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Tremulous Jaw Movements Induced by the VMAT2 Inhibitor Tetrabenazine

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Tremulous Jaw Movements Induced by the VMAT2 Inhibitor Tetrabenazine

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Abstract

Parkinsonism is a movement disorder characterized by several cardinal motor symptoms: resting tremor, akinesia, bradykinesia, rigidity, and postural instability. Parkinsonian resting tremor can be modeled in rodents using the tremulous jaw movement model. Tremulous jaw movements (TJMs) are defined as "rapid vertical deflections of the lower jaw that resemble chewing but are not directed at any particular stimulus." TJMs occur in a frequency range of 3-7 Hz and are induced by a number of pharmacological manipulations that parallel those seen in human Parkinsonism including dopamine (DA) depleting agents, DA antagonists, and cholinomimetic administration. Additionally, TJMs can be attenuated using antiparkinsonian agents including L-DOPA, DA agonists, anticholinergics, and adenosine A_{2A} antagonists. Tetrabenazine (TBZ) is a reversible VMAT2 inhibitor that is approved by the FDA for treatment of chorea associated with Huntington's disease (HD). While TBZ inhibits storage of all monoamines into synaptic vesicles it has been shown to preferentially target DA. Therefore, patients being treated with TBZ often experience depression and Parkinsonism as side effects. The present studies demonstrate the ability of TBZ to induce Parkinsonian tremor using the tremulous jaw movement model. When administered to rats, tetrabenazine (0.25, 0.5, 1.0, 2.0 mg/kg IP) significantly induces tremulous jaw movements in a dose-dependent manner. Freeze frame video analysis reveals these TJMs primarily occur in the 3.0-7.5 Hz frequency range, which falls in the frequency range characteristic of parkinsonian resting tremor. Coadministration of the adenosine A_{2A} antagonist MSX-3 (1.25, 2.5, 5.0, 10.0 mg/kg IP) significantly attenuates TJMs induced by 2.0 mg/kg TBZ in rats. Similarly, coadministration of MSX-3 (2.5, 5.0, 10.0 mg/kg

IP) significantly reduces the number of TJMs induced by 10.0 mg/kg TBZ in mice. To provide a cellular marker of these pharmacological conditions, we examined c-Fos expression in the ventrolateral neostriatum (VLS), the region of the brain most closely associated with the production of TJMs. 2.0 mg/kg TBZ significantly increased the number of c-Fos positive cells in the VLS, while coadministration of 10.0 mg/kg MSX-3 significantly reduced the number of c-Fos positive cells. Taken together, the results indicate that TBZ induces tremor as measured in the tremulous jaw movement model. MSX-3 is capable of attenuating this behavior by blunting the cellular effects caused by TBZ administration, thus lending further support to the use of adenosine A_{2A} antagonists as antiparkinsonian agents.

Chapter 1: General Introduction

Parkinson's Disease and Parkinsonism

Parkinson's Disease (PD) currently affects between 1 and 2 million people in the United States (Ostrem et al., 2010). With several million cases worldwide, it is the second most common neurodegenerative disorder, after Alzheimer's disease (Nussbaum et al., 2003). Age is considered the strongest risk factor for PD, as it affects 1-2% of the population older than 65 years, and over 3% of the population older than 85 years. Given the current aging of the population, the prevalence of PD is expected to increase dramatically over the next decade (Ostrem et al., 2010; Shulman et al., 2011). PD is usually diagnosed between ages 70 and 80 and progresses chronically and slowly until death, on average 15 years after initial diagnosis (Shulman et al., 2011). Upon postmortem examination of brains of PD patients, researchers have identified the

presence of Lewy bodies (ubiquinated protein deposits in the cytoplasm) and Lewy neurites (proteinaceous inclusions within neurites), both of which contain aggregates of the protein α-synuclein (Nussbaum 2003). However, the neuropathological hallmark of PD is the death of dopamine (DA) producing cell bodies in the substantia nigra *pars compacta*, which causes the degeneration of nigrostriatal DA neurons (Hornykiwicz, 1973). In idiopathic PD, this decrease in nigrostriatal input leads to a net increase in inhibitory output from the globus pallidus interna (i.e., medial globus pallidus) and the substantia nigra pars reticulata, ultimately affecting the function of the motor cortex and brainstem motor areas.

