



# Molecular and cellular bases for the protective effects of dopamine D1 receptor antagonist, SCH23390, against methamphetamine-induced neurotoxicity in the rat brain

Geneviève Beauvais

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Molecular and cellular bases for the protective effects of dopamine D<sub>1</sub> receptor antagonist, SCH23390, against methamphetamine-induced neurotoxicity in the rat brain

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Ecole doctorale du médicament  
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## Abstract

Methamphetamine (METH) is a potent psychostimulant known to cause cognitive abnormalities and neurodegenerative changes in the brains of METH abusers. One approach for developing therapies for METH abuse is to understand the molecular mechanisms of toxicity of the drug. Investigations in our laboratory and elsewhere have shown that single intraperitoneal injections of METH (30-40 mg/kg of body weight) can cause damage to striatal and cortical monoaminergic systems and induce neuronal apoptosis in the striatum of rodents via activation of endoplasmic reticulum (ER) and mitochondrial death pathways. Hence, the purpose of this thesis was to investigate if toxic binge METH injections can cause ER- and mitochondria-induced stress in the rat striatum. Recent studies have suggested that dopamine (DA) D<sub>1</sub> and D<sub>2</sub> receptors might mediate neuronal apoptosis in the striatum after single toxic METH doses. We therefore hypothesized that signaling through these two types of DA receptors might activate toxic effects of the binge METH regimen. The role of DA D<sub>1</sub> or D<sub>2</sub> receptors in METH-induced cell death pathways was thus examined by using pharmacological inhibitors of these receptors. In this dissertation, I report that binge METH regimen caused differential changes in immediate early genes (IEGs) that are known to influence synaptic changes in the brain. METH-induced changes in the expression of the IEGs were dependent on DA D<sub>1</sub> receptor stimulation. The second study examined the effects of binge METH on the expression of ER stress- and mitochondrial dysfunction-responsive genes. Pretreatment with the DA D<sub>1</sub> receptor antagonist, SCH23390, caused complete inhibition of METH-induced ER and mitochondrial stresses whereas the DA D<sub>2</sub> receptor antagonist, raclopride, provided only partial blockade. SCH23390 also blocked METH-induced hyperthermia whereas raclopride failed to do so. Interestingly, both antagonists attenuated METH-induced dopaminergic and serotonergic deficits in the striatum. Moreover, SCH23390 but not raclopride blocked METH-induced serotonergic deficits in cortical tissues. I also found that METH treatment induced upregulation of activin  $\beta$ A mRNA, increased TGF- $\beta$  and phosphorylated Smad2 proteins in the rat striatum. SCH23390 pretreatment completely blocked all these effects whereas raclopride did not block METH-induced increases in TGF- $\beta$  expression. In summary, these new data suggest a predominant role of DA D<sub>1</sub> over D<sub>2</sub> receptors in mediating METH toxicity.

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## Abbreviations

AC	Adenylate cyclase
ADHD	Attention deficit and hyperactivity disorder
AIF	Apoptosis inducing factor
AMPH	Amphetamine
AP1	Activating protein 1
Apaf1	Apoptosis protease-activating factor 1
ASK	Apoptosis signal-regulating kinase
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology
BIP	Immunoglobulin binding protein
BMP	Bone morphogenetic protein
bZIP	Basic region leucine zipper
cAMP	Cyclic adenosine monophosphate
Caspases	Cysteine-aspartic proteases
CHOP	C/EBP-homologous protein
CNS	Central nervous system
COMT	Catechol-o-methyltransferase
CRE	calcium/cAMP responsive element
CREB	cAMP response-element binding protein
SOD	Superoxide dismutase
DA	Dopamine
DAT	Dopamine transporter
DAWN	Drug abuse warning network
DEA	Drug enforcement administration
DFF45	DNA fragmentation factor 45
DHBA	2,3- and 2,5-dehydroxybenzoic acid
DIABLO	Direct IAP-associated binding protein with low pI
DOPA	Dihydroxyphenylalanine
DOPAC	Dihydroxyphenylacetic acid
EDEM	Endoplasmic reticulum-degradation-enhancing- $\alpha$ -mannidose-like protein
EGR	Early growth response
eIF2 $\alpha$	$\alpha$ subunit of eukaryotic initiation factor 2

ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERSE	Endoplasmic reticulum stress-response element reporter
ETC	Electron transport chain
FADD	Fas-associated death domain
Fas-L	Fas ligand
FDA	Food and drug administration
GABA	Gamma aminobutyric acid
GAD67	Glutamic acid decarboxylase
GADD34	Growth arrest and DNA damage inducible gene 34
GP	Globus pallidus
GPe	Globus pallidus external
GPi	Globus pallidus internal
GRP	Glucose regulated protein
HSP	Heat shock protein
HtrA2	High temperature requirement A2
5-HT	5-Hydroxytryptophan or serotonin
5-HTT	5-Hydroxytryptophan transporter
HVA	Homovanilic acid
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase-activated deoxyribonuclease
IEG	Immediate early gene
IP3	Inositol 1,4,5-trisphosphate
IRE1	Inositol-requiring enzyme 1
JNK	Jun-N-terminal kinase
MAO	Monoamine oxidase
METH	Methamphetamine
mPTP	Mitochondrial permeability transition pore
NAcc	Nucleus Accumbens
NFAT	Nuclear factor of activated T cells
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
PARP	Poly (ADP-ribose) polymerase
PERK	Protein kinase R-like endoplasmic reticulum kinase
PET	Positron emission tomography



PKA	Protein kinase A
PLC	Phospholipase C
ROS	Reactive oxygen species
Smac	Second mitochondria-derived activator of caspases
SN	Substantia nigra
SNC	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SP	Substance P
SRE	Serum response element
SST	Somatostatin
TGF- $\beta$	Transforming growth factor $\beta$
TH	Tyrosine hydroxylase
TRAF2	Tumor necrosis factor receptor associated factor 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
T $\beta$ IR	TGF- $\beta$ type I receptor
T $\beta$ IIR	TGF- $\beta$ type II receptor
UPR	Unfolded protein response
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
XBP1	X-box binding protein 1

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## **General introduction**

### **1.1 History of methamphetamine**

Methamphetamine (METH) is a potent psychostimulant with varied biochemical and molecular effects in the central nervous system (CNS) (Krasnova and Cadet, 2009). The increases in METH abuse throughout the world is a major public health and social concerns (Kulsudjarit, 2004; Rawson et al., 2002). In the United States, the widespread availability of METH makes it a major drug of abuse (Gonzales et al., 2010). Different factors contribute to the expansion of METH use including inexpensive precursor chemicals, the ease to manufacture and sell the drug. The number of recent new users of methamphetamine among persons aged 12 or older was 105,000 in 2010 (National Survey on Drug Use and Health, September 2011). Additionally, data from the Drug Abuse Warning Network (DAWN) have indicated a constant high number in METH-related visits to emergency rooms from 2007 to 2009 (approximately 65,000). Moreover, evidence shows that in many western United States cities, METH is used extensively by gay males and is frequently associated with high-risk sexual behavior, a major factor in the transmission of HIV and hepatitis (Degenhardt et al., 2010; Gonzales et al., 2006; Gonzales et al., 2008). Epidemiologic data also indicate that METH abuse and trafficking touch different regions in the world including Southeast and East Asia (McKetin et al., 2008), Canada (Callaghan et al., 2007), Australia (Degenhardt et al., 2008), South Africa (i.e., Cape Town) (Kapp, 2008), and parts of Europe (e.g., Czech and Slovak republics) (Griffiths et al., 2008).

From a historical perspective, however, METH has been in existence for many years. Credit for its synthesis is usually given to the Japanese chemist, Akira Ogata (Ogata, 1919). Use of that psychostimulant became popular during World War II, because it was widely distributed to Japanese and German soldiers to help them stay alert and effective (Rasmussen, 2008). After the war, METH became easily available to the public and its abuse rapidly spreads throughout Japan, United Kingdom and different other regions in the world (Matsumoto et al., 2002).

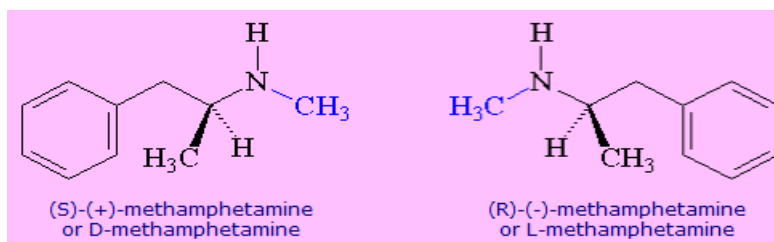
In 1943, Abbott Laboratories requested U.S. Food and Drug Administration (FDA) approval for the use of METH as a treatment for narcolepsy, mild depression, chronic alcoholism and hay fever (Moore, 2010). METH was approved for all these uses and marketed as Desoxyn but later these approvals were removed. METH is currently used to treat attention deficit and hyperactivity

disorder (ADHD) and obesity (FDA, 2007). In the United States, METH was widely abused during the 1960's. *The drug Abuse Control Amendments of 1965* were enacted that limited production and distribution of METH. *The Control Substances Act of 1970* classified METH as a schedule II substance under the Drug Enforcement Administration (DEA) regulations. Nevertheless, clandestine METH laboratories continue to flourish in the USA and throughout the world.

## **1.2 Physical and pharmacokinetic properties of METH**

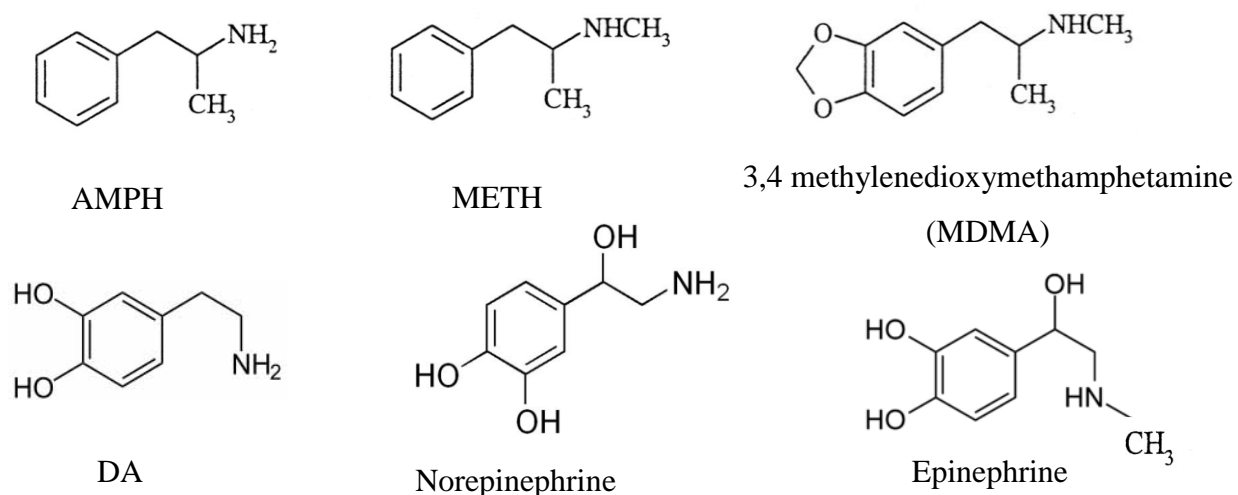
When used illicitly, METH (C<sub>10</sub>H<sub>15</sub>N) is commonly named “speed”, “meth”, “chalk”, “ice”, “glass”, and “crystal” (Derlet and Heischober, 1990). METH can be readily prepared from simple chemical precursors and often impurities are found in these preparations (Derlet and Heischober, 1990; Puder et al., 1988). However, during the past years, one common method of synthesis of the METH form known as “ice” is prepared from l-ephedrine or d-pseudoephedrine (Derlet and Heischober, 1990). The preparation results from the reduction of the β-hydroxyl group on ephedrine with a mixture of iodine and red phosphorous. The product is pure d-METH, which is several times more active than the l form. METH is usually supplied and used as the hydrochloride salt (C<sub>10</sub>H<sub>16</sub>ClN), which is a white crystalline, practically odorless substance (Cook, 1991). METH is easily dissolvable in water, alcohol, chloroform and dimethyl sulfoxide (Freye, 2009).

METH contains a chiral carbon atom, and therefore has two isomers with stereoselective differences in biological action (Figure 1). The drug exists in three forms: dextro (*d*-METH), levo (*l*-METH) and the racemic mixture of *d* and *l*-methamphetamine (*dl*-METH). The levorotary isomer of METH is found in inhalers for nasal decongestion such as Vicks Vapor Inhaler and has four times less stimulant activity than *d*-METH (Mendelson et al., 2008). The *d*-METH form is a known stimulant (DEA schedule II) and is prescribed to patients as Dexedrine/Desoxyn to treat insomnia, ADHD, narcolepsy as well as obesity. The *d*-METH form is the most potent but illicit synthesis of METH for abuse results mostly in the racemic mixture. The racemic mixture of the drug was used in this dissertation.



**Figure 1** Enantiomers of METH

METH and other amphetamine (AMPH) derivatives share a similar phenylethylamine structure with catecholamines, epinephrine, norepinephrine and dopamine (DA) (Figure 2). This similarity is an important determinant of the effects of the drug on monoaminergic systems.



**Figure 2** Structural comparison of METH, AMPH derivatives and catecholamines

METH has a half-time of about 12 hours (Schepers et al., 2003), an important factor in the length of its behavioral actions when compared to other psychostimulants such as cocaine. METH is a weak base with a pKa of 9.9 and is highly lipophilic, thus allowing it to cross the brain barrier easily. METH is primarily metabolized in the liver through aromatic hydroxylation and demethylation, and is excreted mostly in the urine (Cook et al., 1992; Lin et al., 1997). Its metabolism results in the production of the active compound, AMPH as well as in the production of corresponding para-hydroxylated metabolites, p-OH-METH and p-OH-AMPH. Multiple reports demonstrated that there are variations in METH metabolism between species. In humans, the main

metabolite is the drug unchanged, while the major urinary metabolite in rats is 4-hydroxy-METH (Caldwell et al., 1972). Minor metabolites are norepinephrin, 4-hydroxy-norepinephrin and benzoic acid (Caldwell et al., 1972).

### **1.3 Methods of administration of METH**

METH is usually administered orally or by nasal inhalation for medical use. In recreational use, ice or crystal, the METH hydrochloride can be taken orally, snorted, swallowed, smoked, injected or inserted anally (Cho and Melega, 2002; Covey, 2006; Keeling, 2008). The effects are different depending on the route of ingestion. When snorted or orally ingested, METH causes euphoria, but not an intense “rush” (Covey, 2006; Keeling, 2008). Smoking or injecting METH causes a “rush” or “high” effect that has been described to be extremely pleasurable (Covey, 2006; Keeling, 2008). This effect is followed by feelings of euphoria. People who take METH by the intravenous route often develop skin rashes, called speed bumps, and infections at the site of injection (Covey, 2006). In addition, share needles also expose users to different diseases like HIV and hepatitis (Degenhardt et al., 2010; Gonzales et al., 2006; Gonzales et al., 2008; Molitor et al., 1999).

### **1.4 Patterns of abuse and clinical effects including addiction**

METH abuse has three patterns of administration of the drug: low, binge and high intensity (Covey, 2006; Keeling, 2008). Ingestion of a high dose of the drug can be fatal to users (Guharoy et al., 1999; Sribanditmongkol et al., 2000). Low-intensity abusers are not psychologically addicted to METH and take the drug occasionally to stay awake long enough to finish a task (Keeling, 2008). Low-intensity use is characterized by the snorting of powdered METH or ingestions of pills (Covey, 2006). Binge and high-intensity abusers are addicted to METH and prefer to smoke or inject the drug in order to get its effects faster (Keeling, 2008). The binge pattern of abuse has multiple stages during its cycle. Users continually self-administer increasing doses throughout several days, and experience regular “rush” and euphoria. The total drug consumption during a binge is in the range of 2-4 grams. The crystal users will use the drug over a period of three to fifteen days until no “rush” or “high” is experienced (Cho and Melega, 2002; Covey, 2006). Usually, when the sensation of pleasure wears off, the binge METH user becomes irritable and paranoiac because of the lack of sleep and pharmacological effects of the drug. This state is called “tweaking” when the users experience paranoia, hallucinations and violent behavior (Sexton et al., 2010). The METH binge

user eventually crashes because he experiences a drastic drop in mood and energy levels. The individual will subsequently sleep for about one to three days (Keeling, 2008). After crashing, binge users usually return to their premorbid state, with progressive deterioration of both physical and psychological health, accompanied by depression and craving for the drug (Nakama et al., 2008). Withdrawal symptoms lead to repeated cycles of increasing binge ingestion of METH in spite of the negative consequences, well known by the abusers (Kalechstein et al., 2003; McGregor et al., 2005). High-intensity abusers have the impossible goal of never crashing and maintaining a state of euphoria and the perfect rush. The chronic METH users, often called “speed freaks”, consume the drug regularly; they preferentially take regular “hits” of the vaporized compound from pipes as often as every 30 minutes during the day. Estimates of the total drug consumed in a day range from 0.7 to 1 gram (Cho and Melega, 2002). This pattern of METH abuse causes less euphoria with repeated use, leading the user to take greater amount of the drug (Covey, 2006; Keeling, 2008). Tweaking for the high-intensity user is the most dangerous time to confront him because of their violent and unpredictable behavior (Keeling, 2008).

METH is a CNS stimulant and is abused for its desirable effects that include euphoria, increased alertness, improved self-confidence, enhanced energy and increased libido (Homer et al., 2008; Meredith et al., 2005). There are also many adverse health effects associated with METH use. METH is a sympathomimetic drug and will cause increased heart rate and blood pressure, leading to damage of heart vessels (Darke et al., 2008). Therefore, administration of large doses of METH can cause cerebrovascular accidents and cardiac arrhythmias, which in addition to hyperthermia can lead to renal failure and death (Albertson et al., 1999; Darke et al., 2008). Chronic therapeutic use of METH has been shown to cause no changes in blood pressure or heart rate, when administered at the prescribed dosage (about 5 to 10 mg doses) (Mitler et al., 1993). In contrast, illicit chronic use of METH can cause CNS complications such as anxiety, paranoia and chronic psychosis (Brecht et al., 2004; Zweben et al., 2004). Neurological problems include movement disorders, seizures and strokes (Perez et al., 1999). METH addicts can also present with neuropsychological deficits in tests of attention, working memory, and decision making (Sim et al., 2002; Simon et al., 2002; Verdejo-Garcia et al., 2006).

METH is a highly addictive drug. Long-term use of the drug can lead to tolerance, dependence and/or addiction. Tolerance to METH often happens in chronic and binge METH users. Interestingly, it can happen that a METH abuser who had developed tolerance to the drug, become



later more sensitive to some effects. This phenomenon is known as reverse tolerance or sensitization. Chronic use of METH leads to psychological dependence to the drug as evidenced by the compulsive drug use, drug cravings during withdrawal state and inability to stop drug use. Multiple studies have revealed that METH abuse causes plastic changes in the synapses and terminals of the monoamine neurons. These molecular neuroadaptations might constitute physiological components of METH addiction (Barr et al., 2006; Kish, 2008). Although the underlying mechanisms for METH addiction are unknown, it has been suggested that the DA system plays a dominant role, although the 5-hydroxytryptophan (5-HT or serotonin), glutamate and neuropeptides systems have a secondary role.

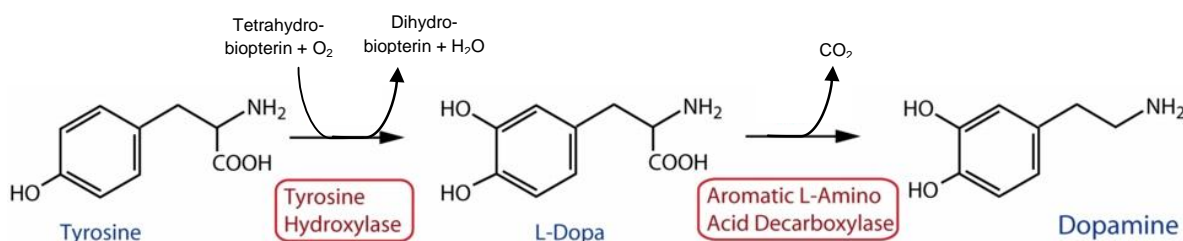
### **1.5 Mechanism of action of METH**

METH is sympathomimetic drug, which mimics catecholamines functions in the brain. METH produces its euphoric effects by inducing release of monoamines in the brain, especially in the basal ganglia (Kish, 2008; Sulzer et al., 2005). METH-induced monoamine release occurs after its entry into monoaminergic terminals by binding to DA and serotonin (5-HT) transporters (DAT and 5-HTT) and by passive diffusion. When inside the terminals, METH binds to vesicular monoamine transporter 2 (VMAT2) and enters monoaminergic vesicles where it disrupts the proton charge in the vesicles and allows redistribution of monoamines in the cytosol (Cubells et al., 1994; Sulzer et al., 2005). In addition, METH inhibits the enzyme monoamine oxidase (MAO), which is responsible for monoamines metabolism, hence resulting in higher availability of the neurotransmitters (Sulzer et al., 2005). METH can also alter the function of the monoamine transporters which induces reverse transport of monoamines into the synaptic cleft (Haughey et al., 2000; Sulzer et al., 2005). This is accompanied by high levels of monoamines in the synaptic cleft that can activate presynaptic receptors and also postsynaptic receptors that are located in the mesolimbic, mesocortical and nigrostriatal pathways (Berke and Hyman, 2000; Moore and Bloom, 1978)(see below). The present dissertation focuses on the neurotoxic effects of METH on the nigrostriatal pathway.

### **1.6 Dopamine hypothesis of addiction**

#### **1.6.1 Dopamine synthesis**

Accumulated evidence suggests that psychostimulants commonly mediate their effects through activation of the DA system (Berke and Hyman, 2000). DA belongs to a group of neurotransmitters called catecholamines (see Figure 2). They all contain a nucleus of a catechol (benzene ring with two adjacent hydroxyl groups) and a side chain of ethylamine or one of its derivatives. DA constitutes about 80% of the catecholamine content of the brain (Vallone et al., 2000). It is mainly synthesized in the substantia nigra (SN) and the ventral tegmental area (VTA), from the amino acid l-tyrosine (Figure 3). The majority of circulating tyrosine originates from dietary sources, but small amounts are derived from hydroxylation of phenylalanine by the liver enzyme phenylalanine hydroxylase. L-tyrosine is transported across the blood-brain barrier of the DA neuron where it is converted to dihydroxyphenylalanine (l-DOPA) by the enzyme tyrosine hydroxylase (TH) (Daubner et al., 2011). This constitutes the rate-limiting step in the synthesis of DA. Thereafter, l-Dopa is converted to dopamine by the aromatic amino acid decarboxylase enzyme (DOPA decarboxylase). DA enters cytoplasmic vesicles via VMAT2 and gets stored into these vesicles until it gets released.



**Figure 3** Dopamine synthesis

### 1.6.2 Dopamine release and stimulation of dopamine receptors

Upon the arrival of an action potential, there is a change in the vesicular membrane protein conformation that allows the influx of calcium ions. Depolarizing stimuli evoke calcium-dependent DA release mainly from cytoplasmic vesicles but newly synthesized DA can also be released (McMillen et al., 1980; Thomas et al., 2008). DA exerts its functions by interacting with specific membrane receptors. DA receptors are classified in two groups based on physiological and biochemical properties fall into two families; the D<sub>1</sub>-like family which includes D<sub>1</sub> and D<sub>5</sub> receptor subtypes, and the D<sub>2</sub>-like family which includes receptor subtypes D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> (Neve et al., 2004; Vallone et al., 2000). Use of pharmacological DA receptor ligands allows the differentiation of these receptors. Table 1 lists some of the selective agonists and antagonists of the DA receptors.

However, some of the drugs do not clearly differentiate between members of the same subfamily; by example, the DA D<sub>2</sub> receptor antagonist, raclopride, has high affinity for both DA D<sub>2</sub> and D<sub>3</sub> receptors. Furthermore, some of the DA receptor ligands can also have significant affinity for other receptors. As an example, the DA D<sub>1</sub> receptor antagonist, SCH23390, inhibited binding of the serotonergic 5-HT<sub>2</sub> receptor antagonist, [3H]spiperone, in rat frontal cortex with an ID<sub>50</sub> of 1.5 mg/kg (Bischoff et al., 1986). However, interaction of SCH23390 with 5-HT<sub>2</sub> receptors was tissue specific as it was characterized in rat frontal cortex, an area rich in both D<sub>1</sub> and 5-HT<sub>2</sub> receptors, but not in the striatum and hippocampus (Bischoff et al., 1986; McQuade et al., 1988). Furthermore, there are contradictory data on the antagonistic properties of SCH23390 indicating that doses of the antagonist up to 5 mg/kg do not affect serotonergic transmission in the cortical tissue (Gandolfi et al., 1988). In this dissertation, rats were administered four doses of 0.5 mg/kg of SCH23390 given at 2-hr intervals in order to antagonize METH-induced DA transmission in the striatum.

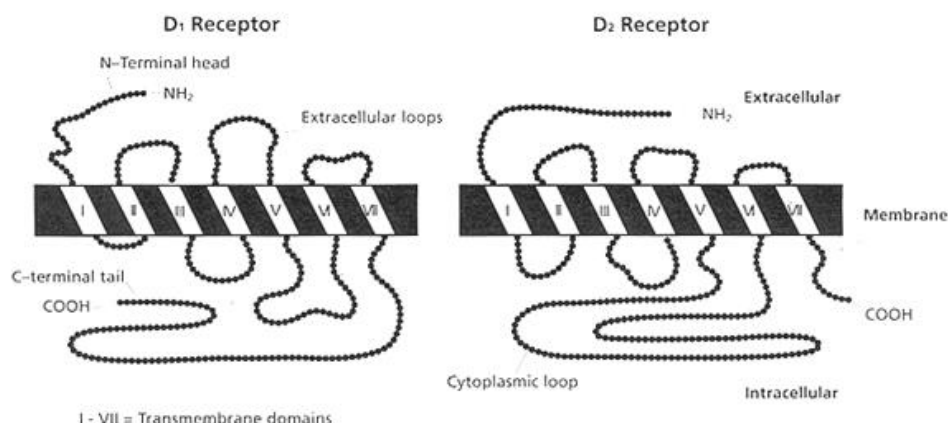
	D <sub>1</sub> -like		D <sub>2</sub> -like		
	D <sub>1</sub>	D <sub>5</sub>	D <sub>2</sub>	D <sub>3</sub>	D <sub>4</sub>
<i>Agonists</i>					
Dopamine	0.9–2340	0.9–261	2.8–474	4–27	28–450
Quinpirole	1900		4.8–576	5.1–24	30–46
Apomorphine	0.7–680	122–163	0.7–24	20–32	4
Bromocriptine	440–672	450	5.3–12.6	5–7.4	290–340
SKF-38393	1–150	0.5–100	150–9560	5000	1000–1800
<i>Antagonists</i>					
SCH23390	0.11–0.35	0.11–0.54	270–1100	314–800	3000–3560
Raclopride	18 000		1–5	1.8–3.5	237–2400
Haloperidol	27–203	33–151	0.6–1.2	2.74–7.8	2.3–5.1
(-)-Sulpiride	20 400–45 000	11 000–77 270	2.5–71	8–206	21–1000
Clozapine	100–261	194–336	56–230	83–620	9–42
Spiperone	99–350	135–4500	0.06–0.37	0.32–0.71	0.05–4

**Table 1** Dopamine receptor agonists and antagonists, K<sub>i</sub> values in nM (K<sub>i</sub> high and K<sub>i</sub> low)

### 1.6.3 Dopamine receptor genes, expression and signaling transduction

All DA receptors have characteristics of G-protein coupled receptors and contain seven transmembrane domains linked by protein loops with an extracellular amino terminus (Civelli et al., 1991; Jackson and Westlind-Danielsson, 1994; Missale et al., 1998). D<sub>1</sub>-like receptors have short third intracellular loop and long carboxyl terminal tail, whereas, the D<sub>2</sub>-like receptors contain

introns in their third intracellular loop and have short carboxyl terminal tail (Figure 4). This characteristic is the source of variants between the receptors.



**Figure 4** Structural features of the DA D<sub>1</sub> and D<sub>2</sub> receptors (Crocker, 1994)

#### D<sub>1</sub>-like receptor subfamily

The D<sub>1</sub>-like receptor genes (D<sub>1</sub> and D<sub>5</sub>) do not contain introns in their coding regions and therefore do not have any variants (Vallone et al., 2000). The D<sub>1</sub> and D<sub>5</sub> receptors have their third intracellular loop and the COOH terminus similar in size but divergent in their sequence. The external loops between transmembrane domains 4 and 5 are different in length (27 and 41 amino acids for D<sub>1</sub> and D<sub>5</sub> receptors) and sequence (Missale et al., 1998). The expression of these genes is not uniformly expressed in the brain. The D<sub>1</sub> receptor is highly expressed in the projection regions of DA neurons such as the striatum, nucleus accumbens (NAcc), frontal cortex, olfactory tubercle and amygdale (Jackson and Westlind-Danielsson, 1994; Missale et al., 1998). It is also found in the island of Calleja and the subthalamic nucleus (Jackson and Westlind-Danielsson, 1994). The D<sub>5</sub> receptor is expressed at much lower levels and is concentrated in the hippocampus and hypothalamus, with lower amount found in the striatum and frontal cortex (Jackson and Westlind-Danielsson, 1994; Meador-Woodruff et al., 1992).

#### D<sub>2</sub>-like receptor subfamily

Gene structures of the D<sub>2</sub>-like receptors contain introns within their coding regions; D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> receptors have six, five and three introns, respectively (Missale et al., 1998). There are short and long variants of D<sub>2</sub> receptor (D<sub>2S</sub> and D<sub>2L</sub>), which are generated by alternative splicing of 29 amino acids in the third intracellular loop (Dal Toso et al., 1989). Spliced variants of the D<sub>3</sub> receptor coding for non-functional proteins has been identified (Fishburn et al., 1993; Giros et al.,

1991). In addition, variations in the D<sub>4</sub> receptor gene were also identified (Van Tol et al., 1992). The D<sub>2L</sub> subtype is more common than the D<sub>2S</sub> subtype (Missale et al., 1998; Vallone et al., 2000). D<sub>2</sub>-like receptors are expressed both at the presynaptic and postsynaptic neurons. Presynaptic D<sub>2</sub> receptors are expressed in the SN pars compacta (SNc) and in the VTA, while D<sub>3</sub> receptor is only found in the SN (Civelli et al., 1991). These presynaptic neurons called autoreceptors play a role in the modulation of nigrostriatal and mesolimbic systems. Highest levels of the D<sub>2</sub> receptors are found in the projection regions such as the dorsal striatum, NAcc, hypothalamus and pituitary (Jackson and Westlind-Danielsson, 1994; Landwehrmeyer et al., 1993). D<sub>3</sub> and D<sub>4</sub> receptors have a lower pattern of expression. D<sub>3</sub> receptors are found in the ventral striatum or NAcc, the islands of Calleja, hypothalamus, thalamus and cerebellum (Bouthenet et al., 1991; Jackson and Westlind-Danielsson, 1994). D<sub>4</sub> receptors are mostly found in the frontal cortex, amygdala, olfactory bulb and hippocampus (Jackson and Westlind-Danielsson, 1994).

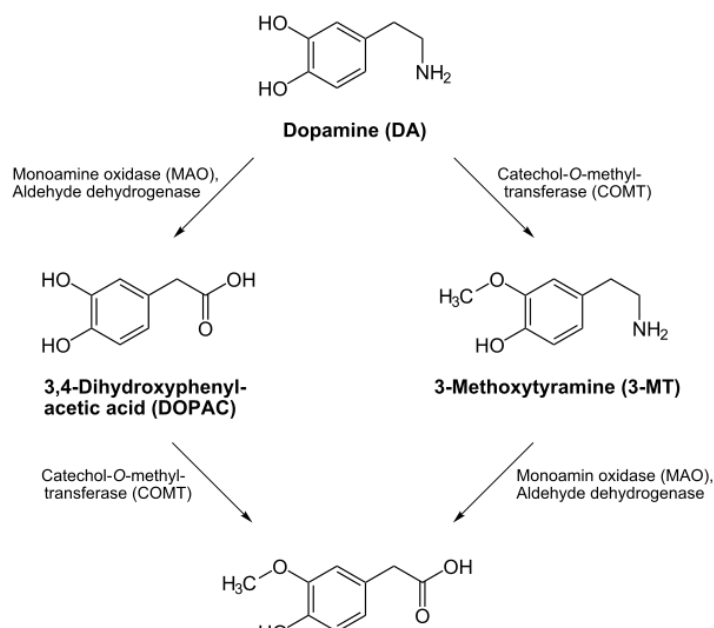
#### Signaling pathways

It is generally assumed that stimulation of D<sub>1</sub>-like receptors, which are coupled to G<sub>s</sub> or G<sub>olf</sub> protein, causes activation of adenylate cyclase (AC) (Herve et al., 1993; Jackson and Westlind-Danielsson, 1994; Monsma et al., 1990; Sunahara et al., 1991). This enzyme causes the conversion of adenosine triphosphate (ATP) to the intracellular second messenger cAMP. The cAMP in turn activates cAMP-dependent protein kinase A (PKA), which phosphorylates numerous substrates such as the transcription factor cAMP response element-binding (Neve et al., 2004). In contrast, D<sub>2</sub>-like receptors inhibit AC (Jackson and Westlind-Danielsson, 1994). The D<sub>2</sub> receptor was first characterized as an inhibitor of intracellular cAMP levels in the pituitary gland, and in striatal cells. However, D<sub>3</sub> receptor weakly inhibits AC (Missale et al., 1998; Robinson and Caron, 1997). D<sub>4</sub> receptor also inhibits cAMP accumulation in the retina and different cell lines (Missale et al., 1998).

Stimulation of DA D<sub>1</sub>-like receptors is also coupled to activation of Gq/phospholipase C (PLC) and causes increases in levels of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) (Jin et al., 2003; Wang et al., 1995a). DA D<sub>1</sub> receptors mediate release of calcium from intracellular stores through activation of PLC and opening of voltage-gated calcium channels (Berridge, 2009; Wu et al., 2006). DA D<sub>2</sub> receptors coupled to G<sub>i/o</sub> protein reduce calcium currents (Jackson and Westlind-Danielsson, 1994; Missale et al., 1998; Yan et al., 1997).

### 1.6.4 Dopamine reuptake and metabolism

Reuptake is the process, by which DA released in the synaptic cleft, is brought back into presynaptic nerve terminals or is internalized by surrounding glial cells (Giros et al., 1992; Mannisto and Kaakkola, 1999; Yu and Hertz, 1982). Reuptake of DA is dependent on the presence of external sodium and chloride and mediated by the DAT, which is localized at the plasma membrane of nerve terminals (Giros et al., 1992). Subsequently, two enzymes metabolize the neurotransmitter: catechol-O-methyltransferase (COMT) localized in the cytoplasm, and MAO expressed on the membrane of mitochondria (Kopin, 1985) (Figure 5). COMT catalyzes the methylation of one group hydroxyl on a DA molecule to produce 3-methoxytyramine. Deamination of DA by MAO produces dihydroxyphenylacetic acid (DOPAC). The combination of both COMT and MAO effects convert DA in homovanillic acid (HVA) (Kopin, 1985; Kopin, 1994). DA is also removed from the extracellular space and metabolized by astrocytes (Mannisto and Kaakkola, 1999; Yu and Hertz, 1982).

