


1-1-2015

# The Role Of E3 Ligase Parkin In Trafficking Of Monoamine Storage Vesicles In Rat Model Of Methamphetamine Neurotoxicity

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**The Role of E3 Ligase Parkin in Trafficking of Monoamine Storage Vesicles in Rat Model of Methamphetamine Neurotoxicity**

by

**Heli Chauhan**

**THESIS**

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**MASTER OF SCIENCE**

2015

MAJOR: PHARMACOLOGY/TOXICOLOGY

Approved By:

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Advisor

\_\_\_\_\_

Date

## ACKNOWLEDGEMENTS

I would like to thank my thesis supervisor Dr. Anna Moszczynska for her guidance throughout my graduate program. I would like to express gratitude to my committee members Dr. Alope Dutta and Dr. Alana Conti for their generous support and guidance towards my thesis.

I am grateful to Dr. Paul Stemmer and the proteomics core facility for their support in the mass spectrometry data. Also, I would not have been able to accomplish this successfully without the love and support of my parents, my brother and friends Nirali Shah, Mrudang Shah and Hardik Doshi.

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## ABBREVIATIONS

- AAV - Adeno associated viral vector
- AR-JP - Autosomal recessive Parkinson's disease
- BBB - Blood brain barrier
- BDNF - Brain derived neurotrophic factor
- BSA – Bovine serum albumin
- CNS - Central nervous system
- DA - Dopamine
- DAergic - Dopaminergic
- DAT - Dopamine transporter
- DCV - Large dense core vesicles
- DDC - Dopa decarboxylase
- DNA - Deoxyribonucleic acid
- DOPAC - 3,4-Dihydroxyphenylacetic acid
- ER - Endoplasmic reticulum
- GABAergic - Gamma amino butyric acid-nergic
- GDNF - Glial cell line derived neurotrophic factor
- Glu - Glutamate
- HSP - Heat shock protein
- 5-HIAA - 5-Hydroxyindoleacetic acid
- 5-HT - 5-Hydroxy tryptophan
- MAO - Monoamine oxidase
- METH - Methamphetamine

NIDA - National institute for drug abuse

NO - Nitric oxide

NT3 - Neurotrophin 3

PET - Positron Emission Tomography

ROS - Reactive oxygen species

SERT - Serotonin transporter

SNAP - Soluble N-ethylmaleimide soluble factor attachment protein

SNARE - Soluble NSF-attachment protein receptor

SNARE - SNAP Receptor complex

SSV - Small synaptic vesicles

TH - Tyrosine hydroxylase

TU - Transducing units

VTA - Ventral tegmental area

WT - Wild type

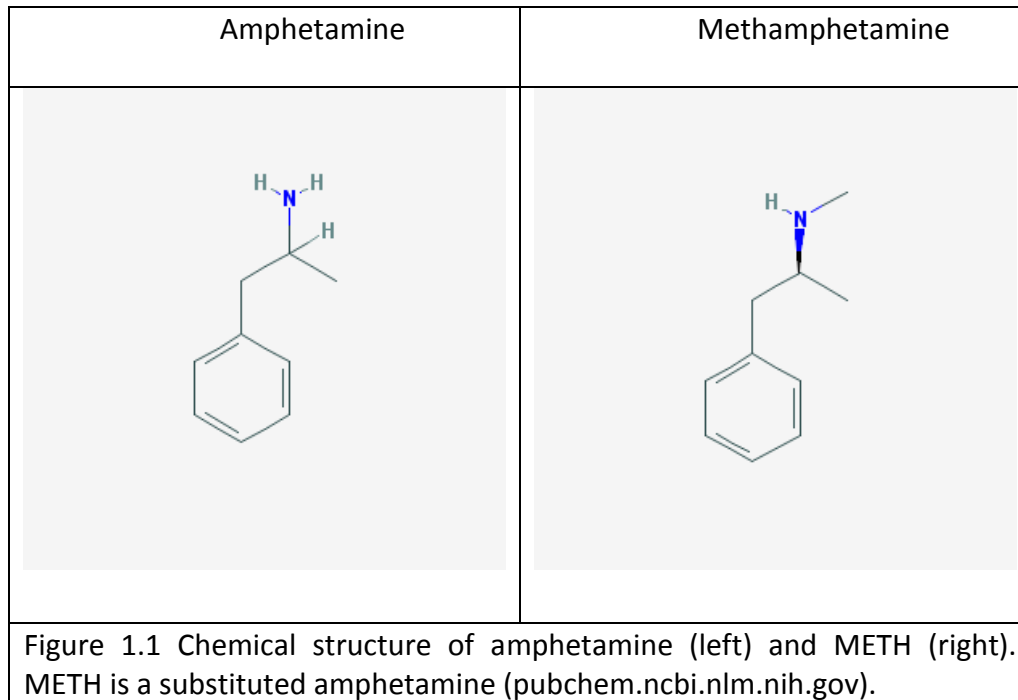
## CHAPTER 1: Introduction

### 1.1 Methamphetamine abuse and its consequences:

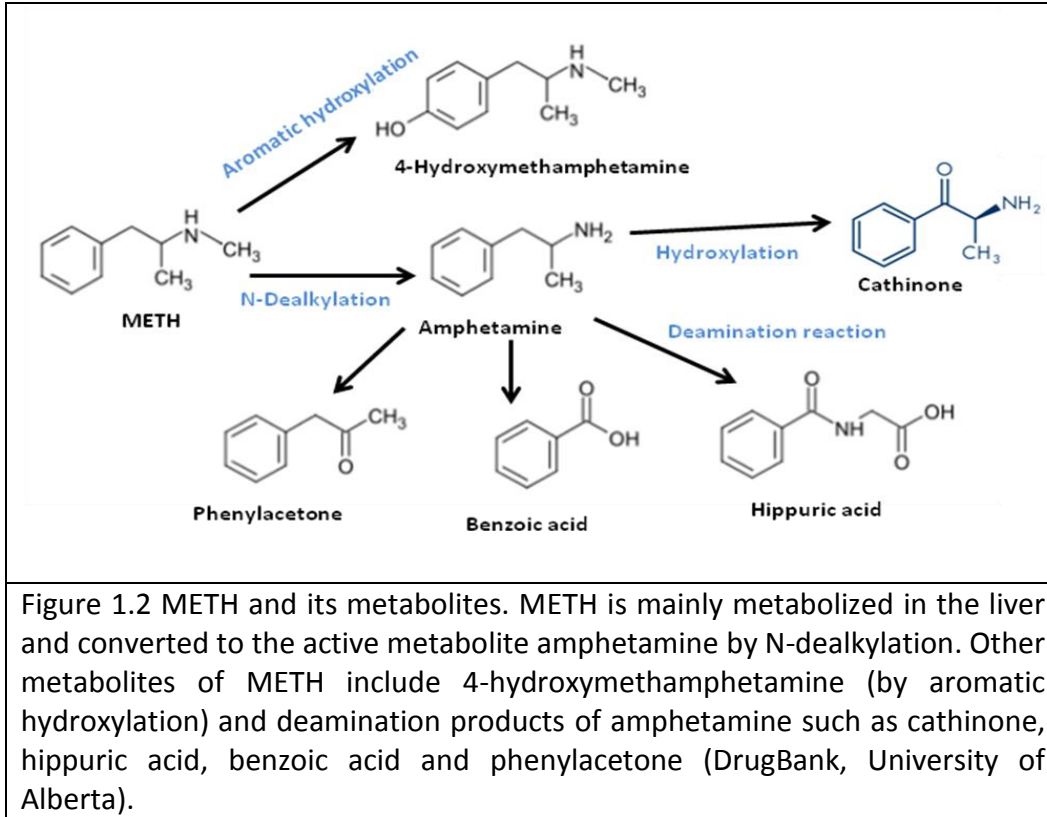
Addiction and drug abuse are currently major problems in the United States. About 100,000 Americans die every year as victims of illicit drug abuse and millions of others suffer from social and economic disruption. Illicit drug use accounts for about \$181 billion per year economically. As per the NIDA definition, “Addiction is defined as a chronic, relapsing brain disease that is characterized by compulsive drug seeking and use, despite harmful consequences”(www.drugabuse.gov).

#### 1.1.1 Properties of Methamphetamine:

Methamphetamine (METH) is a derivative of amphetamine. Both drugs are popular psychostimulants used for their euphoric effect and for recreation. Amphetamine is a methaylated phenylethylamine whereas METH is a double methylated phenylethylamine. Both drugs are toxic (Figure 1.1). However, METH is more potent than amphetamine in the central ervous system (CNS) and its effects manifest at a much faster rate (<http://amphetamines.com/amphetamines-vs-methamphetamines>).



METH crosses the placenta and is secreted into breast milk (Bartu, Dusci et al. 2009). METH is mainly metabolized in the liver with several metabolites found in the urine. Metabolism occurs by aromatic hydroxylation, N-dealkylation and deamination. METH has several metabolites (Figure 1.2). Amphetamine, which is an active metabolite of METH, is also neurotoxic (Torre, Yubero-Lahoz et al. 2012). The half-life of METH is different in humans and rats. In humans, the half-life is about 10-12 h (depending on the route of administration) (Cook, Jeffcoat et al. 1992, Cook, Jeffcoat et al. 1993) as compared to about 1 h in rats (Melega, Williams et al. 1995). Since the half-life of METH in rats is much shorter compared to humans, binge regimen is used to keep the plasma levels of the drug comparable to that in humans.



METH causes a rapid rush of pleasure and euphoria due to release of dopamine (DA) in the nucleus accumbens by acting on DA transporters (Sulzer, Chen et al. 1995, Rothman and Baumann 2003). Other immediate effects are increased wakefulness, increased physical activity, rapid heart rate, elevation of blood pressure, suppression of appetite and increased body temperature. High doses of METH can cause insomnia, psychosis, paranoia, seizures and stroke (Winslow, Voorhees et al. 2007) ([www.drugabuse.gov](http://www.drugabuse.gov)).

#### 1.1.2 History of METH use:

METH has been used as early as the World War I and abuse of the drug reached its peak in 1960s. In the 1930s, amphetamines were freely available as over the counter inhalers and tablets for a variety of conditions such as asthma, narcolepsy and obesity. The non-prescription

amphetamine inhaler Benzedrine became very popular for treating asthma and congestion. Other off label uses for METH included schizophrenia, morphine addiction, alcoholism, migraine, head injuries, hypotension and many others. During the World War II, soldiers used it extensively as it increased wakefulness, suppressed fatigue and appetite. For the same reason, it was also used by athletes and by students to increase attention (Anglin, Burke et al. 2000, Shukla, Crump et al. 2012). Following this, the Drug Abuse Control Amendments of 1965 and the Comprehensive Drug Abuse Prevention and Control Act of 1970 restricted the use of amphetamines, classifying them as Schedule II drugs (Vearrier, Greenberg et al. 2012).

A schedule II drug, today METH is available only through prescription and cannot be refilled. Its medical use is confined to conditions like Attention Deficit Hyperactivity Disorder (ADHD), narcolepsy and obesity (Lambert, Windmiller et al. 1976, Bramble 2000). Illegal METH has different street names such as crystal, chalk, crank, speed, batu, crystal, yaba. It is administered by snorting, smoking, by injection and sometimes orally. Increased monoamine transmission mediated by METH is responsible for most of its abuse potential due to effects like euphoria and increased wakefulness (Vearrier, Greenberg et al. 2012). However, chronic use of METH is associated with neurotoxicity.

### 1.1.3 Epidemiology of METH abuse:

Amphetamines are the second most prevalent illicit drugs in the world after cannabis (Vearrier, Greenberg et al. 2012). According to NIDA, about 13 million people have used METH at least once in their lifetime. As compared to previous years, the use of METH in the United States has declined after 2000. However, it is still abused by a high number of people. For instance, METH use has remained steady, from 530,000 current users in 2007 to 440,000 in

2012 (<http://www.drugabuse.gov/publications/drugfacts/nationwide-trends>). Furthermore, METH abuse might be on a rise again as METH-related visits to United States emergency rooms (ERs) jumped from 67,954 in 2007 to 102,961 in 2011. Out of the total people receiving treatment for METH abuse, 6.7% were pregnant women in 2009 (SAMHSA 2011, TEDS). Also, among pregnant women aged 15-44, the average rate of illicit drug use in 2011-2012 was found to be 5.9% (SAMHSA (2012)).

In addition, the use of amphetamines seems to have increased among 12<sup>th</sup> graders and stopped declining among 8<sup>th</sup> and 10<sup>th</sup> graders from the year 2009. It has been hypothesized that METH is abused as an aid to their studies (Monitoring the Future Study 2013) (Figure 1.3). Along with this, METH abuse is getting increasingly popular in other parts of the world, especially Asian countries (Griffiths, Mravcik et al. 2008, McKetin, Kozel et al. 2008).

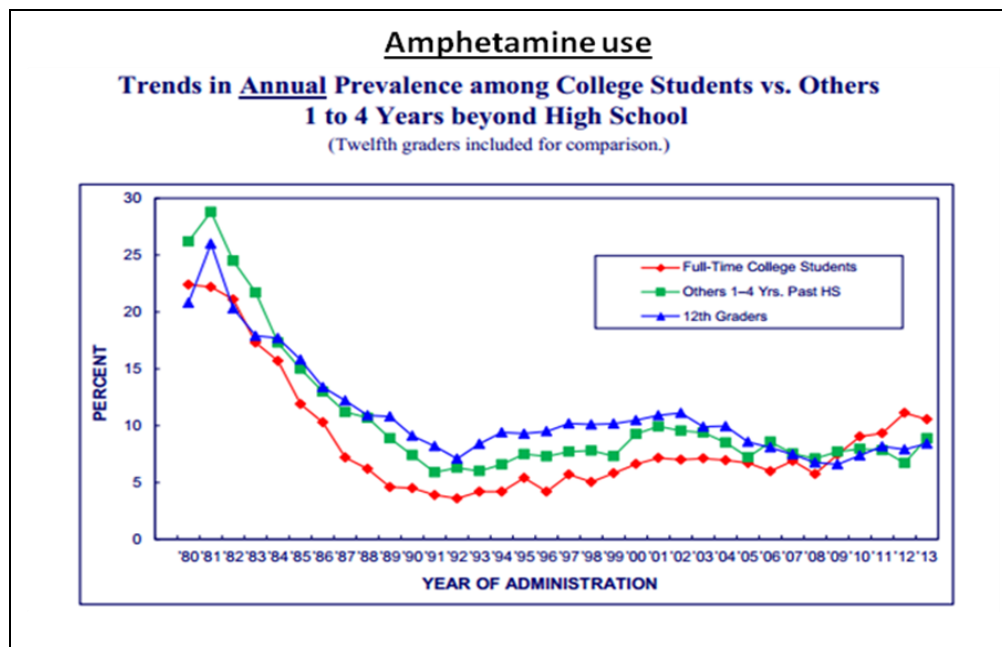


Figure 1.3 Use of amphetamines by college students and high school students. Recent increase in the use of amphetamines by college students has been observed (Monitoring the Future, Institute for Social Research, University of Michigan) (Johnston, O'Malley et al. 2014).



#### 1.1.4 Clinical signs and symptoms of chronic METH abuse:

When taken at high doses, METH causes selective degeneration of dopaminergic (DAergic) and serotonergic (5HTergic) nerve terminals in the brain in both experimental animals and humans (Wilson, Kalasinsky et al. 1996, Quinton and Yamamoto 2006, Krasnova and Cadet 2009). High-dose METH causes a variety of negative behavioral changes in addition to compulsive drug seeking and drug taking, such as cognitive impairments, anxiety, violent behavior and psychosis (Salo, Nordahl et al. 2009, Ares-Santos, Granado et al. 2013). Long-term use of METH causes DA depletion in the striatum along with cognitive impairments and other problems including “METH mouth” (tooth decay) (Hart, Marvin et al. 2012). Neuropsychological effects like decrease in decision making ability (Rogers, Everitt et al. 1999), confusion, hallucination, decreased memory (Braren, D et al. 2014) and psychosis (Flaum and Schultz 1996) also result from chronic use of METH. Long term effects of METH use include tolerance to the effect of METH which causes METH users to escalate their dose to produce the same effects (Gygi, Gygi et al. 1996).

Along with adults, METH has fatal effects on children exposed to the drug in the uterus. METH use by pregnant women has become a major concern due to long term risks to the exposed fetus. Clinical studies in humans have found that exposure to METH during brain development can cause neurobehavioral abnormalities such as aggressive behavior, learning problems and poor social adaptation later in life (LaGasse, Derauf et al. 2012, Diaz, Smith et al. 2014). METH produces a variety of effects on the body. Some of the major effects are:

##### 1. Addiction

2. Cognitive impairments: Low doses of METH improves cognitive performance and memory but long term use of METH causes cognitive impairment, loss of memory and attention (Nordahl, Salo et al. 2003).
3. Violent/aggressive behavior (McKetin, Lubman et al. 2014).
4. Psychotic symptoms: METH use is associated with seizures. Other effects include hallucinations, delusions and paranoia (Zweben, Cohen et al. 2004).
5. Cardiovascular: hypertension and tachycardia (Newton, Garza et al. 2005).
6. Immune system: METH users are more prone to engaging in risky sex as compared to non-METH users. The chance of getting Human Immunodeficiency virus (HIV) increases by engaging in unsafe sex. Heterosexual men abusing METH are more susceptible to infection by HIV. Also, since METH administration by injection is fairly common, the risk of getting HIV through contaminated needles or sharing needles is fairly high (Molitor, Truax et al. 1998, Morb 2006).
7. METH mouth: tooth decay
8. Prenatal exposure: METH abuse by pregnant women leads to adverse effects in infants. Effects like impaired growth of fetus, premature delivery, and low body weight with smaller head circumference of the infant as well as neonatal hepatitis have been observed (Oro and Dixon 1987, Little, Snell et al. 1988, Dahshan 2009).
9. Suicidal tendencies (Richards, Bretz et al. 1999).
10. Risk of developing Parkinson's disease: METH neurotoxicity and Parkinson's disease have similar profiles of brain damage, and there are studies showing correlation between the two. METH users have been found to have double the risk of developing

Parkinson's disease than non-METH users. A population study showed that METH users have increased incidence of Parkinson's disease later in life (Callaghan, Cunningham et al. 2010, Callaghan, Cunningham et al. 2012).

## 1.2 Neurotoxicity of METH

The clinical symptoms of METH are manifestations of its neurotoxicity at the molecular level. The Interagency Committee on Neurotoxicology defined neurotoxicity as:

“Permanent and reversible effects on the structure or function of the nervous system; a toxic effect of the drug that causes one or more of the following:

- Loss of neuronal components (e.g. synthesizing enzymes, receptors, transporters, neurotransmitters, nerve cells and terminals)
- Histological signs of neuronal damage (silver staining, gliosis, swollen axons)
- A loss of the entire neuron and components therein
- A persistent behavioral abnormality associated with the drug.” (From Dr. Moszczynska's presentation).

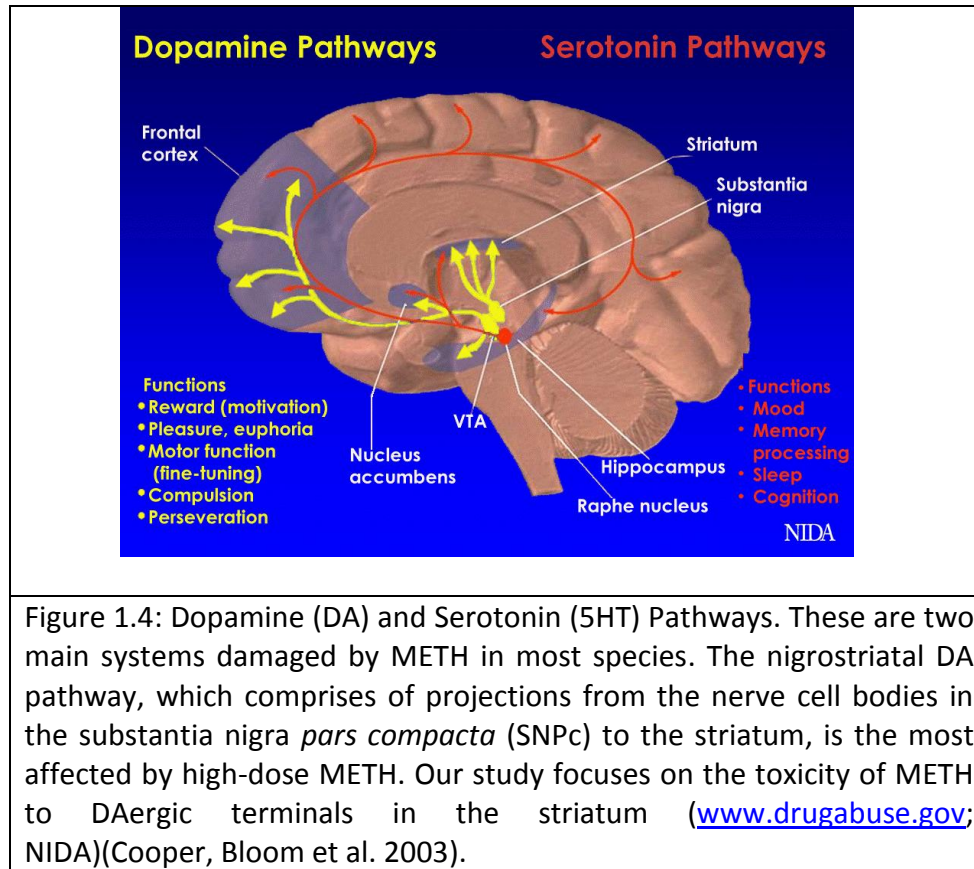
METH toxicity is manifested by one or more of the above conditions. Binge METH (4x5 mg/kg, 4x7.5 mg/kg and 4x10 mg/kg, every 2 h, i.p.) causes significant reduction in DA and 5HT content in the striatum in rats, 7 days following the drug treatment (Matuszewich and Yamamoto 2004, Tata, Raudensky et al. 2007). In mice, METH affects only the DAergic system with majority of previous studies showing a lack of METH-induced toxicity in the 5HTergic system (Jonsson and Nwanze 1982, Anderson and Itzhak 2006). Studies in human chronic METH users showed decreases in DA, TH, DAT and 5HTergic transporter (SERT) levels in the striatum, without affecting the cell bodies. The levels of vesicular monoamine transporter (VMAT2) and

dopa decarboxylase (DDC) remained unchanged, suggesting lack of permanent damage to the nerve terminals (Wilson, Kalasinsky et al. 1996, Chang, Alicata et al. 2007). However, a more recent study found that following oral dose of amphetamine, there is a 5% reduction in VMAT2 binding in the striatum as observed by positron emission tomography (PET) scan (Boileau, Houle et al. 2010). In non-human primates (monkeys), deficiencies in DAergic system are observed as early as 1 week after METH as seen with PET scan and they are persistent. The decreases in DAT, TH and VMAT2 are still observed in striatum one month after METH (Harvey, Lacan et al. 2000). Rats have a toxicity profile that is similar to humans. Although many studies on binge METH effect on DA and its metabolites have looked at 7 days following the drug treatment (Matuszewich and Yamamoto 2004, Tata, Raudensky et al. 2007), decreases in DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) following binge METH (6x5 mg/kg, every 1 h, i.p.) have been observed as early as 24 h after METH (Hodges, Ladenheim et al. 2011). The time course of development of METH toxicity as well as recovery from it varies between species. Mice show astrogliosis as early as 24 h after METH (O'Callaghan and Miller 1994). In rats, microglia activation in the striatum is observed 5 days after and astrocytosis is observed 3 days following METH (Guilarte, Nihei et al. 2003). When single high METH dose (40 mg/kg) is given to rats, significant reduction in TH levels and increased levels of the astrogliosis marker GFAP (glial fibrillary acidic protein) are observed in the striatum as early as at 12 h and 6 h, respectively. The DA depletion with this METH dose was found to occur also at 12 h after the injection of the drug (Cappon, Pu et al. 2000). Granular degeneration of nerve terminals in rat striatum, as detected by silver staining, has been observed at 4 days following high dose binge METH (3x50 mg/kg, every 8 h). No degeneration

was observed in other brain regions such as SNPc and hypothalamus (Ricaurte, Guillery et al. 1982).

As mentioned before, most of the toxicity studies have shown that the damage caused by METH is reversible with time. However, the recovery may or may not be complete and the duration for recovery is different based on the dose of METH and severity of the damage. Also, as mentioned before, there are interspecies differences in the time when recovery takes place. Only one study in rats so far has showed recovery of all the parameters of the DAergic system, 12 months after binge regimen of METH. This study used a single non-toxic dose of 5 mg/kg METH (Manning and Cass 1999). However, studies using higher dose of METH (4x12.5 mg/kg, every 2 h), showed reduction in DA and DOPAC 237 days after the treatment (Friedman, Castañeda et al. 1998). In humans, increased availability of DAT (recovery) is observed after 14 months of abstinence from METH (Volkow, Chang et al. 2001). In monkeys, DAergic markers recover to normal levels after 1.5 years. However, no axonal regeneration has been reported in any of the studies which indicates lack of complete recovery of nerve terminals (Melega, Raleigh et al. 1997, Harvey, Lacan et al. 2000).

#### 1.2.1 Dopaminergic system:



The DAergic system has four different pathways;

- (1) Mesolimbic pathway
- (2) Mesocortical pathway
- (3) Nigrostriatal pathway
- (4) Tuberoinfundibular pathway (Cooper, Bloom et al. 2003).

Of major interest in METH neurotoxicity is the nigrostriatal pathway because the striatum is the brain area most severely affected by METH. The nigrostriatal pathway comprises of the projections from cell bodies in the SNPc to the striatum (also referred to as the dorsal striatum) (Figure 1.4). DAergic projections from the ventral tegmental area (VTA), project to the nucleus accumbens (also referred to as the ventral striatum or limbic striatum) forming the

mesolimbic DA pathway and to the prefrontal cortex forming the mesocortical DA pathway. The tuberoinfundibular pathway connects the hypothalamus with the pituitary gland (Cooper, Bloom et al. 2003). The striatum is the most affected by METH due to its high content of DA. Striatal function is involved in motor coordination and cognitive processes such as working memory (Rolls 1994).

### 1.3 Dopaminergic terminal

#### 1.3.1 Dopaminergic terminal structure and function:

The striatum contains abundant DAergic nerve terminals as compared to other DA-containing regions where axons terminate. As aforementioned, these terminals are the projections of DAergic cell bodies in the SNpc. As seen in the Figure 1.5, the DAergic nerve terminal is structurally similar to other nerve terminals. The nerve terminal can be physically divided into 2 main parts: cytoplasm and the nerve terminal membrane.

Cytoplasm: The cytoplasm of the nerve terminal contains mitochondria and the endoplasmic reticulum (ER). DA storing vesicles as well as vesicles storing other neuronal components are axonally transported to the cytosol from the cell bodies. These vesicles stay in the cytoplasm till being docked at the membrane for exocytosis. The VMAT2 is present on the surface of these synaptic vesicles. The VMAT2 is a proton pump that packages DA into vesicles. There are 3 pools of synaptic vesicles in the presynaptic terminal: (1) reserve pool which is away from the active zone (2) proximal recycling pool that is closer to the active zone (3) the releasable pool of synaptic vesicles that are docked and ready for exocytosis. Proteins alpha-

tubulin, beta-actin and bassoon are present in abundance in the cytosol (Volkandt and Karas 2012).

Membrane: The membrane of the nerve terminal is very dynamic and contains a number of proteins for a variety of functions. Most common proteins observed in the plasma membrane are transporters and channels ( $\text{Na}^+/\text{K}^+$  ATPase; VDACs (voltage dependent anion channel)), cell adhesion molecules, signaling mediators. Apart from this, the presynaptic active zone contains a large number of exocytotic proteins (Volkandt and Karas 2012). Five main proteins are present in the active zone: RIM, Munc13, RIM-BP,  $\alpha$ -liprin and ELKS. These five proteins assemble in one protein complex and mediate the docking of vesicles and recruitment of calcium channels. Piccolo and bassoon make the framework of the active zone guiding the movement of vesicles to the membrane. Filamentous proteins like cell division and control related protein-1 (CDCrel-1) are also present attached to the plasma membrane as well as the vesicles. Moreover, calcium channels and presynaptic neurotransmitter receptor (D2 receptors) are found on the membrane of the nerve terminal (Sudhof 2004). DAT present on the membrane of the terminal plays a very crucial role to reuptake DA from the synapse once it has been released by exocytosis.