Idiopathic PD, however, is just one member of a broader family of movement disorders known as Parkinsonism. In addition to PD, Parkinsonism includes encephalitic, pugilistic, and drug-induced Parkinsonism. Drug-induced Parkinsonism is the second most common cause of Parkinsonism (Alvarez et al. 2007); it can result from administration of pharmacological agents that interfere with DA transmission such as DA antagonists (e.g. haloperidol or pimozide) and DA depleting agents (e.g. reserpine) (Marsden et al., 1975; McEvoy, 1983; Arbaizar et al., 2008). Additionally, several clinical studies have shown that cholinomimetic administration can induce or exacerbate Parkinsonian motor symptoms, including tremor, in humans (Iwasaki et al. 1988; Ott and Lannon, 1992; Kao et al., 1993; Keltner, 1994; Aarsland et al. 2003) Parkinsonism is characterized by several cardinal motor symptoms: resting tremor (3-7 Hz), bradykinesia (slowed movement), akinesia (lack of initiation of spontaneous movement), rigidity (increased muscular tone), and postural instability (Marsden et al., 1975; Findley, 1988; Bergman et al,. 2002, Ostrem et al., 2010; Shulman et al., 2011) Parkinsonian resting

tremor, defined as a "rhythmic, oscillatory, involuntary movement," (Ostrem et al., 2010) is the most common hyperkinetic movement associated with parkinsonism, and occurs in a frequency range of 3-7 Hz, which is distinct from dyskinesias (1-2 Hz), essential tremor (8 Hz), and postural tremors (8-12 Hz) (Findley et al., 1981, Marsden, 1984; Deuschl et al., 1996, 2000). Parkinsonian resting tremor most frequently presents unilaterally in the distal upper extremities as a "pill rolling" movement (Ostrem et al., 2010), but usually spreads bilaterally affecting both the upper and lower limbs, facial muscles, and the jaw, a condition known as "rabbiting" (Weiss et al. 1980; Salamone et al., 1998; Deuschl et al., 2000).

Tremulous Jaw Movements: An Animal Model of Parkinsonian Resting Tremor

Animal models are frequently used to gain insight into the neural circuitry underlying human disorders, and to investigate potential treatments. Several rodent tests are used to assess motor functions related to akinesia/bradykinesia, including locomotor activity and catalepsy, but historically, there for many years there was little focus upon models of tremor. Currently, the tremulous jaw movement model is the most widely used model for studying parkinsonian tremor in rats. Tremulous jaw movements (TJMs) are defined as "vertical deflections of the lower jaw that resemble chewing but are not directed at any particular stimulus" (Salamone et al., 1998, 2005). In rats, the ventrolateral neostriatum (VLS) is the critical striatal region for producing TJMs. This area has been shown to be responsible for orofacial movements and forepaw motor control (Salamone et al., 1990; Jicha and Salamone, 1991; Salamone et al., 1993). Local infusion of the neurotoxin 6-hydroxydopamine (6-OHDA) into the VLS induced TJMs,

while infusion into other striatal areas including the anteroventromedial striatum, dorsolateral striatum, and nucleus accumbens did not (Finn et al., 1998; Jicha and Salamone, 1991). TJMs can also be induced in rodents by administration of pharmacological agents that reduce DA transmission, such as DA-depleting agents (e.g. reserpine) and DA antagonists (e.g. pimozide and haloperidol) (Baskin and Salamone, 1993; Steinpreis et al., 1993; Salamone and Baskin, 1996; Correa et al., 2004; Ishiwari et al., 2005; Salamone et al., 2008; Betz et al., 2009). While reductions in DA transmission are strongly associated with the generation of parkinsonian tremor, acetylcholine (ACh) has also been implicated in TJMs. Muscarinic agonists (i.e. pilocarpine, arecoline) and anticholinesterases (i.e. tacrine, galantamine, physostigmine) increased the number of TJMs in a dose-dependent manner (Salamone et al. 1986; Baskin et al., 1994; Mayorga et al., 1997; Collins et al., 2010a; Collins et al., 2011). Anticholinesterases are the primary treatment for Alzheimer's disease, and therefore patients treated with these drugs may also present with parkinsonian motor side effects, including tremor. Through the use of freeze frame video analysis, researchers have been able to characterize the temporal dynamics of TJMs. This procedure has been extensively employed, and it has been reported that the peak local frequency of TJMs is 3-7 Hz, which corresponds to the frequency of parkinsonian tremor in humans (Salamone et al. 1996, 1998; Finn et al., 1997; Mayora et al., 1997; Cousins et al., 1998; Ishiwari et al., 2005; Collins et al., 2011). EMG recordings from the temporalis muscle in rats show consistent rhythmic bursts of EMG activity in the 3-5 Hz frequency range during bursts TJMs, again consistent with the frequency range of Parkinsonian resting tremor (Cousins et al., 1998; Collins et al., 2011). Furthermore, TJMs in rats can be attenuated using antiparkinsonian agents

