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Adenosine-Dopamine Interactions in the Open Field Arena: Studies Related to Locomotion and Anxiety

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**Adenosine-Dopamine Interactions in the Open Field
Arena: Studies Related to Locomotion and Anxiety.**

The Honors Scholar Thesis of

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I. Acknowledgments

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II. Abstract

Nucleus accumbens dopamine (DA) is an important regulator of locomotion. Recent research has also indicated the neuromodulator adenosine has a role in regulating locomotion. The adenosine A2A receptor subtype is colocalized with DA D2 receptors on medium spiny neurons in the striatum and nucleus accumbens, and interactions between adenosine A2A and DA D2 receptor antagonists are significant for regulating various aspects of motor and motivational function. The adenosine A2A antagonist MSX-3 has been shown to reverse the suppression of locomotion induced by the DA D2 antagonist eticlopride. Recently, the structure of MSX-3 was modified to produce the amino acid ester prodrug MSX-4, which is highly water soluble, and has high oral bioavailability. The present studies sought to elucidate the interactions between DA D2 antagonist eticlopride and the A2A adenosine antagonist MSX-4 by determining if MSX-4 could reverse eticlopride-induced locomotion suppression. Moreover, the induction of anxiety was measured by recording the relative amount of activity in the inner portion of the open field arena. In the current study, rats were injected with eticlopride, MSX-4, saline, or both eticlopride and MSX-4. Locomotion was measured by total activity counts in the open field test during two 5-minute sessions. To determine if the interactions between eticlopride and MSX-4 are related to the induction of anxiety, the relative number of activity counts in the inner portion of the open field was used as the measure of anxiety. In order to provide a neural marker of the interaction between eticlopride and MSX-4, histological studies measured the expression of c-Fos and DARPP-32 (THR34) immunoreactivity, which are commonly used as a marker of neural activity. Eticlopride significantly suppressed locomotion and increased c-Fos and DARPP-32 expression in the nucleus accumbens as compared to vehicle animals. The A2A antagonist MSX-4 reversed the locomotion suppression induced by eticlopride, and decreased the eticlopride-

induced expression of c-Fos and DARRP-32 in the nucleus accumbens. MSX-4 produced no significant increase in the anxiety index. These results indicate that the effects of MSX-4 are consistent with the general antiparkinsonian profile of adenosine A2A antagonists, and suggest that signs of angiogenesis as measured by the open field were not produced as a side effect of this drug. This research may be relevant for the development of novel drug therapies for the treatment of parkinsonism and psychomotor dysfunctions in depression.

III. Introduction

Locomotor activity is regulated by complex neurochemical interactions which occur in the circuitry of the basal ganglia. Considerable evidence has implicated nucleus accumbens dopamine (DA) in the regulation of locomotor activity (Annett et al. 1989, Baldo et al. 2007, Duvauchelle et al. 2000, Gong et al. 1999, Hemby et al. 1992, Kelly et al. 1989, Pijenburg et al. 1973, Pijenburg et al. 1976). Administration of DA antagonists either systemically or directly into the nucleus accumbens reduces drug-induced, novelty-induced, food-induced and spontaneous locomotion (Ahlenius et al. 1987, Correa et al. 2002, Correa et al. 2000, Kelly et al. 1976, Koob et al. 1978, McCullough et al. 1992). The death of DA containing neurons leads to the hypokinetic disorder Parkinson's disease and thus DA is considered particularly important in the regulation of movement (Davie 2008).

Recent research has also indicated that the neuromodulator adenosine interacts with DA in the regulation of various behavioral functions, including locomotion (Antoniou et al. 2005, Ferre et al. 1997, Fuxe et al. 2003, Fuxe et al. 2007, Hauber et al. 1998, Hauber et al. 1997, Ishiwari et al. 2007, Nagel et al. 2003, Stromber et al. 2000, Svenningsson et al. 1999, Barraco et al. 1993). A₁ and A_{2A} adenosine receptor subtypes are involved in striatal function. The A₁ receptor is distributed more widely throughout the brain while anatomical studies have shown that adenosine A_{2A} receptors have a high level of expression within both the neurostriatum and the nucleus accumbens (Svenningsson et al. 1999; Wang et al. 2000; Hettinger et al. 2001; Chen et al. 2001, Fastbom et al 1987). Physiologically, adenosine and DA receptors have been shown to overlap in their expression in these regions, and stimulation of adenosine receptors changes the binding affinities of DA receptors (Schiffman et al. 1991; Fink et al. 1992; Rosin et al. 1998; Svenningsson et al. 1999; Hillion et al. 2002; Chen et al. 2001; Fuxe et al. 2003, 2007; Mingote et al. 2008). Adenosine A₁ and DA D₁ receptors colocalize on substance P striatonigral neurons

and the A_{2A} and DA D2 receptor subtypes colocalize on the same enkephalin-positive ventral and dorsal striatopallidal neuron (Schiffman et al. 1991; Fink et al. 1992; Rosin et al. 1998; Svenningsson et al. 1999; Hillion et al. 2002; Chen et al. 2001; Fuxe et al. 2003, 2007; Mingote et al. 2008). Adenosine A_{2A} and DA D2 receptors are thought to form heteromeric complexes that influence neurotransmitter binding such that stimulation of A_{2A} receptors decreases the affinity of DA D2 receptor agonists (Ferré, 1997; Svenningsson et al. 1999; Ferré et al. 2008). In addition to heteromeric interactions, these receptors also interact through signal transduction mechanisms coupled to G-protein-coupled c-AMP second messenger systems (Ferré, 1997; Svenningsson et al. 1999; Ferré et al. 2008).

