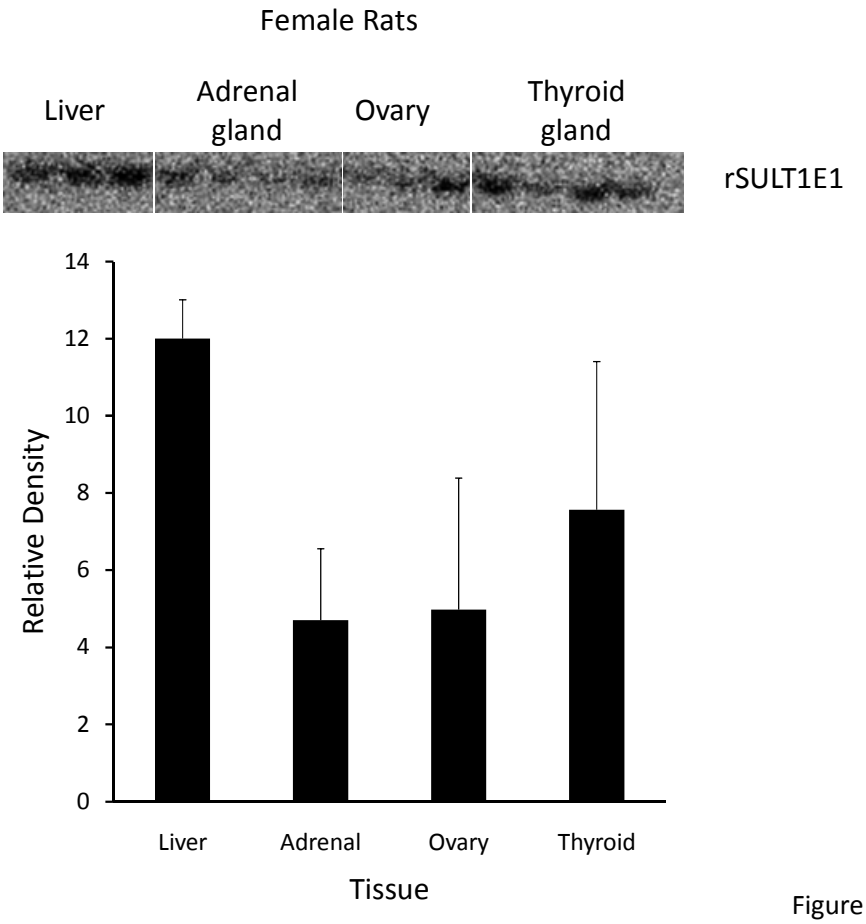
Female Rats
Liver
Adrenal gland
Ovary
Thyroid gland
rSULT1E1
Relative Density
14
12
10
8
6
4
2
0
Liver
Adrenal
Ovary
Thyroid
Tissue

Figure 1b

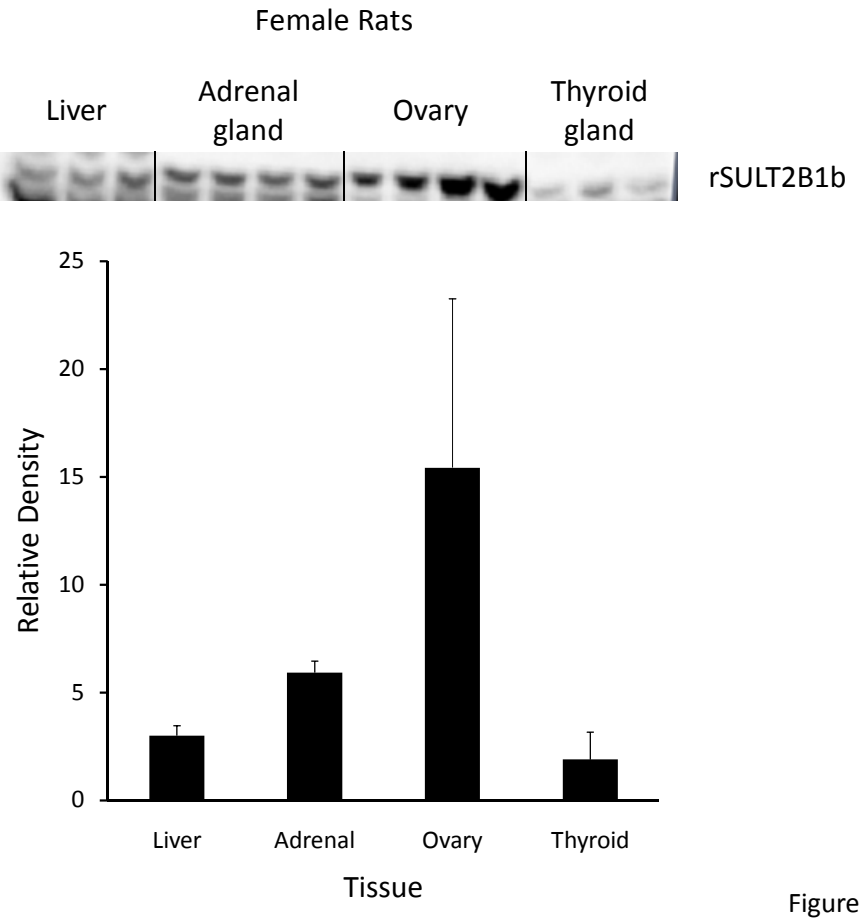
Female Rats
Liver
Adrenal gland
Ovary
Thyroid gland
rSULT2B1b
Relative Density
25
20
15
10
5
0
Liver
Adrenal
Ovary
Thyroid
Tissue

Figure 1c

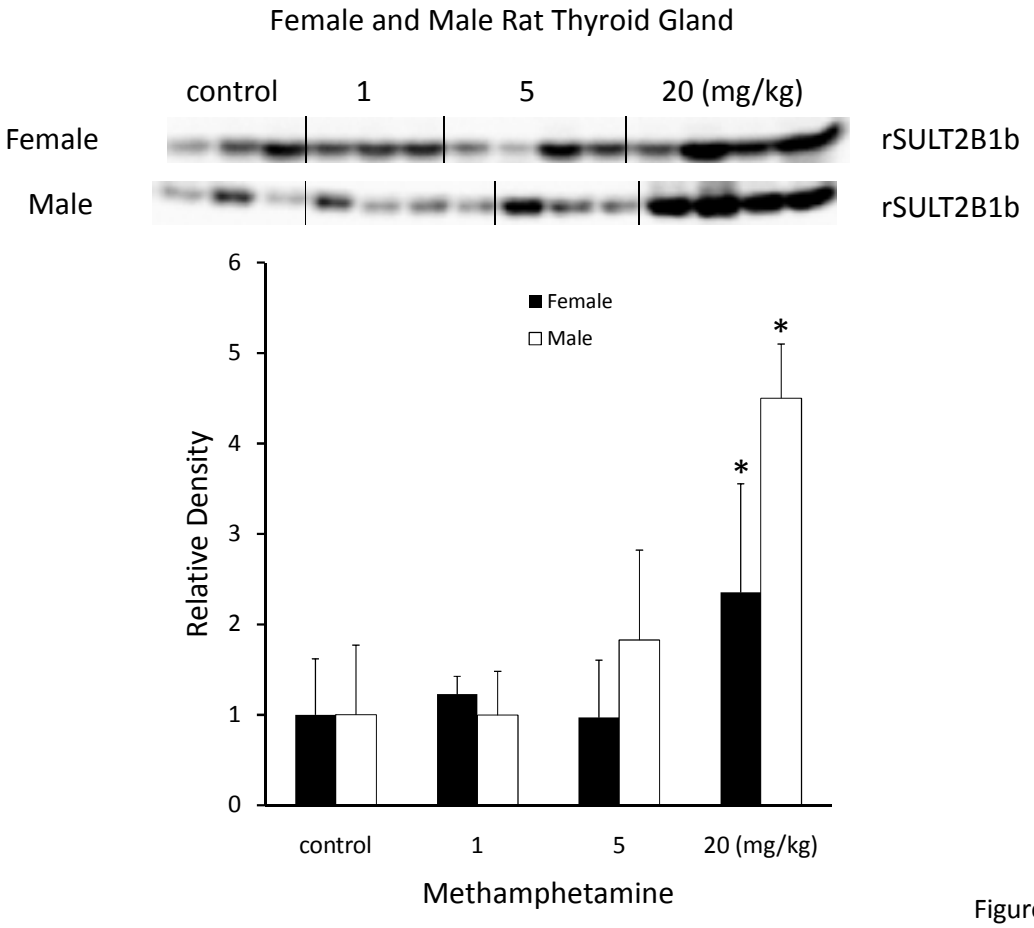
Female and Male Rat Thyroid Gland
control
1
5
20 (mg/kg)
Female
rSULT2B1b
Male
rSULT2B1b
Female
Male
Relative Density
0
1
2
3
4
5
6
*
*
control
1
5
20 (mg/kg)
Methamphetamine