including L-DOPA (Cousins and Salamone, 1996; Cousins et al. 1997) DA agonists (Baskin and Salamone, 1993; Cousins et al., 1997; Salamone et al., 2005) amantadine (Cousins et al., 1997), muscarinic antagonists (Steinpreis et al., 1993; Cousins et al., 1997; Mayorga et al., 1997; Betz et al., 2009) and adenosine A_{2A} antagonists (Correa et al. 2004; Salamone et al., 2008; Betz et al., 2009; Collins et al., 2010a, 2011, 2012; Santerre et al., 2012). Thus, tremulous jaw movements meet a reasonable set of criteria for use as a valid animal model of parkinsonian tremor (Salamone et al., 1998; Cenci et al., 2002; Collins-Praino et al. 2011).

More recently, researchers have extended the TJM model to mice, thereby providing a model for examining the effects of various genetic manipulations on parkinsonian tremor. Similarly to rats, TJMs are induced in mice after administration of DA-depleting agents (Lopez-Cruz et al., in prep), muscarinic agonists (Salamone et al., 2012) and anticholinesterases (Podurgiel et al., in prep, see appendix 1), and also have been observed to occur primarily in bursts with a peak frequency of 3-7 Hz (Salamone et al., 2012). Recent research using this model has shown that the adenosine A_{2A} antagonist MSX-3 is capable of attenuating TJMs induced by the muscarinic agonist pilocarpine (Salamone et al., 2012), and the anticholinesterase galantamine (Podurgiel et al., in prep, see appendix 2). Furthermore, adenosine A_{2A} receptor knockout (KO) mice show a reduction in TJMs after administration of pilocarpine (Salamone et al., 2012) compared to wild type (wt) controls. Thus, extending the TJM model to mice has given researchers means to investigate a new dimension of Parkinsonism and tremorogenesis, and future studies should continue to validate and utilize this model.

Tetrabenazine as a Tremorogenic Agent

Tetrabenazine (TBZ) is a monoamine-depleting agent that was first introduced as an antipsychotic drug in the 1960's (Quinn et al., 1959). Pharmacologically, TBZ reversibly inhibits vesicular monoamine transporter 2 (VMAT2), which is expressed in the brain and responsible for transporting catecholamines from the cytoplasm into synaptic vesicles. TBZ has little effect on 5-HT transport, but more selectively depletes storage of DA and norepinephrine (NE), and has the highest binding density in DA-rich areas of the brain including the caudate, putamen, and nucleus accumbens (Pettibone, 1984a, 1984b; Thibaut, 1995). TBZ has a high affinity for VMAT2, but not VMAT1, which is found in the periphery as well as the brain (Fasano, 2009; Guay, 2010). In addition to inhibiting VMAT2, TBZ blocks DA D2 receptors, but with a much lower affinity (Reches et al., 1983). Reserpine and TBZ differ in their mechanism of action; reserpine is an irreversible and nonselective inhibitor of both VMAT1 and VMAT2 inhibitor, while TBZ is reversible in its selective actions as an inhibitor of VMAT2. Additionally, reserpine is more potent than TBZ, it depletes monoamine stores to a greater extent, and has a longer recovery of monoamine stores compared to TBZ (Guay, 2010).

