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Behavioral and Neurochemical Characterization of the Effects of the Novel Adenosine Antagonist MSX-4

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**Behavioral and Neurochemical Characterization of the Effects of the Novel
Adenosine Antagonist MSX-4**

Jessica Lee Santerre

B.S. University of Connecticut, 2009

A Thesis

Submitted in Partial Fulfillment of the

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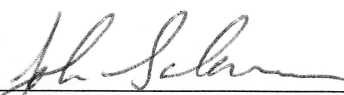
Master of Arts Thesis

Behavioral and neurochemical characterization of the effects of the novel adenosine antagonist MSX-4

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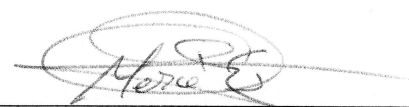
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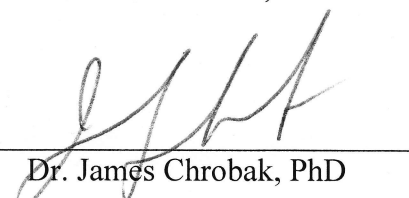
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Behavioral and Neurochemical Characterization of the Effects of the Novel Adenosine Antagonist MSX-4

Abstract

Organisms frequently make effort-related decisions based upon assessments of motivational value and response costs. Dopamine (DA) and DA receptors (DA-Rs), particularly in nucleus accumbens (NAc), are known to regulate such effort-related choice behavior processes. For instance, DA-R antagonists as well as NAc DA depletion can decrease choice behavior. Moreover, previous studies have shown that systemic administration of adenosine A_{2A} antagonists reverses the behavioral effects of DA-R D2 antagonists in rats responding on the concurrent choice task. MSX-3, a selective adenosine A_{2A} antagonist, is a prodrug of MSX-2 with better water solubility. MSX-3 produces a substantial dose-related reversal of the effects of DA-R D2 antagonists on lever pressing and chow intake. MSX-4 is a novel A_{2A} antagonist that is an amino acid ester prodrug of MSX-2, and is thought to have better oral bioavailability than MSX-3. In the present studies, we investigated the effects of MSX-4 on choice behavior using the commonly employed fixed ratio 5 (FR5)/chow feeding procedure. MSX-4 (2.0-8.0 mg/kg IP) produced a dose-related reversal of the effects of 0.08 mg/kg eticlopride on lever pressing and chow intake, with a similar time course as MSX-3. Following oral administration, both MSX-3 and MSX-4 showed a reversal of motivational impairments induced by eticlopride at comparable doses. Furthermore, eticlopride-induced increases in c-Fos expression in NAc were reversed by co-administration of MSX-3 and MSX-4. Overall, these data suggest that both adenosine A_{2A} antagonist pro-drugs induce similar behavioral effects, and similar actions

on cellular markers of neural activity in NAc. Additionally, this research further supports the hypothesis that DA and adenosine interact in the regulation of effort-related choice behavior. Further understanding this interaction between DA and adenosine and may have implications for understanding the energy-related motivational dysfunctions, such as psychomotor slowing, fatigue, and apathy, all of which are seen in patients with depression, Parkinson's disease, and other central nervous system disorders.

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Chapter 1: General Introduction

Motivation and Goal Directed Behavior

Organisms are constantly required to make effort-related decisions based upon cost/benefit analyses, allocating responses according to assessments of motivational value and work requirements (Farrar et al. 2007). These motivational values can be defined as the set of processes through which organisms regulate the probability, proximity and availability of significant stimuli, including food, water, sex and other stimuli (Salamone 1992; Salamone and Correa 2002). An important aspect of motivation is that organisms are able to overcome work-related response costs that separate them from motivationally relevant stimuli (Salamone, 1992; Salamone et al., 1991, 1997, 2003, 2007; Salamone and Correa, 2002; van den Bos et al., 2006; Niv et al., 2007). Furthermore, motivation can then be broken down into directional and activational components. Directional aspects of motivations refer to the fact that motivated behavior is either directed towards or away from a particular stimulus (Salamone and Correa 2002). In addition, goal directed behavior frequently is characterized by a high degree of activity, vigor, or persistence in work output (Salamone and Correa 2002). These activational aspects of motivation allow organisms to overcome work-related response costs or constraints that separate them from significant stimuli (Salamone et al. 1997, 2007; Salamone and Correa 2002; Van den Bos et al. 2006). The work requirements for obtaining reinforcing stimuli differ substantially depending upon the instrumental task, and these requirements can vary along several distinct dimensions (e.g. numbers of responses, force or distance requirements; Collier and Jennings 1969; Aberman and Salamone 1999; Ishiwari et al. 2004a; van den Bos et al. 2006). A lack of behavioral activation can be maladaptive in organisms. For instance, in

humans, symptoms such as psychomotor slowing, anergia and fatigue are fundamental aspects of depression, as well as other psychiatric and neurological disorders (Demyttanaere et al. 2005; Salamone et al. 2006, 2007; Yurgelum-Todd et al. 2007; Capuron et al. 2007; Majer et al. 2008). A vast amount of evidence is consistent with the idea that dopamine is involved in normal and pathological aspects of motivation (Salamone et al. 2007).

Dopaminergic Involvement in Effort and Effort-Related Decision Making

Extensive research has implicated the nucleus accumbens (NAc) dopaminergic system in effort related processes. In particular, NAc dopamine (DA) is thought to be involved in an organism's ability to overcome work-related response costs in instrumental behavior (Salamone et al. 1997, 2005, 2007; Barbano and Cador 2007; Phillips et al 2007; Robbins and Everitt 2007). Several studies have indicated that DA in the NAc is essential in mediating the activational aspects of motivation and effort-related processes. This idea has been supported through studies assessing the ability of DA antagonists, administered both systemically and intracranially, as well as NAc DA depletions, which reduce the tendency of animal to expend effort to obtain motivationally relevant stimuli. NAc DA depletions have little effect on some aspects of food motivation, such as food intake (Koob et al. 1978; Salamone et al. 1993). Furthermore, manipulations affecting NAc DA transmission have contrasting effects on instrumental tasks with different ratio requirements. For example, it has been shown that NAc DA depletions had little or no effect on response schedules that have low-to-moderate ratio requirements, like FR1 (McCullough et al. 1993; Aberman and Salamone 1999; Salamone et al. 2001; Ishiwari et al. 2004a). Conversely, higher response

ratios, such as FR5-64, showed greater impairments with NAc DA depletions (Aberman and Salamone, 1999; Salamone et al. 1991; Salamone et al. 1993; Ishiwari et al. 2004a). Studies using these high ratio requirements reported that animals with NAc DA depletions ceased to respond once the ratio requirements became too high (i.e., FR 300; see Salamone et al., 2001). The magnitude of the ratio requirement appears to be a critical determinant of sensitivity to the effects of NAc DA depletions. These data bolster the idea that DA, particularly in the NAc, is involved in regulating the effort expended by organisms to obtain access to motivational stimuli. Taken together, these data suggest that DA in the NAc is necessary for activational components of motivation, but less important for directional aspects (e.g. food intake or appetite; Aberman and Salamone 1999; Salamone et al. 1993, 2003; Ishiwari et al. 2004a; Mingote et al. 2005).

Not only is NAc DA, involved in exertion of effort, but it also is thought to be involved in an organism's ability to make choices based on cost/benefit assessments (Salamone et al. 1991, 1997, 2003, 2005, 2007; Walton et al. 2006; Phillips et al. 2007). As discussed above, organisms are constantly required to make effort-related decisions based upon cost/benefit analyses, allocating responses according to assessments of motivational value and work requirements (Farrar et al. 2007). One of the choice tasks used to study this type of function is the concurrent lever pressing/chow feeding procedure (Salamone et al., 1991). In this task, animals can choose between pressing a lever to receive a preferred food (palatable operant pellets) or approaching and eating a less preferred but freely available rodent chow that also is in the chamber. If the effort requirement is low (i.e, FR1 or FR5), rats typically will receive most of their food from lever pressing, and will consume very little of the chow. In free feeding experiments it was shown that rats considerably

preferred the operant pellets over the standard lab chow (Salamone 1991). An expanse of research has shown that DAergic manipulation disrupts choice behavior in this procedure. Low-to-moderate doses of systemically administered DA antagonists that act on D₁ or D₂ family receptors (e.g. SCH23390, SCH39166, haloperidol, eticlopride) suppress lever pressing for food while increasing consumption of the less preferred, freely available lab chow (Salamone et al. 1991, 2002; Cousins et al. 1994; Koch et al. 2000; Sink et al. 2008). Interestingly, when free chow is no longer available in the operant chamber, DA-depleted animals will show higher levels of lever pressing (Cousins and Salamone, 1994). Another important concept is that food intake and preference are not affected by these pharmacological manipulations in a free choice experiment (Salamone et al. 1991; Koch et al. 2000). 1993; Cousins and Salamone, 1994). Taken together, these data indicate that interference with DA transmission does not alter an animal's appetite, or the motivational salience of the food, yet it appears to reduce the effort they're willing to exert to obtain it.