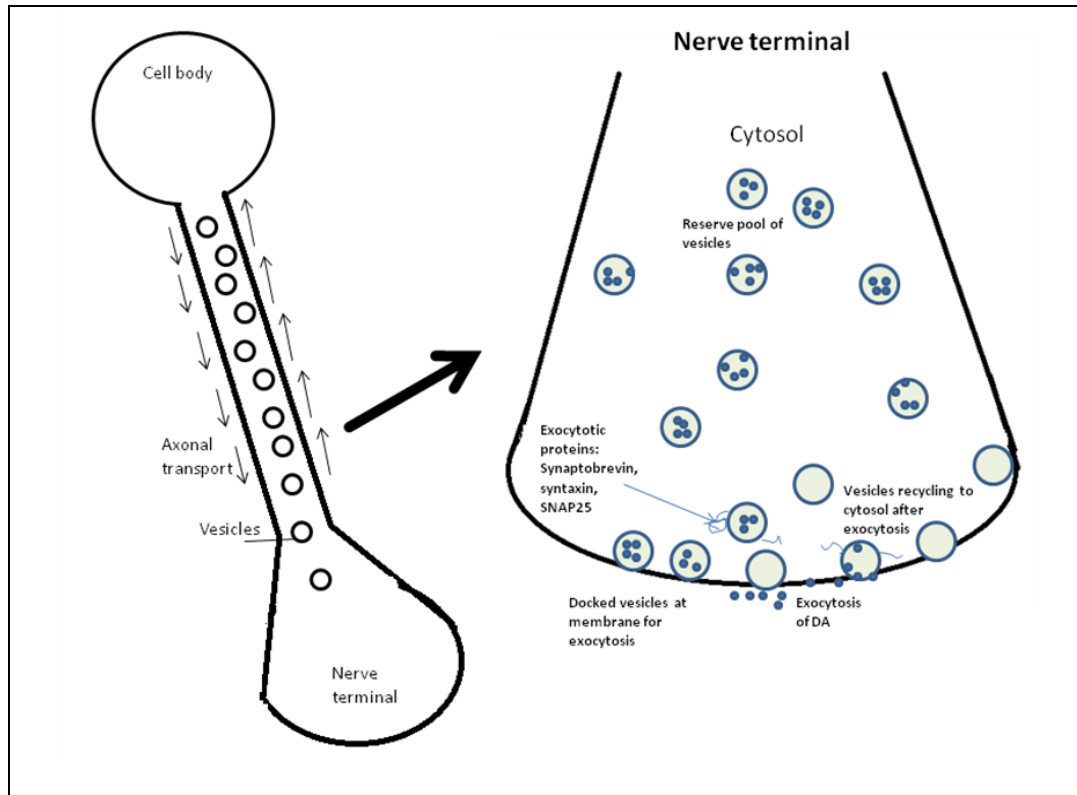


Figure 1.5: Dopaminergic (DAergic) nerve terminal. DA packaged in vesicles is transported axonally from the cell body to the nerve terminal. At the terminal, DA is released from the vesicles by exocytosis. Once released in the synapse, DA reuptake occurs via dopamine transporter (DAT) into the cytoplasm of the nerve terminal. Free DA in the cytosol is then packaged into recycled vesicles by the vesicular monoamine transporter 2 (VMAT2) residing in their membrane (Cooper, Bloom et al. 2003).

### 1.3.2 Dopamine synthesis, storage, uptake, release and recycling:

DA is synthesized in the DAergic cell bodies as well as in DAergic terminals. Like other catecholamines, it is synthesized from phenylalanine by a series of steps. Tyrosine hydroxylase (TH) is the rate limiting enzyme in the synthesis of DA (Daubner, Lea et al. 2011). Once synthesized in cell bodies, DA is packaged into synaptic vesicles and transported down to the striatum (nerve terminals) by axonal transport. DA is also synthesized locally (Demarest and Moore 1979). However, it is important that DA be stored inside the synaptic vesicles. At the pH

of cytosol (pH 7.4, normal physiological pH), DA autooxidizes and causes oxidative damage to the terminal (LaVoie and Hastings 1999). The pH inside the storage vesicles is acidic (between 5-5.7) which prevents autooxidation (Pothos, E Mosharov et al. 2002, Markov, Mosharov et al. 2008). Action potential leads to opening of calcium channels, which leads to docking and fusion of the DA storage vesicles at the membrane of the nerve terminal for exocytosis. Docking of vesicles occurs by formation of the 7S complex. The 7S complex is the protein complex formed by the interaction between vesicle proteins v-SNARE and synapptotagmin along with membrane proteins SNAP-25 and syntaxin (Scheller 1995). The post-synaptic effect of released DA is terminated by reuptake or negative feedback mechanism. The released DA acts on D2 autoreceptor and negatively regulates its own exocytotic release. Following exocytosis and post-synaptic action, DA reuptake into the nerve terminal occurs via DAT. Once inside the terminal, the reloading of vesicles with DA occurs after the recycling of vesicles following exocytosis. The vesicles are refilled with DA in one of the 3 ways: (1) vesicles are refilled with DA without undocking (these vesicles constitute the readily releasable pool of DA) (2) the vesicles undock and are refilled with DA (3) the vesicles undock, undergo clathrin mediated endocytosis and are refilled with DA after fusion with endosomes (Sudhof 2004). Apart from vesicle mediated exocytosis, somatodendritic release of DA occurs in brain regions other than striatum (SNPc and VTA). Most of the neurotransmitters in the brain have point-to-point synaptic contacts for signaling. However, DA signaling varies in this regard. Since most of the DA receptors are extrasynaptic, a 'spillover' of DA from the synapse followed by diffusion into the extrasynaptic space is commonly observed. This is known as volume transmission. As the diffusion rate of DA is much faster than the reuptake rate, DAT cannot prevent the

extrasynaptic spillover of DA. However, DAT does regulate the DA concentration in space and time and also affects the active lifespan of DA. Thus, DAT plays an important role in regulating the radius of DA effect (Cragg and Rice 2004, Rice, Patel et al. 2011).

#### 1.4 Mechanisms of METH Neurotoxicity:

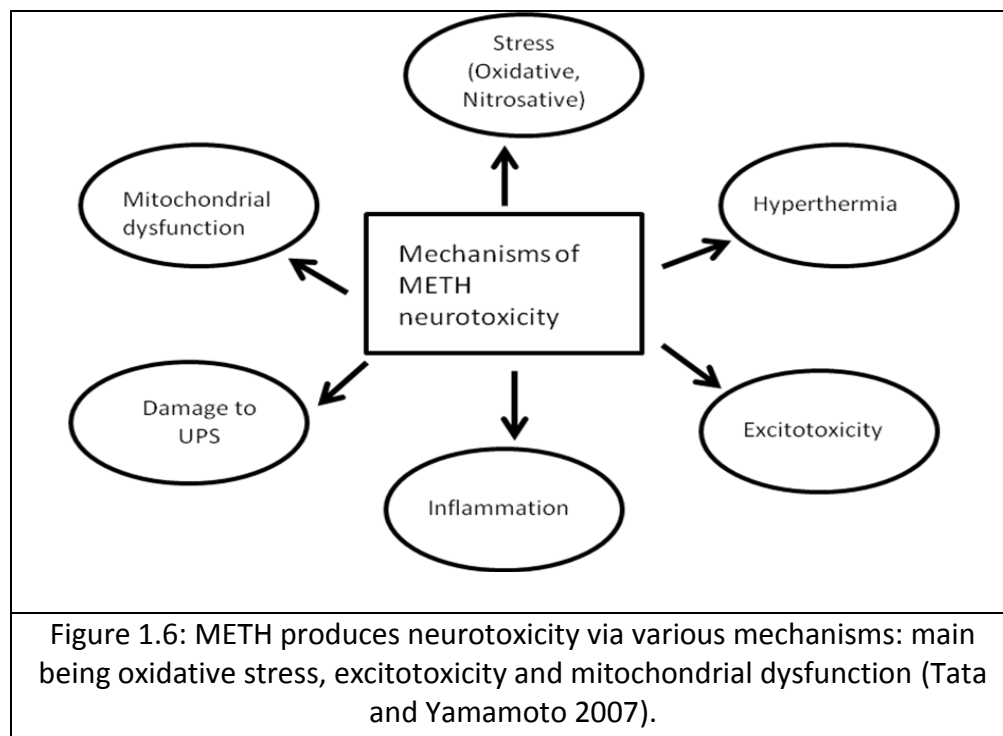
METH enters the DAergic terminal partially by diffusion but mostly via DAT, which sees METH as a substrate (Schuldiner, Steiner-Mordoch et al. 1993). After entering the terminal by DAT-mediated active translocation, METH disrupts the function of DAT by various mechanisms. METH is known to cause DA efflux in the synapse by DAT reverse transport (Figure 1.6). It has also been shown to cause internalization of DAT into endosomes (Kahlig, Binda et al. 2005, Fleckenstein, Volz et al. 2007). Once in the terminal, METH releases DA from the vesicles. This free DA auto-oxidizes and produces free radicals which oxidize and inactivate DAT (Berman, Zigmond et al. 1996, Fleckenstein, Metzger et al. 1997). Fleckenstein and colleagues (Fleckenstein, Haughey et al. 1999) have shown that METH decreases the function of DAT in vivo. Two theories exist about the mechanism by which METH releases vesicular DA:

- (1) Weak base hypothesis: METH enters the vesicles via lipophilic diffusion.

The pH of the vesicles is maintained at 5-5.7 at all times. This acidic pH is very important for transport of DA into the vesicles from the cytosol against the concentration gradient. Like all amphetamines, METH is a weak base which protonates in acidic medium. Once in protonated state, it accumulates in acidic environment. Due to protonation of METH, there is depletion of protons in the vesicles which alters the acidic pH of the vesicles (Lee, Gubernator et al. 2010, Sulzer 2011).

(2) Substrate of VMAT2: This hypothesis states that METH acts as a substrate of VMAT2 and enters the vesicles. It is not completely clear whether METH is a direct substrate of VMAT2; however, METH does competitively inhibit the uptake of DA by VMAT2 into the vesicles. Thereafter, it disrupts the vesicular pH gradient and releases DA. This theory is supported by several previous studies which show preferential binding of VMAT2 towards substituted amphetamines (Schuldiner, Steiner-Mordoch et al. 1993, Gonzalez, Walther et al. 1994, Peter, Jimenez et al. 1994, Reith and Coffey 1994).

Apart from affecting DAT and VMAT2, METH is known to decrease the activity of TH (Hotchkiss and Gibb 1980) and inhibit DA metabolism by antagonizing the actions of monoamine oxidase (MAO), an enzyme responsible for metabolism of monoamines. This way there is more of cytosolic DA for reverse transport as well as autooxidation (Sulzer 2011).



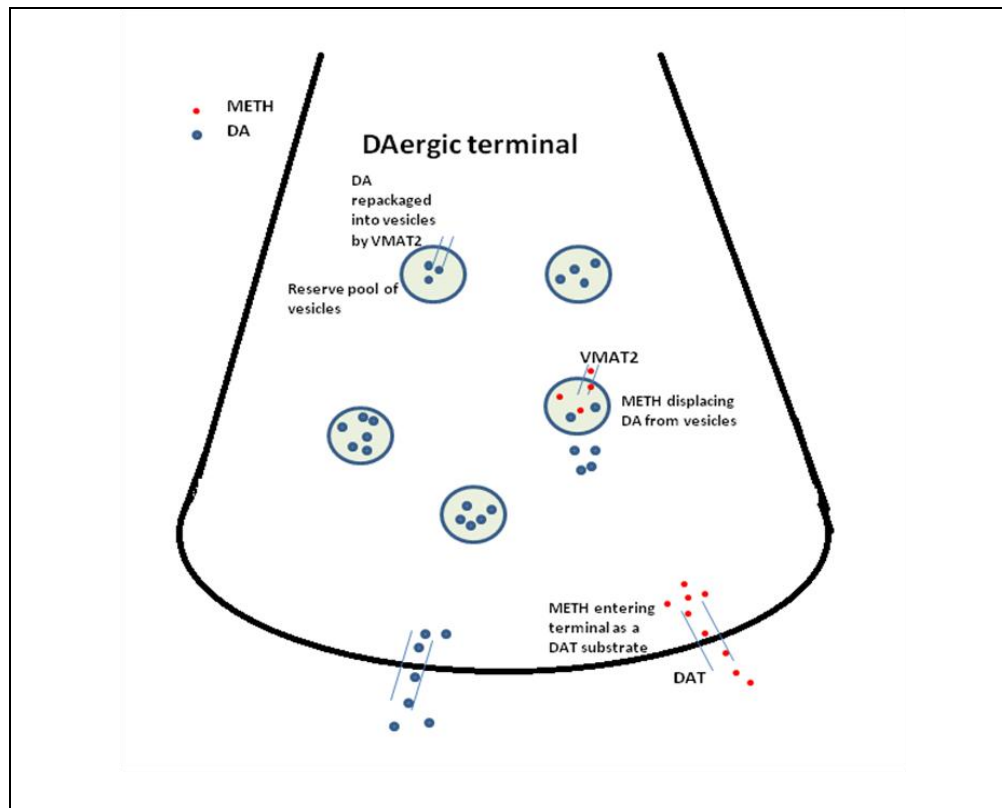
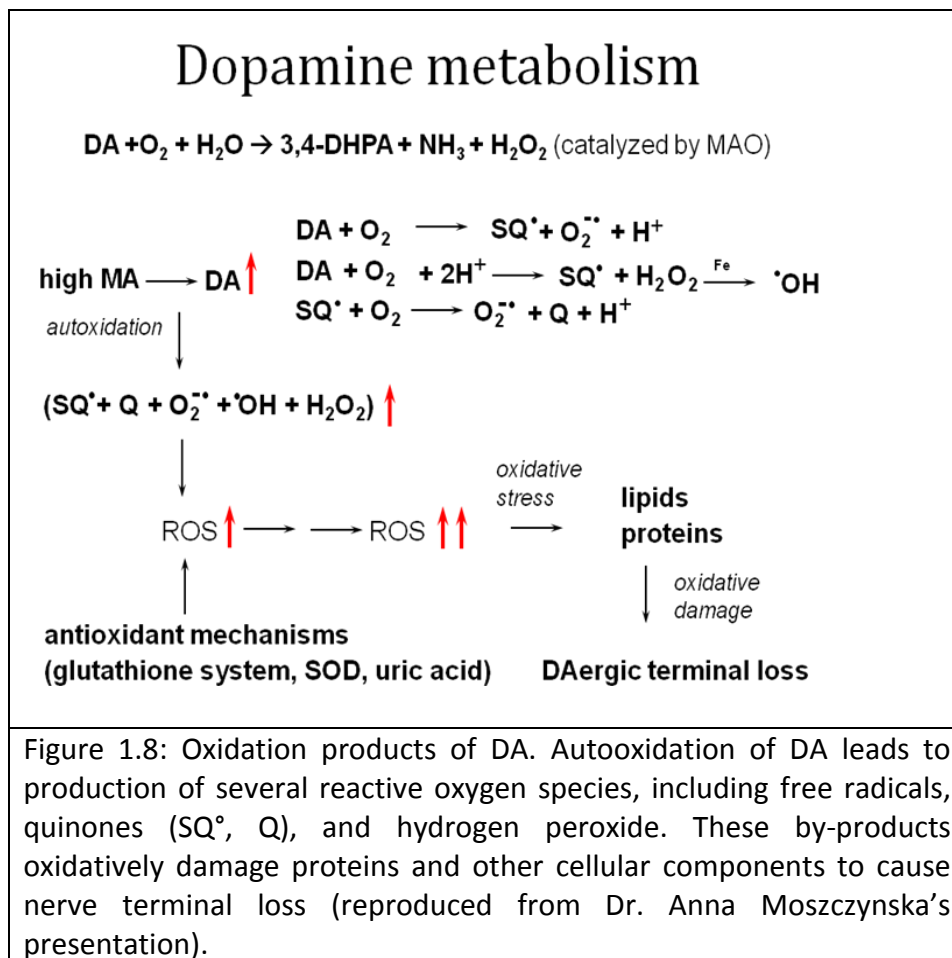


Figure 1.7: Mechanism of METH neurotoxicity. METH enters the dopaminergic (DAergic) nerve terminal as a substrate of dopamine transporter (DAT) and via passive diffusion. Once inside the terminal, it releases DA from the storage vesicles by impairing the function of vesicular monoamine transporter 2 (VMAT2) and via collapsing proton gradient across the vesicular membrane after diffusing into the vesicles (Sulzer 2011).

An early event of METH neurotoxicity is autooxidation of released DA with the production of several reactive oxygen species (ROS) ( $\text{H}_2\text{O}_2$ ,  $\text{O}_2$ ,  $\text{O}_2^{\cdot-}$ ,  $\text{OH}$ ,  $\text{OH}^-$  and DA quinones). Along with oxidative stress, nitrosative stress also plays role in METH neurotoxicity. The generated free radicals lead to accumulation of oxidized proteins and lipid peroxidation products. Moreover, the DA quinones covalently modify cysteinyl residues of proteins which cause selective toxicity to DAergic terminals (LaVoie and Hastings 1999, Tata and Yamamoto 2007).



**A. Oxidative stress and DA:** Owing to the selective toxicity of METH to DAergic and 5HT terminals, DA mediated oxidative stress is believed to be one of the major mechanisms of METH toxicity. METH acts by producing ROS, reactive nitrogen species (RNS) and lipid peroxidation products. Moreover, as per the weak base hypothesis, amphetamines disrupt the proton gradient of the vesicles and stimulate efflux of neurotransmitters from the vesicles (Sulzer and Rayport 1990, Pifl, Drobny et al. 1995). As a result, free DA is released in the cytoplasm of the nerve terminal. Free DA autooxidizes to produce ROS and quinones which lead to oxidative stress (Yamamoto, Moszczynska et al. 2010) (Figure 1.8). This theory has been proved by the protective

effect produced by pretreatment with antioxidants like ascorbic acid and vitamin E (Vito and Wagner 1989). Increased levels of ROS as well as increase in antioxidants like glutathione (both reduced and oxidized form) as a compensatory mechanism at 2 h after binge METH has been reported in rats (Harold, Wallace et al. 2000). However, another study that used higher doses of binge METH reported a decrease in total glutathione levels at 3 h after the last METH injection, suggesting overutilization of GSH (Moszczynska, Turenne et al. 1998).

**B. Excitotoxicity:** METH causes an increased release of glutamate in the striatum which, in turn, causes increased glutamate receptor activation (Nash and Yamamoto 1992). Glutamate release causes activation of NMDA and non-NMDA receptors and increases production of nitric oxide (NO) and superoxide radicals via calcium mediated pathway (Gunasekar, Kanthasamy et al. 1995). Nitric oxide and superoxide radicals interact to produce the neurotoxin peroxynitrite (Pacher, Beckman et al. 2007). End process involves nitration of proteins and damage to mitochondria, endoplasmic reticulum and deoxyribonucleic acid (DNA) (Yamamoto, Moszczynska et al. 2010).

**C. Hyperthermia:** Apart from being a stressor by itself, hyperthermia is an important component augmenting METH neurotoxicity. Hyperthermia-mediated production of free radicals, disruption of DAT, TH and ubiquitin proteasome system increase the severity of METH toxicity. Increase in the rates of all enzymatic processes at high temperature would explain the above effect (Bowyer, Davies et al. 1994, Yamamoto, Moszczynska et al. 2010). This is consistent with the finding that inhibiting

hyperthermia reduces free radical formation and subsequent damage to DAergic terminals (Fleckenstein, Wilkins et al. 1997).

**D. Mitochondrial dysfunction** is another contributor to METH neurotoxicity. After diffusion into the mitochondria, METH accumulates there. Here, it alters mitochondrial membrane potential, decreases the function of the mitochondrial complexes and hinders the production of ATP (Davidson, Gow et al. 2001). This is followed by the release of cytochrome c to the cytosol as well as activation of caspase-mediated apoptotic pathways in both cell bodies (SNPc, striatum) and terminals (striatum). These effects, along with low ATP levels in the brain, cause mitochondrial damage and toxicity (Burrows, Gudelsky et al. 2000).

**E. Inflammation** is also one of the several mechanisms of METH toxicity. METH-induced inflammatory response is mediated by several pathways. METH releases glutamate, which activates microglia in rat striatum. Moreover, DA quinones, which are products of autooxidation of DA, also activate microglia. These activated microglia release pro-inflammatory molecules. Increased glutamate receptor activation enhances production of interleukins (IL-1 $\beta$  and IL-6) and tumor necrosis factor (TNF- $\alpha$ ) (Yamamoto, Moszczynska et al. 2010).

**F. Damage to the Ubiquitin proteasome System (UPS):**

The ubiquitin proteasome system serves as a scavenger for damaged and misfolded proteins or those proteins which are no longer needed. In this system, the proteins to be degraded are tagged with a 76 amino acid protein ubiquitin. This process is brought about by 3 enzymes:



- Ubiquitin activating enzyme E1
- Ubiquitin conjugating enzyme E2
- Ubiquitin ligase E3, which ligates ubiquitin to the substrate protein.

There are different types of E3 ligases in various regions of the body. After the first ubiquitin is ligated, a polyubiquitin chain is formed on the substrate, which marks it for degradation by the 26S proteasome.

The mammalian 26S proteasome comprises of two components:

1. A 20S core with catalytic sites for proteases. It can even degrade oxidized proteins in the ubiquitin-independent manner.
2. Two 19S regulatory caps that deubiquitinate the substrate preparing it for degradation in the 20S core (Hershko A 1998).

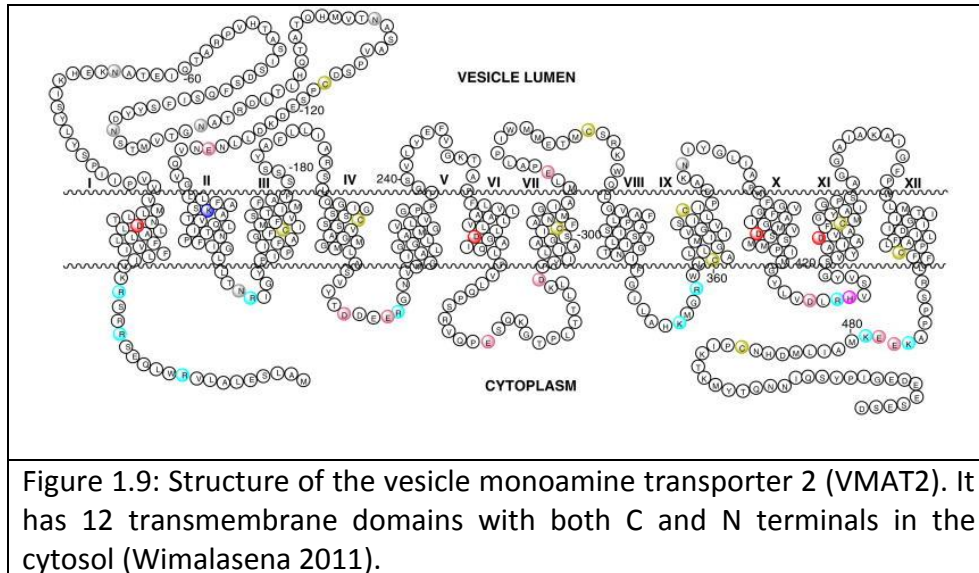
High doses of binge METH decrease the activity of 26S proteasome in striatal synaptosomes from adult rats. Furthermore, binge METH oxidatively damages parkin and decreases its levels in these rats (Moszczynska and Yamamoto 2011) as well as in late adolescent rats exposed to binge METH (Killinger, Shah et al. 2014). Out of the 600 E3 ligases present in neurons, parkin is known to be greatly affected by METH. Parkin has many substrates such as alpha-synuclein, CDCrel-1, alpha-tubulin, synaptotagmin and others. Without parkin, there would be an abnormal accumulation of the above substrates leading to toxicity. Thus, parkin deficit and dysfunction of the proteasome can lead to build up of damaged proteins and contribute to METH neurotoxicity. In support of this notion, overexpression of parkin was found to protect DAergic terminals in the striatum from METH neurotoxicity (Liu, Traini et al. 2013).

## 1.5 Vesicular monoamine transporter 2 in METH neurotoxicity:

### 1.5.1 VMAT2 structure and function:

VMAT2 is a member of the TEXANs (toxin extruding antiporter system) family. VMAT2 has a role of extruding free DA and other monoamines from the cytoplasm into the vesicles in exchange of protons (Martin, Schafer et al. 2013). VMAT2 is derived from the gene SLC18A2. It is an acidic glycoprotein with 3 isoforms: native form (45 kDa), partially glycosylated form (55 kDa) and fully glycosylated form (75 kDa). It is a transmembrane protein containing 12 transmembrane domains with both C and N terminals in the cytosolic part of the vesicle (Cruz-Muros, Afonso-Oramas et al. 2008, Wimalasena 2011) (Figure 1.9). VMAT2 is expressed in various monoaminergic cells in the brain, mast cells and histamine containing gut cells (Weihe, Schafer et al. 1995). Within the brain, striatum and hypothalamus are rich in VMAT2. The primary function of VMAT2 is to sequester monoamines from the cytosol of the nerve terminal and package it into synaptic vesicles. It maintains a high neurotransmitter concentration inside the vesicles by transporting monoamines against the concentration gradient. The energy for this transport is derived from the acidic pH gradient maintained inside the vesicles (Wimalasena 2011). The function of VMAT2 is extremely important for proper presynaptic function. Evidence for this is obtained by studies on homozygous VMAT2 knockout mice, which do not survive more than few days after birth due to improper monoamine storage and exocytotic release. Also, in VMAT2 heterozygous mice, decreased extracellular DA levels and high sensitivity to METH and other psychostimulants is observed (Wang, Gainetdinov et al. 1997). Another study showed that VMAT heterozygous mice had more neurotoxicity as compared to wild type mice

(Fumagalli, Gainetdinov et al. 1999). Thus, VMAT2 plays an important role in neurotransmitter release and the mechanism by which psychostimulants produce neurotoxicity.



### 1.5.2 VMAT2 and the synaptic vesicle cycle:

Synaptic vesicles loaded with neurotransmitters travel to the membrane in response to action potential. At the membrane, they dock and prime to release DA in the synapse. After docking, DA is released by exocytosis. Following exocytosis, one of the three phenomena can occur:

- (1) The empty vesicles do not undock and they are loaded with neurotransmitter again. These vesicles constitute the readily releasable pool of vesicles.
- (2) The empty vesicles undock and recycle back to the cytosol where they are loaded with neurotransmitter
- (3) The vesicles undergo clathrin-mediated endocytosis and get filled with neurotransmitter again via an endosomal compartment.

Recycling of VMAT2 vesicles is important as this leads to reloading of vesicles for the new exocytotic cycle (Sudhof 2004).