In May of 2008, tetrabenazine was approved by the Food and Drug Administration (FDA) for treatment of chorea associated with Huntington's disease (HD) (de Tommaso et al., 2011). HD is a neurodegenerative disorder caused by an abnormally expanded CAG region on exon 1 of the HTT gene that affects approximately 30,000 patients in the Unites States (Poon et al., 2010). HD patients display motor abnormalities, cognitive impairments, and various degrees of psychosis (Iversen et al., 2009). Chorea

refers to abnormal, involuntary movement and is the most common of the motor abnormalities associated with HD (Iversen et al., 2009). Research on human HD patients has revealed that TBZ is effective in treating chorea in both the short and long-term (Kenney et al. 2007a, 2007b; Frank 2009; Poon et al. 2010; de Tommaso et al. 2011; Chen et al. 2012). Frank (2009) revealed that after 80 weeks of TBZ treatment, chorea significantly improved from baseline with a mean reduction in the total maximal chorea (TMC) score of 4.6 units in 45 HD patients. TBZ has also been shown to be effective at reducing chorea within hours after a single dose. Kenney et al. (2007) observed a 42.4% average decrease in the Unified Huntington's Disease Rating Scale (UHDRS) chorea score in 10 HD patients who were monitored every 2 hours after TBZ administration. In animal studies, TBZ treatment has been shown to alleviate motor deficits and reduce striatal cell loss in the YAC128 mouse model of HD (Wang et al., 2010).

While TBZ does show promise for reducing chorea in HD patients, there are also adverse events (AEs) associated with TBZ treatment. The most common AEs reported in human studies include drowsiness, Parkinsonism, and depression (Kenney et al., 2007; Frank, 2009). While Frank (2009) reported a significant average decrease in chorea after long-term TBZ treatment, the mean parkinsonism score increased 2.1 UHDRS units in 45 patients assessed between baseline and week 80. Given the fact that TBZ depletes DA, it is not surprising that Parkinsonism is induced. Research on Parkinsonism could therefore utilize TBZ in animal models. More specifically, TBZ could be used as an agent for inducing parkinsonian tremor in the jaw movement model, and provide a means for assessing possible therapeutic treatments for Parkinsonian tremor.

Adenosine A_{2A} Antagonists: Treatment for Parkinson's Disease

In recent years, researchers have examined the role of adenosine in basal ganglia circuitry and its relevance to Parkinsonism. Adenosine is an endogenous purine neuromodulator that has shown to be involved in the regulation of sleep, arousal, neuroprotection, epilsepy, and the brain's response to ethanol and opiates (Iversen et al., 2009). Adenosine receptors are linked to g-proteins, and four subtypes are present in the body: A_1, A_{2A}, A_{2B}, and A_3. A_1 and A_{2A} are the most common subtypes in the brain, and have a higher affinity for adenosine than the A_{2B}, and A_3 subtypes (Ferre et al., 1997; Iversen et al., 2009) A_1 receptors are widely distributed throughout the brain, and are linked to the g-proteins G_i/G_o (Cieslak et al. 2008; Iversen et al., 2009). Therefore, activation of A_1 receptors inhibits adenylate cyclase. In the striatum, A_1 receptors are colocalized with DA D1 receptors on substance-P containing "direct pathway" medium spiny neurons (Ferre et al., 1997, 2001; Cieslak et al., 2008). Because D1 receptors are linked to the g-protein G_s, stimulation of these receptors induces actions on the cAMP-associated signal transduction pathway that are opposite to those produced by A_1 stimulation. A_{2A} receptors are very densely concentrated in the striatum, where they are colocalized with DA D2 receptors on enkephalin-postive "indirect pathway" medium spiny neurons (Fink et al., 1992; Ferre et al., 1997, 2001; Cieslak et al., 2008). These DA D2 receptors and adenosine A_{2A} receptors have been shown to form heterodimers (Fuxe et al., 2003). Additionally, research has shown that these receptors converge on the same cAMP-associated signal transduction mechanism and exert opposing effects. DA D2 receptors are linked to the g-protein G_i and inhibit adenylate cyclase, while A_{2A} receptors are linked to the g-proteins G_s/G_{olf} and activate adenylate cyclase (Ferre et al., 1997;

2001, 2008; Iversen et al., 2009). It is this interaction that has led researchers to investigate the use of adenosine A_{2A} antagonists as possible anti-parkinsonian agents.