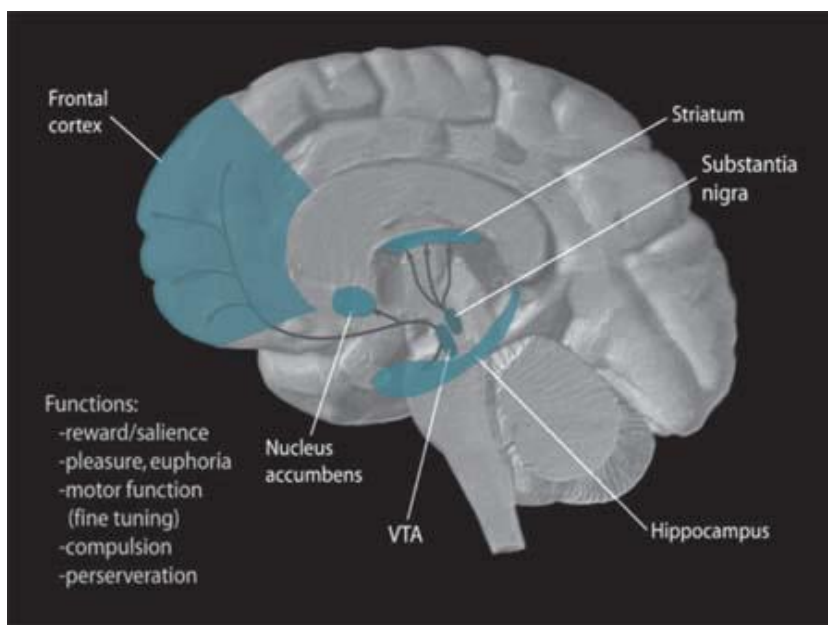


**Figure 5** Metabolism of DA

### 1.6.5 Dopamine pathways in the CNS

In the CNS, there are four central dopaminergic systems (Figure 6):

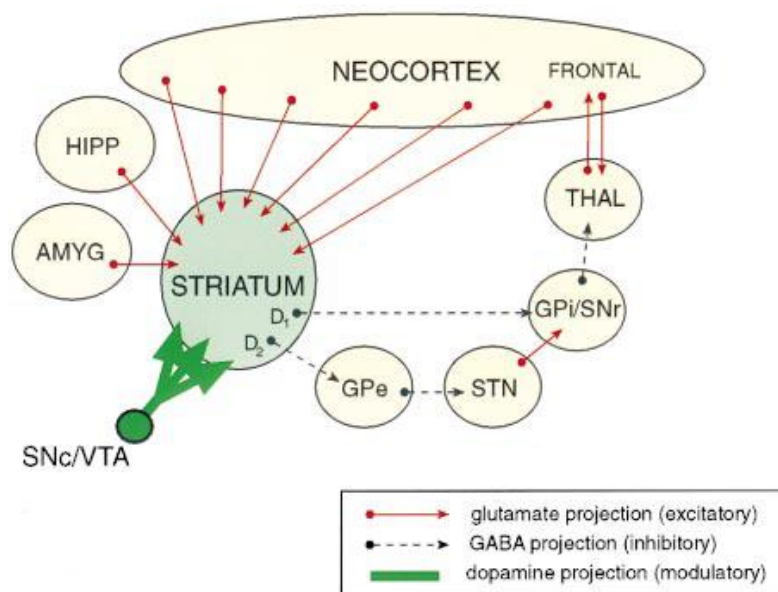
- (1) The nigrostriatal pathway has 80% of DA neurons in human brain. The cell bodies of the neurons are localized in the SNc. The projections of these neurons end into the dorsal striatum, constituted of caudate and putamen nuclei (Joel and Weiner, 2000; Moore and Bloom, 1978). In rodents, these regions consist of one nucleus named the dorsal striatum or caudate putamen (Joel and Weiner, 2000). This pathway controls motor activity (Gerfen, 1992b).
- (2) The mesolimbic pathway begins in the VTA and terminates in different limbic area, such as NAcc, olfactory tubercle, amygdala and hippocampus (Moore and Bloom, 1978; Swanson, 1982). The mesolimbic pathway is responsible for motivation, reward, drug addiction, motor activity (Le Moal and Simon, 1991).
- (3) The mesocortical pathway also originates in the VTA and terminates in the cortical structures (Moore and Bloom, 1978; Swanson, 1982). This pathway controls cognitive functions and learning (Le Moal and Simon, 1991).
- (4) The tuberoinfundibular pathway goes from the hypothalamus to the hypophysis, regulating neuroendocrine function (Moore and Bloom, 1978).



**Figure 6** Illustration of the dopamine pathways within the human brain. Obtained from <http://www.drugabuse.gov/>

### 1.6.6 Dopamine and the neural circuits of the basal ganglia

The striatum is part of the basal ganglia, a complex of nuclei that has important activity in the brain. The basal ganglia contain the dorsal striatum or caudate-putamen, the NAcc, globus pallidus internal and external (GPi and GPe) (Graybiel, 2000). The basal ganglia function in conjunction to other CNS regions including cortex and thalamus; together, they form a sensorimotor circuit “cortico-striato-thalamo-cortico” (Alexander et al., 1990; Brown and Marsden, 1998) (Figure 7). In this neural circuit, the striatum plays a central role; it receives glutamatergic inputs from the cortex and thalamus, dopaminergic inputs from the midbrain (SN pars compacta and VTA), cholinergic inputs from the brainstem and serotonergic inputs from the dorsal raphe nucleus. It also sends gamma aminobutyric acid (GABA) outputs to the GP, SN pars reticulate (SNr), and subsequently to the thalamus and the frontal cortex involved in motor activities. Dysregulations in the function of the basal ganglia and allied nuclei can result in disorders of movement programming and learning processes, and characterize some neurodegenerative diseases such as Parkinson’s disease and drugs addiction (Kreitzer, 2009).



**Figure 7** Simplified anatomy of cortex–basal ganglia circuits (Berke and Hyman, 2000)

In the striatum, DA fibers originating from the SNc modulate both projection neurons and interneurons. The striatum is primarily composed of medium spiny neurons which synthesize GABA (90 %) and are projections neurons. Activation of the dopaminergic nigrostriatal pathway



stimulates DA D<sub>1</sub> and D<sub>2</sub> receptors expressed in two different populations of GABAergic neurons involved in the striatonigral/direct and striatopallidal/indirect pathways, respectively (Figure 6). However, there is evidence that a portion of 10-20% of DA D<sub>1</sub> and D<sub>2</sub> receptors colocalizes in the striatal tissue (Surmeier et al., 1992).

The direct pathway originates from GABA spiny medium projection neurons that also express the neuropeptides, substance P (SP) and dynorphin. The membrane of these neurons contains DA D<sub>1</sub> receptors coupled to AC. The projections go to two different regions of the basal ganglia, the GPi (primates)/entopeduncular nucleus (rodents) and SNr. In normal state, these two brain regions inhibit the thalamus by sending GABA outputs. However, when the direct pathway is activated, release of GABA from the striatum inhibits tonic activity of the GABA neurons in the GPi and SNc, desinhibiting the thalamic networks. The activated thalamus releases glutamate in the cortex, then the cortex can stimulate motor activity and send glutamate outputs to the striatum.

The indirect pathway involves GABA medium spiny neurons that are also enkephalin-positive. These neurons express DA D<sub>2</sub> receptors at their membrane. Their axons send GABA outputs in the GPe (primates)/GP (rodents), which subsequently desinhibits glutamatergic neurons in the subthalamic nucleus. Indeed, this region stimulates the GPi and the SNc, reinforcing their inhibitory effects on the thalamus and causing a decrease in motor functions.

Furthermore, DA neurotransmission also affects GABA and acetylcholine spiny interneurons in the striatum, which represent 10% of striatal neurons. Despite few number of interneurons, they play an important role in regulating striatal outputs (Kreitzer, 2009). The large cholinergic interneurons (< 5% of striatal neurons) are identified by the presence of the enzyme, choline acetyltransferase. These neurons receive glutamatergic inputs from the thalamus. Cholinergic interneurons express both D<sub>2</sub> and D<sub>5</sub> receptors and are responsive to their activity (Bergson et al., 1995; Yan and Surmeier, 1997). Striatal cholinergic interneurons also express the SP receptor and release acetylcholine in response to its activation (Kawaguchi, 1997).

Medium-sized GABA interneurons are divided in three subtypes: parvalbumin-positive; somatostatin-, neuropeptide Y-, and nitric oxide synthase-positive (SST-NPY-NOS); and calretinin-positive (Kawaguchi et al., 1995). Neurons containing the calcium binding protein parvalbumin, constitute between 3-5% of striatal cells and are slightly larger than the medium-sized spiny GABA-positive cells. They receive powerful excitation from the cerebral cortex. Somatostatin-containing interneurons represent 1-2% of striatal neurons. Somatostatin/NOS-positive cells receive

direct cortical inputs, which might be glutamatergic. Calretinin, which is, as noted, expressed in a population of smaller aspiny neurons, is a calcium-binding protein with strong homology with calbindin. These cells express GABA and glutamic acid decarboxylase (GAD<sub>67</sub>). The modulation of these interneurons by DA is not clearly characterized, but it is known that the GABAergic aspiny interneurons express DA D<sub>5</sub> receptors (Bergson et al., 1995; Centonze et al., 2003). Although the role of the striatal interneurons is not fully understood, some researchers have investigated their implication in METH toxicity. It has been shown that the intraventricular administration of the neuropeptide NPY or NPY receptor agonist protected the mouse striatum from METH-induced apoptosis, and this effect was dependent on DA D<sub>1</sub> receptor (Thiriet et al., 2005). It was suggested that METH treatment caused an interaction between the axon collaterals of striatonigral projection neurons and NPY/ positive interneurons, knowing that striatonigral neurons express SP and DA D<sub>1</sub> receptors, whereas NPY-positive interneurons express the neurokinin-1 receptor for SP (Gerfen, 1992a; Kawaguchi, 1997). In addition, Zhu et al (2006) demonstrated that multiple injections of METH induced apoptosis in about 45% of GABA-parvalbumin-positive neurons in the dorsal striatum, and 29% of cholinergic neurons in the dorsal–medial striatum (Zhu et al., 2006a).

## **1.7 METH-induced neurotoxicity**

### **1.7.1 Human data**

Brain imaging studies of METH abusers showed that METH causes long-term damages to the brain. Magnetic resonance imaging studies gave evidence of brain structural abnormalities including a significant loss of gray matter in the cortical and limbic systems, reduction in hippocampal volume, significant white-matter hypertrophy, medial temporal lobe damage and striatal enlargement (GP and putamen) (Baicy and London, 2007; Chang et al., 2005; Thompson et al., 2004a). In addition, positron emission tomography (PET) studies showed abnormalities in glucose metabolism in brain of METH users, which have increased metabolism in the parietal cortices but decreased metabolism in the thalamus plus striatum (Volkow et al., 2001c). These structural alterations after chronic use of METH have been linked to motor and cognitive impairments. By example, METH addicts exhibit deficits in attention, working memory, decision-making and motor activity (Paulus et al., 2002; Thompson et al., 2004a; Volkow et al., 2001d). However, striatal enlargement is thought to be as a compensatory response to repeated METH-induced striatal injury, because METH users with the smallest striatal volumes had the greatest

cumulative lifetime of METH use and poorest cognitive performance (Chang et al., 2005). It was suggested that this effect is related to the induction of gliosis and neurotrophic factors, because activated macrophages and microglia after striatal injury caused wounding of the striatum and dopaminergic fibers sprouting (Batchelor et al., 1999).

In addition, analysis of brain of METH users showed neurochemical changes affecting the dopaminergic and serotonergic systems. PET studies revealed that DAT was decreased in the caudate-putamen, the NAcc and the prefrontal cortex (McCann et al., 1998; Sekine et al., 2001). Post mortem analysis also showed a significant decrease in DA, DAT and TH density even after as much as three years of abstinence from the drug (Wilson et al., 1996). Furthermore, the density of 5-HTT was found decreased in the midbrain, striatum, thalamus and cortex of brain of METH abusers (Sekine et al., 2006). Another study confirmed the reduction in 5-HTT levels in orbitofrontal and occipital cortices (Kish et al., 2009). There is also evidence of a decrease in DA D<sub>2</sub> receptors in the striatal tissue (both dorsal and ventral striatum) of METH addicts (Volkow et al., 2001a). Importantly, there is proof of partial recovery from the structural abnormalities caused by METH abuse in rodents (Cass and Manning, 1999; Friedman et al., 1998), nonhuman primates (Harvey et al., 2000) and humans (Volkow et al., 2001b; Wang et al., 2004) after protracted abstinence. By example, loss of DAT in METH abusers recovers after abstinence of twelve months or longer in the striatum, without any improvement in slower motor function and decreased memory (Volkow et al., 2001b). However, abstinence of nine months or longer affects thalamic glucose metabolism (but not striatal glucose metabolism) and correlated with an improvement in neuropsychological performance (Wang et al., 2004). Administration of METH to vervet monkeys also caused long-term but reversible deficits in TH, DAT and VMAT2 in the striatum (Harvey et al., 2000). Studies in rats revealed that METH-induced decreases in presynaptic monoaminergic markers such as 5-HT (Friedman et al., 1998), DA and its metabolites (Cass and Manning, 1999; Friedman et al., 1998) recover to normal levels in the striatum between six and twelve months postinjection. The findings from these studies have implications in the treatment of METH abusers and the elucidation of the mechanisms underlying the apparent reversibility of METH neurotoxicity is needed.

### 1.7.2 Animal studies

#### 1.7.2.1 Patterns of use of METH

Use of animal models is an efficient tool to study the neurotoxic effects of METH abuse (Kita et al., 2003). Different models to study METH neurotoxicity exist, which mimic human METH abuse patterns and include chronic, high-dose and multiple daily (binge) exposure to the drug. In our experiments, we have used a one-day METH dosing regimen that better models the acute overdose pathologies seen in humans. None of the models is perfect; each one can contribute to understand the neuropathological effects of METH. However, it is important to select the most appropriate dosing regimen for a study. Multiple animals were used including guinea pigs, cats, monkeys, mice and rats (Krasnova and Cadet, 2009). The use of these numerous animal models has demonstrated a major role played by dopaminergic system in METH neurotoxicity.

#### 1.7.2.2 Monoamines deficits

Animal studies have demonstrated neurodegenerative effects on the monoaminergic systems in rat and mice brains (Krasnova and Cadet, 2009). Single and multiple high doses of METH caused long-term decreases in DA levels in rat striatum (Cappon et al., 2000; Chapman et al., 2001; Ricaurte et al., 1980). High doses of METH have also been shown to cause lasting depletions of 5-HT in the striatum (Cappon et al., 2000; Ricaurte et al., 1980; Richards et al., 1993), but also in the NAcc, hippocampus and frontal cortex (Friedman et al., 1998; Green et al., 1992; Richards et al., 1993). METH also caused reductions in the activity of TH and TPH, the rate-limiting enzymes for DA and 5-HT synthesis. TH activity was decreased in the striatum (Cappon et al., 2000; Chapman et al., 2001; Kogan et al., 1976), while TPH activity decreased in the striatum, cortex, NAcc and hippocampus (Bakhit et al., 1981; Hotchkiss and Gibb, 1980; Morgan and Gibb, 1980).

Administration of toxic doses of METH to mice resulted in similar dopaminergic nerve terminals destruction. Studies have shown significant reductions in DA in the striatum (Green et al., 1992; Ladenheim et al., 2000; O'Callaghan and Miller, 1994) and cortex (Fantegrossi et al., 2008; Ladenheim et al., 2000), decreases in DAT proteins levels (Deng et al., 1999; Fumagalli et al., 1999) and binding (Ladenheim et al., 2000; Xu et al., 2005; Zhu et al., 2005) in the striatum, and loss of TH immunoreactivity (Bowyer et al., 2008; Deng et al., 1999) and protein levels (O'Callaghan and Miller, 1994; Xu et al., 2005; Zhu et al., 2005) in the striatum. However, the serotonergic neurons seem resistant to the effects of METH in mice.

METH toxicity also caused terminal damage of monoaminergic neurons characterized by a loss of the functions of DAT, 5-HTT and VMAT2 in rats (Guilarte et al., 2003). It was established that METH causes a decrease in DAT binding in striatum (Guilarte et al., 2003). METH also caused decrease in 5-HTT binding in the striatum, anterior cingulate, NAcc, amygdala, hippocampus, somatosensory cortex, hypothalamus, thalamus and septum (Armstrong and Noguchi, 2004; Guilarte et al., 2003). It have also been reported to cause decreases in VMAT2 binding (Guilarte et al., 2003; Segal et al., 2005) and immunoreactivity (Eyerman and Yamamoto, 2007) in the rat striatum.

#### 1.7.2.3 Hyperthermia

Exposure to METH causes hyperthermia, which can cause mortality. Studies by our lab and others have shown that METH causes an increase of the core body temperature of animals. It has also been established that this effect is modulated by the dose used and the ambient temperature (Bowyer et al., 1992; Brown et al., 2003b). Mice administered a single toxic dose of 45 mg/kg of METH at a room temperature of 22°C exhibited an increase in core temperature up to 39.5°C from a baseline of 38°C; however, at ambient temperature of 6°C, the animals had a decrease in rectal temperature from 37°C to 35.5°C (Xie et al., 2000).

Many reports have suggested that there is a correlation between METH-induced hyperthermic responses and the depletion of monoamine concentrations in the striatum. By example, Bowyer et al (1992, 1994) have shown that the extent of the DA depletion after METH administration is dependent on the degree of hyperthermia. Rats injected with multiple doses of METH (at an ambient temperature of 23-24°C) exhibited higher depletion in DA content in the brain when their core temperature was more than 41°C (Bowyer et al., 1992; Bowyer et al., 1994). Furthermore, when METH is administered at low ambient temperature of 4°C, there was protection against depletion of striatal monoamines content in mice (Albers and Sonsalla, 1995; Ali et al., 1994) and rats (Bowyer et al., 1992). Mechanisms underlying these thermoregulations are unknown.

#### 1.7.2.4 Neuronal apoptosis

Investigations in our laboratory and elsewhere have shown that the use of doses of METH that cause damage to nigrostriatal and cortical monoaminergic systems can also cause neuronal apoptosis in the striatum and the cortex but not in the SN nor in the VTA (Cadet et al., 2007).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunohistochemistry demonstrated that METH caused neuronal apoptotic in the striatum (Jayanthi et al., 2005), and prefrontal cortex (Kadota and Kadota, 2004) of rats. Fluoro-Jade immunohistochemistry also demonstrated neuronal death in somatosensory cortices (Eisch and Marshall, 1998; O'Dell and Marshall, 2000) of rats. METH can also cause neuronal death in the striatum, frontal and parietal cortices, hippocampus and olfactory bulb of mice (Deng et al., 1999; Deng et al., 2001; Deng et al., 2007; Zhu et al., 2005; Zhu et al., 2006a; Zhu et al., 2006b). METH-induced neuronal apoptosis in the striatum affects populations of medium spiny projection neurons that are enkephalin positive, as well as cholinergic and parvalbumin-positive interneurons (Thiriet et al., 2005; Zhu et al., 2006a). Moreover, there was loss of calbindin interneurons in the cortex and hippocampus of rats 30 days after escalating dose-binge METH regimen (Kuczenski et al., 2007).

### **1.8 Role of dopamine system in METH neurotoxicity**

Many studies have demonstrated an important role of DA in METH-induced neurotoxicity. Combining pharmacological and genetic approaches in animal studies, it has been possible to demonstrate the involvement of DA, its transporter and receptors in response to METH.

#### **1.8.1 Dopamine overflow and oxidative stress**

The mechanisms via which METH induced its neurotoxic effects are not fully known, however, many reports suggest that oxidative stress has a principal role in them. Oxidative stress involves the excessive formation of free radicals or reactive oxygen species (ROS) containing unpaired electron, such as the hydroxyl radicals, hydrogen peroxide and superoxide radicals. Oxidative stress has been implicated in a number of neurodegenerative diseases including Alzheimer's (Sompal et al., 2008) and Parkinson's (Chin et al., 2008).

After METH enters the brain and displaces DA in the vesicles out in the cytosol and synaptic cleft, the excess of DA can be autooxidized (Kita et al., 2003). The formation of DA quinones and semiquinones can generate toxic radicals like superoxide, hydrogen peroxide and nitrogen radicals (Cadet and Brannock, 1998; Krasnova and Cadet, 2009; Larsen et al., 2002; LaVoie and Hastings, 1999; Stokes et al., 1999). LaVoie and Hastings (1999) found that administration of neurotoxic doses of METH to rats caused formation DA quinones which bind to cysteinyl residues on proteins, leading to an increase in protein cysteinyl-DA levels in the striatum. By example, formation of

hydroxyl radicals has been revealed in striatal dialysate and tissue by an increase in 2,3- and 2,5-dehydroxybenzoic acid (DHBA), the stable product of the reaction of salicylate with hydroxyl radicals, after treatment with a neurotoxic dose of METH (Giovanni et al., 1995). In addition, during synthesis and metabolism of DA, TH and MAO produce hydrogen peroxide, which interacts with iron (Cadet et al., 1994b; Melega et al., 2007). It has been shown also that METH induces lipid peroxidation (Gluck et al., 2001; Jayanthi et al., 1998). METH-induced increases in free radicals and lipid peroxidation can damage cell membranes and nuclear DNA, they may be a possible cause of mitochondrial dysfunction and apoptosis in the striatum (this idea will be discussed later).

Parallel to the increase in radicals, METH also causes a decrease in the detoxifying enzymes that play an important role in the metabolism of ROS. For example, METH administration causes decreases in the levels of copper-zinc superoxide dismutase (CuZn SOD), catalase, glutathione, and peroxiredoxins in the brain (Chen et al., 2007; Gluck et al., 2001; Jayanthi et al., 1998; Li et al., 2008). Transgenic mice that overexpressed CuZn SOD, the enzyme that catalyzes the breakdown of superoxide radicals, were protected against METH toxicity (Cadet et al., 1994b; Hirata et al., 1996). In addition, administration of antioxidants such as bromocriptine (Kondo et al., 1994) and L-carnitine (Virmani et al., 2003) protected against METH-induced neurotoxicity.

### 1.8.2 DAT and VMAT2

As mentioned earlier, METH binds strongly to DAT and VMAT2 to release DA into the cytosol of DA neurons and in the synaptic cleft (Sulzer et al., 2005). This theory implicates that alteration in VMAT2 functions might also contribute to METH toxicity. In support of that, VMAT2-deficient mice exhibited increased degeneration of DA neurites and accumulation of oxygen radicals in mouse ventral midbrain neuronal cultures (Larsen et al., 2002). Another study showed that impaired VMAT2 function in heterozygote VMAT2 knockout mice potentiates METH-induced neurotoxicity to dopaminergic neurons (Fumagalli et al., 1999). Use of the VMAT2 inhibitor, reserpine, exacerbated METH neurotoxicity, demonstrated by the fact that striatal DA content in reserpine pretreated mice was significantly lower than in control mice (Albers and Sonsalla, 1995; Kuhn et al., 2008; Thomas et al., 2008).

Furthermore, many studies have demonstrated that the dopaminergic transporters play an important role in METH neurotoxicity. Pretreatment with the DAT inhibitor, amphetamine, protected against METH-induced neostriatal DA depletion (Marek et al., 1990) and decrease in

striatal TH activity (Schmidt and Gibb, 1985). In addition, posttreatment with the DAT inhibitor, methylphenidate, 1 hour after METH treatment protected also against long-term decreases in vesicular DA uptake, reductions in VMAT2 ligand binding and decreases in VMAT2 immunoreactivity (Sandoval et al., 2003). Furthermore, DAT knockout mice were protected against METH-induced DA depletion (Fumagalli et al., 1998). In contrast, increased DAT function correlated with intracellular accumulation of METH and increased neurotoxicity in embryonic mesencephalic cells placed at 37°C (Xie et al., 2000). These data also suggest that increased temperature mediates the role of DAT in METH-induced neurotoxicity.

### 1.8.3 Dopamine receptors

Excess of DA in the synaptic cleft, after METH administration, causes overstimulation of DA receptors. This suggests a role of DA receptors in mediating METH neurotoxicity. Multiple studies have given evidence that DA receptor antagonists can protect against METH-induced toxic effects to the monoamine systems (Angulo et al., 2004; Jayanthi et al., 2005; O'Dell et al., 1993; Sonsalla et al., 1986). For example, pretreatment with the DA D<sub>1</sub> receptor antagonist, SCH23390 or D<sub>2</sub> receptor antagonist, eticlopride, prior to each injection of binge METH regimen attenuated acute DA overflow and lasting DA depletion in the striatum (O'Dell et al., 1993). In addition, pretreatment with SCH23390 or the D<sub>2</sub> receptor antagonist, raclopride, before a single toxic dose of METH, attenuated striatal DA deficits (Jayanthi et al., 2005), reductions in DAT binding and depletion in TH protein levels (Xu et al., 2005). SCH23390 also caused complete protection against decreases of TH and DA in the striatum, as well as TPH and 5-HT deficits in mouse striatum and cortex after multiple injections of METH (Sonsalla et al., 1986). However, the D<sub>2</sub> receptor antagonist, sulpiride, blocked the effects of METH on only the dopaminergic system (Sonsalla et al., 1986). Another study showed that SCH23390 and eticlopride attenuated reduction in striatal DA and 5-HT contents after binge METH injections (4x10 mg/kg) at an ambient temperature of 24°C (Broening et al., 2005). However, when the drugs are administered at 33°C, eticlopride failed to prevent 5-HT decreases (Broening et al., 2005). These data suggest that DA D<sub>2</sub> receptors-mediated their effects are dependent on thermoregulation.

Both DA receptor subtypes contribute also to METH-induced neuronal apoptosis. Pretreatment with DA D<sub>1</sub> and D<sub>2</sub> antagonists SCH23390 and raclopride provided protection against neuronal apoptosis in the striatum, induced after a single toxic dose of METH (Xu et al., 2005). The



protection afforded by SCH23390 against METH-mediated cell death may depend on the Fas ligand (Fas L)/Fas death pathway because pretreatment with the antagonist caused significant inhibition of increases in the expression of Fas L and caspase 3 caused by METH treatment in rat striatal cells (Jayanthi et al., 2005). Recently, our laboratory has shown that pretreatment with SCH23390 provided partial protection against activation of ER stress pathways in rats injected with a single toxic dose of METH (Jayanthi et al., 2009).

These reports overall show the involvement of both types of DA receptor in mediating METH-induced monoaminergic damage and neuronal apoptosis. However, METH neurotoxicity activates a mosaic of complex signaling pathways, in which the specific role of each DA receptor subtype has to be elucidated. This dissertation focuses primarily on the effects of SCH23390 on METH-induced regulation of gene expression and activation of cellular stresses.

## **1.9 Molecular mechanisms of METH neurotoxicity**

### **1.9.1 METH-induced regulation of immediate early genes expression**

Neuronal responses to psychostimulants involve a regulatory mechanism of gene expression in different brain regions receiving monoamine inputs such as the striatum, cortex and hippocampus. These changes in gene expression represent molecular mechanisms underlying behavioral response, neuroadaptations and addiction to drugs of abuse (Berke and Hyman, 2000). Exposure to METH causes changes in the striatal expression of genes including immediate early genes (IEGs), ER stress-related genes and genes that participate in apoptotic events (Cadet et al., 2010b; Jayanthi et al., 2005; Jayanthi et al., 2009). IEGs include AP1 family of transcription factors that is composed of homodimers and heterodimers of basic region leucine zipper (bZIP) proteins that include cFos, FosB, Fra1, and Fra2, cJun, JunB, and JunD (Hughes and Dragunow, 1995). Several studies have demonstrated that METH-induced changes in these genes are dependent on DA D<sub>1</sub> receptor-mediated phosphorylation of the transcription factor cAMP response-element binding protein (CREB). By example, METH caused rapid increases in the IEG cfos in rodent striatum (Cadet et al., 2001; Wang et al., 1995b), increases which were completely blocked by SCH23390 (Cadet et al., 2010b). The role of the transcription factor cFos in METH neurotoxicity has been investigated in different studies. Heterozygote and homozygote cfos knockout mice injected with binge METH regimen, showed exacerbation of DA terminals damage in the striatum characterized by severe decrease in DAT binding, DAT protein levels and TH immunoreactivity (Deng et al., 1999). In

addition, both genotypes of cfos knockout mice showed increased apoptotic DNA fragmentation in the striatum and cortex 3 days after METH treatment. Microarray analyses revealed that METH induced increases in the expression of several DNA repair genes such as APEX, PolB, LIG1, nibrin, DDB1 and DNA mismatch repair proteins MSH3 and PMS1, but these increases were absent in cfos knockout mice (Cadet et al., 2002). These reports support a protective role for cfos against METH damage, especially against DNA damage. METH treatment also causes upregulation of fosB in rodent striatum (Cadet et al., 2001; Cadet et al., 2010b). Binge METH injections (4x10 mg/kg, 2 hours intervals) administered to fosB -/- mutant mice caused increased number of degenerated neurons in the cortex and striatum as determined by Fluoro-Jade B staining (Kuroda et al., 2010). In contrast to results obtained with cfos knockout mice (Deng et al., 1999), there was no exacerbation of striatal dopaminergic neuron degeneration or neuronal apoptosis in fosB mutant mice. This report suggests that fosB mediates neuroprotection against METH neurotoxicity as a negative feedback of calcium signaling and in supporting microglial function. Moreover, single METH injection (40 mg/kg) also caused substantial DA D<sub>1</sub>-dependent increases in the other members of the fos family, fra1 and fra2, at 2 and 4 hours posttreatment (Cadet et al., 2010b; Jayanthi et al., 2009).

In contrast to the Fos proteins, the transcription factor cJun seems to mediate activation of pro-death signalings after METH administration. Single injection of METH (40 mg/kg) caused significant increases in cjun, junB and junD mRNA of rodent striata which peaked at 2 hours after the drug injection (Cadet et al., 2001; Cadet et al., 2010b; Jayanthi et al., 2002). METH-induced increases in these genes were totally blocked by pretreatment with the D<sub>1</sub> receptor antagonist, SCH23390 (Cadet et al., 2010b). The single METH injection was also associated with increases in the protein expression of cJun, phosphorylated cJun at Ser63 and Ser73 and phosphorylated Jun-N-terminal kinases (JNK) at Thr183 and Tyr185 in mice striata (Jayanthi et al., 2002). Deng et al (2002) showed that cjun knockout mice exhibited a reduction in DNA fragmentation, caspase 3 activity and cleaved poly (ADP-ribose) polymerase (PARP) after METH treatment. However, deficit in cjun gene did not attenuate dopaminergic terminal damage (Deng et al., 2002b). These findings suggest that cjun only plays a role in the mediation of neuronal apoptosis in striatal cells. In addition, METH-induced phosphorylated cJun protein colocalized with markers of DNA fragmentation and cell death in mice striata (Deng et al., 2002b). Pretreatment with the JNK-specific inhibitor, SP600125, attenuated METH-induced JNK phosphorylation, activation of

caspase 3 and cell death in human neuroblastoma cells, while pretreatment with antioxidant vitamin E prevents METH-associated JNK phosphorylation (Wang et al., 2008). These findings suggest that oxidative stress-activated cJun/JNK signaling pathway is involved in METH-induced cell death.

METH treatment also affects the expression of the early growth response (Egr) family of IEGs, which groups Egr1 (Krox-1, NGF1A, Zif268), Egr2 (Krox20, NGF1B), Egr3 (Pilot), and Egr4 (NGF1C) (Beckmann and Wilce, 1997). Several studies have shown that METH caused induction of egr1 transcript in the striatum (Wang et al., 1995b). METH-induced egr1 transcript was markedly attenuated in the CuZn SOD transgenic mice (Hirata et al., 1998). This finding suggests that oxidative mechanism mediate the induction of egr1 after METH treatment. Recently, our laboratory has demonstrated that METH also influences the expression of other members of the family. Single dose of 40 mg/kg of METH caused increases in egr1, egr2 and egr3 in rat striatum that were blocked by SCH23390 (Cadet et al., 2010b). It is now necessary to investigate the role of each gene in response to brain injury.

In summary, different regimen doses of METH administered to an animal model will cause specific striatal transcriptional profiles. The pattern of genes induced or repressed would be a molecular hallmark of activated signaling pathways and of synaptic plasticity in response to the drug.

### 1.9.2 Mechanisms of METH-induced activation of death pathways

Apoptosis plays a critical role in mammalian development; dysregulation of apoptotic processes have been documented in models of cancer formation, in diabetes, and in neurodegenerative disorders (Cadet et al., 2007; Culmsee and Landshamer, 2006). As reviewed elsewhere (Cadet et al., 2007), we have demonstrated that METH-induced neuronal apoptosis involves a complex interaction between death pathways that have previously been identified in rodent models of neurodegenerative disorders (Culmsee and Landshamer, 2006). Because METH abusers show cognitive abnormalities and neurodegenerative changes in their brains (Ernst et al., 2000; Volkow et al., 2001d) and because identification of the processes involved might be of therapeutic value, we have been seeking to decipher the molecular and cellular mechanisms involved in METH-induced neurotoxicity.

#### 1.9.2.1 Death receptor pathway

Apoptosis is mediated by various molecular pathways that cause the activation of several members of the cysteine-aspartic proteases (caspases) family of proteolytic enzymes (Schultz and

Harrington, 2003). Jayanthi et al (2005) have shown that single toxic dose of 40 mg/kg of METH causes neuronal apoptosis in rat striatum via activation of the FasL/Fas death pathway. Activation of this programmed cell death is initiated by binding of the tumor necrosis factor, Fas L to its receptor Fas. Fas contains a cytoplasmic death domain where Fas-associated death domain (FADD) can bind in presence of Fas L, and recruits procaspase 8 for subsequent activation in caspase 8. Activated caspase 8 then activates downstream caspases, such as caspase 3. Activated caspase 3 cleaves the inhibitor of caspase-activated deoxyribonuclease (ICAD)/ DNA fragmentation factor 45 (DFF45) and inhibits its binding to CAD. The latter now enters the nucleus and degrades the cell's chromosomal DNA, leading to DNA fragmentation and cell death.

It has been shown that METH caused increases in Fas L mRNA and protein in striatal neurons that stained for GAD and enkephalin (Jayanthi et al., 2005). In addition, there was also evidence of METH-induced cleavage of procaspase 8 and induction of the effector caspase 3 that colocalized with Fas L. Pretreatment with SCH23390 blocked increases in caspases 8 and 3. Fas was also increased in the same cell as caspase 3. It appears that METH-induced activation of Fas L/Fas apoptotic pathway in the striatum depends on upregulation of calcineurin activity and activation of the transcription factors nuclear factor of activated T cells (NFAT) (Jayanthi et al., 2005). Calcineurin, a calmodulin-dependent phosphatase, causes dephosphorylation of NFAT proteins and their translocation to the nucleus (Beals et al., 1997; Jain et al., 1993). NFATs bind to AP1 and Egr families of transcription factor to regulate the expression of Fas L (Li-Weber and Krammer, 2002). METH induced upregulation of calcineurin levels, nuclear shuttling of NFATc3 and NFATc4 that were blocked by SCH23390 (Jayanthi et al., 2005). In addition, METH caused increases in different members of AP1 and Egr families. These findings suggest that the DA system via stimulation of DA D<sub>1</sub> receptors is involved in activation of the Fas L/Fas death pathway, after METH administration.

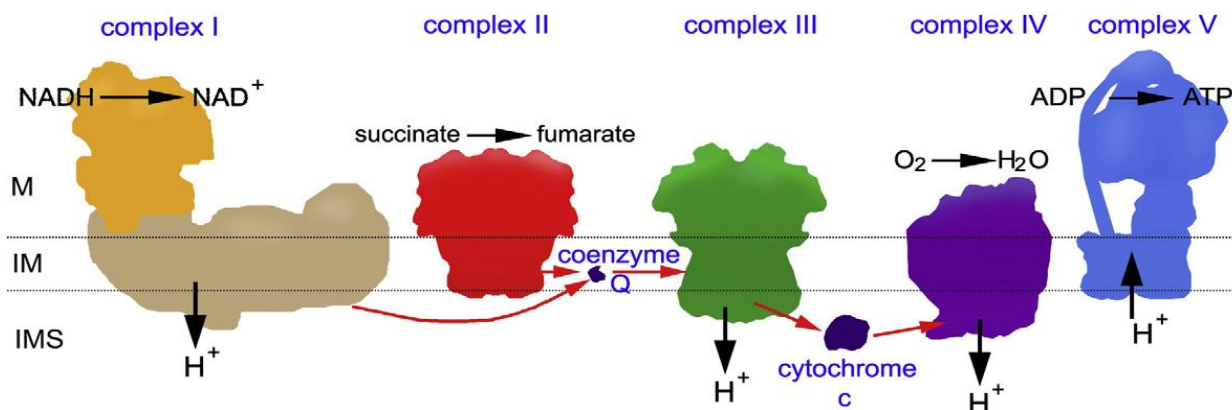
#### 1.9.2.2 Mitochondrial dysfunction

##### Mitochondria and energy metabolism

Mitochondria are the primary site of energy metabolism through the production of ATP necessary for function and maintenance of eukaryotic cells (Dudkina et al., 2010). They are double-membraned organelles; the outer mitochondrial membrane is permeable to ions and small proteins of molecular weights < 10 kDa, while the inner mitochondrial membrane houses the multimeric

enzyme complexes of the electron transport chain. Although the mitochondria contain their own DNA, most of the mitochondrial proteins of respiratory chain complexes are imported and encoded by nuclear DNA.

ATP production is usually carried out at the level of the inner mitochondrial membrane through oxidative phosphorylation involving the reduction of oxygen to water by multimeric enzyme complexes. The electron transport chain (ETC) is composed of five enzyme complexes (Figure 8): NADH-ubiquinone oxidoreductase (complex I), succinate dehydrogenase-CoQ oxidoreductase (complex II), cytochrome reductase (complex III), cytochrome oxidase (complex IV), and ATP synthase, which is sometimes referred to as complex V. During oxidative phosphorylation, ETC complexes are usually involved in reduction and oxidation reactions which generate a proton gradient across the inner mitochondrial membrane space that is used by the ATP synthase complex to synthesize ATP from ADP and inorganic phosphate Pi. The transfer of protons from the matrix to the inner mitochondrial membrane leads to the generation of a mitochondrial membrane potential  $\Delta\Psi_m$  of 150-180 mV.



**Figure 8** Representation of the oxidative system. The position of the matrix (M), the intermembrane space (IMS) and cristae or inner membrane (IM) has been indicated (Dudkina et al., 2010).

### Mitochondria and apoptosis

The ETC is an important intracellular source of ROS. During the generation of ATP via oxidative phosphorylation by the ETC components, electrons can randomly interact with oxygen ( $O_2$ ) resulting in the partial reduction of  $O_2$  to form superoxide anion  $O_2^-$ , a majority of which is generated from complex I and complex III of the ETC (Turrens, 1997). In addition to oxidative

stress, elevated cytosolic calcium represents a threat to mitochondrial function, because calcium can diffuse easily in the mitochondria through mitochondria–endoplasmic reticulum (ER) contacts, and accumulate in the matrix (Hayashi et al., 2009). Both oxidative stress and calcium deregulation can cause mitochondrial dysfunction by causing mutations of the mitochondrial DNA, depolarizing the mitochondria, altering the oxidative phosphorylation and activating apoptotic pathways (Chan, 2006; Mammucari and Rizzuto, 2010). This might cause a perturbation in ATP formation. In addition, the transient opening of the mitochondrial permeability transition pore (mPTP) will cause release of cytochrome c and other pro-apoptotic proteins (Saelens et al., 2004). Released cytochrome c can complex with apoptosis protease-activating factor 1 (Apaf-1) and procaspase 9 to form the apoptosome. The apoptosome then activates downstream effector caspases, which then initiate the apoptotic cascade.