### 1.5.3 VMAT2 in neuroprotection and neurotoxicity:

VMAT2 is an integral protein of the membrane of the monoamine storage synaptic vesicles. Its main function is to sequester monoaminergic neurotransmitters like DA, 5HT, norepinephrine and histamine from cellular cytosol into vesicles (Eiden, Schafer et al. 2004). At molecular level, one of the key manifestations of METH toxicity is the dysfunction of VMAT2 in the striatum. Thus, a decrease in vesicular DA uptake capacity of striatal VMAT2 has been observed as early as 1 h after binge METH in rats (Brown, Hanson et al. 2000). In mice, binge METH caused a decrease in VMAT2 ligand binding 6 days after METH treatment (Hogan, Staal et al. 2000). Toxic doses of METH also cause an altered subcellular distribution of VMAT2. At 1 h following METH, immunoreactivity of the partially glycosylated form of VMAT2 (~55 kDa) decreases in cytosolic fraction of synaptosomes with either an increase or no change in the membrane fraction in mice (Ugarte, Rau et al. 2003) and rats (Riddle, Topham et al. 2002), respectively. Compared to saline controls, the glycosylated form of VMAT2 (~68 kDa) also decreases in the cytosolic fraction at 1 h after the last METH injection in the rat striatum. At 24 h after binge METH, the immunoreactivity of the glycosylated isoform of VMAT2 was found to decrease in all synaptosomal fractions (Eyerman and Yamamoto 2005). These results suggest either degradation of VMAT2 or its mobilization to a non-synaptosomal compartment, potentially by a retrograde transport of damaged VMAT2 to the SNPc. A suggested possibility was damage to VMAT2 by nitrosative stress. Eyerman and colleagues showed nitrosylation of VMAT2 (~68 kDa) 1 h after METH and that the short term (1 h) and long term (7 days) decreases

in immunoreactivity of VMAT2 could be reversed by pretreatment with a nitric oxide synthase inhibitor (Eyerman and Yamamoto 2007). Thus, a number of studies have shown dysfunction of VMAT2 following binge METH regimen.

Especially in the DAergic systems, VMAT2 plays a neuroprotective role by sequestering free DA from cellular cytosol into storage vesicles at nerve terminals. The acidic pH in these storage vesicles protects the terminals from the oxidative damage caused by free radical products of DA autooxidation. VMAT2 transfection into PC12 cells lowered the cell death caused by METH (Vergo, Johansen et al. 2007). Further studies have shown that DA receptor agonists like pramipexole (a D2/D3 receptor agonist) and apomorphine (a D1/D2 receptor agonist) which are used to treat symptoms caused by DAergic nerve degeneration, increase the DA uptake capacity of VMAT2. This means that restoring or enhancing the function of VMAT2 can be used as a treatment for neurodegenerative conditions (Truong, Rau et al. 2003, Truong, Hanson et al. 2004). In METH toxicity study, the VMAT2 ligand lobeline was found to inhibit METH-mediated release of DA from the synaptic vesicles in vivo (Eyerman and Yamamoto 2005). Moreover, pre-treatment by lobeline was found to decrease METH self-administration in rats (Harrod, Dwoskin et al. 2001). Thus, VMAT2 plays an important role in neuroprotection and is an emerging target for development of treatments for METH toxicity and other neurodegenerative conditions (Harrod, Dwoskin et al. 2001, Dwoskin and Crooks 2002).

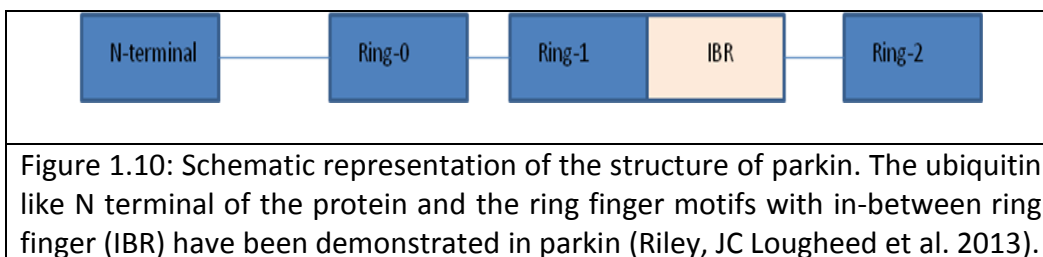
## 1.6 Parkin in METH neurotoxicity:

### 1.6.1 Parkin structure and function:

Parkin is a ring-between-ring ring protein (RBR), which belongs to a group of 12 multidomain enzymes. The parkin gene encodes for the protein parkin, which has 5 domains, 4 out of which are zinc binding domains and one is homologous to ubiquitin (Byrd and Weissman 2013, Spratt, Walden et al. 2014). The N terminal of parkin shows homology to ubiquitin and the C terminal has two ring finger motifs with an in-between ring finger domain (IBR) (Morett and Bork 1999). The IBR and ring finger motifs are believed to be binding sites for the ubiquitin-conjugating enzymes E2 (Figure 1.10). Parkin is made up of 465 amino acids and has a molecular weight of 52 kDa. Expression of parkin occurs in multiple organs like brain, heart, testis and skeletal muscle. Within the brain, it is found in many regions such as striatum, SNPC, cerebellum and hippocampus (Oliveras-Salva, Rompuy et al. 2011). Parkin functions primarily as an E3 ligase in the ubiquitin proteasome system. E3 ligases ligate the polyubiquitin chains to substrate proteins and mark them for degradation by the proteasome. Parkin self-ubiquitinates itself and regulates its own levels. A number of parkin substrates have been identified. Of major interest for us being the septin CDCrel-1. Parkin directly interacts with CDCrel-1 via its ring finger domain and controls the turnover of the protein (Zhang, J Gao et al. 2000). Other substrates of parkin are Pael-R, alpha synuclein, synphilin-1, alpha-tubulin and many others (Cookson 2003). Previous studies have shown that METH oxidatively damages parkin as well as the 26S proteasome along with reducing parkin mRNA levels (Nakahara, Kuroki et al. 2003, Moszczynska and Yamamoto 2011).

Parkin knockout mice show no differences in brain morphology, weight and size as compared to controls (Itier, Ibáñez et al. 2003). No loss of DAergic neurons or their striatal projections was observed until 24 months of age; however, decreases in DAT and VMAT2

binding activity were observed. Small differences were observed in the DAergic neurotransmission and variable data exists regarding DA metabolism in different studies. Also, inhibition of METH-induced DA release was observed. This suggests lowering of DAergic density in the absence of parkin. Parkin knockout rats also showed no pathological changes. However, at 8 months of age, they showed a 20% reduction in TH positive neurons in SNPc (Itier, Ibáñez et al. 2003, Sato, Chiba et al. 2006, Oliveras-Salva, Rompuy et al. 2011, Dave, Silva et al. 2014).



#### 1.6.2 Parkin in neurotoxicity and neuroprotection:

METH induced oxidative stress causes neurotoxicity via multiple mechanisms. Loss of parkin function has been related to Parkinson's disease (Matsumine, Saito et al. 1997) and METH neurotoxicity. Parkin has been implicated in both autosomal recessive juvenile Parkinson's disease (AR-JP) and idiopathic Parkinson's disease. Loss of parkin function due to mutation in parkin gene leads to AR-JP (Shimura, Hattori et al. 1999), whereas loss of parkin function and accumulation of parkin substrate alpha-synuclein and formation of Lewy bodies is believed to be one of the causes for idiopathic form of the disease (Choi, Golts et al. 2001). METH damages parkin and decreases its levels (Moszczynska and Yamamoto 2011). The DA released by METH covalently modifies parkin and disrupts its function (LaVoie, Ostaszewski et al. 2005). Oxidative stress is also known to alter the solubility of parkin and cause parkin monomers to aggregate into insoluble non-functional polymers (LaVoie, Ostaszewski et al.

2005). Binge METH has been shown to oxidatively damage parkin as early as 1 h after the last dose of METH. These decreases in the immunoreactivity of parkin persisted up to 24 h returning to basal levels at 48 h (Moszczynska and Yamamoto 2011).

Parkin protects against a number of stresses. Overexpression of parkin in cells treated with proteasomal inhibitors showed significant protection from damage caused by dysfunctional proteasome (Petrucci, O'Farrell et al. 2002). Other studies have shown that overexpression of parkin protects cells from death due to excitotoxicity and toxicity caused by Pael-R or alpha-synuclein (Feany and Pallanck 2003). Darios and colleagues showed that parkin protects from mitochondrial death by prevention of mitochondrial swelling and release of cytochrome c (Darios, Corti et al. 2003). Parkin has also been found to play a neuroprotective role in the toxicity produced by METH. Enhancing parkin function could play a protective role by scavenging damaged protein aggregates. More recently, our lab published that parkin overexpression in rat nigrostriatal system protects against METH induced decreases in striatal TH (Liu, Traini et al. 2013). The molecular mechanism by which parkin protects against METH toxicity is unknown.

## 1.7 Septins:

### 1.7.1 Structure and function:

Septins are a family of proteins that function as GTPases. They have a GTP binding domain present in each protein. They play role in a variety of biological conditions like cytokinesis, vesicle dynamics & exocytosis, neoplasia, infection, hemostasis, neuropathology and neurodegenerative diseases. Septin polypeptides are found to complex and form filament



or ring like higher order structures (Roeseler, Sandrock et al. 2009). Septins have been found to interact with the rSEC6/8 exocyst complex (a multi-protein complex which interacts with vesicular docking proteins to play a role in release of neurotransmitters), which is believed to play a regulatory role in exocytosis (Hsu, Ting et al. 1996, Hsu, Hazuka et al. 1998). CDCrel-1 is a septin, which is mostly expressed in the brain and present in very small amounts in non-neuronal cells. It comprises about 0.1 percent of the total proteins in the brain. In mouse brain, CDCrel-1 has been observed to localize around the presynaptic vesicles (Kinoshita, Noda et al. 2000). Within the human brain, the protein CDCrel-1 has been found in all regions with higher expression in cortical areas and lower in sub-cortical regions such as hippocampus, putamen and thalamus (Caltagarone, Rhodes et al. 1998). Despite the fact that it lacks a transmembrane domain, CDCrel-1 is found attached to membrane and synaptic vesicles. It has also been found that CDCrel-1 binds to syntaxin, which is a part of the soluble N-ethylmaleimide sensitive fusion protein (NSF) attachment protein (SNAP) Receptor complex (SNARE). By this interaction, it prevents docking of vesicles to the membrane and prevents exocytosis (Beites, Xie et al. 1999). Interestingly, CDCrel-1 is a substrate of the E3 ligase parkin. Parkin interacts with CDCrel-1 via its ring finger domain and augments its turnover mediated by the UPS (Zhang, J Gao et al. 2000).

#### 1.7.2 CDCrel-1 in neurotoxicity:

CDCrel-1 inhibits exocytosis by interacting with the SNARE complex. Role in exocytosis is believed to be the primary function of CDCrel-1 in mammals (Beites, Xie et al. 1999). Under basal conditions, the levels of CDCrel-1 are maintained at a very low level in DAergic neurons

as compared to GABAergic neurons (Kinoshita, Noda et al. 2000). However, under abnormal conditions, such as diminished function of parkin, this protein accumulates in cells (Zhang, J Gao et al. 2000). The overexpression of CDCrel-1 showed DAergic neuron specific loss in the SNpc and decline of striatal DA in rat brain. No damage by CDCrel-1 expression was observed in the globus pallidus. This DAergic selective loss could be prevented by co-expression of parkin (Zhizhong Dong 2003). It is not clear how the accumulation of CDCrel-1 can be selectively toxic to DAergic system. Mechanisms have been suggested for this phenomenon. It was observed that inhibition of TH and DA synthesis prevented the neurodegeneration by CDCrel-1. This means that CDCrel-1 produces DA-dependent neurotoxicity and that augmentation of DA synthesis could be the cause for its damaging effect. This speculation would account for why only DAergic specific toxicity is observed by CDCrel-1 overexpression. Also, CDCrel-1 inhibits the exocytosis of DA. This impairment in the neurotransmission of DA and possible oxidative damage due to increased free DA in the terminal could also be responsible for neurodegeneration (Dong, Ferger et al. 2003). None of the above speculations have been proven to be the mechanism by which CDCrel-1 causes DAergic toxicity.

## 1.8 Rationale:

Despite the extensive research in the area of METH neurotoxicity, no treatment regimen or medication to cure METH-induced brain damage are available. Current medications used in emergency rooms only provide symptomatic relief from METH overdose. There are no treatments to prevent, inhibit or reverse METH toxic effects on the brain. Thus, there is an immediate need for effective treatment to cure METH damage to the brain. Consequently, new molecular drug targets are needed to develop new pharmaceuticals.

E3 ligase parkin protects striatal DAergic terminals against METH neurotoxicity. Therefore, parkin is a potential drug target for therapeutic interventions in human METH users, particularly those who take METH in high doses. Furthermore, no studies to date examined the role of parkin in regulation of VMAT2-bearing vesicle trafficking in vivo. The important role of proper VMAT2 function and VMAT2 vesicle trafficking in DA neuron homeostasis has been established.

Many studies have shown the dysfunction of VMAT2 function and trafficking following METH. Although the decrease in cytosolic fraction was shown, none of the studies observed a decrease in the VMAT2 immunoreactivity at the membrane. Previous study has shown a redistribution of VMAT2 from membrane to cytosol upon D2 receptor activation (Truong, Newman et al. 2004). Since METH indirectly acts as a D2 agonist by releasing DA, we would expect a decrease in VMAT2 at the membrane following METH treatment. This suggests VMAT2 might be retained at the membrane of the terminal, possibly trapped via filamentous proteins (Hirokawa, Sobue et al. 1989). However, whether VMAT2 is trapped at the plasma membrane and by what mechanism has not been determined.

As an E3 ligase, parkin regulates the turnover of many proteins and thereby protecting the cell against damage by aggregated or misfolded proteins. CDCrel-1 is one of the several substrates of parkin. Following insult by METH, there is a decrease in parkin levels which could lead to accumulation of CDCrel-1 (Dong, Ferger et al. 2003, Moszczynska and Yamamoto 2011). Why accumulation of CDCrel-1 is responsible for neurodegeneration is unclear. Accumulation of other substrates of parkin such as Pael-R and alpha-synuclein is also found to cause

neurodegeneration (Dusonchet, Bensadoun et al. 2009, Perren, Toelen et al. 2014). Parkin is one of several hundred E3 ligases in neurons. To the best of our knowledge, the regulation of CDCrel-1 by an E3 ligase other than parkin has not been found. The mechanism by which CDCrel-1 accumulation leads to DAergic neurodegeneration needs to be determined.

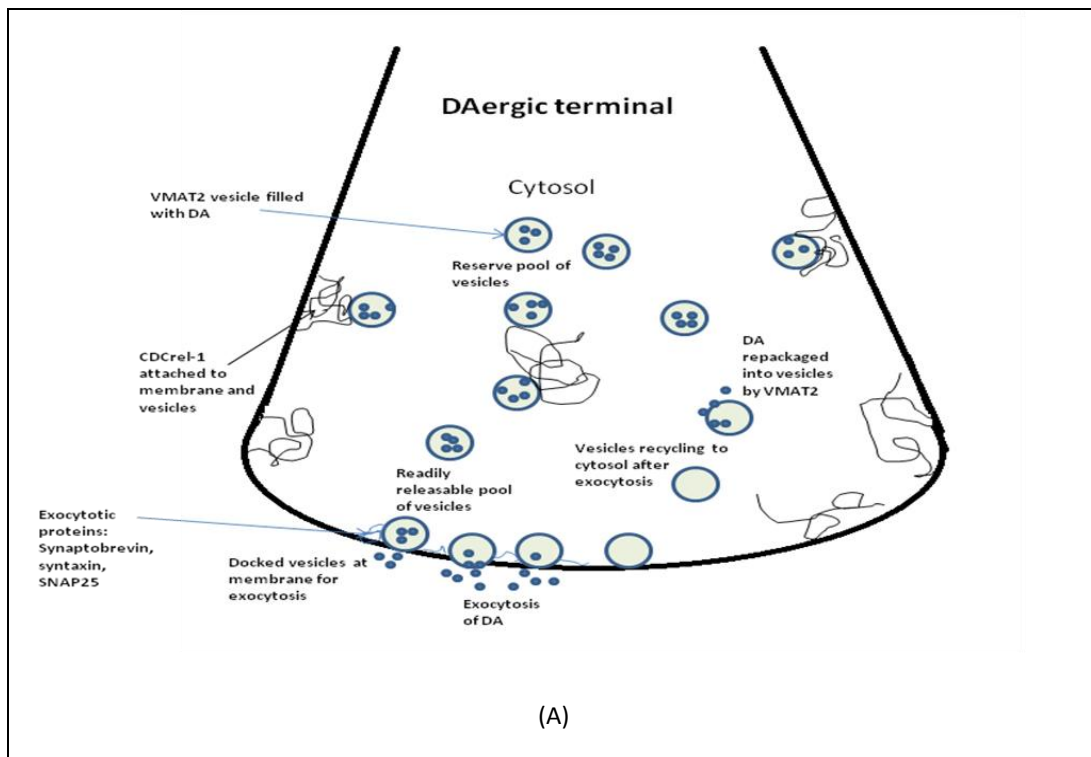
### 1.9 Hypothesis and specific aims:

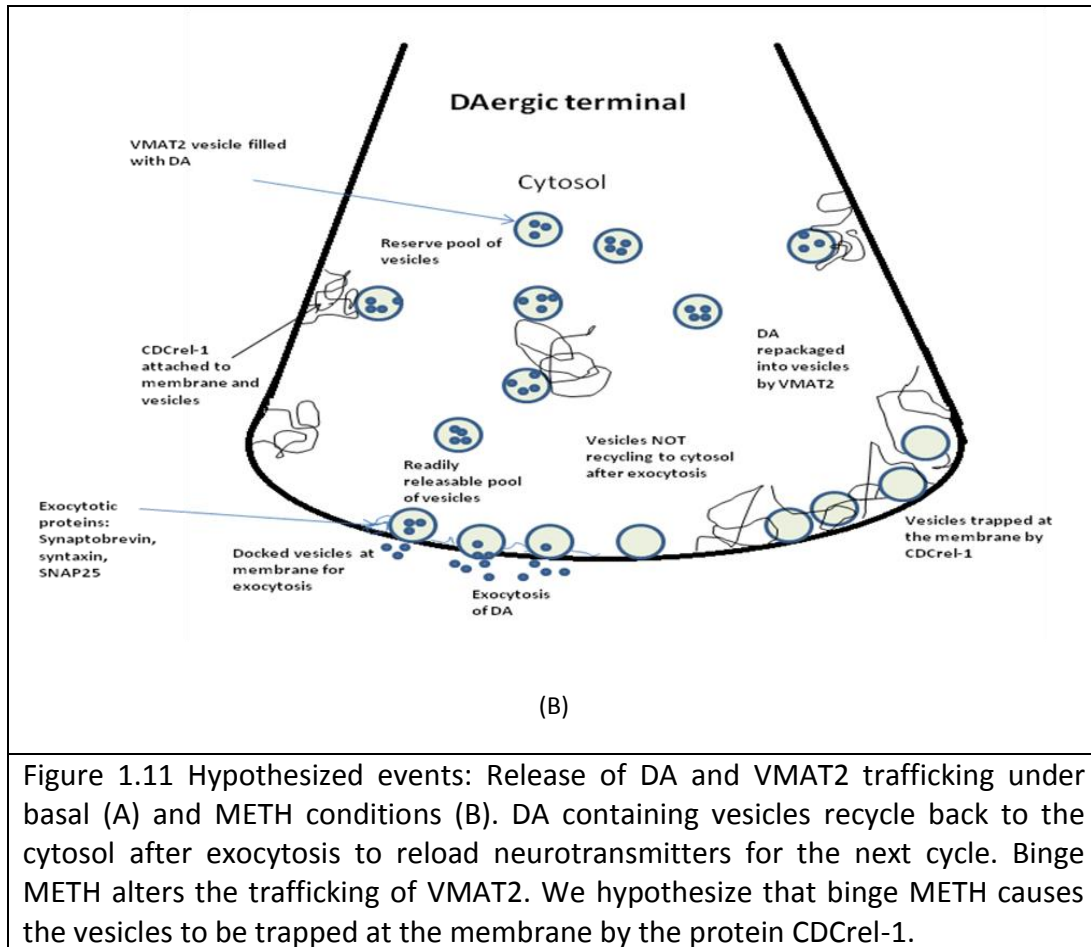
METH, a widely used drug of abuse, is neurotoxic to DAergic terminals in the striatum. This neurotoxicity, mainly, in part, is due to lack of cytosolic DA sequestration by synaptic vesicles. The autooxidation of this unsequestered DA leads to oxidative stress and neurodegeneration of the terminals. VMAT2 plays a neuroprotective role by transporting cytoplasmic DA into vesicles for storage and protection from oxidation. It has previously been shown that METH toxicity is associated with impaired VMAT2 trafficking and a decrease in the levels of E3 ligase parkin in striatal synaptosomes at 1 h and 24 h time points (Riddle, Topham et al. 2002, Moszczynska and Yamamoto 2011). Parkin regulates the levels of CDCrel1, a protein found to inhibit exocytosis and induce neurodegeneration to DA neurons (Zhang, J Gao et al. 2000, Chu, Hadlock et al. 2010). At present, the interactions between CDCrel-1, VMAT2 and parkin in METH toxicity are not known. Since the impaired trafficking of VMAT2 as well as the deficit in parkin mediated by binge METH have been studied at 1 h and 24 h after the last injection of the drug, we decided to limit our study to these two time points.

The goal of the present study was to test the hypothesis that METH-mediated parkin deficit results in accumulation of CDCrel-1 within DAergic terminals, which in turn causes an entrapment of VMAT2 vesicles at the plasma membrane, preventing their recycling and proper

sequestration of cytosolic DA. As mentioned previously, septins form filamentous structures that might be responsible for entrapment of VMAT2 by physical occlusion (Figure 1.11). Based on the available data, the following chain of events was hypothesized to occur after binge METH administration:

- (1) The levels of parkin decrease 1 h and 24 h following the last METH dose
- (2) The levels of CDCrel-1, the substrate of parkin, will elevate at the same times following METH
- (3) VMAT2 vesicles will be entrapped in CDCrel-1 filaments and will not be recycled back to the cytosol at 1 h and/or 24 h
- (4) Intracellular DA sequestration will decrease following METH neurotoxicity
- (5) Overexpression of parkin will decrease CDCrel-1 levels and restore VMAT2 trafficking





### Specific Aims:

- (1) To determine the immunoreactivity of parkin, CDCrel-1 and VMAT2 in synaptosomal fractions from the striatum following binge METH
- (2) To elucidate whether VMAT2 interacts with CDCrel-1 and whether VMAT2 remains trapped at the membrane following METH
- (3) To establish whether parkin plays a protective role in METH induced neurotoxicity via restoration of proper VMAT2 trafficking.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Animals

Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) (PND>60 days), were pair-housed under a 12 h light/dark cycle in a temperature- and humidity-controlled room. Food and water were available *ad libitum*. Temperature of the rats was measured via a rectal probe digital thermometer (Thermalert TH-8; Physitemp Instruments, Clifton, NJ). All animal procedures were conducted in strict accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Wayne State University (# A06-03-10 and # A05-07-13).

### 2.2 Drug Treatment

(+)-Methamphetamine, free-base (METH) (Sigma- Aldrich, St. Louis, MO) or saline were administered to rats in the following binge regimen: METH: (8 mg/kg) or saline (1 ml/kg) was administered to rats in four successive intraperitoneal (i.p.) injections, 2 hours apart. Rats were sacrificed by decapitation at 1 h or 24 h after the last injection of METH or saline. Core body temperature was monitored before the treatment as well as 1 h after each dose of METH or saline. Striata and cerebella were harvested immediately following the euthanasia and stored at -80°C until analyzed.

### 2.3 Preparation and fractionation of synaptosomes

The harvested tissue samples were homogenized in 500 µl of ice cold 0.32 M sucrose solution with 13 strokes, using a dounce homogenizer (Wheaton, USA). The homogenates were first centrifuged at 800 x *g* for 24 min at 4°C. Nuclei and other debris (P1) were discarded and

the supernatants (S1) were again centrifuged (22,000 x *g* for 17 min, 4°C) to obtain total synaptosomal pellets (P2). The P2 pellets were resuspended in 150 µl of distilled deionized water (ddH<sub>2</sub>O) giving total synaptosomal fractions. A volume of 50 µl of this total synaptosomal fraction was aliquoted from each sample for further analysis. The remaining 100 µl was centrifuged (22,000 x *g* for 17 min, 4°C) to obtain cytosol-vesicular (S3) and membrane-endosomal (P3) fractions. The P3 pellets were resuspended in 100 µl of ddH<sub>2</sub>O. Synaptosomal preparations were analyzed for protein concentration by Bradford protein assay using bovine serum albumin (BSA) as the standard. All samples and resulting fractions were kept on ice at all times prior to analyses. For all future references, P2 fraction is referred to as the total synaptosomal or total fraction, P3 is referred to as the membrane fraction and S3 is referred to as the vesicular or cytosolic fraction.

## 2.4 Electrophoresis and Western Blotting

Synaptosomal fractions (total, membrane and cytosolic) were subjected to reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing and reducing conditions (with beta-mercaptoethanol added as a reducing agent to the sample loading buffer). The amount of 10-20 µg proteins were loaded per lane on 4-12% Bis-Tris gels (Life Technologies, Grand Island, NY). The gels were run for 1 h at room temperature at 150V using an Invitrogen apparatus. All proteins were transferred to PVDF membranes (EMD Millipore, Billerica, MA) at 400 mA and then blocked for 1 h at room temperature with 3% non-fat dried milk dissolved in TBST (10 mM Tris, 150 mM NaCl, and 0.05% Tween-20). The membranes were then probed with either mouse monoclonal primary antibodies against CDCrel-1 (1:1000, overnight at 4°C) (MAB5358; Chemicon International, Temecula, CA), parkin



(1:1,000; overnight at 4°C) (Prk8; Cell Signaling Technology, Danvers, MA), actin (1:1,000; 1 h at room temperature) (8H10D10; Signaling Technology, Danvers, MA), alpha-tubulin (1:1000, overnight at 4°C) (sc58668; Santa Cruz Biotech, Santa Cruz, CA, USA), or rabbit polyclonal primary antibody against VMAT2 (1:3000, overnight at 4°C) (NBP1-69750H, Novus Biologicals). The incubations with primary antibodies were followed by washing (3 times for 5 min) with TBST followed by incubations with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000 for 1 h at room temperature). Blots were developed using ECL detection and LAS4000 bioimager (GE Healthcare, Piscataway, NJ). For standardization across blots, each blot contained all experimental groups. The western blot data was expressed as relative optical density units on each gel normalized to saline controls. This approach normalized differences in the development of the blot and across blots.

## 2.5 Loading Control

Actin and alpha-tubulin are components of microtubules and present in high abundance in cells. Consequently, they are widely used as loading controls. We observed a change in the levels of actin at 1 h after binge METH in both membrane as well as vesicular fractions. So, actin did not prove to be a good loading control for our study. There was no change in actin levels at 24 h time point. Along with actin, we also assessed the levels of alpha-tubulin at 1 h and 24 h after METH. Alpha-tubulin levels also changed following METH. To be accurate and consistent, we used Ponceau S (P7170, Sigma- Aldrich, St. Louis, MO) as a loading control for the duration of the entire study. The detailed description of these findings is included in the Results section.

## 2.6 Immunofluorescence

Synaptosomes were isolated from the striatum of saline-treated and METH-treated rats. The total synaptosomal pellet was resuspended in permeabilization buffer (0.32M sucrose, 0.01% digitonin, 1 x HALT, 1mg/mL BSA) and gently inverted at 4°C for 1 h. Antibodies to parkin (1:250) (Prk8; Cell Signaling Technology, Danvers, MA) and TH (1:250) (AB152, EMD Millipore Corp., Billerica, MA) were subsequently added to the re-suspended synaptosomes and incubated under gentle agitation at 4°C overnight. Samples were then centrifuged at 22,000 x g for 5 min at 4°C and the supernatant carefully discarded. The resulting pellet was gently washed in permeabilization buffer 3 times for 10 min at 4°C. Between each wash the samples were centrifuged at 22,000 x g for 5 min to pellet the synaptosomes and supernatant was carefully discarded. Following the final wash, the synaptosomal pellet was re-suspended in permeabilization buffer containing Alexa 488 donkey anti-mouse (1:150) (A-21202, Life Technologies Corp., Carlsbad, CA) and donkey anti-rabbit Alexa 594 (1:150) (A-21207, Life Technologies Corp., Carlsbad, CA). Following the addition of secondary antibody all samples were protected from light until imaging. Samples were incubated with secondary antibody under gentle agitation at 4°C for 2 h. The samples were then washed as previously described. The final synaptosomal pellet was then re-suspended in 300 µl of permeabilization buffer and 5 µL samples were aliquoted onto frosted microscope slides (Fisherbrand, Thermo Fisher Scientific Inc., Waltham, MA). The samples were allowed to settle and dry for 4 h at room temperature. The slides were then mounted with Fluoromount-G™ mounting media (0100-01, SouthernBiotech, Birmingham, AL) and allowed to dry overnight. Slides were then examined

for fluorescence using confocal microscopy (Leica TCS SPE, Leica Microsystems, Wetzlar, Germany).