The relation between adenosine and DA has been of particular interest in recent years due to research on Parkinson's disease. Parkinson's disease is a neurological disorder associated with DA depletions in the basal ganglia, specifically the caudate putamen (Salamone et al. 1998). "Parkinsonism" is a term referring to a broad spectrum of motor disorders whose symptoms include akinesia (lack of movement), bradykinesia (slowed movement), impairments in motor control and locomotion, rigidity and tremor. Parkinsonian symptoms have been produced in rats using DA antagonists, the same drugs used as a current treatment for psychosis (Ferre 1997, Salamone et al. 1998). Animal models looking at catalepsy, rotation, tremor and locomotion have demonstrated that antagonism of adenosine A_{2A} receptors could be an effective therapy for parkinsonism (Ferré et al. 2001, Hauber et al. 2001, Ishiwari et al. 2007, Kanda et al. 1998, Matsuya et al. 2007, Pinna et al. 2005, Salamone et al. 2007, Salamone et al. 2008, Bara-Jimenez et al. 2003, Ferré et al. 2004, Simola et al. 2006, Tronci et al. 2007). Recent research has demonstrated the importance of A_{2A} receptor antagonist in the reversal of DA antagonist induced locomotor suppression and tremulous movement, which were induced by haloperidol and reversed after injections of the adenosine A_{2A} antagonist KF17837 (Correa et al. 2004, Wardas et

al. 2001). The interactions between adenosine and dopamine receptor antagonists have differential effects depending on their selectivity profile. MSX-3, an adenosine A_{2A} antagonist significantly reverses the suppression of locomotion induced by the DA D2 antagonist eticlopride; however, the adenosine A1 antagonists DCPCX and CPT were unable to reverse the locomotor impairments elicited by eticlopride (Collins et al. 2010b).

Clearly the antagonistic relationship between adenosine and DA has implications for the treatment of parkinsonism. Furthermore, it has been suggested that adenosine A_{2A} receptors could be effective at treating psychomotor slowing and anergia in depression and other disorders (El Yacoubi et al. 2003; Hanff et al. 2010, Hodgson et al. 2009, Salamone et al. 2007, 2010). A number of papers have investigated the ability of adenosine A_{2A} antagonists to reverse the locomotor suppression that results from interference of DA transmission. In one particular study, locomotor suppression induced by the DA depleting agent reserpine was reversed by the administration of the adenosine A_{2A} antagonist KW6002 (Shiozaki et al. 1999). This drug was also used to rescue locomotor suppression expressed by DA D2 receptor deficient mice (Aoyama et al. 2000). Injections of MSX-3 either systemically or directly into the nucleus accumbens core increased locomotor activity in haloperidol-treated rats (Ishiwari et al. 2007). In addition, adenosine A_{2A} antagonists have been shown to have psychomotor stimulant properties, and to reverse many of the behavioral effects of DA D2 antagonists such as decreases in operant lever pressing and effort-related choice behavior (Randall et al. 2011, Font et al. 2008, Mingote et al. 2008). Based on this preclinical research, it has been suggested that adenosine A_{2A} antagonists could be useful as antiparkinsonian drugs, antidepressants, or as a treatment for motivational or effort-related symptoms such as psychomotor retardation, anergia, apathy and fatigue, which are core symptoms of depression and other psychiatric disorders (Ferré et al. 1997; Svenningsson et al. 1999; Wardas et al. 2001; Morelli and Pinna 2001; Hodgson et al. 2009; Salamone et al.

2008a,b., El Yacoubi et al. 2003; Hanff et al. 2010, Hodgson et al. 2009, Salamone et al. 2007, 2010, Marin, 1996; Demyttenaere et al. 2005; Salamone et al. 2006; Friedman, 2009).

With the growing clinical interest in adenosine A_{2A} antagonists, developing and testing novel adenosine A_{2A} antagonists is becoming a primary research priority (Le Witt et al. 2008; Pinna 2009; Hodgson et al. 2009; Salamone, 2010a). Developing novel ligands that have useful *in vivo* characteristics is an important aspect in drug development (Santerre et al. submitted). Many drugs have low absorption properties, or low solubility and blood brain barrier penetrability which make them difficult to use clinically (Müller, 2009). Prodrugs are an effective way of ameliorating or alleviating some of these absorption issues. Prodrugs are bioreversible derivatives of a drug that is administered in its inactive form and is enzymatically transformed to its active form (Rautio et al., 2008; Müller, 2009). Prodrugs are developed by attaching an inactive promoiety to the pharmacologically active parent drug via a covalent bond that is easily altered chemically or enzymatically (Rautio et al., 2008). Ideally, when the prodrug is cleaved it will produce a high ratio of the parent drug, and the promoiety will be non-toxic (Rautio et al., 2008). When the drug and promoeity are cleaved, the pharmacologically active parent drug is released and absorbed into the system (Rautio et al., 2008). Essentially, the use of prodrugs enhances the clinical relevance of a drug without changing the pharmacological activity of the parent drug (Santerre et al. submitted). One example of an adenosine A_{2A} antagonist parent drug that has been used in prodrug development is MSX-2. MSX-2 has poor water solubility (Müller, 2009). One approach to increase water solubility is to attach a promoiety to the drug. MSX-3 was produced as a prodrug of MSX-2 (Müller, 2009). As mentioned earlier, previous studies have shown that MSX-3 can reverse the behavioral effects of DA D2 antagonists (Collins et al. 2010). Recently, a new prodrug of MSX-2 was developed (Vollmann et al. 2008). This new prodrug, MSX-4, is much more water soluble than MSX-3. MSX-4 was also found to be stable in artificial

gastric acid and readily cleaved by pig liver esterase into MSX-2 suggesting that it could be bioavailable (Vollmann et al. 2008).