Apoptosis can also occur without the opening of the mPTP and involves the Bcl-2 family of proteins. The Bcl-2 family group anti-apoptotic members and pro-apoptotic members that share homology in up to four conserved regions termed Bcl-2 homology (BH) 1–4 domains. The anti-apoptotic members include Bcl-2 and Bcl-X<sub>L</sub> that share homology throughout all four BH domains. The multi-domain pro-apoptotic members such as Bax, Bok and Bak comprised of BH1, BH2 and BH3 domains. The “BH3-only” pro-apoptotic members such as Bid, Bik, Bad, Bim share homology only in the BH3 domain. Pro-apoptotic members can induce mitochondrial membrane permeability, which results in the release of mitochondrial matrix proteins, while anti-apoptotic members such as Bcl-2 preserve mitochondrial integrity. The pro-apoptotic “the BH3 only” proteins activate directly multi-domain pro-apoptotic species (Letai et al., 2002; Wei et al., 2000) or indirectly by disrupting the function of anti-apoptotic Bcl-2 family members (Letai et al., 2002). Pro-apoptotic members of this family, Bax and Bad have been shown to form pores in the outer mitochondrial membrane (Donovan and Cotter, 2004), and that can lead to release of cytochrome c, apoptosis inducing factor (AIF) and endonuclease G. AIF and endonuclease G are involved in caspase-independent cell death and contribute to DNA fragmentation and subsequent chromosomal condensation, which are hallmark features of apoptosis (Donovan and Cotter, 2004). Other proteins released upon mitochondrial outer membrane permeabilisation include second mitochondria-derived activator of caspases (Smac)/direct IAP-associated binding protein with low pI (DIABLO) and Omi/high temperature requirement A2 (HtrA2) which antagonize inhibitor of apoptosis proteins (IAPs), a

family of cellular caspase inhibitors, thereby promoting caspases activation (Du et al., 2000; Suzuki et al., 2001).

#### METH-induced mitochondrial dysfunctions

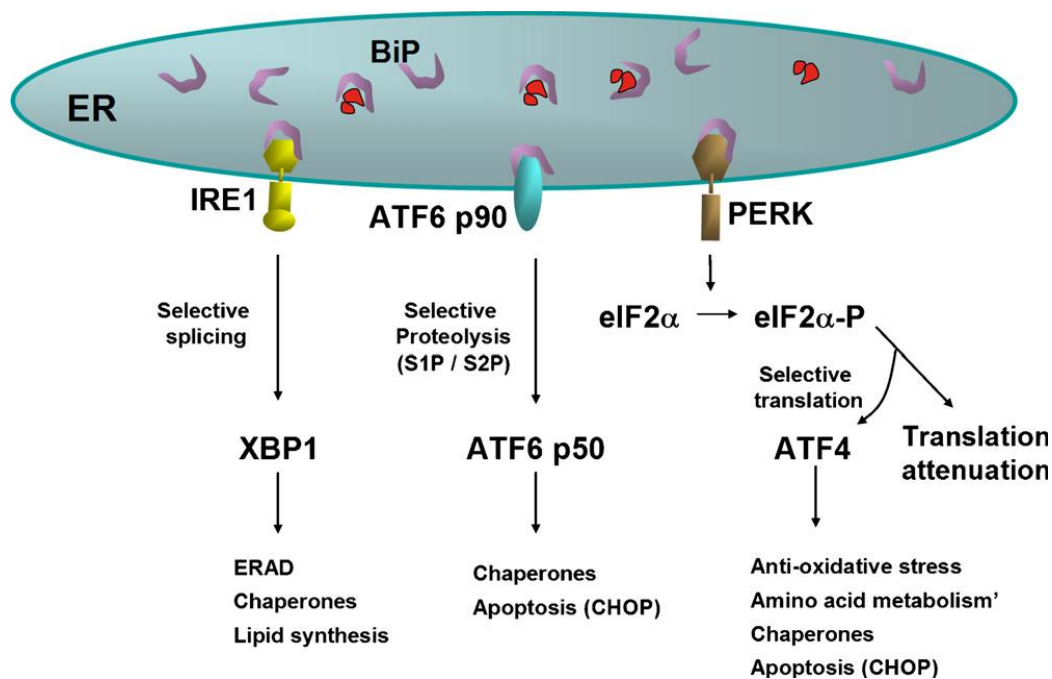
There is evidence that METH causes activation of mitochondrial-dependent death pathway (Jayanthi et al., 2001; Jayanthi et al., 2004). METH is a cationic lipophilic molecule that can diffuse easily into the mitochondria. Accumulation of positively charged particles in the mitochondria can interfere with the electrochemical gradient necessary for the ETC, and therefore, inhibit ATP synthesis. METH has been shown to cause rapid decreases in the activity of complex II (Brown et al., 2005) and complex IV of the mitochondrial ETC in the striatum (Burrows et al., 2000), causing energy deficit (Chan et al., 1994). In addition, mitochondrial dysfunction exacerbates oxidative stress in the cell, by allowing formation of ROS that cause damage to lipids (Jayanthi et al., 1998). Another hypothesis is that the displacement of calcium molecules stored in the ER to the mitochondria activates these apoptotic pathways. It is also consistent with our demonstration that METH causes cell death through interaction of the mitochondria- and ER-dependent death pathways (Jayanthi et al., 2004).

METH-induced mitochondrial dysfunction is a major determinant of lethal cell injury. Our lab has shown that METH caused increases in pro-apoptotic proteins, Bax, Bad and Bid, but decreases in antiapoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> in immortalized rat striatal cells (Deng et al., 2002a) and in mouse neocortex (Jayanthi et al., 2001). The imbalance in the expression of these genes may cause a decrease in the mitochondrial membrane potential and formation of channels in the membrane. There is evidence that a single toxic dose of METH (40 mg/kg) treatment causes release of cytochrome C, AIF and Smac/DIABLO in mouse striatum (Jayanthi et al., 2004). The release of these molecules results in activation of caspases cascade and the proteolysis of several target proteins, including PARP, lamins and CAD/DFF45 (Deng et al., 2002a; Jayanthi et al., 2004). The possibility that binge METH injections cause mitochondrial dysfunction is investigated in this thesis, as well as the effects of pretreatment with DA D<sub>1</sub> and D<sub>2</sub> receptor antagonists, SCH23390 and raclopride.

#### 1.9.2.3 Endoplasmic reticulum stress pathway

The cellular organelle, ER, is an interconnected network of tubules, vesicles, and cisternae that connects the nucleus to the Golgi. The ER participates in the synthesis, post-translational modification and the folding of membrane and secretory proteins with the assistance of chaperones (Hartl and Hayer-Hartl, 2002). Secreted and membrane associated proteins pass through the ER on their way to the Golgi for further processing. The ER also acts as a storage location for calcium, glycogen, steroids, and other macromolecules.

ER stress event occurs when there is an alteration in ER homeostasis due, by example, to disturbances of calcium level. This will cause an accumulation of misfolded proteins in the ER. Cells have developed signaling pathways to respond to ER stress, which are called the unfolded protein response (UPR) (Figure 9) and result in three different responses: upregulation of molecular chaperones to increase folding activity and reduce protein aggregation, translational attenuation to prevent accumulation of misfolded proteins, ER associated degradation (ERAD) to promote clearance of misfolded proteins by the ubiquitin-proteasome pathway (Schroder and Kaufman, 2005).



**Figure 9** ER and the unfolded protein response (Malhotra and Kaufman, 2007)



### The UPR

The UPR is initiated by three membrane sensor proteins, inositol-requiring enzyme 1 (IRE1), PERK-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Kim et al., 2006). These transmembrane proteins are normally inactive due to association of their N-terminal domains with GRP78 (glucose regulated protein 78 kDa)/BiP (immunoglobulin binding protein), an ER-resident chaperone.

#### *The IRE1-XBP1 pathway*

There are two mammalian forms of IRE1: IRE1 $\alpha$  and IRE1 $\beta$ . IRE1 $\alpha$  is expressed ubiquitously, whereas IRE1 $\beta$  is expressed only in intestinal epithelial cells (Bertolotti et al., 2001). IRE1 $\alpha$  is an ER transmembrane kinase/endoribonuclease. Its N-terminal luminal domain acts as a sensor for ER stress. Upon dissociation from BiP, IRE1 $\alpha$  dimerizes and undergoes trans- autophosphorylation to become active. Activation of the kinase domain leads to the activation of its endoribonuclease function and splicing of the mRNA of the transcription factor X-box binding protein 1 (XBP1). Spliced XBP1 encodes a basic b-ZIP transcription factor, which translocates to the nucleus and binds the ER stress-response element reporter (ERSE) DNA region in order to activate BiP, XBP1, foldases such as heat shock proteins (HSPs) and genes encoding ER-degradation-enhancing- $\alpha$ -mannidose-like protein (EDEM) (Yamamoto et al., 2004; Yoshida et al., 2003). ER chaperones transport out the misfolded proteins through the ER membrane protein translocator Sec61 complex to the cytosol. The misfolded proteins are ubiquitinated marking them for degradation by the 26S proteasome.

#### *The PERK/ATF4 pathway*

PERK, a second transducer of the UPR, regulates protein synthesis during ER stress. Like IRE1 $\alpha$ , it is an ER transmembrane kinase and its N-terminal luminal domain is sensitive to ER stress. When released from BiP, PERK dimerizes and undergoes trans-autophosphorylation via its cytoplasmic kinase domain. Activated PERK directly phosphorylates Ser51 on the  $\alpha$  subunit of eukaryotic initiation factor 2 (eIF2 $\alpha$ ). This inhibits the formation of ribosomal initiation complexes and recognition of AUG initiation codons, inhibiting general protein synthesis (Blais et al., 2004). However, there is a selective translation of UPR target genes, such as ATF4, a b-ZIP transcription factor that regulates UPR targets such as C/EBP-homologous protein (CHOP) and growth arrest and DNA damage inducible gene 34 (GADD34). Translational recovery is mediated by GADD34, a phosphatase which dephosphorylates eIF2 $\alpha$  (Novoa et al., 2001).

### *The ATF6 pathway*

ATF6 is the third transducer of the UPR and mediator of transcriptional induction. ATF6 senses stress in its N-terminal luminal domain and is dissociated from BiP upon ER stress. ATF6 goes to the Golgi where it is proteolytically cleaved by site 1 (S1) and site 2 (S2) proteases, generating an active b-ZIP factor (Hong et al., 2004). ATF6 translocates to the nucleus where it binds to several promoter elements and upregulates UPR targets such as BiP, XBP1, glucose regulated protein 94 (GRP94), HSPs, CHOP and controls expression of genes involved in ERAD (Yoshida et al., 2003).

### ER stress and caspases-dependent apoptotic pathways

In case of severe ER stress or excessive activation of the UPR, the damaged cells undergo apoptosis. There are three known pathways involved in ER stress-dependent apoptosis which are initiated by the transcription factor CHOP, the kinase JNK, and ER-associated caspase 12 in rodents (Kim et al., 2006).

### *CHOP pathway*

PERK/ATF4 and ATF6 signaling transductions activate transcription of CHOP, which is a b-ZIP transcription factor that is a member of the C/EBP family. CHOP regulates the expression of various genes, such as Bcl-2 family proteins. One proposed mechanism for the role of CHOP in mediating apoptosis is by the repression of the Bcl-2 gene promoter, potentiating the effects of the proapoptotic Bcl-2 proteins (McCullough et al., 2001). This will cause release of mitochondrial proteins such as cytochrome c, and subsequent caspases activation. Thus, this ER apoptotic pathway is mitochondrial-dependent.

### *JNK pathway*

IRE1 $\alpha$  can also initiate ER stress-mediated apoptosis through a XBP1-independent pathway. Chronic activation of IRE1 $\alpha$  leads to the recruitment of tumor necrosis factor receptor associated factor 2 (TRAF2), an adaptor protein that couples JNK activation to plasma membrane receptors (Urano et al., 2000; Yoneda et al., 2001). Moreover, an apoptosis signal-regulating kinase (ASK1) is required for TRAF2-dependent JNK activation during TNF-induced apoptosis (Nishitoh et al., 2002). Activation of JNK has been shown to lead to phosphorylation of Bcl-2 and Bcl-X<sub>L</sub> and consequently inhibition of their anti-apoptotic functions (Molton et al., 2003). Therefore, IRE1/TRAF2/ASK1/JNK-signaling pathway is important for ER stress-induced apoptosis.

### *Caspase 12 pathway*

The third apoptotic pathway is initiated by activation of caspase 12 from procaspase 12. Instead of other caspases, caspase 12 pathway is independent of mitochondria-mediated apoptotic signals (Szegezdi et al., 2003). This is consistent with the fact that caspase 12  $-/-$  mice are resistant to ER stress-specific apoptosis, but not to apoptosis from other cell death stimuli (Nakagawa et al., 2000). It is thought that caspase 12 is activated by calcium-dependent cysteine proteases, calpains, caspase 7 and TRAF2 (Nakagawa and Yuan, 2000; Yoneda et al., 2001). When caspase 12 is activated, it cleaves procaspase 9 to render caspase 9 active, which in turn activates caspase 3. Humans, however, lack functional caspase 12, but it has been suggested that human caspase 4 substitutes the function of caspase 12.

### ER stress and METH-induced cell death

Our lab first gave evidence that METH causes activation of ER stress in mice striata (Jayanthi et al., 2004). It has been shown that a single toxic dose of METH (40 mg/kg) causes activation of the UPR with increased expression of chaperones and the three ER transmembrane sensor proteins in rat striatum (Jayanthi et al., 2009). There was also activation of pro-death pathways involving the CHOP, JNK and calpain/caspase 12 pathways (Jayanthi et al., 2002; Jayanthi et al., 2004; Jayanthi et al., 2009). There was participation of the mitochondria releasing cytochrome C, AIF and Smac/DIABLO in the cytosol of striatal neurons (Jayanthi et al., 2004). Jayanthi et al (2009) also showed that single toxic dose of METH caused ER stress pathway in a DA D<sub>1</sub> receptor-dependent fashion. In this thesis, we devote one chapter to determine if binge METH injections cause activation of ER stress pathways. Thus, the possibility exists that the DA D<sub>1</sub> antagonist, SCH23390, might also protect against METH-induced these effects. We therefore examine the effects of pretreatment with SCH23390 and raclopride.

### **1.10 Methamphetamine and trophic factors**

Nerve growth factors determine survival or death of cells by activating different signaling pathways. Analysis of cDNA arrays revealed that binge METH injections caused upregulation of several trophic factors including GDF, FGF, EGF in mice striata (Cadet et al., 2002). Most studies investigate the effects of exogenous trophic factors on METH neurotoxicity. By example, an intrastriatal injection of members of GDNF family of trophic factors given 1 day before METH

treatment prevented METH-induced reductions in striatal DA levels (Cass, 1996; Cass et al., 2006). In addition, cultures of rat primary cortical neurons exposed to brain-derived neurotrophic factor (BDNF) before METH were protected against METH-induced neuronal death (Matsuzaki et al., 2004). Moreover, BMP-7 also prevented METH-induced apoptosis in vitro as well as decreases in TH-immunoreactivity in vitro and in vivo (Chou et al., 2008). This study also demonstrated that heterozygote BMP-7 +/- mice were more sensitive to METH treatment. These findings support a role of these neurotrophic factors in the protective mechanisms against METH toxicity. The effects of METH on the signal transduction of activin A and TGF- $\beta$  trophic factors are investigated in this dissertation, as well as the influence of DA neurotransmission on these signaling.

#### 1.10.1 Activins and TGF- $\beta$ s

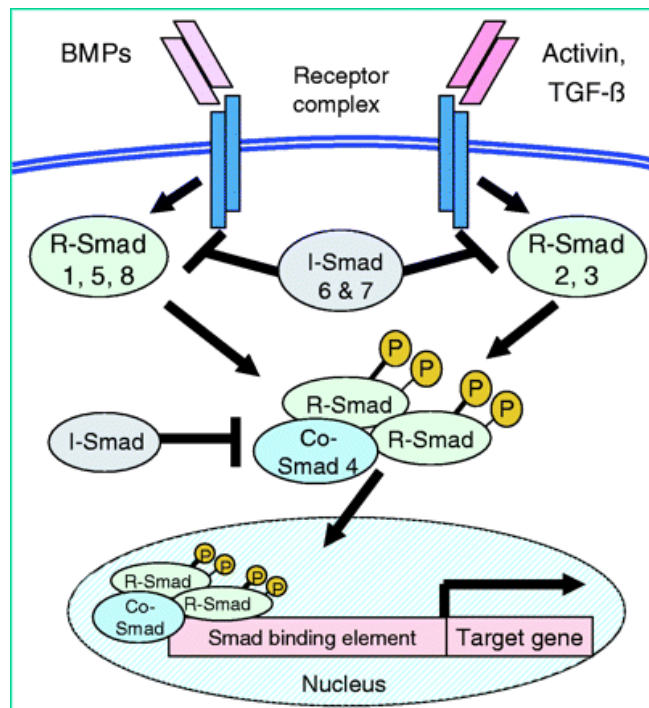
Transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily comprises nearly 30 proteins in mammals, including GDNF, TGF- $\beta$ s (isoforms 1, 2 and 3), activins and inhibins, nodal and bone morphogenetic proteins (BMPs). These factors mediate signals through serine/threonine kinase receptors (Derynck, 1994). The focus in this thesis is the regulation of expression of activin and TGF- $\beta$  after toxic doses of METH.

The mammalian brain expresses activin  $\beta$ A and  $\beta$ B mRNA. These translated subunits are combined to form activin A ( $\beta$ A- $\beta$ A), activin B ( $\beta$ B- $\beta$ B) and activin AB ( $\beta$ A- $\beta$ B) (Thompson et al., 2004b). Basal expression of activin  $\beta$ A mRNA is broadly distributed on neurons with particular predominance in the olfactory bulb, frontal cortex and striatum (Roberts et al., 1996). Activin A binds to type II receptors, and the complex recruits and activates the activin-like type IB receptor, to cause intracellular signals (Abe et al., 2004; Carcamo et al., 1994).

TGF- $\beta$  family consists of three isoforms: TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3. TGF- $\beta$ 2 and TGF- $\beta$ 3 mRNAs are found in all areas of the CNS including cortex, hippocampus, striatum, brainstem and cerebellum (Bottner et al., 2000; Gomes et al., 2005). There are very few anatomical studies of the distribution of TGF- $\beta$ 1 in the brain. One study reported that TGF- $\beta$ 1 mRNA is constitutively expressed in some brain regions such as the hippocampus, cortex and hypothalamus (Vivien and Ali, 2006). TGF- $\beta$  binds to TGF- $\beta$  type I receptors (T $\beta$ RI) and TGF- $\beta$  type II receptors (T $\beta$ RII) in order to propagate downstream signals (Carcamo et al., 1994).

The signaling pathway of TGF- $\beta$  family is well conserved and involves three subclasses of Smad proteins (Moustakas et al., 2001): Receptor-regulated Smads (R-Smads) grouping Smad1,

Smad2, Smad3, Smad5 and Smad8, the common mediator or co-Smad (Smad4), and two inhibitory Smads (I-Smads) (Smad6 and Smad7). Activated activin or TGF- $\beta$  type I receptor results in phosphorylation and activation of R-Smads. TGF- $\beta$  and activin receptors phosphorylate Smad2 and Smad3, while BMP receptors phosphorylate Smad1, Smad5 and Smad8. Phosphorylated R-Smad then forms a complex with the co-Smad, Smad4, which translocates into the nucleus to undergo transcriptional regulation of target genes. I-Smads interfere with the receptor activation or complex formation of R-Smads (Figure 10). In this dissertation, the striatal expression of phosphorylated Smad2 (P-Smad2) used as a marker for activin A/TGF- $\beta$  response, is measured after METH and SCH23390 treatments.



**Figure 10** TGF- $\beta$  superfamily signaling by Smads (Itman et al., 2006)

#### 1.10.2 Activin A and TGF- $\beta$ s in brain injury

Activin A was found to promote survival of neuronal cells in vitro. It supported retinal neurons (Schubert et al., 1990), as well as midbrain dopaminergic neurons (Kriegstein et al., 1995) and hippocampal neurons (Iwahori et al., 1997) in culture. Activin A also protected cortical neurons in culture from hydrogen peroxide-free radical stress (Mukerji et al., 2007), highlighting its role as an acute response to oxidative stress. Several reports have shown the induction of activin A in several

models of acute brain injury (Florio et al., 2007; Munz et al., 1999). Activin  $\beta$ A mRNA was increased in models of hypoxia/ischemia (Mukerji et al., 2007; Wu et al., 1999), mechanical brain injury (Lai et al., 1997) and excitotoxicity (Tretter et al., 1996). These findings suggest that increased activin A is an endogenous response to acute brain injury. There is evidence that this trophic factor supports a protective role in vivo. Exogenous activin A reduced neuronal death in hippocampus and dorsolateral striatum of infant rats, caused by ischemic brain injury (Wu et al., 1999). Additionally, a role for endogenous activin A in preventing neuronal cell death is suggested with increased neuronal death following kainic acid lesions in transgenic animals expressing dominant negative activin type IB receptor in neurons (Muller et al., 2006). This report suggests that activin A protects against excitotoxic injury.

TGF- $\beta$ s 1-3 also promote survival and protection of mesencephalic dopaminergic neurons in vitro (Kriegstein et al., 1995; Poulsen et al., 1994). In models of neuronal death, TGF- $\beta$  proteins have been reported to be up-regulated after models of ischemia (Ata et al., 1999; Wiessner et al., 1993). The effects of TGF- $\beta$ 1 on brain damages have been investigated using exogenous application of the factor. Accordingly, intra-cerebroventricular injection of TGF- $\beta$ 1 before the induction of focal ischemia in mice (Prehn et al., 1993) or global ischemia in rats (Henrich-Noack et al., 1996) leads to a moderate reduction of brain lesions. TGF- $\beta$ 1 protected hippocampal and cerebellar neurons against N-methyl-D-aspartate (NMDA) and kainate toxicity (Prehn et al., 1994; Prehn and Miller, 1996). In addition, it protected cortical neurons against NMDA (Bruno et al., 1998). Use of a soluble receptor as TGF- $\beta$  antagonist markedly exacerbates transient focal ischemic damages in rats, highlighting the role of endogenous TGF- $\beta$  in protective response of the brain (Ruocco et al., 1999).

TGF- $\beta$ s and activin A both are involved in response to alterations in calcium levels and oxidative stress. Toxic doses of METH also cause oxidative stress and excitotoxicity marked by increase calcium in the neuron (Cadet et al., 2007; Krasnova and Cadet, 2009). Therefore, we hypothesized a response to METH toxicity may be an induction of TGF- $\beta$ s and activin A striatal expression. Recently, our lab shows that METH injection caused increases in activin  $\beta$ A mRNA in the intact and denervated sides of the striatum in a model of hemiparkinsonian rats (Cadet et al., 2010a). In this thesis, we investigated the effects of toxic binge regimen of METH and pretreatment with SCH23390 or raclopride on activin A and TGF- $\beta$  signals transduction in rat striatum.

### 1.11 Research aims

As outline in the previous paragraphs, much work has been done seeking to decipher the molecular and cellular mechanisms involved in METH-induced neurotoxicity. Considering the efficiency of the DA D<sub>1</sub> receptor antagonist, SCH23390, to block some of the toxic effects of METH, it is important to elucidate the basic mechanisms in the protective effects of the dopaminergic antagonist, SCH23390. This thesis examined the multiple signaling pathways activated after toxic binge METH injections in rats and possible neuroprotective agents (SCH23390 and raclopride).

The manuscript 1 examines if the neuroprotective effects of SCH23390 pretreatment, prior to binge METH injections, is due to a blockade of the regulation of striatal IEGs expression observed after METH administration

The aim of the manuscript 2 was to examine whether SCH23390 also provides protection against binge METH-induced ER and mitochondrial stress pathways. We also test the effects of the DA D<sub>2</sub> receptor antagonist, raclopride, on these stress pathways.

In the manuscript 3, we investigate the effects of the SCH23390 and raclopride on METH-induced changes in striatal and cortical DA, 5-HT and their metabolites. We also examine whether the antagonists attenuate METH-induced hyperthermia.

The aim of the manuscript 4 was to assess if response to METH toxicity involves the neurotrophic factors, activin A and TGF- $\beta$ s and activation of their transduction pathways.

## **Manuscript 1**

**Differential effects of methamphetamine and SCH23390 on the expression of members of IEG families of transcription factors in the rat striatum**



**Differential effects of methamphetamine and SCH23390 on the expression of members of IEG families of transcription factors in the rat striatum**

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## Abstract

Methamphetamine (METH) is a psychostimulant that can cause long-lasting neurodegenerative effects in humans and animals. These toxic effects appear to occur, in part, via activation of dopamine (DA) D<sub>1</sub> receptors. This paper assessed the possibility that the DA D<sub>1</sub> receptor antagonist, SCH23390, might inhibit METH-induced changes in the expression of several members of immediate early genes (IEGs) which are known to control more delayed expression of other genes. We found that injections of METH (4x10 mg/kg, given at 2 h intervals) caused significant increases in c-fos and fra-2 expression which lasted from 30 min to 4 h. Pre-treatment with SCH23390, given 30 min before each METH injection, completely blocked METH-induced expression of c-fos, but only partially inhibited fra-2 mRNA expression. These results were confirmed by western blot analysis which showed METH-induced changes in c-Fos protein expression that were blocked by pretreatment with SCH23390. There were also delayed METH-induced DA D<sub>1</sub> receptor-dependent effects on fosB mRNA expression. Even though fra-1 expression was not affected by pretreatment with METH alone, the repeated injections of SCH23390 caused substantial decreases in fra-1 mRNA expression in both the presence and absence of METH. The repeated injections of METH caused no changes in the mRNAs for c-jun, junB or junD. However, there were significant increases in the phosphorylation of c-Jun protein (ser63). Phosphorylation of c-Jun occurred in a delayed fashion (16 and 24 h after the last METH injections) and was attenuated by SCH23390 pretreatment. Interestingly, SCH23390 given alone caused significant decreases in phospho-c-Jun at all time-points. The METH injections also caused delayed induction in the expression of members of the Egr family of transcription factors in a DA D<sub>1</sub> receptor-dependent fashion. Repeated injections of SCH23390 caused substantial suppression of basal striatal egr-1 and egr-2 mRNA expression but not of that of egr-3. Both *crem* and *arc* mRNA levels were induced by METH in a SCH23390-sensitive fashion. Moreover, multiple injections of SCH23390 given alone caused marked inhibition of basal *arc* expression. These results show that multiple injections of METH can differentially affect the expression of several IEGs, some of which occurred in a DA D<sub>1</sub> receptor dependent fashion. The SCH23390-mediated suppression of basal fra-1, egr-1, and egr-2 mRNA levels suggests that their basal expression in the striatum might be dependent on tonic stimulation of the DA D<sub>1</sub> receptor.

## Introduction

Methamphetamine (METH) is an addictive drug that causes long-term motor and cognitive abnormalities (Volkow et al., 2001; Gold et al., 2009). Imaging studies using positron emission tomography and post-mortem studies of the brains of METH-abusers have provided evidence for loss of striatal dopamine (DA) nerve terminals (Wilson et al., 1996; Volkow et al., 2001). Studies in rodents have shown that administration of toxic doses of METH, either as single or multiple injections, results in long-term changes in the brain (Krasnova and Cadet, 2009). These abnormalities include decreases in the levels of striatal DA and serotonin (5-HT) and of their metabolites as well as decreases in the activity of their biosynthetic enzymes (Kita et al., 2003). The use of toxic doses of METH can also cause neuronal apoptosis in the striata and cortices of rodents (Deng et al., 2002; Jayanthi et al., 2005; Thiriet et al., 2005; Cadet et al., 2005).

The pharmacological and neurotoxic effects of METH depend on the release of DA from striatal DA terminals and stimulation of DA receptors in the striatum which contains high densities of DA D<sub>1</sub> and D<sub>2</sub> receptors (Russell et al., 1992, O'Dell et al., 1991, 1993). The toxic effects of the drug depend, in part, on activation of D<sub>1</sub> receptors because inhibition of DA D<sub>1</sub> receptors protects against METH-induced toxicity in the striatum (Jayanthi et al., 2005; Xu et al., 2005). These protective effects of DA D<sub>1</sub> antagonism might occur through inhibition of DA D<sub>1</sub> receptor-mediated induction of genes that might be involved in pro-toxic cascades (Cadet et al., 2005; Jayanthi et al., 2005, 2009). To test the idea, we measured the effects of multiple METH injections on the expression of immediate early genes (IEGs), including several members of AP-1 and Egr families of transcription factors, in the absence and presence of the DA D<sub>1</sub> receptor antagonist, SCH23390 (Iorio et al., 1983). We also measured the effects of SCH23390 on METH-induced changes in arc and crem mRNA levels because Arc is an effector gene involved in synaptic plasticity (Bramham et al., 2008) and because Crem participates in the regulation of neuroadaptive processes (Hughes and Dragunow, 1995). Our experiments show that multiple injections of METH doses which are known to cause long-term DA depletion and neuronal apoptosis (Deng et al., 1999; Ladenheim et al., 2000; Jayanthi et al., 2005) are associated with marked increases in the expression of several IEGs in a SCH23390-sensitive fashion.

## Experimental procedures

## **Animals and Drug Treatment**

Male Sprague-Dawley rats (Charles River Labs, Raleigh, NC, USA), weighing 250-300g were maintained in a room at temperature of 22 °C and had free access to food and water. They were divided into four groups of animals: [i) saline (C), ii) SCH23390 (0.5 mg/kg x 4 injections every 2 h) (S), iii) METH (10 mg/kg x 4 injections every 2 h) (M), and iv) SCH23390 plus METH (S + M)]. All injections were given intraperitoneally. The SCH23390 injections were given 30 min before each METH injection. For quantitative PCR, rats were euthanized at 30 min, 2, and 4 h after drug injections. For Western blot analysis, animals were euthanized at 30 min, 2, 4, 16, and 24 h after the last drug injection. All animal use procedures were according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

## **Isolation of RNA**

After animals were euthanized, striatal tissues were rapidly dissected, placed on dry ice and stored at -80°C (n = 6 per group). Total RNA for the 30 min, 2 h and 4 h samples was extracted with Qiagen RNeasy Midi kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Analysis of samples for quality and quantity was assessed using an Agilent 2100 Bioanalyzer 2 (Agilent, Palo Alto, CA, USA).

## **Reverse transcription and mRNA quantitation**

The expression levels of several immediate genes were studied by real-time quantitative polymerase chain reaction (qPCR). Total RNA for each sample was reverse-transcribed using oligo (dT) into cDNA using Advantage RT for PCR kit (Clontech, Mountain View, CA, USA). For PCR amplification of cDNA, primer Sequences for rat were generated by the LightCycler probe design software v. 2.0 (Roche, Indianapolis, IN, USA) and purchased from Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD, USA). The primer list is shown in Table 1. PCR experiments were performed on Lightcycler 480 II (Roche, Indianapolis, IN, USA), using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA). The relative amounts of messenger RNA were normalized to clathrin mRNA and then quantified.

## **Western Blot**

Protein for striatal tissues were homogenized in a buffer containing 320 mM sucrose, 5 mM HEPES, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin. Homogenates were

centrifuged at 5000g for 5 min, and the supernatant fractions were subsequently centrifuged at 30,000g for 30 min. The resulting pellet was resuspended in the sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 50 mM dithiothreitol). Protein concentration was quantified with the BCA protein assay kit (Thermo scientific, Rockford, IL, USA). The lysates were denatured in sample buffer at 100 °C, and separated by SDS-PAGE. After the proteins were electrophoretically transferred on PVDF membranes, and membrane blocking, primary and secondary antibody incubations, and chemiluminescence reactions were carried out according to the protocol described by individual antibody suppliers. The membranes were incubated with c-Fos, c-Jun (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and phospho-c-Jun (Ser63) (New England Biolabs, Beverly, MA, USA) (1:1000) antibodies, at 4 °C overnight. The blots were re-probed with  $\alpha$ -Tubulin antibody (1:4000; Sigma, 2 h at room temperature). For quantification, the signal intensity was normalized over the signal intensity of  $\alpha$ -Tubulin. Signal intensity was measured densitometrically with LabWorks version 4.5 (BioImaging Systems analysis software, BioImaging System, UVP Inc., Upland, CA, USA).

### **Statistical Analysis**

For analysis of the qPCR data, the values used consist of a ratio of the fluorescence values, normalized to the values of the endogenous gene clathrin. Values represent means  $\pm$  SE (6 animals/group). The fold changes in gene expression were generated from normalized data from the various in comparison to the control group. Statistical analysis for the q-PCR and western blot data was carried by a one-way ANOVA followed by Fisher's protected least square difference (PLSD) test using StatView (SAS Institute, Cary, NC, USA). The null hypothesis was rejected at  $p < 0.05$ .

### **Results**

#### **Multiple injections of METH caused differential changes in the expression of fos and jun families of IEGs**

Fig. 1 shows the effects of METH and SCH23390 on members of the fos family of transcription factors. Repeated injections of SCH23390 alone caused no changes in c-fos expression (Fig. 1A). METH injections caused rapid and substantial increases in c-fos expression which were apparent at 30 min and lasted for the 4 h duration of the study. Injections of saline before each of

the four METH injections gave identical results to the injections of METH alone (data not shown). Injections of SCH23390 before each METH administration caused total inhibition of METH-induced c-fos expression (Fig. 1A). mRNA levels were measured according to a standard curve for each gene. We used 6 replicates for each reaction. These reactions yielded a standard curve with a slope of -3.3 and the efficiency value was  $\approx 2$ . The amplification curve for a replicate of c-fos mRNA is shown in Fig. 1B. Similar curves were generated for each gene at all time-points and are available on request. The protein level of c-Fos was also assessed and showed also increased at 30 min, 2 h and 4 h (Fig. 2). In contrast to the rapid METH-induced changes in c-fos expression, METH caused somewhat more delayed increases in fosB expression occurring at the 4 h time-point (Fig. 1C). Pretreatment of the animals with SCH23390 also blocked the METH effects of fosB expression (Fig. 1C). Unexpectedly, repeated injections of METH caused no changes in fra-1 expression in the rat striatum (Fig. 1D). In contrast, repeated injections of SCH23390 caused significant decreases in fra-1 expression at the 4 h time-point in both the absence and presence of METH, suggesting that the latter effects were due solely to DA D<sub>1</sub> receptor antagonism. Fig. 1D shows that the METH-induced effects on fra-2 expression are somewhat similar to those observed for c-fos expression. Specifically, fra-2 expression was rapidly induced by METH, peaked at 2 h then started to revert towards normal by the 4 h time-point (Fig. 1E). Interestingly, SCH23390 pretreatment blocked the effects of METH on fra-2 expression only at the 30-min time-point, suggesting that METH might cause the more delayed increases via mechanisms other than stimulation of DA D<sub>1</sub> receptors.

The repeated injections of METH caused no significant changes in the expression of c-jun (Fig. 3A), junB (Fig. 3B) and junD (Fig. 3C). Repeated injections of saline before each METH injection did not affect these results (not shown). Injections of SCH23390 alone caused no significant decreases in basal expression of these genes when compared to controls. As previously reported, c-Jun protein exerts its action via phosphorylation at serine-63 or -73 though the actions of c-Jun N-terminal kinases (JNKs) (Raivich, 2008), a process that seems to be involved in METH-induced toxicity since a single large toxic dose of METH can cause increased c-Jun phosphorylation at ser-63 (Jayanthy et al., 2002). Similarly, repeated injections of METH used in the present study also caused c-Jun phosphorylation at 16- and 24-h after the last METH injection (Fig. 4). In contrast, SCH23390 caused significant decreases in the basal levels of phosphorylated c-Jun. These changes

are not due to increased c-Jun protein expression since, similar to the PCR results, METH caused no changes in total c-Jun protein expression.

### **Effects of toxic doses of METH on egr expression**

The effects of METH on members of the Egr family of transcription factors are shown in Fig. 5. Repeated injections of SCH23390 alone caused significant suppression of the basal egr-1 expression (Fig. 5A). SCH23390 caused decreases in basal egr-1 expression at 60 min after the last injection of SCH23390 (30 min after the last METH injection) in both the absence and presence of METH. METH caused significant increases in egr-1 expression at the 4-h time-point. Injections of saline before each METH injection caused identical changes to those observed in the METH alone group (not shown). SCH23390 pretreatment blocked the METH-induced changes. Repeated injections of SCH23390 also suppressed basal egr-2 expression at all the time-points studied. METH caused time-dependent increases in egr-2 expression which become significant at the 2-h time-point (Fig. 5B). Pretreatment with SCH23390 also blocked METH-mediated increases in egr-2 expression. Injections of SCH23390 alone caused no significant changes in egr-3 expression (Fig. 5C). METH caused time-dependent increases in egr-3 expression. These increases were of greater magnitude than the changes observed in egr-1 and egr-2 expression (compare Fig. 5C to 5A and 5B). SCH23390 pretreatment also blocked the METH-induced increases in egr-3 expression.

### **Effects of METH and SCH23390 on arc and crem expression in the striatum**

Fig. 6 shows the effects of METH and SCH23390 on arc and crem mRNA levels. Administration of SCH23390 alone caused suppression of basal arc mRNA (Fig. 6A). METH injections caused small increases in arc expression at the 30-min and 2-h time-points, with reversal towards normal at the 4-h time-point. SCH23390 pretreatment blocked the METH-induced increases in arc expression (Fig. 6A). Interestingly, the levels of arc mRNA in the groups that got the combined SCH23390 and METH treatments were comparable to the levels observed in the SCH23390 alone groups (compare the S groups to the M+S groups in Fig. 6A).