Subsequently, studies were conducted in order to ascertain the relevance of DA, specifically in the NAc, using this effort-related choice procedure. DA depletions in different regions of the striatum were used to assess the role of each region in effort-related processes. DA depletions in anteroventromedial and ventrolateral neostriatal areas did not produce the same behavioral effects on the choice procedure as systemic administration of DA antagonists (Cousins et al. 1993). Anteroventromedial DA depletions had no effect on the concurrent choice procedure. However, ventrolateral depletions severely impaired motor capabilities, which reduced both lever pressing and chow intake (Cousins et al. 1993). These animals were shown to have motor impairments that disrupted their ability to handle food (Cousins et al. 1993). Studies using DA depletions or intracranial injections

of a D₂ antagonist targeting the NAc produced results that more closely resembled data obtained from systemic studies of the choice paradigm. Injections of the D₂ antagonist eticlopride into either dorsomedial shell, medial core, or lateral core subregions of the NAc have been shown to shift behavior away from lever-pressing and towards consumption of the freely available lab chow (Salamone et al. 1991; Sokolowski and Salamone 1998; Kock et al. 2000; Nowend et al. 2001; Farrar et al. 2010). Immunohistochemical studies have also been used as a method to link these motivational functions to the NAc. Intracumbens injections of the D₂ antagonist eticlopride, at the same dose used in behavioral studies, induced a robust and significant increase in c-Fos immunoreactivity in NAc core neurons relative to vehicle-treated animals (Farrar et al. 2010). This finding is consistent with several earlier reports indicating that systemic administration of D₂ DA antagonists can increase c-Fos expression in striatal areas, including NAc (Dragunow et al., 1990; Miller, 1990; Robertson and Fibiger, 1992; Fibiger, 1994; MacGibbon et al., 1994; Wan et al., 1995; Pinna et al., 1999). As a whole, these findings strengthen the hypothesis that DA transmission, specifically within the NAc, is essential for the conductance of effort-related processes and effort-related choice.

Adenosine

Adenosine is a purine nucleoside, which exerts neuromodulatory effects throughout various regions of the peripheral and central nervous system. Adenosine is present not only within in the cell, where it acts as a metabolic intermediate (Lopes et al. 2011), but also extracellularly, where it functions as a neuromodulator. Intracellular and extracellular production of adenosine occurs via dephosphorylation of adenosine monophosphate

(AMP) by nucleotidases (Svenningsson et al., 1999). In the extracellular space, this dephosphorylation is the last event of an enzymatic cascade that converts ATP into adenosine. Bidirectional transporters maintain the concentrations of adenosine in the extracellular and the intracellular spaces. The mechanisms that control adenosine release as a neuromodulator in the central nervous system are not well known, although changes in extracellular concentrations of adenosine are partly dependent on ATP release (Svenningsson et al., 1999). Adenosine is released into the extracellular space by astrocytes and neurons, and binds to one of four G-protein-coupled adenosine receptors (A_1 , A_{2A} , A_{2B} , and A_3). An emphasis should be placed upon the A_1 and A_{2A} subtypes, as they are predominantly found in the central nervous system. Adenosine has differential effects depending upon whether A_1 or A_{2A} receptors are stimulated (Ribeiro et al. 2002). Adenosine A_1 receptors are associated with G_i mediated signaling, while adenosine A_{2A} receptors are directly coupled with G_s . Adenosine exerts much of its effects by modulation of the glutamatergic, cholinergic, dopaminergic and GABAergic neurotransmitter systems (Kurokawa et al., 1996; Latini et al., 1996; Mori and Shindou, 2003; Popoli et al., 2003). Pharmacological manipulations of adenosine receptors have been shown to influence sleep and arousal, cognition and memory as well as neuronal degeneration. Due to this wide variety of actions, pharmacological agents acting on adenosine receptors may have therapeutic benefits for various central nervous system disorders. The A_{2A} receptor in particular has been implicated in goal directed behaviors and effort related decision-making.

Adenosine and Effort Related Choice

As discussed earlier, adenosine also plays a pivotal role in effort-related processes. Within the last few years, increasing evidence has accumulated indicating that central adenosine neurotransmission plays an important role in modulating the functional circuitry of the basal ganglia (Ferré et al., 1997; Svenningsson et al., 1999; Hauber et al., 2001). Several subtypes of adenosine receptors are involved in striatal function, and anatomical studies have demonstrated that the adenosine A_{2A} receptor subtype has a relatively high degree of expression within both the neostriatum and the NAc (Svenningsson et al., 1999; Wang et al., 2000; Chen et al., 2001; Hettinger et al., 2001). In addition, most of the A_{2A} receptors are colocalized with D₂ DA receptors, and are found mainly in enkephalin-positive neurons that project to pallidal structures such as globus pallidus and ventral pallidum (i.e., striatopallidal neurons; Schiffmann et al., 1991; Fink et al., 1992; Svenningsson et al., 1999; Hillion et al., 2002). Adenosine A_{2A} receptors are also found presynaptically on glutamatergic afferents as well as on local axon collaterals of medium spiny neurons. Multiple biochemical and behavioral approaches also suggest that a postsynaptic interaction exists between adenosine A_{2A} and DA D₂ receptors located on striatopallidal neurons. Several possible molecular mechanisms for this interaction have been proposed, among these include the finding that D₂ receptors and A_{2A} receptors (Hillion et al., 2002; Fuxe et al., 2003) have been shown to form heteromeric receptor complexes. Also, signal transduction pathways have been studied as possible mechanisms for striatal interactions between D₂ receptors and A_{2A} receptors. Stimulation of A_{2A} receptors leads to activation of adenylyl cyclase via G_s or G_{olf} proteins, while stimulation of D₂ receptors results in inhibition of adenylyl cyclase activity via G_i protein, suggesting that

within the indirect basal ganglia pathway, DA and adenosine mediate functionally antagonistic actions at the second-messenger level (Fuxe et al., 1998; Ferré et al., 2001).

Evidence suggests that these DA-adenosine interactions are involved in NAc-mediated behavioral functions. Stimulation of NAc adenosine A_{2A} receptors by local injections of the agonist CGS 21680 decreased locomotor activity (Barraco et al., 1993, 1994), and also suppressed instrumental lever pressing and altered effort-related choice behavior in a manner that mimicked the effects of accumbens DA D₂ receptor antagonism (Mingote et al., 2008; Font et al., 2008). The suppression of locomotion induced by the DA antagonist haloperidol was reversed by injections of the adenosine A_{2A} receptor antagonist MSX-3 (see Hockemeyer et al., 2004) into the NAc core, but not into the shell or the ventrolateral neostriatum (Ishiwari et al., 2007). As mentioned above, stimulation of NAc A_{2A} receptors produces actions that closely resemble the effects of intra-accumbens DA antagonism, and intra-accumbens A_{2A} receptor antagonism has been shown to reverse the behavioral effects of DA receptor antagonism. Consistent with these behavioral studies, neurochemical assays have highlighted the interactions between DA D₂ receptor antagonists and adenosine A_{2A} receptor antagonists. As stated earlier, administration of the D₂ antagonist eticlopride increases c-Fos positive cells in the NAc core (Dragunow et al., 1990; Miller, 1990; Robertson and Fibiger, 1992; Fibiger, 1994; MacGibbon et al., 1994; Wan et al., 1995; Pinna et al., 1999; Farrar et al. 2010). Concurrent administration of the A_{2A} antagonist MSX-3, reversed increased c-Fos expression at similar doses used in behavioral studies (Farrar et al. 2010). Because of the importance of DA adenosine interactions, there have been several attempts to develop novel adenosine A_{2A} receptor

antagonists; such drugs could be highly useful as research tools, and also may have clinical utility for the treatment of parkinsonism, depression and other disorders.

Adenosine Antagonist Pro-drugs

One of the main limitations in drug discovery is developing ligands with the ability to have a therapeutic advantage in a clinical setting. For example, many drugs have difficulties in absorption properties, solubility and blood brain barrier penetrability (Muller, 2009). An effective method in alleviating these issues is the use of a pro-drug approach. Prodrugs are described as bioreversible derivatives of drug molecules that must undergo a chemical or enzymatic biotransformation to the active forms, prior to exerting a pharmacological action (Rautio et al. 2008a). Pro-drugs are traditionally developed by attaching a non-active promoiety to a pharmacologically active parent drug. This drug-promoiety combination alone is pharmacologically inactive. The drug and promoiety are covalently linked via bioreversible groups that are chemically enzymatically labile. The ideal prodrug yields the parent drug with high recovery ratios, with the promoiety being non-toxic (Rautio et al, 2008b). Ultimately, the drug and promoiety are cleaved by chemical and/or enzymatic transformations, thus releasing the pharmacologically active parent drug. By employing this methodology, the clinical relevance of a drug molecule may be enhanced without modifying the pharmacological activity of a parent drug (Rautio et al. 2008).

MSX-2, like other adenosine receptor antagonists and xanthine derivatives, in particular, suffer from poor water solubility (Muller, 2009), effectively eliminating its clinical relevancy. One approach to increase water solubility is by attaching a polar moiety to the

drug, which can be cleaved off by an enzymatic reaction to release the active drug. To allay this issue with MSX-2, a pro-drug approach was applied. MSX-3 is a pro-drug of the adenosine A_{2A} antagonist MSX-2 (Muller, 2009), and many of the experiments mentioned beforehand employed MSX-3 to ascertain the interactions between dopamine and adenosine in the NAc. In the development of MSX-3, a phosphoric acid group is added to MSX-2 acting as a promoiety. This combination of drug and promoiety increases the water solubility of the compound. This large increase in water solubility can be attributed to the introduction of 2 negative charges. When the compound enters the system- the two Na ions dissociate leaving the two negative oxygens. MSX-3 is then dephosphorylated, or has the phosphate group enzymatically removed- leaving the pharmacologically active MSX-2. Effectively, the water solubility increases from less than 0.1 mg/ml for MSX-2, to 9.0 mg/ml for MSX-3 (Muller, 2009). However, with respect to the clinical relevancy of MSX-3, peroral application of phosphate prodrugs was less favorable because it is highly unlikely that the intact- highly polar- prodrug could be absorbed. Phosphoric acids esters usually undergo enzymatic hydrolysis prior to absorption and therefore phosphate prodrugs are typically used for intravenous administration (Muller, 2009). Therefore, a new approach was developed to increase the clinical utility of MSX-2. Muller and colleagues chose to utilize an amino acid ester prodrug approach. This time, rather than adding a phosphate acid group to the parent drug, MSX-2, a valine ester amino acid promoiety was selected,. This approach would maintain the water solubility that was seen with MSX-3, but also was intended to enhance peroral absorption and release of the drug not in the intestine, but after absorption (Vollmann, 2008). The new compound, MSX-4, was developed by condensing the carboxylate function of valine with the OH group of MSX-2, resulting in a prodrug with a

basic amino group that can be protonated, resulting in a large increase in water solubility. MSX-4 was found to be stable in artificial gastric acid, but readily cleaved by pig liver esterase. Unlike MSX-3, MSX-4 may provide a better treatment option for motivational deficits associated with depression in a clinical setting. Although MSX-3 has been well characterized *in vivo*, MSX-4 has yet to be characterized in terms of its behavioral effects.