Figure 2

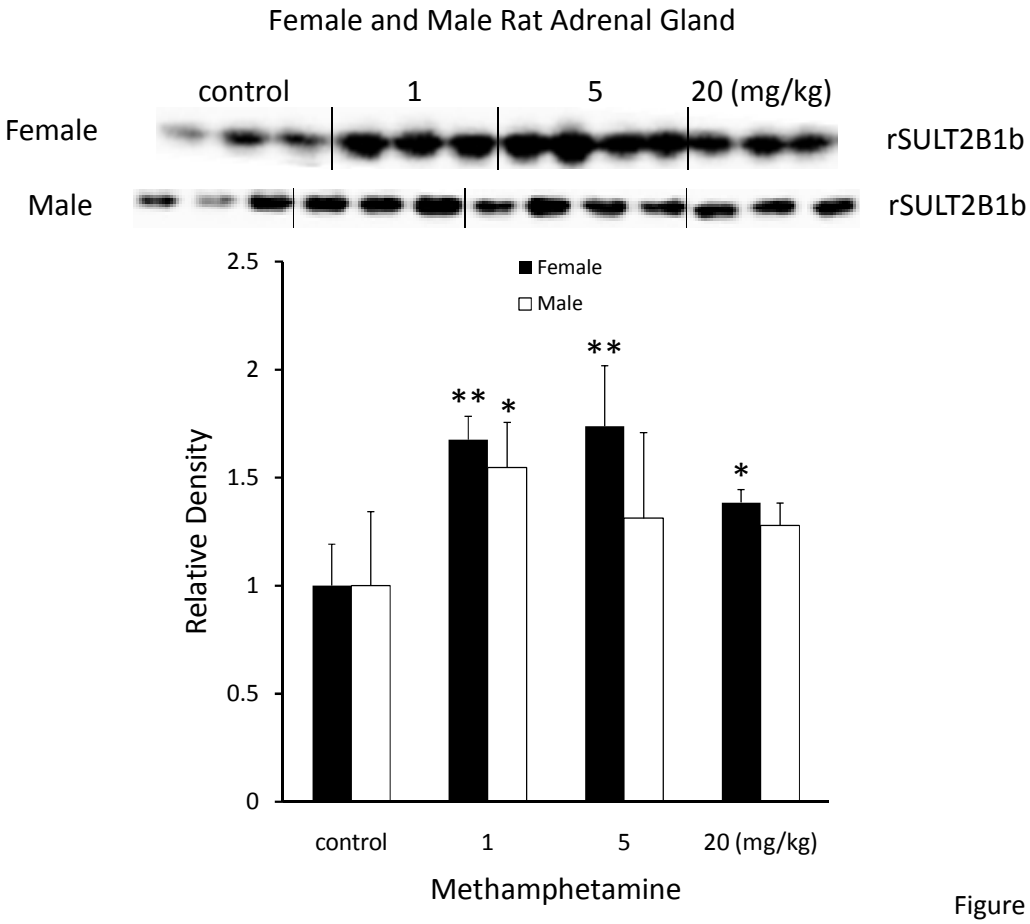
Female and Male Rat Adrenal Gland
control
1
5
20 (mg/kg)
Female
rSULT2B1b
Male
rSULT2B1b
Female
Male
Relative Density
2.5
2
1.5
1
0.5
0
**
*
**
*
control
1
5
20 (mg/kg)
Methamphetamine

Figure 3a

Male Rat Adrenal Gland

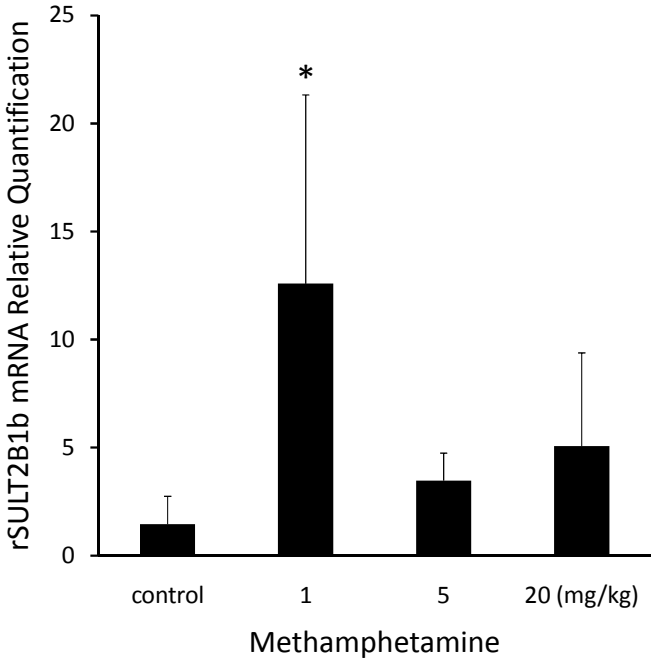

Figure 3b

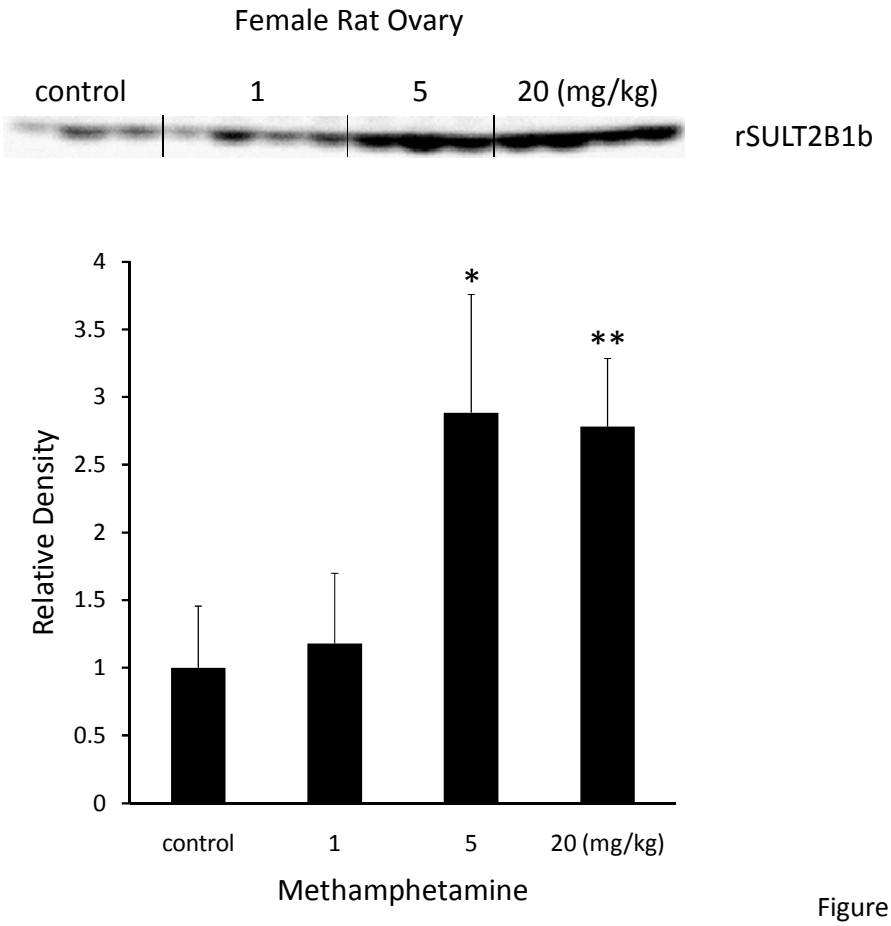
Female Rat Ovary
control
1
5
20 (mg/kg)
rSULT2B1b
Relative Density
4
3.5
3
2.5
2
1.5
1
0.5
0
*
**
control
1
5
20 (mg/kg)
Methamphetamine