Repeated injections of SCH23390 had no effects on basal crem expression in comparison to the control group (Fig. 6B). Repeated injections of METH caused increases in crem mRNA levels which were observable at the 30-min time-point and lasted for the duration of the study.

Administration of SCH23390 prior to the injections of METH also blocked METH-mediated increases in *crem* expression (Fig. 6B).

## Discussion

The main findings of this paper are that (i) multiple injections of METH caused substantial increases in the expression of multiple transcription factors in the rat striatum; (ii) the DA D<sub>1</sub> receptor antagonist, SCH23390, blocked these METH-induced changes in a time-dependent fashion; and that (iii) repeated injections of SCH23390 can suppress the basal levels of *egr-1*, *egr-2* and *arc* mRNAs. Unexpectedly, the repeated injections of METH caused no changes in the levels of *c-jun*, *junB* or *junD* mRNAs. The observations of METH-induced increases in IEG mRNA levels and their dependency, for the most part, on stimulation on DA D<sub>1</sub> receptors are consistent with the report that four injections of METH, given according to a schedule similar to the one used in the present study, can cause increases in DA efflux that stayed elevated for 16 hours after the last METH injection (O'Dell et al., 1991). The results of the present study are also consonant with and extend those of previous papers that have reported that single injections of METH (40 mg/kg) caused significant increases in *c-fos*, *fosB*, *fra-2*, *c-jun*, *junB* and *junD* in the mouse striatum (Cadet et al., 2001; Jayanthi et al., 2009). We also extended our previous results by showing that the effects of multiple METH injections occur mostly via stimulation of DA D<sub>1</sub> receptor. These results are consistent with previous observations that acute administration of cocaine (Berretta et al., 1992) and amphetamine (Wang et al., 1995) can cause increases in a number of IEGs in a DA D<sub>1</sub> receptor-dependent fashion.

The early induction of *c-fos* observed within 30 min of the last METH injection was not surprising, given the fact that *c-fos* is an IEG that is activated within minutes of neuronal stimulation (Hughes and Dragunow, 1995). This observation is consistent with those of other investigators who have reported that single injections of variable doses of METH can cause rapid increases in the expression of *c-fos* in the rodent brain (Wang et al., 1995, Thiriet et al., 2001). Because METH toxicity is exacerbated in *c-fos* knock-out mice (Deng et al., 1999), we had proposed previously that METH-induced increases in *c-fos* expression might constitute an attempt for the organism to protect striatal neurons against METH-induced neuronal apoptosis. The observation that the repeated injections of the DA D<sub>1</sub> receptor antagonist, SCH23390, can block



METH-induced increases in c-fos is consistent with observations of other authors who have reported that cocaine and METH-induced c-fos protein expression occurs via DA D<sub>1</sub> receptor-mediated mechanisms (Young et al., 1991, Yoshida et al., 1995). Our observations of METH-induced increases in fosB and fra-2 expression are also consistent with previous demonstrations that cocaine can also affect the expression of other fos-related proteins (Nye et al., 1995) and that METH can cause increases in fosB expression in mice (Cadet et al., 2002). Similar to c-fos, the METH-induced increases in fosB might be involved in a neuroprotective loop since METH toxicity is exacerbated in fosB knockout mice (Kuroda et al., 2009).

Our observations of METH-induced increases in fra-2 expression are consistent with the previous demonstration that administration of METH doses, similar to those used in our study, caused substantial increases in Fra-2 protein in the mouse striatum at 3 days post-drug (Pennypacker et al., 2000). The timing in protein expression reported in the Pennypacker paper was, however, more delayed than the changes in mRNA levels as might be expected. The current study extends those results further by showing that the early but not the delayed effects of METH on fra-2 mRNA are dependent on stimulation of striatal DA D<sub>1</sub> receptors. The lack of inhibiting effects of SCH23390 on METH-induced changes on fra-2 at the 2-h and 4-h time-points suggests that METH might induce fra-2 via other mechanisms which involve oxidative stress (Kranova and Cadet, 2009). When taken together, these results suggest the possibility that several members of the fos family of transcription factors might work in tandem to protect the brain against METH toxicity.

Fos proteins are known partners of Jun members of the AP-1 family of transcription factors (Raivich, 2008). These transcription factors participate in the regulation of neurotransmission via their influence on genes that code for various neurotransmitters (Hughes and Dragunow., 1995). Although our PCR results did not show any effects of METH on the expression of c-jun, junB and junD, we found that repeated injections of METH did cause c-Jun phosphorylation at ser-63 which is an important activating mechanism for that protein (Derijard et., 1994; Raivich, 2008). We identified significant increases in phospho-c-Jun (Ser63) at the 16 and 24-h time-points, which were dependent on DA D<sub>1</sub> receptor stimulation since pretreatment with SCH23390 blocked these changes. The manner by which stimulation of DA D<sub>1</sub> receptors can cause phosphorylation of c-Jun might occur via its effects on the cAMP/PKA cascade which is known to interact with the SAPK pathway to activate JNK (Disa et al., 2000; Zhen et al., 1998).

Egr family transcription factors are expressed in several brain regions including the striatum and can be induced in responses to various stimuli to promote memory formation, tolerance to the effects of drugs, and to promote synaptic plasticity (Alberini, 2009). As shown above, SCH23390 treatment alone caused significant decreases in basal egr-1 and egr-2 expression, findings that are consistent with those of Mailleux et al (1992) who had used in situ hybridization technique and reported that SCH23390 can cause significant decreases in basal egr-1 expression in the rat striatum. These observations, taken together, indicate that basal levels of egr-1 and egr-2 mRNA are dependent on baseline stimulation of DA D<sub>1</sub> receptors. Our observations that repeated injections of METH caused significant time-dependent increases in egr-1, egr-2 and egr-3 expression are compatible with those of earlier studies that had reported METH-induced egr-1 expression after single injections of METH (Thiriet et al., 2001, Wang et al., 1995). Jayanthi et al (2005) had previously reported that a single large dose of METH can also cause changes in the expression of these IEGs and that they were involved in the activation of the FasL/Fas death pathway in the rat striatum. Our present observations that multiple injections of METH can also result in egr expression are consistent with these results and support the idea that these transcription factors might indeed play a role in mediating METH-induced degeneration of intrinsic striatal cells (Jayanthi et al., 2005). Nevertheless, because these IEGs are known to influence processes that lead to synaptic plasticity and memory formation (Bozon et al., 2002; Ko et al., 2005, Li et al., 2007), it is possible that these transcription factors might also be involved in METH-induced neuroadaptive changes in the brain. This discussion is also consistent with the fact that Egr-1 and Egr-3, which are induced by METH, can regulate the expression of Arc (Li et al., 2005), an METH-inducible effector gene that is involved in long-term potentiation (LTP) and neuronal plasticity (Bramham et al., 2008). It is thus not farfetched to suggest that the METH-induced increases in striatal arc mRNA expression might play a role in the development of structural plasticity observed after chronic exposure to psychostimulant (Robinson and Kolb., 2004).

The regulation of the expression of several transcription factors that are influenced by direct and indirect DA agonists is mediated, in part, by phosphorylation of the cAMP Response Element Binding (CREB) protein (Hyman et al., 1995, Hughes and Dragunow., 1995) although there are many IEGs whose expression is independent of stimulation of DA D<sub>1</sub> receptors (Jayanthi et al., 2009). CREB function is regulated, in part, by ICER, which is an inducible product of the CREM gene (Borlikova and Endo., 2009, Mioduszevska et al., 2003). ICER is a powerful repressor which

is important for the transient nature of cAMP- induced gene expression (Mioduszevska et al., 2003). Thus, the observed METH-induced changes in CREM expression might serve to maintain cellular homeostasis by suppressing CREB-mediated gene expression.

In summary, the present data show that administration of METH caused differential changes in the expression of several IEGs, for the most part, in a DA D<sub>1</sub> receptor-dependent fashion. The time-course of these changes and their dependency, for the most part, on stimulation of DA D<sub>1</sub> receptors, are consistent with the fact that repeated injections of METH can cause prolonged elevation (up to 16 h after the last METH injection) of the levels of DA in the extracellular space (O'Dell et al., 1991). In any case, the observations that SCH23390 can block the METH-induced increases in the expression of the majority of the mRNAs measured in the present study provide a partial explanation for the protective effects that pretreatment with the DA antagonist, SCH23390, affords against METH-induced cell death in the striatum (Xu et al., 2005). Finally, these observations further implicate DA D<sub>1</sub> receptor-mediated mechanisms as important targets for therapeutic interventions against METH addiction and toxicity.

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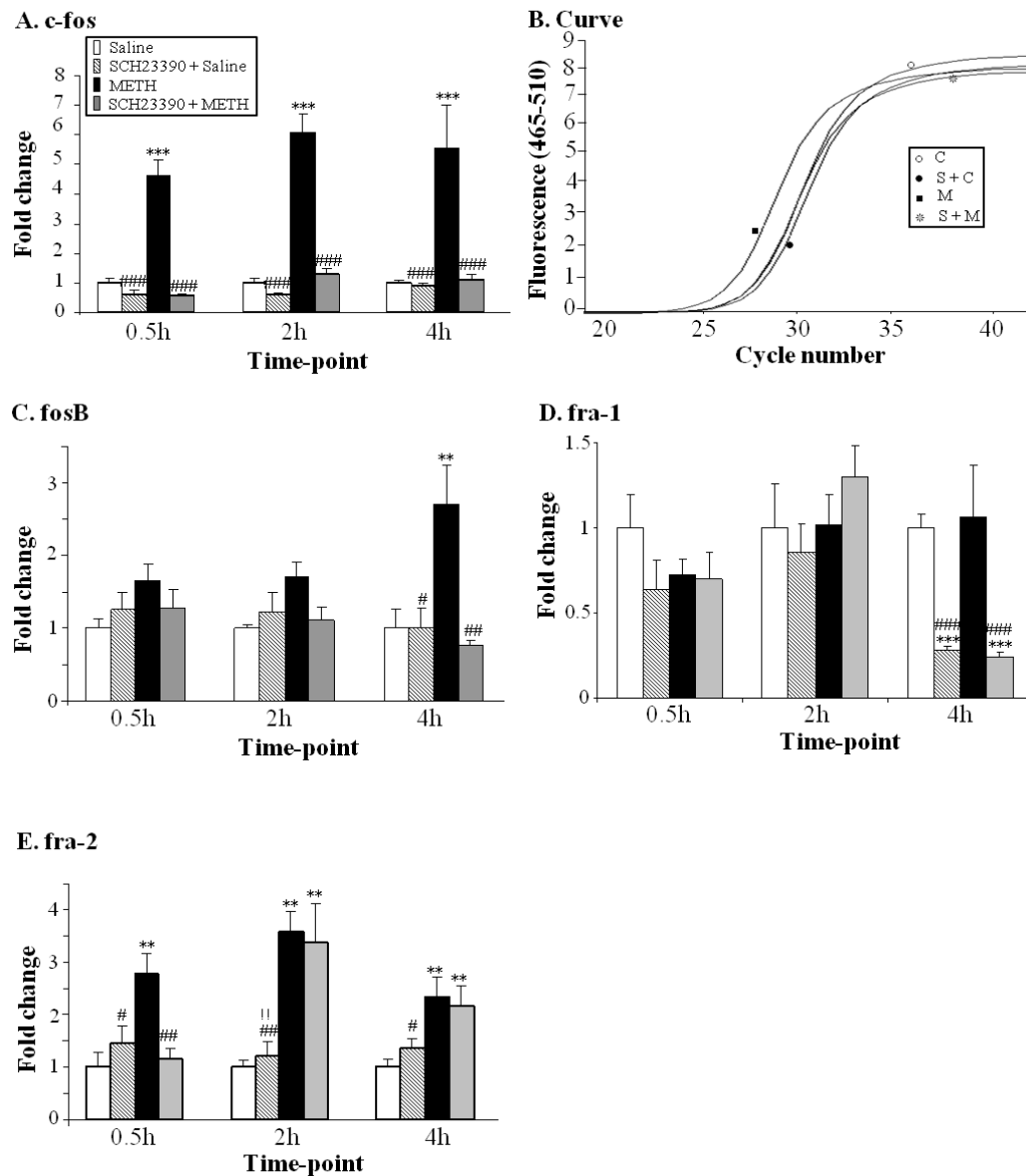
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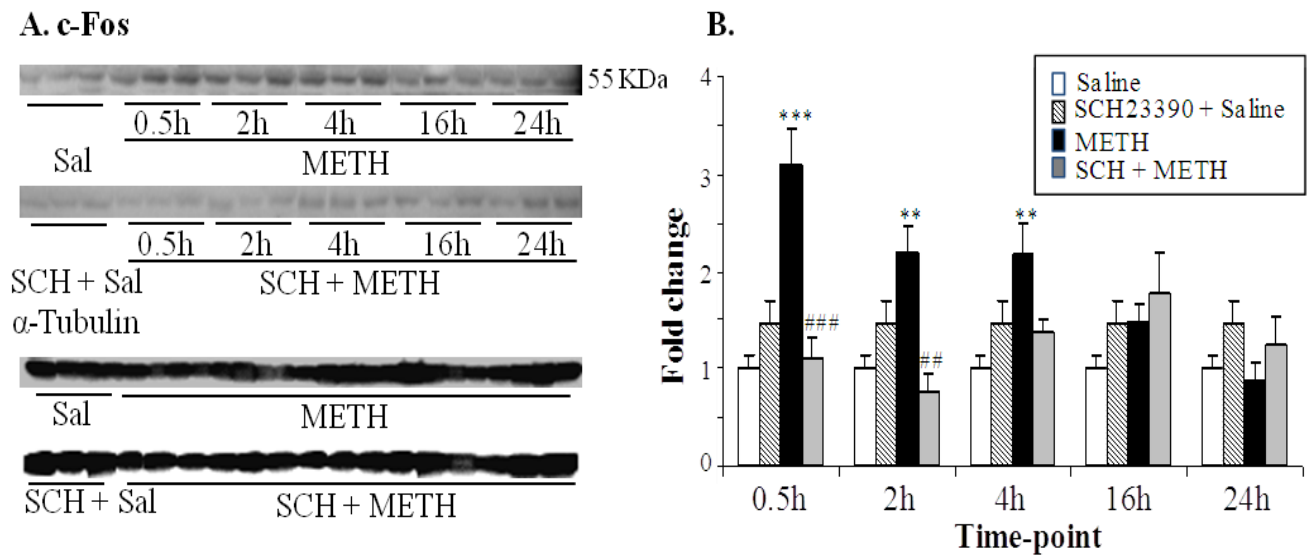
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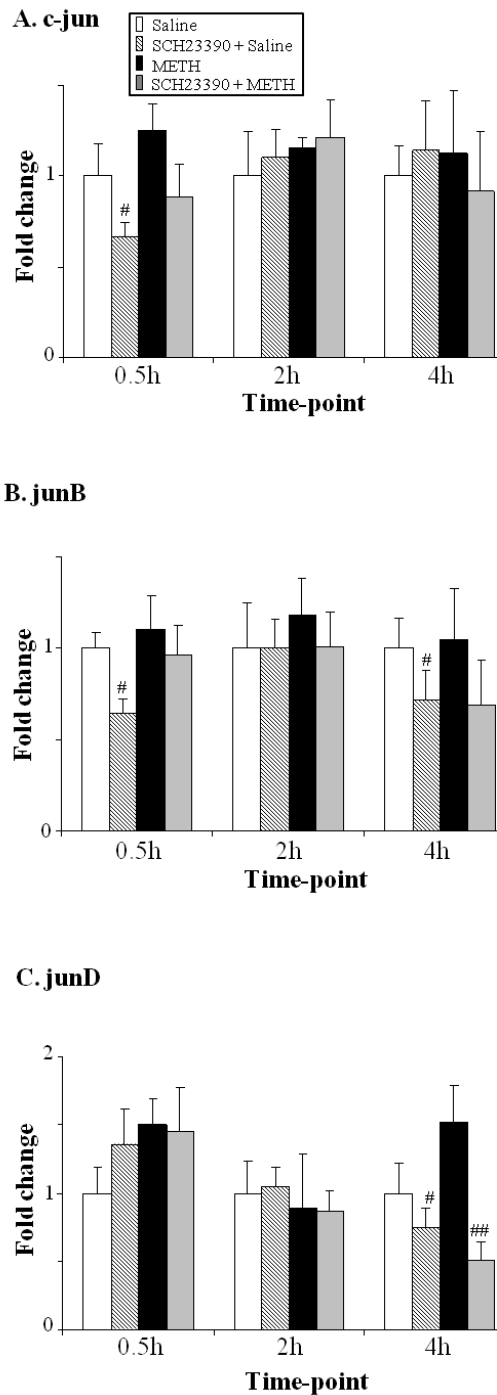


**Fig. 1. Effects of METH and SCH23390 on the expression of the fos family genes.** METH administration caused induction of (A) c-fos, (C) fosB and (E) fra-2, but did not influence fra-1 expression (D). Amplification curve for c-fos gene expression for the 30 min time-point is shown in fig. 1B and was reproducible. The levels of mRNA were normalized to clathrin mRNA levels. Data were obtained from RNA isolated from six animals per group and quantitation determined individually. Statistical significance was determined by ANOVA followed by protected least-squares difference (PLSD). Key to statistics: \*, \*\*, \*\*\* =  $p < 0.05$ ,  $0.01$ ,  $0.001$ , respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05$ ,  $0.01$ ,  $0.001$ , respectively in comparison to the METH (M) group. !,  $p < 0.01$  in comparison to the SCH23390 + METH (S + M) group.

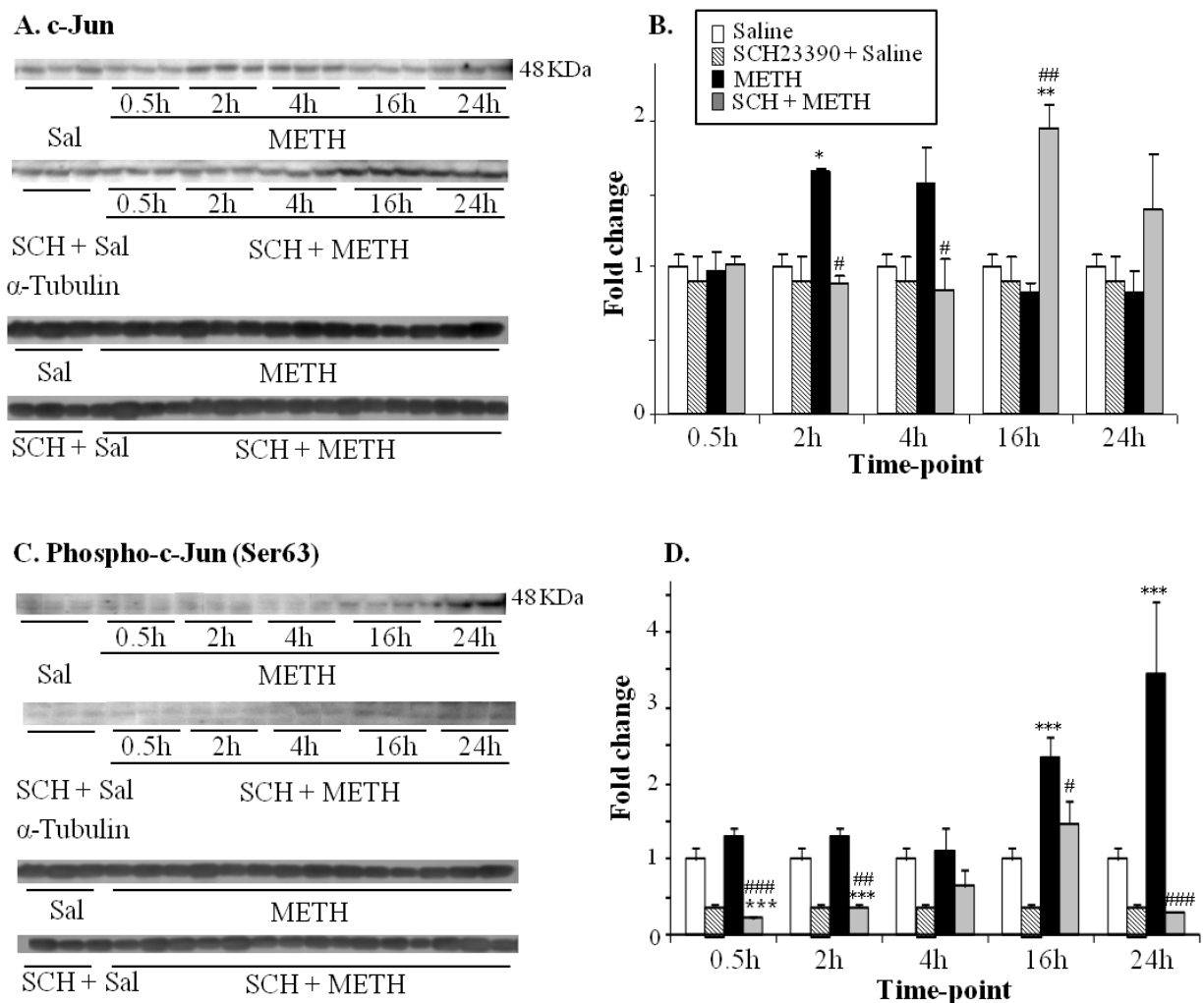




**Fig. 2. Effects of METH and SCH23390 on c-Fos protein level.** (A) Representative photomicrograph of results showing the effects of METH and SCH23390 pre-treatment on striatal c-Fos protein levels at different time-points. (B) The quantitative data of the western blots represent means  $\pm$  SEM ( $n = 3$ ). The experiments were repeated three times for each protein. For quantification, the signal intensity was normalized over the signal intensity of  $\alpha$ -tubulin. (B) METH caused significant DA D<sub>1</sub> receptor-dependent increases in c-Fos protein. Statistics are as described in Fig. 1. Key to statistics: \*, \*\*, \*\*\* =  $p < 0.05$ , 0.01, 0.001, respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05$ , 0.01, 0.001, respectively in comparison to the METH (M) group.

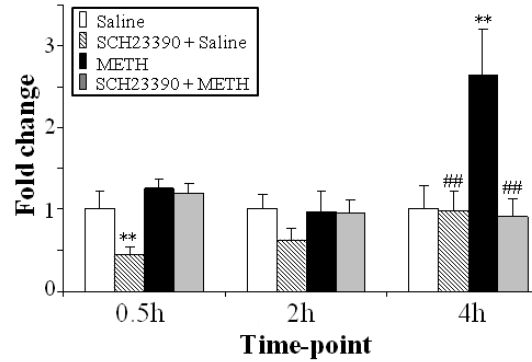


**Fig. 3. Effects of METH and SCH23390 on the levels of jun transcripts.** The expression of (A) c-jun, (B) junB and (C) junD mRNA expression was not affected by METH treatment although SCH23390 pretreatment caused significant decreases in junD at 4 h post drug treatment (C). Normalization, quantification, and statistics are as described in Fig. 1. Key to statistics: #, ## =  $p < 0.05$ ,  $0.01$ , respectively in comparison to the METH (M) group.

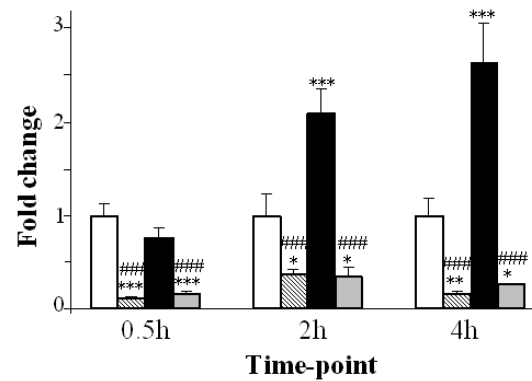


**Fig. 4. Effects of METH and SCH23390 on c-Jun and phospho-c-Jun protein levels.** (A and C) show representative photomicrographs of results showing the effects of METH and SCH23390 pre-treatment on the levels of c-Jun and phospho-c-Jun in rat striatal tissues at different time-points. (B and D) show the quantitative data of the western blots represent means  $\pm$  SEM (n = 3). The experiments were repeated three times for each protein. For quantification, the signal intensity was normalized over the signal intensity of  $\alpha$ -tubulin. (C) METH-induced changes in c-Jun were seen only at the 2-h time-point and this was blocked by SCH23390 pre-treatment. (D) Phospho-c-Jun showed significant increases at 16 h and 24 h after METH treatment. Pretreatment of SCH23390 blocked METH-induced changes in phospho-c-Jun. Normalization, quantification, and statistics are as described in Fig. 1. Key to statistics: \*, \*\*, \*\*\* =  $p < 0.05$ ,  $0.01$ ,  $0.001$ , respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05$ ,  $0.01$ ,  $0.001$ , respectively in comparison to the METH (M) group.

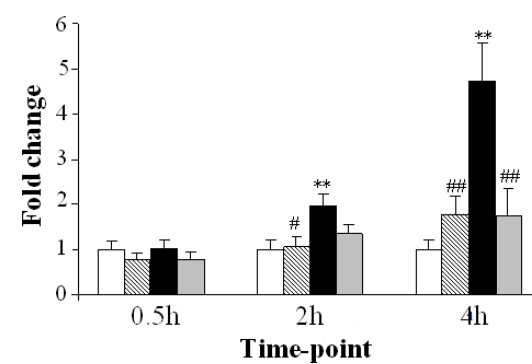
### A. egr-1



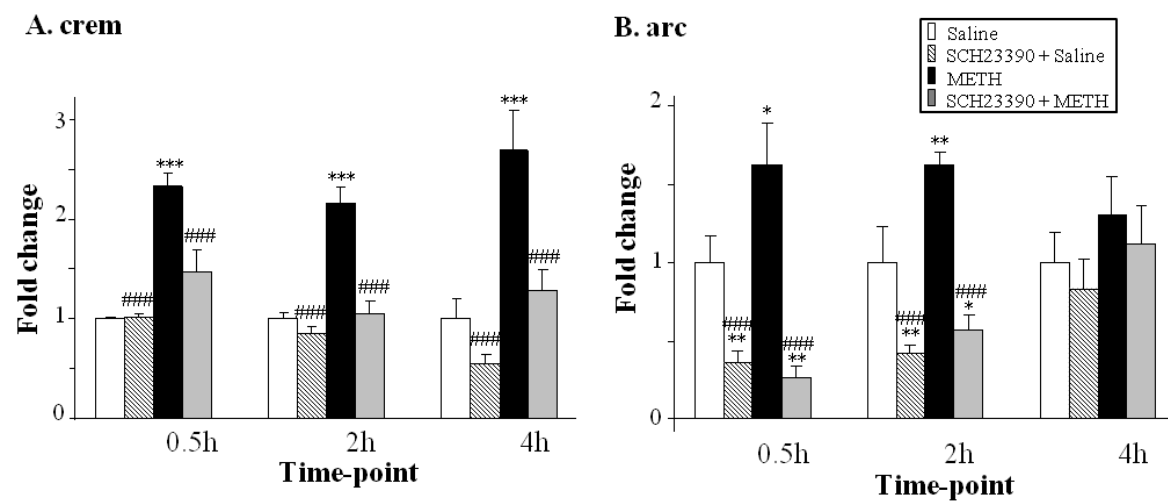
### B. egr-2



### C. egr-3



**Fig. 5. METH caused induction of striatal egr mRNA levels.** (A, B, C) Time-dependent induction of egr-1, egr-2 and egr-3 was observed after METH injections. SCH23390 blocked METH-induced changes in these genes. Normalization, quantification, and statistics are as described in Fig. 1. Key to statistics \*, \*\*, \*\*\* =  $p < 0.05$ ,  $0.01$ ,  $0.001$ , respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05$ ,  $0.01$ ,  $0.001$ , respectively in comparison to the METH (M) group.



**Fig. 6. METH caused rapid induction of *crem* and *arc* mRNA levels.** (A) METH induced rapid increases in the *crem* transcript levels. (B) *arc* expression was also induced by METH. Normalization, quantification, and statistics are as described in Fig. 1. Key to statistics: \*, \*\*, \*\*\* =  $p < 0.05$ ,  $0.01$ ,  $0.001$ , respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05$ ,  $0.01$ ,  $0.001$ , respectively in comparison to the METH (M) group.

## **Manuscript 2**

**The dopamine D<sub>1</sub> receptor antagonist, SCH23390, but not the D<sub>2</sub> receptor antagonist, raclopride, protects against methamphetamine toxicity by preventing methamphetamine-induced hyperthermia**

**The dopamine D<sub>1</sub> receptor antagonist, SCH23390, but not the D<sub>2</sub> receptor antagonist, raclopride, protects against methamphetamine toxicity by preventing methamphetamine-induced hyperthermia**

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## **Abstract**

Methamphetamine (METH) toxicity is dependent on normally functioning dopaminergic systems. Activation of dopamine D<sub>1</sub> and D<sub>2</sub> subtypes of receptor participates in the effects of the drug in the brain. The present study investigated the potential role of dopamine D<sub>1</sub> or D<sub>2</sub> receptors in METH neurotoxicity in the cortex and striatum. The dopamine D<sub>1</sub> receptor antagonist, SCH23390, attenuated METH-induced depletion of dopamine, serotonin and their metabolites in both striatal and cortical tissues. SCH23390 also attenuated METH-induced hyperthermia. The dopamine D<sub>2</sub> receptor antagonist, raclopride, also attenuated METH-induced decreases in dopamine and its metabolites in both striatal and cortical regions. However, raclopride prevented METH-induced serotonin depletion only in the striatum. Unexpectedly, raclopride failed to block METH-induced hyperthermia.

The results of the present studies indicate that SCH23390 exerts widespread protection against METH toxicity by preventing METH-induced hyperthermia. In contrast, the protective effects of raclopride are more limited and do not depend on temperature regulation.

**Keywords:** Methamphetamine; Hyperthermia; Toxicity; Monoamines; Striatum; Cortex



## **Introduction**

Methamphetamine (METH) administration causes hyperthermia and monoamine depletion in the brain (Bowyer et al., 1994; Kiyatkin and Sharma, 2009; Krasnova and Cadet, 2009). This is manifested by dopamine (DA) and serotonin (5-HT) loss in various brain regions (Krasnova and Cadet, 2009; O'Callaghan and Miller, 1994; Ricaurte et al., 1980; Sonsalla et al., 1986). These toxic effects are depending on oxidative stress, excitotoxicity and mitochondrial dysfunctions (Krasnova and Cadet, 2009). Although a relationship seems to exist between METH-induced hyperthermia and METH toxicity, not all pharmacological or genetic manipulations depend on temperature regulation to protect against or exacerbate METH toxicity (Deng et al., 1999; Deng et al., 2002b; Krasnova and Cadet, 2009; Xu et al., 2005). DA D<sub>1</sub> receptor antagonists have been shown to attenuate METH toxicity (Broening et al., 2005; Sonsalla et al., 1986), in part, by influencing METH-induced hyperthermia (Albers and Sonsalla, 1995; Broening et al., 2005). However, the effects of DA D<sub>2</sub> antagonism on METH-induced high temperature are not clear.

In the present study, we investigated the relative effects of a D<sub>1</sub> and a D<sub>2</sub> receptor antagonist on METH-induced depletions of DA and 5-HT levels in both striatal and cortical tissues. We also report on the effects of these drugs on METH-induced changes in temperature in animals treated under the same conditions.

## **Material and Methods**

### **Drugs**

(±)-METH hydrochloride was provided by the National Institute on Drug Abuse pharmacy; SCH23390 hydrochloride was purchased from TOCRIS bioscience (Ellisville, MO, USA) and raclopride was from Sigma Aldrich (St. Louis, MO, USA). All drugs were diluted with 0.09% saline.

### **Treatments and temperature monitoring**

Male Sprague-Dawley rats (Charles River Labs, Raleigh, NC, USA), weighing 250-300g were housed two per cage in a temperature-controlled room at 22 °C. Rats had free access to food and water. On the day of the experiment, the rats were housed individually. The animals were divided

into six different groups of treatments on a daily schedule ( $n = 7$  animals per group). They were pretreated with four injections of either saline (1 ml/kg), SCH23390 (0.5 mg/kg) or raclopride (0.5 mg/kg) with a 2-hr interval between each injection. Saline (1 ml/kg) or METH (10 mg/kg) was administered 30 minutes after each pretreatment injection. The animals were then split into six treatment groups: saline + saline (Control), saline + METH (METH), SCH23390 + saline (SCH), SCH + METH (SCH + METH), raclopride + saline (Rac) and raclopride + METH (Rac + METH). Body temperatures of the rats were measured with a Vet-Temp Instant Animal Ear Thermometer inserted into the ear of the animal. Temperature was recorded half-hour after each pretreatment plus treatment patterns of injections and at two hours after the last injection. Rats were euthanized by decapitation seven days later. Striatal and cortical tissues were collected and stored at  $-80^{\circ}\text{C}$  until assayed for monoamine levels.

### **Measurements of monoamines and of their metabolites**

Striatal and cortical DA, 5-HT and their metabolites were measured by high-performance liquid chromatography (HPLC) with electrochemical detection. Each sample was sonicated in 0.6 ml of 0.1 M perchloric acid buffer and then centrifuged at 14 000 g for 15 minutes. An aliquot of the supernatant was injected onto the HPLC system. The analytical column was Bondapak C-18 (5 mm, 4.6  $\times$  250.0 mm; Waters, Milford, MA), and the mobile phase contained 0.08 M sodium acetate, 1 mM sodium EDTA, 5 mM heptanes sulfonic acid, 4.0% acetonitrile, pH 4.0, flow rate 1.5 ml/min,  $25^{\circ}\text{C}$ . The installation consisted of Waters 717 Plus automated injection system, Waters piston pump, Waters manometric module, and Bioanalytical Systems LC-4C amperometric detector. The glassy carbon electrode was used at a potential of 0.75 V. DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT, and 5-HIAA were separated and their relative peaks compared to synthesized standards. Sample concentrations were calculated with a Hewlett Packard integrator.

### **Statistics**

Data analysis was conducted with StatView 5.0.1 statistics software (SAS Institute, Cary, NC, USA). We had compared all treatments at a time-point by a one-way ANOVA followed by post-hoc Fisher's protected least square difference (PLSD) test. Significance was set at  $p < 0.05$ .

## Results

### Effects of SCH23390 on METH-induced monoamine depletion in the striatum and cortex

Binge METH injections caused significant decreases in DA levels and its metabolites in the striatum (Fig. 1). Striatal DA levels were reduced ( $F_{(5, 30)} = 19.708$ ,  $p < 0.0001$ ) to 37% of control (Fig. 1A). METH also caused significant decreases in DOPAC ( $F_{(5, 30)} = 5.732$ ,  $p = 0.0008$ ) (Fig. 1B) and HVA ( $F_{(5, 28)} = 6.656$ ,  $p = 0.0003$ ) (Fig. 1C). Pretreatment with SCH23390 significantly protects against METH-induced depletion of DA (Fig. 1A), DOPAC (Fig. 1B) and HVA (Fig. 1C). Binge METH injections also caused significant reductions in DA and metabolites in the cortex (Fig. 1D-F). METH also reduced cortical DA levels to 55.4% of control ( $F_{(5, 28)} = 2.721$ ,  $p = 0.0399$ ) (Fig. 1D). DOPAC was reduced to 58.4% ( $F_{(5, 30)} = 5.339$ ,  $p = 0.0013$ ) (Fig. 1E) while HVA was less affected (70.9%) ( $F_{(5, 31)} = 3.249$ ,  $p = 0.0179$ ) (Fig. 1F). SCH23390 pretreatment caused complete inhibition of METH-induced reductions in the levels of DA (Fig. 1D) and of its metabolites (Fig. 1E-F) in the cortex.

Fig. 2 shows the effects of METH and SCH23390 on 5-HT and 5-HIAA levels in the striatum and the cortex. METH caused significant reduction of striatal 5-HT levels ( $F_{(5, 27)} = 6.758$ ,  $p = 0.0003$ ) (Fig. 2A) and 5-HIAA ( $F_{(5, 29)} = 4.746$ ,  $p = 0.0027$ ) (Fig. 2B). The levels of 5-HT and 5-HIAA reached 39% and 37% of control, respectively. SCH23390 completely blocked the toxic effects of METH on both substances (Fig. 2). In addition, METH caused decreases in the levels of cortical 5-HT ( $F_{(5, 30)} = 18.359$ ,  $p < 0.0001$ ) (Fig. 2C) and 5-HIAA ( $F_{(5, 31)} = 9.119$ ,  $p < 0.0001$ ) (Fig. 2D). After the METH injections, the values of 5-HT and 5-HIAA reached 31.6% and 43.6% of control, respectively. SCH23390-pretreated rats were protected against METH-induced depletion in cortical 5-HT (Fig. 2C) and 5-HIAA (Fig. 2D).

### Effects of raclopride on METH-induced monoamine depletion in the striatum and cortex

Fig. 3 shows the effects of binge METH injections and raclopride on the dopaminergic system in the striatum and the cortex. Raclopride completely prevented METH-induced reductions in striatal DA ( $p < 0.0001$ ) (Fig. 3A) and its metabolites, DOPAC ( $p = 0.0004$ ) (Fig. 3B) and HVA ( $p = 0.0002$ ) (Fig. 3C). Fig. 3 shows that raclopride also provided significant protection against METH-induced decreases in cortical levels of DA ( $p = 0.0449$ ) (Fig. 3D), DOPAC ( $p = 0.0007$ ) (Fig. 3E), and HVA ( $p = 0.0012$ ) (Fig. 3F).

Fig. 4 shows the effects of raclopride on striatal and cortical 5-HT and 5-HIAA levels. Raclopride blocked METH-induced decreases in 5-HT ( $p = 0.0029$ ) (Fig. 4A) and 5-HIAA ( $p = 0.0037$ ) (Fig. 4B). In contrast to the protection against METH-induced striatal 5-HT and 5-HIAA depletion, raclopride failed to protect against cortical 5-HT (Fig. 4C,  $p < 0.0001$ ) and 5-HIAA (Fig. 4D,  $p = 0.0052$ ) depletion.