Examining the behavioral profile of a novel drug is the next step in drug development. Although MSX-3 has been widely used *in vivo*, the behavioral effects of MSX-4 have not been studied. The present study focused on characterizing the behavioral actions of MSX-4 in relation to locomotion. However, current research suggests that adenosine is also related to anxiety (Correa and Font, 2008, Imaizumi et al. 1994). For example, caffeine, a commonly used stimulant, has been shown to induce anxiety in humans and animals (Greden 1974, Uhde et al. 1984, Bruce 1990). Recent research has concentrated on the role of adenosine in animal models of anxiety. Studies have found that adenosine A₁ and A_{2A} receptor subtypes are the most closely related to anxiety, and that blocking one or both of these receptors will produce an anxiogenic effect in rats (Kulkarni et al. 2007, Jain et al. 1995). Adenosine plays a parallel role in both maintaining homeostasis and modulating transmitters at the synaptic level (Cunha 2001). On its own, it has been shown that adenosine administration induces anxiolysis (i.e., anxiety-relieving) while adenosine antagonism produces anxiogenic effects (i.e., increases in anxiety-related behaviors; refs. Kulkarni et al. 2007, Jain et al. 1995). It was shown that caffeine, a non-selective adenosine A₁ and A_{2A} antagonist, decreases the time spent in the open arms of the elevated plus maze, causing a particular preference for closed sections of the maze (El Yacoubi et al. 2000). It is still unknown whether the anxiogenic effect of caffeine is A₁ or A_{2A} specific but research has indicated that these properties are due to the simultaneous blockade of A₁ - A_{2A} receptors (El Yacoubi et al. 2000, Fredholm et al. 1999).

Many of the early behavioral studies looking at the effects of adenosine on anxiety have used non-selective adenosine antagonists. Data obtained from pharmacological and genetic

research of both selective A1 receptor A_{2A} receptor antagonists have yielded contradictory results. Adenosine A1 receptor knock-out mice have normal levels of locomotion but show more anxiety than wild-type mice (Johansson et al. 2001, Gimenez-Llort et al. 2001). Research using A1 receptor antagonists show that in different studies, the same drug has anxiogenic effects or no effect. DPCPX decreased the time spend in the light zone of the dark/light test in mice, and in the elevated plus maze (Imaizumi et al. 1994, Prediger et al. 2006). However, in multiple other studies, DPCPX had no effect on the anxiety-like behaviors of mice in the elevated plus maze, dark/light box, or open field arena (El Yacoubi et al. 2000, Griebel et al. 1991, Jain et al. 1995, Prediger et al. 2006). Several limited studies of selective adenosine A1 agonists have suggested that A1 agonists seem to produce anxiolysis. The selective adenosine A1 receptor agonist CPA decreased anxiety-like behaviors in the elevated plus maze (Jain et al. 1995). CCPA, an analogue of CPA has also shown anxiolytic effects in the plus maze and the dark/light test (Prediger et al. 2004, Florio et al. 1998, Prediger et al. 2006). From this previous research it is clear those adenosine A1 antagonists do not show a clear pattern of effects on anxiety; however A1 agonists seem to be mostly anxiolytic.

Due to the concentration of A_{2A} receptors in the striatum, it has been suggested that the involvement of adenosine in anxiety is mainly due to A1 activation (Jain et al. 1995). Nonetheless, A_{2A} receptors are also present in the extended amygdala and hypothalamus which are brain regions involved in the regulation of anxiety and stress responses (Moreau et al. 1999). In addition, genetic studies in humans suggest that a polymorphism in the A_{2A} receptor gene may render susceptibility to the development of panic disorder (Deckert et al. 1997). Pharmacological data in animals, like the A1 data, does not point to a clear involvement of A_{2A} receptor in the regulation of anxiety. DMPX, an adenosine A_{2A} receptor antagonist did not have a significant effect on anxiety-like behavior in an elevated plus maze, open field, or Vogel conflict test in rats

(Jain et al. 1995, Thorsell et al. 2007). However, in genetic animal studies, A_{2A} receptor knock-out mice display more anxiety and reduced locomotion than wild-type mice (Yacoubi et al. 2000, Ledent et al. 1997). This is consistent with the knowledge that adenosine A_{2A} receptors seem to regulate propiomelanocortin (POMC) gene expression (Jegou et al. 2003). Increased POMC expression leads to hyperactivity of the pituitary-adrenocortical axis than in wild-type mice (Jegou et al. 2003). In light of the research relating adenosine to anxiety, it is important to look for side effects when testing a novel drug. Returning to the original discussion, if the adenosine A_{2A} antagonist MSX-4 induces anxiety that is too severe, it may outweigh the benefits of using MSX-4 as a potential treatment for disorders such as Parkinsonism, psychomotor slowing, and depression.

In order to study the neurochemical interactions between dopamine and adenosine as they relate to locomotion and anxiety, several behavioral assays have been developed. The typical paradigms used to measure locomotion include locomotion chambers and the open field arena which look at overall rodent movement. For anxiety-like behaviors, tests such as the open field, the elevated plus maze and the light/dark box have been most commonly used in the laboratory.. While many of these paradigms test either locomotion or anxiety, the open field arena can actually test both behaviors.

The open field arena consists of a square base that is surrounded by four tall walls (Figure 5). The base is divided into a five by five grid. A lamp emitting red light is placed above the center of the arena. The animal is placed in the middle of the arena and is allowed to move around for a ten minute session. Two experimenters observe the session and tally the number of inner and outer crossings that the animal makes. Crossings were defined as the movement of both front paws from one square to another. Outer crossings were defined as movement into one of the squares adjacent to the walls, while inner crossings were defined as movement into one of

the central squares. The total activity of the animal can be calculated by adding the number of inner crossings with the number of outer crossings.

In terms of measuring anxiety-like behavior, it is well known that rodents prefer small dark spaces as opposed to bright open ones (Montgomery 1958). In the open field, the areas near the wall, or the outer edged of the arena, are darker and more closed off than the arena in the center of the arena. Animals which explore more around the outer edged of the arena are considered to show higher anxiety-like behaviors than animals which explore in the inner part of the arena (Prut and Belzung, 2003). This model for anxiety has been validated using the benzodiazepine inverse agonist FG7142 which was shown to increase the time spent in the outer edges of the open field (Sink et al. 2010).