Summary of Experiments

The present studies were conducted in order to characterize the behavioral effects of the novel adenosine A_{2A} antagonist, MSX-4. For these experiments, the concurrent choice procedure described above was used. MSX-3 and MSX-4 were compared in terms of their ability to reverse impairments in the choice procedure after administration of the D2 antagonist eticlopride. The first group of experiments (2.1-2.2) focused on the optimal doses of MSX-4 for intraperitoneal administration. In particular, it was important to compare the potency (i.e., effective doses) of MSX-3 and MSX-4. For this set of experiments the general hypothesis, based on promoiety absorption and solubility properties, was that MSX-3 would be more potent than MSX-4 in experiments employing intraperitoneal administration (Muller 2009; Vollmann et al., 2008). The second set of experiments (2.3-2.4) was carried out to determine the differences in time course properties between MSX-3 and MSX-4. For these experiments, the general hypothesis was that, MSX-4 would produce a longer therapeutic window than MSX-3, due to the fact the parent drug is released later in the absorption process (Muller, 2009; Vollmann, et al, 2008). The third experiment (2.5) was designed to assess the oral absorption properties of MSX-3 and MSX-4. For these sets of experiments the general hypothesis was that MSX-4 would be orally active, and may gain

potency compared to MSX-3 after oral, as opposed to IP, administration. This hypothesis was based on data proposing that an amino acid ester prodrug approach, like that used in the development of MSX-4, would increase the peroral availability over a phosphate ester approach (MSX-3; Muller, 2009; Vollmann et al, 2008). The last experiment assessed the differences in immunohistochemical expression after administration of MSX-4. The general hypothesis for this set of experiments was that expression of c-Fos positive cells induced by eticlopride, would be reversed after administration of MSX-4, in a manner similar to MSX-3 as shown in previous studies (e.g. Farrar et al., 2010).

Chapter 2: Functional interactions between dopamine D2 receptor antagonism and adenosine A2A receptor antagonism: Behavioral comparison between two novel pro-drugs of the adenosine A2A receptor antagonist MSX-2: MSX-3 and MSX-4.

Introduction

Organisms often are required to make effort-related decisions based upon cost/benefit analyses, allocating responses according to assessments of motivational value and work requirements (Salamone and Correa, 2002; Salamone et al., 1997, 2003, 2005; Denk et al., 2005; Rushworth et al., 2004; Ernst and Paulus, 2005, Farrar et al. 2007). Goal directed behavior frequently is characterized by a high degree of activity, vigor, or persistence in work output (Salamone and Correa 2002). An important aspect of motivation is that organisms are able to overcome work-related response costs that separate them from motivationally relevant stimuli (Salamone, 1992; Salamone et al., 1991, 1997, 2003, 2007; Salamone and Correa, 2002; van den Bos et al., 2006; Niv et al. 2007). Extensive research has implicated nucleus accumbens DA in effort related processes. In particular, accumbens DA is thought to be involved in an organism's ability to overcome work-related response costs in instrumental behavior (Salamone et al. 1997, 2005, 2007; Barbano and Cador 2007; Phillips et al 2007; Robbins and Everitt 2007). One of the tasks used to study this type of behavior is the concurrent lever pressing/chow feeding procedure (Salamone et al., 1991). In this task, animals can choose between pressing a lever to receive a preferred food (palatable operant pellets) or approaching and eating a less preferred but freely available rodent chow that also is in the chamber. If the effort requirement is low (i.e, FR1 or FR5), rats typically will receive most of their food from lever

pressing, and will consume very little of the chow. A large amount of research has shown that DAergic manipulations disrupt choice behavior in this procedure. Interference with DA transmission by administration of low doses of DA antagonists or depletions of accumbens DA cause a dramatic shift in behavior by reducing lever pressing for food but increasing chow intake (Salamone et al., 1991, 2002; Cousins et al., 1993; Koch et al., 2000; Nowend et al., 2001).

The purine nucleoside adenosine also plays a pivotal role in these effort-related processes. Considerable evidence indicates that there is a functional interaction between DA and adenosine A_{2A} receptors in striatal areas, including the nucleus accumbens (Svenningsson et al., 1999; Wang et al., 2000; Hettinger et al., 2001; Chen et al., 2001). Data suggests that these DA-adenosine interactions are involved in accumbens-mediated behaviors such as motivation and effort. Due to these interactions, considerable research has attempted to develop novel adenosine A_{2A} receptor antagonists capable of attenuating these motivational deficits in rats, which are potentially related to depression and psychomotor slowing. One of the main limitations in drug discovery is developing ligands with the ability to have a therapeutic advantage in a clinical setting. Adenosine receptor antagonists, in general, and xanthine derivatives, in particular, suffer from poor water solubility (Muller, 2007). For this reason it is important to develop drugs which can enhance water solubility. Techniques such as pro-drug applications have been employed to develop more clinically relevant ligands. The present studies were conducted in order to better characterize two novel pro-drugs of the adenosine A_{2A} receptor antagonist MSX-2. Both MSX-3 and MSX-4 were examined in terms of their ability to reverse the effects of the DA antagonist eticlopride on FR5 lever pressing and response allocation using the

concurrent lever pressing/chow feeding task. Neurochemical changes induced by administration of eticlopride, and eticlopride co-administered with MSX-3 and MSX-4 also were examined, in order to assess differences between the two pro-drugs. The first two experiments focused on the optimal doses of MSX-3 and MSX-4 for intraperitoneal administration. The second set of experiments was carried out to determine the differences in time course properties between MSX-3 and MSX-4. The last behavioral experiment assessed the oral absorption properties of both MSX-3 and MSX-4. Finally, the last experiment tested the ability of MSX-3 and MSX-4 to reverse the expression of cFos positive cells in the nucleus accumbens core induced by administration of the D₂ antagonist eticlopride.

EXPERIMENTAL PROCEDURES

Subjects

Adult male drug-naïve Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed in a colony maintained at 23°C with 12 hour light/dark cycles (lights on at 0:700 h). Rats (N=81) weighed 290-340 g at the beginning of the study, and were initially food deprived to 85% of their free-feeding body weight for operant training. Rats were fed supplemental chow to maintain the food restriction throughout the study, with water available ad libitum in the home cages. Despite food restriction, rats were allowed most weight gain throughout the experiment. All animals were approved by the University of Connecticut institutional animal care and use committee, and followed NIH guidelines.

Pharmacological Agents

Eticlopride (S(-)-3-chloro-5-ethyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxybenzamide hydrochloride) was obtained from Sigma Chemical Co. (St. Louis, MO). Eticlopride was dissolved in 0.9% saline and was also used as the vehicle control. The adenosine A_{2A} antagonists used were MSX-3 ((E)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl] propyl] ester disodium salt) and MSX-4, which were generously donated by the laboratory of Dr. Christa Muller (Pharmazeutisches Institut; Universität Bonn; Bonn, Germany). MSX-3 ((E)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl] propyl]ester disodium salt) was synthesized and generously donated by the Pharmazeutisches Institut, Universität Bonn (Bonn, Germany). MSX-3 was dissolved in 0.9% saline, and the pH of the MSX-3 solution was adjusted by adding 1.0 N NaOH until the drug was completely in solution (pH 7.1–7.4). Selection of MSX-4 was dissolved in de-ionized water. MSX-3 and MSX-4 are both pro-drugs of the active adenosine A_{2A} antagonist, MSX-2.

Selection of Doses

Doses of eticlopride used for the experiments were based upon previous research (Sink et al. 2008; Worden et al. 2009, Nunes et al. 2010). Previous studies were also conducted to select the doses of MSX-3 (Ishiwari et al., 2007; Farrar et al. 2006; Worden et al. 2009). The dose of MSX-3 used for experiment 2.3 was calculated based off of the drugs ED₅₀ for reversal of chow consumption. Based on pilot data assessing the oral potency of MSX-3, doses were selected for experiment 2.5. The dose range of MSX-4 used in experiment 2.2 was based upon extensive pilot work. The dose of MSX-4 used in

experiment 2.4 was calculated based off of the drugs ED50 for reversal of chow consumption. Subsequent doses of MSX-4 used in experiment 2.6 were based upon the results of the first experiment. Doses of eticlopride used for experiments 2.7-2.8 were based upon previous research (Sink et al. 2008; Worden et al. 2009, Nunes et al. 2010). Previous studies were also conducted to select the doses of MSX-3 (Ishiwari et al., 2007; Farrar et al. 2006; Worden et al. 2009). The dose of MSX-4 used in experiment 2.8 was based upon extensive pilot work and data obtained in experiment 2.2.

Behavioral Procedures:

Behavioral sessions were conducted in operant conditioning chambers (28cm x 23cm x 23cm; Med Associates). Rats were initially trained to lever press on a continuous reinforcement schedule (30-min sessions; 45-mg pellets, Bioserve, Frenchtown, NJ, were used for all operant behavior tests) and then were shifted to the FR5 schedule (30-min sessions, 5 days/week) and trained for several additional weeks. Rats were then trained on the concurrent F55/chow-feeding procedure. In this task weighed amounts of lab chow (Lab Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO; typically 15-20 g, three large pieces) were concurrently available on the floor of the chamber during the FR5 sessions. At the end of the session, rats were immediately removed from the chamber and food intake was assessed by weighing the remaining food (including spillage). Rats were trained until they reached stable levels of baseline lever pressing and chow intake (i.e., consistent responding over 1200 lever presses per 30 minutes), after which drug testing began. For most baseline day's rats did not receive supplemental feeding, however, over weekends and after drug tests, rats usually received supplemental chow in the home cage. On baseline

and drug treatment days, rats normally consumed all the operant pellets administered through lever pressing during each session.