### **Effects of SCH23390 and raclopride pretreatment on METH-induced hyperthermia**

In order to test for possible contributions of hyperthermia to our results, we also measured temperature in all groups of animals. As expected, binge METH injections caused significant increases in rat body temperature (Fig. 5). Hyperthermia became obvious 30 min after the first injection of METH (10 mg/kg), stayed elevated, and was still present 2 hours after the last injection (Fig. 5A). The average temperature of rats increased from 37.9 °C to 39.3 °C ( $p < 0.0001$ ) with the highest temperature reaching 41.08 °C ( $p < 0.0001$ ) after the third METH injection. Pretreatment with SCH23390 completely blocked METH-induced hyperthermia (Fig. 5A).

Fig. 5B shows the effects of raclopride on METH-induced hyperthermia in rats. Post-hoc analysis revealed that raclopride combined with METH caused a higher increase in temperature than METH alone (+ 1 °C,  $p = 0.0243$ ) after the first injection of METH (Fig. 5B). That group showed slightly lower but not significant decreases in temperature in comparison to the METH alone group, except for the last time-point. At all time-points, the METH plus raclopride group showed much higher levels of body temperature than the control group (Fig. 5B).

## **Discussion**

The purpose of the present study was to test whether the respective DA  $D_1$  and  $D_2$  receptor antagonists, SCH23390 and raclopride, would have differential effects on METH-induced hyperthermia and toxicity in the striatum and cortex. Because the effects of hyperthermia have not been widely reported on METH-induced toxicity in the cortex, we chose to also study this structure. Consistent with previous studies, we found that METH caused decreases in DA, 5-HT and their metabolites in the striatum and cortex (Ali et al., 1996; Broening et al., 2005; Graham et al., 2008), decreases that were accompanied by hyperthermia.

Several studies have reported a contributing role for hyperthermia in the neurodegenerative effects of METH in the striatum (Ali et al., 1996; Bowyer et al., 1994). For example, Bowyer and colleagues (1994) have shown that higher hyperthermic responses were associated with greater DA depletion. In contrast, lowering environmental temperature reduced METH-induced toxicity (Ali et al., 1995; Ali et al., 1996; Bowyer et al., 1994). Nevertheless, other studies have failed to show a relationship between METH-induced hyperthermia and monoamine depletion. By example, administration of the drug, reserpine caused hypothermia in rats but did not protect against METH toxicity (Albers and Sonsalla, 1995). In addition, exacerbation of METH-induced neuronal apoptosis in cFos heterozygous knockout mice (Deng et al., 1999) and reduction of apoptosis in cJun heterozygote mice (Deng et al., 2002b) were not dependent on thermoregulation. It is important to point out that both a dose of SCH23390 (0.1 mg/kg) that did not block hyperthermia in mice after administration of a toxic single dose of METH (Xu et al., 2005) and a dose of SCH23390 (0.5 mg/kg) that did block hyperthermic response in rodents treated with multiple injections of METH (Albers and Sonsalla, 1995; Broening et al., 2005), were both able to protect against METH-induced reductions in tyrosine hydroxylase, tryptophan hydroxylase, DA, 5-HT, and metabolites (Albers and Sonsalla, 1995; Broening et al., 2005; O'Dell et al., 1993; Sonsalla et al., 1986; Xu et al., 2005), as well as in levels of cortical 5-HT and 5-HIAA (Sonsalla et al., 1986). Taken together, these data might indicate that the attenuation of hyperthermia by SCH23390 is a dose-dependent effect whereas protection against monoamine depletion might not be. Data collected from pharmacological studies and genetical manipulations suggest the involvement of D<sub>1</sub> and D<sub>2</sub> receptors in METH-increased body temperature. As an example, blockade of DA D<sub>1</sub> and D<sub>2</sub> receptors by SCH23390 and eticlopride, respectively, prevented METH-induced hyperthermia (Albers and Sonsalla, 1995; Broening et al., 2005). Ito et al. (2008) used D<sub>1</sub> and D<sub>2</sub> knockout mice to study the hyperthermic and lethal effects of METH. METH-induced hyperthermia was prevented in D<sub>2</sub> knockout mice whereas there was a slight but significant increase in body temperature in D<sub>1</sub> knockout mice (Ito et al., 2008). In addition, there was more lethality in wild type mice (27%), followed by D<sub>1</sub> (7%) and D<sub>2</sub> (4%) knockout mice. These observations are consistent with those of another study that showed that administration of METH to D<sub>2</sub> homozygote knockout mice did not cause hyperthermia (Granado et al., 2011). Inactivation of D<sub>2</sub> receptor also blocked DA depletion in the striatum. These data support a role of D<sub>2</sub> receptor to mediate METH-induced hyperthermia and neurotoxicity.

The role of DA receptors in regulation of body temperature has also been studied in experiments that did not involve METH administrations. However, the exact role of each type of receptor is still not understood. Selective agonists of D<sub>1</sub> and D<sub>2</sub> receptors were shown to cause hypothermia in rodents whereas D<sub>3</sub> and D<sub>4</sub> are not involved in thermoregulation (Chaperon et al., 2003; Salmi et al., 1993; Salmi and Ahlenius, 1997). By example, use of the D<sub>2</sub>/D<sub>3</sub> receptor agonist, PD 128907, or the selective D<sub>2</sub> receptor agonist, R(-)-2, 10, 11-trihydroxy-N-propyl-noraporphine hydrobromide (TNPA), caused hypothermia in rat (Chaperon et al., 2003). The effects of antagonists of different subtypes of DA receptors on hypothermia induced by PD 128907 were also investigated. The selective D<sub>3</sub> antagonist, A-437203, and the selective D<sub>4</sub> receptor antagonist, L-745,870, were without any effects on the hypothermic responses while the selective D<sub>1</sub> receptor antagonist, SCH 23390, and the selective D<sub>2</sub> receptor antagonist, L-741,626, prevented hypothermia. It has been proposed that there is a cooperative/synergistic action of DA D<sub>1</sub> and D<sub>2</sub> receptors in thermoregulation. However, another study gave evidence that D<sub>1</sub> and D<sub>2</sub> receptors have independent actions on thermoregulation in rats (Salmi et al., 1993). The D<sub>1</sub> receptor agonist A68930 caused hypothermia which was sensitive to the D<sub>1</sub> receptor antagonist, SCH 23390, but not to the D<sub>2</sub>/D<sub>3</sub> receptor antagonist, raclopride whereas the D<sub>2</sub> receptor agonist, quinpirol, caused hypothermia that was antagonized only by raclopride (Salmi et al., 1993).

Interestingly, our present data also show that METH does not affect cortical and striatal monoaminergic systems in an identical fashion. For example, both DA D<sub>1</sub> and D<sub>2</sub> receptor antagonists protect against METH-induced DA depletion in the striatum but only SCH23390 protected against cortical 5-HT levels. Our study is consistent, in part, with the findings of two previous studies. For example, the DA D<sub>2</sub> receptor antagonist sulpiride (up to 80 mg/kg) did not protect against binge METH toxicity in either cortical or striatal serotonergic systems (Sonsalla et al., 1986). In contrast, another group reported that pretreatment with the D<sub>2</sub> receptor antagonist, eticlopride (4x0.5 mg/kg) could attenuate METH-induced reduction of striatal 5-HT (Broening et al., 2005). This paper did not report on the effects of eticlopride on cortical 5-HT (Broening et al., 2005).

In summary, the DA D<sub>1</sub> and D<sub>2</sub> receptor antagonists, SCH23390 and raclopride, attenuate METH-induced reductions in striatal and cortical DA and its metabolites. However, only SCH23390 prevented hyperthermia and damage to cortical serotonergic systems. Our findings suggest that

more investigations are necessary to truly clarify the impact of hyperthermia on METH toxicity in various brain regions.

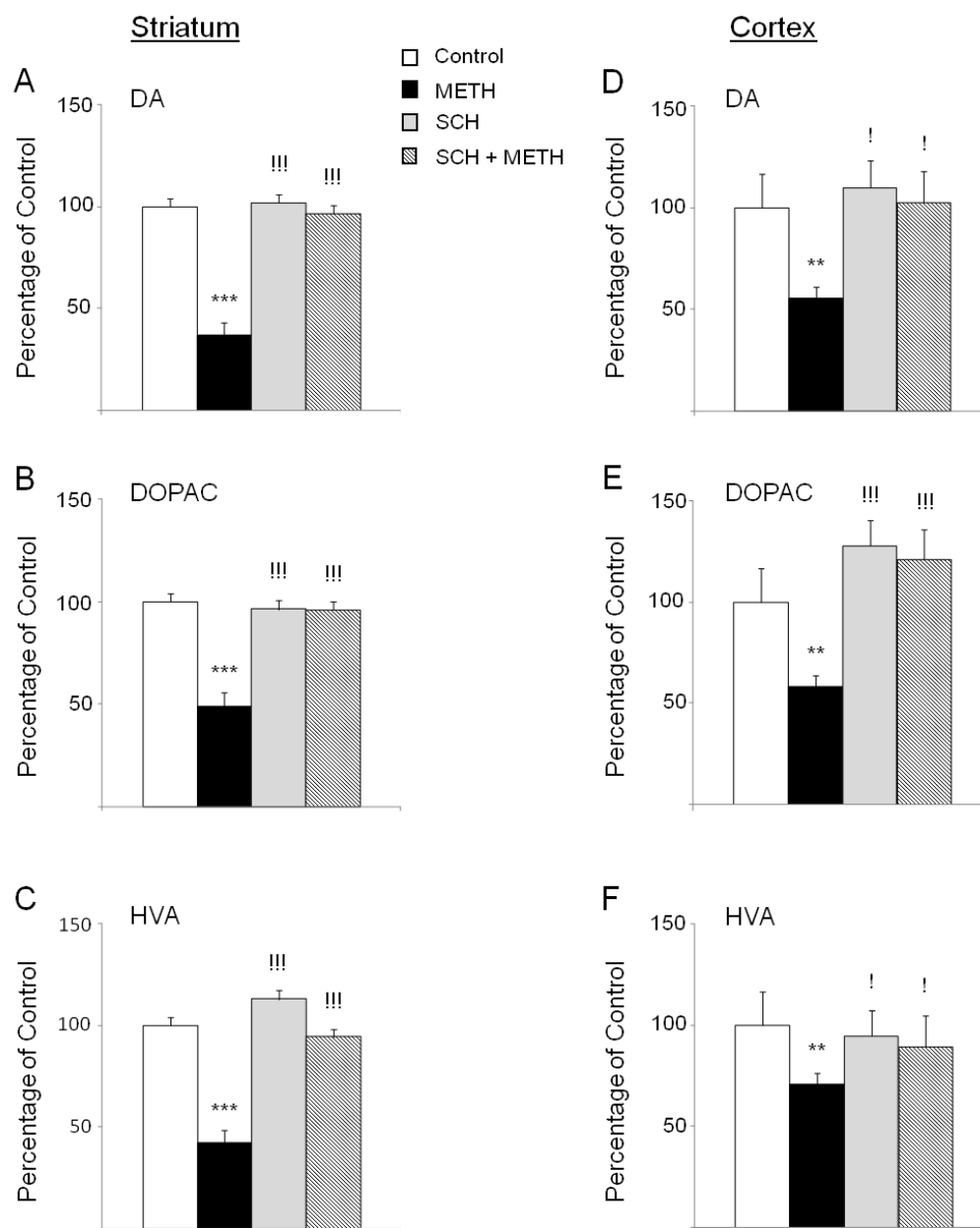
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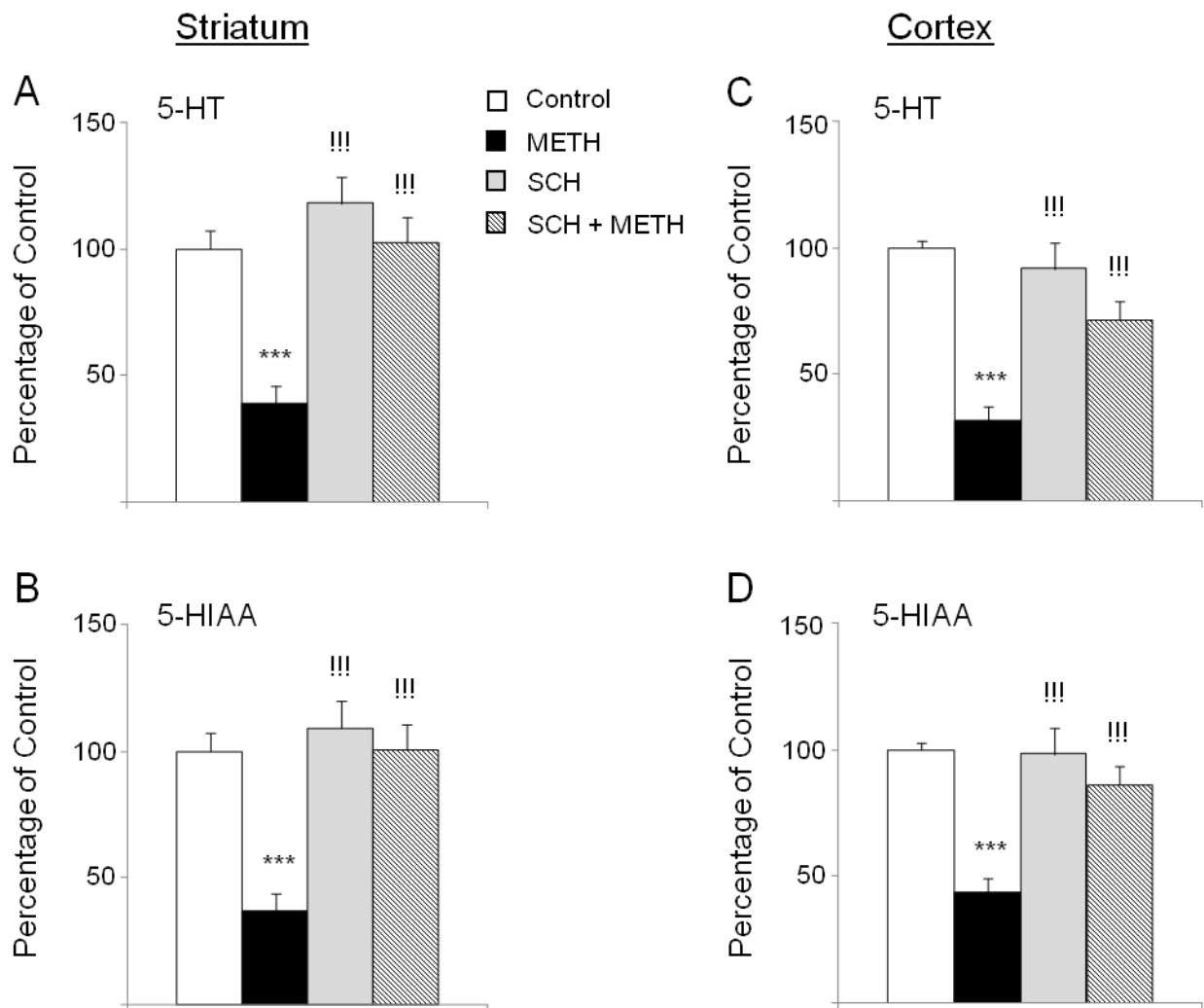
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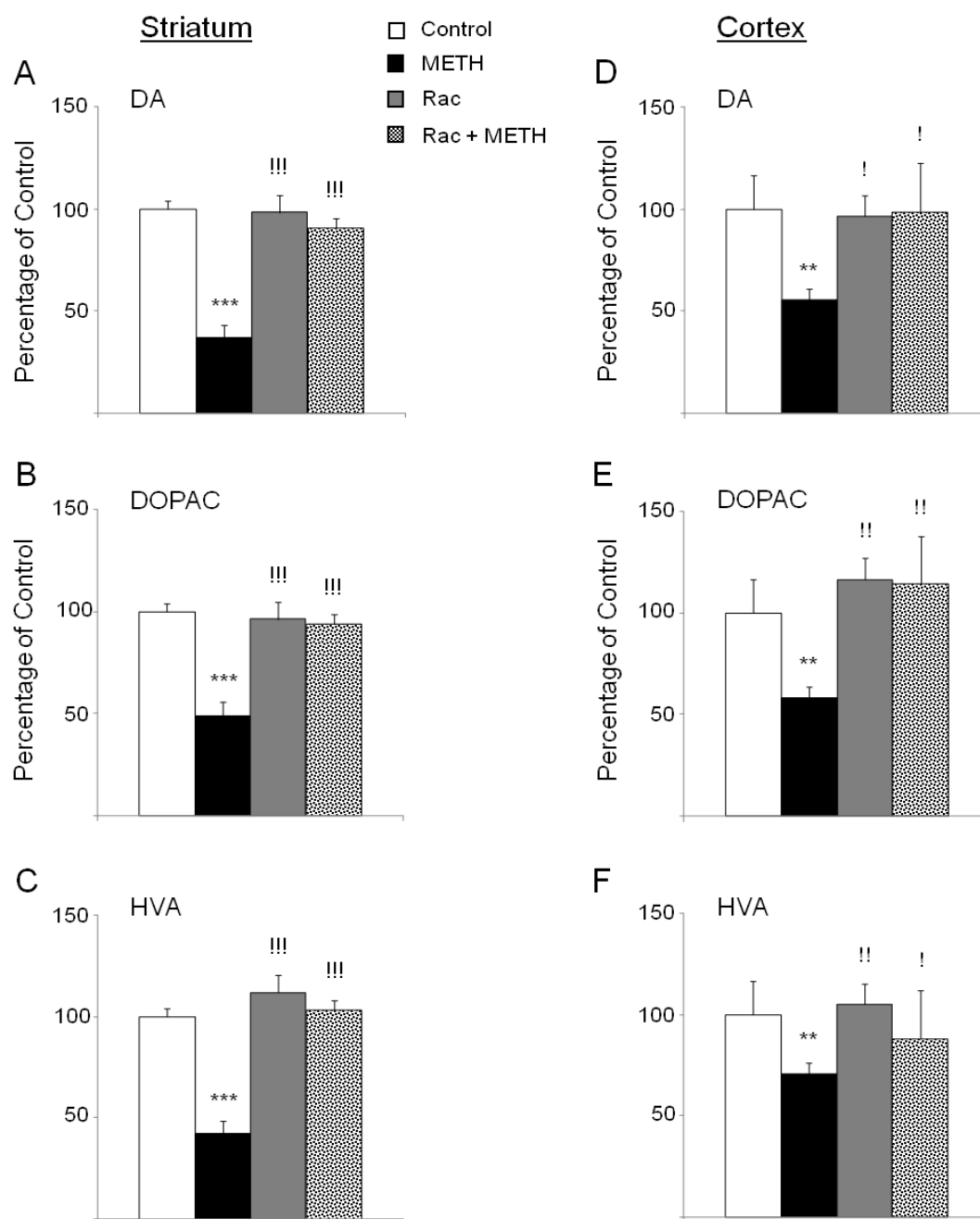
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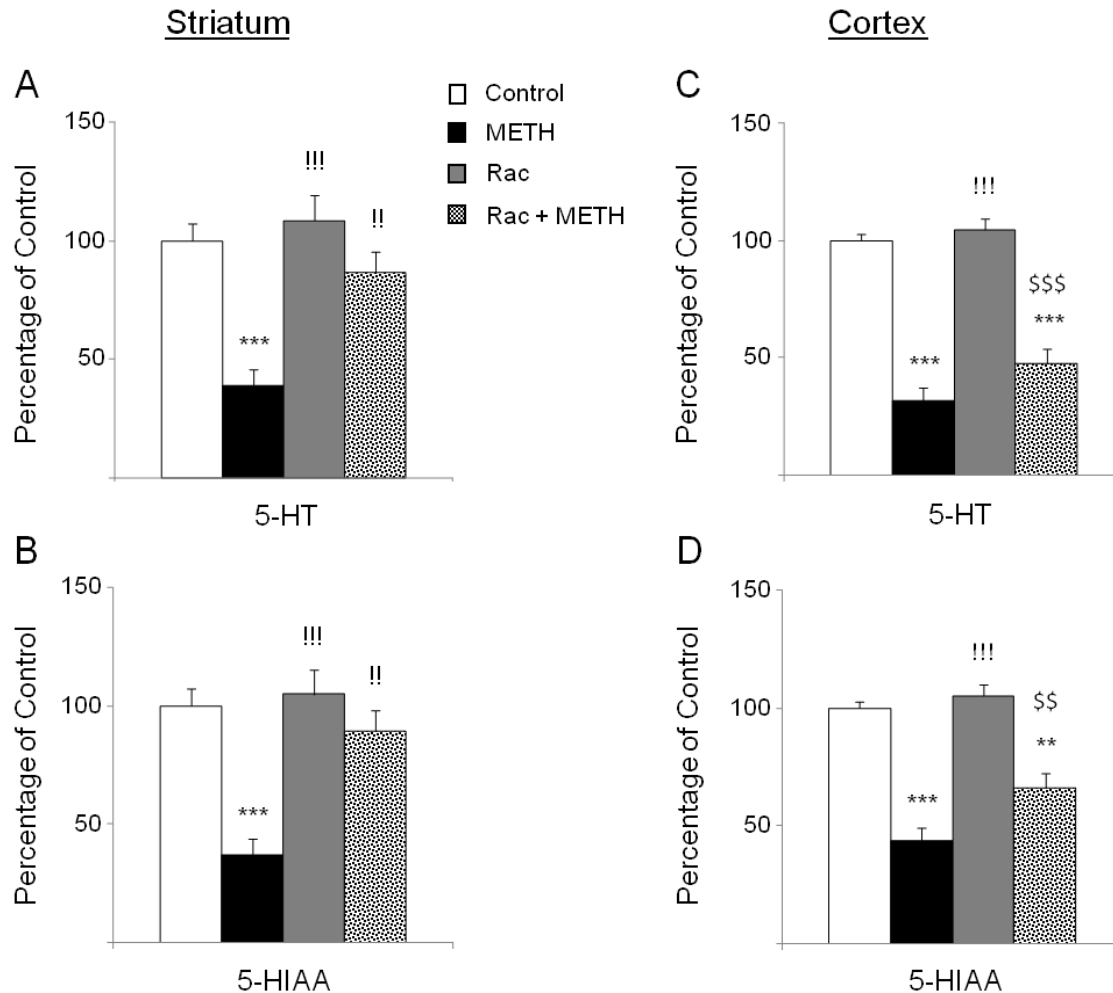
**Fig. 1. Effects of SCH23390 on METH-induced changes in striatal and cortical DA, DOPAC and HVA concentrations.** Rats received 0.5 mg/kg of SCH23390 (i.p) before each injection of 10 mg/kg of METH, given four times a day at 2-h intervals. Animals were killed one week later and HPLC performed on brain tissues to measure DA (A) and its metabolites DOPAC (B) and HVA (C). Cortical tissues were also used to measure DA (D), DOPAC (E) and HVA (F) levels. Results are percentages of the control group. Key to statistics: \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Control group. ! =  $p < 0.05$ ; !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison to the METH group (post-hoc test).



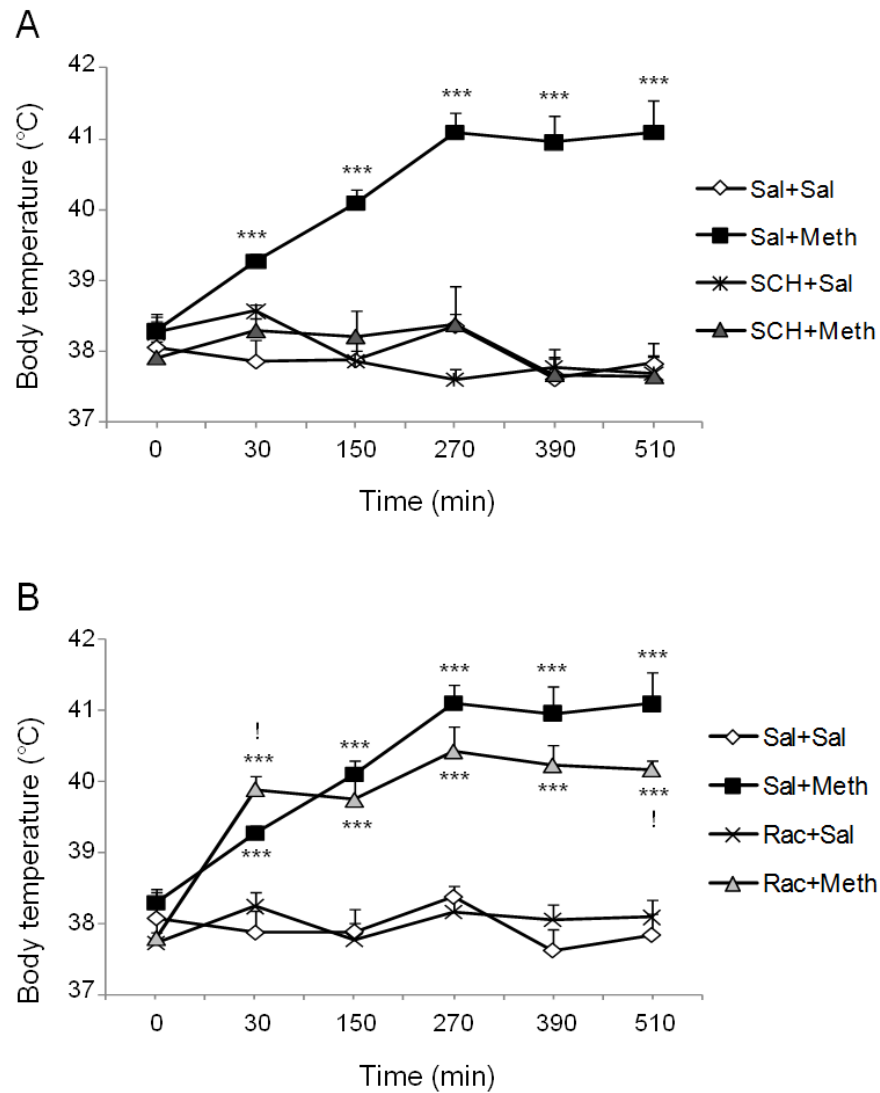
**Fig. 2. Effects of SCH23390 on METH-induced changes in 5-HT and 5-HIAA in rat striatal and cortical tissues.** Animals were treated as in Fig. 1. METH caused significant decreases in striatal and cortical 5-HT (2A, 2C) and 5-HIAA (2B, 2D) levels. Key to statistics: \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Control group. ! =  $p < 0.05$ ; !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison to the METH group (post-hoc test).



**Fig. 3. Effects of raclopride on METH-induced neurochemical changes of striatal and cortical DA systems.** Raclopride was administered 30 min prior each injection of METH, for a total of four injections of METH administered at 2-hr intervals. Levels of striatal (A-C) and cortical (D-F) DA (A, D), DOPAC (B, E) and HVA (C, F) were determined by HPLC analysis. Key to statistics: \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Control group. ! =  $p < 0.05$ ; !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison to the METH group (post-hoc test).



**Fig. 4. Effects of raclopride on METH-induced depletion of striatal and cortical 5-HT and 5-HIAA levels.** The animals were treated as in Fig. 3. METH caused significant decreases in striatal (A, B) and cortical (C, D) 5-HT (A, C) and 5-HIAA (B, D) levels. The values were normalized to the control group. Key to statistics: \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Control group. !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison to the METH group. \$\$ =  $p < 0.01$ ; \$\$\$ =  $p < 0.001$ , for comparison between Rac and Rac + METH groups (post-hoc test).



**Fig. 5. Effects of SCH23390 and raclopride on METH-induced hyperthermia.** SCH23390 (A) or raclopride (B) was administered 30 min before each saline or METH injection. Animals were treated as described above. Temperature was recorded 30 min after each of these injections (30, 150, 270 and 390 min) and two hours after the last one (510 min). Data represent the means of seven rats per group. Statistical differences in temperature were considered significant at p values less than 0.05. Key to statistics: \*\*\* =  $p < 0.001$ , in comparison to the Control group. ! =  $p < 0.05$ , in comparison to the METH group (post-hoc test).

### **Manuscript 3**

**Involvement of dopamine receptors in binge methamphetamine-induced  
activation of endoplasmic reticulum and mitochondrial stress pathways**

## **Involvement of dopamine receptors in binge methamphetamine-induced activation of endoplasmic reticulum and mitochondrial stress pathways**

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## Abstract

Single large doses of methamphetamine (METH) cause endoplasmic reticulum (ER) stress and mitochondrial dysfunctions in rodent striata. The dopamine D<sub>1</sub> receptor appears to be involved in these METH-mediated stresses. The purpose of this study was to investigate if dopamine D<sub>1</sub> and D<sub>2</sub> receptors are involved in ER and mitochondrial stresses caused by single-day METH binges in the rat striatum. Male Sprague-Dawley rats received 4 injections of 10 mg/kg of METH alone or in combination with a putative D<sub>1</sub> or D<sub>2</sub> receptor antagonist, SCH23390 or raclopride, respectively, given 30 min prior to each METH injection. Rats were euthanized at various time-points afterwards. Striatal tissues were used in quantitative RT-PCR and western blot analyses. We found that binge METH injections caused increased expression of the pro-survival genes, BiP/GRP-78 and P58<sup>IPK</sup>, in a SCH23390-sensitive manner. METH also caused up-regulation of ER-stress genes, Atf2, Atf3, Atf4, CHOP/Gadd153 and Gadd34. The expression of heat shock proteins (HSPs) was increased after METH injections. SCH23390 completely blocked induction in all analyzed ER stress-related proteins that included ATF3, ATF4, CHOP/Gadd153, HSPs and caspase-12. The dopamine D<sub>2</sub>-like antagonist, raclopride, exerted small to moderate inhibitory influence on some METH-induced changes in ER stress proteins. Importantly, METH caused decreases in the mitochondrial anti-apoptotic protein, Bcl-2, but increases in the pro-apoptotic proteins, Bax, Bad and cytochrome c, in a SCH23390-sensitive fashion. In contrast, raclopride provided only small inhibition of METH-induced changes in mitochondrial proteins. These findings indicate that METH-induced activation of striatal ER and mitochondrial stress pathways might be more related to activation of SCH23390-sensitive receptors.

## Introduction

Methamphetamine (METH) addiction is very prevalent throughout the world. The accumulated evidence suggests that the acute effects of METH on neurons post-synaptic to striatal dopamine (DA) terminals are due to DA release (O'Dell et al., 1993) and subsequent stimulation of DA receptors in the brain (Jayanthi et al., 2005; Krasnova and Cadet, 2009; Xu et al., 2005). Chronic METH abuse is associated with medical, neurologic and neurodegenerative complications (Gold et al., 2009; Krasnova and Cadet, 2009; McGee et al., 2004; Volkow et al., 2001d; Wilson et al., 1996). These neuropsychiatric adverse events include secondary depression, psychotic states and psychomotor impairments (Lan et al., 1998; Simon et al., 2000). Post-mortem studies have revealed that the brains of METH addicts show depletion of DA, serotonin (5-HT) and of their metabolites in the striatum (Wilson et al., 1996). There are also losses in dopamine transporter (DAT) (Volkow et al., 2001d) and serotonin transporter (5-HTT) (Sekine et al., 2006) in the brains of METH abusers. In rodents, METH induces similar degeneration of monoaminergic systems in various regions of the brain including the striatum, cortex and hippocampus (Deng et al., 2001; Krasnova and Cadet, 2009; Sonsalla et al., 1986). The effects of METH on DA system include reductions in the neurotransmitter, its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the DA synthesis enzyme, tyrosine hydroxylase (TH), and the vesicular transporter (VMAT2) (Krasnova and Cadet, 2009; Sonsalla et al., 1986; Xu et al., 2005). The serotonin system is also affected by METH and experiences reduction in the levels of 5-HT, its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), and of the 5-HT synthesis enzyme, tryptophan hydroxylase (TPH) (Sonsalla et al., 1986). METH-induced biochemical and structural changes in monoaminergic terminals are dependent on normal dopaminergic functions. Specifically, DA D<sub>1</sub> and D<sub>2</sub> receptors antagonists were shown to attenuate the toxic effects of METH on DA and 5-HT systems (Sonsalla et al., 1986; Xu et al., 2005). In addition, the essential role of DA in METH toxicity was elegantly demonstrated in studies in which depletion of DA provided protection against METH-induced damage of DA terminals whereas increasing DA promoted these toxic effects (Kuhn et al., 2008).

METH also causes cell death of neurons located post-synaptic to monoaminergic terminals (Deng et al., 1999; Deng et al., 2001; Jayanthi et al., 2005; Krasnova and Cadet, 2009; Thiriet et al., 2005; Xu et al., 2005). Cell death appears to occur in enkephalin-positive cells (Thiriet et al., 2005) that

express D<sub>2</sub> receptors (LaVoie and Hastings, 1999) and in other neurons (Xu et al., 2005) that express D<sub>1</sub> receptors (LaVoie and Hastings, 1999). Although there are multiple classes of DA receptors in the striatum, the most abundant subtypes are the D<sub>1</sub> and D<sub>2</sub> receptors (Beaulieu and Gainetdinov, 2011; Lachowicz and Sibley, 1997). In the dorsal striatum, the D<sub>1</sub>-like subtype of DA receptors is thought to be mainly responsible for METH-induced changes in gene expression and, possibly, for METH-induced neuronal apoptosis (Cadet et al., 2010b). These ideas are consistent with the recent demonstration that activation of endoplasmic reticulum (ER) stress pathways in rat striatum by a single large METH dose is inhibited by the DA D<sub>1</sub> receptor antagonist, SCH23390 (Jayanthi et al., 2009). SCH23390 also blocked METH-induced cell death in the rodent brain (Jayanthi et al., 2005; Xu et al., 2005). The DA D<sub>2</sub>-like receptor might also be involved in METH-induced cell death because the DA D<sub>2</sub> receptor antagonist, raclopride, was reported to also inhibit this process to a great degree (Xu et al., 2005). Nevertheless, it is still not clear how inhibition of DA D<sub>1</sub> and D<sub>2</sub> receptors might interfere with intracellular death pathways in order to protect against METH-induced neuronal apoptosis.

METH is known to exert its toxic effects, in part, by causing oxidative stress (Cadet et al., 1994a; Jayanthi et al., 1998; Krasnova and Cadet, 2009). Oxidative stress can increase the expression of ER resident chaperones, such as BiP/GRP-78, P58<sup>IPK</sup>, and heat shock proteins (HSPs) that are important regulators of aberrant protein folding (Malhotra and Kaufman, 2007). Under severe ER stress, the ER-located trans-membrane proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (Ire-1), and PKR-like ER kinase (PERK) regulate the unfolded protein response (UPR). ATF6 acts as a transcription factor for UPR induction (Adachi et al., 2008).

Phosphorylation of Ire-1 induces ER-resident proteins, such as BIP/GRP-78, GRP94 and C/EBP homologous protein (CHOP)/growth arrest-and DNA damage-inducible gene 153 (Gadd153) (Bertolotti et al., 2000). On the other hand, PERK can induce phosphorylation of eukaryotic initiation factor-2 $\alpha$  (eIF2 $\alpha$ ) to inhibit total translation but increases translation of ATF4, which is also a transcription factor for UPR induction (Harding et al., 2000). ER stress-induced cellular demise is also mediated, in part, by calpain-mediated activation of the protease caspase-12 (Momoi, 2004).

METH administration can also trigger alterations in the expression of the Bcl-2 family of proteins and secondary activation of mitochondria stress-mediated death pathways (Cadet et al., 1997; Deng et al., 2002a; Jayanthi et al., 2001). Because DA D<sub>1</sub> and D<sub>2</sub> antagonists have been shown to provide

significant protection against METH-induced cell death (Jayanthi et al., 2005; Xu et al., 2005), we investigated the molecular mechanisms of this protection on METH-induced activation of ER and mitochondrial-dependent pathways in the brain. To pursue this idea further, we used the commonly implemented binge patterns of METH injections employed in toxicity studies for purpose of comparison with previous observations with single METH injections. It was important to do this because single and binge METH injections have been reported to differentially affect striatal neurons (Zhu et al., 2006b). Thus, the first aim of our study was to test the possibility that binge METH administration might cause concomitant changes in ER and mitochondria stresses proteins in the rat striatum. If so, we also wanted to delineate the timing of these METH-induced changes. The second aim was to investigate if the DA D<sub>1</sub>-like receptor antagonist, SCH23390, might block METH-induced changes in expression of genes and proteins involved in both stress pathways. Although SCH23390 can also bind to 5-HT receptors (Bischoff et al., 1986; Bischoff et al., 1988), the 5-HT system has not been shown to play an important role in METH toxicity (Gandolfi et al., 1988; Thomas et al., 2010a). The third aim was to test if the DA D<sub>2</sub>-like receptor antagonist, raclopride, had any influence on METH-induced alterations in the expression of several proteins involved in ER and mitochondria stress pathways.

## Results

### **METH causes early dopamine D<sub>1</sub> sensitive-up-regulation in mRNA levels of ER stress markers, BiP/GRP-78 and P58<sup>IPK</sup>**

Figure 1 shows the effects of METH injections on the expression of the ER stress genes, BiP/Grp-78 (Otero et al., 2010) and P58<sup>IPK</sup> (van Huizen et al., 2003). Repeated injections of METH caused very early increases in BiP/Grp-78 mRNA levels which were apparent 30 min after the METH injections and peaked at 2 hr time-point (Fig. 1A). The METH-induced changes were normalized at 16 hr after the last METH injection. METH also caused increases in P58<sup>IPK</sup> mRNA levels which were also apparent 30 min after the injections, peaked at 4 hr, and then normalized at 16 hr after the binge METH injections (Fig. 1B). SCH23390 prevented the METH-induced increases in BiP/Grp-78 and P58<sup>IPK</sup> mRNA levels, but had no effects when administered alone (Figs. 1A and 1B). As a result of these observations, we sought to identify other ER stress proteins whose expression might

be influenced by the METH injections. We also examined if these changes might be affected by pretreatment with D<sub>1</sub>-like or D<sub>2</sub>-like receptor antagonists.