Figure 4

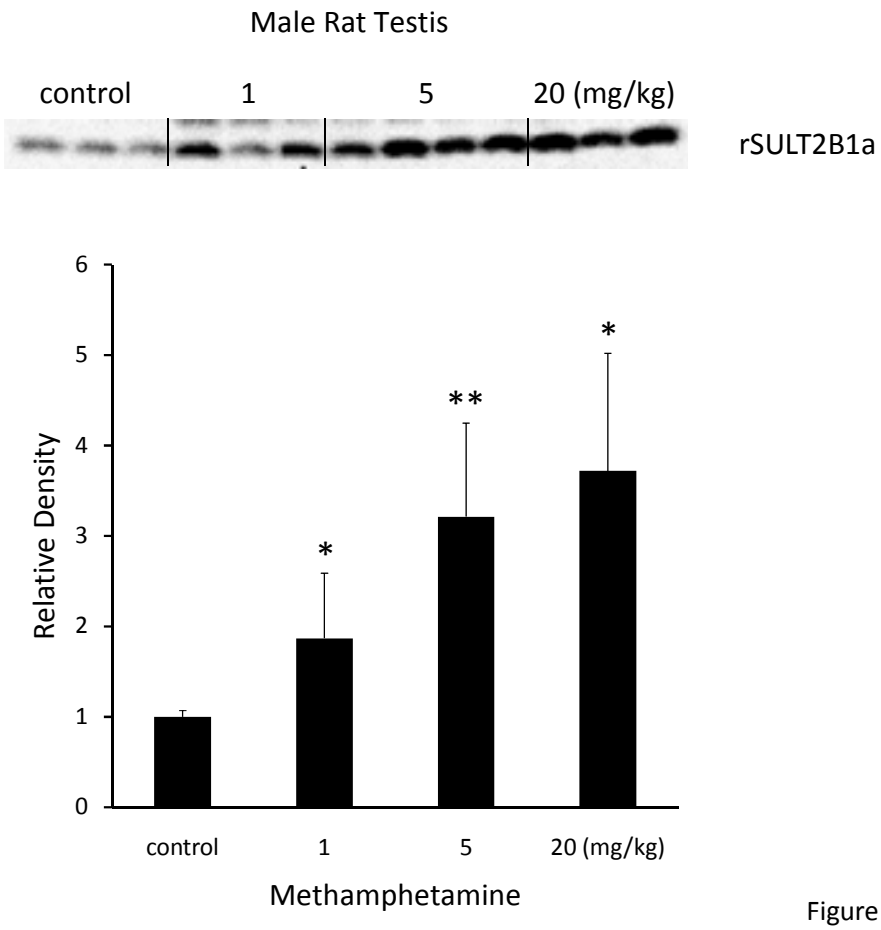
Male Rat Testis
control
1
5
20 (mg/kg)
rSULT2B1a
Relative Density
0
1
2
3
4
5
6
*
**
*
control
1
5
20 (mg/kg)
Methamphetamine

Figure 5a

Male Rat Testis

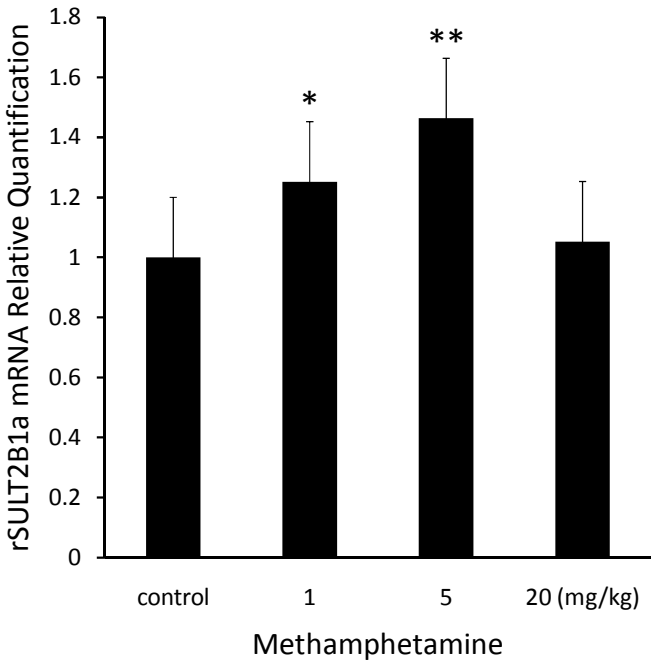

Figure 5b

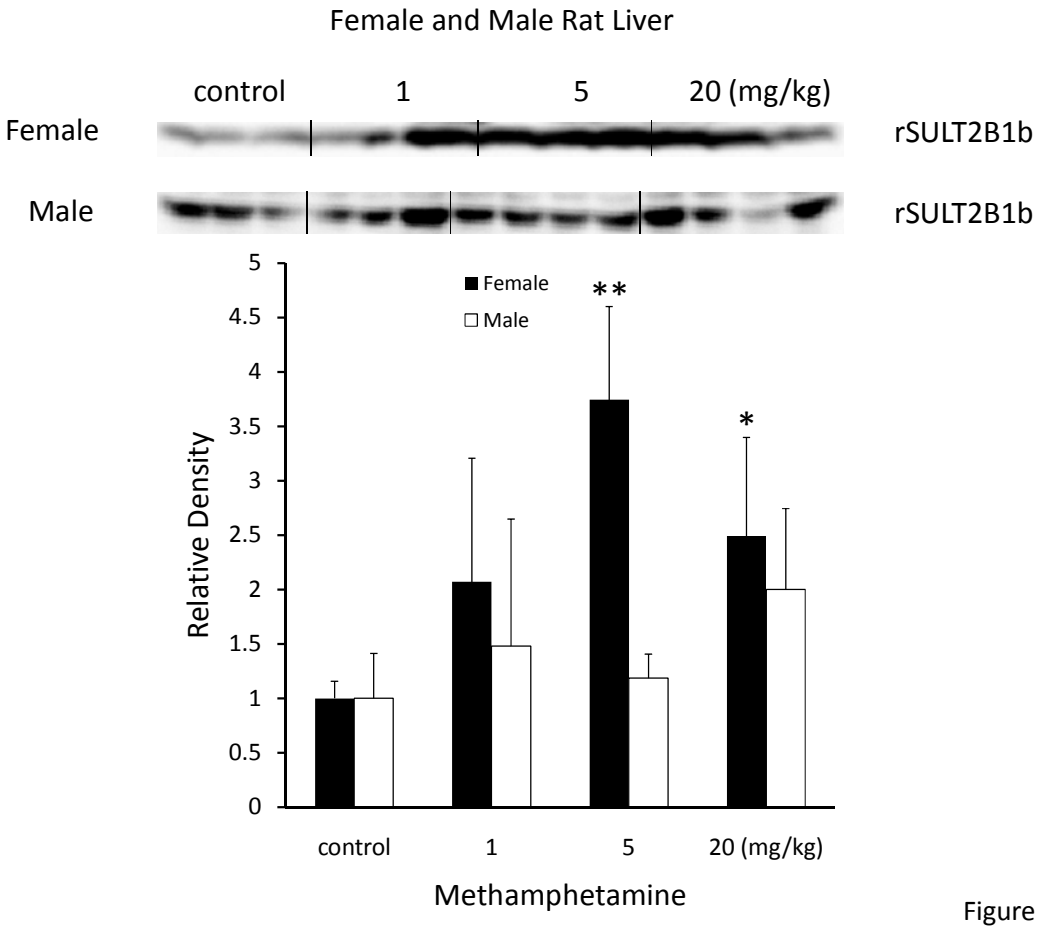
Female and Male Rat Liver
control
1
5
20 (mg/kg)
Female
rSULT2B1b
Male
rSULT2B1b
Female
Male
**
*
Relative Density
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
control
1
5
20 (mg/kg)
Methamphetamine