Experimental Procedures

Rats were trained on the concurrent FR5/chow-feeding procedure (as described above) before testing began, and each experiment employed different groups of rats. The first four experiments used a within-group design with each rat receiving all combined IP drug treatments or time manipulations for their particular experiment in a randomly varied order (one treatment per week, with none of the treatment sequences repeated across different animals in the same experiment). The final experiment employed a between-subject design with each rat receiving only one orally administered drug treatment for their particular experiment, chosen randomly. Baseline (i.e., non-drug) sessions were conducted four additional days per week. The specific treatments and testing times for each experiment are listed below.

Experiment 2.1 and 2.2: Ability of the A_{2A} antagonists MSX-3 and MSX-4 to reverse the effects of the D_2 antagonist eticlopride. On the test day, trained rats ($n=7$) received the following treatments: 0.9% saline vehicle (30 min before testing) plus another saline vehicle IP (20 min before testing), 0.08 mg/kg eticlopride IP (30 min before testing) plus 0.9% saline vehicle IP (20 min before testing), 0.08 mg/kg eticlopride IP (30 min) plus 0.25 mg/kg MSX-3 IP (20 min), 0.08 mg/kg eticlopride IP (30 min) plus 0.5 mg/kg MSX-3 IP (20 min), 0.08 mg/kg eticlopride IP (30min) plus 1.0 mg/kg MSX-3 IP (20 min), .08 eticlopride IP (30 min) plus 2.0 mg/kg MSX-3 IP (20 min). For the MSX-4/eticlopride experiment, trained rats ($n=12$) received the following treatments: saline vehicle (30 min before

testing) plus deionized water (DI H₂O) vehicle IP (40 min before testing), .08 mg/kg eticlopride IP (30 min before testing) plus DI H₂O vehicle IP (40 min before testing), 0.08 mg/kg eticlopride IP (30 min) plus 1.0 mg/kg MSX-4 IP (40 min before testing), 0.08 mg/kg eticlopride IP (30 min) plus 2.0 mg/kg MSX-4 (40 min), 0.08 mg/kg eticlopride IP (30 min) plus 4.0 mg/kg MSX-4 IP (40 min), 0.08 mg/kg eticlopride IP (30 min) plus 8.0 mg/kg MSX-4 IP (40 min).

Experiment 2.3 and 2.4: Time course of the ability of the A_{2A} antagonists MSX-3 and MSX-4 to reverse the effects of the D₂ antagonist eticlopride. On test day in the MSX-3/eticlopride time course experiment trained rats (n=6) received the following treatments: 0.09% saline vehicle IP (30 minutes before testing) plus saline vehicle IP (20, 40, 80, 160 or 230 minutes before testing), 0.08 mg/kg eticlopride IP (30 min before testing) plus saline vehicle IP (20, 40, 80, 160 or 320 min before testing), 0.08 mg/kg eticlopride IP (30 min) plus 1.5 mg/kg MSX-3 IP (20, 40, 80, 160 or 320 minutes before testing). For the MSX-4/eticlopride time course experiment trained rats (n=8) received the following treatments: 0.09% saline vehicle IP (30 minutes before testing) plus deionized water (DI H₂O) vehicle IP (20, 40, 80, 160 or 230 minutes before testing), 0.08 mg/kg eticlopride IP (30 min before testing) plus DI H₂O vehicle IP (20, 40, 80, 160 or 320 min before testing), 0.08 mg/kg eticlopride IP (30 min) plus 6.0 mg/kg MSX-4 IP (20, 40, 80, 160 or 320 minutes before testing).

Experiment 2.5: Oral Availability of the A_{2A} antagonists MSX-3 and MSX-4. Reversal of the effects of the D₂ antagonist eticlopride. On test day trained rats (n=42) received the following treatments in the eticlopride/MSX-3 oral administration experiment: 0.09% saline vehicle IP (30 min before testing) plus saline vehicle OA (80 min before testing), 0.08

mg/kg eticlopride IP (30 min) plus saline vehicle OA (80 min), 0.08 mg/kg eticlopride IP (30 min) plus 4.0 mg/kg MSX-3 OA (80 min), 0.08 mg/kg eticlopride IP (30 min) plus 8 mg/kg MSX-3 OA (80 min), 0.08 mg/kg eticlopride IP (30 min) plus 4 mg/kg MSX-4 OA (80 min), or 0.08 mg/kg eticlopride IP (30 min) plus 8.0 mg/kg MSX-4 OA (80 min).

Experiment 2.6: Effect of eticlopride alone and eticlopride plus MSX-4 on immediate early gene expression (*c-Fos* immunoreactivity) in nucleus accumbens neurons. Experimentally naïve rats (n=6) were randomly assigned to the following treatment conditions: 0.09% saline vehicle IP (90 min before perfusion) plus deionized water (DI H₂O) vehicle IP(90 min before perfusion), 0.08 mg/kg eticlopride IP (90 min) plus DI H₂O vehicle IP (90 min), 0.08 mg/kg eticlopride IP (90 min) plus 8.0 mg/kg MSX-4 IP (90 min before perfusion). All animals were anesthetized and perfused with physiological saline followed by 3.7% formaldehyde 90 min after the IP injections and stored at 4 °C in formaldehyde for one day. Brains were then put on .9% sucrose until saturated.

c-Fos Visualization and Quantification

The processing for c-Fos visualization was performed using a standard immunohistochemistry protocol modified for the detection of c-Fos in free-floating sections. Free floating coronal sections (50 μ m) were serially cut using a microtome cryostat (Weymouth, MA, USA), rinsed in 0.01 M PBS (pH 7.4) and incubated in 0.3% hydrogen peroxide (H₂O₂) for 30 min to block endogenous staining. Sections were then rinsed in PBS (3 \times for 5 min) and transferred into the primary antibody, anti-c-Fos (Calbiochem, Germany) for a 48 h incubation. Following the primary antibody treatment,

the sections were rinsed in PBS and incubated in the secondary antibody, anti-rabbit HRP conjugate, envision plus (DAKO, Denmark) for 2 h. The immunohistochemical reaction was developed using diaminobenzidine (DAB) as the chromagen. Processed sections were then mounted to gelatin-coated slides, air dried, and cover-slipped using Cytoseal 60 (Thermo Scientific) as a mounting medium. The sections were examined and photographed using a Nikon Eclipse E600 (Melville, NY, USA) upright microscope equipped with an Insight Spot digital camera (Diagnostic Instruments, Inc). Images of the region of interest were magnified at 20X and captured digitally using SPOT software; the target area was the Nucleus Accumbens core region. Brains with extensive damage or uneven staining across sections were excluded from statistical analysis. Cells that were positively labeled for c-Fos were quantified with the aid of ImageJ software (v.1.42, National Institutes of Health sponsored image analysis program). The analysis was carried out on two sections per animal and the average value for both sections was used for statistical analysis.

Statistical Analyses:

For experiments 2.1-2.4, total number of lever presses and gram quantity of chow intake from the 30-minute sessions were analyzed with repeated measures of analysis of variance (ANOVA). A computerized statistical program (SPSS 12.0 for Windows) was used to perform these analyses. When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each treatment with the eticlopride vehicle control group (Keppel 1991). To assess these comparisons, α level was kept at 0.05. The total number of comparisons was restricted to the number of treatments minus one (Keppel, 1991). This method of calculation allows each condition

that combined eticlopride and adenosine antagonists to be compared to the eticlopride vehicle condition. Effect size calculations (R^2 values; Keppel 1991) were performed to measure the magnitude of the reversal effect; these analyses were conducted by removing the vehicle plus vehicle condition, and calculating the R^2 value for the four treatments that included an injection of the D_2 antagonist eticlopride. With this type of calculation, the magnitude of the treatment effect is independent of the number of animals, and is expressed as the proportion of the total variance accounted for by the treatment variance. (For example $R^2=.03$ reflects 30% of the variance explained across experiments and measures). Data for experiments 2.5 were analyzed using a one-factor (drug treatment) between subjects ANOVA. Non-orthogonal planned comparisons were used to compare the eticlopride condition to both the vehicle and MSX-3 or MSX-4 plus eticlopride conditions.

RESULTS

Experiment 2.1: The results of experiment 2.1 are shown in Figure 2.1. There was an overall significant effect of drug treatment on lever pressing (Figure 2.1A; $F(5, 30) = 11.466$, $p < 0.001$). Planned comparisons showed that eticlopride produced a significant reduction in lever pressing compared to vehicle control ($p < 0.05$). In addition, co-administration of MSX-3 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with the 0.5, 1.0 and 2.0 mg/kg doses of MSX-3 producing significant differences relative to eticlopride plus vehicle ($p < 0.05$). There also was an overall significant effect of drug treatment on chow intake (Figure 2.2B; $F(5,30) = 9.225$, $p < 0.001$). Planned comparisons indicated that eticlopride produced a significant increase in chow intake compared to vehicle control ($p < 0.05$). Co-administration of MSX-3

with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 0.5, 1.0 and 2.0 mg/kg dose of MSX-3 being significantly different from eticlopride plus vehicle ($p < 0.05$).

Experiment 2.2: The results of experiment 2.2 are shown in Figure 2.2. There was an overall significant effect of drug treatment on lever pressing (Figure 2.2A; $F(5, 55) = 13.477$, $p < 0.001$). Planned comparisons showed that eticlopride produced a significant reduction in lever pressing compared to vehicle control ($p < 0.05$). In addition, co-administration of MSX-4 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with the 1.0, 2.0, 4.0 and 8.0 mg/kg doses of MSX-4 producing significant differences relative to eticlopride plus vehicle ($p < 0.05$). There also was an overall significant effect of drug treatment on chow intake (Figure 2.2B; $F(5, 55) = 7.658$, $p < 0.001$). Planned comparisons indicated that eticlopride produced a significant increase in chow intake compared to vehicle control ($p < 0.05$). Co-administration of MSX-4 with haloperidol produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 8.0 mg/kg dose of MSX-4 being significantly different from eticlopride plus vehicle ($p < 0.05$).