### **Effects of METH, SCH23390, and raclopride on HSP40 and HSP70 in the rat striatum**

We used western blot experiments in order to determine if METH or the D<sub>1</sub> receptor antagonist, SCH23390, had any effects on the expression of the chaperones, HSP40 and HSP70. Figure 2 shows that binge METH injections caused significant and prolonged increases in the expression of HSP40 (Figs. 2A and 2B) and HSP70 (Figs. 2A and 2C) protein levels in the rat striatum. SCH23390 pretreatment completely blocked METH-induced increases in HSP40 (Fig. 2B) and HSP70 (Fig. 2C) at all time-points.

The effects of the D<sub>2</sub>-like receptor antagonist, raclopride, on METH-induced HSPs proteins were also investigated in a different group of rats. METH alone caused rapid and persistent increases in HSP40 and HSP70 (Fig. 3). Raclopride pretreatment caused significant attenuation of METH-induced changes in HSP40 expression at all time-points examined in the present study (Fig. 3B). However, injections of raclopride in combination with METH only slightly attenuated the METH-induced increases in HSP70 at 30 min, but were ineffective afterwards (Fig. 3C).

### **Effects of METH and SCH23390 on mRNA levels of ER stress responsive genes**

We also measured the effects of METH on the mRNA levels of several members of the activating transcription factor (ATF) family (Fig. 4). Injections of METH caused delayed increases in Atf1 (16 and 24 hr) (Fig. 4A) and Atf5 (16 hr) (Fig. 4E) mRNA levels. There were bimodal increases in Atf3 (Fig. 4C) (0.5 and 16 hr) and Atf4 (Fig. 4D) transcripts that occurred at 4 and 16 hr after METH. SCH23390 pretreatment blocked these METH-induced increases (Figs. 4A and 4C-4E). The mRNA levels of Atf2 and Atf6 (Figs. 4B and 4F) were not affected by any of the drug combinations.

METH injections also caused biphasic changes in the mRNA levels of CHOP, consisting of rapid increases 30 min after METH injection, peaking at 2 hr, and returning to normal by 4 hr after METH administration. Unexpectedly, there were also delayed METH-induced increases in CHOP mRNA levels at 24 hr after the last drug injection (Fig. 5A). As shown in Figure 5A, SCH23390 pretreatment blocked METH-induced changes in CHOP mRNA levels. METH injections also

caused increases in Gadd34 mRNA expression that were apparent at 16 and 24 hr, in a SCH23390-sensitive manner (Fig. 5B).

### **Effects of METH, SCH23390, and raclopride on ER stress-related proteins in the rat striatum**

Figure 6 shows the effects of METH on ATF3 and ATF4 protein levels which were determined by western blot. Binge METH injections caused somewhat delayed increases in ATF3 (Figs. 6A and 6B) and ATF4 protein levels at 4, 16, and 24 hr (Figs. 6A and 6C). Pretreatment with SCH23390 prevented METH-induced changes in both ATF3 and ATF4 protein levels (Figs. 6A-6C). Figures 6A and 6D show that there were also METH-induced increases in CHOP protein levels which remained elevated from 4 hr after the last METH injection and thereafter. As shown in Figure 6D, SCH23390 pretreatment blocked METH-induced changes in CHOP protein levels. Figure 6E illustrates the effects of binge METH injections on cleaved caspase-12 protein expression. There were METH-induced increases in cleaved caspase-12 at all time-points after the last injection of the drug (Figs. 6A and 6E). SCH23390 pretreatment also prevented the METH-induced changes in caspase-12 protein.

In the experiments testing the effects of raclopride, we confirmed the METH-induced increases in ATF3, ATF4, CHOP and caspase-12 protein expression (Fig. 7). Pretreatment with raclopride had no significant effects on the METH-induced increases in ATF3 and ATF4 protein levels (Figs. 7B and 7C). Raclopride pretreatment caused partial attenuation of the METH-induced effects on CHOP and caspase-12 expression (Figs. 7D and 7E).

### **Effects of METH, SCH23390, and raclopride on proteins involved in mitochondria-dependent stress pathways**

Binge METH injections caused significant decreases in Bcl-2 protein levels at 30 min, 2 hr and 4 hr in rats treated with METH alone (Figs. 8A and 8B). METH also caused significant increases in Bax protein levels (Fig. 8C). The drug also caused more delayed increases in Bad protein expression which were apparent at 4 hr (Fig. 8D). Rats pretreated with the D<sub>1</sub> antagonist, SCH23390, showed significant attenuation of the METH-induced changes in Bcl-2, Bax and Bad protein expression (Figs. 8A-8D). We also measured the effects of METH on cytochrome c because this protein is involved in the mitochondria-dependent death pathway and is released from mitochondria into the cytoplasmic during the process of apoptosis caused by various agents (Kluck

et al., 1997; Smith et al., 2008). METH caused increases in cytosolic cytochrome c protein levels at 16 hr and 24 hr time-points that were inhibited by pretreatment with SCH23390 (Fig. 8E).

We also tested the effects of METH and raclopride on proteins that are involved in mitochondria-dependent cellular stress. Rats injected with METH showed significant decreases in Bcl-2 protein levels at both 30 min and 2 hr after the last injection of METH (Fig. 9B). METH caused increases in Bax protein levels that lasted throughout the experiments (Fig. 9C). Bad protein levels were also increased (Fig. 9D). The expression of cytochrome c was also increased in the cytosol at 4 hr and 16 hr after METH injections (Fig. 9E). Raclopride caused only small attenuation of the effects of METH on Bcl-2 expression (Fig. 9A). In addition, the D<sub>2</sub>-like receptor antagonist attenuated the METH-induced early effects but not its later effects on Bax expression (Fig. 9B). Raclopride had some preventive effects on METH-induced Bad protein (Fig. 9D) but failed to impact METH-induced increases in cytosolic cytochrome c protein levels (Fig. 9E).

### **Effects of SCH23390 and raclopride pretreatment on METH-induced hyperthermia**

In order to test for possible contributions of hyperthermia to our results, we also measured temperature in all experimental groups. As expected, binge METH injections caused significant increases in rat body temperature (Fig. 10). Hyperthermia was apparent 30 min after the first injection of METH (10 mg/kg), stayed elevated, and was still present 2 hr after the last injection (Fig. 10A). The average temperature of rats increased from 37.9°C to 39.3°C ( $p < 0.0001$ ) with the highest temperature reaching 41.08°C ( $p < 0.0001$ ) after the third METH injection. Pretreatment with SCH23390 completely blocked METH -induced hyperthermia (Fig. 10A).

Figure 10B shows the effects of raclopride on METH -induced hyperthermia in rats. Post-hoc analysis revealed that raclopride combined with METH caused a higher increase in temperature than METH alone (+ 1°C,  $p = 0.0243$ ) after the first injection of METH (Fig. 10B). That group showed slightly lower but not significant decreases in temperature in comparison to the METH alone group, except for the last time-point. At all time-points, the METH plus raclopride group showed much higher levels of body temperature than the control group (Fig. 10B).

## Discussion

METH-induced excessive release of DA results in the formation of reactive oxygen species that damage terminals of DA neurons (Cadet et al., 1994a; LaVoie and Hastings, 1999). METH also causes neuronal apoptosis in neurons post-synaptic to DA terminals (Deng et al., 2001; Jayanthi et al., 2005). These deleterious effects appear to be mediated, in part, by oxidative stress as well as by mitochondrial and ER stresses (Krasnova and Cadet, 2009; Kuperman et al., 1997; Riddle et al., 2006), that are secondary to increased DA overflow in the synaptic cleft (O'Dell et al., 1993). In addition to the processes described before, METH-induced toxicity and molecular events, appear to also depend mainly on stimulation of DA D<sub>1</sub>-like receptors (Cadet et al., 2010b), with DA D<sub>2</sub>-like receptors also playing a role in preventing METH-induced neuronal apoptosis (Xu et al., 2005). It was, therefore, important to attempt to dissect the role of these subtypes of receptors on signaling mechanisms that have been shown to participate in METH-induced demise of striatal neurons located post-synaptic to DA nerve endings (Krasnova and Cadet, 2009).

In the present report, we used the relatively selective DA D<sub>1</sub> receptor antagonist, SCH23390 (Andersen and Gronvald, 1986) and the somewhat, more selective DA D<sub>2</sub> receptor antagonist, raclopride (Hall et al., 1988). In addition to its antagonistic properties on the D<sub>1</sub>-like receptor, SCH23390 has high affinity for other receptors. For example, SCH23390 binds to the DA D<sub>5</sub> receptor (Bourne, 2001), but this receptor has lower level of expression than the D<sub>1</sub> receptor in the striatum (Meador-Woodruff et al., 1992). SCH23390 has high affinity (IC<sub>50</sub>= 30 nM) for 5-HT<sub>2</sub> receptors that are abundant in the striatum, being 4.5-23 times less potent than the reference 5-HT<sub>2</sub> receptor antagonists (Bischoff et al., 1988). Injections of increasing doses of SCH23390 (0.03-10.0 mg/kg intra-peritoneally) blocked binding of the serotonergic receptor antagonist, (<sup>3</sup>H)-spiperone, to 5-HT<sub>2</sub> receptors in vivo in the frontal cortex but not in the striatum of rats (Bischoff et al., 1986). Importantly, single (0.1 mg/kg or 5 mg/kg) or repeated administration of SCH23390 (0.1 mg/kg daily for 21 days) did not alter the kinetic characteristics of 5-HT<sub>2</sub> receptors, 5-HT levels, or 5-HT turnover (Gandolfi et al., 1988). Since we used a total of 2 mg/kg of SCH23390 per animal in the present experiment, it is possible that the drug might be affecting mainly striatal DA D<sub>1</sub> receptors. Most importantly, METH-induced biochemical and structural abnormalities in the striatum do not appear to depend on 5-HT neurotransmission because 5-HT<sub>2</sub> and 5-HT<sub>3/4</sub> receptor antagonists did not prevent METH-induced reductions in markers of monoaminergic neurons and of TH or TPH



activities (Johnson et al., 1994). Moreover, increasing 5-HT levels by using the 5-HT precursor, 5-hydroxytryptophan, or decreasing 5-HT with the reversible inhibitor of TPH, p-chlorophenylalanine, did not influence METH-induced reductions of DA, TH or DAT levels (Thomas et al., 2010a). Furthermore, deletion of the TPH2 gene that caused marked 5-HT depletion in mice also did not impact the toxic effects of METH on striatal dopaminergic markers (Thomas et al., 2010a). Thus, when taken together with these observations, our present results suggest that SCH23390 might be exerting its protective effects against ER and mitochondria stresses mainly by inhibiting striatal DA D<sub>1</sub>-like but not 5-HT<sub>2</sub> receptors. Further studies using knockout mice might help to clarify these issues further. Similar to our use of SCH23390, we used raclopride in our experiments based on its specificity to antagonize DA D<sub>2</sub> receptors. However, raclopride has higher affinity at D<sub>2</sub> than at D<sub>3</sub> receptors (Goto et al., 1993; Hai et al., 1999; Oyadomari and Mori, 2004). Although D<sub>2</sub> and D<sub>3</sub> receptors are members of the D<sub>2</sub>-like family of receptors, they have differential anatomical distribution in the brain. D<sub>2</sub> receptors are abundant in the dorsal part of the striatum, containing the caudate and putamen; while D<sub>3</sub> receptors are more concentrated in limbic areas like the nucleus accumbens, the ventral part of the striatal nucleus (Ma et al., 2002; Ohba et al., 2003). Because the present deals with the dorsal striatum, it is likely that our results are due to D<sub>2</sub> receptor antagonism.

The ER is a highly versatile protein synthesis factory that maintains cellular homeostasis via tight regulation of constitutive and inducible ER resident chaperones (Gregersen and Bross, 2010; Simmen et al., 2010). BiP/GRP-78, a glucose-regulated and calcium binding ER chaperone protein, is a central regulator of the UPR (Otero et al., 2010). Increased availability of BiP/GRP-78 in the lumen of the ER helps or influences translocation of new synthesized proteins (Kang et al., 2006). In the present study, we observed early increases in BiP/Grp-78 mRNA levels, in a fashion consistent with similar changes reported after a single injection of a large dose (40 mg/kg) of METH (Jayanthi et al., 2004; Jayanthi et al., 2009), after METH self-administration (Hayashi et al., 2010), and after multiple injections of amphetamine (AMPH) (Thomas et al., 2010b). We also identified similar SCH23390-sensitive increases in the mRNA levels of the ER membrane chaperone, P58<sup>IPK</sup>. Because up-regulation of P58<sup>IPK</sup> mRNA is a common response to ER stress events (Rutkowski et al., 2007; van Huizen et al., 2003), the present findings suggest that binge METH injections might cause ER stress. P58<sup>IPK</sup> is thought to be a co-chaperone which interacts with BiP/GRP-78 and other cytosolic chaperones including HSPs to promote co-translocational ER

protein degradation (Melville et al., 1997; Melville et al., 2000; Rutkowski et al., 2007). P58<sup>IPK</sup> can also independently bind to and inhibit the ER stress-inducible eIF2 $\alpha$  kinase, PERK, in order to attenuate the UPR cascade via negative feedback (van Huizen et al., 2003). Thus, our observations of METH-induced simultaneous increases in BiP/Grp-78 and P58<sup>IPK</sup> suggest that the organism was triggering compensatory responses to fight against METH-induced ER stress events.

We found, in addition, that binge METH injections caused significant increases in HSP40 and HSP70, chaperones that function to assist in the folding of stress-denatured proteins and have anti-apoptotic properties (Young, 2010). Our observations of METH-induced early increases in the levels of these two proteins are analogous to those reported in previous studies which showed that single toxic METH injections can cause increases in HSP70-like proteins (Bowyer and Davies, 1999; Kiyatkin and Sharma, 2010; Kuperman et al., 1997). Moreover, binge patterns of AMPH injections were also found to cause significant increases in the expression of both HSP40 and HSP70 proteins in the vasculature surrounding the forebrain (Thomas et al., 2010b). Thus, our observations extend these findings by showing that both relatively selective D<sub>1</sub> and D<sub>2</sub> receptor antagonists can attenuate METH-induced expression of HSPs, suggesting the involvement of both subtypes of DA receptors in mediating these increases. Previous studies have shown that the HSP induction after METH administration depends on METH-induced hyperthermia (Goto et al., 1993; Kuperman et al., 1997; Yu et al., 1999). These results are consistent with our present observations that the METH injections cause both hyperthermia and HSP induction. METH-induced changes in HSPs can be blocked by preventing hyperthermia in mice treated with ibogaine (Yu et al., 1999). In addition, lowering ambient temperature to 18°C attenuated the hyperthermic response to METH and blocked HSP72 induction (Goto et al., 1993). Our results thus suggest that the blocking effects of SCH23390 for METH-induced HSP40 and HSP70 chaperones might be dependent, in part, on the prevention of METH-induced hyperthermia by SCH23390, since pretreatment with raclopride, which did not prevent METH-induced increases in body temperature, still provided some degree of inhibition of HSP induction by METH. The blocking effects of raclopride might be due, in part, to its inhibitory effects on the ER stress pathway because activation of that pathway can also result in increased HSP mRNA levels (Schroder and Kaufman, 2005). Taken together, our present observations suggest that multiple factors might be involved in HSP regulation. This suggestion might explain the biphasic induction of HSP70 observed after METH since HSP70 mRNA levels

were elevated at 30 min post-METH treatment, became normalized in the intervening hours, and then increased again at 16 and 24 hr after the last METH injection.

We found that binge METH injections caused biphasic pattern of induction of Atf1, Atf3 and Atf4 genes. Members of the activating transcription factor (ATF) family have been implicated in various stress responses (Ameri and Harris, 2008; Jiang et al., 2004). Members of the ATF/CREB family are immediate early responsive genes that are regulated through a cAMP responsive element (CRE) consensus binding site (Ameri and Harris, 2008). The transcription factors in the ATF and activating protein-1 (AP-1) families can dimerize through their basic leucine-zipper domain and regulate their own expression (Hai and Curran, 1991). Thus, it is possible that the early and transient induction of transcription of Atf1 and Atf3, after METH administration, might be due to their regulation by CREB and AP-1 proteins. ATF1 has a high degree of homology with CREB with which it can heterodimerize. However, the role of ATF1 transcription factor in response to ER stress has not been investigated (Hurst et al., 1991). In contrast, there are several reports of the role of ATF3 and ATF4 in the ER stress response pathway (Jiang et al., 2004; Schroder and Kaufman, 2005). Our results showed a delayed induction of Atf3 gene, an effect that might be regulated by ATF4, as previously reported in other ER stress models (Jiang et al., 2004). There was also a rapid induction of Atf4 mRNA at 4h, followed by some degree of normalization, and then a delayed induction at 24 h after the last METH injection. The changes in Atf3 and Atf4 mRNA levels were also associated with increases in both ATF3 and ATF4 protein levels, findings consistent with our observations following a single large dose of METH (Jayanthi et al., 2009). It is also important to point out that the induction of these members of the ATF/CREB family might be regulated by CREB that is downstream of the D<sub>1</sub>-cAMP-PKA cascade (Sands and Palmer, 2008). This idea is consistent with our findings that putative blockade of the DA D<sub>1</sub> receptor by SCH23390 can completely block METH-induced ATF3 and ATF4 protein expression. ATF4 also regulates the expression of CHOP/Gadd153 during the UPR (Fawcett et al., 1999). The CHOP promoter contains C/EBP-ATF and ER stress responsive element (ERSE) sites that are essential for CHOP induction during ER stress (Ma et al., 2002; Oyadomari and Mori, 2004). During ER stress, ATF4 binds to C/EBP-ATF on the CHOP promoter, providing a partial explanation of the biphasic nature of METH-induced CHOP induction (Fawcett et al., 1999; Oyadomari and Mori, 2004). This discussion suggests that multiple mechanisms might contribute to METH-induced ER stress, since we also observed changes in SCH23390-sensitive cleaved caspase-12 proteins after METH

administration, with only partial inhibitory effects observed after pre-treatment with raclopride. The potential role of temperature regulation in these METH-induced changes needs to also be taken into consideration since SCH23390, but not raclopride, was able to block METH-induced hyperthermia.

Mitochondrial dysfunctions have been reported to influence METH toxicity (Cadet et al., 2003; Jayanthi et al., 2004). METH-induced cell death involves the release of the apoptogenic molecules cytochrome c and apoptosis inducing factor (AIF) from mitochondria, upregulation of pro-death members of the Bcl-2 family of mitochondrial proteins, as well as downregulation of anti-death proteins (Jayanthi et al., 2001; Jayanthi et al., 2004). Over-expression of Bcl-2, an anti-apoptotic gene, was able to protect against METH-induced apoptosis in immortalized neural cells (Cadet et al., 1997). The present findings of METH-induced decreases in the anti-apoptotic protein, Bcl-2, but increases in the pro-apoptotic proteins, Bax and Bad, are consistent with those observed after single large doses of METH (Jayanthi et al., 2001). Our results also suggest that these changes seem to be dependent on SCH23390-sensitive receptors but not on raclopride-sensitive ones.

Taken together, our findings suggest that SCH23390 and raclopride provide differential inhibition on METH-induced changes in proteins involved in ER and mitochondria cell death pathways. Our observations might provide a partial explanation for the previous report that SCH23390 provided almost total protection against cell death at a relatively low dose (0.1 mg/kg) given 30 min before an injection of METH (30 mg/kg) (Xu et al., 2005). However, comparatively higher dose of raclopride (1 mg/kg) was required to observe similar protective effects (Xu et al., 2005). Therefore, it is possible that the dosage of raclopride used in the present study might not have been enough to block METH-induced changes in ER stress-related genes and mitochondrial proteins, so that higher raclopride doses might have been more effective. However, use of such higher doses might cause a loss of the D<sub>2</sub>/D<sub>3</sub> specificity of the drug. The possibility also exists that differences in paradigms used in the two different studies, [single injection in the previous study (Xu et al., 2005) and multiple injections in our study], might have caused some of the discrepancies in the observations. It also needs to be pointed out that measurements of TUNEL-positive cells (Xu et al., 2005) are not equivalent to measures of ER- and mitochondria-dependent pathways (present study). Blockade of ER-dependent pathways by higher doses of raclopride might be sufficient to block the appearance of TUNEL-positive cells (Xu et al., 2005). This remains to be determined.

In summary, we report, for the first time, that binge METH injections can cause substantial increases in the expression of proteins that participate in ER- and mitochondria-dependent stress responses. These METH-induced changes appear to be secondary, for the most part, to stimulation of receptors that are more sensitive to inhibition by SCH23390 that almost completely blocked the METH-induced alterations in proteins involved in both ER and mitochondrial stresses. In contrast, the D<sub>2</sub>-like receptor antagonist, raclopride, had small to moderate effects on ER stress proteins but had no significant effects on mitochondria-dependent cellular stress proteins. When taken together, our results and those of other investigators suggest that the protective effects of SCH23390 might be due to inhibition of multiple death pathways in various subtypes of striatal neurons (Cadet et al., 2005; Xu et al., 2005) whereas raclopride might attenuate METH-induced activation of ER stress in enkephalin-positive GABA neurons (Thiriet et al., 2005; Xu et al., 2005) that express mainly the DA D<sub>2</sub> receptor subtype (LaVoie and Hastings, 1999).

## **Materials and Methods**

### **Animals, Drug Treatment and tissue collection**

The drugs used are (+/-)-methamphetamine HCL (NIDA Pharmacy), SCH23390 hydrochloride (TOCRIS bioscience, Ellisville, MO, USA) and raclopride (Sigma Aldrich, St. Louis, MO, USA). All drugs were diluted with 0.9% saline. All experiments were according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

Male Sprague-Dawley rats (Charles River Labs, Raleigh, NC, USA), weighing 250-300g, were housed individually in cages in a temperature-controlled room (22°C) and had free access to food and water. To test the effects of the D<sub>1</sub> receptor antagonist, SCH23390, animals were divided in four treatment groups. One group received four intra-peritoneal injections of saline given at 2-hr intervals and followed each by a dose of 10 mg/kg of METH 30 min later. Another group received saline alone according to the same schedule. The third and fourth groups received injections of SCH23390 (0.5 mg/kg) 30 min before each injection of saline or METH. The dose of SCH23390 used, was based on its high affinity for D<sub>1</sub> receptors ( $K_i=0.14$  nM) (Andersen and Gronvald, 1986). Thus, the four groups were: Saline + Saline (Control), SCH23390 + Saline (SCH), Saline + METH (METH) and SCH23390 + METH (SCH + METH), administered as patterns repeated four times at intervals two hours. Tympanic temperatures of the rats were measured with a Vet-Temp Instant

Animal Ear Thermometer. Temperature was recorded half-hour after each pattern of injections, and two hours after the last injection. Rats were sacrificed by decapitation at 30 min, 2, 4, 16 and 24 hr after the last saline or METH injections. Their brains were rapidly removed; striatal tissues were dissected, placed on dry ice, and then stored at -80°C until further assays. One side of the brain was used for quantitative PCR and the other side for western blot analyses.

Studies on the effects of D<sub>2</sub> receptor antagonism were conducted in a second group of rats. Experiments were, for the most part, similar to the ones described for SCH23390, except for the fact that we used the D<sub>2</sub>-like receptor antagonist, raclopride ( $K_D=1$  nM) at a dose of 0.5 mg/kg administered four times (Hall et al., 1988; Kohler et al., 1985). We also focused mostly on protein expression since protein products are the responsible agents in biochemical pathways. There were four groups of animals: Saline + Saline (Control), raclopride + Saline (Rac), Saline + METH (METH) and raclopride + METH (Rac + METH). Tympanic temperatures of the animals were also measured at the times mentioned earlier.

### **Quantitative RT-PCR analysis**

Total RNA was extracted from striatal samples and used for quantitative PCR to measure the expression of ER stress genes. We used the Qiagen RNeasy Midi kit (Qiagen, Valencia, CA, USA) to isolate total RNA. Analysis of samples for quality and quantity was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). A total of 1 µg RNA per sample was reverse-transcribed using oligo (dT) into cDNA using Advantage RT for PCR kit (Clontech, Mountain View, CA, USA). Sequences for gene-specific primers were designed by the LightCycler probe design software v. 2.0 (Roche, Indianapolis, IN, USA) and purchased from Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD USA). These sequences are listed in the Table 1. PCR experiments were performed using Lightcycler 480 II (Roche, Indianapolis, IN, USA) and iQ SYBR Green Supermix (Roche, USA). We have used a total of six animals per group in our experiment and have replicated each PCR running two or three times. Quantitation of our samples was determined using the second derivative crossing-points analysis. We have used the light chain of clathrin as internal control because of its stable expression across tissues and treatments. Fold changes in gene expression were calculated as ratios of normalized values for each group over those of the saline group.

## Western Blot

Cytoplasmic and nuclear fractions from striatal tissues were prepared using the NE-PER nuclear and cytoplasmic Extraction kit (Thermo scientific Pierce, Rockford, IL, USA). Protein concentration of cell lysates was quantified with the BCA protein assay kit (Thermo scientific Pierce, Rockford, IL, USA). For each protein studied, we have performed western blot analysis using six samples per group, and the experiment was replicated twice. Striatal protein lysates were separated by SDS-PAGE and electrophoretically transferred on PVDF membranes. Subsequently, the membranes were incubated overnight at 4°C with the following antibodies: HSP40, HSP70, Bad, Bax, Bcl-2, cytochrome c (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA), caspase-12 (1:1000; Biovision, Mountain View, CA, USA), ATF3, ATF4 and CHOP (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After incubation with the antibodies, the blots were washed with tris-buffered saline with 0.1% Tween-20. Afterwards, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse secondary antibody (1:1500; Cell Signaling Technology Inc., Danvers, MA, USA) for 1 hr at room temperature. To confirm equal protein loading, the blots were re-probed with  $\alpha$ -Tubulin antibody (1:4000, 2-hr at room temperature; Sigma-Aldrich, St. Louis, MO, USA<sup>34</sup>). LumiGLO chemiluminescent reagents (Cell Signaling Technology Inc., Danvers, MA, USA) were used to detect protein expression. Signal intensity was measured densitometrically with Carestream Molecular Imaging software (Carestream Health, Rochester, NY, USA).

## Statistical analysis

Statistical analysis for the qPCR and western blot data was carried out by a one-way ANOVA followed by post-hoc Fisher's protected least square difference (PLSD) test using StatView version 5.0.1 (SAS Institute, Cary, NC, USA). P values less than 0.05 were considered significant.

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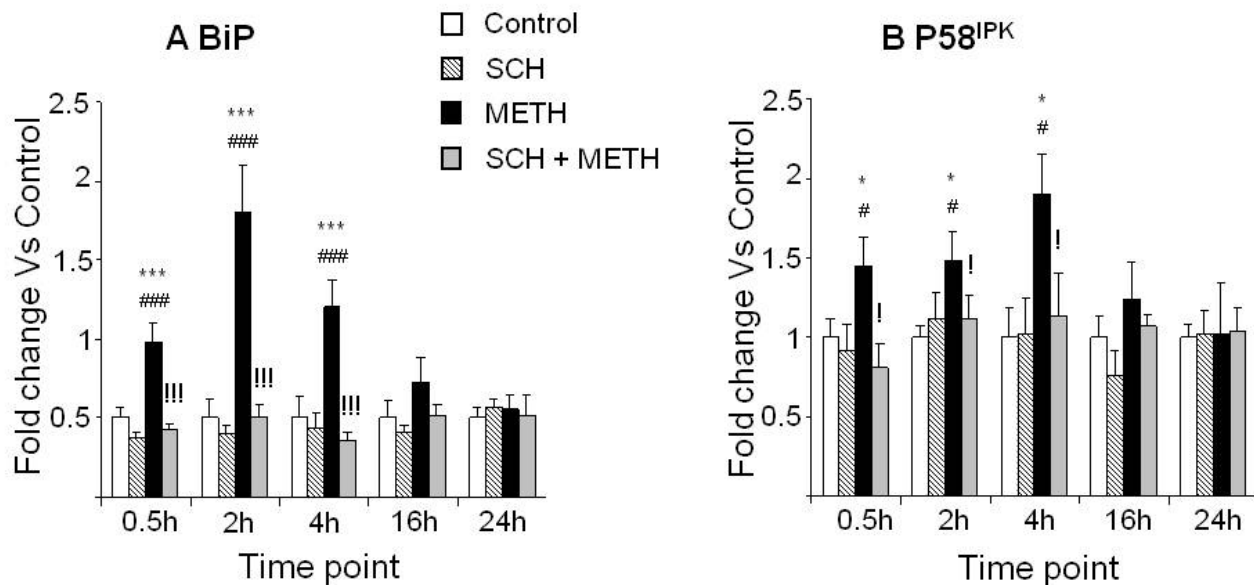
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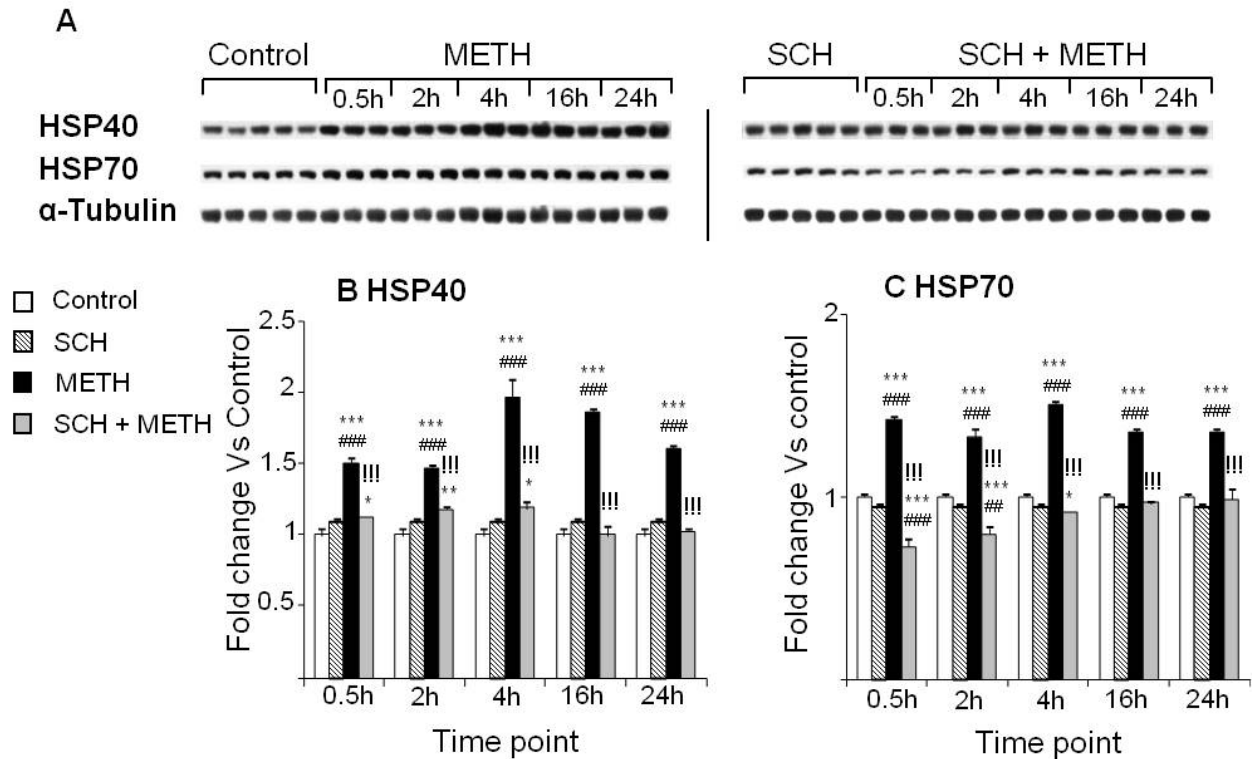
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**Table 1. Primer sequences**

<b>Gene</b>	<b>Forward primer</b>	<b>Reverse primer</b>
<b>Atf1</b>	GAT GCT CAA GGA AAC GGA	CAC ACA ACA CAC ACA GAA
<b>Atf2</b>	TCA TAA AGA TTG CCC TGT AAC	GAA CTG ACT CCA TTG GAC
<b>Atf3</b>	TGG AGT CAG TCA CCA TCA A	CAT TCA CAC TCT CCA GTT
<b>Atf4</b>	TCG GCC CAA ACC TTA TGA	TAG CTC CTT ACA CTC GC
<b>Atf5</b>	AGA AGA GAG ACC AGA ATA AG	CAT ACT GGA TCT CCC GT
<b>Atf6</b>	AAG TGA AGA ACC ATT ACT TTA TAT C	TTT CTG CTG GCT ATT TGT
<b>BiP/GRP-78</b>	TAC TCG AAT TCC AAA GAT TCA G	TCA AGC AGA ACC AGG TC
<b>P58<sup>IPK</sup></b>	GAG CCC GAC AAT GTA AA	AAT AAT CCC GCT TCT GTG
<b>CHOP/Gadd153</b>	GGA AGT GCA TCT TCA TAC ACC ACC	TGA CTG GAA TCT GGA GAG CGA GGG
<b>Gadd34</b>	TGA ATG TTG AGA GAA GAA CC	TTG TTT AGA AGT CGC TCT G
<b>Clathrin</b>	AAG TAT CCG TAA GTG GAG	GGG GTT AAA GTC ACA CAG

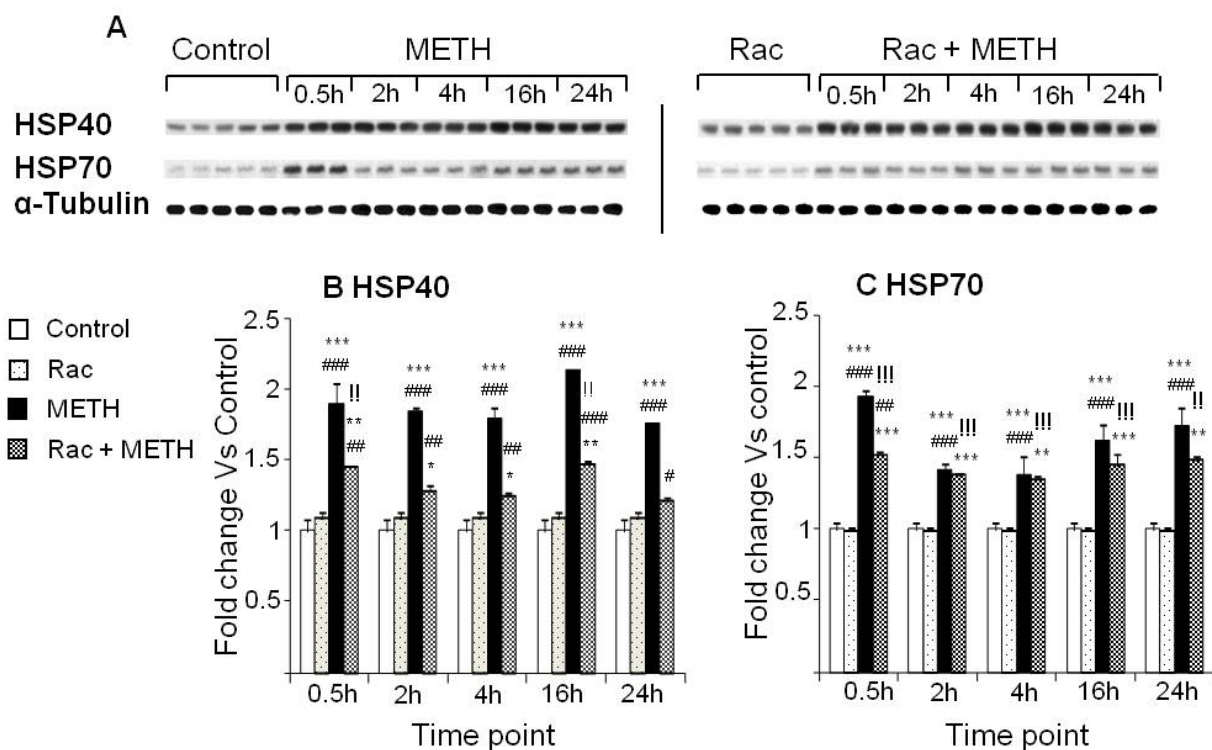


**Fig.1. Binge METH injections caused time-dependent increases in the expression of the ER chaperone, BiP/GRP-78, and of the co-chaperone, P58<sup>IPK</sup>.** Levels of (A) BiP/GRP-78 and (B) P58<sup>IPK</sup> transcripts were rapidly increased at 30 min after METH injections. RT-PCR was performed on total RNA isolated from the striatal tissue. Data were obtained from RNA isolated from six animals per group and determined individually. The levels of mRNA were normalized to clathrin mRNA levels. Values obtained for the treatment groups were compared by analysis of variance (ANOVA) followed by post-hoc analyses when ANOVA revealed significant changes. Key to statistics: \* =  $p < 0.05$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. # =  $p < 0.05$ ; ### =  $p < 0.001$ , in comparison to the SCH group. ! =  $p < 0.05$ ; !!! =  $p < 0.001$ , in comparison of METH group to the SCH + METH group.