Figure 6a

Male Rat Liver

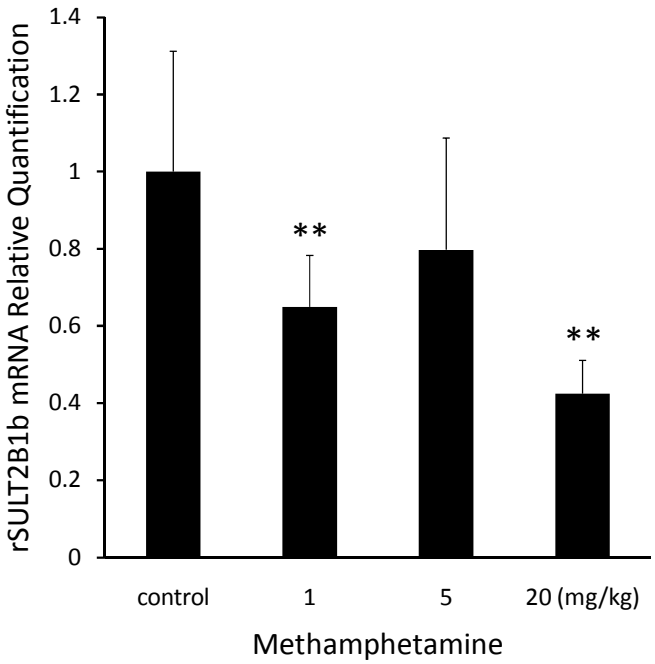

Figure 6b

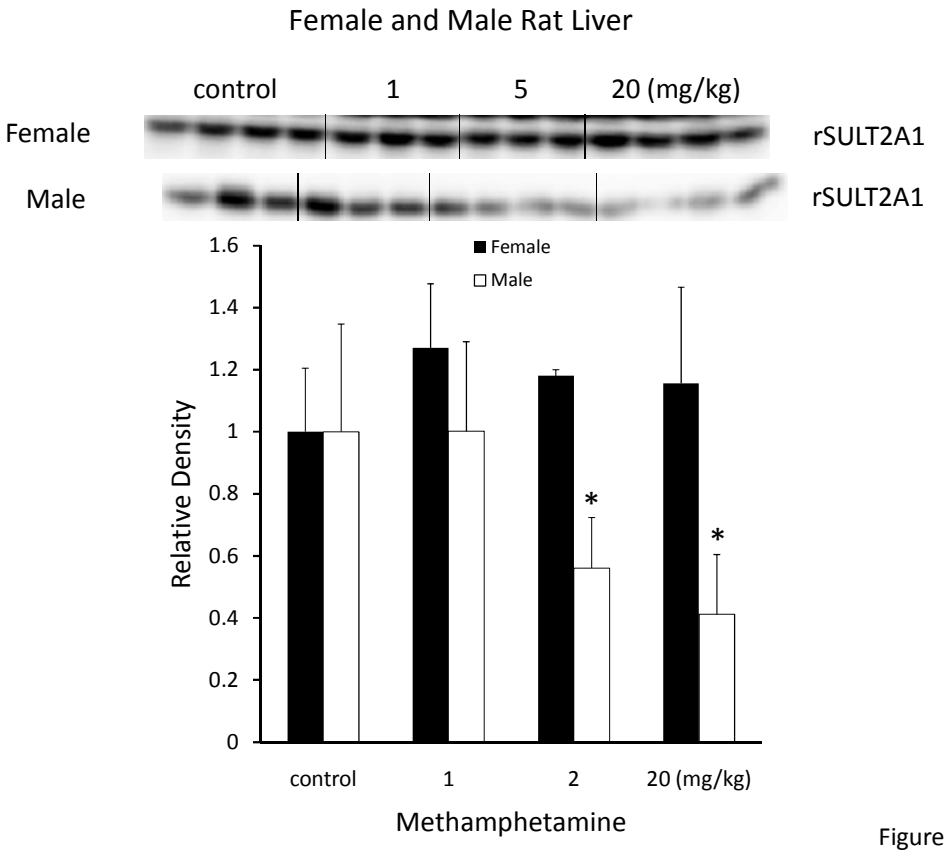
Female and Male Rat Liver
control
1
5
20 (mg/kg)
Female
rSULT2A1
Male
rSULT2A1
Female
Male
Relative Density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
control
1
2
20 (mg/kg)
Methamphetamine

Figure 7a

Female Rat Liver

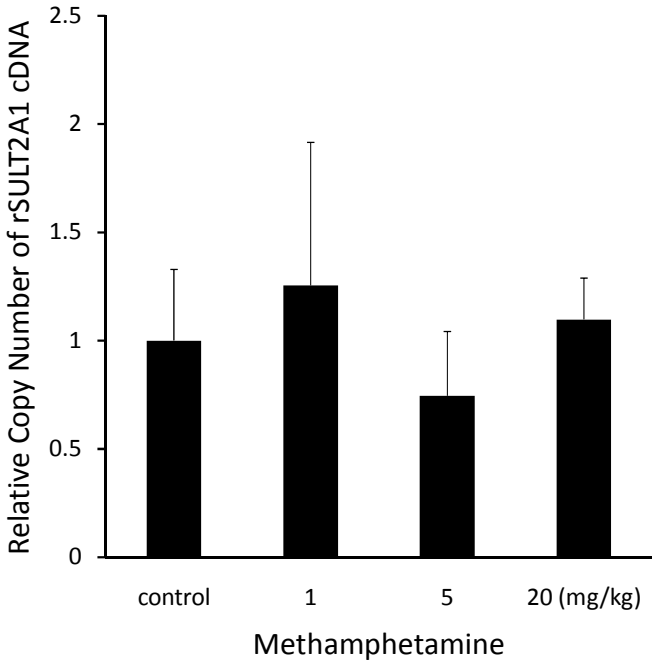

Figure 7b

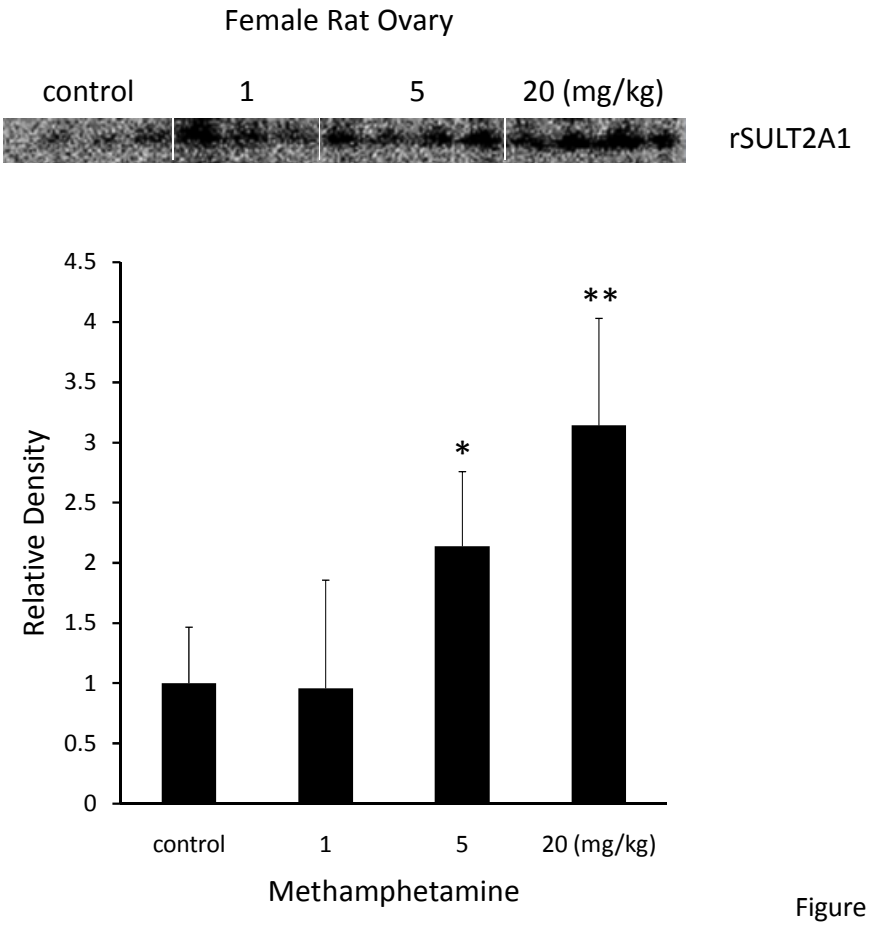
Female Rat Ovary
control
1
5
20 (mg/kg)
rSULT2A1
Relative Density
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
**
control
1
5
20 (mg/kg)
Methamphetamine