Experiment 2.3: The results of experiment 2.3 are shown in Figure 2.3. There was an overall significant effect of drug treatment on lever pressing (Figure 2.2A; $F(6, 30) = 16.263$, $p < 0.001$). Planned comparisons showed that eticlopride produced a significant reduction in lever pressing compared to vehicle control ($p < 0.05$). In addition, co-administration of 1.5 mg/kg MSX-3 with eticlopride produced a significant increase in lever

pressing compared to eticlopride plus vehicle, with the 20 minute, 40 minute, and 80 minute lead times of administration producing significant differences relative to eticlopride plus vehicle ($p < 0.05$). There also was an overall significant effect of drug treatment on chow intake (Figure 2.3B; $F(6,30) = 11.464$, $p < 0.001$). Planned comparisons indicated that eticlopride produced a significant increase in chow intake compared to vehicle control ($p < 0.05$). Co-administration of 1.5 mg/kg MSX-3 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with 20 minute, 40 minute, and 80 minute lead times of administration being significantly different from eticlopride plus vehicle ($p < 0.05$).

Experiment 2.4: The results of experiment 2.4 are shown in Figure 2.4. There was an overall significant effect of drug treatment on lever pressing (Figure 2.4A; $F(6, 42) = 16.338$, $p < 0.001$). Planned comparisons showed that eticlopride produced a significant reduction in lever pressing compared to vehicle control ($p < 0.05$). In addition, co-administration of 6.0 mg/kg MSX-4 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with the 20 minute, 40 minute, and 80 minute lead times producing significant differences relative to eticlopride plus vehicle ($p < 0.05$). There also was an overall significant effect of drug treatment on chow intake (Figure 2.2B; $F(6,42) = 12.127$, $p < 0.001$). Planned comparisons indicated that eticlopride produced a significant increase in chow intake compared to vehicle control ($p < 0.05$). Co-administration of 6.0 mg/kg MSX-4 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 20 minute, 40 minute, and 80 minute lead times of administration being significantly different from eticlopride plus

vehicle ($p < 0.05$).

Experiment 2.5: The results of experiment 2.5 are shown in Figure 2.5. There was an overall significant effect of drug treatment on lever pressing (Figure 2.5A; $F(5, 36) = 20.6$, $p < 0.001$). Planned comparisons showed that eticlopride produced a significant reduction in lever pressing compared to vehicle control ($F(1,36) = 28.0$, $p < 0.01$). In addition, oral co-administration of MSX-3 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with the 8.0 mg/kg dose of MSX-3 producing significant differences relative to eticlopride plus vehicle ($F(1,36) = 32.5$, $p < 0.01$). Also, oral co-administration of MSX-4 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with the 8.0 mg/kg dose of MSX-4 producing significant differences relative to eticlopride plus vehicle ($F(1,36) = 44.2$, $p < 0.01$). There also was an overall significant effect of drug treatment on chow intake (Figure 2.5B; $F(5,36) = 7.6$, $p < 0.001$). Planned comparisons indicated that eticlopride produced a significant increase in chow intake compared to vehicle control ($F(1,36) = 15.0$, $p < 0.01$). Oral co-administration of MSX-3 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 8.0 mg/kg dose of MSX-3 producing significant differences relative to eticlopride plus vehicle ($F(1,36) = 9.0$, $p < 0.01$). In addition, oral co-administration of MSX-4 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 8.0 mg/kg dose of MSX-4 being significantly different from eticlopride plus vehicle ($F(1,36) = 12.2$, $p < 0.01$).

Experiment 2.6: The results of experiment 2.6 are shown in Fig. 2.6. There was a overall

effect of drug treatment on the number of c-Fos positive cell counts. Eticlopride increased c-Fos positive cell counts relative to vehicle alone. cFos positive cells increased from 70.25 cells in the vehicle/vehicle condition, to 166.66 upon administration of eticlopride., Co-administration of MSX-4 reduced c-Fos positive cell counts relative to eticlopride alone, with only 58.75 cFos positive cells in NAc core.

FIGURE 2.1

Lever Pressing (A) and Chow Consumption (B): Eticlopride and MSX-3

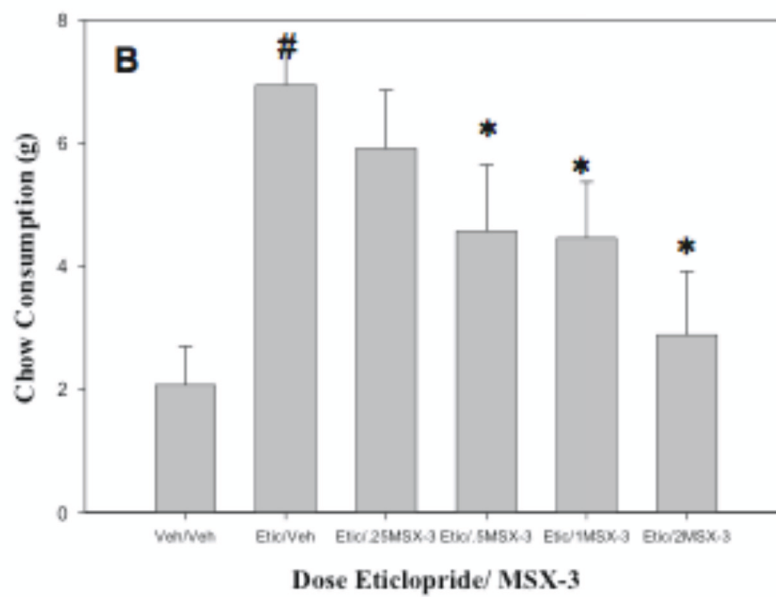
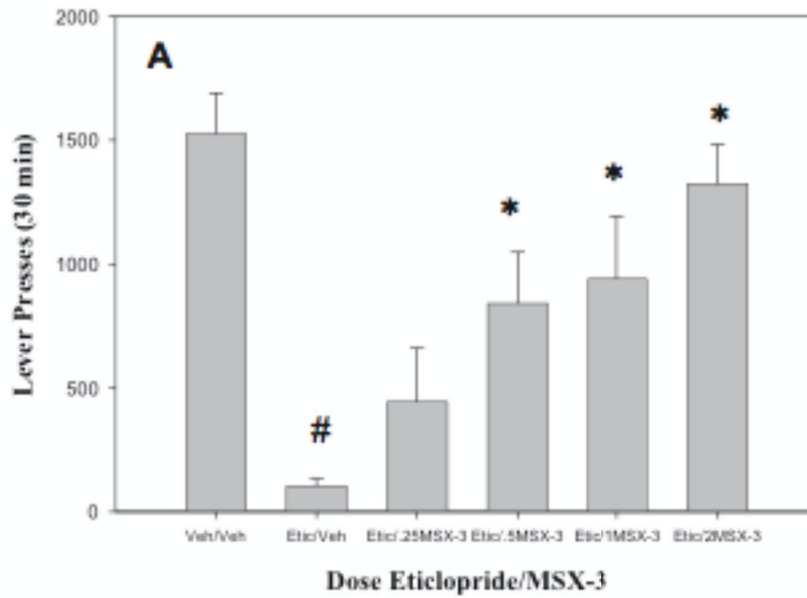


Figure 2.1. The effects of the adenosine A_{2A} antagonist MSX-3 on eticlopride induced changes in performance on the concurrent lever pressing/chow feeding procedure. Rats received IP injections of vehicle plus vehicle (Veh/Veh), 0.08 mg/kg eticlopride plus vehicle (Etic/Veh), and eticlopride plus 0.25, 0.5, 1.0 or 2.0 mg/kg doses of MSX-3. A. Mean (SEM) number of lever presses (FR5 schedule) during the 30 minute session. B. Mean (SEM) gram quantity of chow intake. Eticlopride significantly decreases lever pressing and increased chow intake relative to vehicle ($\#p < 0.05$). Co-administration of MSX-3 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with the 0.5, 1.0 and 2.0 mg/kg doses of MSX-3 producing significant differences relative to eticlopride plus vehicle ($*p < 0.05$). Also, co-administration of MSX-3 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 0.5, 1.0 and 2.0 mg/kg dose of MSX-3 being significantly different from eticlopride plus vehicle ($*p < 0.05$).