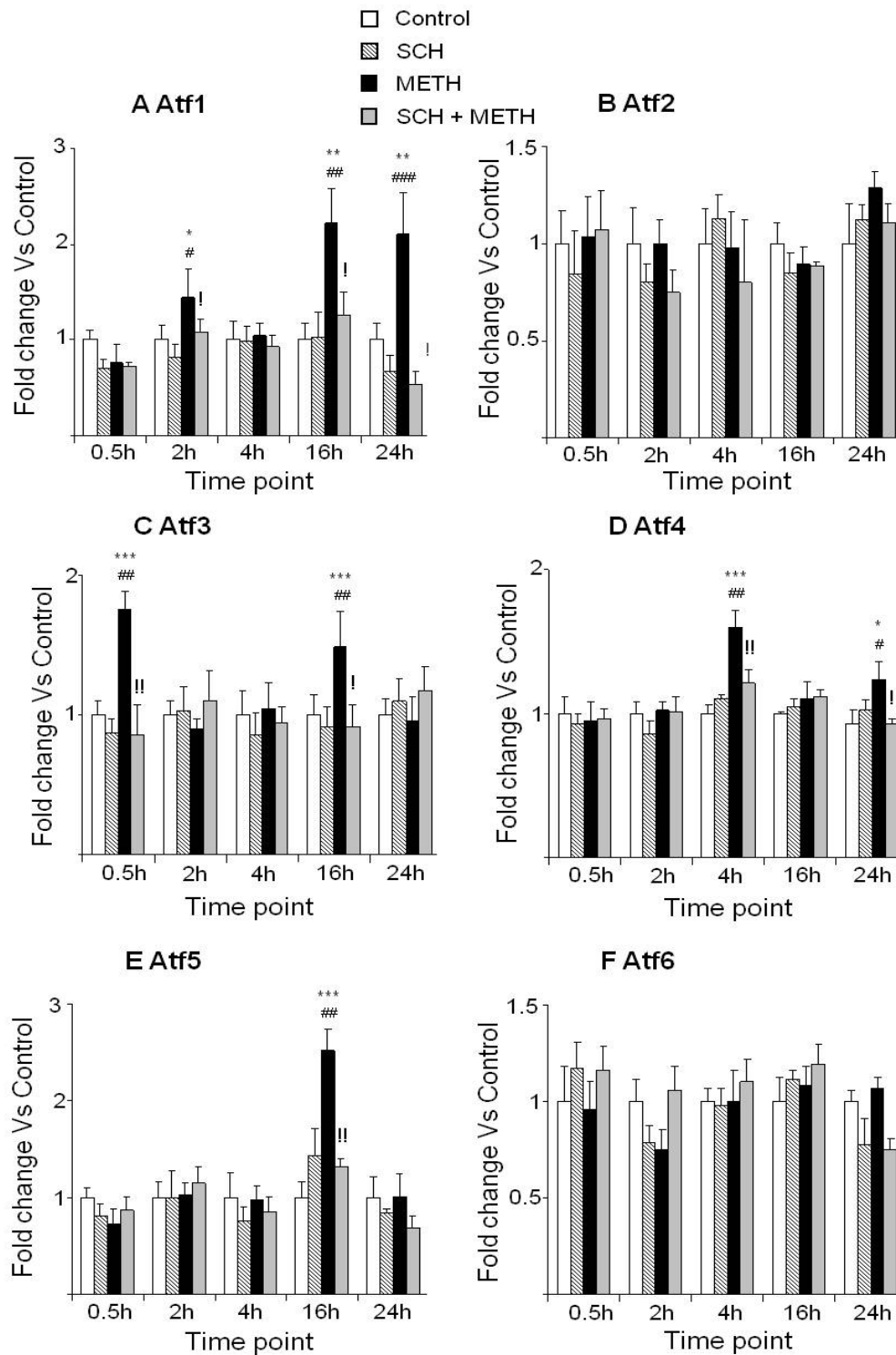


**Fig. 2. Effects of METH and SCH23390 administration on the expression of cytosolic chaperones HSPs.** (A) Representative western blot bands (1 band for Saline or SCH representing each time-point, 3 bands for METH and SCH + METH). METH administration caused rapid and stable induction of the chaperones HSP40 (B) and HSP70 (C). Pretreatment with SCH23390 prevented these increases. Protein expression was normalized to  $\alpha$ -Tubulin. Key to statistics: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the SCH group. !!! =  $p < 0.001$ , in comparison of METH group to the SCH + METH group.



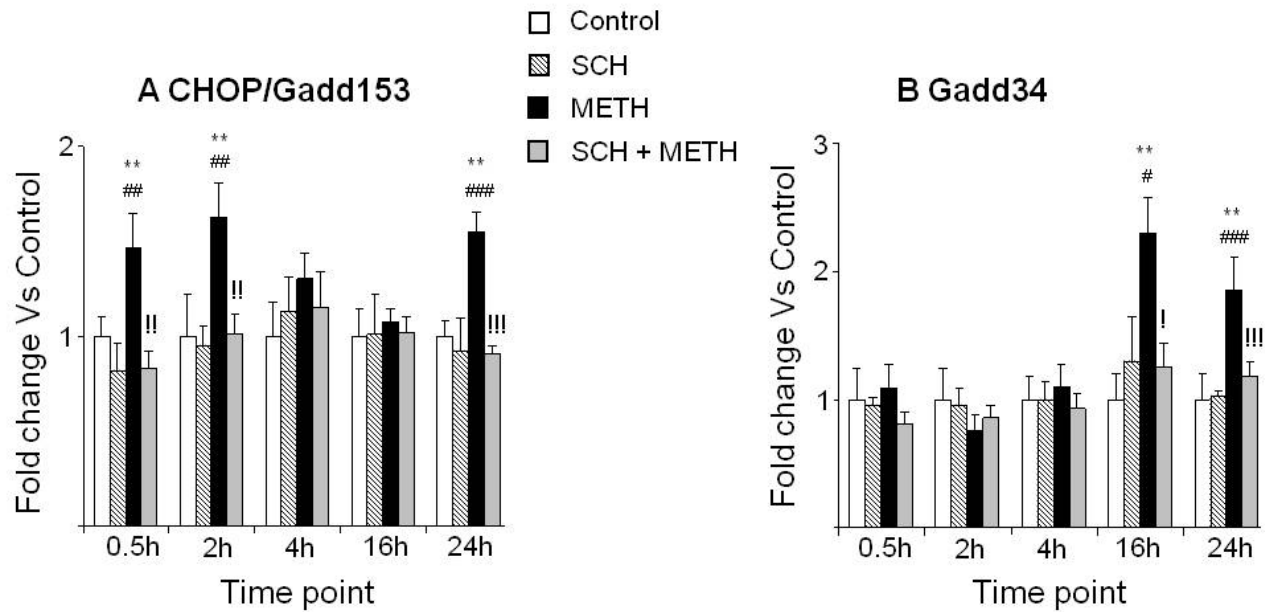


**Fig. 3. Effects of raclopride on METH-induced HSP40 and HSP70.** (A) Representative immunoblots of the effects of the drugs. (B, C) Quantitative analysis of the proteins. Protein expression was normalized to  $\alpha$ -Tubulin. Key to statistics: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. # =  $p < 0.05$ ; ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the Rac group. !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison of METH group to the Rac + METH group.

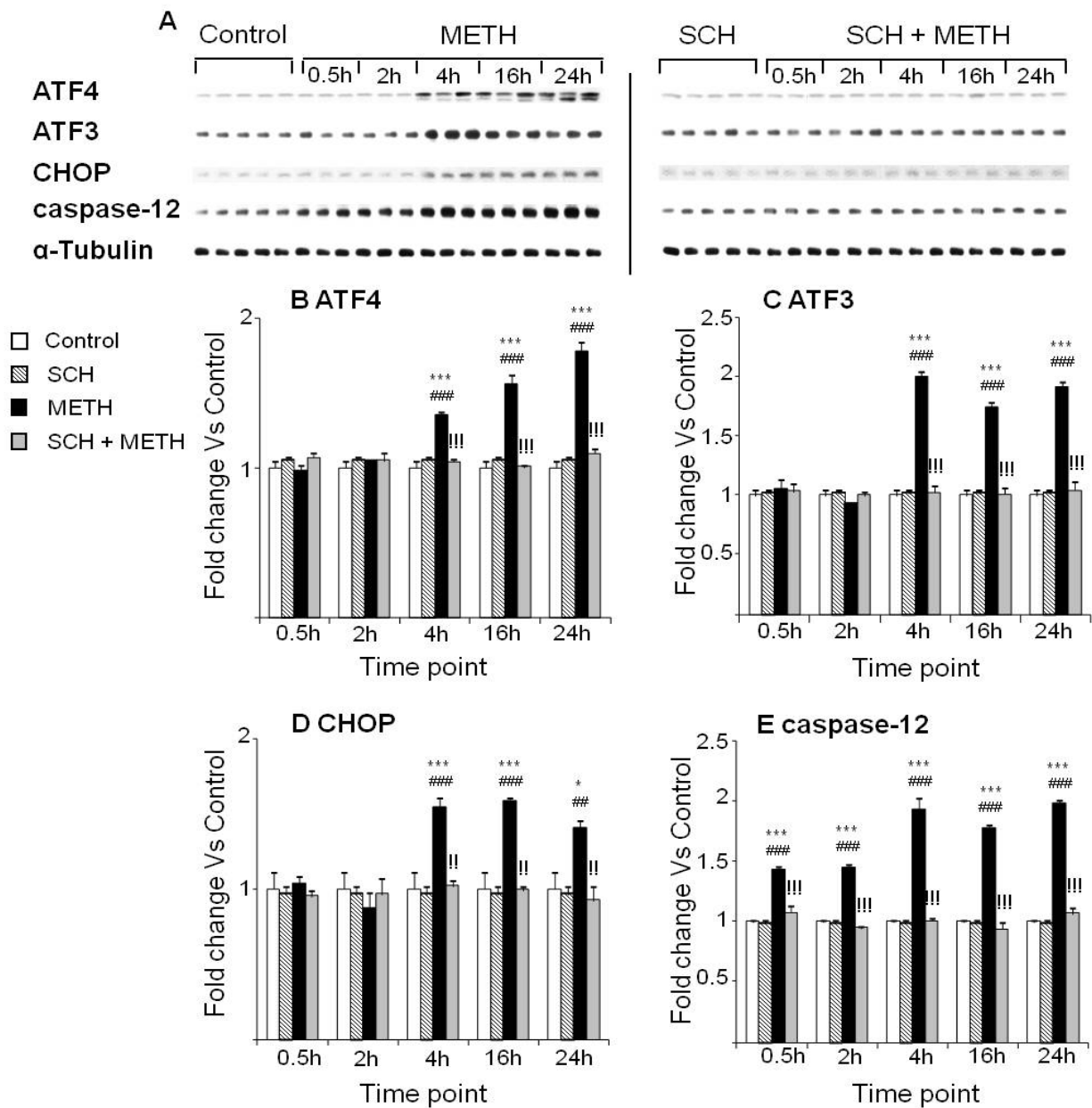


**Fig. 4. METH caused differential effects on ER stress genes.** Binge toxic doses of METH have differential effects on the members of the ATF family of transcription factors (A-F). Key to statistics: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. # =  $p < 0.05$ ; ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the SCH group.

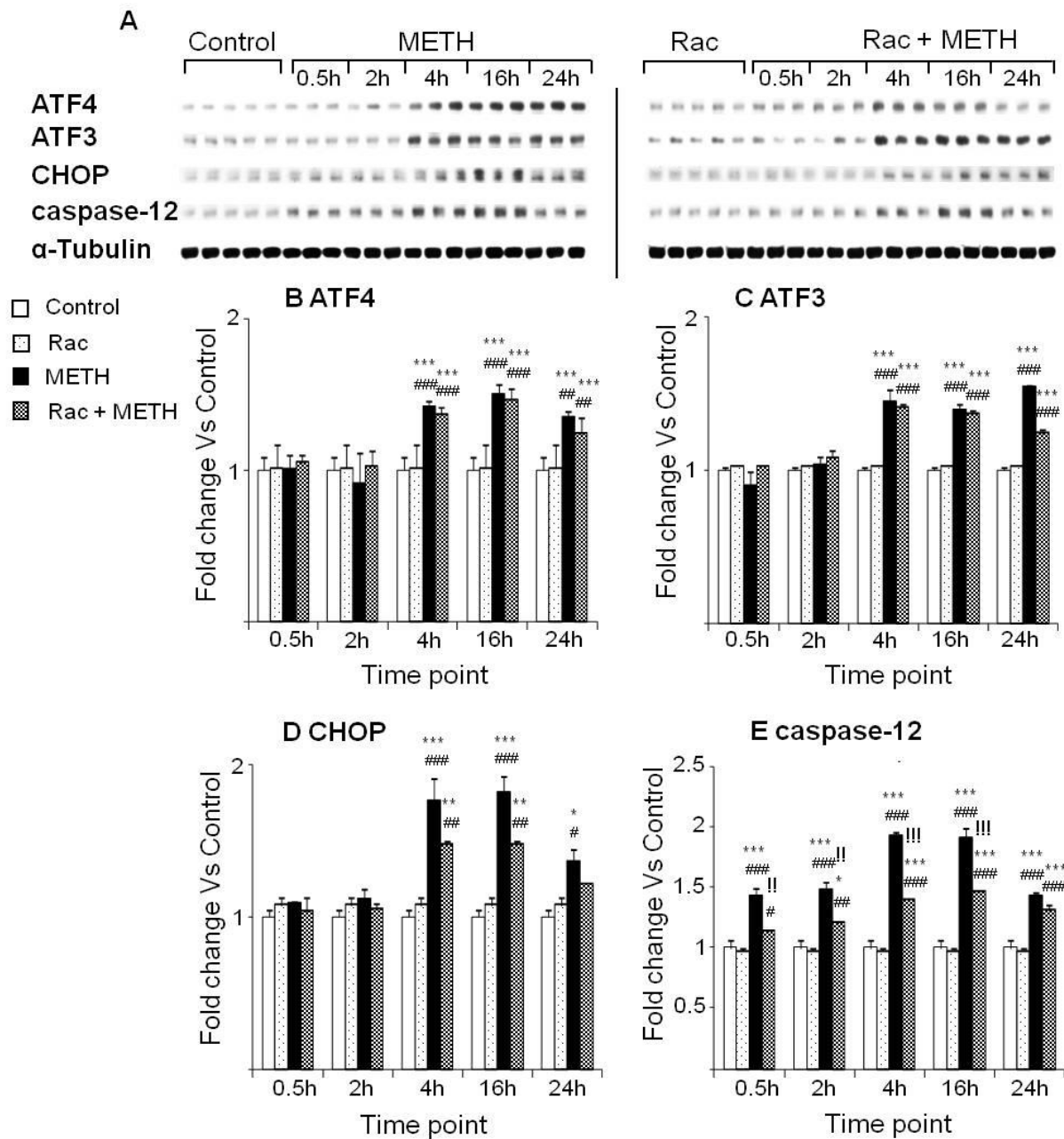
0.05; ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the SCH group. ! =  $p < 0.05$ ; !! =  $p < 0.01$ , in comparison of METH group to the SCH + METH group.



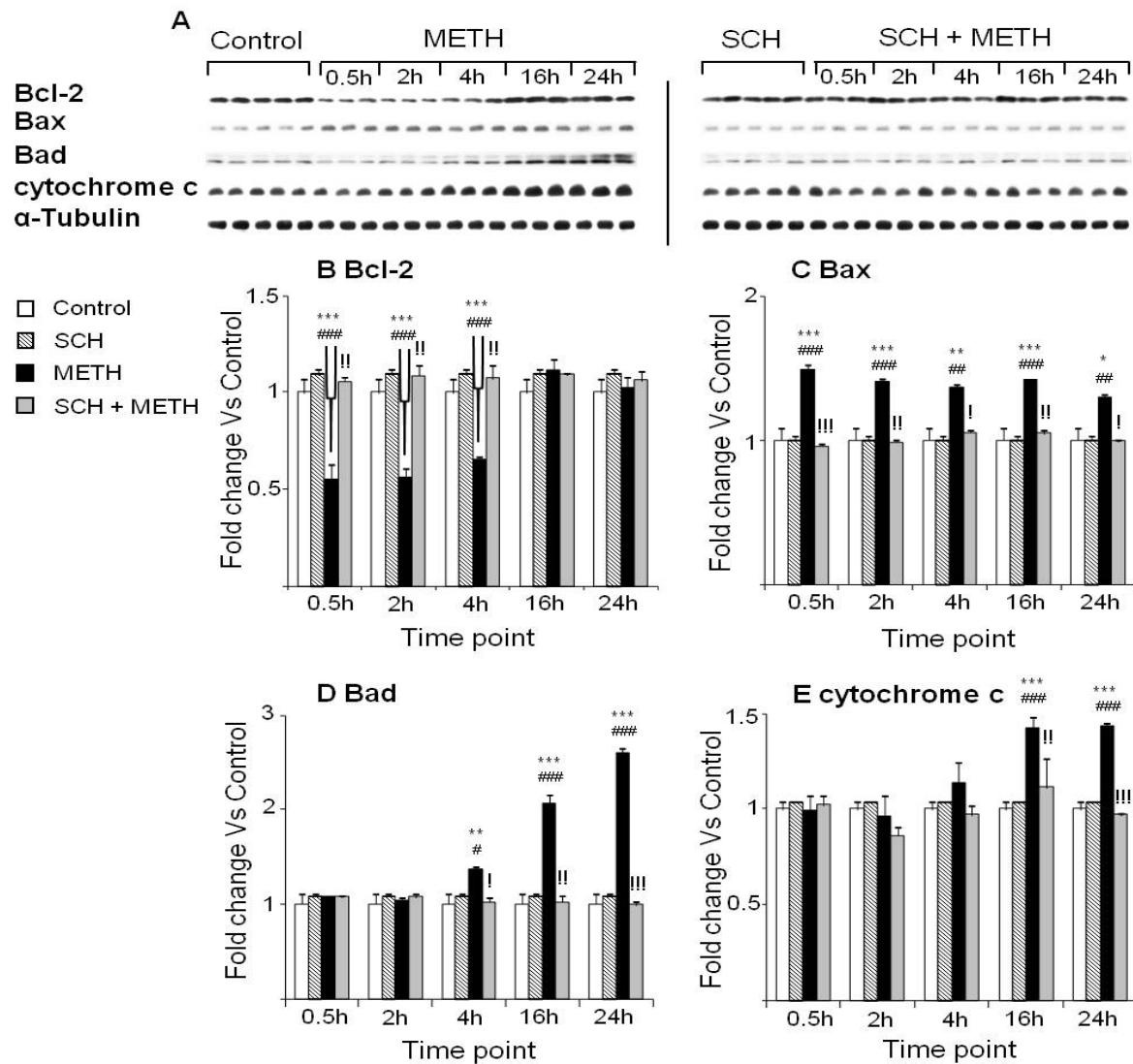
**Fig. 5. Effects of METH on the transcript levels of pro-death genes.** (A) METH caused rapid induction in Chop/Gadd153 mRNA levels. (B) Gadd34 was up-regulated at late time-points. Key to statistics: \*\* =  $p < 0.01$ , in comparison to the Saline group. # =  $p < 0.05$ ; ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the SCH group. ! =  $p < 0.05$ ; !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison of METH group to the SCH + METH group.



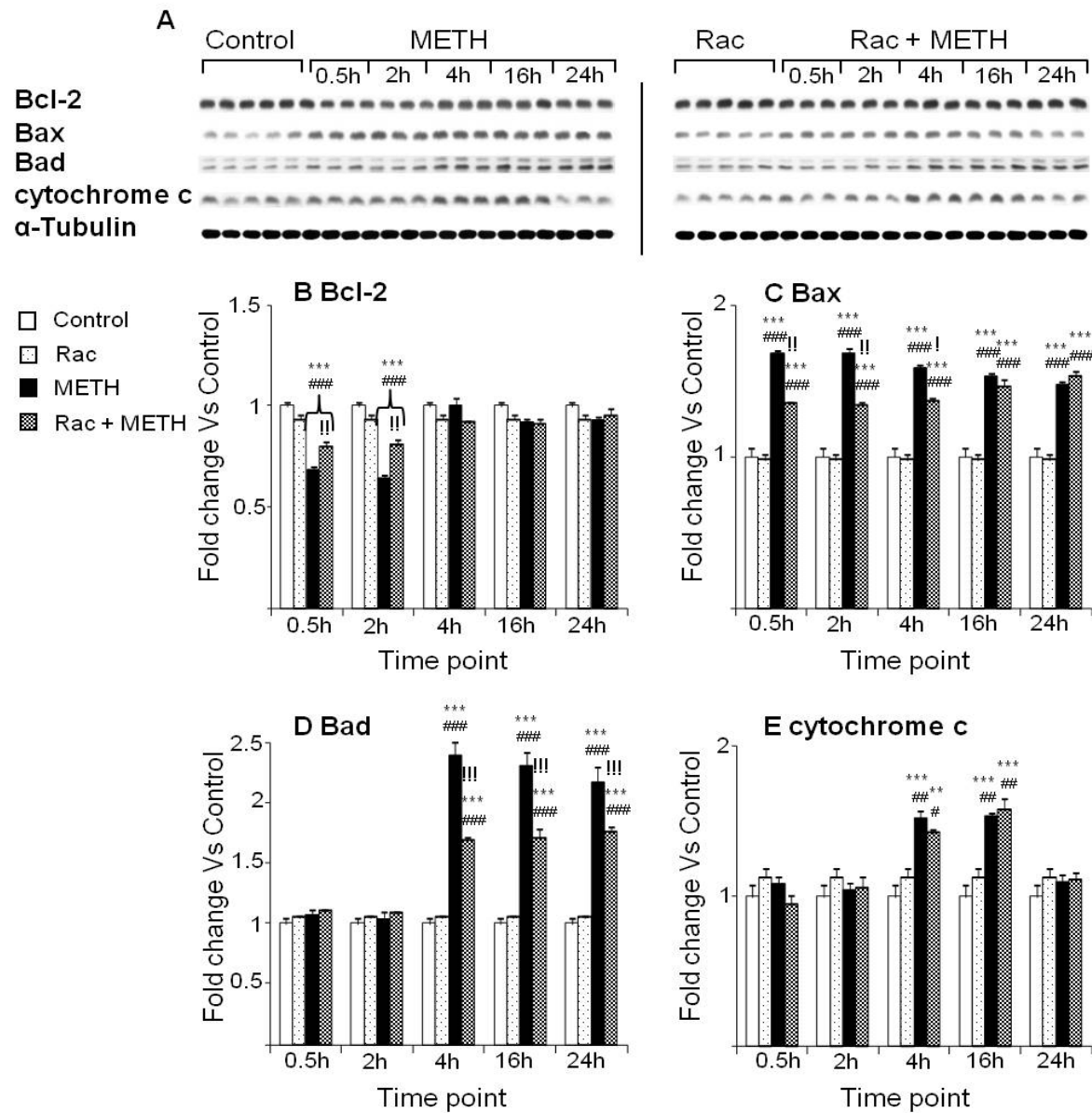
**Fig. 6. Effects of METH injections and SCH23390 treatment on the expression of stress response regulated proteins.** (A) Representative immunoblots showing the effects of METH and SCH23390. (A-E) Pretreatment with SCH23390 blocked the METH-induced changes on ATF3 (B), ATF4 (C), CHOP (D) and caspase-12 (E). Protein expression was normalized to  $\alpha$ -Tubulin. Key to statistics: \* =  $p < 0.05$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the SCH group. !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison of METH group to the SCH + METH group.



**Fig. 7. Raclopride did not block METH-induced ATF4 and ATF3 expression.** (A) Representative immunoblots of ATF4, ATF3, CHOP and caspase-12. Quantification of ATF4 (B), ATF3 (C), CHOP (D) and caspase-12 (E) are shown. Key to statistics: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. # =  $p < 0.05$ ; ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the Rac group. !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison of METH group to the Rac + METH group.

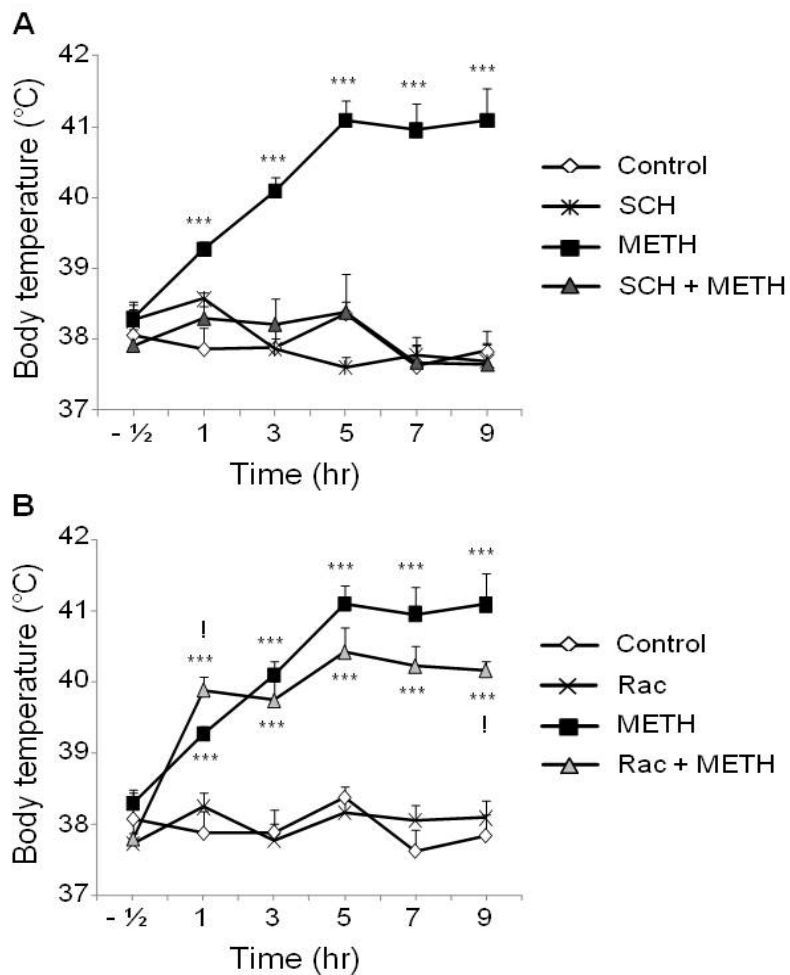


**Fig. 8. Effects of METH and SCH23390 treatments on the expression of mitochondrial dysfunction-related proteins.** (A) Representation of immunoblots of Bcl-2, Bax, Bad and cytochrome c. (B) METH injections caused rapid SCH23390-sensitive decreases in Bcl-2 protein levels. METH caused rapid increases in (C) Bax and (D) Bad protein levels that were inhibited by SCH23390 pretreatment. (E) METH injections were associated with release of cytochrome c from mitochondrial to cytoplasmic compartments, as shown by increases in cytochrome c levels in cytoplasmic fractions. Pretreatment with SCH23390 blocked cytochrome c release. Key to statistics: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. # =  $p < 0.05$ ; ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the SCH group. ! =  $p < 0.05$ ; !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison of METH group to the SCH + METH group.



**Fig. 9. The effects of METH and raclopride on the expression of the Bcl-2 family of proteins and cytochrome c.** (A) Representative immunoblots. (B) Pretreatment with raclopride attenuated METH-induced decreases in Bcl-2 protein levels, and (C, D) increases in Bax and Bad expression. In contrast, raclopride was ineffective to block cytochrome c induction (E). Protein expression was normalized to  $\alpha$ -Tubulin. Key to statistics: \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. # =  $p < 0.05$ ; ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the Rac group. ! =  $p < 0.05$ ; !! =  $p < 0.01$ ; !!! =  $p < 0.001$ , in comparison of METH group to the Rac + METH group.





**Fig. 10. Effects of SCH23390 and raclopride on METH-induced hyperthermia.** Animals in the control group received an injection of saline followed 30 min later by another injection of saline, this pattern of injections was repeated four times at 2-hr intervals. The METH treatment group of rats received four injections of saline at 2-hr intervals, each saline injection being followed by an injection of METH (10 mg/kg). (A) Two other groups of animals were pretreated with SCH23390 30 min before each of four saline or METH injections given according to the intervals described above. (B) Two other groups were pretreated with raclopride and treated with either saline or METH injections as above. Temperature was recorded 1 hr prior to the first injection (-1 hr), 30 min after each combined set of injections (shown in arrows), and 2 hr after the final injection. Statistical differences in temperature were considered significant at p values less than 0.05. Key to statistics: \*\*\* =  $p < 0.001$ , in comparison to the control group. ! =  $p < 0.05$ , in comparison to the METH group (post-hoc test).

## **Manuscript 4**

**Binge methamphetamine injections cause SCH23390-sensitive increases in  
nuclear Smad2 phosphorylation**

## **Binge methamphetamine injections cause SCH23390-sensitive increases in nuclear Smad2 phosphorylation**

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## Abstract

Methamphetamine (METH) abuse causes long-term degeneration of monoamine terminals, neuronal apoptosis and changes in gene expression in various brain regions. The present study was undertaken to identify transcriptional responses in the rat striatum after binge METH injections. Male Sprague-Dawley rats were administered METH (10 mg/kg, i.p.) or saline four times in one day at 2-hour intervals. Rats were euthanized at various time-points after the last injection of METH or saline. Microarray and RT-PCR analyses showed that METH caused early induction of several genes including activin  $\beta$ A, TGF- $\beta$ , and follistatin. The METH injections also caused increased phosphorylation of Smad2 in cytoplasmic and nuclear fractions obtained from the striatum. Pretreatment with the dopamine D<sub>1</sub> receptor antagonist, SCH23390, completely blocked METH-induced changes in TGF- $\beta$  expression and Smad2 phosphorylation. In contrast, the dopamine D<sub>2</sub> receptor antagonist, raclopride, only partially blocked the effects of METH on TGF- $\beta$  expression and Smad2 phosphorylation. These observations suggest that the TGF- $\beta$ -Smad2 signaling pathway might be involved in the molecular effects of binge METH administration in the dorsal striatum.

## Introduction

(METH) is abused worldwide because of its powerful stimulant properties (Meredith et al., 2005). Long-term use of the drug has negative effects on several cognitive and motor functions (Homer et al., 2008; Volkow et al., 2001d). Findings had identified neurochemical changes in the brain of METH addicts that affect the dopaminergic and serotonergic neurons. For instance, the striatum exhibit decreases in the levels of dopamine (DA), dopamine transporters, and vesicular monoamine transporter-2 (Kitamura et al., 2007; Wilson et al., 1996). Serotonin transporters (Sekine et al., 2006; Volkow et al., 2001d) levels are also decreased. METH treatment also leads to neurodegeneration of monoaminergic terminals in the rodent brain (Marshall et al., 2007; Ricaurte et al., 1980). Single and binge toxic doses of METH also cause cell death of postsynaptic neurons localized in various brain regions, including the striatum, cortex, hippocampus and olfactory bulb (Deng and Cadet, 2000; Deng et al., 2002a; Deng et al., 2007; Ladenheim et al., 2000; Zhu et al., 2005; Zhu et al., 2006b).

The present study used microarray analysis to identify changes in gene expression after binge METH injections. We found increases in activin  $\beta$ A/inhibin  $\beta$ A, transforming growth factor 1 (TGF- $\beta$ 1) and follistatin (see below). The changes in TGF- $\beta$ 1 are consistent with our observations of increases in TGF- $\beta$ 1 in the striatum after METH treatment in two previous microarray studies (Cadet et al., 2001; Cadet et al., 2002). Although we had not pursued these observations further, several members of the TGF- $\beta$  superfamily of trophic factors behave as early response genes after various brain injuries that include ischemia and excitotoxicity (Florio et al., 2007; Kriegstein et al., 2002; Mukerji et al., 2009; Vivien and Ali, 2006). They appear to determine, in part, the survival or death of neurons of interest to the present study. Exogenous administration of two of these factors, glial cell line-derived neurotrophic factor (GDNF) and bone morphogenetic protein-7 (BMP7) was shown to prevent degeneration of DA nerve terminals in vivo (Cass, 1996; Chou et al., 2008) and to block neuronal apoptosis of striatal neurons in vitro (Chou et al., 2008). In order to further investigate the effects of METH on the TGF- $\beta$  signaling, we used RT-PCR to confirm the changes in gene expression. We also found increases in TGF- $\beta$  protein and in phosphorylated Smad2 (P-Smad2). Pretreatment with the DA D<sub>1</sub> receptor antagonist, SCH23390, completely blocked METH-induced changes in TGF- $\beta$  and phosphorylated Smad2 expression whereas the DA D<sub>2</sub> receptor antagonist, raclopride, had partial inhibitory effects.

## **Methods**

### **Animals and Drugs treatment**

All experiments were done according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee. Male Sprague-Dawley rats (Charles River Labs, Raleigh, NC, USA), weighing 250-300g, were used in this study. During treatment, they were housed one per cage in a temperature-controlled room (22°C) and had free access to food and water. The animals were injected with the following drugs: (+/-)-methamphetamine HCL (NIDA Pharmacy), SCH23390 hydrochloride (TOCRIS bioscience, Ellisville, MO, USA) and raclopride (Sigma Aldrich, St. Louis, MO, USA). All drugs were diluted with 0.9% saline.

The animals were injected with four intra-peritoneal (i.p.) injections of saline given at 2-hr intervals and followed each by a dose of 0 (saline + saline or control group) or 10 mg/kg of METH 30 min later (saline + METH or METH group). To test the effects of the D<sub>1</sub> receptor antagonist, SCH23390, animals received four i.p. doses of SCH23390 (0.5 mg/kg) at 2-hr intervals, each injection followed 30 min by an injection of saline (SCH23390 + saline or SCH group) or 10 mg/kg METH (SCH23390 + METH or SCH + METH group). The effects of the DA D<sub>2</sub> receptor antagonist, raclopride, were investigated by treating animals with four injections of raclopride (0.5 mg/kg), followed each by an injection of saline (raclopride + saline or Rac group) or METH (10 mg/kg) (raclopride + METH or Rac +METH group) 30 min later. Rats were sacrificed by decapitation 30 min, 2, 4, 16 and 24 hrs after the last injection. The brains were rapidly removed; striatal tissues were dissected, placed on dry ice, and then stored at -80°C until further assays. One side of the brain was used later to extract RNA and perform RT-PCR and microarray experiments, while the other side was used for western blot analyses.

### **RNA extraction**

We used the Qiagen RNeasy Midi kit (Qiagen, Valencia, CA, USA) to isolate total RNA from samples of four treatment groups: Control, METH, SCH and SCH +METH. Analysis of quality and quantity of extracted RNA were assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA), before performing microarray experiment and quantitative RT-PCR.

## **Microarray analysis**

Illumina's RatRef-12 Expression BeadChips arrays (22, 227 probes) were used in the microarray analysis (Illumina Inc., San Diego, CA, USA). We have analyzed samples from control group and METH group at 2-hrs time-point. A 600 ng aliquot of total RNA from each striatal sample was amplified using Ambion's Illumina RNA Amplification kit (cat. no. IL1791; Ambion, Austin, TX, USA). Single stranded RNA (cRNA) was generated and labeled by incorporating biotin-16-UTP (Roche Diagnostics GmbH, Mannheim, Germany, cat. no. 11388908910). 750 ng of amplified cRNA were hybridized on the Chips at 55°C overnight according to the manufacturer's protocol. Hybridized biotinylated cRNA was detected with Cyanine3- streptavidine (cat. #146065; Amersham Biosciences, Piscataway, NJ, USA) and quantified using Illumina's BeadStation 500GX Genetic Analysis Systems scanner.

The Illumina BeadStudio software was used to measure fluorescent hybridization signals. Data were extracted by BeadStudio (Illumina, San Diego, CA, USA) and then analyzed using GeneSpring software v. 7.3.1 (Silicon Genetics, Redwood City, CA, USA). Raw data were imported into GeneSpring and normalized using global normalization. METH-responsive genes were kept if they have a 1.5-fold change and the P value < 0.05.

The list of significant genes at 2-hrs time-point was used to identify connections between them using the Ingenuity Pathways Analysis (IPA 6.5 software, Ingenuity Systems). These connections were representing as molecular networks. The canonical pathway analysis compared our list of genes to established molecular pathways to indicate which pathways were activated in response to METH. The statistical significance was set at  $P < 0.05$ .

## **RT-PCR analysis**

An aliquot of 1 µg RNA sample was reverse-transcribed using Advantage RT for PCR kit (Clontech, Mountain View, CA, USA). Sequences for gene-specific primers were designed by the LightCycler probe design software v. 2.0 (Roche, Indianapolis, IN, USA) and purchased from Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD, USA). The following PCR primers were used: rat inhibin  $\beta$ A subunit: forward: GCT TCA TGT GGG TAA AGT, reverse: CTG CCT TCC TTG GAA ATC; follistatin: forward: CCC TTG TAA AGA AAC GTG, reverse: CAC ATT CGT TGC GGT AG; rat activin receptor type IIA (acvrIIA): forward: TAG AGG ATT GGC ATA TTT ACA TGA, reverse: CAA ACT TTA AGG CCA ACC. PCR

experiments were performed using Lightcycler 480 II (Roche, Indianapolis, IN, USA) and iQ SYBR Green Supermix (Roche, Indianapolis, IN, USA). We have used a total of six animals per group in our experiment and have replicated each PCR run two or three times. Quantitation of our samples was determined using the second derivative crossing-points analysis. The expression of the housekeeping gene clathrin was used to normalize the expression of the other genes. Fold changes in gene expression were calculated as ratios of normalized values for each group over those of the saline group.