Figure 8a

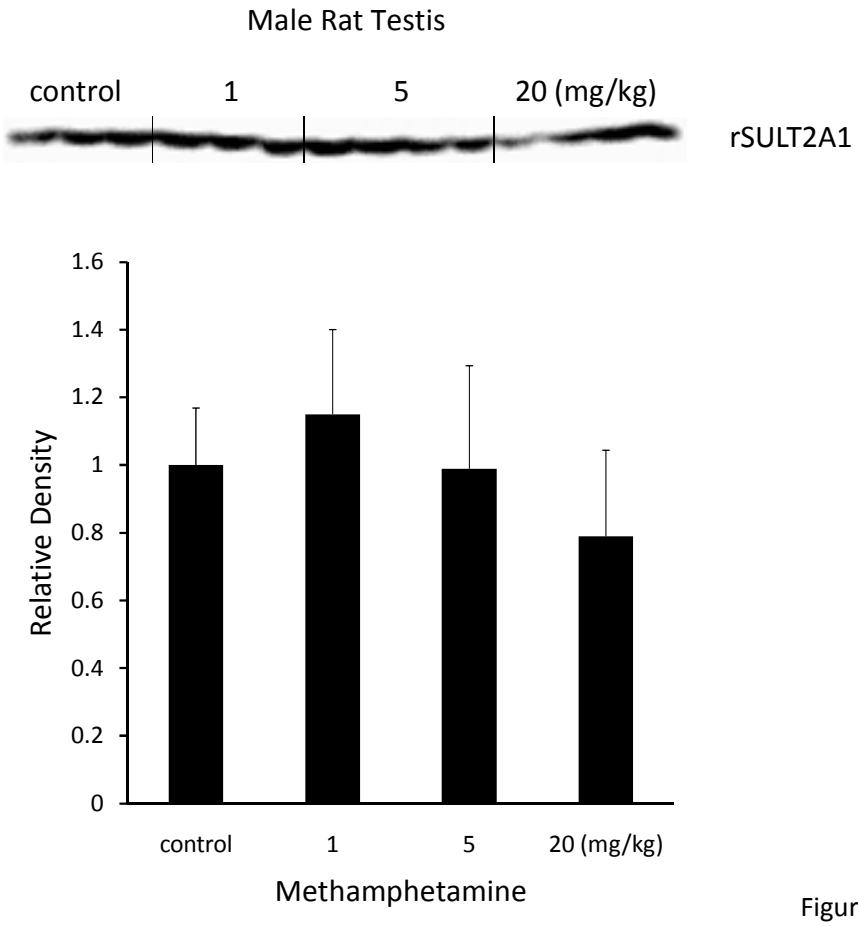
Male Rat Testis
control
1
5
20 (mg/kg)
rSULT2A1
Relative Density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
1
5
20 (mg/kg)
Methamphetamine

Figure 8b

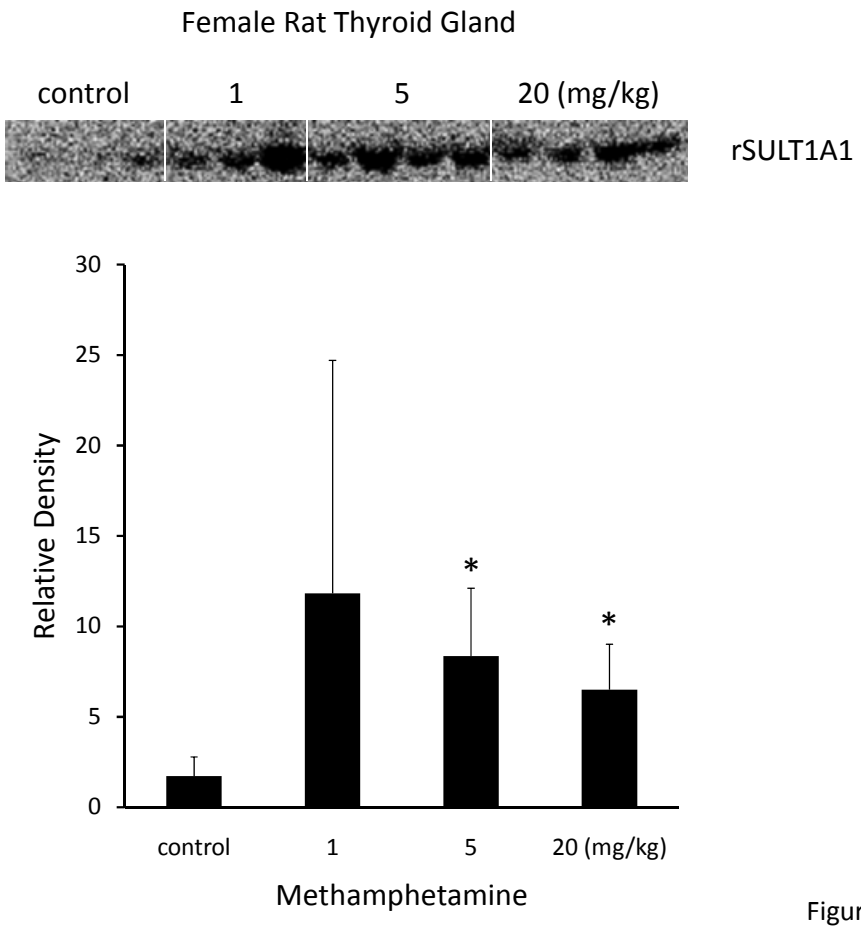
Female Rat Thyroid Gland
control
1
5
20 (mg/kg)
rSULT1A1
Relative Density
30
25
20
15
10
5
0
*
*
control
1
5
20 (mg/kg)
Methamphetamine

Figure 9a

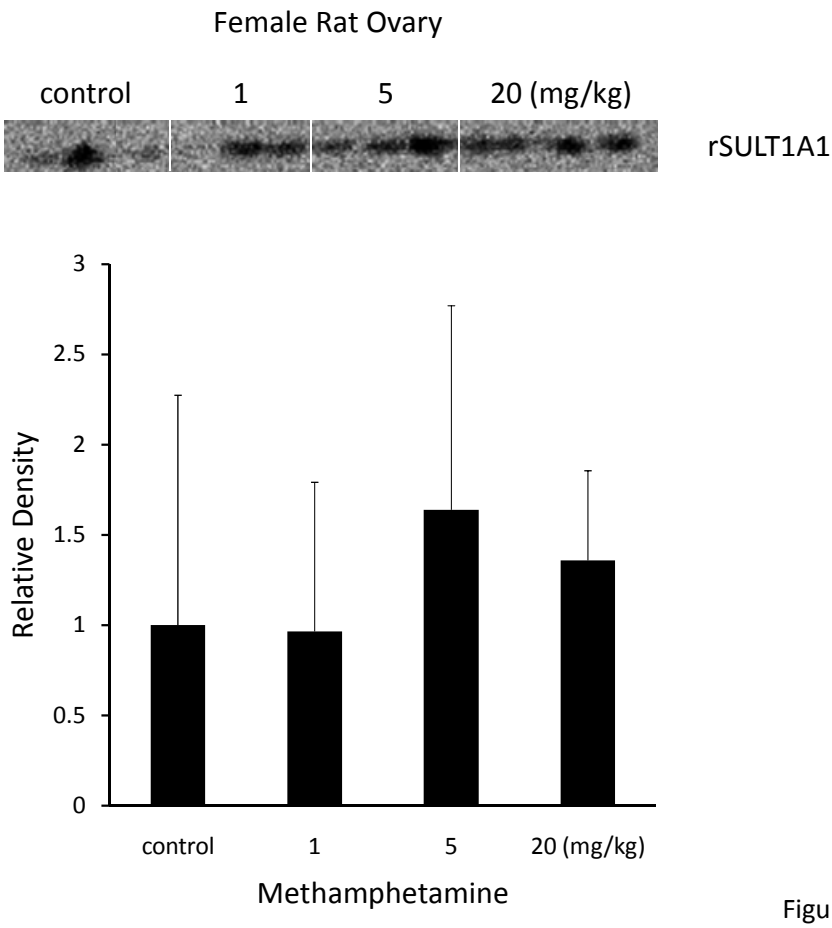
Female Rat Ovary
control
1
5
20 (mg/kg)
rSULT1A1
Relative Density
3
2.5
2
1.5
1
0.5
0
control
1
5
20 (mg/kg)
Methamphetamine