The present study sought to elucidate the interactions between DA D2 antagonist eticlopride and the A_{2A} adenosine antagonist MSX-4 by determining if MSX-4 could reverse eticlopride-induced locomotion suppression. Moreover, the induction of anxiety was measured by recording the relative amount of activity in the inner portion of the open field arena. To provide a cellular marker of the interaction between eticlopride and MSX-4, a second study to study the immunoreactivity of two markers of signal transduction, c-Fos and DARPP-32 (Thr34), was determined in brain sections of nucleus accumbens. It was hypothesized that MSX-4 would show similar behavioral and neurochemical characteristics as the adenosine A_{2A} antagonist MSX-3, by reversing the suppression of locomotion induced by eticlopride and attenuate eticlopride-induced increases in c-Fos and DARPP-32 expression.

IV. Materials & Methods

Animals

A total of 29 adult male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) with no prior drug experience and minimum handling were paired-housed in a colony maintained at 23°C, with a 12 hour light/dark cycle. The rats weighed between 300-390 grams at the beginning of the experiment and had ad libitum access to lab chow and water. These studies were conducted according to University of Connecticut and NIH guidelines for animal care and use.

Drugs and Dose Selection

This study used intraperitoneal (IP) injections of eticlopride (Sigma) and MSX-4 (Muller laboratory). Eticlopride (S(-)-3-chloro-5-ethyl-N-[(ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxybenzamide HCL), a D2 antagonist, was dissolved in 0.9% saline and injected at a dose of 0.08mg/kg. MSX-4 (L-valine-3-{8-[(E)-2-[3-methoxyphenyl]ethenyl]-7-methyl-1-propargylxanthine-3-yl}propyl ester hydrochloride), the A_{2A} antagonist prodrug that is converted into MSX-2 *in vivo*, was dissolved deionized H₂O, and injected at a dose of 8.0 mg/kg.

Behavioral Procedures

Open Field Maze: The open field consisted of a Plexiglas-covered black floor (115×115 cm) with red lines spaced 23 cm apart, dividing the floor into a five-by-five grid. Walls around the perimeter measured 44 cm in height (Figure 5). Testing was performed in a very dimly lit room with a single red light situated above the center of the box. The apparatus was novel to the subject at the time of testing, and each subject was tested only once. Two experimenters who were blind to the treatment condition observed the session, and tallied the number of inner and outer line crossings. Crossings were defined as movement of both front paws from one square to another. Outer crossings were defined as movement into one of the squares adjacent to the walls, while inner crossings were defined as movement into one of the central squares. Each 10-min

session was divided into two 5-min bins. Testing was conducted during the light part of the light–dark cycle.

c-Fos and pDARPP-32 (THR34) Immunohistochemistry

Animals were anesthetized with CO₂ and perfused with physiological saline followed by formalin ninety min after IP injection. The brains were stored in formalin and then were cryoprotected for two days before being sliced into 50-μm sections using a cryostat and stored in wells containing Dulbecco's phosphate buffered saline (PBS). Sections for c-Fos visualization were incubated in 0.3% hydrogen peroxide (H₂O₂) for 30 min to block endogenous staining and sections for DARPP-32 visualization were incubated in a solution of 0.1% triton-X, 5% normal donkey serum, and PBS for 30 min to block endogenous staining. c-Fos sections were incubated in the primary antibody, anti-c-Fos (1:5000, Calbiochem, Germany), and DARPP32 sections were transferred into the primary antibody anti-pDARPP32 at a concentration of 1:1000 (Santa Cruz Biotechnology, USA) at 4 °C for 24 h. Subsequently, the sections were incubated in the secondary antibody, anti-rabbit horseradish peroxidase conjugate, Envision Plus (DAKO, Denmark) for 1.5 h. The visualization of c-Fos and DARPP-32 expression was completed using the chromagen diaminobenzidine (DAB). Tissue from the different treatment groups was processed together in batches in order to control for variability in the immunohistochemical reaction. Cover-slipped slides were examined microscopically to determine and digitally capture marked cells in the regions of interest. The sections were 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 (nucleus accumbens core) were magnified at 20x and captured digitally using SPOT software. Cells that were positively labeled for c-Fos or DARPP-32 were quantified with ImageJ software (v.1.42, National Institutes of

Health sponsored image analysis program) and a macro written to automate particle counting within the region of interest. The size of the region of interest counted was 1000x1000 μ m. For each animal, cell counts were at levels that correspond to 1.70 mm through 0.70 mm relative to bregma (Paxinos and Watson, 1997) bilaterally from at least three sections, and counts were averaged across sides and sections.

Experiment 1: *Ability of MSX-4 to reverse the effects of eticlopride in the open field.*

Naïve rats (total n=29; 7-8 per group) received one of the following treatments: saline vehicle IP (30 min before testing) plus deionized water vehicle IP (40 min before testing), 0.08 mg/kg eticlopride IP (30 min before testing) plus deionized water vehicle IP (40 min before testing), 0.08 mg/kg eticlopride IP (30 min) plus 8.0 mg/kg MSX-4 IP (40 min), or saline vehicle IP (30 min) plus 8.0 mg/kg MSX-4 IP (40 min). Rats were then tested in the open field as described above, and were assessed for both total locomotor activity and relative inner activity.

Experiment 2: *Ability of MSX-4 to reverse the effects of eticlopride on c-Fos and DARPP-32 immunoreactivity in nucleus accumbens neurons.* Experimentally naïve rats (n=24; 6 per group) were randomly assigned to the following IP treatments: 0.9% saline vehicle plus deionized water vehicle, 0.08 mg/kg eticlopride plus deionized water vehicle, 0.08 mg/kg eticlopride plus 8.0 mg/kg MSX-4, or saline vehicle plus 8.0 mg/kg MSX-4. All animals were anesthetized with CO₂ and perfused 90 min after both injections with physiological saline followed by 3.7% formaldehyde; brains were removed and stored at 4 °C in formaldehyde for one day, and then were put in 0.9% sucrose.

V. Results

In experiment 1, there was an overall significant effect of drug treatment on total locomotor activity (Figure 1; $F(3,22) = 5.6$, $p < 0.005$). Planned comparisons showed that eticlopride produced a significant reduction in locomotion relative to vehicle control ($p < 0.05$). In addition, co-administration of 8.0 mg/kg MSX-4 with eticlopride produced a significant increase in locomotor activity compared to eticlopride plus vehicle ($p < 0.05$). However, there were no significant effects of drug treatment on relative inner activity (Figure 2; $F(3,22) = 0.88$, n.s.), which is used as a marker of anxiety-related effects of drugs.