## 2.7 Co-Immunoprecipitation

Striatal synaptosomal fractions were prepared from rats euthanized at 1 h time point following saline or METH treatment. Dynabeads (Life Technologies, Grand Island, NY) were incubated in the presence of 2  $\mu$ l of either a rabbit polyclonal VMAT2 primary antibody (NBP1-69750H, Novus Biologicals), mouse monoclonal CDCrel1 primary antibody (MAB5358; Chemicon International, Temecula, CA), parkin monoclonal primary antibody (Prk8, 1:1000; Cell Signalling Technology), or radioimmunoprecipitation assay (RIPA) buffer (negative control) for 6–12 h at 4°C, followed by the addition of synaptosomal fractions (100-200  $\mu$ g) and second incubation (12 h at 4°C). For all quantitative co-immunoprecipitation studies, equal protein content of synaptosomal fraction was incubated with beads for each representing group (saline or METH). Following each incubation, the beads were washed three times using phosphate buffered saline with Tween-20 (PBST, 0.02% Tween-20). Following immunoprecipitation, Parkin, CDCrel1 or VMAT2 protein was dissociated from the beads using SDS Tris–Glycine sample buffer (Bio-Rad, Hercules, CA, USA) and heating (10 min at 70°C). The supernatants from the beads were run on 4-12% Bis-Tris gels under reducing conditions and subjected to western blot analysis using parkin, VMAT2 and CDCrel1 antibodies and appropriate secondary antibodies, as described above (section 2.4).

## 2.8 Mass Spectrometry

Membrane fractions of synaptosomes were incubated with Dynabeads conjugated with VMAT2 antibody and processed as described for co-immunoprecipitation. The resulting eluted samples were run on a gel and the gel was stained with Sypro Ruby stain. Gel slices were excised from a Sypro Ruby-stained gel and the slices were washed and digested. The gel pieces were first washed with water and 25 mM  $\text{NH}_4\text{HCO}_3$ , 50% acetonitrile for 15 min each. The liquid was removed and the gel pieces were dehydrated in 100% acetonitrile for 5 min. Once the liquid was removed, the gel pieces were rehydrated in 50 mM  $\text{NH}_4\text{HCO}_3$ . After 5 min, an equal volume of 100% ACN was added and the gel pieces were left to incubate in the solution for another 15 min. All liquid was then removed and the gel pieces were dehydrated once again in 100% acetonitrile for 5 min. Once the liquid was removed after the incubation, the gel pieces were speed vacuum dried for 5 min. The following reactions were then performed: reduction with 5 mM dithiothreitol in 50 mM  $\text{NH}_4\text{HCO}_3$ , alkylation with 15 mM indoleacetic acid in 50 mM  $\text{NH}_4\text{HCO}_3$ , and overnight digestion with sequencing-grade trypsin (Promega, Madison, WI) in 25 mM  $\text{NH}_4\text{HCO}_3$ /10% ACN. Following digestion, peptides were extracted from the gel plugs using 50% acetonitrile/0.05% formic acid. The free peptides were then speed vacuumed to dryness and solubilized in 2% acetonitrile/0.1% formic acid.

The peptides were separated by a reverse phase chromatography (Acclaim PepMap RSLC C18 column, Thermo Scientific, Waltham, MA), followed by ionization with the Nanospray Flex Ion Source (Thermo Scientific), and introduced into a Q Exactive mass spectrometer (Thermo Scientific). Abundant species were fragmented with high-energy collision-induced dissociation (HCD). Data analysis was performed using Proteome Discoverer 1.4 (Thermo

Fisher, Waltham, MA), which incorporated the Mascot algorithm (Matrix Science). The Swiss Prot\_2013\_03 database was searched against rat protein sequences and a reverse decoy protein database was run simultaneously for false discovery rate (FDR) determination. Secondary analysis was performed using Scaffold 4.2.1 (Proteome Software). Minimum protein identification probability was set at  $\geq 95\%$  with 2 unique peptides at  $\geq 99\%$  minimum peptide identification probability. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.020 daltons and a parent ion tolerance of 10 parts per million. Carbamidomethylation of cysteine was specified in Mascot and X! Tandem as a fixed modification. Glu->pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, gln->pyro-Glu of the N-terminus, oxidation of methionine, and acetylation of the N-terminus were specified in X! Tandem as variable modifications. Oxidation of methionine and acetylation of the N-terminus were specified as variable modifications as well.

Mass spectrometry processing was done by the Proteomics Core facility of Wayne State University. The Wayne State University Proteomics Core Facility is supported by the NIH Center Grants P30 ES06639, P30 CA22453 and P60 DK020572.

## 2.9 Parkin Overexpression

Parkin was overexpressed in the rat striatum using the adeno-associated viral vector (AAV2/6). The rat parkin-coding (AAV2/6-parkin) and the non-coding vector (AAV2/6) were obtained from Dr. Bernard Schneider from the Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland. Virus-loaded dose of the vector ( $2 \times 10^7$  transducing units, TUs) in 2  $\mu$ l volume were injected in the brain as per Liu et al (Liu, Traini et al. 2013). Briefly, rats were

allowed to acclimatize for one week and then the parkin-coding or empty vector ( $2 \times 10^7$  TUs) was microinjected in the SNpc on the left side of the brain using a stereotaxic apparatus. For the stereotaxic surgery, mixture of xylazine (20 mg/ml) and ketamine (100 mg/ml) was given intraperitoneally to anesthetize the rats. Unilateral microinjection was performed on the left side of brain in the SNpc with either the parkin-coding or non-coding AAV vector. The right side of the brain for each rat would act as a control for the injected side (left). The microinjection was done using the following coordinates: – 5.6 mm (AP) from Bregma, – 2 mm (ML) from Bregma, – 7.6 mm (V) from the dura according to the Paxinos and Watson's rat brain atlas. Vector (coding or non-coding) was injected at a rate of 0.15  $\mu$ l/min using a syringe pump with a 5  $\mu$ l Hamilton syringe (Harvard Apparatus, Holliston, MA, USA). The syringe was left in place for 5 min at – 7.6 mm than withdrawn to – 6.6 mm (5 min) and then – 5.5 mm (for 5 min). The syringe was slowly raised out of the brain over 2 min.

Three weeks following the microinjections, the rats were divided into 2 groups. One group was treated with binge METH (4x8 mg/kg, every 2 h, i.p.) and the other group was treated with saline (4x1 mg/ml, every 2 h, i.p.). Core body temperature was measured before the treatment and 1 h after each dose of METH or saline. After the drug or saline treatment, rats were euthanized 1 h following the last dose. After euthanasia, striata were harvested by brain dissection and were stored at  $-80^{\circ}\text{C}$  till further analysis.

For assessing the immunoreactivity, both the left (injected side) and the right (non-injected side) of the striatum were processed independently by density centrifugation as described in section 2.3 to obtain the total, membrane and cytosol synaptosomal fractions. All the samples

were maintained on ice at all times. The appropriate fractions were analyzed by western blotting (as per section 2.4) using antibodies against parkin, CDCrel-1 and VMAT2.

### 2.10 Chromatography:

Dopamine (DA) Content: Synaptosomal membrane pellet was obtained as per method described by Chu et al (Chu, Hadlock et al. 2010). Briefly, striatal tissue was homogenized using a dounce homogenizer (13 strokes) in 0.32M sucrose solution. The homogenate was then fractionated at 800xg for 24 min at 4<sup>0</sup>C to obtain the supernatant S1. The supernatant S1 was further fractionated at 22,000xg for 17 min at 4<sup>0</sup>C to obtain the synaptosomal pellet. The pellet was resuspended in 75 ul of water and osmolarity was immediately restored by adding equal volume of pH 7.5 buffer containing 25mM HEPES and 100mM potassium tartarate. The resulting solution was fractionated at 22,000xg for 17 min at 4<sup>0</sup>C to obtain membrane pellet and cytosolic fraction. The membrane pellet obtained was re-suspended in perchloric acid (final concentration 0.3N). The resulting solution was then centrifuged at 22,000xg for 30 min to obtain the precipitated protein pellet. The supernatent was analyzed for DA content using High Performance Liquid Chromatography (HPLC). The protein pellet was resuspended in 1 N sodium hydroxide and its concentration was determined using the bicinchoninic acid (BCA) assay. The DA content obtained by HPLC was normalized per protein precipitated from that particular sample.

### 2.11 Statistical Analysis:

Data analysis was performed using GraphPad Prism program (GraphPad Software Inc.,San Diego, CA, USA). Data with two treatment groups were analyzed using two-tailed,

unpaired Student's t-test. Data from more than two treatment groups were analyzed using one-way ANOVA. Correlations between immunoreactivities, temperatures and different parameters was done using Pearson's correlation test. All data are expressed as mean  $\pm$  standard errors of mean (SEM). Significance was set at  $p < 0.05$  (95% confidence interval).



## CHAPTER 3 RESULTS

### 3.1 Establishing the validity of loading controls:

Loading controls are used to account for the uneven loading of gels during electrophoresis and to normalize bands per loading control. Mostly, cytoskeletal proteins like actin or tubulin are used as loading controls due to their high abundance in cells. However, METH is known to affect the levels of actin and tubulins (Maeno, Iwasaa et al. 2000). Therefore, we decided to assess if the effect of METH on the levels of two cytoskeletal proteins- actin and alpha-tubulin.

#### 3.1.1 Actin as a loading control

In order to validate actin as a loading control, we assessed the immunoreactivity of actin across fractions of striatal synaptosomes at 1 h and 24 h after treatment with METH. We found that binge METH decreased actin immunoreactivity in all 3 fractions: total (-13%), membrane-endosomal (-14%) and vesicle-cytosolic (-21%) fractions, at 1 h after the last dose (Student's t-test,  $p < 0.05$ ). At 24 h, the levels of actin returned to the control levels across all fractions. All the immunoreactivities were normalized to total protein levels determined by Ponceau S staining.

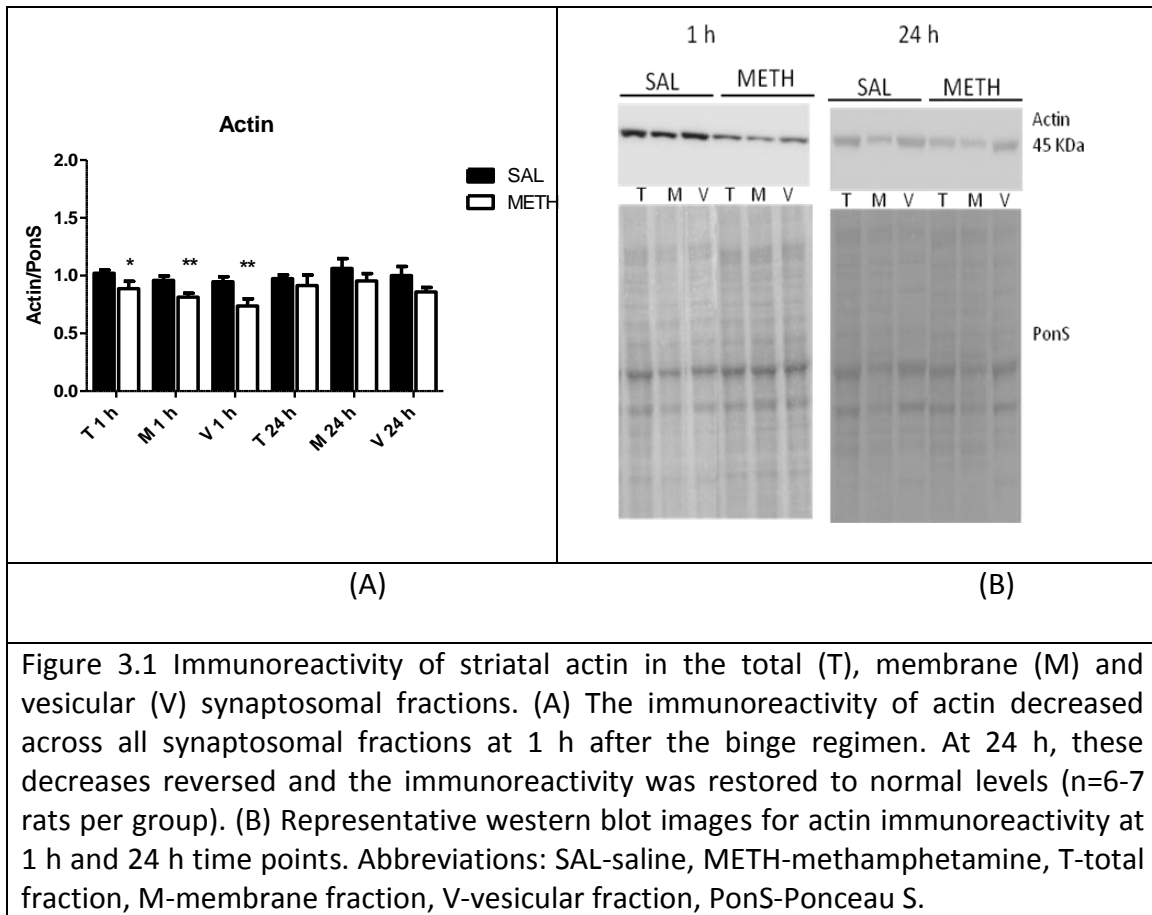
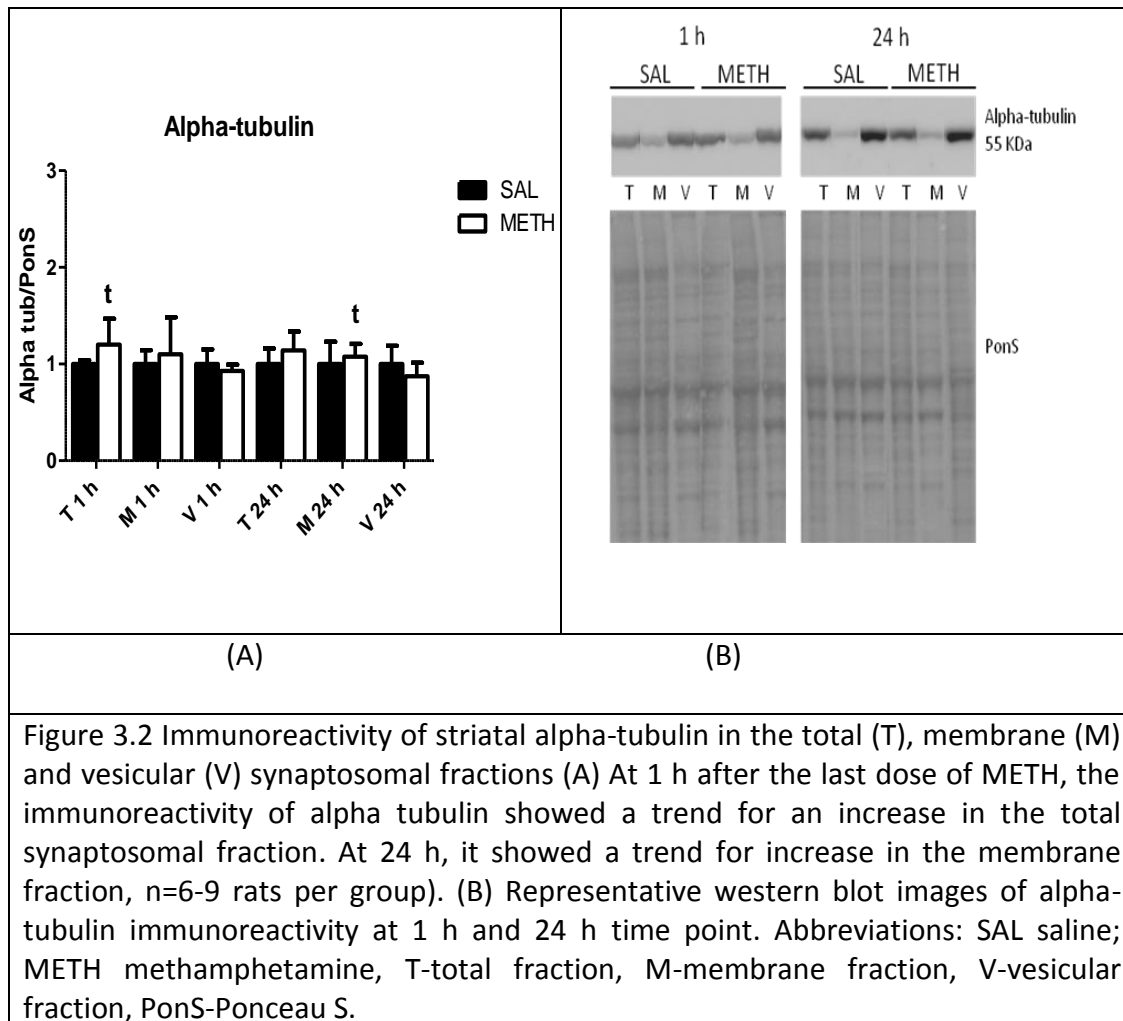


Figure 3.1 Immunoreactivity of striatal actin in the total (T), membrane (M) and vesicular (V) synaptosomal fractions. (A) The immunoreactivity of actin decreased across all synaptosomal fractions at 1 h after the binge regimen. At 24 h, these decreases reversed and the immunoreactivity was restored to normal levels (n=6-7 rats per group). (B) Representative western blot images for actin immunoreactivity at 1 h and 24 h time points. Abbreviations: SAL-saline, METH-methamphetamine, T-total fraction, M-membrane fraction, V-vesicular fraction, PonS-Ponceau S.

### 3.1.2 Alpha-tubulin as a loading control

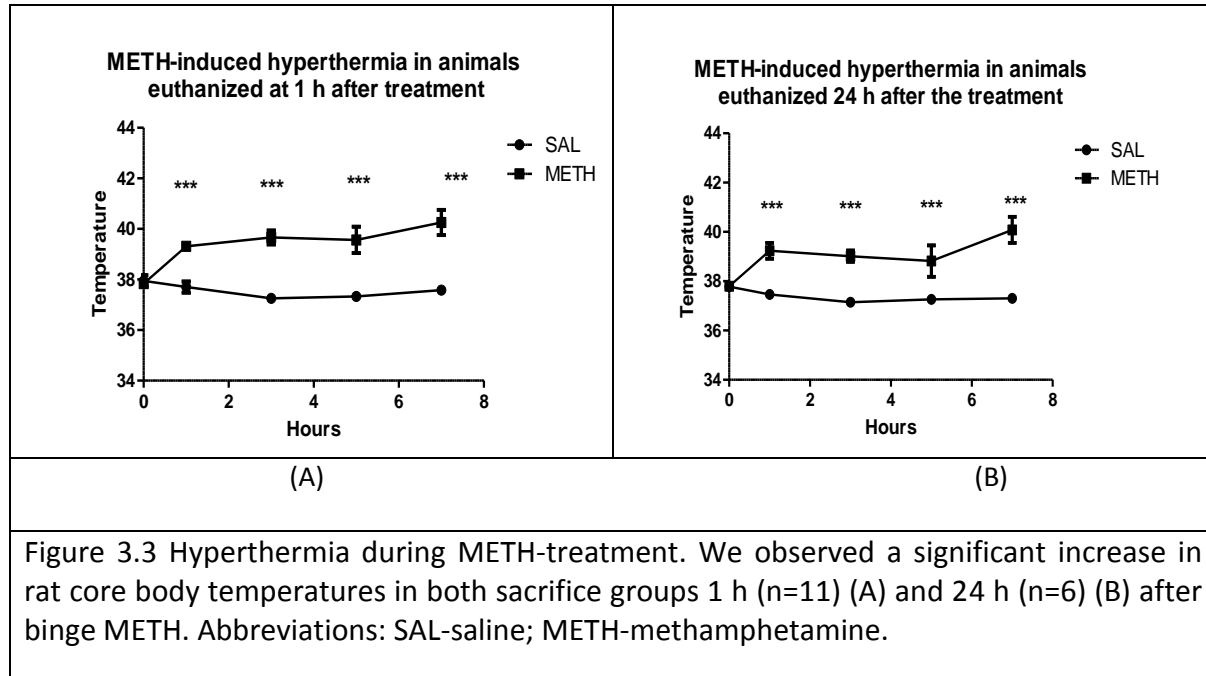
Alpha-tubulin is another cytoskeletal protein that is widely used as a loading control. It is also a substrate of E3 ligase parkin. To determine if METH affects its levels, we assessed its immunoreactivity in synaptosomal fractions using electrophoresis and western blotting. The immunoreactivity of alpha-tubulin showed a statistical trend for an increase in the total synaptosomal fraction at 1 h (20%, Student's t test,  $p=0.0561$ ). At 24 h, a trend to increase was observed in the membrane fraction (7%, Student's t test,  $p=0.0621$ ).



### 3.2 Validation of METH neurotoxicity:

In experimental animals, high doses of METH are known to cause hyperthermia, which is one of the factors mediating the neurotoxicity of the drug. Increased core body temperature after METH increases toxicity and lower body temperature protects against METH-induced toxicity (Bowyer, Davies et al. 1994, Yamamoto, Moszczynska et al. 2010). In order to confirm that METH induces hyperthermia in our model, core body temperature was measured throughout the METH treatment. Rectal temperature was measured before the start of the treatment and one hour following each dose of METH or saline. As seen in Figure 3.3, significant hyperthermia at each measurement throughout the treatment was observed in animals in the 1

h and 24 h time point groups (two-way ANOVA with repeated measures, 1 h,  $p < 0.01$ ,  $F = 12.25$ ,  $dfn = 4$ ,  $dfd = 80$ ; 24 h,  $p < 0.01$ ,  $F = 7.10$ ,  $dfn = 4$ ,  $dfd = 40$ ).

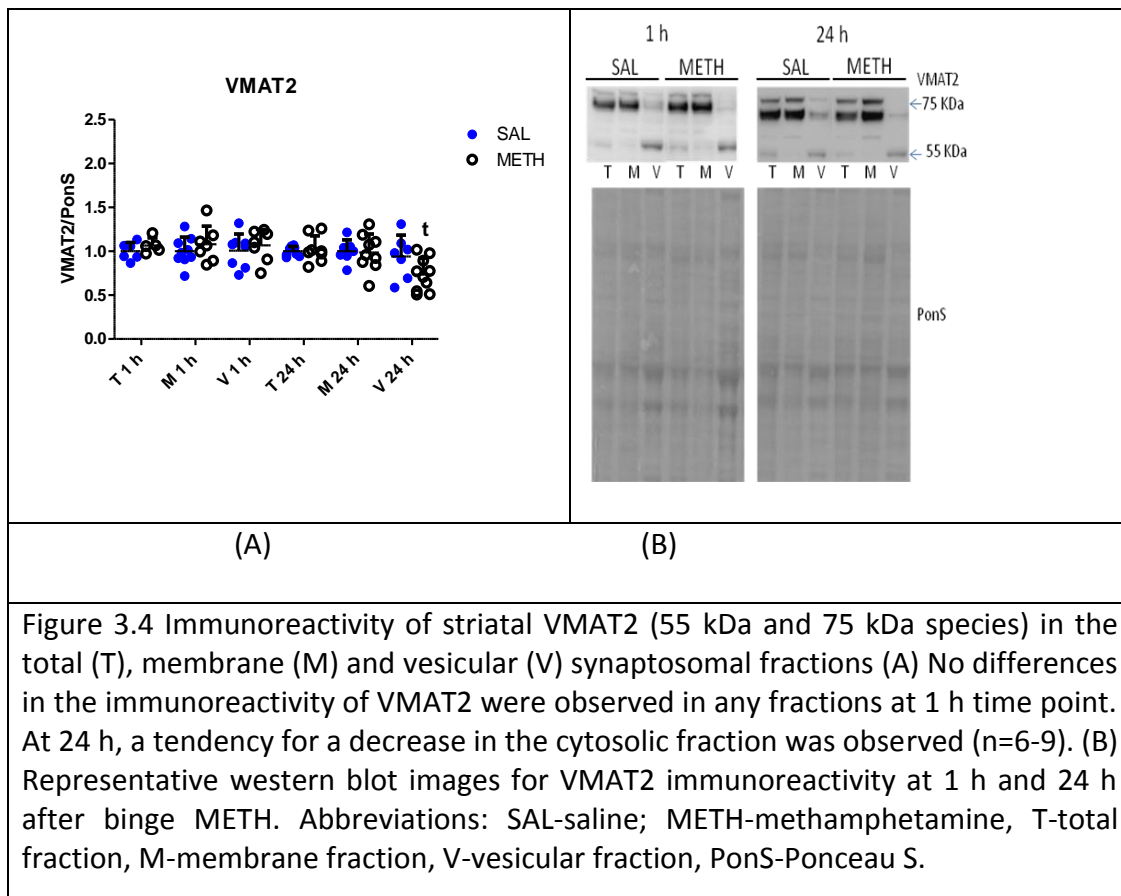


### 3.3 The effect of binge METH on trafficking of Vesicular Monoamine Transporter 2 (VMAT2) in DAergic terminals:

Previous studies have reported changes in striatal VMAT2 immunoreactivity after high dose METH at 1 h and 24 h following the treatment. Changes in partially glycosylated VMAT2 (~55 kDa) in the membrane and vesicular synaptosomal fractions were observed at 1 h time point (Riddle, Topham et al. 2002, Ugarte, Rau et al. 2003). A decrease in glycosylated VMAT2 (~68 kDa) in vesicular fraction at 1 h and decreases across all synaptosomal fractions at 24 hours has also been shown (Eyerman and Yamamoto 2005, Eyerman and Yamamoto 2007).

To determine whether trafficking of VMAT2 is impaired in our neurotoxicity model, we assessed immunoreactivity of VMAT2 in synaptosomal fractions at 1 h and 24 h after binge

METH. Of the 3 isoforms of VMAT2, the partially glycosylated form (55 kDa) and the glycosylated form (75 kDa) were identified by our antibody. The unglycosylated VMAT2 (45 kDa) was detected at very low levels in terminal endings. As compared to saline controls, no change was observed in the immunoreactivity of VMAT2 at 1 h time point. Trend for a decrease in the immunoreactivity of both isoforms of VMAT2 in the cytosolic fraction was seen at 24 h time point (-20%, Student's t test,  $p=0.0666$ ). In our fractionation protocol, we did not spun down vesicles to concentrate the sample. As a result of this, we observed a weaker signal for VMAT2 in cytosolic fraction as compared to other fractions in the western blot image. We expected a decrease in VMAT2 at 1 h due to the activation of DA D2 receptors (Truong, Newman et al. 2004) by METH-released DA.



### 3.4 The effect of binge METH on the levels of parkin:

#### 3.4.1 Parkin in striatal synaptosomes:

Binge METH (4x10 mg/kg, every 2 h, i.p.) decreases the levels of E3 ligase parkin in striatal synaptosomes by DA-mediated oxidative damage (Moszczynska and Yamamoto 2011). To evaluate the effect of METH on the E3 ligase parkin in our model of METH neurotoxicity (4x8 mg/kg, every 2 h, i.p.), we assessed immunoreactivity of parkin at 1 h and 24 h after binge METH. As assessed by western blotting, the immunoreactivity of parkin in the total synaptosomal fraction showed a statistical trend for a decrease at 1 h after the last dose of METH (-19%, Student's t test,  $p=0.0629$ ,  $n=8-12$  rats per group). No statistically significant changes were observed in any other fractions at 1 h. The Figure 3.5 shows 2 subpopulations of rats in the membrane ( $p=0.44$ ) and vesicular fraction ( $p=0.10$ ) as well. These decreases might become statistically significant if sample sizes are increased. The immunoreactivity remained unchanged across all fractions at 24 h time point. The small deficit observed in the immunoreactivity of parkin could have been a result of two subpopulations of rats, which showed different effects. As can be seen in the scatter dot plot, one subpopulation of rats shows deficit in parkin whereas the other subpopulation shows no change in the levels of parkin following METH (Figure 3.5 A). Since the rats used in this study are outbred rats, the variability in effect observed could be due to individual differences between the rats.