FIGURE 2.2

Lever Pressing (A) and Chow Consumption (B): Eticlopride and MSX-4

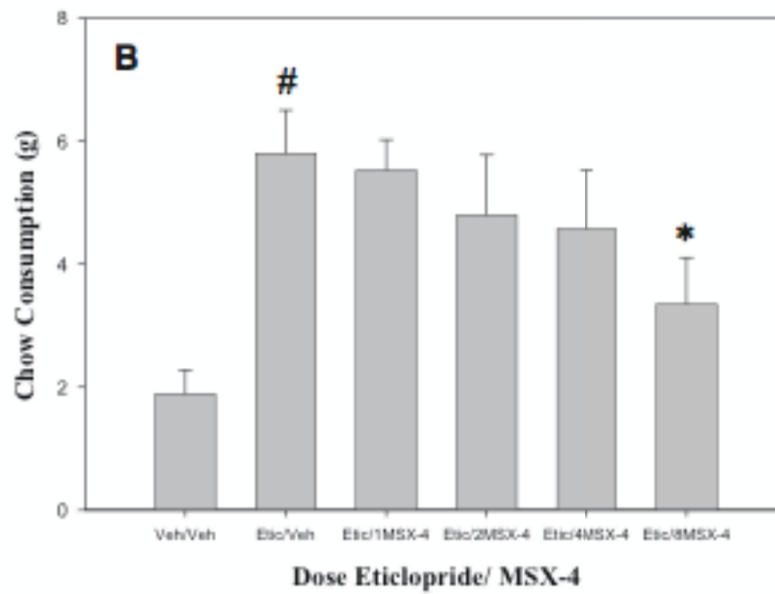
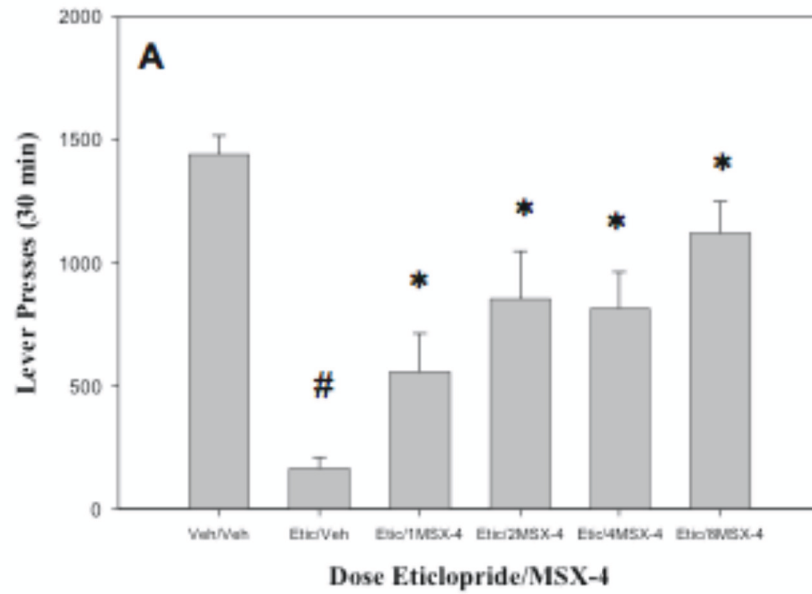


Figure 2.2. The effects of the adenosine A_{2A} antagonist MSX-4 on eticlopride induced changes in performance on the concurrent lever pressing/chow feeding procedure. Rats received IP injections of vehicle plus vehicle (Veh/Veh), 0.08 mg/kg eticlopride plus vehicle (Etic/Veh), and eticlopride plus 1.0, 2.0, 4.0 and 8.0 mg/kg doses of MSX-4. A. Mean (SEM) number of lever presses (FR5 schedule) during the 30 minute session. B. Mean (SEM) gram quantity of chow intake. Eticlopride significantly decreases lever pressing and increased chow intake relative to vehicle ($\#p < 0.05$). Co-administration of MSX-4 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with the 1.0, 2.0, 4.0 and 8.0 mg/kg doses of MSX-4 producing significant differences relative to eticlopride plus vehicle ($*p < 0.05$). Also, co-administration of MSX-4 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 8.0 mg/kg dose of MSX-4 being significantly different from eticlopride plus vehicle ($*p < 0.05$).

FIGURE 2.3

Lever Pressing (A) and Chow Consumption (B): Eticlopride and MSX-3

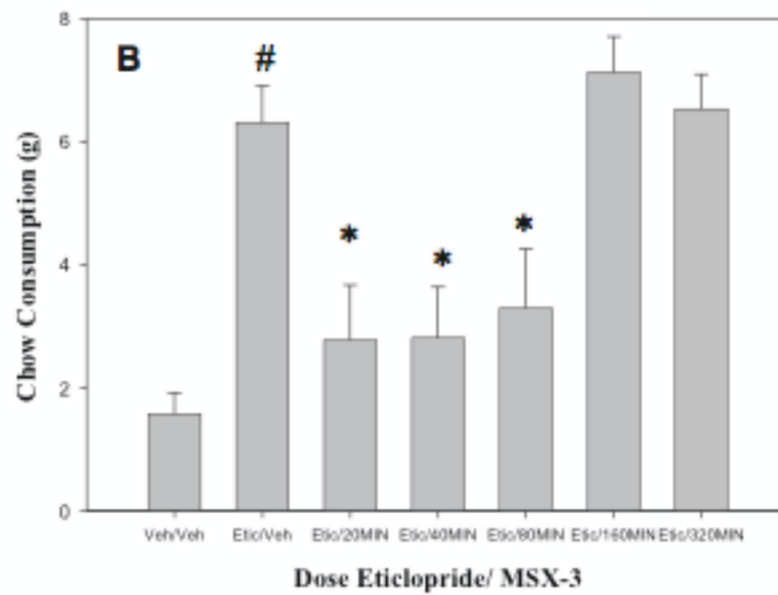
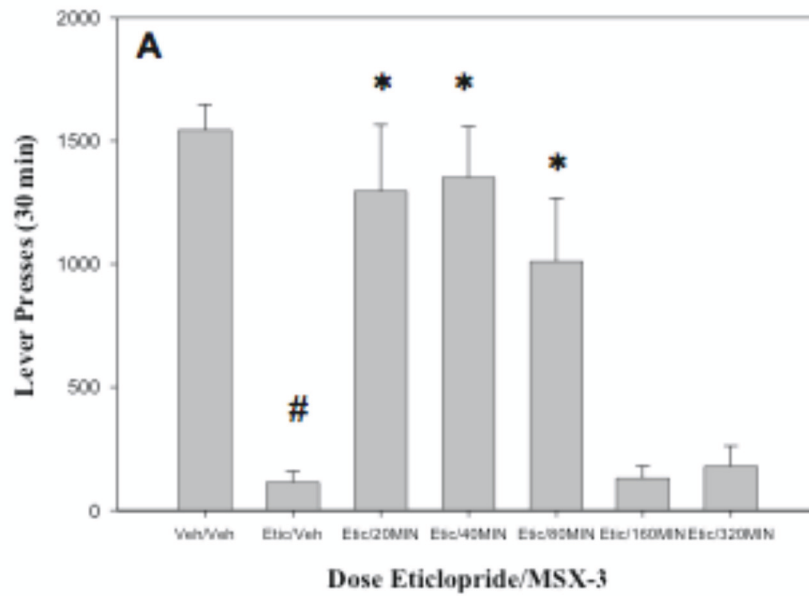


Figure 2.3. The effects of the adenosine A_{2A} antagonist MSX-3 on eticlopride induced changes in performance on the concurrent lever pressing/chow feeding procedure after varied time courses of administration. Rats received IP injections of vehicle plus vehicle (Veh/Veh), 0.08 mg/kg eticlopride plus vehicle (Etic/Veh), and eticlopride plus 1.5 mg/kg of MSX-3 at 20, 40, 80, 160 and 320 minutes prior to testing. A. Mean (SEM) number of lever presses (FR5 schedule) during the 30 minute session. B. Mean (SEM) gram quantity of chow intake. Eticlopride significantly decreases lever pressing and increased chow intake relative to vehicle ($\#p < 0.05$). Co-administration of MSX-3 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with MSX-3 administered at 20, 40 and 80 minutes prior to testing producing significant differences relative to eticlopride plus vehicle ($*p < 0.05$). Also, co-administration of MSX-3 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 20, 40 and 80 minute lead times being significantly different from eticlopride plus vehicle ($*p < 0.05$).

FIGURE 2.4.

Lever Pressing (A) and Chow Consumption (B): Eticlopride and MSX-4

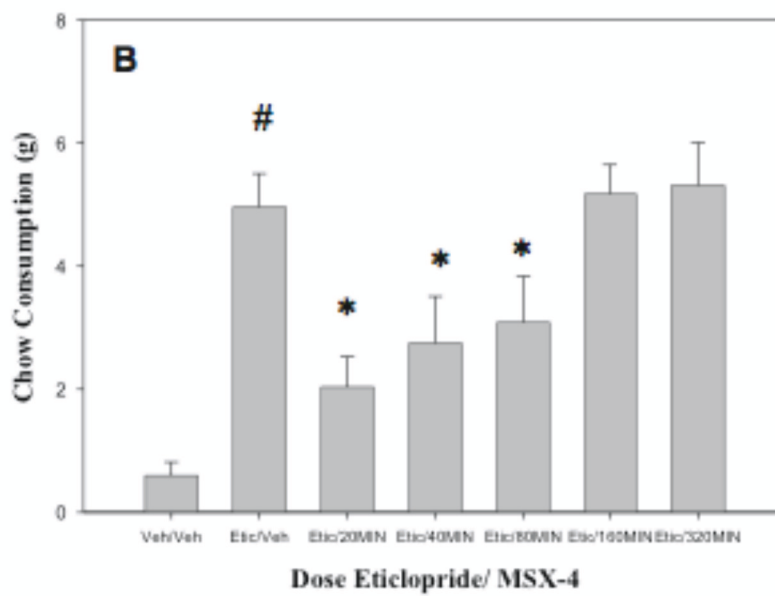
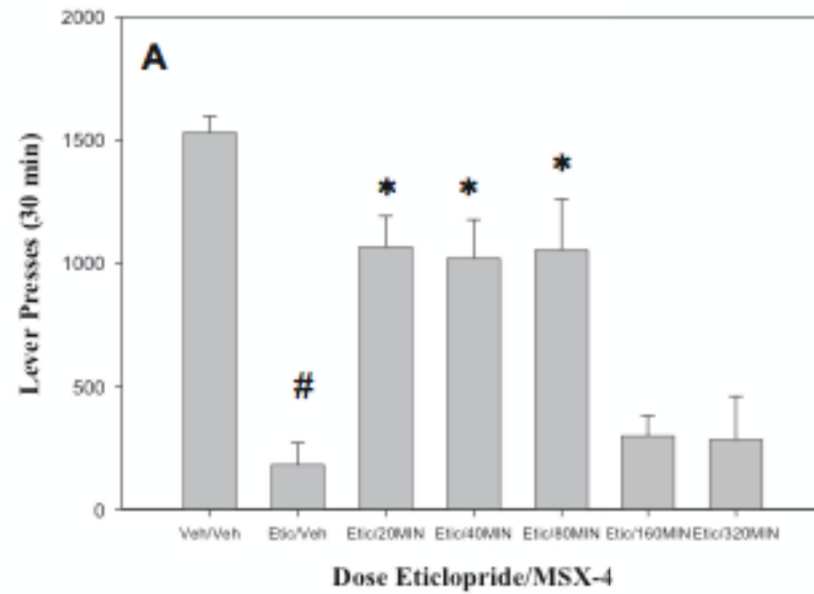


Figure 2.4. The effects of the adenosine A_{2A} antagonist MSX-4 on eticlopride induced changes in performance on the concurrent lever pressing/chow feeding procedure after varied time courses of administration. Rats received IP injections of vehicle plus vehicle (Veh/Veh), 0.08 mg/kg eticlopride plus vehicle (Etic/Veh), and eticlopride plus 6.0 mg/kg of MSX-4 at 20, 40, 80, 160 and 320 minutes prior to testing. A. Mean (SEM) number of lever presses (FR5 schedule) during the 30 minute session. B. Mean (SEM) gram quantity of chow intake. Eticlopride significantly decreases lever pressing and increased chow intake relative to vehicle ($\#p < 0.05$). Co-administration of MSX-4 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with MSX-4 administered at 20, 40 and 80 minutes prior to testing producing significant differences relative to eticlopride plus vehicle ($*p < 0.05$). Also, co-administration of MSX-4 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 20, 40 and 80 minute lead times being significantly different from eticlopride plus vehicle ($*p < 0.05$).