Figure 9b

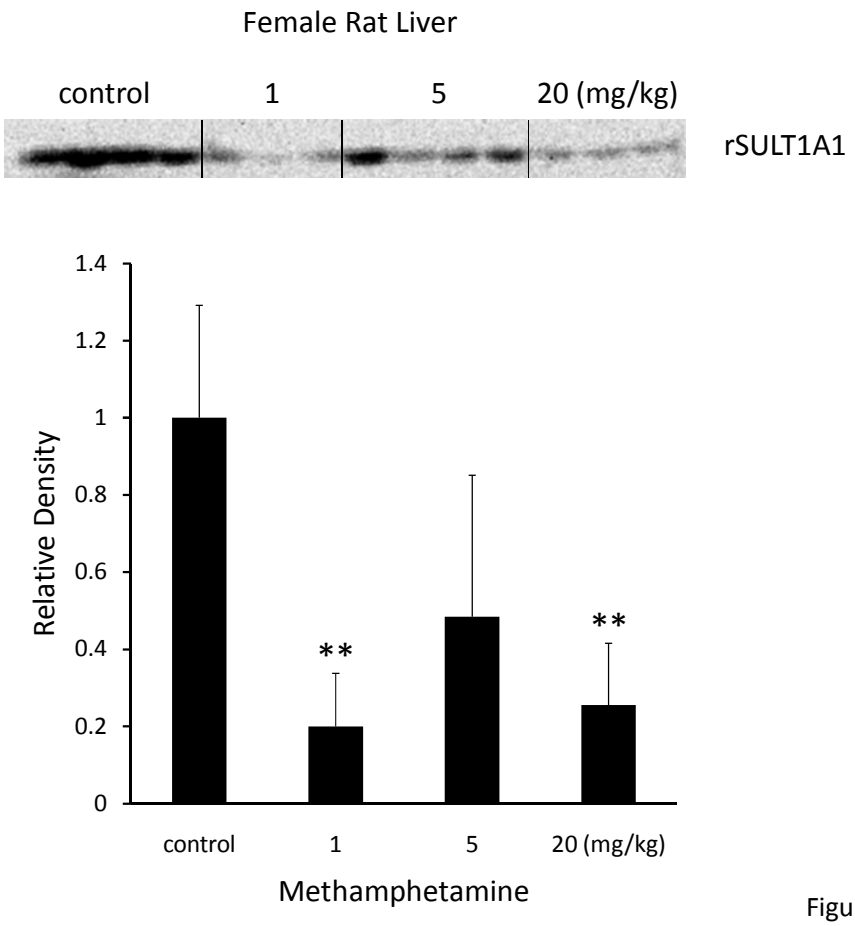
Female Rat Liver
control
1
5
20 (mg/kg)
rSULT1A1
Relative Density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
control
1
5
20 (mg/kg)
Methamphetamine

Figure 9c

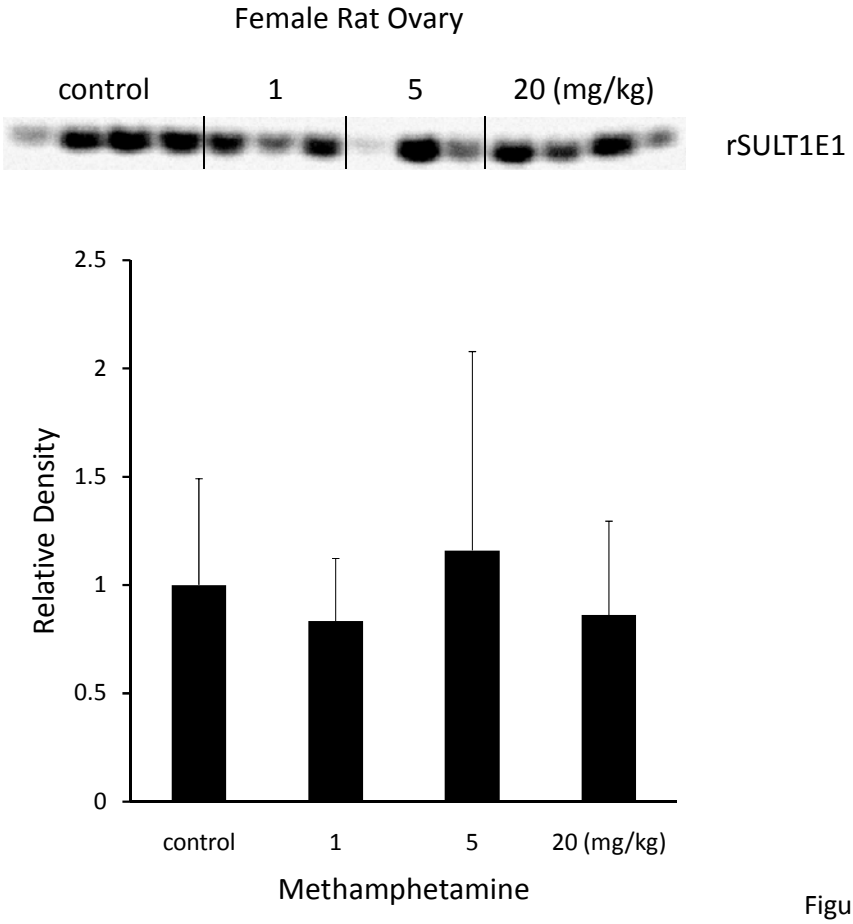
Female Rat Ovary
control
1
5
20 (mg/kg)
rSULT1E1
2.5
2
1.5
1
0.5
0
Relative Density
control
1
5
20 (mg/kg)
Methamphetamine

Figure 10

CHAPTER IV

DISCUSSION

Methamphetamine affects hormonal secretions of the thyroid gland, the adrenal gland and the testis/ovary either by directly interacting with specific receptors on the cell surface or indirectly through the increase of dopamine level in the nervous system (Melis & Argiolas, 1995; Agmo & Fernandez, 1989; Giuliano & Allard, 2001; Power, Mani, Codina, Conneely, & O'Malley, 1991; Sprague *et al.*, 2003; Grace *et al.*, 2010; Williams *et al.*, 2006; Williams *et al.*, 2000; Schaefer *et al.*, 2008). The phase II drug-metabolizing enzyme cytosolic sulfotransferases (SULTs) have been shown to be involved in regulating the activities of these endogenous hormones in the existence of sulfatases (Luu-The *et al.*, 1996; Chapman *et al.*, 2004). Some steroid hormones are the targets of cytosolic sulfotransferases (Luu-The *et al.*, 1996; Chapman *et al.*, 2004). However, the information regarding the effects of METH on the expression of SULTs in these endocrine glands is very limited. In the present study, we investigated protein expression and mRNA transcription changes of selected SULT isoforms (rSULT1A1, rSULT1E1, rSULT2A1, rSULT2B1) in the thyroid gland, the adrenal gland, the ovary/testis

of female and male Sprague-Dawley rats 24 hours after acute methamphetamine exposure. We also investigated the effects of METH on those SULT isoforms in the liver of female and male rats in the same treatment. Because the liver is the main detoxification organ in the body and is one of the targeting tissues of endocrine hormones, we were interested in studying the effects of METH on SULTs in the liver.

The data show that the cholesterol sulfotransferase, rSULT2B1b, expression was significantly induced 24 hours after a single dose treatment of METH in all of the three endocrine glands studied in female rats. rSULT2B1b expression in the thyroid gland and the adrenal gland of male rats was also significantly induced by METH. According to Kohjitani *et al.* (Kohjitani, Fuda, Hanyu, &Strott, 2005) and our experimental results, rSULT2B1a is the only SULT2B isoform expressed in the testis of rats. These results show that the expression of rSULT2B1a was induced significantly in the testis by one day METH treatment. The reverse transcription qRT-PCR results of rSULT2B1b and rSULT2B1a agreed with Western blotting data in the adrenal gland and the testis of male rats (We failed to save enough samples of female rats for reverse transcription qRT-PCR). This suggests that the regulation of rSULT2B1a and rSULT2B1b by METH in the adrenal gland and the testis of male rats might occur at the transcriptional level. The expression of rSULT2A1 was shown by Western blotting to be significantly induced in the ovary of female rats after the METH treatment. The expression of rSULT2A1 was inhibited in the liver of male rats by METH. rSULT2A1 expression was not changed significantly in the testis of male rats or in the liver of female rats. mRNA transcription of rSULT2A1 in the liver of female rats also did not change significantly after the drug treatment. The expression of rSULT1A1 was induced significantly in the thyroid gland of

male rats after the drug treatment. Surprisingly, rSULT1A1 was inhibited significantly in the liver of female rats by METH according to our Western blotting results. The expression of rSULT1A1 in the ovary of female rats was not affected by METH. For rSULT1E1, we only got results in the ovary of female rats by Western blotting, and the expression of this enzyme in the ovary was not affected by METH treatment.