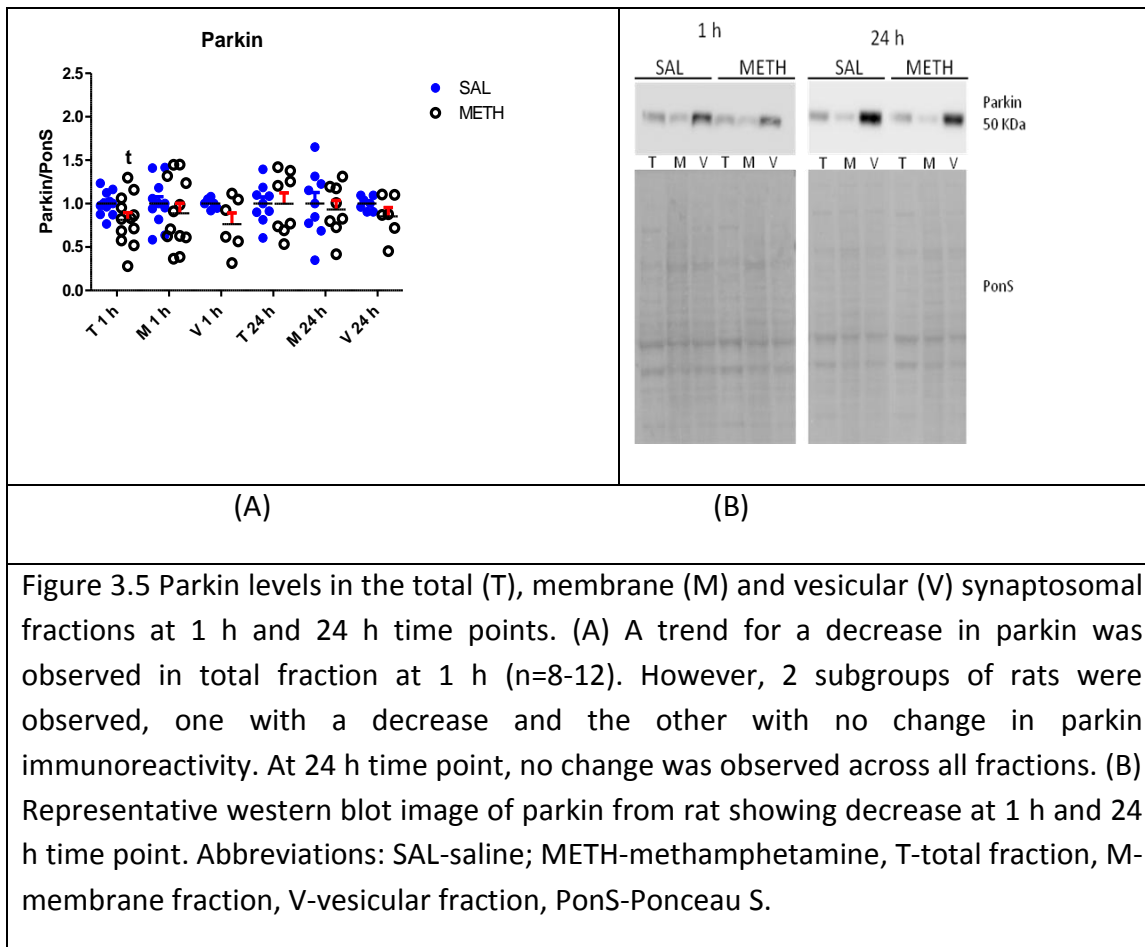
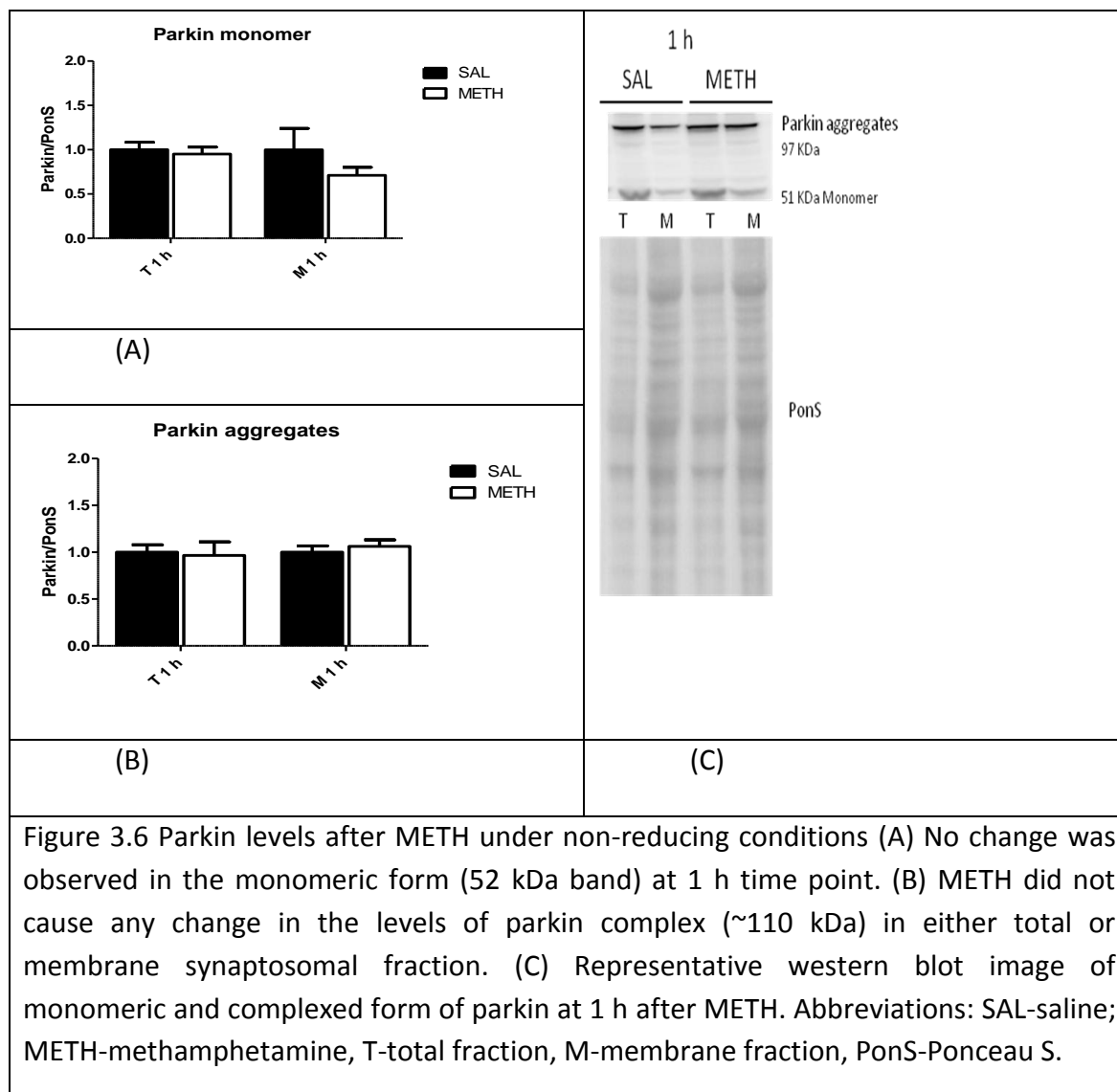


Figure 3.5 Parkin levels in the total (T), membrane (M) and vesicular (V) synaptosomal fractions at 1 h and 24 h time points. (A) A trend for a decrease in parkin was observed in total fraction at 1 h (n=8-12). However, 2 subgroups of rats were observed, one with a decrease and the other with no change in parkin immunoreactivity. At 24 h time point, no change was observed across all fractions. (B) Representative western blot image of parkin from rat showing decrease at 1 h and 24 h time point. Abbreviations: SAL-saline; METH-methamphetamine, T-total fraction, M-membrane fraction, V-vesicular fraction, PonS-Ponceau S.

### 3.4.2 Parkin monomers and oligomers:

METH releases DA and free DA is known to covalently modify parkin, causing it to aggregate. These aggregates of parkin lack their usual E3 ligase function (LaVoie, Ostaszewski et al. 2005). Since the deficit in parkin observed under our conditions was small and occurred only in a subpopulation of rats, we decided to assess whether there was an increase in the levels of parkin aggregates accompanied by a decrease in monomeric form, without a change in the total levels of parkin. To determine this, we performed electrophoresis under non-reducing conditions to prevent the aggregates from breaking down. This was followed by western blotting using the parkin antibody. Parkin aggregates are usually observed as tetramers around 200 kDa as observed by previous studies (LaVoie, Ostaszewski et al. 2005). We did not observe

any such high molecular weight aggregates of parkin. However, our antibody detected a complexed form of parkin around 110 kDa. This form has been found by another study, which suggested that the 110 kDa form is a protein complex where parkin occurs bound to other interacting proteins. This study also found that the levels of this 110 kDa form do not change with the change in the expression of parkin (VanHumbecq, Waelkens et al. 2008). This explains our finding that there is no change in the levels of the complexed form of parkin (110 kDa) after METH, as compared to saline.

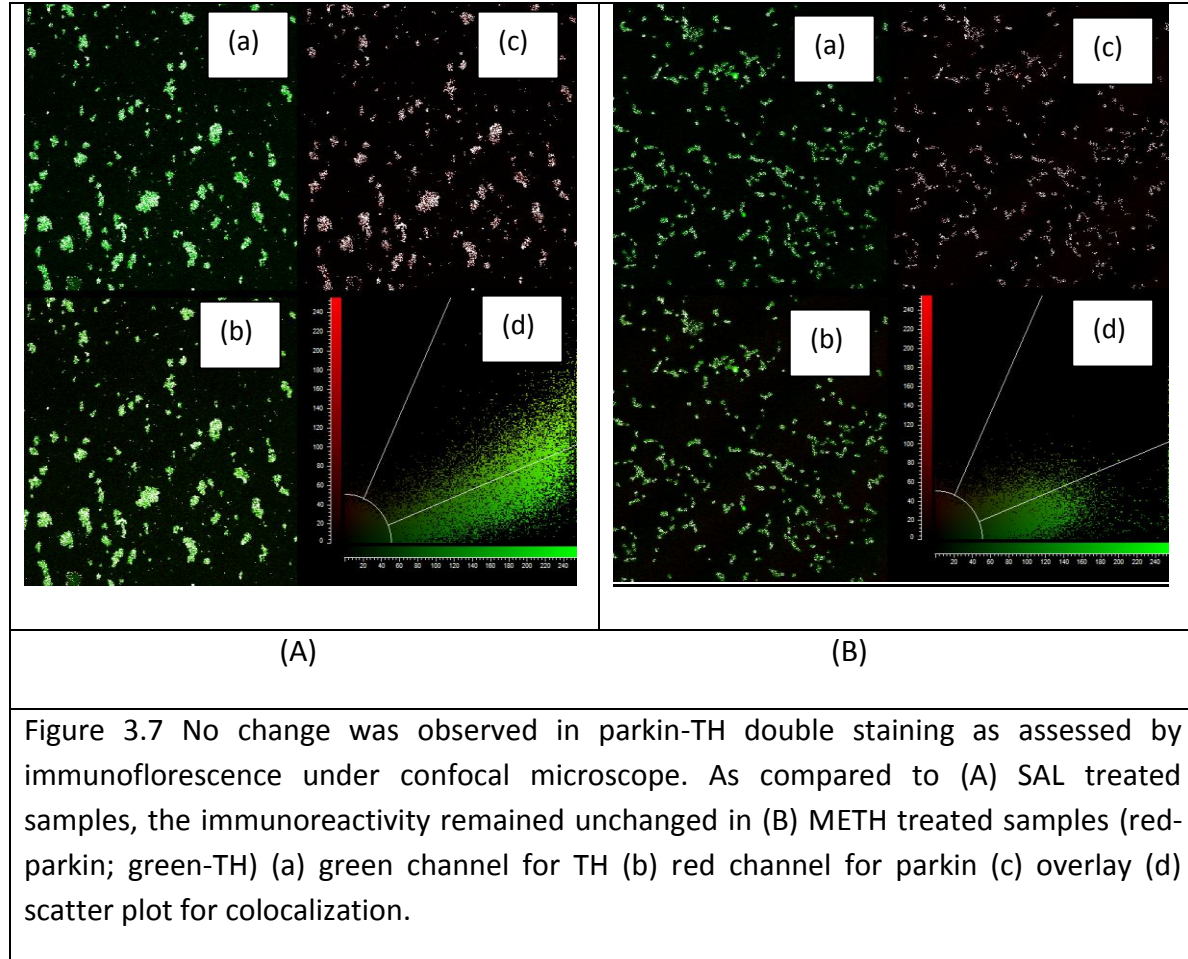




### 3.4.3 Parkin in DAergic terminals:

DAergic terminals constitute ~21% of the total nerve terminals in the striatum. This suggests that even if there was a deficit in parkin in these terminals, we would not see it by western blotting due to dilution of the effect. To determine whether there was any decrease in parkin in DAergic terminals, we performed immunofluorescence on striatal synaptosomes plated on microscopic slides and double labeled with antibodies against parkin and the catecholaminergic marker TH.

To ensure unbiased results, all the slides were numbered and the person taking the images was blinded i.e. the identity of slides was kept unknown until the final analysis of colocalization. Multiple areas were imaged per slide. The Leica software calculated Pearson's coefficient for colocalization in each area and these values were normalized per TH intensity for that area. The normalized values were averaged per animal and statistical analysis was performed using Student's t-test. No statistically significant changes were observed between saline and METH treated brains. (SAL,  $0.005 \pm 0.0007$  N=4; METH,  $0.004 \pm 0.0005$ , N=4; Student's t test,  $p=0.5136$ ).



#### 3.4.4 Parkin in cerebellar synaptosomes:

Cerebellum has negligible DA content. To determine whether the trend for decrease in parkin in total fraction observed previously was specific to DA-rich striatal synaptosomes, we assessed the immunoreactivity of parkin in total and membrane fraction of synaptosomes from cerebellum harvested at 1 h after the last dose of METH. As compared to saline controls, an increase in parkin immunoreactivity was observed in total (37%, Student's t-test,  $p < 0.05$ ) and membrane (31%, Student's t-test,  $p < 0.05$ ) fractions.

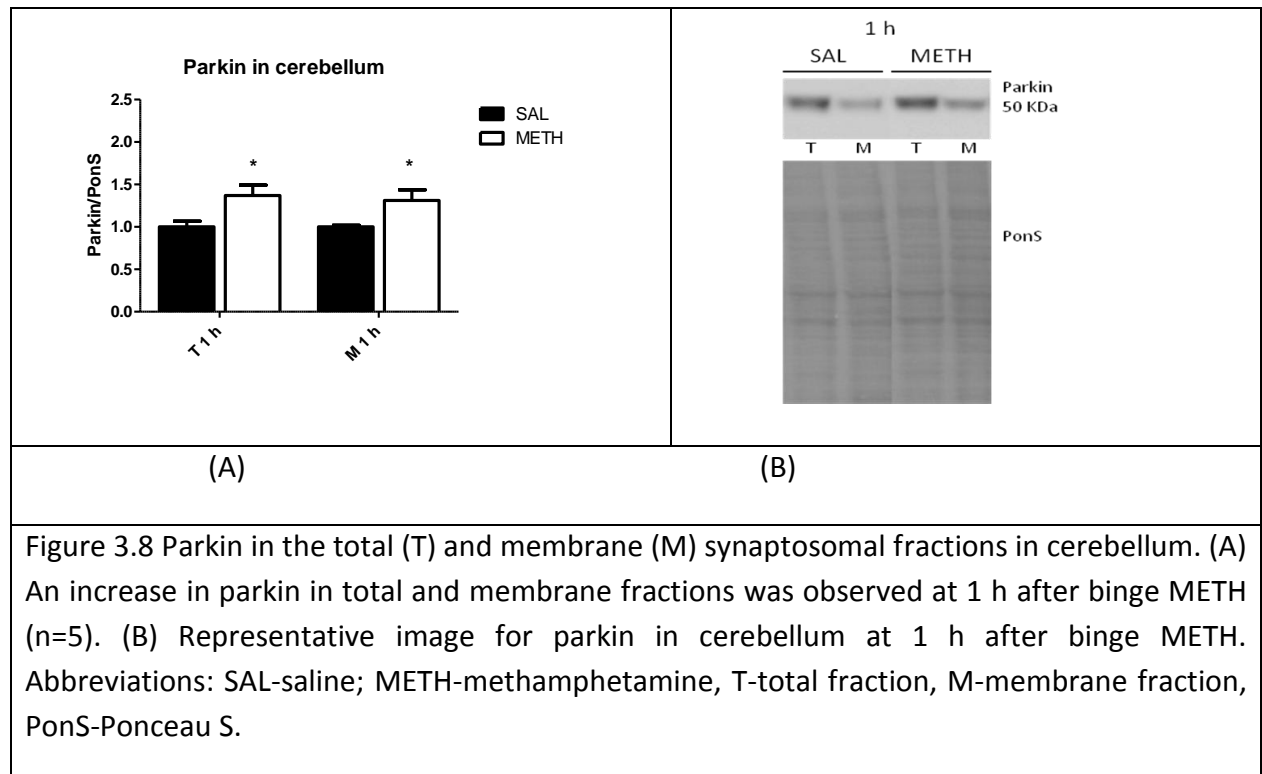


Figure 3.8 Parkin in the total (T) and membrane (M) synaptosomal fractions in cerebellum. (A) An increase in parkin in total and membrane fractions was observed at 1 h after binge METH (n=5). (B) Representative image for parkin in cerebellum at 1 h after binge METH. Abbreviations: SAL-saline; METH-methamphetamine, T-total fraction, M-membrane fraction, PonS-Ponceau S.

### 3.5 The effect of binge METH on the levels of CDCrel-1:

Excess of CDCrel-1 damages DAergic neurons in rodents (Zhizhong Dong 2003). CDCrel-1 is a substrate of parkin (Yi Zhang 2000). Consequently, a deficit in parkin levels or its function can result in increased CDCrel-1 levels. Therefore, we next assessed the levels of CDCrel-1 protein in synaptosomal fractions at 1 h and 24 h after METH.

#### 3.5.1 CDCrel-1 in striatal synaptosomes

Using western blotting, we observed that in a subpopulation of rats, METH caused a trend for increased immunoreactivity of CDCrel-1 in total synaptosomal fraction at 1 h after binge regimen (35%, Student's t test,  $p=0.0542$ ). Also, a trend for a decrease was observed in the cytosolic fraction at 24 h time point (-15%, Student's t test,  $p=0.0698$ ). As with parkin, we

observed two subpopulations of rats, one with an increase in the immunoreactivity of CDCrel-1 and the other with no change after binge METH (Figure 3.9 A).

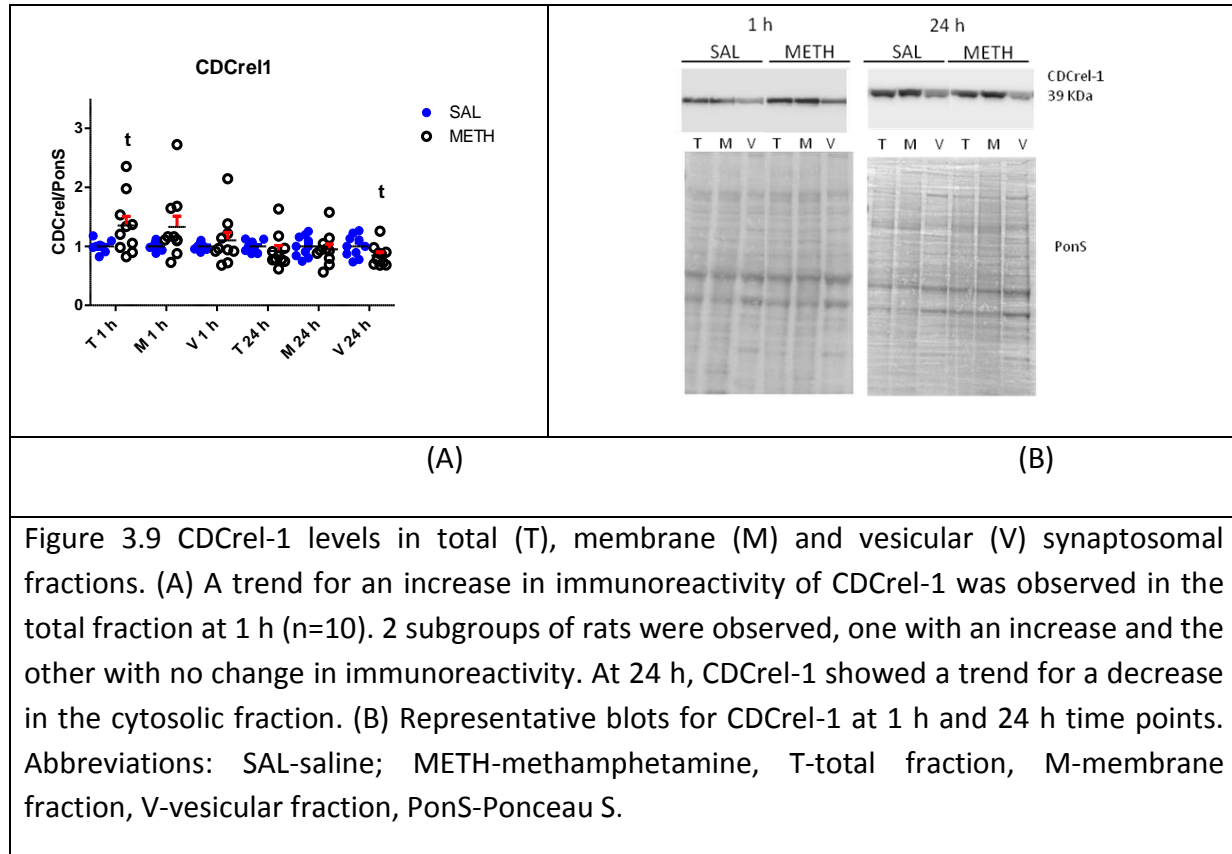


Figure 3.9 CDCrel-1 levels in total (T), membrane (M) and vesicular (V) synaptosomal fractions. (A) A trend for an increase in immunoreactivity of CDCrel-1 was observed in the total fraction at 1 h (n=10). 2 subgroups of rats were observed, one with an increase and the other with no change in immunoreactivity. At 24 h, CDCrel-1 showed a trend for a decrease in the cytosolic fraction. (B) Representative blots for CDCrel-1 at 1 h and 24 h time points. Abbreviations: SAL-saline; METH-methamphetamine, T-total fraction, M-membrane fraction, V-vesicular fraction, PonS-Ponceau S.

### 3.5.2 CDCrel-1 in cerebellar synaptosomes:

As cerebellum has minimal DAergic innervations, the levels of CDCrel-1 in the cerebellum were assessed to determine whether METH affects other non-DAergic nerve terminals there. Unlike striatum, the synaptosomes from cerebellum show no increase in the immunoreactivity of CDCrel-1 after binge METH. This serves as a negative control to the effect observed in striatal synaptosomes.

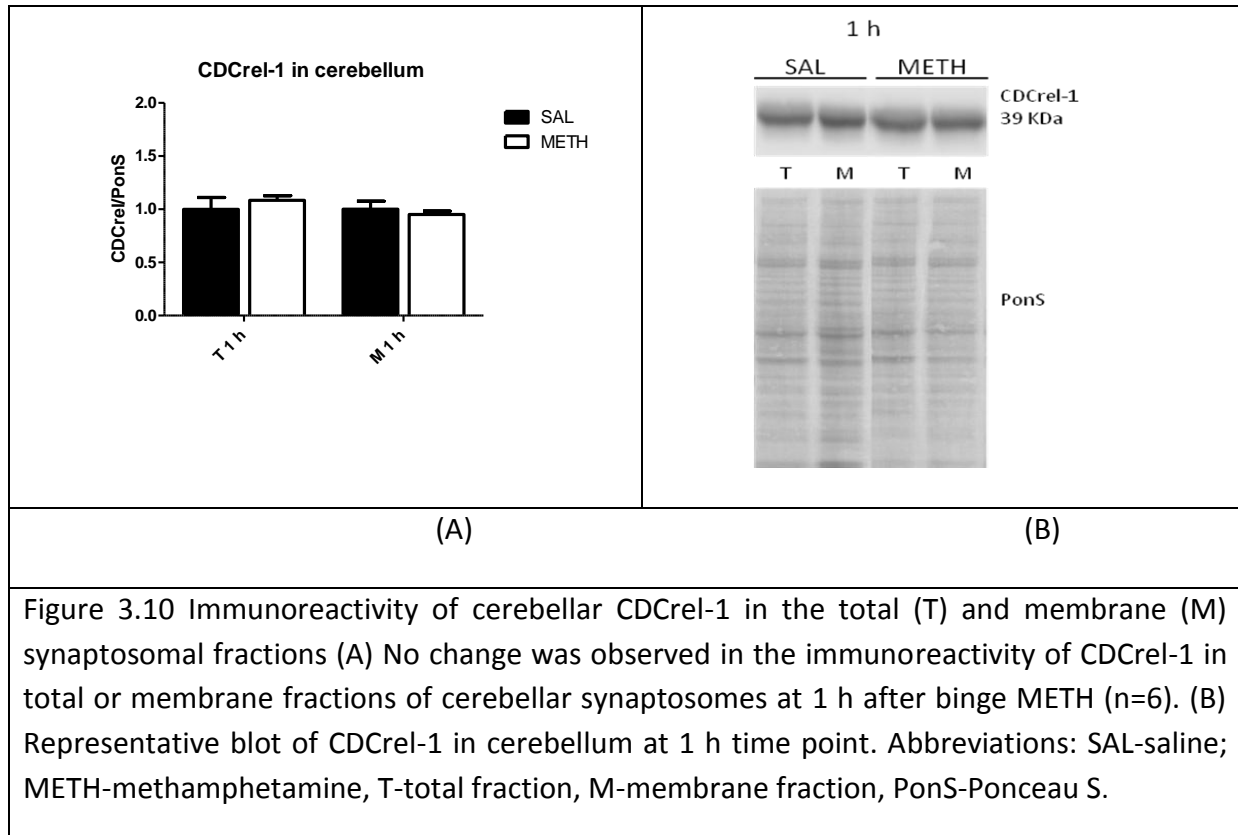


Figure 3.10 Immunoreactivity of cerebellar CDCrel-1 in the total (T) and membrane (M) synaptosomal fractions (A) No change was observed in the immunoreactivity of CDCrel-1 in total or membrane fractions of cerebellar synaptosomes at 1 h after binge METH (n=6). (B) Representative blot of CDCrel-1 in cerebellum at 1 h time point. Abbreviations: SAL-saline; METH-methamphetamine, T-total fraction, M-membrane fraction, PonS-Ponceau S.