FIGURE 2.5.

Lever Pressing (A) and Chow Consumption (B): Eticlopride and MSX-3 or MSX-4

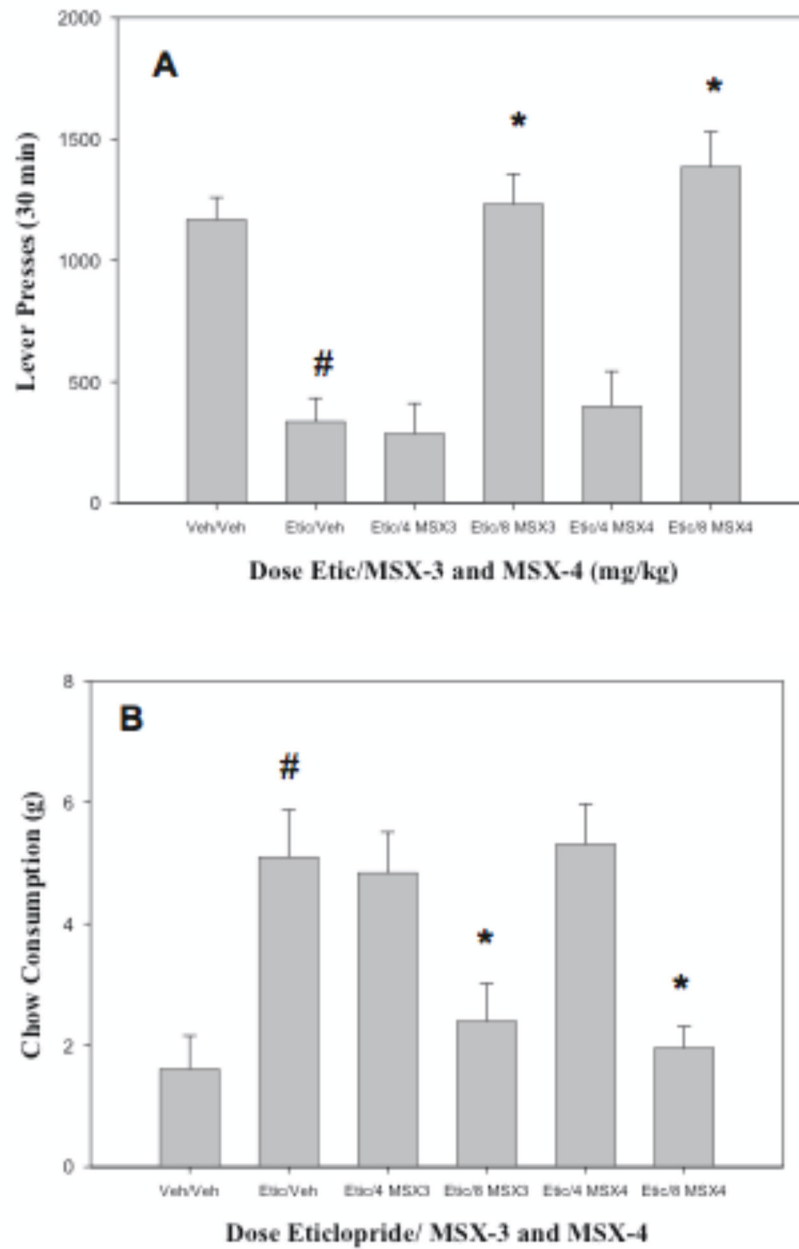


Figure 2.5. The effects of the adenosine A_{2A} antagonists MSX-3 and MSX-4 on eticlopride induced changes in performance on the concurrent lever pressing/chow feeding procedure after oral administration. Rats received IP injections of eticlopride or vehicle, in combination with oral administration of 4.0, and 8.0 mg/kg doses of either MSX-3 or MSX-4 prior to testing. A. Mean (SEM) number of lever presses (FR5 schedule) during the 30 minute session. B. Mean (SEM) gram quantity of chow intake. Eticlopride significantly decreases lever pressing and increased chow intake relative to vehicle ($p < 0.05$). Co-administration of MSX-3 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with MSX-3 at the 8.0 mg/kg dose producing significant differences relative to eticlopride plus vehicle ($p < 0.05$). Also, co-administration of MSX-3 with eticlopride produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 8.0 mg/kg dose being significantly different from eticlopride plus vehicle ($p < 0.05$). In addition, co-administration of MSX-4 with eticlopride produced a significant increase in lever pressing compared to eticlopride plus vehicle, with MSX-4 at the 8.0 mg/kg dose producing significant differences relative to eticlopride plus vehicle ($p < 0.05$). Co-administration of MSX-4 with eticlopride also produced produced a significant decrease in chow intake relative to eticlopride plus vehicle, with the 8.0 mg/kg dose being significantly different from eticlopride plus vehicle ($p < 0.05$).

FIGURE 2.6.

c-Fos Immunocytochemistry, A. Vehicle/Vehicle B.Eticlopride/Vehicle C.

Eticlopride/MSX-4

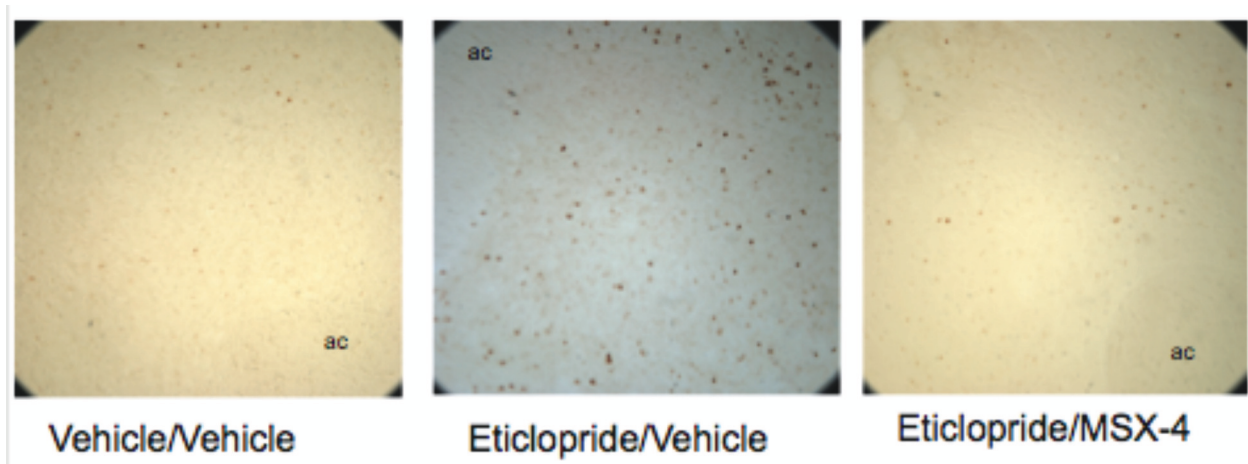


Figure 2.6. The effects of systemic MSX-3 on eticlopride-induced c-Fos expression in nucleus accumbens. Rats received of I.P. injections of vehicle plus vehicle (Veh-Veh), vehicle plus 0.08 mg/kg eticlopride plus eticlopride (Etic-Veh), and 0.08 mg/kg eticlopride plus 8 mg/kg MSX-4. Eticlopride alone increased c-Fos expression relative to vehicle. MSX-4 reduced the c-Fos expression induced by eticlopride.

DISCUSSION

The present studies were conducted in order to characterize the novel adenosine A_{2A} antagonist MSX-4. In particular, these studies were designed in order to assess MSX-4's ability to reverse impairments in effort-related choice induced by the DA D_2 receptor antagonist, eticlopride. To better understand the properties of MSX-4, we compared it to another pro-drug of the A_{2A} antagonist MSX-2, MSX-3. Both drugs were compared in terms of their ability to reverse the motivation deficits induced by eticlopride, the optimal time course for this effect, and for oral activity. Lastly, we conducted a small study on the effects of MSX-4 on c-Fos immunoreactivity in the NAc. MSX-3 reversed the behavioral effects induced by eticlopride in the concurrent FR5 lever pressing/chow choice procedure at doses of 0.5-2.0 mg/kg, whereas MSX-4 was less potent, reversing the effects of eticlopride at 2.0-8.0 mg/kg. Both MSX-3 and MSX-4 had similar time courses in their ability to reverse the behavioral effects induced by systemic administration of eticlopride in that they increased lever presses and decreased chow intake between 20-80 minutes after systemic administration. Oral administration studies indicated that both MSX-3 and MSX-4 were capable of reversing behavioral effects induced by eticlopride at 8.0 mg/kg. Finally, systemic administration of eticlopride increased number of c-Fos positive cells compared to vehicle alone in the NAc core. However, co-administration of MSX-4 attenuated c-Fos positive cells in the NAc compared to eticlopride alone.