The results of experiment 2 are shown in Figures 3 and 4. There was an overall effect of drug treatment on the number of c-Fos positive cells (Figure 3; $F(3,20) = 34.1$, $p < 0.001$). Planned comparisons showed that eticlopride increased c-Fos positive cell counts relative to vehicle alone ($p < 0.05$), and that the combination of MSX-4 plus eticlopride significantly reduced the number of c-Fos positive cells as compared with eticlopride alone ($p < 0.05$). There also was an overall effect of drug treatment on the number of DARPP-32 positive cells (Figure 4; $F(3,20) = 27.8$, $p < 0.001$). Planned comparisons showed that eticlopride increased DARPP-32 positive cell counts relative to vehicle alone ($p < 0.05$), and that the combination of MSX-4 plus eticlopride significantly decreased DARPP-32 positive cells as compared with eticlopride alone ($p < 0.05$).

VI. Discussion

The present study was conducted to determine the ability of the novel adenosine A_{2A} antagonist prodrug MSX-4 to reverse the locomotor suppression induced by the D2 antagonist eticlopride. This study also looked at the ability of MSX-4 to attenuate the signal transduction effects of eticlopride-induced expression of c-Fos and DARPP-32 in the nucleus accumbens. MSX-4 significantly attenuated the suppression of open field locomotion induced by eticlopride. This is consistent with previous studies showing that adenosine A_{2A} antagonists such as MSX-3, KF 17837, and KW 6002 could reverse the suppression of locomotion induced by D2 antagonists in the open field and small stabilimeter cages (Correa et al. 2004, Ishiwari et al. 2007; Salamone et al. 2008a; Collins et al. 2010, 2012). MSX-4 had no effect on locomotion when administered alone, which is consistent with previous research showing that KF 17837 did not increase locomotor activity when administered alone (Correa et al. 2004). It is possible that a higher dose of MSX-4 would have increased open field activity, or that a different test, such as a low response rate operant schedule, would be more sensitive to the stimulant effects of MSX-4 (Randall et al. 2011).

MSX-4 did not induce anxiety-like behavior in the open field. MSX-4 had no significant effect on relative inner activity in the open field, which is considered an indicator of anxiogenic behavior. Rodents that spend less time exploring the inner portion of the arena are considered to have higher levels of anxiety (Prut and Belzung, 2003). As explained at the beginning, it is important to examine the effects of adenosine A_{2A} antagonists on measures of anxiety, like the open field, because of their potential anxiogenic effects (Correa and Font, 2008). These anxiogenic side-effects would be highly undesirable if MSX-4 is used clinically. Future research should assess the effects of MSX-4 using other anxiety-related tasks such as the elevated plus maze. Nonetheless, the involvement of adenosine in anxiety is still in question. Interactions of

adenosine with other neurotransmitter systems, basal levels of adenosine, and baseline levels of anxiety-like behavior all have an effect on the anxiety-like behaviors exhibited (Correa and Font, 2008). Future animal studies looking at the effects of adenosine on anxiety should consider looking at rodent models of behavior inhibition (Qi et al. 2010) and anxiety to see if concentrations of basal adenosine differ for animals that show high and low levels of anxiety and stress. In addition, it would be interesting to determine if differing baseline levels of anxiety is reflective of expression of A₁ and A_{2A} receptors in the extended amygdala and other regions related to anxiety and stress response.

Immunohistochemistry for c-Fos and DARPP-32 were used to provide neural markers of the ability of MSX-4 to reverse the cellular actions of eticlopride. In the present study, MSX-4 reversed the increase in both c-Fos and DARPP-32 expression that was induced by the DA D2 antagonist eticlopride in the nucleus accumbens core. In previous research, DA D2 antagonists were shown to increase c-Fos immunoreactivity in striatal regions and that DA D2 antagonism activates the cAMP/DARPP-32 signal transduction cascade in medium spiny neurons in the striatum that express D2 and A_{2A} receptors (Segovia et al. 2012, 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, Bonito-Olivia et al. 2011). Other research has shown that systemic administration of A_{2A} antagonists can reverse c-Fos expression induced by eticlopride, haloperidol, and pimozide which are all DA D2 antagonists (Boegman and Vincent, 1996; Pinna et al. 1999; Betz et al. 2009; Farrar et al., 2010). Nucleus accumbens core was the area of focus for c-Fos and DARPP-32 expression as previous research has shown that nucleus accumbens core is a critical region for D2/ A_{2A} interactions in regulating locomotion and effort-related choice behavior (Ishiwari et al. 2007; Font et al. 2008; Farrar et al. 2010; Pardo et al. 2012). The immunohistochemistry results suggest that MSX-4 reversed the suppression of

locomotion induced by eticlopride due to weakening the cellular effects of D2 antagonism. This is consistent with other studies showing that D2 and A_{2A} receptors are colocalized on the same medium spiny neurons, and interact via the formation of heteromers onto the same signaling pathways (Svenningsson et al. 1999; Ferré et al. 2008; Farrar et al. 2010).

In summary, MSX-4 induced behavioral and cellular effects that match the profile of an adenosine A_{2A} antagonist. Recent research determined that MSX-4 is pharmacologically active in vivo and that its prodrug form allows it to be bioavailable. MSX-4 matches the behavioral profile of an antiparkinsonian and further suggests the use of adenosine A_{2A} antagonists as a therapeutic. Although much of the previous research on adenosine A_{2A} antagonists focuses on alleviating symptoms of parkinsonism, these drugs could also be used as treatments of motivational symptoms like psychomotor retardation, anergia, and fatigue in depression, schizophrenia, and other disorders.