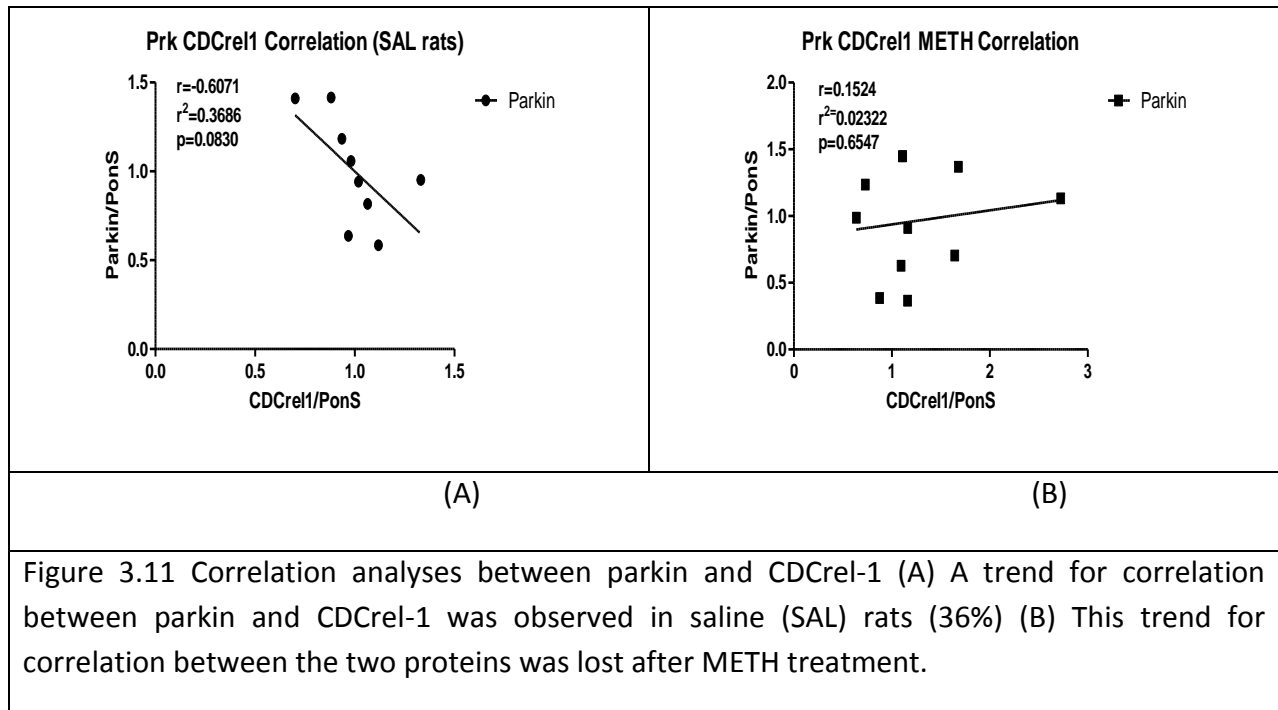
### 3.6 Parkin CDCrel-1 protein-protein interactions:

CDCrel-1 is known to be a substrate of parkin *in vitro* (Yi Zhang 2000). To determine whether the two proteins interact with each other under our *in vivo* conditions and whether this interaction is affected by METH treatment, we performed correlation analyses and coimmunoprecipitation studies.

#### 3.6.1 Correlation analyses:

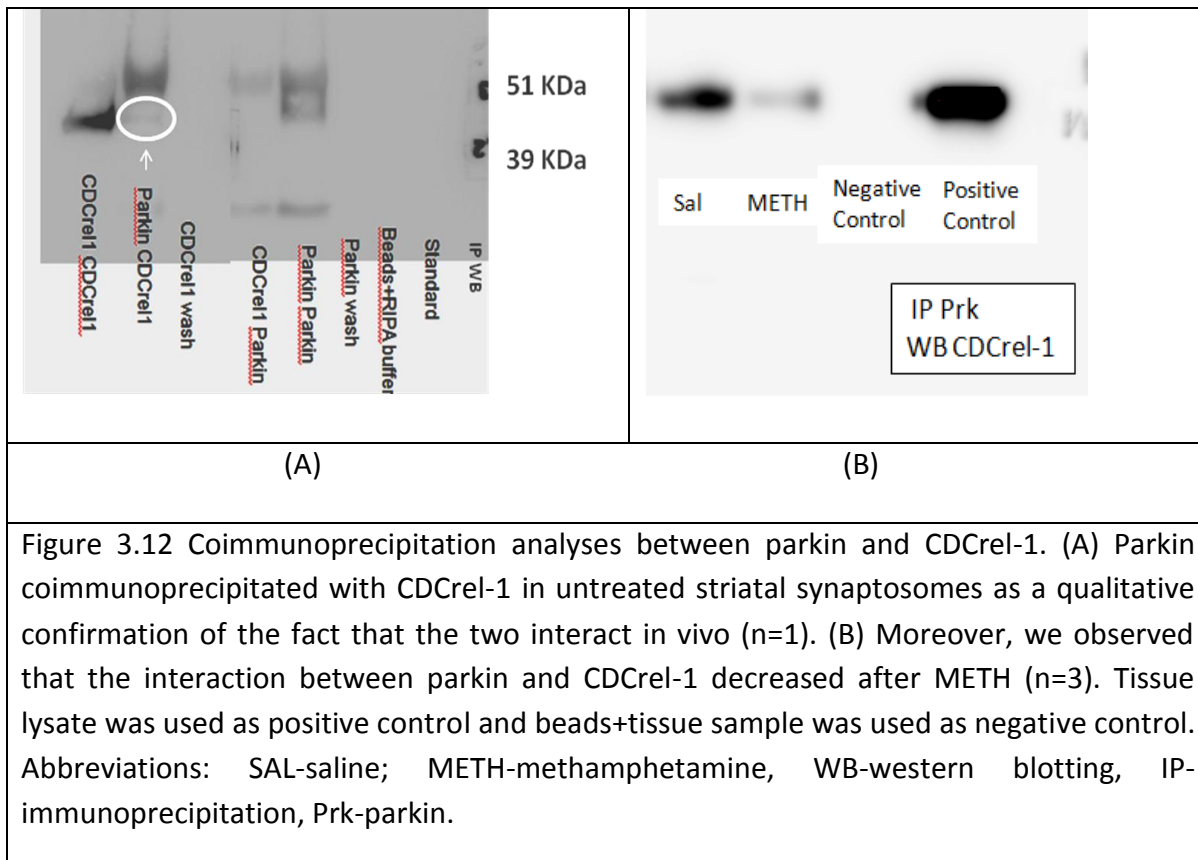
As described previously, we assessed immunoreactivity of both parkin and CDCrel-1 in striatal synaptosomes from rats killed 1 h after METH or saline. Although CDCrel-1 is a substrate of parkin, we did not know whether changes in their levels are dependent on each other under our treatment conditions. To determine this, we correlated the immunoreactivity of parkin and

CDCrel-1 from saline- as well as METH-treated rats. We observed a trend for a correlation between the two proteins in saline-treated rats (36%, Pearson's correlation analysis,  $p=0.0830$ ,  $r^2=0.3686$ ), which decreased after the treatment with METH (2.32%,  $p=0.6547$ ,  $r^2=0.02322$ ).



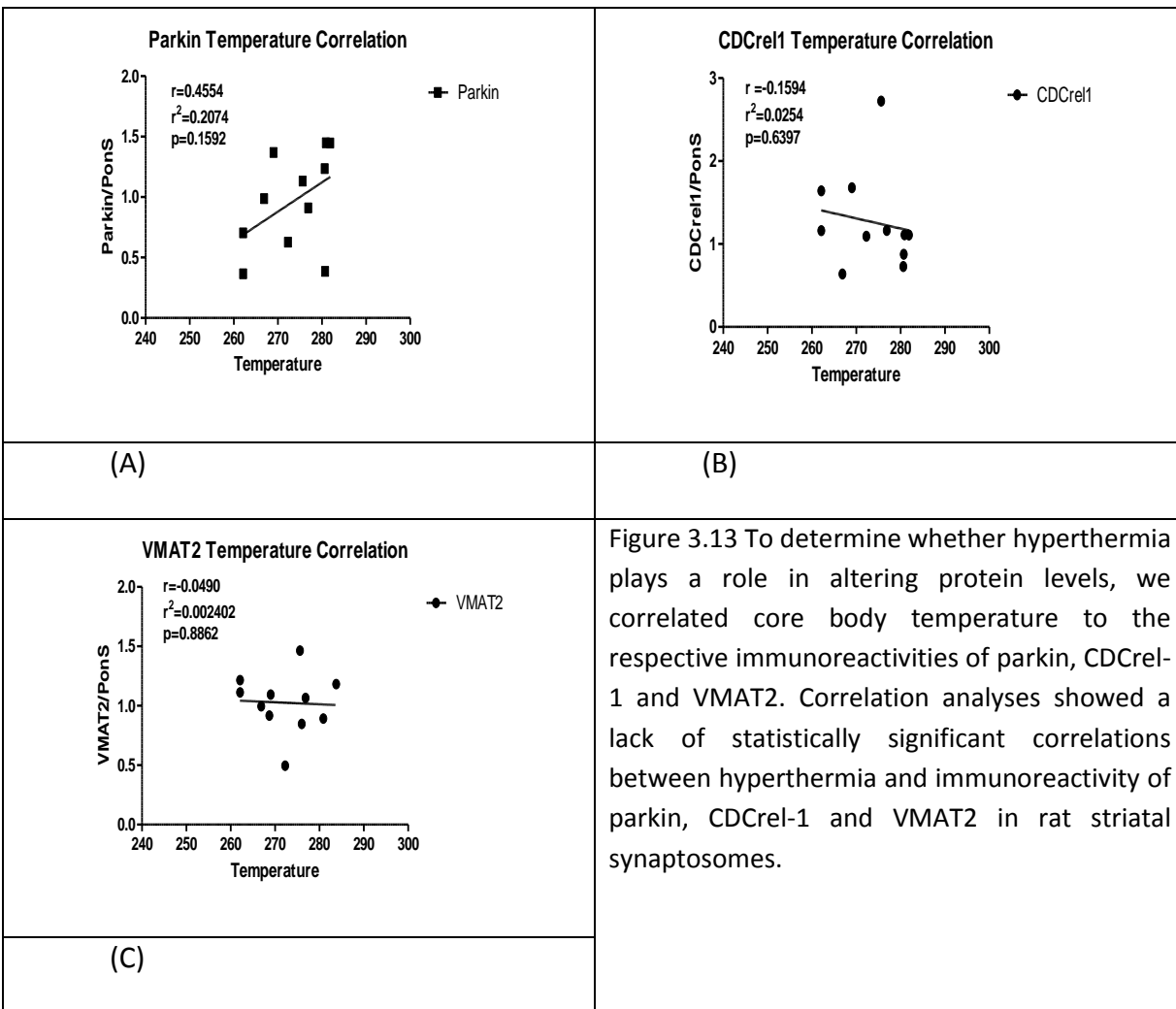
### 3.6.2 Coimmunoprecipitation:

To establish the interaction between parkin and CDCrel-1 in rat striatum, we employed coimmunoprecipitation technique. Striatal synaptosomes from saline- and METH-treated rats were subjected to coimmunoprecipitation using antibodies against CDCrel-1 and parkin. First, we set to determine in an untreated brain whether the two proteins coimmunoprecipitate with each other. After this, we compared the immunoprecipitation between saline and METH treatment conditions (Figure 3.13 A). We detected a decreased interaction between the two proteins after METH (Figure 3.13 B).



### 3.7 Correlation between parkin, CDCrel-1, VMAT2 and hyperthermia

Hyperthermia plays an important role in the toxicity of METH. Moreover, increased toxicity of METH is observed with increased core body temperatures (Bowyer, Davies et al. 1994). To determine whether the changes in immunoreactivity of parkin, CDCrel-1 and VMAT2 after METH are dependent on hyperthermia, we correlated their immunoreactivities to core body temperature of rats. We found that hyperthermia has no effect on the immunoreactivity of CDCrel-1 (-2.5%, Pearson's correlation analysis,  $p=0.6397$ ,  $r^2=0.025$ ) or VMAT2 (0.2%, Pearson's correlation analysis,  $p=0.8862$ ,  $r^2=0.002$ ). Hyperthermia had some effect on the immunoreactivity of parkin (20%, Pearson's correlation analysis,  $r^2=0.2074$ ), however the correlation was not statistically significant ( $p=0.1592$ ).



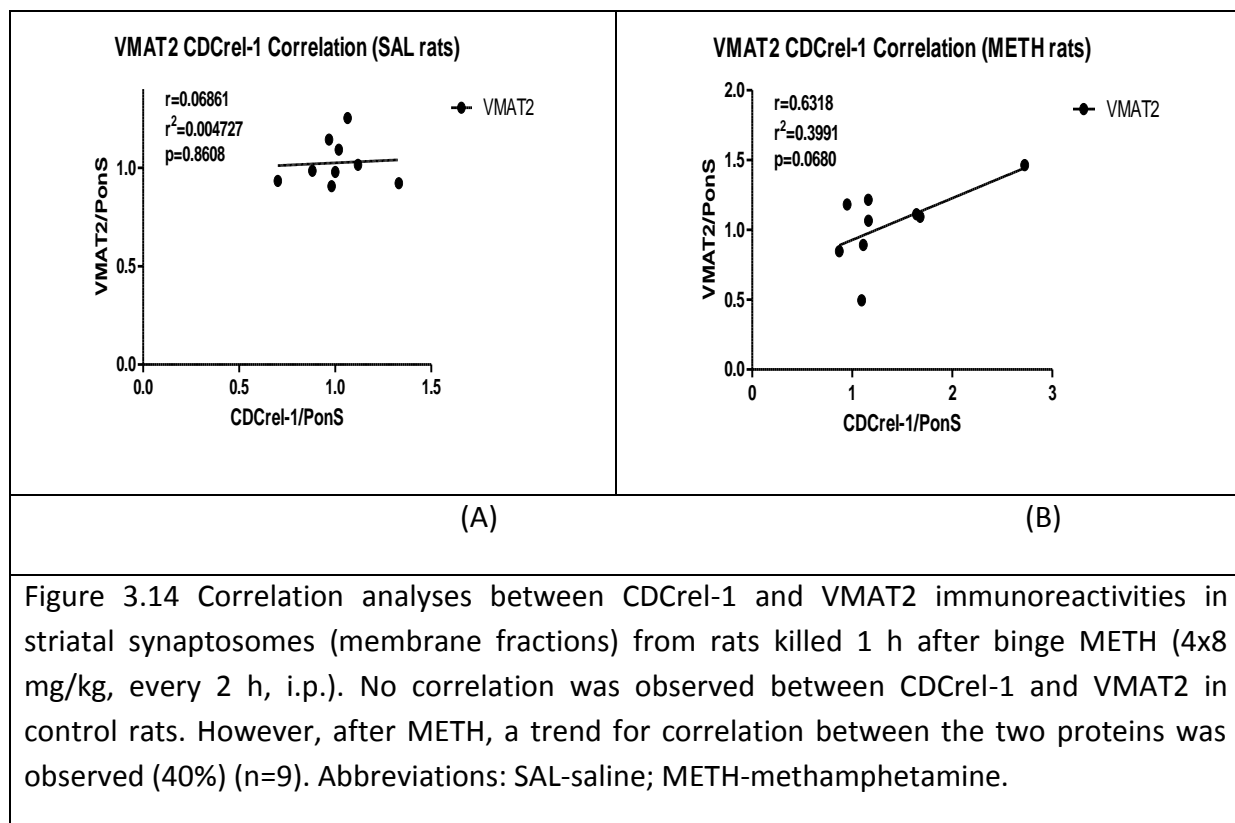
### 3.8 CDCrel-1 VMAT2 protein-protein interactions:

We hypothesized that the increased CDCrel-1 after METH entraps VMAT2 at the membrane of the nerve terminal. To assess whether CDCrel-1 interacts with VMAT2, we performed correlation, immunoprecipitation and mass spectrometry studies in synaptosomes from saline as well as METH treated rats.

#### 3.8.1 Correlation analyses



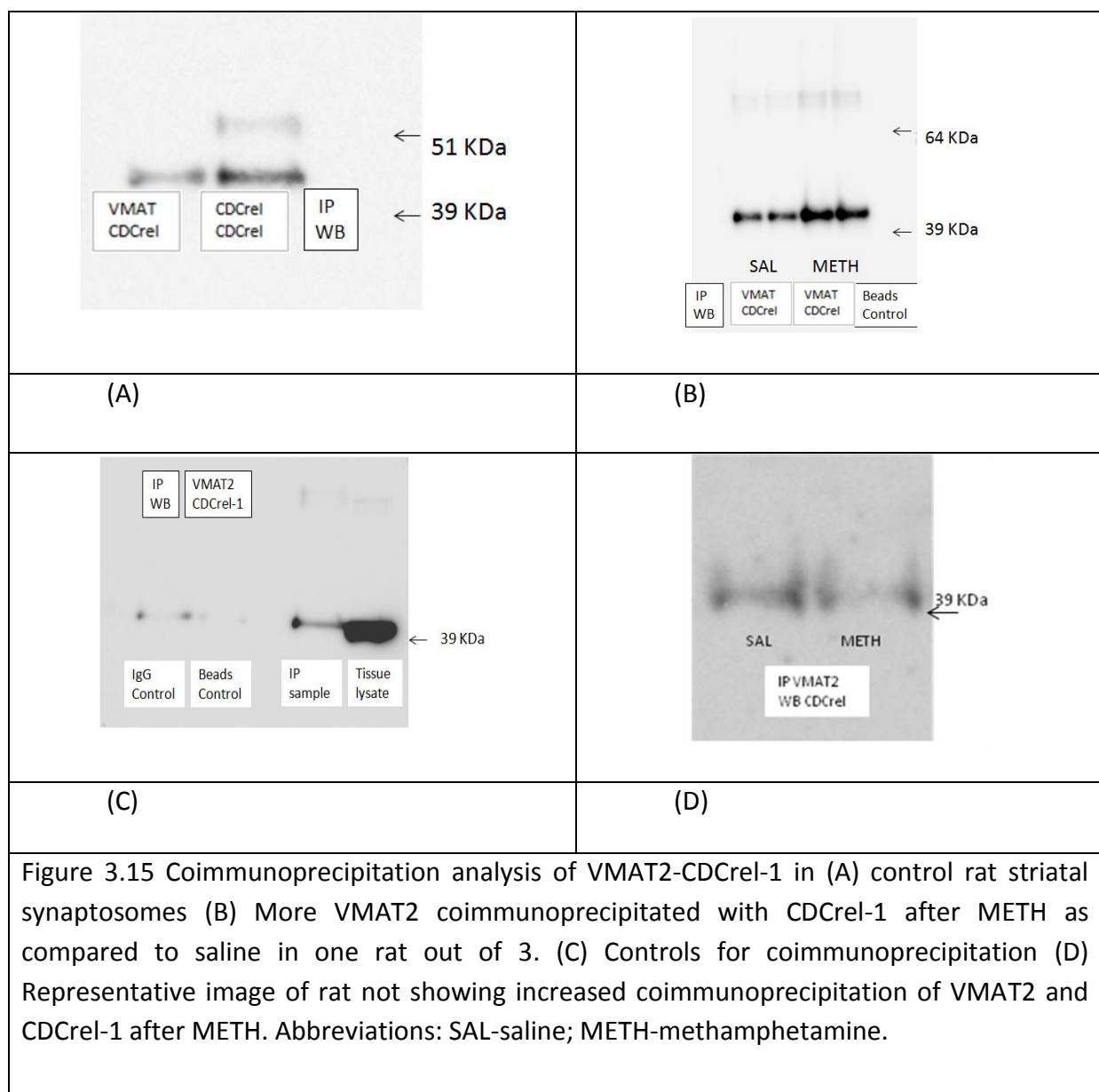
Using SDS-PAGE and western blotting, we determined the immunoreactivity of CDCrel-1 and VMAT2 in the synaptosomal membrane fraction after saline and METH treatment. We then plotted the immunoreactivity of both these proteins to perform a correlation study. We observed that there was no correlation between the two proteins in saline treated rats. However, after METH treatment, there was a statistical trend for a correlation between CDCrel-1 and VMAT2 (40%, Pearson's correlation analysis,  $r^2=0.3991$ ,  $p=0.0680$ ).



### 3.8.2 Coimmunoprecipitation

In order to establish their interaction, we performed coimmunoprecipitation of VMAT2 and CDCrel-1 in the membrane fraction of synaptosomes. First, we assessed whether CDCrel-1 coprecipitates with VMAT2 under basal conditions in untreated rat striatal total synaptosomes (Figure 3.16 A). Thereafter, we performed immunoprecipitation to compare the interactions of

the two proteins under saline and METH treatment in the membrane fraction of synaptosomes. Concurrent with the correlation analysis, we observed that there was more interaction between VMAT2 and CDCrel-1 after METH as compared to saline in one rat (Figure 3.16 B) but not all three rats (Figure 3.16 D). The validation of coimmunoprecipitation was done using proper controls such as IgG to specific antibody species and unconjugated beads incubated with the sample (Figure 3.16 C).



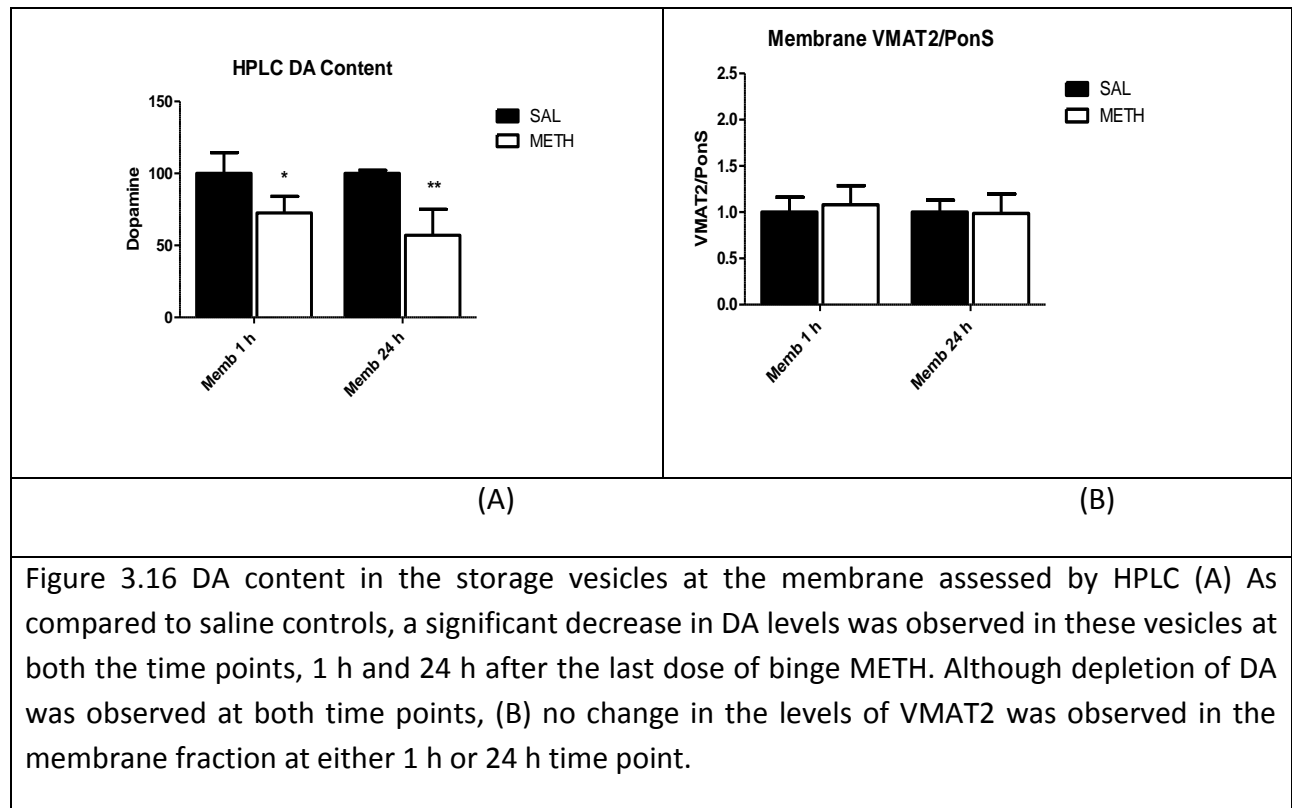
### 3.8.3 Mass Spectroscopy

To validate the interaction between VMAT2 and CDCrel-1 observed by coimmunoprecipitation, we employed the mass spectrometry technique. In this method, magnetic beads conjugated to the VMAT2 antibody were incubated with the membrane fraction of the synaptosomal preparation to pool VMAT2 from the sample. This experiment was carried out in synaptosomal preparations from both saline- and METH-treated rats. To detect the proteins that coimmunoprecipitated with the pooled VMAT2, the eluent from the beads was run on a gel and subjected to mass spectrometry analysis. Along with many other exocytotic proteins, we observed that the protein Septin 5 (CDCrel-1) coimmunoprecipitated with VMAT2 in both samples, saline and METH. No statistically significant differences were observed in the amount of VMAT2 protein that coimmunoprecipitated in METH-treated rats as compared to saline controls. However, we observed increase in the amount of CDCrel-1 coprecipitated with VMAT2 in one rat as compared to the other two. This variable effect seen could be due to individual differences between these outbred rats. The coimmunoprecipitation of the pre-synaptic marker protein bassoon indicates the abundance of pre-synaptic synaptosomes in our sample. Due to the hydrophobic nature and low abundance of VMAT2, it was not detected in the initial run. To validate our method and ensure that VMAT2 was pooled by the beads, we repeated the experiment using double quantity of beads. This subsequent experiment led to detection of VMAT2 by mass spectrometry.

Protein coimmunoprecipitated with VMAT2	Molecular weight (kDa)	Abundance of protein						p value, Student's t-test
		Saline			METH			
Septin 5 (CDCrel-1)	43	3.58E+08	2.63E+08	3.53E+08	5.80E+08	1.62E+08	1.42E+08	0.8478
VMAT2	56	1.39E+08	1.04E+08	1.38E+08	4.21E+08	8.37E+07	1.61E+08	0.4076

### 3.9 High Performance Liquid Chromatography (HPLC):

To determine to what extent METH causes depletion of DA in striatum, DA content was assessed in membrane fraction of synaptosomes using HPLC. There was a significant decrease in the levels of DA at both time points, 1 h and 24 h after binge METH (Figure 3.17 A). Although the levels of DA at both 1 h and 24 h were depleted, no change in the levels of the protein VMAT2, which is an integral part of DA storage vesicles, was observed at the membrane of the nerve terminal (Figure 3.17 B). This implies that there is no physical loss of vesicles at the membrane. The loss in DA could be a result of functional impairment of the DA sequestering vesicles despite a lack of change in their levels.



### 3.10 Influence of parkin on trafficking of VMAT2:

Parkin is known to play a protective role in several neurodegenerative diseases as an E3 ligase (Wilhelmus, Nijland et al. 2012). Also, parkin protect DAergic terminals of the striatum from the toxic effects of METH (Liu, Traini et al. 2013). To determine whether overexpression of parkin reverses the accumulation of its substrate CDCrel-1 and impaired trafficking of VMAT2, we assessed immunoreactivity of these proteins by western blotting in rat brain overexpressing parkin in the nigrostriatal DA system on the left side of the brain.