The results of these studies highlight the ability for A_{2A} antagonism to reverse the effects of D_2 antagonism on the concurrent choice procedure. In addition, it is demonstrated that different pro-drugs of the same parent drug, have differential effects on the effort related choice procedure in terms of potency after varying routes of

administration. Moreover, results support DA-adenosine interactions in the NAc as an important region in behavioral changes induced through manipulation of effort-related processes. Furthermore, the results indicate that MSX-4 is a useful pro-drug tool for probing the functional significance of adenosine A_{2A} receptor blockade. These results, and their general significance are discussed further below.

DA-Adenosine Interactions and Effort-Related Processes

Consistent with previous studies (Salamone et al. 1991, 2002; Cousins et al 1994; Sink et al. 2008), the present results showed that D₂ antagonism produced a substantial effect on the behavior of rats performing on the concurrent FR5/chow feeding choice task. In all 5 behavioral experiments, eticlopride significantly reduced lever pressing and increased chow intake. This paradigm illustrates that rats receiving low doses of DA antagonists retain their tendency to acquire and consume food. Although these animals perform poorly in lever pressing, rats are capable of engaging in food-motivated behavior and producing alternative methods in order to obtain food. Taken together, these results indicate that D₂ antagonism interferes with exertion of effort in food-seeking behavior as well as effort-related decision making (Salamone et al., 1997, 2003, 2005; Koch et al., 2000; Salamone and Correa, 2002; Denk et al., 2005).

The current studies focused upon the ability of two adenosine A_{2A} antagonist pro-drugs, MSX-3 and MSX-4, to reverse the effects of DA D₂ antagonism. MSX-3 is a well characterized drug that has been used in several published papers (Farrar et al., 2007, 2010; Salamone et al. 2008a,b, 2009; Mott et al., 2009; Worden et al. 2009). In contrast, MSX-4 is a novel compound that was developed very recently (Vollmann et al., 2008). We

demonstrated that systemic administration of either MSX-3 or MSX-4 was capable of reversing deficits in the concurrent choice procedure induced by systemic administration of the D₂ antagonist eticlopride. MSX-3 produced a full reversal of the suppressive effects of eticlopride on lever pressing, as well as chow consumption. In comparison, MSX-4, which possesses the same parent drug as MSX-3 in combination with a different promoiety, also reversed the eticlopride-induced shift in motivational and goal directed behaviors. These results are consistent with previous data showing that adenosine A_{2A} receptor antagonists can reverse the behavioral effects of DA antagonists on tests involving locomotion, rigidity, catalepsy and tremor (Hauber et al., 2001; Wardas et al., 2001; Correa et al., 2004; Collins et al., 2010). In addition to these findings, the present study revealed that MSX-3, administered systemically is four times more potent than systemically administered MSX-4. These results support that hypothesis that by possessing a phosphate acid ester, MSX-3 would maintain a higher potency due to increased water solubility. MSX-3 is readily cleaved by phosphatases liberating the active parent drug, MSX-2 (Mueller, 2009). MSX-3 displays a water solubility of 9.0 mg/ml, while MSX-4 displays a lower solubility of 7.2 mg/ml, which bolsters the results showing MSX-3 to be more potent after systemic administration.

Time course studies demonstrated that systemic administration of either MSX-3 or MSX-4 was capable of reversing deficits in the concurrent choice procedure induced by systemic administration of the D₂ antagonist eticlopride after different times of administration. MSX-3 increased lever pressing and decreased chow intake in eticlopride-treated rats, reversing the eticlopride-induced shift in motivational and goal directed behaviors. MSX-3 produced a full reversal of the suppressive effects of eticlopride on lever

pressing, as well as chow consumption. In comparison, MSX-4 increased lever pressing and decreased chow intake in eticlopride-treated rats, reversing the eticlopride-induced shift in motivational and goal directed behaviors. MSX-4 produced a substantial though partial reversal of the eticlopride-induced deficits. The present study revealed that systemically administered MSX-3 and MSX-4 display similar time courses in behavioral efficacy. Both drugs display a therapeutic window from 20 minutes to 80 minutes after systemic administration. This is not in agreement with our original hypothesis in that MSX-4 would be longer active than MSX-3 due to the parent drug being released later in the absorption process. However, it is possible that in order to achieve differences in time course, oral administration should be used to capture differences in therapeutic windows between MSX-3 and MSX-4.

It was hypothesized that MSX-4 would be orally active, and would gain potency compared to MSX-3 in tests that involved oral administration. Peroral application of phosphate prodrugs, such as MSX-3, is thought to be less favorable because it is highly unlikely that the intact- highly polar- prodrug can be absorbed. Phosphoric acids esters usually undergo enzymatic hydrolysis prior to absorption and therefore phosphate prodrugs are typically used for intravenous administration (Muller, 2009). Therefore, we assessed whether oral administration of either MSX-3 or MSX-4 was capable of reversing deficits in the concurrent choice procedure induced by systemic administration of the D₂ antagonist eticlopride. MSX-3 produced a full reversal of the suppressive effects of eticlopride on lever pressing, as well as chow consumption, at 8.0 mg/kg. Similarly, MSX-4 produced a full reversal of the eticlopride-induced deficits. Although MSX-4 did not prove to be more orally available than MSX-3, it did produce reversal of the effort related choice

paradigm at the same dose that was effective for MSX-3. In systemic studies MSX-3 was four times more potent than MSX-4. Thus, after oral administration this disparity in potency appears to be abolished; after oral dosing, MSX-3 is four times less potent, while MSX-4 maintains the same potency shown in earlier experiments. This supports the hypothesis that an amino acid ester does lend to a higher oral availability as compared to IP administration. This increased oral availability is consistent with previous data using valacyclovir and valganciclovir, valine esters of the potent antiviral drugs acyclovir and ganciclovir which display better oral absorption when paired with the valine ester (MacDougall et al. 2004).

Lastly, to determine the effects of eticlopride and MSX-4 on neural activity, we gathered preliminary data using c-Fos expression in NAc core as a marker. Systemic injections of eticlopride, increased c-Fos positive cells in NAc core relative to vehicle-treated animals. This data is consistent with previous data showing that systemic and intra accumbens administration of DA D₂ antagonists can increase c-Fos immunoreactivity in NAc core (Dragunow et al, 1990., Miller, 1990; Robertson and Fibiger, 1992; Fibiger, 1994; MacGibbon et al., 1994; Wan et al. 1995; Pinna et al. 1999, Betz et al. 2009; Farrar et al. 2010). DA D₂ receptors are negatively coupled to cAMP production, while adenosine A_{2A} receptors, which are co-localized with D₂ receptors on striatopallidal neurons (e.g. Ferre, 1997), are positively coupled to cAMP production. Administration of the A_{2A} receptor antagonist, CGS21690 mimics the effects of D₂ antagonists on induction of c-Fos expression (Pinna et al. 1997). Previous data has also shown that systemic administration of A_{2A} receptors antagonists can partially reverse the increases in c-Fos expression induced by the DA D₂ antagonist haloperidol (Boegman and Vincent, 1996; Pinna et al. 1999).

Additionally, recent data from our laboratory has shown that induction of c-Fos through intracranial administration of the D₂ antagonist eticlopride was attenuated after co-administration of MSX-3 (Farrar et al., 2010). In the present study, the ability of MSX-4 to attenuate eticlopride-induced increases in c-Fos immunoreactivity were assessed in a small group of animals. While systemic injections of eticlopride led to apparent increases in c-Fos positive cells in the nucleus accumbens core, MSX-4 co-administration showed a trend towards attenuating the increase in c-Fos positive cells. Doses of MSX-4 and eticlopride were the same as those used in the previous behavioral experiments. Thus, it can be shown that the same doses that induce changes in the effort related choice paradigm also have a direct effect on neuronal expression. The c-Fos immunohistochemistry methods used in the present studies appear to provide a useful neural marker of the interaction between drugs that act on DA and adenosine receptors. Future studies with more animals should be conducted to provide a detailed characterization of the neurochemical effects of MSX-4.

General Conclusions:

The present experiments were designed to characterize some of the behavioral effects of the novel adenosine A_{2A} antagonist MSX-4. In order to better understand the potential therapeutic utility of MSX-4, it was important to assess this drug using a well-known paradigm. The interaction between DA D₂ antagonists and adenosine A_{2A} antagonists has been shown to be highly relevant for regulating goal-directed behaviors. MSX-3, a well-characterized adenosine A_{2A} antagonist, has proved effective in reversing the impairments in goal-directed behaviors induced by D₂ antagonism. MSX-2, the active drug in both MSX-3 and MSX-4, is a potent A_{2A} antagonist as determined by *in vitro* studies. However, MSX-2 exhibits poor water solubility and limited peroral availability, limiting its usefulness in

vivo. Both MSX-3 and MSX-4 were derived to enhance water solubility and oral availability through the adherence of a pharmacologically inactive promoiety. Overall, MSX-3 and MSX-4 showed similar characteristics in their ability to reverse effort-related impairments induced by the D₂ antagonist eticlopride. Moreover, the results described above support the hypothesis that adenosine/DA interactions are an important aspect of the overall neurochemical processes regulating effort-related aspects of motivation. Interest in prodrugs such as MSX-3 and MSX-4 has grown immensely over the past 20 years. Prodrugs help overcome various barriers to drug formulation and delivery such as poor aqueous solubility, chemical instability, insufficient oral absorption, rapid pre-systemic metabolism, inadequate brain penetration, toxicity and local irritation. MSX-4, with its clear biological activity and oral bioavailability, may be a useful pharmacological tool for research in this area, and could prove to be beneficial as a treatment for motivational deficits associated with depression in a clinical setting.

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