VII. References

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VII. Figure Captions

Figure 1. Total locomotor activity counts measured in the open field arena. Rats received IP injections of vehicle plus vehicle (Veh/Veh), 0.08 mg/kg eticlopride plus vehicle (Etic/Veh), 0.08 mg/kg eticlopride plus 8.0 mg/kg MSX-4, or vehicle plus 8.0 mg/kg MSX-4. # eticlopride plus vehicle significantly differed from vehicle/vehicle, $p < 0.05$; * significantly different from eticlopride plus vehicle $p < 0.05$.

Figure 2. Relative inner activity measured in the open field arena ((inner counts/total counts)*100). Rats received IP injections of vehicle plus vehicle (Veh/Veh), 0.08 mg/kg eticlopride plus vehicle (Etic/Veh), 0.08 mg/kg eticlopride plus 8.0 mg/kg MSX-4, or vehicle plus 8.0 mg/kg MSX-4.

Figure 3. Results of the c-Fos immunohistochemistry. Rats received IP injections of vehicle plus vehicle (Veh/Veh), 0.08 mg/kg eticlopride plus vehicle (Etic/Veh), 0.08 mg/kg eticlopride plus 8.0 mg/kg MSX-4, or vehicle plus 8.0 mg/kg MSX-4. A. Representative schematics showing regions of interest for photomicrographs in the nucleus accumbens core (for clarity, regions are marked on only one side of the brain; Figure is modified from Paxinos and Watson, 1998). B. Photomicrographs from representative rats, showing c-Fos expression in nucleus accumbens core across each drug treatment condition. C. Quantification of number of c-Fos positive cells in the accumbens core across the different treatment conditions. # eticlopride plus vehicle significantly differed from vehicle/vehicle, $p < 0.05$; * significantly different from eticlopride plus vehicle $p < 0.05$.

Figure 4. Results of the DARPP-32 immunohistochemistry. Rats received IP injections of vehicle plus vehicle (Veh/Veh), 0.08 mg/kg eticlopride plus vehicle (Etic/Veh), 0.08 mg/kg eticlopride plus 8.0 mg/kg MSX-4, or vehicle plus 8.0 mg/kg MSX-4. A. Representative schematics showing regions of interest for photomicrographs in the nucleus accumbens core (for clarity, regions are marked on only one side of the brain; Figure is modified from Paxinos and Watson, 1998). B. Photomicrographs from representative rats, showing DARPP-32 expression in nucleus accumbens core across each drug treatment condition. C. Quantification of number of DARPP-32 positive cells in the accumbens core across the different treatment conditions. # eticlopride plus vehicle significantly differed from vehicle/vehicle, $p < 0.05$; * significantly different from eticlopride plus vehicle $p < 0.05$.

Figure 5. The open field consisted of a Plexiglas-covered black floor (115×115 cm) with red lines spaced 23 cm apart, dividing the floor into a five-by-five grid. Walls around the perimeter measured 44 cm in height. Testing was performed in a very dimly lit room with a single red light situated above the center of the box (not shown here).