The effects of methamphetamine on endocrine hormones have been studied in the past few years. METH exposure increased both instant and chronic release of corticosteroids in human (Grace *et al.*, 2010; Williams *et al.*, 2006; Williams *et al.*, 2000; Schaefer *et al.*, 2008). It has also been reported that thyroxine (T_4) level had been significantly induced after a single high dose treatment (40 mg/kg) of a methamphetamine derivative MAMA (3,4-methylenedioxymethamphetamine) (Sprague *et al.*, 2003). METH exposure significantly increases dopamine levels in the synapse by blocking dopamine reuptake by the neuron cells (Brennan *et al.*, 2010; Melo *et al.*, 2010). The female sex hormones, estrogens, are reported to be able to diminish the amount of striatal dopamine depletion caused by METH exposure (Dluzen & McDermott, 2002). The sex-dependent estrogen level might be the reason for the different METH-induced neurotoxicity in male and female individuals (Yu & Liao, 2000). According to our experimental results (Figures 2, 3, and 4), the expression levels of rSULT2B1b in these three endocrine glands were induced significantly by a single dose 24 hours methamphetamine treatment in both female and male rats. In the testis, rSULT2B1a expression was also significantly induced by METH in the same treatment.

Though the primary antibody we used in the testis was the same primary antibody as we used for rSULT2B1b in the other tissues, we believed that the result we got in the

testis from this antibody was rSULT2B1a. First, because the sequences of rSULT2B1a and rSULT2B1b are 99% identical, the antibody that recognizes rSULT2B1b should also recognize rSULT2B1a, and the band obtained was approximately 41 KDa, which is the molecular weight for rSULT2B1a. Secondly, we could not get a rSULT2B1b amplification product using either reverse transcription qRT-PCR or traditional reverse transcription PCR, while rSULT2B1a could be amplified with reverse transcription qRT-PCR. Finally, Kohjitani *et al.* also failed to amplify rSULT2B1b in the testis of male rats (Kohjitani *et al.*, 2006). rSULT2B1a is specific for sulfation of pregnenolone, which is the precursor of testosterone and estrogens. SULT2B1b is also known as the cholesterol sulfotransferase, specifically catalyzing the sulfation of cholesterol as well as most cholesterol derivatives (Fuda *et al.*, 2007). SULT2B1b has been reported not to directly participate in detoxification of xenobiotics but to regulate the signaling pathway of endogenous hormones signaling pathway (Chen *et al.*, 2007; Glass & Saijo, 2008; Fuda *et al.*, 2007). Chen *et al.* reported that overexpression of rSULT2B1b would attenuate the liver X receptors (LXRs) signaling in cultured mammalian cell lines by adding the sulfuryl groups to the oxysterol LXR ligands (Chen *et al.*, 2007). LXRs are nuclear receptors regulating the fatty acid and cholesterol metabolism *in vivo* complementarily with another nuclear receptor sterol regulatory element binding protein (SREBP) (Glass & Saijo, 2008). SREBP independently controls the cholesterol synthesis and uptake, while LXRs are activated by binding with oxysterol ligands to bind and promote the transcription of genes limiting the amount of cholesterol in the cell (Glass & Saijo, 2008). Oxysterols are the newly-found specific substrate for SULT2B1b (Chen *et al.*, 2007). Sulfated oxysterols lose the ability to bind with LXRs and make LXRs unable to promote

the transcription of the genes limiting cholesterol synthesis and uptake. These will trigger the subsequent cholesterol accumulation and lipid metabolizing pathway (Glass & Saijo, 2008).

The adrenal cortex synthesizes adrenal steroid hormones (corticosteroids), that regulate salt and water balance in the body, response to stresses, and are also involved in the immune system, and sexual development and function of the body (Maggio & Segal, 2010). Steroid hormones are derived from the same precursor, cholesterol, through a complex series of biochemical reactions (Hardy & Cooper, 2010). The significant induction of rSULT2B1b expression in the adrenal gland after METH treatment shown by our results (Figure 3) may contribute to the long term increase in the adrenal hormone production. Under normal physiological conditions, LXRs work with SREBP complementarily to maintain the cholesterol level in the cell. When rSULT2B1b expression is induced by METH, the enzyme will sulfate the free oxysterols in the cell and make it unable to bind with LXRs. The inactive LXRs cannot bind and promote the transcription of the genes limiting the amount of cholesterol in the cell, resulting in the accumulation of cholesterol in the adrenal gland cells. Since cholesterol is the precursor of all steroid hormones synthesized by the adrenal gland, the accumulation of cholesterol in the adrenal cortex may contribute to the increased production of steroid hormones after METH treatment. On the other hand, the rapid release of adrenal hormones after METH exposure may result from another pathway which is downstream of the binding of METH to the dopamine receptors on the surface of the adrenal gland cells. The instantly increased adrenal hormones may play a role in the induction of rSULT2B1b expression, and the increased rSULT2B1b expression then further increases the hormone production

through the pathway we discussed above. It is clear that defining the mechanism of rSULT2B1b induction will help better understand the whole METH regulating pathway. The mechanism of rSULT2B1b induction after METH treatment in the adrenal cortex is being investigated in our lab now. We strongly believe that rSULT2B1b is involved in the induced secretion process of steroid hormones in the adrenal cortex.

LXRs also play an important role in regulating lipid metabolism and thyroid hormone status. Synthetic LXR agonist has been reported to significantly decrease the thyronine/thyroxine (T_3/T_4) ratio in plasma (Davies *et al.*, 2008). And this decrease happened together with the decreased expression of deiodinase 1 (DIO1) and 2(DIO2) mRNA in the thyroid gland (Davies *et al.*, 2008). In addition, expression of SREBP-1c was markedly increased in the thyroid gland (Davies *et al.*, 2008). These demonstrated that T_4 production was actually increased after deactivating LXRs by using the LXR agonist. Our results show that rSULT2B1b expression was significantly induced by high dose METH injection (20 mg/kg). The induced expression of rSULT2B1b will sulfate oxysterols and prevent them from binding with LXRs, and that will make LXRs inactive. We believe the LXRs deactivated through the pathway we proposed will have the same effect of the inactive LXRs deactivated by synthetic LXR agonist, that is, increasing T_4 production in the thyroid gland. The increased T_4 level is the effect that Sprague *et al.* monitored after a single high dose treatment of a methamphetamine (40 mg/kg) derivative MAMA (3,4-methylenedioxymethamphetamine) (Sprague *et al.*, 2003).Thus, we believed that rSULT2B1b is involved in the increase of T_4 production in the thyroid gland after METH treatment.