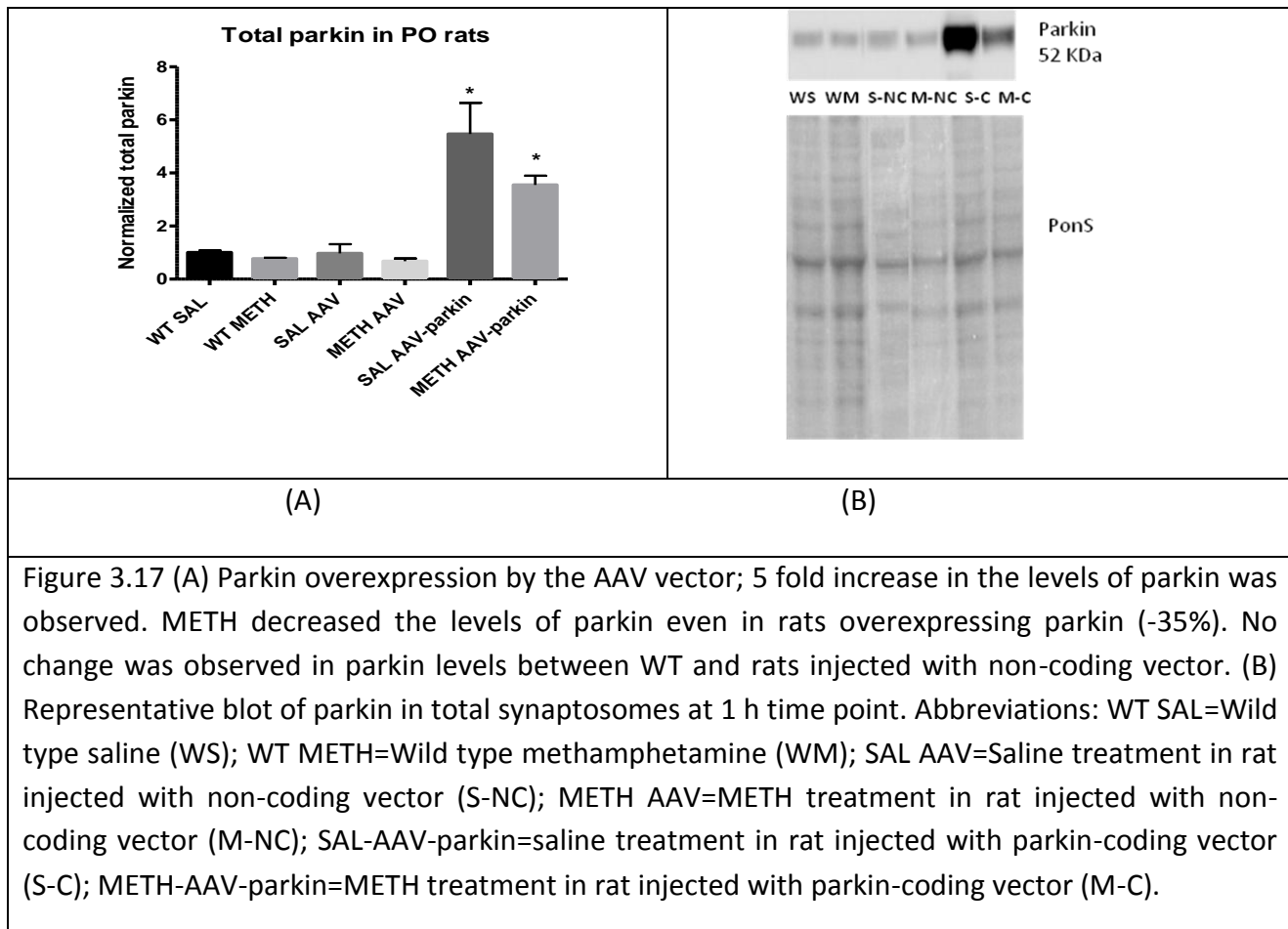
#### 3.10.1 Establishing the overexpression of parkin:

Parkin-coding adeno associated viral vector (AAV) was microinjected in the left SNPc of rat brain. Control group of rats were microinjected with non-coding AAV. The immunoreactivity of parkin was evaluated in the striatum after 3 weeks. To study the effect of binge METH (4x8

mg/kg) on parkin overexpressing rats, rats were treated with either saline or METH (4x8 mg/kg) and killed 1 h after the last dose.

Western blot analysis revealed that there was a significant overexpression of parkin in the striatum 3 weeks after the microinjection (5 fold increase) (one-way ANOVA,  $p < 0.05$ , F value 24.18,  $r^2 = 0.8580$ ). Bonferroni's multiple comparison (post-hoc) test revealed significant increase in the level of coding vector injected rats vs. wild type rats. Two-way ANOVA would have been more appropriate for this data, but small sample size and unequal number of subjects in each group did not allow us to use two-way ANOVA. Since t-test would not be appropriate considering we are comparing more than two groups, we decided to use one-way ANOVA for our analysis.

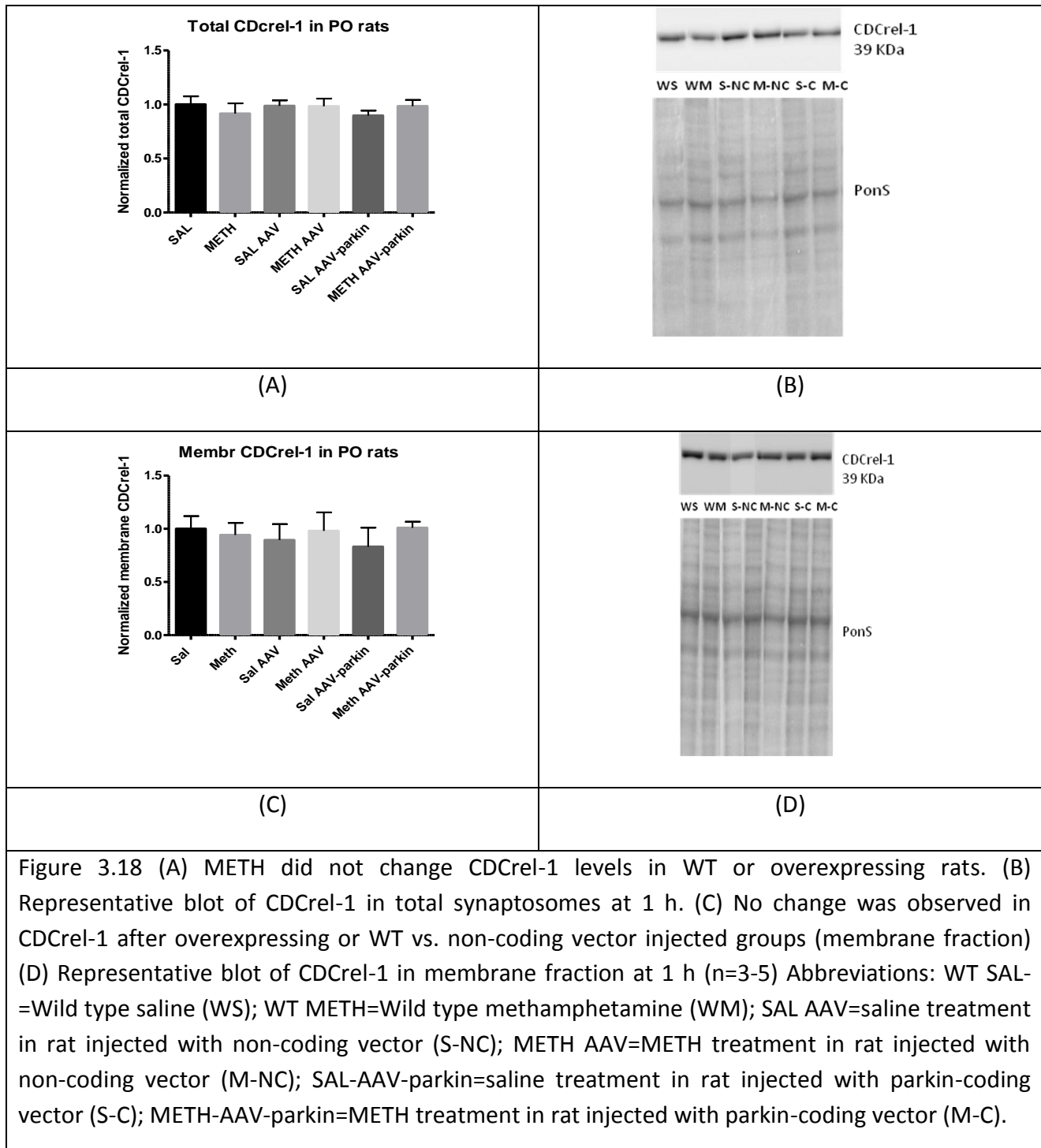
We observed a 23% ( $p = 0.029$ ) and 30% ( $p = 0.3932$ , not statistically significant by t test) decrease in parkin levels in METH-treated rats as compared to saline controls in WT and non-coding vector microinjected rats respectively. This is consistent with our previous results in wild type animals as described in section 3.4.1. No change was observed in the parkin levels between WT rats and rats injected with non-coding vector, suggesting that the vector alone does not affect the levels of parkin. Moreover, the levels of parkin in METH-treated parkin-AAV injected rats were much lower than that of saline-treated parkin-AAV injected rats (35% decrease). This suggests that 4x8 mg/kg METH causes decrease in levels of parkin in the striatum.



### 3.10.2 Effect of parkin overexpression on CDCrel-1 levels:

We found a trend for an increase in the levels of CDCrel-1 in total fraction of synaptosomes in wild type rats. To determine whether overexpression of parkin led to a decrease in CDCrel-1 levels, we assessed immunoreactivity of CDCrel-1 in the total and membrane fraction of synaptosomes. As described previously, data is divided based on 6 treatment groups. Contrary to our expectation, no statistically significant changes were observed in the immunoreactivity of CDCrel-1 in either total (one way ANOVA,  $p=0.8992$ ,  $F$  value= 0.3132,  $r^2=0.0726$ ) or membrane fraction (one way ANOVA,  $p=0.9559$ ,  $F$  value= 0.2064,  $r^2=0.0490$ ) of synaptosomes after overexpression of parkin ( $n=3-5$ ). No change was observed in

total fraction between the WT and rats injected with non-coding vector. Thus, the vector does not affect the levels of CDCrel-1. As compared to SAL-AAV-parkin group, CDCrel-1 levels did not change following METH administration (METH-AAV-parkin rats) in total or membrane fraction.





### 3.10.3 Effect of parkin overexpression on VMAT2 levels and trafficking:

The main aim of the parkin overexpression study was to determine whether parkin plays a role in protecting against the impaired trafficking of VMAT2 caused by METH (Riddle, Topham et al. 2002, Ugarte, Rau et al. 2003). Parkin was overexpressed in rats using the AAV gene transfer vector. The levels of VMAT2 were assessed across all three fractions of synaptosomes (total, membrane and cytosolic). One-way ANOVA with Bonferroni's multiple comparison test was used to compare the VMAT2 levels amongst multiple groups. No statistically significant changes were observed in the total (one-way ANOVA,  $p=0.8454$ ,  $F \text{ value}=0.3965$ ,  $r^2=0.0901$ ); membrane (one way ANOVA,  $p=0.7546$ ,  $F \text{ value}=0.5250$ ,  $r^2=0.1160$ ) or cytosolic fraction (one way ANOVA,  $p=0.5785$ ,  $F \text{ value}=0.7759$ ,  $r^2=0.1625$ ). Across all fractions, no differences in levels of VMAT2 following METH treatment were observed in either METH-AAV-parkin vs. SAL-AAV-parkin or METH-AAV vs. SAL-AAV. We assessed the levels of parkin only in total synaptosomal fractions to validate whether the overexpression of parkin was successful.

The results obtained from this parkin overexpression study suggest that parkin does not play a role in the trafficking of VMAT2. However, we accept the drawback of our study, which is a small number of subjects per group. Thus, we might observe different results after adding more rats to the study.

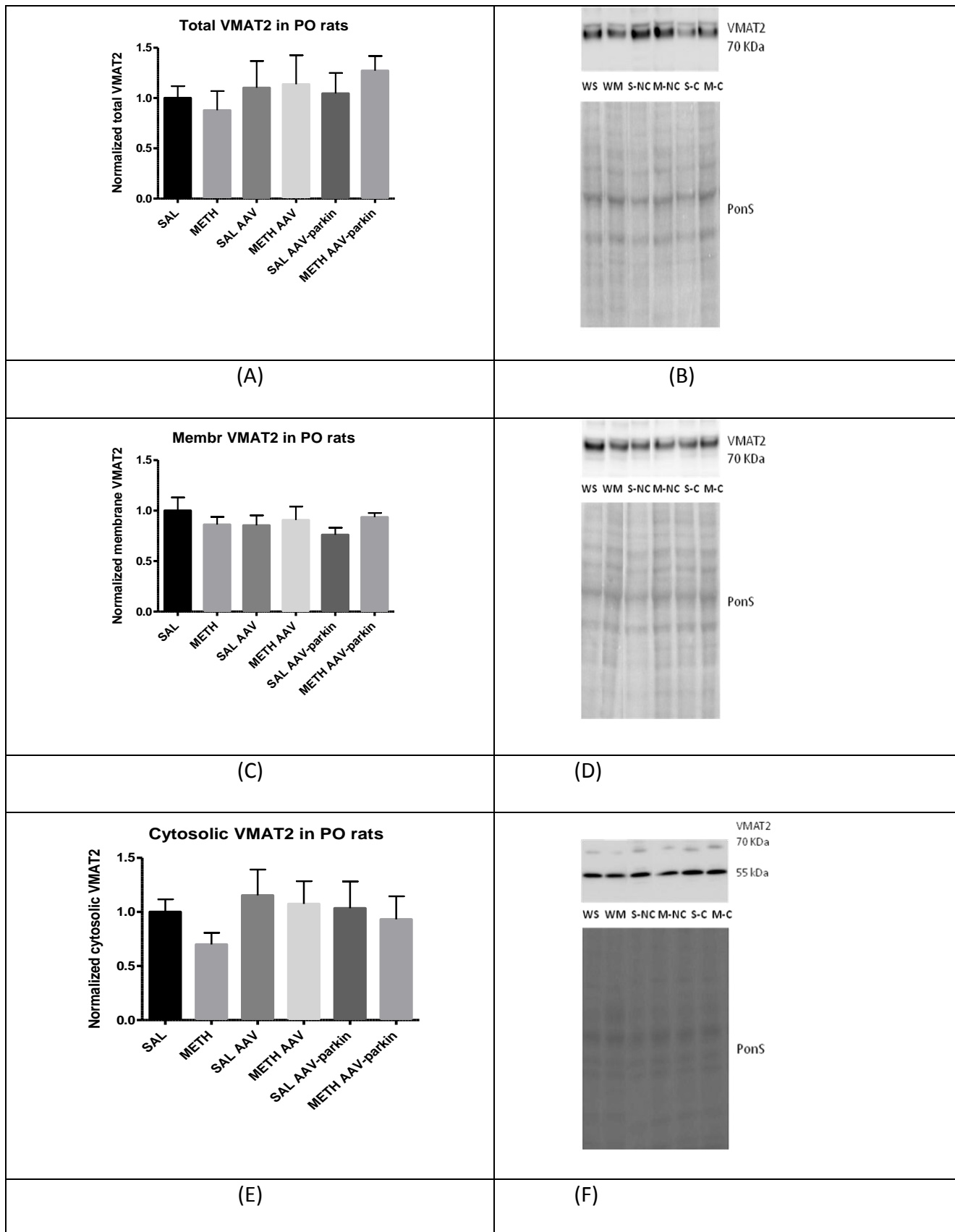


Figure 3.19 The immunoreactivity of VMAT2 (55 kDa and 70 kDa) in total (A), membrane (C) and vesicular (E) synaptosomes at 1 h after binge METH was not different from the wild type rats (n=3-5). Representative blot of VMAT2 in total (B), membrane (D) and vesicular fraction (F) of synaptosomes at 1 h time point. As compared to saline, a 30% decrease (not statistically significant) in WT METH-treated group was observed in the cytosolic fraction. Abbreviations: WT SAL=Wild type saline (WS); WT METH=Wild type methamphetamine (WM); SAL AAV=saline treatment in rat injected with non-coding vector (S-NC); METH AAV=METH treatment in rat injected with non-coding vector (M-NC); SAL-AAV-parkin=saline treatment in rat injected with parkin-coding vector (S-C); METH-AAV-parkin=METH treatment in rat injected with parkin-coding vector (M-C).

## Chapter 4 Discussion

### 4.1 Loading controls:

Microtubule dynamics play a role in many of the active processes in the brain. It has been shown that actin polymerization is responsible for METH associated memory (the memory responsible for relapse to drug usage); and its depolymerization causes disruption of this memory in self administering rats and mice (Young, Aceti et al. 2014). Previous study in mice showed up-regulation of G-actin and F-actin proteins after chronic METH administration (Shibasaki, Mizuno et al. 2011). This study demonstrated that actin dynamics is very responsive to METH administration. Another study on levels of tubulins after single as well as chronic injections of METH, found no change in the expression of alpha tubulin mRNA. A single dose of METH was shown to induce the rearrangement of neuronal network with increased length and density of dendritic spines (Ujike, Takaki et al. 2006). This rearrangement is brought about by cytoskeletal protein dynamics. Moreover, METH disrupts blood brain barrier (BBB) by mobilization of tight junction protein occludin from plasma membrane to endosomes. One study has shown that this disruption of BBB can be prevented by inhibition of actin nucleation (Park, Kim et al. 2013). The above literature suggests that actin and cytoskeletal rearrangement play an important role in the effects produced by METH. Actin and alpha tubulin are widely used loading controls. Based on the above mentioned literature on effect of METH on cytoskeletal proteins (Shibasaki, Mizuno et al. 2011, Young, Aceti et al. 2014) as well unpublished results from Dr. Moszczynska's unpublished results on levels of alpha-tubulin after METH, we decided to assess its effect on actin and alpha-tubulin in our model. Consistent with previous findings, we observed a change in levels of actin and tubulin after METH treatment. At

1 h time point, we observed a significant decrease in actin immunoreactivity across all synaptosomal fractions. The levels of alpha tubulin in total synaptosomes had a trend for an increase at 1 h.

#### 4.2 Binge METH neurotoxicity:

Binge regimen of METH means repeated administration of the drug at regular intervals of time. Several models of binge METH regimen have been studied and all produce different intensity of toxicity. A dose of 4x5 mg/kg in rats produced ~27% loss of striatal DA 7 days after the treatment (Fornai, Torracca et al. 1996). This decrease was even more severe (80% DA loss, 70% loss of DOPAC, 60% loss of HVA) 3 days after the treatment of mice with 4x5 mg/kg dose (Boireau, Bordier et al. 1995). Hyperthermia produced by this dose was about 38-39<sup>0</sup>C (Bowyer, Tank et al. 1992). In a different study, administration of 4x15 mg/kg regimen of METH to mice produced about 75% loss in striatal DA 3 days after the treatment (Green, Souza et al. 1992). In rats, 4x7.5 mg/kg dose produced about 26% depletion of striatal DA (Nash and Yamamoto 1992) whereas 4x10 mg/kg dose produced a 56% DA depletion in the striatum 7 days after METH (Wallace, Gudelsky et al. 1999). This dose was also found to decrease immunoreactivity of parkin at 1 h and 24 h after the last dose (Moszczynska and Yamamoto 2011). The 4x10 mg/kg dose produced a hyperthermia of 40-41<sup>0</sup>C as observed by previous studies (Wallace, Gudelsky et al. 1999, Moszczynska and Yamamoto 2011). Bowyer and colleagues found a correlation between hyperthermia and METH induced toxicity (Bowyer, Tank et al. 1992). Our study used 4x8 mg/kg dose and we observed significant hyperthermia in our rats. Since our dose was lesser than the 4x10 mg/kg regimen, our toxicity profile was not as severe, on average, as in some other studies, e.g. Moszczynska and Yamamoto's study, which

observed about 40% deficit in parkin at 1 h after binge METH. Moreover, we observed a deficit in parkin in only a subpopulation of rats and not extending beyond the 1 h time point. Thus, based on the previous literature and hyperthermia as seen in our study, the toxicity in our model is on a threshold of serious damage caused by METH.

#### 4.3 Impaired trafficking of VMAT2 after binge METH:

The basic function of VMAT2 is to sequester intracellular DA into synaptic vesicles. To the best of our knowledge, no study so far has published about VMAT2 being internalized to DA storage vesicles after administration of amphetamines or oxidative stress. So, as far as we know, VMAT2 remains on the surface of the synaptic vesicle and the levels and localization of VMAT2 represent the levels and localization of the vesicles in the nerve terminal. Previous studies have shown that METH alters the levels of VMAT2 and localizes them to a region not retained in the synaptosomal preparation within 1 h (Riddle, Topham et al. 2002, Ugarte, Rau et al. 2003). We did not observe this effect in our study. One reason for this could be our lower METH dose (4x8 mg/kg) as compared to the dose used in these studies (4x10 mg/kg). In terms of the effect of binge METH on VMAT2 expression at the membrane, one study has shown an increase whereas the other shown no change in the levels of VMAT2 (~55 kDa) with a decrease in the cytosolic fraction at 1 h time point (Riddle, Topham et al. 2002, Ugarte, Rau et al. 2003). These findings suggested impaired trafficking of VMAT2 vesicles to the cytosol. We did not observe any change in the levels of VMAT2 at the membrane as compared to saline controls. Our results are similar to those of Riddle and colleagues who observed a lack of change in VMAT2 at the membrane fraction. Yamamoto group has shown a decrease in the glycosylated form of VMAT2 (~68 kDa) in the vesicular fraction at 1 h and decreases across all three

synaptosomal fractions at 24 h time point following METH (Eyerman and Yamamoto 2005). Our antibody detected both isoforms of VMAT2 and we did not observe any statistically significant changes in either of the forms in any of the synaptosomal fractions. As mentioned before, one reason for this discrepancy could be the higher dose used by all the previous studies (4x10 mg/kg). Dose dependency of METH effects has been established with different doses of METH producing different severity of toxicity (Nash and Yamamoto 1992, Boireau, Bordier et al. 1995, Wallace, Gudelsky et al. 1999). Another factor that could be a possible contributor to the different effects observed by us is hyperthermia. Hyperthermia is an important component of METH toxicity. We observed lower hyperthermia in our rats (39-40<sup>0</sup>C) as compared to the previous studies (40-41<sup>0</sup>C) (Bowyer, Tank et al. 1992, Eyerman and Yamamoto 2007). Lower METH dose would have produced less hyperthermia and oxidative damage to proteins, including VMAT2, resulting in a less impaired trafficking of VMAT2 and its recovery within 24 h after binge METH.

D2 receptor agonists induce redistribution of VMAT2 vesicles from the plasma membrane to the cytosol. A single administration of the non selective D2 receptor agonist quinpirole led to a decrease in the immunoreactivity of VMAT2 in the membrane with a corresponding increase in the cytosolic fraction 3 h after the dose (Truong, Newman et al. 2004). Since METH is an indirect D2 agonist by releasing DA in the synapse, we expected to see redistribution of VMAT2 from membrane to cytosol after METH. However, we did not observe mobilization of VMAT2 from membrane to cytoplasm. We hypothesized that this lack of redistribution of VMAT2 could be due to a possible entrapment of VMAT2 at the membrane as a manifestation of METH toxicity and that it could lead to a dysfunction of VMAT2. This notion

is supported by a study that found a decrease in vesicular DA uptake at 1 h and 24 h after binge METH (Brown, Hanson et al. 2000). Thus, VMAT2 could be partially entrapped at the membrane by CDCrel-1, which is further discussed in the section 4.6.

#### 4.4 Parkin deficit in striatum following binge METH:

The parkin gene and the E3 ligase protein parkin have been widely studied in Parkinson's disease (Matsumine, Saito et al. 1997, Hattori, Kitada et al. 1998, Leroy, Anastasopoulos et al. 1998). Dysfunction and damage to parkin due to METH and oxidative stress has also been studied in vitro and in vivo (LaVoie, Cortese et al. 2007, Moszczynska and Yamamoto 2011). Our previous study on rat synaptosomes after binge regimen of METH (4x10 mg/kg) has shown decrease in parkin levels in total synaptosomes as early as 1 h after the last dose of METH. Parkin levels were still decreased at 24 h but returned to the control levels at 48 h after METH. Our current study, using 4x8 mg/kg METH, found a trend for a decrease (-19%) in parkin in total synaptosomes at 1 h and no change at 24 h time point. In addition, two subpopulations of rats were observed in our study at 1 h time point: one with a decrease in parkin and the other with no change. These two sub groups of rats with different effects led to the lower overall deficit observed in parkin. We tried to deduce the possible cause for the two subpopulations of rats in our results. For this, we correlated the immunoreactivity of parkin to different indices such as core body temperature and immunoreactivity of CDCrel-1. However, we could not find any specific correlations between the subpopulations of rats and other indices. We could not determine what led to different levels of decrease in parkin levels in response to METH insult.



One of the most obvious parameters that was different between our study and the previous one was the dose of METH. A number of models for different regimen and dosing of METH have been studied. Binge regimens such as 4x10 mg/kg every 2 h, 4x7.5 mg/kg every 2 h, 4x5 mg/kg every 2 h have been shown to produce different toxic effects (Imam, el-Yazal et al. 2001, Zhu, Xu et al. 2006, Phillips, Kamens et al. 2008, Good and Radcliffe 2011). A single dose of METH produced dose-dependent reductions in striatal DA levels: to 71% (at 20 mg/kg), 49% (at 30 mg/kg) and 29% (at 40 mg/kg) of control levels, 3 days following the treatment with METH (Fukumuraa, Cappona et al. 1998). Previous studies on binge METH neurotoxicity showed that a dose of 4x10 mg/kg produced 56% toxicity as observed by DA depletion at 7 days in the striatum, 4x7.5 mg/kg produced 26%, whereas 4x5 mg/kg produced 27% toxicity (Fornai, Torracca et al. 1996, Wallace, Gudelsky et al. 1999). Thus, different METH doses produce different levels of toxicity, which is directly proportional to the dose of METH. More toxicity is observed at higher dose. This loss of DA content is associated with the increase in DA quinones production by METH. Oxidative stress caused by METH is one of the major mechanisms of METH toxicity and the DA ROS produced are known to damage parkin (LaVoie, Ostaszewski et al. 2005, LaVoie, Cortese et al. 2007, Moszczynska and Yamamoto 2011). Most of the previous studies on binge METH toxicity have used 10 mg/kg of METH, free-base. Under our conditions, this dose was found to be very toxic and led to high mortality rates amongst the rats. Due to this reason, we used 8 mg/kg of free-base METH. This relatively lower dose of 8 mg/kg might be responsible for the lesser decrease in parkin levels than observed by previous study, which used 4x10 mg/kg regimen (Moszczynska and Yamamoto 2011). Also, at 4x10 mg/kg dose, the effect of METH on parkin reverses by 48 h; this time might be earlier for the lower METH dose of 4x8

mg/kg. This could be the reason why we see no effect at the 24 h time point as the effect of METH would be washed off by that time.

We analyzed multiple possible causes for the discrepancy of our results with the previously observed deficit in parkin (Moszczynska and Yamamoto 2011) at 1 h and 24 h:

(1) Hyperthermia: Increase in core body temperature as a result of METH administration is a common manifestation of METH toxicity. Hyperthermia is known to augment the toxic effects of METH such as DA depletion (Sandoval, Hanson et al. 2000, Tata, Raudensky et al. 2007). For example, one study performed an experiment to observe the toxicity of high dose METH (4x10 mg/kg) in a low temperature environment (23<sup>0</sup>C) in mice. They observed only 20% decrease in DA as compared to 80% depletion at room temperature (Ali, Newport et al. 1994). As described previously, the range for hyperthermia found in most studies is 39-41<sup>0</sup>C. The core body temperatures of the rats in the previous study were more towards the upper limit of the range (40-41.5<sup>0</sup>C) (Moszczynska and Yamamoto 2011). These higher body temperatures as compared to our model could be another reason for the discrepancy between our results. Moreover, we observed a positive correlation between core body temperature and the immunoreactivity of parkin in our study, suggesting that parkin levels increased with rising body temperature. It is possible that parkin is involved in thermoregulation and thereby acting as a protective agent against psychostimulant drugs (Takamatsu, Shiotsuki et al. 2011). Also, we found a significant increase in total and membrane synaptosomal fractions from cerebellum in our study. This finding also suggests that

there might be an adaptational increase in parkin levels as a result of the mild oxidative stress from our METH regimen of 4x8 mg/kg. Although no study directly shows effect of hyperthermia on parkin, indirect evidence and our correlation between parkin and body temperatures supports the argument that hyperthermia could play a role in regulation of parkin levels and explain our finding.

(2) Loading control: Loading controls are used to normalize protein bands from electrophoresis considering their high abundance and lack of change in their levels regardless of drug treatment. Moszczynska and colleagues observed about 40% decrease in the levels of parkin in total synaptosomes at 1 h. Their study used alpha-tubulin as the loading control (Moszczynska and Yamamoto 2011). In order to validate different loading controls, we assessed levels of two loading controls: actin and alpha-tubulin after binge METH. As observed from our results, METH causes a trend for an increase in the levels of alpha-tubulin in total synaptosomes 1 h after the last dose of METH. This suggests that METH causes change in alpha-tubulin levels and since Moszczynska and colleagues used alpha-tubulin as a loading control, they could have detected an exaggerated deficit in parkin than the actual effect. They compared the levels of alpha-tubulin in total striatal synaptosomal fraction between saline- and binge METH-treated rats and found a small and statistically non-significant increase in the METH group (personal communication with Dr. Moszczynska). In our study, we used Ponceau S as a loading control where no difference in staining was observed under saline and METH treatment conditions.