VII. Figures

Figure 1. Open field total locomotor activity

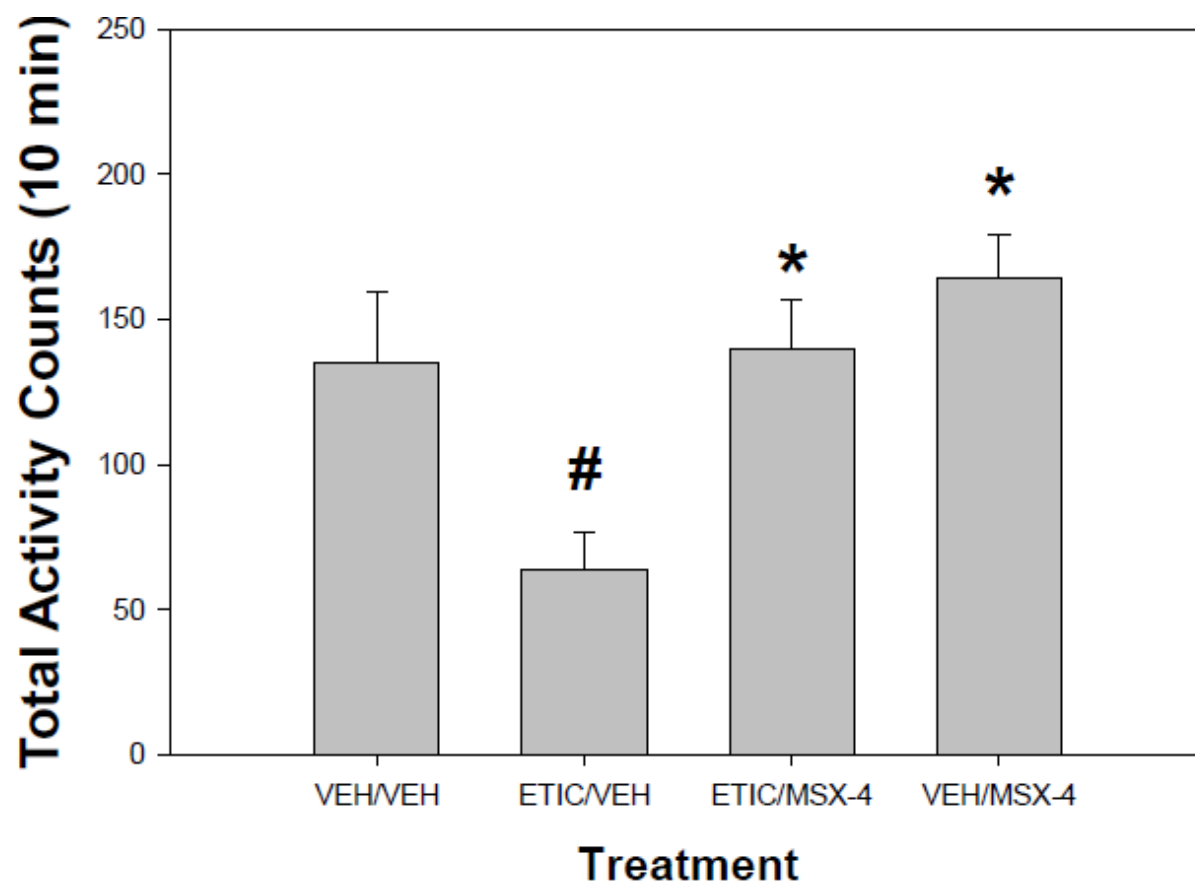


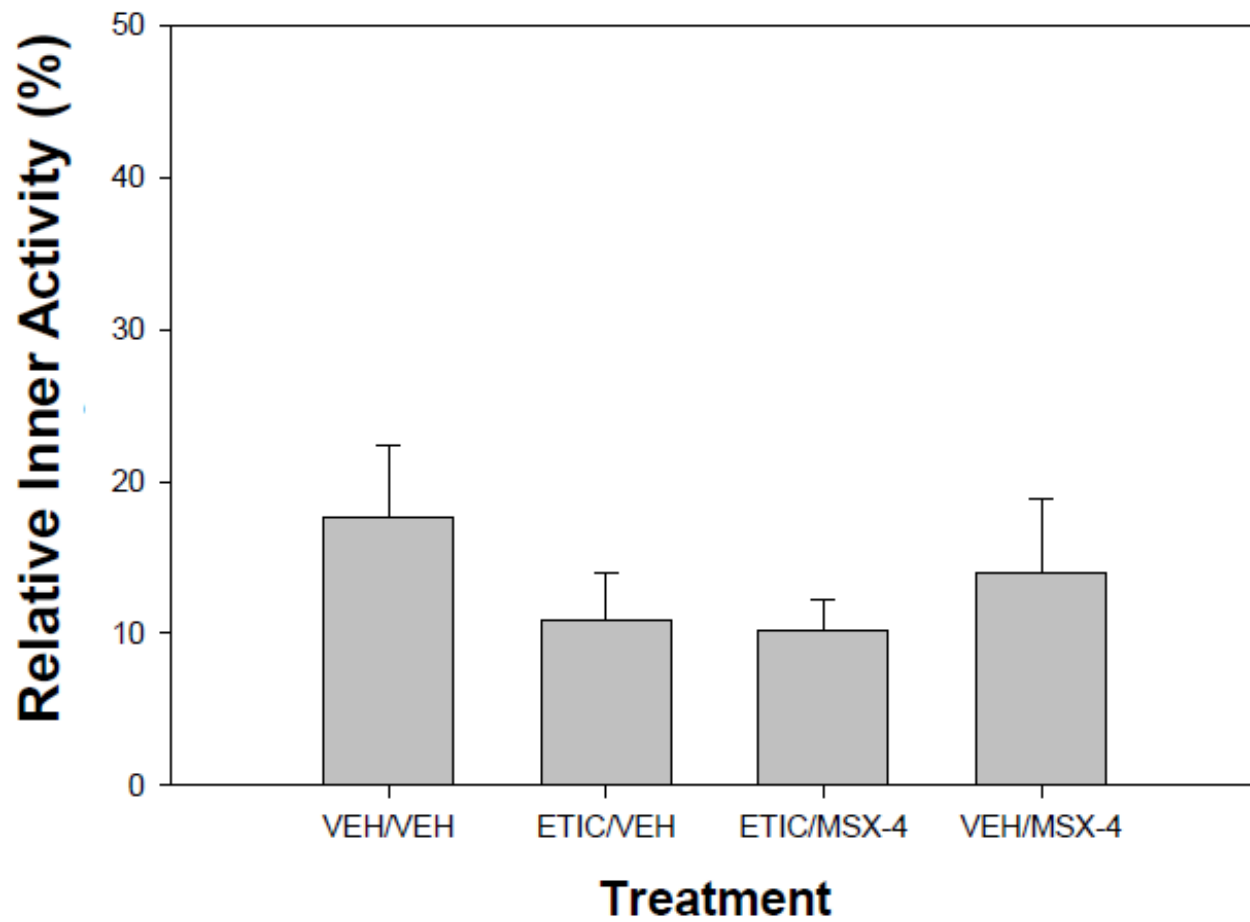
Figure 2. Open field anxiety index