Estrogens are synthesized in the ovary from the precursor cholesterol. Estrogen is reported to diminish the amount of striatal dopamine depletion by METH (Dluzen & McDermott, 2002). They are present in significantly higher level in women than in men. The sex-dependent expression of estrogen has been reported to be the reason for the sexual differences of METH-induced striatal neurotoxicity (Yu & Liao, 2000). METH can induce more serious neurotoxicity in men than in women (Yu & Liao, 2000). Furthermore, ovariectomized female rats were less likely to abuse METH when allowed free-choice access to the drug, as determined by decreased intake of METH (Kucerova, Vrskova, & Sulcova, 2009). These all indicate that constantly expressed estrogen can reduce the dopamine depletion after METH treatment and decrease the neurotoxicity induced by METH. Our results show that the expression of rSULT2B1b was induced by METH in the ovary of female rats. The increased rSULT2B1b enzyme may cause an accumulation of cholesterol in the ovary cells through the pathway discussed above in the adrenal and thyroid gland paragraph. Since cholesterol is also the precursor of all three estrogens, the increased cholesterol level may result in more estrogen synthesis which will further protect the neuron cells from METH's neurotoxic effects. In the testis, estrogens are produced far less in levels than those produced in the ovary. In addition, according to our experimental results, protein expression and mRNA transcription of rSULT2B1a were both induced in testis of rats. rSULT2B1a is specific for sulfation of pregnenolone, which is the precursor of estrogens and testosterone. This will further decrease the amount of estrogens in male rats. Furthermore, testosterone is also reported to counteract the neurotoxicity of METH similar to estrogen (Dluzen & McDermott, 2006). The increase of rSULT2B1a will also decrease testosterone in the testis, which

will intensify the neurotoxicity caused by METH treatment. These may answer why female individuals are reported to exhibit less neurotoxic effects resulting from METH than males. The induction of both protein expression and mRNA transcription of rSULT2B1a suggests that the induction of rSULT2B1a by METH may occur on the transcriptional level. The mechanism of the induction of rSULT2B1b and rSULT2B1a in the ovary and the testis is under investigation in our lab now. We believe the mechanism of rSULT2B1b and rSULT2B1a induction in the ovary and the testis is involved in the sex-dependent neurotoxicity in female and male individuals.

The expression changes of other SULT isoforms are not consistent throughout the three endocrine glands studied, suggesting that the roles other SULT isoforms play in those glands vary from tissue to tissue. Since SULT2A1 is responsible for the sulfation of hydroxysteroids and may be involved in eliminating excess hormones from the body (Pacifici, 2005), it is more likely to have increased expression in those endocrine glands and decreased expression in the hormone target organs, such as kidney and liver. Our results show that the expression of rSULT2A1 was increased in the ovary of female rats. However, the expression of rSULT2A1 has been significantly inhibited by METH in the liver of male rats while not significantly changed in the liver of female rats. This different regulation of rSULT2A1 by METH may result from the mediation of certain nuclear receptors (Chen *et al.* 2006; Fang *et al.* 2007; Kin *et al.* 2004; Seely *et al.* 2005). In ovary, METH may induce the expression of rSULT2A1 by binding to certain nuclear receptors. And in liver, METH may inhibit the expression of rSULT2A1 through endogenous hormones that competitively bind to nuclear receptors. Further studies are needed to uncover the mechanisms of SULT regulation by METH. We believe the

unveiling of these mechanisms will uncover the mystery of how METH works to change the level of hormones produced throughout the body.

CHAPTER V

SUMMARY AND CONCLUSIONS

In present study, we have investigated the comparative levels of different SULT isoforms and the effects of methamphetamine on sulfotransferases in the endocrine glands and the liver of rats. The results can be summarized as follows:

1. Distribution experiments showed that rSULT1E1, rSULT2B1b, and rSULT2A1 are expressed in the thyroid gland, the adrenal gland, the ovary and the liver of female rats.
2. The highest dosage of METH (20 mg/kg) significantly induced the expression of rSULT2B1b in the thyroid gland of both female and male rats.
3. METH significantly induced the expression of rSULT2B1b in the adrenal gland of female rats.
4. The lowest dosage of METH (1 mg/kg) significantly induced the mRNA transcription and protein expression of rSULT2B1b in the adrenal gland of male rats. This indicates that the regulation of rSULT2B1b in the adrenal gland of male rats may occur at the transcriptional level.

5. METH (5 and 20 mg/kg) significantly induced the expression of rSULT2B1b in the ovary of female rats.
6. METH significantly induced the mRNA transcription and the protein expression of rSULT2B1a in the testis of male rats. This indicates that the regulation of rSULT2B1a in the testis of male rats may occur on the transcriptional level.
7. METH (5 and 20 mg/kg) significantly induced the expression of rSULT2B1b in the liver of female rats.
8. 5 mg/kg and 20 mg/kg treatment of METH significantly inhibits the expression of rSULT2A1 in the liver of male rats.
9. 5 mg/kg and 20 mg/kg treatment of METH significantly induces the expression of rSULT2A1 in the ovary of female rats.
10. 5 mg/kg and 20 mg/kg treatment of METH significantly induces the expression of rSULT1A1 in the thyroid gland of female rats.
11. METH significantly inhibits the expression of rSULT1A1 in the liver of male rats.

In summary, METH significantly induced the expressions of rSULT2B1b in the thyroid gland and the adrenal gland of both female and male rats. This suggests that rSULT2B1b may be responsible for the increase of thyroid and adrenal hormone productions after METH exposure through the LXR regulating signaling pathway, because rSULT2B1b is strongly related to the activation and deactivation of LXRs. METH also significantly induced the expression of rSULT2B1b in the ovary and the expression of rSULT2B1a in the testis of rats. This suggests that rSULT2B1b and

rSULT2B1a may be involved in the expression change of estrogens and testosterone in the ovary and testis of rats caused by METH treatment. Sex-dependent sex hormone levels may serve as one of the reasons for the different METH-induced neurotoxicity in female and male individuals.

CHAPTER VI

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VITA

TENG SUN

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF METHAMPHETAMINE ON THE EXPRESSION OF SULFOTRANSFERASES IN ENDOCRINE GLANDS AND THE LIVER OF RATS

Major Field: Veterinary Biomedical Sciences (Toxicology and Enzymology)

Biographical:

Education:

Completed the requirements for the Master of Science in Veterinary Biomedical Sciences at Oklahoma State University, Stillwater, Oklahoma in December, 2010.

Completed the requirements for the Bachelor of Science in Molecular Biology at Jilin University, Changchun, Jilin, China in 2008.

Experience:

Served as a research assistant in Dr. Guangping Chen's lab, Department of Physiological Sciences, School of Veterinary Biomedical Sciences at Oklahoma State University, August 2008 to July 2010.

ADVISER'S APPROVAL: Guangping Chen

Name: Teng Sun Date of Degree: December, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECTS OF METHAMPHETAMINE ON THE EXPRESSION OF SULFOTRANSFERASES IN ENDOCRINE GLANDS AND THE LIVER OF RATS

Pages in Study: 74 Candidate for the Degree of Master of Science

Major Field: Veterinary Biomedical Sciences (Toxicology and Enzymology)

Scope and Method of Study: My objectives were to: 1) To investigate the effects of METH on protein expression of different SULT isoforms in the adrenal gland, the thyroid gland, and the ovary/testis of rats. 2) To investigate the effects of METH on mRNA transcriptions of different SULT isoforms in the adrenal gland, the thyroid gland, and the ovary/testis of rats. 3) To investigate the effects of METH on protein expression and mRNA transcription of SULTs in the liver of rats. Male and Female Sprague-Dawley rats (Harlan, Indianapolis, IN), 10 to 11 weeks old and 200-300 g body weight were used in this investigation. Rats were housed in a temperature- and humidity-controlled room and supplied with rodent chow and water for at least 1 week before use. Rats were divided randomly into groups of four. Methamphetamine was dissolved in saline and administrated by intraperitoneal injection at 1, 5 and 20 mg/kg to 3 separate groups of rats. The control rats received only the saline vehicle. The treatment was single dose treatment. The rats were euthanized 24 h after the drug treatment. The livers, the thyroid gland, the adrenal glands, and the testes/ovaries were collected from each rat and were snap-frozen in liquid nitrogen. Samples were stored at -80 ℃ until use.

Findings and Conclusions: I found that METH significantly induced the expressions of the rat cholesterol sulfotransferase (rSULT2B1b) in the thyroid gland and the adrenal gland of both female and male rats. This suggests that rSULT2B1b may play a role in the increase of thyroid and adrenal hormone productions after METH exposure through the Liver X Receptor (LXR) regulating pathway, because rSULT2B1b is strongly related to the activation and deactivation of LXRs. I also found that METH significantly induced the expression of rSULT2B1b in the ovary and the expression of the rat pregnenolone sulfotransferase (rSLUT2B1a) in the testis of rats. This suggests that rSULT2B1b and rSULT2B1a may be involved in the expression change of estrogens and testosterone in the ovary and testis of rats caused by METH treatment. Sex-dependent sex hormone levels may serve as one of the reasons for the different METH-induced neurotoxicity in female and male individuals.

ADVISER'S APPROVAL: Guangping Chen