### **Western blotting experiments**

Protein lysates from striatal tissues were extracted as well as cytoplasmic and nuclear fractions were separated using the NE-PER nuclear and cytoplasmic Extraction kit (Thermo scientific Pierce, Rockford, IL, USA). Protein concentrations were determined by the BCA protein assay kit (Thermo scientific Pierce, Rockford, IL, USA). For each protein studied, we have performed western blot analysis using six samples per group, and the experiment was replicated twice. Striatal protein lysates were separated by SDS-PAGE and electrophoretically transferred on PVDF membranes. Subsequently, the membranes were incubated overnight at 4°C with the following antibodies: TGF- $\beta$  (1:250; Cell Signaling Technology Inc., Danvers, MA, USA), Smad2 (Antibody dilution: 1:1000), and P-Smad2 (antibody dilution: 1:250) (Cell Signaling Technology Inc., Danvers, MA, USA). Smad2 and P-Smad2 were measured in nuclear and cytoplasmic lysates. After incubation with the antibodies, the blots were washed with tris-buffered saline with 0.1% Tween-20. Afterwards, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse secondary antibody (1:1500; Cell Signaling Technology Inc., Danvers, MA, USA) for 1 hr at room temperature. To confirm equal protein loading, the blots were re-probed with  $\alpha$ -Tubulin antibody (1:4000, 2-hr at room temperature; Sigma-Aldrich, St. Louis, MO, USA<sup>34</sup>). LumiGLO chemiluminescent reagents (Cell Signaling Technology Inc., Danvers, MA, USA) were used to detect protein expression. Signal intensity was measured densitometrically with Carestream Molecular Imaging software (Carestream Health, Rochester, NY, USA).



## Statistics

Statistical analysis for the qPCR and western blot data was carried out by a one-way ANOVA followed by post-hoc Fisher's protected least square difference (PLSD) test using StatView version 5.0.1 (SAS Institute, Cary, NC, USA). P values less than 0.05 were considered significant.

## Results

### Binge METH administration caused varied changes in gene expression

To identify new signaling pathways that are induced after binge METH injections, we compared striata tissues of rats treated with either saline or METH using microarray analysis with Rat Illumina arrays that allowed for query of 22,227 transcripts. We found that METH caused changes in the expression of 536 genes (351 upregulated, 185 downregulated) at 2 hrs after the last injections. These genes included different transcription factors, regulators of cell cycle and differentiation, endoplasmic reticulum stress-responsive genes, neuropeptides and genes effectors in signaling transductions. Among those, there were several genes related to the TGF- $\beta$  signaling pathway, including TGF- $\beta$ , activin  $\beta$ A and follistatin (Table 1). Analysis with the IPA software showed that the canonical TGF- $\beta$  signaling pathway was significantly affected (Fig. 1). Genes of interest include TGF- $\beta$  and activins (shown in pink). We chose to further investigate the effects of METH on TGF- $\beta$ .

Quantitative PCR was used to confirm METH's effects on the expression of activin  $\beta$ A and follistatin genes (Fig. 2). Consistent with the array data, METH caused rapid and transient increases in activin  $\beta$ A mRNA 30 min after drug treatment, and normalization was observed at 16 hrs ( $p < 0.0001$ ) (Fig. 2A). METH also caused increases in the expression of follistatin that functions as an antagonist of activin A (Abe et al., 2004). Follistatin was induced at 30 min time-point ( $p < 0.0001$ ), and returned to normal at 16 hrs (Fig. 2B). In addition, we have measured the expression of acvrIIA in the striatum after METH administration. METH also caused rapid and significant increases in acvrIIA from 0.5 hr to 4 hrs ( $p < 0.0001$ ) (Fig. 2C).

### **METH caused SCH23390-sensitive increases in striatal TGF- $\beta$ protein**

To test if METH caused changes in TGF- $\beta$  expression, western blot analysis was used to measure the expression of striatal TGF- $\beta$  and analyze the effects of SCH23390 and raclopride pretreatments. TGF- $\beta$  protein increased at 30 min after binge METH injections by 1.6-fold (Figs. 3A and 3B). TGF- $\beta$  returned to control levels at 16-hrs time-point. SCH23390 completely blocked METH-induced changes in TGF- $\beta$  expression (Figs. 3A and 3B). Raclopride pretreatment only partially blocked the METH-induced increases in TGF- $\beta$  protein expression (Figs. 3C and 3D).

### **Effects of METH and pretreatment with SCH23390 and raclopride on Smad2 phosphorylation**

To investigate whether the induction in TGF- $\beta$  expression caused activation of its signaling transducer, western blot was used to examine the phosphorylation state of Smad2 in both cytoplasmic and nuclear fractions of striatal cell lysates. The phosphorylation of Smad2 was increased almost 4-fold in the cytosol after METH administration at 30 min, 2 and 4 hrs ( $p < 0.0001$ ) (Figs. 4A and 4B). The increases in the cytoplasmic fraction were correlated to increases in the striatal nuclear fraction from 30 min to 4 hrs ( $p < 0.0001$ ) (Figs. 4C and 4D). SCH23390 pretreatment suppressed METH-mediated phosphorylation of Smad2 in both cytoplasmic and nuclear fractions (Figs. 4A-4D). In contrast, raclopride pretreatment partially blocked the METH-induced Smad2 phosphorylation moderated the induction in cytoplasmic phosphorylated Smad2 following METH treatment at 30 min, 2 hrs and 4 hrs time-points (Figs. 5A and 5B). Raclopride also attenuated METH-induced increases of the phosphorylation of Smad2 in the nuclear fractions at 30 min and 2 hrs time-points, while it completely blocked the induction at 4 hrs (Figs. 5C and 5D).

### **Discussion**

The main findings of the present experiments are that binge METH injections caused early activation of several members of the TGF- $\beta$  superfamily of trophic factors. The TGF- $\beta$  superfamily consists of TGF- $\beta$ s, activins, inhibins, GDNF and bone morphogenic proteins (BMPs). These ligands play central roles in neuronal development, differentiation, apoptosis, and survival

(Krieglstein et al., 2002). Of interest to our present study, binge METH injections (4x10 mg/kg) caused decreases in BMP7 mRNA in the substantia nigra and the striatum of mice (Chou et al., 2008). BMP7 +/- mutant mice were more sensitive to METH neurotoxicity (Chou et al., 2008). In contrast, administration of exogenous BMP7 protected against METH-induced toxicity in DA terminals in mice striata (Chou et al., 2008).

Our present results demonstrate for the first time that binge METH injections are associated with increases in inhibin  $\beta$ A expression. Inhibin  $\beta$ A homodimerizes or dimerizes with inhibin  $\beta$ B subunit to form activin A or activin AB (Brown et al., 2003a). The biological activities of activins are mediated by an activin receptor type I (acvrI)/activin receptor type II (acvrII) heterocomplex (Abe et al., 2004). In addition to the receptors, activins also bind to follistatin which acts as an antagonist (Harrison et al., 2005). Our findings of increased expression of acvrIIA and follistatin suggest that binge METH perturbs various molecules involved in the activin pathway. Multiple studies reported early upregulation of activin  $\beta$ A subunit mRNA after brain damages such as stroke, seizure, excitotoxicity or ischemia (Florio et al., 2007). Oxidative injury caused by hydrogen peroxide administration also caused increased activin  $\beta$ A mRNA in cortical neurons in culture (Mukerji et al., 2007). Addition of activin A provided protection against methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-induced death of DA neurons (Krieglstein et al., 1995). Taken together, our observations suggest that increased activin A might be part of a protective cascade against METH toxicity.

In a previous microarray analysis, this laboratory found increased cortical TGF- $\beta$ 1 mRNA at 4 and 16 hrs in mice after administration of a single dose of 40 mg/kg of METH (Cadet et al., 2001). TGF- $\beta$ 1 is a known injury-responsive gene that is upregulated after ischemia, stroke and excitotoxicity (Vivien and Ali, 2006). Another important finding in this report was that METH treatment also caused phosphorylation of Smad2 which is an important intracellular ingredient of the TGF- $\beta$  signaling pathway. Indeed, both TGF- $\beta$  and activin signaling pathways can lead to Smad2 phosphorylation (Gomes et al., 2005; Moustakas and Heldin, 2009). Smad2 is a transcriptional activator that complexes with co-activators or repressors, and together they bind to Smad binding element (SBE) to mediate TGF- $\beta$  response (Massague et al., 2005; Moustakas et al., 2001).

In summary, we found that binge METH injections affect the expression of activin A and TGF- $\beta$  in the rat striatum. These changes were also associated with increased Smad2 phosphorylation,

indicating that METH might activate Smad2-target genes. Future studies are necessary to test these ideas.

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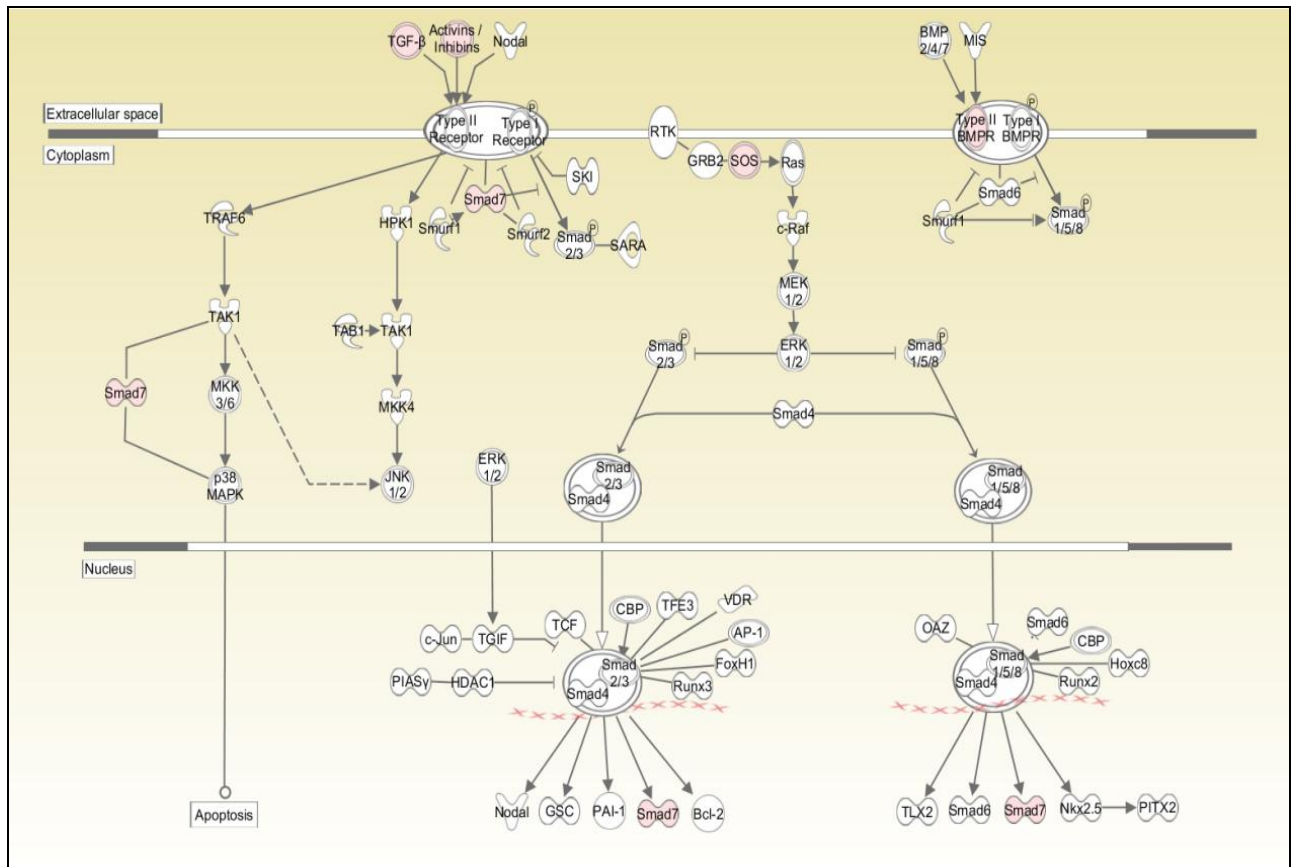
**Table 1 METH administration caused changes in genes involved in the TGF- $\beta$  signaling pathway**

Accession No.	Gene symbol	METH/Control
<b>Transcription factors</b>		
NM_012953	Fosl1	10.39
NM_012760	Plagl1	3.73
NM_053349	Sox11	3.49
NM_031135	Klf10	3.35
NM_031059	Msx1	3.07
NM_022685	Rem2	2.83
NM_024125	Cebpb	2.74
NM_024360	Hes1	2.44
NM_017334	Crem	2.29
NM_021693	Snf1lk	2.10
XM_343378	Pou2f3	2.10
XM_234025	Mycn	1.78
NM_030858	<b>Smad7</b>	1.76
NM_031345	Tsc22d3	1.70
NM_199268	Pde8b	1.64
NM_133290	Zfp36	1.62
NM_053328	Bhlhb2	1.62
<b>Metabolism</b>		
NM_031341	Slc7a7	18.48
NM_053551	Pdk4	9.87
NM_012681	Ttr	9.62
NM_144743	Ces6	8.59
NM_022215	Gpd1	3.90
XM_232202	Adamts9_predicted	2.59
NM_023964	Gapdhs	2.04
NM_012935	Cryab	2.03
NM_031823	Wfs1	1.97
NM_138974	Gstp2	0.67
NM_080783	Gale	0.60
NM_138877	Cyb5r3	0.56

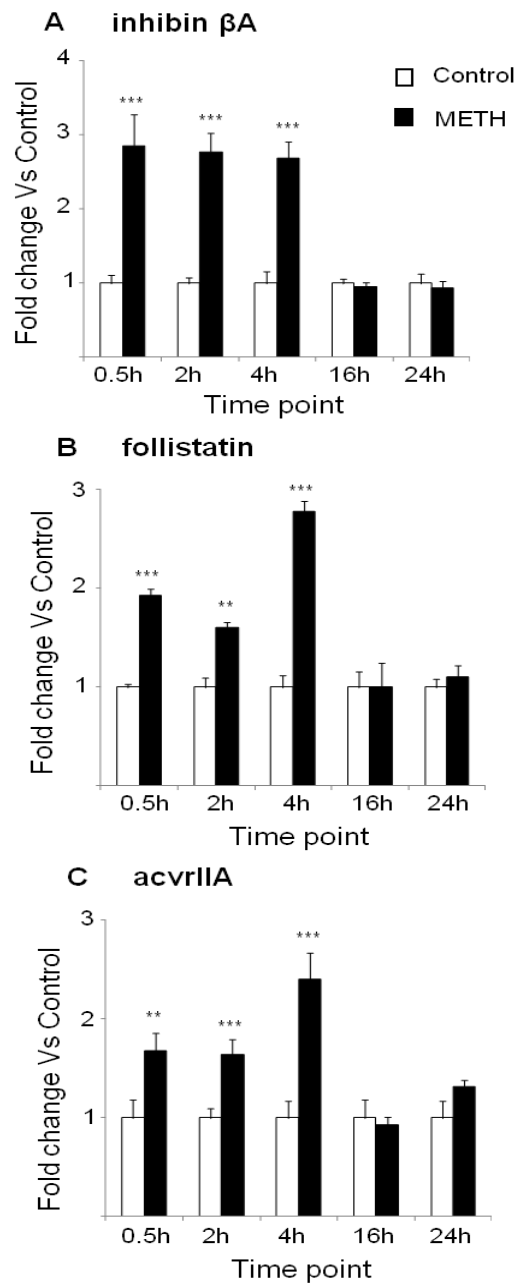
Signaling pathway		
NM_022239	Nmu	15.77
NM_012492	Adrb2	15.51
NM_001009518	V1rc38	15.37
NM_153737	Sostdc1	14.75
NM_145881	Rims2	7.14
XM_216884	Nts_predicted	6.28
XM_343250	Trib1	5.92
NM_017128	<b>Inhba</b>	4.80
XM_223560	Dusp18_predicted	3.94
XM_221901	Nptx2_predicted	2.53
NM_019374	Pdyn	2.38
NM_021578	<b>Tgfb1</b>	2.203
XM_233820	<b>Sos1</b>	2.866
NM_030584	<b>Sost</b>	2.189
NM_012561	<b>Fst</b>	2.15
NM_012800	P2ry1	2.13
NM_019348	Sstr2	2.07
XM_225726	Mapk4	2.01
NM_031606	Pten	1.78
NM_012666	Tac1	1.67
NM_001008284	Crkl	1.64
NM_053429	Fgfr3	1.56
NM_024400	Adamts1	1.50
NM_012750	Gfra2	0.57
NM_207588	Oxgr1	0.08
NM_023968	Npy2r	0.04
Apoptosis		
NM_017019	Il1a	15.50
NM_181084	Trp53inp1	2.75
XM_218543	Plekhf1_predicted	2.00
XM_343065	Nfkbia	1.85
NM_019143	Fn1	1.74
NM_012580	Hmox1	1.70
NM_053734	Ncf1	1.52
XM_343773	Rbm3	0.43
NM_172322	Pycard	0.41



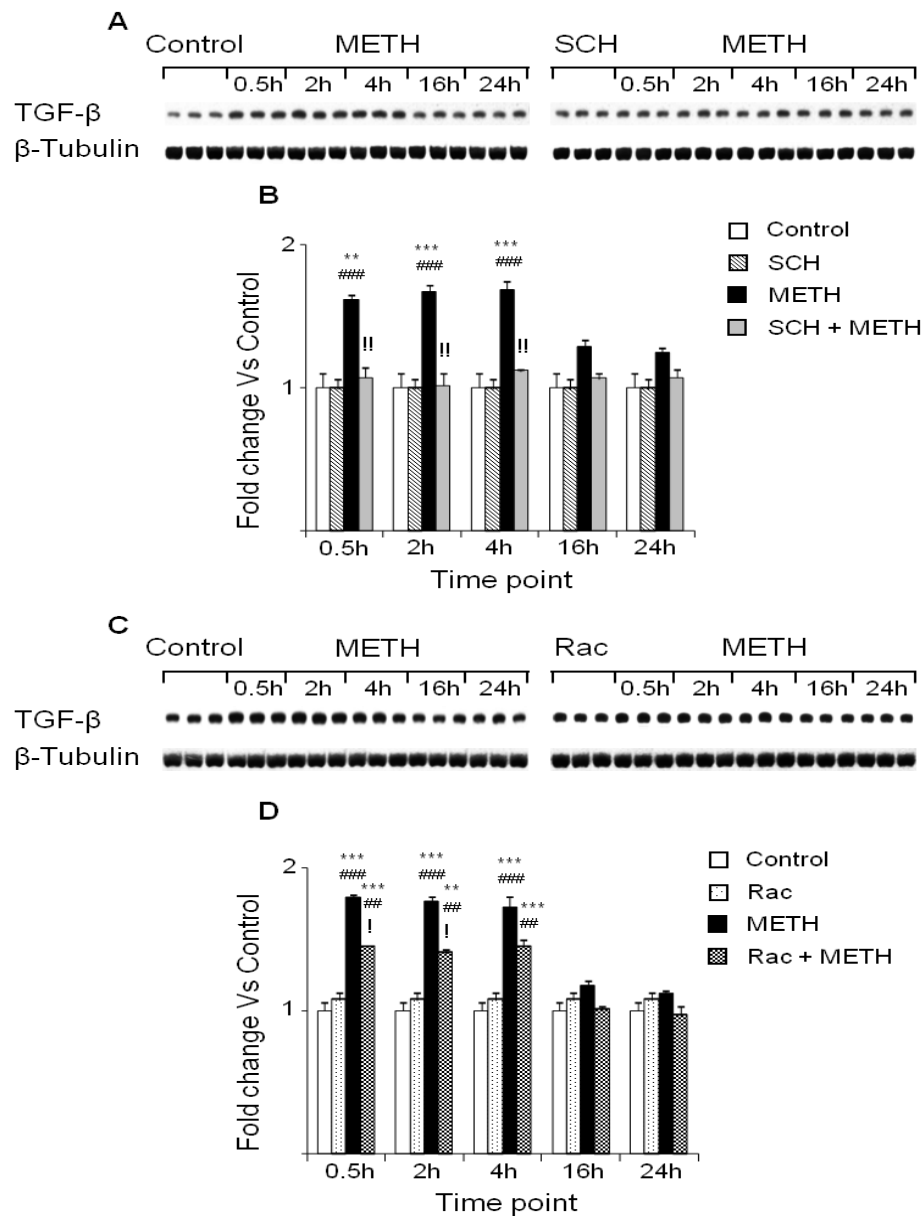
<b>Immune response</b>		
NM_012553	Ela2	14.12
NM_031336	Kl	2.01
XM_228909	Tlr7_predicted	1.63
NM_031116	Ccl5	0.60
XM_215303	RT1-S3	0.52
NM_134350	Mx2	0.39
<b>Stress responses</b>		
XM_237999	Gadd45g	2.70
NM_001009541	Ier2	2.03
NM_031666	Syt10	1.92
XM_223680	Ahsa2_predicted	1.85
XM_341663	Dnajb1_predicted	1.81
NM_001008321	Gadd45b	1.68
NM_031147	Cirbp	0.53
NM_001007554	Fblim1	0.43
<b>Cell adhesion</b>		
XM_343126	Itgb8_predicted	41.36
NM_053933	Pcdha4	12.59
XM_213560	Pkp2	2.03
XM_233602	Mfap2_predicted	2.02
NM_138544	Fat3	1.71
XM_214400	Col4a1	1.63
<b>Cell cycle</b>		
NM_001004232	Fscn3	16.06
NM_030998	Amhr2	2.51
XM_226165	Cables1_predicted	2.03
XM_218759	Chsy1_predicted	1.90
XM_342888	Plk3	1.89
NM_138855	Spdy1	0.46
<b>Cell differentiation</b>		
XM_344544	Angpt2	4.29
NM_173125	Pdlim7	1.77
NM_017336	Ptpro	1.66
NM_021846	Mcl1	1.62
NM_021751	Prom1	0.63
NM_012995	Ocm	0.51



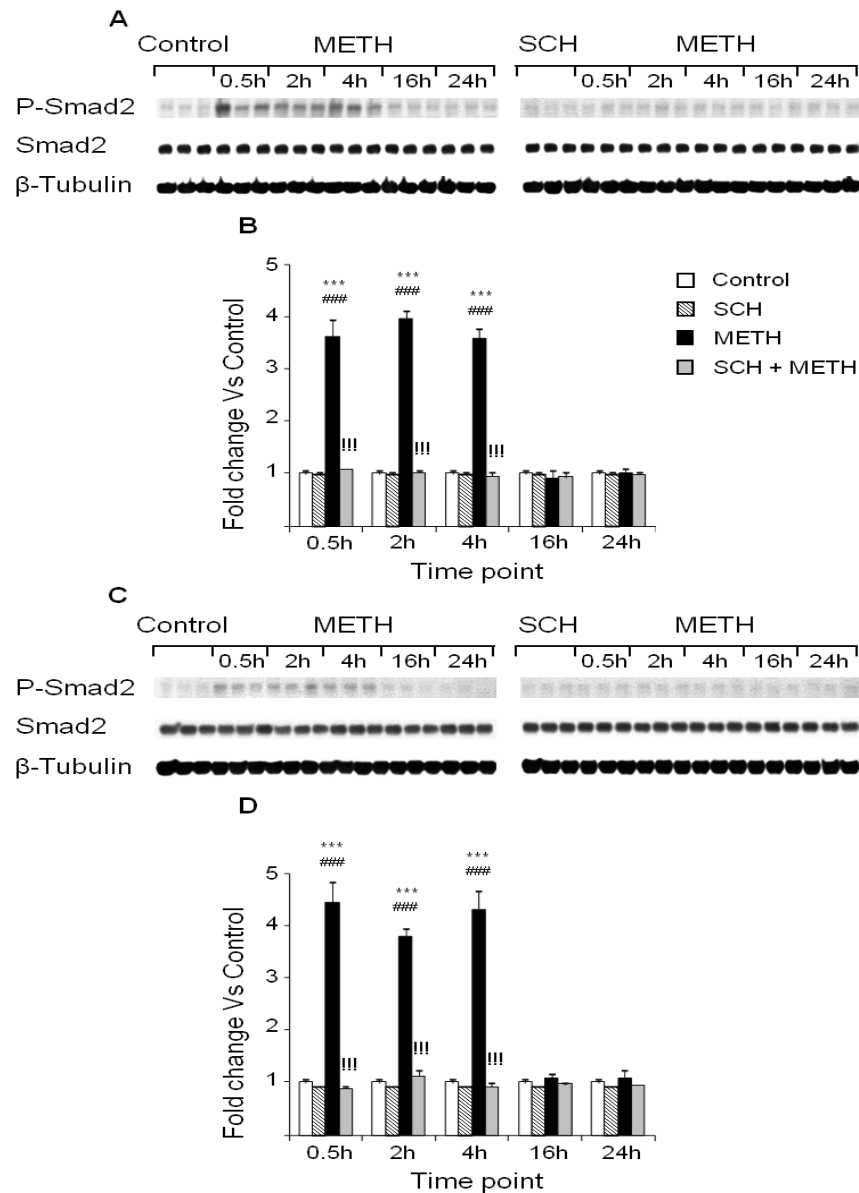
**Fig. 1. Networks of related genes were identified using Ingenuity Pathway Analysis (IPA) software.** Molecular network showing affected genes that are involved in TGF-β signaling. Relationships are shown as lines and arrows. Genes colored in pink were up-regulated after binge METH injections.



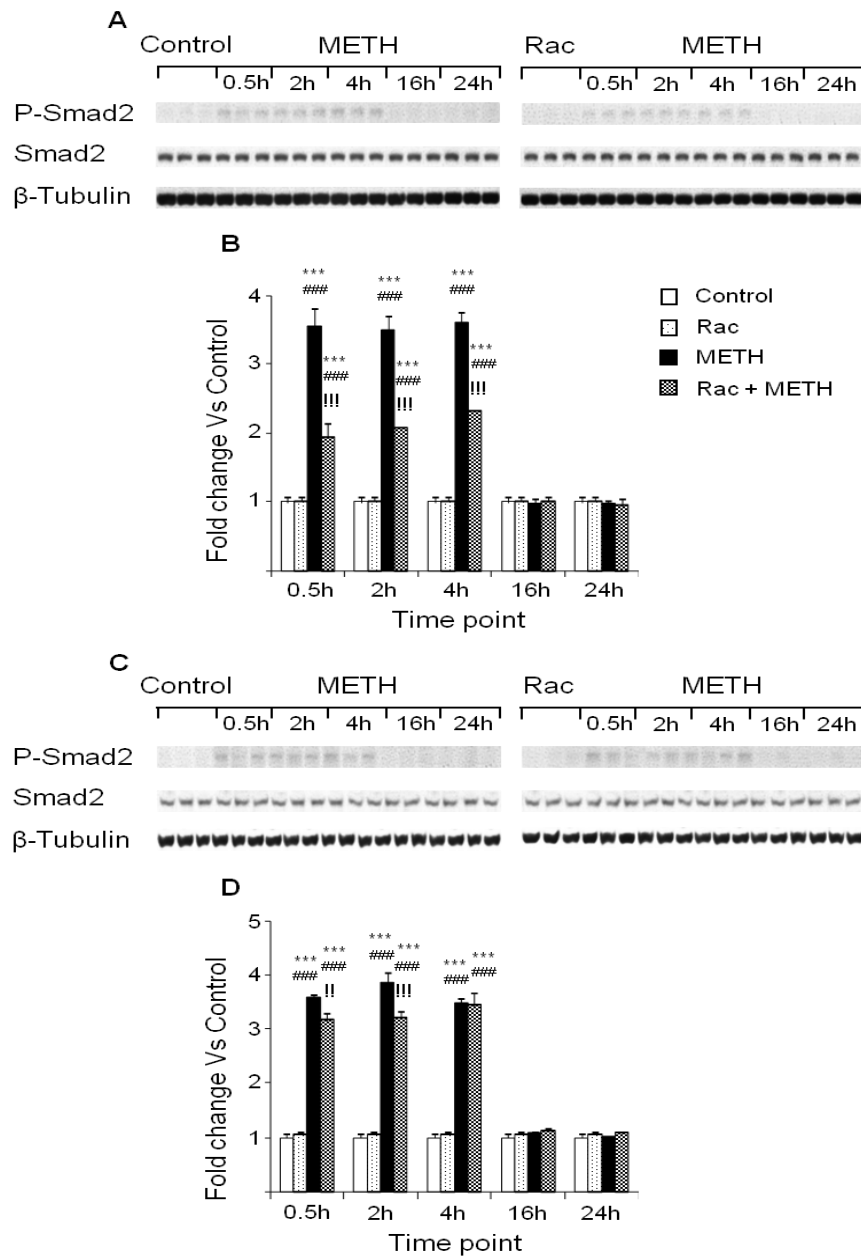
**Fig. 2. Effects of METH on different genes related to TGF- $\beta$  signaling.** Rats were treated with saline or binge METH injections (4x10 mg/kg). Striatal tissues of these animals were used to perform RT-PCR experiments. Quantitative measurement was used to verify METH-induced the expression of activin  $\beta$ A (A), follistatin (B) and acvrII (C) mRNAs. Key to statistics: \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group.



**Fig. 3. METH-induced TGF- $\beta$  protein expression is dependent to DA D<sub>1</sub> receptor activation.** The effects of binge METH injections (4x10 mg/kg, 2-hr apart, i.p) on striatal TGF- $\beta$  protein were tested, as well as the impact of a pretreatment with SCH23390 of raclopride (4x0.5 mg/kg, 30 min before each METH injection). (A-B) TGF- $\beta$  was significantly increased at 0.5 hr to 4 hrs after METH injections. SCH23390 pretreatment attenuated these effects. (C-D) In contrast, pretreatment with raclopride failed to prevent these effects. \*\* =  $p < 0.001$ ; \*\*\* =  $p < 0.001$ , in comparison to the Saline group. ## =  $p < 0.01$ ; ### =  $p < 0.001$ , in comparison to the SCH group. ! =  $p < 0.05$ ; !! =  $p < 0.01$ , in comparison of METH group to the SCH + METH group.



**Fig. 4. SCH23390 blocked METH-induced activation and nuclear translocation of P-Smad2 in rat striata.** Rats Sprague Dawley received four i.p doses of 10 mg/kg of METH, at 2 hr intervals. Each METH injection was either pretreated with a dose of 0.5 mg/kg of SCH23390 or raclopride. (A-B) P-Smad2 expression was increased early after METH administration in the cytoplasmic fraction, and these changes were sensitive to SCH23390 pretreatment. (C-D) Nuclear expression of P-Smad2 was also increased after METH injections and was blocked by SCH23390. Key to statistics: \*\*\* =  $p < 0.001$ , in comparison to the Saline group. ### =  $p < 0.001$ , in comparison to the SCH group. !!! =  $p < 0.001$ , in comparison of METH group to the SCH + METH group.



**Fig. 5. Raclopride did not affect the increases in P-Smad2 after METH injections.** Rats were either treated with binge toxic doses of METH (4x10 mg/kg) or a combination of raclopride (0.5 mg/kg) plus METH (10 mg/kg) given four times. Striatal expression of P-Smad2 was determined in the cytoplasmic and nuclear fractions after treatments. (A-B) Activated P-Smad2 was increased in both cytoplasmic (A-B) and nuclear (C-D) fractions. Pretreatment with raclopride did not affect these effects (A-D). Key to statistics: \*\*\* =  $p < 0.001$ , in comparison to the Saline group. ### =  $p < 0.001$ , in comparison to the SCH group. !! =  $p < 0.001$ ; !!! =  $p < 0.001$ , in comparison of METH group to the SCH + METH group.

## Conclusion and future perspectives

The purpose of this dissertation was to determine the basic mechanisms in the protective effects of the dopaminergic antagonist, SCH23390, against METH-induced neurotoxicity in rat striatum. Pretreatment with a single dose of SCH23390 (0.5 mg/kg) prevented neuronal apoptosis in rat striatum after a single 40 mg/kg dose of METH (Jayanthi et al., 2005). SCH23390 (0.1 mg/kg) also blocked neuronal apoptosis after administration of 30 mg/kg of METH to mice (Xu et al., 2005). Our studies utilized a dosing regimen in which rats were given four injections of 10 mg/kg of METH at two-hour intervals. This model is commonly used to document METH toxicity (Ali et al., 1996; Deng et al., 1999; Kuroda et al., 2010). It was important to characterize the molecular mechanisms of binge METH toxicity in comparison to the single METH dose, because a single dose of 30 mg/kg of METH caused the appearance of more apoptotic cell death than after binge METH regimen (4x10 mg/kg, 2-hours intervals) (Zhu et al., 2006b).

The manuscript 1 investigated the effects of METH and SCH23390 on IEGs expression. Consistent with previous findings, our data showed that DA D<sub>1</sub> receptors mediate induction in fos, crem, egr and arc after METH administration (Cadet et al., 2010b). These data suggest that the transcription factor CREB might mediate the changes in gene expression through calcium/cAMP responsive element (CRE). However, METH-induced increases in Fra2 mRNA were insensitive to pretreatment with SCH23390 (Beauvais et al., 2010). It is not surprising that Fra2 might be regulated differently because in contrast to cFos proteins that have transient induction, Fra2 have prolonged kinetics, suggesting protracted regulation of target genes (Pennypacker et al., 2000a; Pennypacker et al., 2000b). METH induced Fos expression early (Cadet et al., 2010b), while Fra2 protein was detected in striatal tissue 5 days post-treatment (Pennypacker et al., 2000b). The promoter region of fra2 contains CRE, serum element response (SRE) and AP1 binding sites (Yoshida et al., 1993). It is possible that calcium/calmodulin-dependent kinase I (CAMKI) that is activated by calcium influx through NMDA receptors can target SRE domain on fra2 gene promoter. Interestingly, the temporal induction and pattern of IEGs affected by METH are different in the single bolus dose and multiple injections regimen of METH (Cadet et al., 2010b; Jayanthi et al., 2002). By example, Jayanthi, et al. (2002) reported that a single injection of METH (40 mg/kg) caused significant increases in cjun mRNA which peaked at 2 hours, whereas we did not find changes in cjun expression at 2 and 4 hours (Beauvais et al., 2010). However, gene expression

seems to cause similar synaptic changes in both models of METH toxicity, with induction of *cfos* which may be a pro-survival response (Deng et al., 1999), and *egr* genes which may activate the Fas L/Fas death pathway (Jayanthi et al., 2005). In addition, we have found increases in phosphorylated cJun known to activate the JNK pathway (Jayanthi et al., 2002).

The manuscript 2 investigated whether SCH23390 and raclopride blocked METH-induced monoaminergic damages in striatal and cortical tissues, as well as the role of hyperthermia in this protection. Accordingly to previous reports, our data confirmed that both types of DA receptors are involved in neurodegeneration of striatal dopaminergic neurons. Several laboratories have shown that hyperthermia plays an important role in mediating the long-term neurotoxic effects of METH on striatal DA neurons (Albers and Sonsalla, 1995; Bowyer et al., 1994). Collectively, these experiments demonstrate a link between hyperthermia and METH-induced neurotoxicity, suggesting that the attenuation of METH-induced hyperthermia could play a role in the protection afforded by SCH23390 or raclopride against the neurotoxic effects of the stimulant. Thus, it was important to determine whether the prevention of neuronal apoptosis in the striatum after METH treatment, was due to attenuation of hyperthermia. Our data indicated that the protective properties of SCH23390, but not raclopride, might be dependent on prevention of hyperthermia. However, Xu et al (2005) demonstrated that the dose of SCH23390 (0.1 mg/kg) that completely blocked METH-induced apoptosis in the mice striatum, did not suppress hyperthermia. These discrepancies might depend on the dose of SCH23390, the dosing regimen of METH and the animal model used. Taken together, these data indicate that hyperthermia may play a role, but is not a major component of the protective mechanisms of SCH23390 and raclopride.

The manuscript 3 determined the effects of SCH23390 and the DA D<sub>2</sub> receptor antagonist, raclopride, on METH-induced ER and mitochondrial stress pathways. Single dose of 1 mg/kg of raclopride prevented neuronal apoptosis in mice striata, caused by a dose of 30 mg/kg of METH (Xu et al., 2005). As expected, we found that METH induced the expression of ER stress pathway-related genes, such as the chaperone BiP, foldases HSPs, ATFs and CHOP. Jayanthi et al (2009) conducted microarray experiments on striatal tissues after an injection of METH (40 mg/kg) and found increases in different chaperones, foldases and the three ER stress transmembrane sensor proteins, PERK, IRE1 $\alpha$  and PERK. Similar to our data, these increases were completely blocked by SCH23390. However, our data are the first to investigate the influence of DA D<sub>2</sub> receptors in these events. The results suggest that these receptors are not involved as much as D<sub>1</sub> receptors to cause



upregulation of ER stress-responsive genes. In addition, mitochondrial perturbations were determined by the decrease in Bcl-2 levels, increases in the proapoptotic Bax and Bad proteins as well as accumulation of cytochrome C in the cytosol.

The manuscript 4 demonstrated the involvement of TGF- $\beta$  family of trophic factors in response to toxic doses of METH. Our observation of an activin A and TGF- $\beta$  response to METH toxicity is consistent with previous reports demonstrating upregulation of these trophic factors in neurons following brain injury (Florio et al., 2007; Vivien and Ali, 2006). The early rise and fall of activin  $\beta$ A mRNA and TGF- $\beta$  protein suggest that changes in activin A and TGF- $\beta$  are transient responses. Our data indicate that increases in activin A and/or TGF- $\beta$  are accompanied by P-Smad2 phosphorylation. Detailed understanding of the molecular mechanisms that underlie induction of multiple members of the TGF- $\beta$  family of trophic factors is needed. It will be important to do further studies to identify which populations of neurons respond to the TGF- $\beta$  signals, and which cells survived after injection of toxic doses of METH.

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