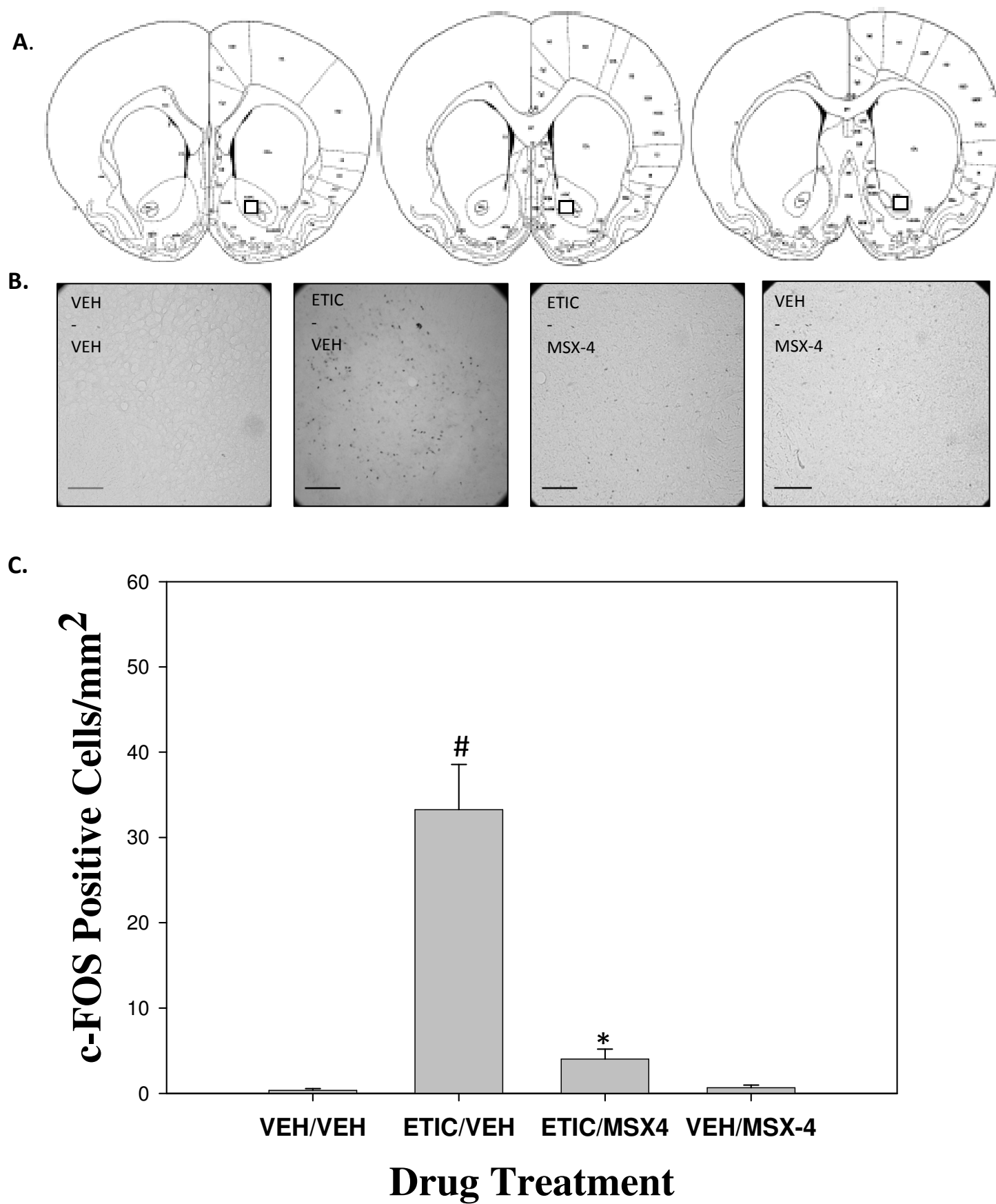
Figure 3. c-Fos immunohistochemistry

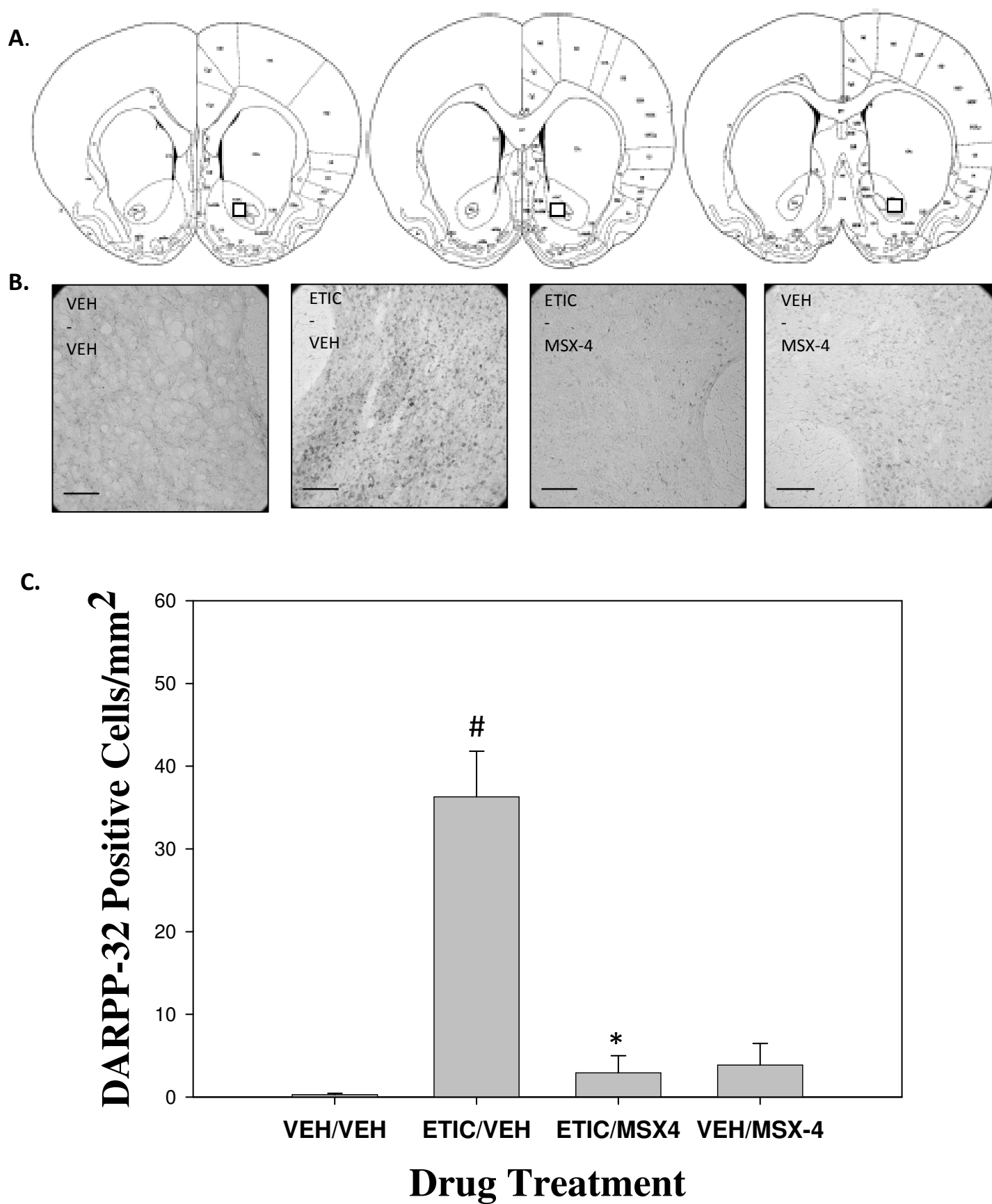
Figure 4. pDARPP32-THR34 immunohistochemistry

Figure 5.Example of the open field arena.