(3) Compensatory increase in glutathione: Endogenous antioxidants like glutathione are known to protect the cell from oxidative stress. By conjugation with prooxidants, glutathione prevents oxidative stress and even acts as a peroxidase. The levels of reduced glutathione (GSH) increase in the event of any insult to the cell for defense. However, at higher damage by METH, the levels of GSH and its related enzymes such as glutathione peroxidase and glutathione reductase decrease (Moszczynska, Turenne et al. 1998, Açikgöz, Gönenç et al. 2001, Pompella, Visvikisa et al. 2003, Mirecki, Fitzmaurice et al. 2004). Cell culture studies have also shown that METH initially increases the reduced form of glutathione followed by decrease in its levels at higher doses of METH (Silva, Silva et al. 2013). Since we used a lower dose than some in vivo studies (Moszczynska, Turenne et al. 1998, Açikgöz, Gönenç et al. 2001), it is possible that the toxicity produced under our conditions is not severe enough and leads to a compensatory increase in glutathione levels. These increased glutathione levels would dampen the oxidative stress and lead to less damage to parkin.

(4) Compensatory increase in parkin after oxidative stress: Dysfunction of parkin has been observed in both Parkinson's disease as well as METH toxicity (Leroy, Anastasopoulos et al. 1998, Moszczynska and Yamamoto 2011). Moreover, parkin has been found protect from oxidative stress. One study showed that dysfunction of parkin is associated with increased oxidative stress and lipid peroxidation (Su, Duan et al. 2013). It is possible that at the toxicity produced by our dose, there is a compensatory increase in parkin levels for neuroprotection. Mild oxidative stress is known to increase the activity of proteasome to scavenge damaged proteins (Elkon, Melamed et al. 2004).

Thus, it is possible that our dose released DA to a threshold which does not produce toxicity but damages proteins to activate the UPS and thereby parkin. Another possibility is that there is increased synthesis of parkin in the SNpc, which is axonally transported to the terminals in the striatum upon mild oxidative stress. However, there is no published literature supporting this. Thus, it is not unlikely that the METH-induced toxicity in our model acts as a threshold for the adaptational increase in brain's defense system leading to increased parkin levels in the striatum.

(5) Parkin monomer modified by HNE: Parkin is known to be more susceptible to DA mediated oxidative damage than other E3 ligases (LaVoie, Cortese et al. 2007). A number of studies have shown the covalent modification of parkin and its aggregation by DA and its oxidation products (LaVoie and Hastings 1999, Winklhofer, Henn et al. 2003). For example, DA quinone formation leads to oxidative damage to parkin in rats (LaVoie, Ostaszewski et al. 2005, LaVoie, Cortese et al. 2007). Our previous study shows that parkin is covalently modified by oxidative damage by binding of lipid peroxidation product 4-HNE (4-hydroxynonenal). However, no aggregates of parkin were reported in this study (Moszczynska and Yamamoto 2011). Another study has shown that oxidative and nitrosative stress alters the solubility of parkin and destroys its ability for neuroprotection in cell culture as well as in vivo in mice (Wang, Ko et al. 2005). This study by Wang and colleagues showed increased amounts of insoluble parkin fractions 24-48 h after insult by an oxidative stressor. Damaged parkin monomer can be degraded or sequestered into aggregates or both. Aggregation of parkin renders it in a detergent-insoluble form. These oligomers of parkin are not functional

(Winklhofer, Henn et al. 2003). As we did not observe a significant decrease in the total levels of parkin, we expected to see an increase in the oligomeric form with concomitant decrease in the monomeric form of parkin. However, we did not observe any signs of increased aggregation of parkin following METH in our model. Most of the studies on the aggregation of parkin by oxidative stress were conducted in vitro (LaVoie, Cortese et al. 2007). The results from these studies could be different from the results obtained from in vivo studies. We speculate that in our rats, although the parkin levels are not altered greatly, actual damage to the protein and loss of its protective function could have occurred by one of the above-mentioned causes. Previous study has published about covalent modification of parkin by oxidative stress (LaVoie, Ostaszewski et al. 2005). Similarly, it could be possible that oxidative stress causes conformational change in the structure of parkin. To the best of our knowledge, no study has reported such conformational change in parkin. However, if that is the case, the activity of parkin might be affected but we will not detect a change in parkin levels due to the denaturing conditions of our electrophoresis.

(6) DAergic terminals form a small population in the striatum: The nigrostriatal system comprises of innervations arising from the SNPc to the striatum. The striatum is the main input centre for basal ganglia. It receives innervations from multiple nerve cells (Cooper, Bloom et al. 2003). Our study focused on the DAergic terminals in the striatum. However, the DAergic terminals form a very small population of all the nerve terminals in the striatum. The glutamatergic terminals are present in much higher levels than the DAergic ones. So, it is a possibility that any effect on deficit

in parkin which is only in the DAergic terminals will be diluted by the vast majority of parkin present in the glutamatergic and GABAergic terminals (Smith, Bevan et al. 1998, Henny, Brown et al. 2012). Moreover, striatum also receives substantial glutamatergic input from the subthalamic nucleus (Ribak, Vaughn et al. 1979, Yelnik 2002). Thus, it is very difficult to estimate the change in parkin levels exclusively in the DAergic terminals. To determine any change in parkin levels in DAergic terminals after binge METH, we double stained synaptosomes with antibodies against parkin and DAergic marker TH. No difference in immunofluorescence was observed between synaptosomes from saline and METH treated rats. This might not be completely accurate since TH is also present in noradrenergic terminals. However, noradrenergic terminals are more concentrated in the spinal cord and olfactory bulb than striatum (Sotiriou, Vassilatis et al. 2010). So, it is possible the results we observe are more or less representative of DAergic terminals and that METH has no effect on the levels of parkin in DAergic nerve terminals. Also, as a negative control for DAergic innervation, we assessed parkin levels in total and membrane fractions of synaptosomes from cerebellum. To our surprise, we found that METH caused an increase in the levels of parkin in both these fractions from cerebellum. This suggests that in nerve terminals other than the DAergic population, parkin increases following insult by METH. This increase could be a compensatory mechanism to protect that brain region from the toxic effects of METH.

(7) No degradation by 20S or 26S proteasome: Binge METH (4x10 mg/kg) has been found to reduce the activity of the UPS in a dose and time dependent manner (Moszczynska and Yamamoto 2011). Thus, if the activity of UPS is impaired, the

degradation of parkin decreases. The same study has also shown that binge METH (4x10mg/kg) decreases the activity of the 26S proteasome which is responsible for ubiquitin-mediated degradation of proteins. On the other hand, the activity of 20S increases following oxidative stress (Grune, Reinheckel et al. 1997, Kurepa, Toh-E et al. 2008). Since parkin turnover is mediated by ubiquitination and the activity of 26S decreases after METH, it can be envisioned that damaged parkin is not effectively scavenged and so we do not see a decrease in the total levels of parkin. However, this possibility is unlikely, as we do not observe parkin aggregates. It is possible that 20S proteasome, which degrades oxidized proteins (Hershko A 1998, Davies 2001), takes over 26S function. On the other hand, the previous study reported deficit in parkin along with an increase in the 20S after binge METH (Moszczynska and Yamamoto 2011).

(8) Another less likely reason for the lack of deficit in parkin observed in our study, which is in contrast to the study of Moszczynska and Yamamoto, could be genetic differences between outbred rats used in our study vs. rats used in their study (Phillips, Kamens et al. 2008).

At 24 h time point, no change in parkin after binge METH was observed. First reason for not seeing any effect at 24 h could be the dose. Since we do not see a significant deficit in parkin at 1 h at our dose, it is possible that, by 24 h, parkin returned to the control levels by de novo synthesis. Another explanation could be that the deficit in parkin occurred between 1 h and 24 h and was confined to DAergic terminals. Also, due to higher number of rats in our study (n=11) as compared to Moszczynska and Yamamoto study (n=5-7), we saw emerging subpopulations of rats that differently respond to binge METH. Thus, a deficit in parkin due to



damage by oxidative stress could be masked by the compensatory synthesis. METH is also known to affect microtubules but not many studies have focused on the effect of METH on axonal transport (Maeno, Iwasaa et al. 2000). Since parkin is neuroprotective, a theoretically explanation could be that there is a compensatory increase in its local synthesis or its anterograde transport from the cell bodies in the SNpc. However, no literature supporting this has been published. It is possible is that even though the levels are not affected by METH, its activity as an E3 ligase might be disrupted, leading to dysfunction of the UPS and subsequent neurotoxicity.

In summary, based on literature and our findings, the discrepancy in our results and those from the previous study can be attributed mostly to the lower dose of METH employed in our study and the relatively lower hyperthermia observed in our rats. Consequently, the oxidative stress and hyperthermia were not very severe in our model and so could have failed to cause damage to parkin and the protein synthesis machinery. This might have led to the lower damage to parkin, which could have been balanced by the increased protein synthesis as a compensatory mechanism. Since adaptation to the insult by METH can differ between animals, we see two subpopulations of rats where some rats show higher damage due to lower compensatory increase of parkin in response to METH.

#### 4.5 Parkin overexpression in the striatum:

As described previously, METH causes impaired trafficking of VMAT2 and its mobilization to a non-synaptosomal compartment. This decrease in the levels of cytosolic VMAT2 has been found at both 1 h and 24 h after binge regimen of METH (Riddle, Topham et

al. 2002, Ugarte, Rau et al. 2003, Eyerman and Yamamoto 2005). Also, METH has been found to decrease the vesicular uptake capacity and substrate binding of VMAT2 at 1 h time point (Brown, Hanson et al. 2000). VMAT2 plays a very important role of sequestering cytosolic DA into synaptic vesicles. In absence of such sequestration, free DA undergoes auto-oxidation and causes neurodegeneration via oxidative stress (Miyazaki and Asanuma 2008, Guillot and Miller 2009).

Parkin is an E3 ligase in the UPS (Shimura, Hattori et al. 2000). Parkin plays a protective role by regulating the turnover of its substrates as accumulation of many of parkin substrates such as CDCrel-1, alpha-synuclein and Pael-R is known to cause selective degeneration of DAergic neurons (Dong, Ferger et al. 2003, Feany and Pallanck 2003, Perren, Toelen et al. 2014). Moreover, overexpression of parkin has been shown to protect against METH induced neurotoxicity as assessed by reduction in depletion of TH after METH; about 50% of DAergic axons destined for damage were protected in the striatum by parkin overexpression. The same study also determined that this protection by parkin is not mediated by thermoregulation or increased 20S activity (Liu, Traini et al. 2013). Thus, although the protective nature of parkin in METH neurotoxicity has been established, the mechanism of protection remains to be determined.

#### 4.5.1 Validation of parkin overexpression and METH effect on parkin levels:

We used the AAV2/6 gene transfer vector to overexpress parkin in rat nigrostriatal DA system. The AAV2/6 vector has been shown to effectively overexpress proteins in previous studies (Dong, Ferger et al. 2003, Liu, Traini et al. 2013). Previous study by Liu and colleagues

observed an eight fold increase in expression of parkin in rat striatum using the AAV2/6 parkin-coding vector. To assess parkin overexpression in our study, we measured the levels of parkin in total synaptosomes by western blotting. We found a five-fold increase in the levels of parkin. This could be due to the new formulation of the AAV vector. In this experiment, binge METH decreased parkin levels in all groups: by 23%, 30% and 35% in WT, non-coding vector-microinjected and and parkin overexpressing rats as compared to saline counterparts. Only the 35% deficit was statistically significant; nevertheless this finding is consistent with the previous study that showed a decrease in the levels of parkin (-48%) in striatal synaptosomes at 1 h following binge METH (4x10 mg/kg) (Moszczynska and Yamamoto 2011) and confirms dose-dependent effect of binge METH on parkin levels. No change was observed in parkin levels between WT rats and rats injected with non-coding vector, which suggests that the vector alone was non-toxic and did not affect the levels of parkin. Thus any increase observed in the expression of parkin was due to the parkin-encoding vector.

#### 4.5.2 Parkin overexpression on CDCrel-1 levels:

Once the overexpression of parkin was established, we next assessed the levels of CDCrel-1 in total and membrane fractions of striatal synaptosomes. CDCrel-1 was shown to be a substrate of parkin in vitro (Zhang, J Gao et al. 2000). Consistent with this study, we observed a coimmunoprecipitation between parkin and CDCrel-1 in our in vivo study, along with a trend for correlation between the levels of the two proteins, in WT saline-treated rats. This led us to believe that CDCrel-1 could be a substrate of parkin in our in vivo model. However, we observed no change in the levels of CDCrel-1 in rats overexpressing parkin, which suggested that CDCrel-1 might not be a substrate of parkin, or is a substrate for multiple E2 ligases, in vivo.

Our results from WT rats revealed a decrease in coimmunoprecipitation of parkin and CDCrel-1 after METH, supporting the idea that parkin is, in part, involved in regulation of CDCrel-1 levels. In vivo conditions are different and E3 ligase-substrate dynamics are much more complex in animals as compared to cell culture. Moreover, coimmunoprecipitation study is not an accurate measure to determine whether CDCrel-1 is a substrate of parkin. Coimmunoprecipitation indicates that the two proteins interact directly or in a protein complex. To establish substrate specificity, ubiquitination assay in presence and absence of parkin would be a better measure. To date, majority of studies on parkin and CDCrel-1 protein-protein interaction has been conducted in vitro (Beites, Xie et al. 1999, Zhang, J Gao et al. 2000). Thus, one explanation for lack of change in CDCrel-1 levels in parkin overexpressing rats could be that it is not a substrate of parkin in vivo. On the other hand, parkin was overexpressed only in the nigrostriatal DA system, not in the whole striatum. Therefore, it is possible that CDCrel-1 levels changed in DAergic terminals upon overexpression of parkin but the change was not detected due to the fact that DAergic terminals constitute only a small percentage of total synaptosomal population in the striatum. Against this hypothesis is a finding from a study on parkin knock out mice that showed unaffected levels of CDCrel-1 (Goldberg, Fleming et al. 2003). This lack of expected increase in CDCrel-1 due to absence of parkin indicates that even though parkin might regulate the levels of CDCrel-1, parkin is not exclusive in that function in vivo. These results support our findings from parkin overexpression study that change in the expression of parkin does not cause changes in the levels of CDCrel-1.

#### 4.5.3 Parkin overexpression on trafficking of VMAT2:

Parkin has been shown to be neuroprotective against METH toxicity. Various studies have shown neuroprotection by overexpression of parkin suggested different mechanisms responsible for its protective action (Bian, Liu et al. 2012, Liu, Traini et al. 2013, Rana, Rera et al. 2013). The objective of our study was to evaluate whether parkin plays a protective role by redistribution of VMAT2 from membrane to cytoplasm after METH. The impaired trafficking of VMAT2 after METH has been well established. A number of studies have shown a decrease in VMAT2 in the cytoplasmic fraction of striatal synaptosomes 1 h following METH (Riddle, Topham et al. 2002, Ugarte, Rau et al. 2003, Eyerman and Yamamoto 2007). We obtained inconsistent results. In the first experiment, a decrease in synaptosomal cytosolic VMAT2 at 1 h time point was not observed. In the second experiments, we observed such decrease in WT but not in microinjected rats. No previous study has looked at the trafficking of VMAT2 after overexpression of parkin. We expected a redistribution of VMAT2 from the plasma membrane to the cytosol as a result of parkin overexpression. This hypothesis was based on the assumption that increased parkin would lead to more degradation of CDCrel-1, the protein we thought to be responsible for entrapment of VMAT2 at the membrane. The results that we observed were contrary to our hypothesis. We did not observe any redistribution of VMAT2 or a change in its levels in total, membrane or cytosolic fractions upon overexpression of parkin. The possible reason for this could be that parkin expression does not affect the levels of CDCrel-1 and so the VMAT2 remains trapped at the membrane. Previous study on parkin-CDCrel-1 interaction by Zhang and colleagues was in vitro which might not hold true for in vivo studies. Also, one study has shown no change in levels of CDCrel-1 in parkin-null mice indicating low regulation of parkin of CDCrel-1 levels (Zhang, J Gao et al. 2000, Goldberg, Fleming et al. 2003).

According to our hypothesis, the protective role of parkin is mediated by down-regulation of CDCrel-1. Since no change was observed in the levels of CDCrel-1 in parkin overexpressing rats, it might explain, at least in part, why we did not see any improvement in the trafficking of VMAT2. Alternatively, VMAT2 trafficking is impaired/changed due to a reason other than “physical” entrapment at the membrane by CDCrel-1. Furthermore, VMAT2 trafficking might not depend on parkin at all. In support of this conclusion, control striata overexpressing parkin did not statistically differ with respect to VMAT2 levels in synaptosomal fraction from control striata.

#### 4.6 CDCrel-1 in METH toxicity:

The septin CDCrel-1 has been widely implicated in the neurodegeneration of DAergic neurons. Under basal conditions the levels of CDCrel-1 are maintained low in the striatum as compared to other brain regions. Overexpression of CDCrel-1 in rat brain leads to selective DAergic cell degeneration in the SNpc (Dong, Ferger et al. 2003). Due to its selectivity for damage to the DAergic system, we hypothesized its involvement in METH toxicity. We expected that the tendency for a decrease in parkin would lead to a lack of effective turnover and accumulation of CDCrel-1 in the striatum. We observed a trend for an increase in the levels of CDCrel-1 in total synaptosomes at 1 h after the last dose of METH and only in a subpopulation of rats. The moderate increase in CDCrel-1 in total synaptosomes at 1 h was not observed at 24 h and the levels of CDCrel-1 returned to normal. Moreover, in our second experiment (parkin overexpression), CDCrel-1 levels did not increase after binge METH either in total or membrane fraction at 1 h, despite the more pronounced parkin deficits. As discussed for parkin, the 8 mg/kg dose of METH used in our study could lose its effect by 24 h and so not much toxicity

was observed at 24 h time point. Our data suggest that binge METH administration does not lead to an increase in CDCrel-1 levels in DAergic terminals unless an increase was selective to these terminals or CDCrel-1 was not detected by our antibody due to its aggregation or post-translational modification (Garcia, Rodrigues et al. 2008, Hernández-Rodríguez and Momany 2012). Assessment of CDCrel-1 levels from total and membrane synaptosomal fractions from cerebellum showed no change in its levels in any of the rats. This suggests that there are regional differences in the effect of METH on CDCrel-1 levels and confirms a dependence of CDCrel-1 toxicity on DA (Dong, Ferger et al. 2003).

Our data indicate that CDCrel-1 is regulated by an E3 ligase other than parkin in rat brain. Parkin is not the only E3 ligase present in neurons. About 616 E3 ligases with RING finger domain are known to be present in humans (Deshaies and Joazeiro 2009). So, if parkin has been damaged by METH then it is possible that some other E3 ligase takes over the degradation of CDCrel-1. Several other RING finger domain E3 ligases such as Siah-1 and Siah-2 are known to share substrates with parkin (Zhang, J Gao et al. 2000, Qi, Kim et al. 2013). Interestingly, we observed a correlation between parkin levels and CDCrel-1 levels in saline but not METH-treated rats. We observed an interaction of parkin with CDCrel-1 in control rats and a decrease in the interaction after METH treatment also by a second method, a coimmunoprecipitation. These results suggest that parkin, at least in part, regulates CDCrel-1 levels in the absence of METH, CDCrel-1 levels might be regulated, in part, by METH-induced hyperthermia. Coexpression of heat shock protein 70 (HSP70) provided about 20% protection against neurodegeneration caused by CDCrel-1 overexpression in rats as observed by loss of TH-positive terminals in striatal sections (Jung, Fitzsimons et al. 2008). This fact is relevant as the

HSP proteins are upregulated with stress such as increase in body temperature. Since METH induced hyperthermia, which acts as a stress, it might cause increase in the HSPs. These increased HSPs could be responsible for regulating the levels of CDCrel-1. Thus, the lack of increase in CDCrel-1 after treatment with METH could be due to the protective effects of HSPs elevated in response to METH induced hyperthermia. Against this possibility, we did not detect correlation between CDCrel-1 immunoreactivity and core body temperatures.

#### 4.7 VMAT2 CDCrel-1 interaction:

Impaired trafficking of VMAT2 following high-dose METH has been established. METH is known to cause redistribution of VMAT2 from cytosol to a fraction not retained in the synaptosomes (Riddle, Topham et al. 2002, Ugarte, Rau et al. 2003). Also, D2 agonist has been shown to mobilize VMAT2 from membrane to cytosol, shortly after its administration (Truong, Newman et al. 2004). Since METH causes release of DA from the terminals, it acts as an indirect D2 agonist (Kahlig, Binda et al. 2005). CDCrel-1 is a septin with an intricate role in exocytosis. Moreover, electron microscopy study has revealed that vesicles are linked to each other as well as the plasma membrane through filamentous structures. Since septins are filamentous in nature, they could be responsible for hindering the movement and recycling of vesicles to and from the membrane (Hirokawa, Sobue et al. 1989). We hypothesized an entrapment of VMAT2-associated vesicles by CDCrel-1 at the membrane. We observed coimmunoprecipitation between VMAT2 and CDCrel-1 in both METH- as well as saline-treated rats. One out of 3 rats showed increased interaction between the two proteins after METH. On assessment by mass spectrometry, we observed similar results with one out of 3 rats showing much higher interaction of CDCrel-1 with VMAT2 after METH. Our results suggest that METH might not play



a role in the interaction between VMAT2 and CDCrel-1. But since we see two subpopulations of rats, one of which shows increased CDCrel-1 levels, CDCrel-1 could be at least partly responsible in holding VMAT2 at the membrane. Moreover, regression analysis of CDCrel-1 and VMAT2 levels in METH-treated rats showed correlation between the two proteins in 3 rats, which had higher CDCrel-1 levels than the other rats. This result is consistent with a previous study which showed decrease in exocytosis and secretion of growth hormone from cells at higher concentration of CDCrel-1 (Beites, Xie et al. 1999). Similar to the results for CDCrel-1, we did not observe a correlation between VMAT2 levels and METH-induced hyperthermia. Since no correlation of hyperthermia was seen with any of the two proteins, we concluded that hyperthermia might not play a role in the interaction between CDCrel-1 and VMAT2. Based on the above results and existing literature, it is likely CDCrel-1 entraps VMAT2 only if the levels of CDCrel-1 are above a certain threshold; and that even without direct interaction with VMAT2, CDCrel-1 could be responsible for trapping it at the membrane through its filamentous network. Alternatively, VMAT2 vesicles are entrapped at the membrane via other filamentous protein, e.g. actin or fodrin (Hirokawa, Sobue et al. 1989).

#### 4.8 Dopamine (DA) Content:

Using HPLC, we assessed DA levels in the membrane fraction of the synaptosomes. We observed DA depletion at the membrane of the nerve terminal at 1 h (-30%) and more depletion at 24 h (-45%) after the last dose of binge METH. METH releases DA from VMAT2 storage vesicles into the cytoplasm. Subsequently, DA is released to the synaptic cleft via reverse DAT transport (Sulzer, Chen et al. 1995). The released DA is metabolized which results in decreased total tissue levels of the neurotransmitter. It has been determined that at 1 h after

high-dose binge METH, the total levels of DA drop by 81% (Eyerman and Yamamoto 2005). We found a lower deficit in DA levels at 1 h (30%). This lower depletion of DA in our study can be attributed to a few causes. Firstly, our study used a binge regimen of 4x8 mg/kg as compared to the Eyerman and Yamamoto study, which used a dose of 4x10 mg/kg. As discussed earlier, higher dose of METH produces higher toxicity. Another reason for small DA depletion in our study could be that we measured DA content in the membrane fraction of synaptosomes. The previous study assessed total DA content. Thus, their results represent overall depletion of DA whereas our study represents depletion of DA at the membrane of the nerve terminal.

At 24 h after binge METH (3x10 mg/kg), a ~45% decrease in striatal DA levels was found in a previous study (Graham, Noailles et al. 2008). The DA depletion observed in our study was similar to this result as we also observed a ~45% decrease in DA content. Our dosing regimen (4x8 mg/kg) was different than theirs (3x10 mg/kg) but the cumulative METH dose was very similar. Interestingly, the previous study measured DA content in striatal homogenate, whereas we measured DA content in synaptosomal membrane fraction. Possibly, METH depletes the vesicles in the cytosol to the same extent as vesicles at the membrane. Despite the depletion of DA, there was no concurrent decrease in the levels of VMAT2 at the membrane at both time points. One reason for this could be that the VMAT2 vesicles that are entrapped at the membrane by CDCrel-1 or other fibrous protein lose their ability to sequester DA. The readily releasable pool of vesicles sits very close to the membrane and sequester DA from there (Sudhof 2004). Although no studies have shown entrapment of VMAT2 at the membrane leading to loss of function, one study showed that septins could restrict the movement of

vesicles at the membrane by forming filamentous network around them (Hirokawa, Sobue et al. 1989).

#### 4.9 Future Directions

Our results suggest that, in rat striatum, parkin does not play a role in regulation of VMAT2 trafficking or regulation of CDCrel-1 levels in rat striatum and that CDCrel-1 is not responsible for lack of VMAT2 recycling to the cytosol after METH-mediated activation of DA autoreceptors. The major limitation of our model was a very modest parkin deficit. In addition, the number of rats per group in the parkin overexpression study was small; therefore, it is difficult to definitively conclude that parkin does not play a role in regulation of VMAT2. These limitations rendered our main scientific question – does parkin protects against METH neurotoxicity via regulation of VMAT2 vesicle trafficking - not definitely answered. Future experiments should include repetition of the study using doses of binge METH that produce significant parkin deficit and larger sample sizes. It would be also beneficial to isolate DAergic synaptosomes from other synaptosomal populations. Unfortunately, the techniques available today have serious limitations. Subsequent experiments should determine what mechanism is responsible for altered trafficking of VMAT2-bearing storage vesicles and whether VMAT2 vesicles “entrapped” at the membrane have lowered capacity to uptake DA than the vesicles in the cytosol.

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**ABSTRACT****THE ROLE OF E3 LIGASE PARKIN IN TRAFFICKING OF MONOAMINE STORAGE VESICLES IN RAT MODEL OF METHAMPHETAMINE NEUROTOXICITY**

by

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Methamphetamine (METH), a psychostimulant, is a widely used drug of abuse. METH is toxic to dopaminergic (DAergic) and serotonergic (5-HT) nerve terminals in the striatum when administered at high doses. METH releases Dopamine (DA) from vesicular monoamine transporter 2 (VMAT2) containing synaptic vesicles and induces oxidative stress by auto-oxidation of DA. The VMAT2 plays a neuroprotective role by sequestering cytoplasmic DA into vesicles for storage and protection from auto-oxidation. It has previously been shown that METH toxicity is associated with impaired VMAT2 trafficking and oxidative damage to the E3 ligase parkin. CDCrel1, a protein found to inhibit exocytosis, is regulated by parkin. We hypothesized that METH-mediated decrease in parkin causes accumulation of the parkin substrate CDCrel1, which entraps VMAT2 vesicles at the plasma membrane, preventing their recycling to the cytosol. We observed inability of the VMAT2 vesicles to mobilize to the cytosol due to a possible CDCrel-1 mediated entrapment following METH. Our findings suggest that CDCrel1 could partially entrap VMAT2 vesicles at the membrane, impairing their normal



recycling to the cytosol. We executed a parkin overexpression study to evaluate whether parkin protects against the METH-induced dysfunction of VMAT2 trafficking. We found that parkin does not reverse the impaired trafficking of VMAT2 caused by binge METH.

Keywords: Methamphetamine, parkin, VMAT2, CDCrel-1

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