There is a large body of research showing that DA and adenosine interact in an antagonistic manner throughout the striatal complex, in both neostriatum and nucleus accumbens. This interaction is mostly due to the selective interactions between specific receptor subtypes, namely the D2 and A_{2A} subtypes on enkephalin-positive neurons, and D1 and A_1 subtypes on substance P-positive neurons (Ferre et al., 1997, 2001). Behaviorally, adenosine antagonists produce many of the same motor effects as DA agonists, and adenosine agonists induce many of the same motor effects as DA antagonists (Ferre et al., 2001; Font et al., 2008; Mingote et al., 2008; Randall et al., 2011). Adenosine antagonists increase motor activities, and can be counteracted by DA depletion or pharmacological antagonism of DA receptors (Ferre et al., 2001). Adenosine A_{2A} antagonists reverse behavioral deficits induced by D2 antagonists, and those induced by D1 antagonists, albeit to a lesser degree (Hauber et al., 2001; Worden et al., 2009; Nunes et al., 2010). Behavioral research has examined the anti-parkinsonian effects of A_{2A} antagonists using tests of motor function that model the cardinal motor symptoms of Parkinsonism. Hauber et al. (1998) showed intracerebral infusion of the adenosine A_{2A} antagonist MSX-3 into the striatum increases locomotion and is capable of attenuating catalepsy induced by systemic injection of the D1 antagonist SCH23390 or the D2 antagonist raclopride. Follow up research revealed that systemic administration of the adenosine A_{2A} antagonist CSC reversed catalepsy induced by systemic administration of the D2 antagonist raclopride, and catalepsy induced by microinfusion of the D2 antagonist sulpiride into the striatum is attenuated by co-microinfusion of MSX-3

(Hauber et al., 2001). MSX-3 has also been shown to reverse locomotor suppression induced by the D1 antagonist SCH 39166, and the D2 antagonists eticlopride and haloperidol (Ishiwari et al., 2007; Collins et al., 2010). KF17837, another A_{2A} antagonist, has been to attenuate catalepsy induced by haloperidol and resperine (Kanda et al., 1994), as well as locomotor suppression and the induction of TJMs induced by haloperidol (Correa et al., 2004). Other A_{2A} antagonists, SCH58261 and ST1535, were found to attenuate impairments in sensorimotor integration and initiation of movement seen in rats with a unilateral 6-OHDA lesion (Pinna et al., 2007). A_{2A} receptor knockout mice display a reduction in catalepsy (Chen et al. 2001) and significantly fewer pilocarpine-induced tremulous jaw movements (Salamone et al., 2012) compared to wild-type mice. A_{2A} antagonists have also been shown to attenuate TJMs induced by DA antagonists, DA depleting agents, muscarinic agonists, and anticholinesterases (Correa et al. 2004; Salamone et al., 2008; Betz et al., 2009; Collins et al., 2010, 2011, 2012; Santerre et al., 2012). Salamone et al. (2008) have shown that the adenosine A_{2A} antagonists MSX-3 and KW6002 are capable of attenuating catalepsy, locomotion suppression, and TJMs induced by reserpine or DA antagonism, and thus display antiparkinsonian characteristics. In 2005, Istradefylline (KW 6002) became the first A_{2A} antagonist to undergo clinical trials for treatment of humans with PD, and continued research has shown that A_{2A} antagonism reduces PD symptoms in humans (LeWit et al., 2008).

Present Work

The present experiments sought to establish a rodent model of parkinsonian tremor using the VMAT2 inhibitor TBZ. The first experiment examined the effect of

various doses of TBZ on the induction of TJMs. Experiment 2 investigated the local frequency characteristics of the TJMs induced by the peak dose of TBZ from experiment 1. This experiment was done to determine if the TJMs occur in the 3-7 Hz frequency range, and thus could serve as a model of human Parkinsonian resting tremor and not another form of tremor that occurs at a different frequency range. Experiments 3 and 4 evaluated the ability of the adenosine A_{2A} antagonist MSX-3 to reverse TJMs induced by TBZ in rats and mice. Since adenosine A_{2A} antagonists have been shown to be antiparkinsonian agents, it was hypothesized that MSX-3 should be able to attenuate TBZ-induced TJMs. Finally, experiment 5 examined the ability of MSX-3 to reverse the effects of TBZ on c-Fos in VLS, thus providing evidence at the cellular level that MSX-3 is reversing the effects of TBZ due to the convergence of D2 and A_{2A} receptors on the c-AMP associated signal transduction pathway. The VLS was chosen as the region of interest for this study because it is the neostriatal region most closely associated with the induction of TJM activity (Salamone et al., 1990, 1998, 2008; Jicha and Salamone 1991; Finn et al., 1998; Collins-Praino et al., 2011), and has been used previously in studies of c-Fos immunoreactivity associated with TJMs (Betz et al., 2009). The VLS is thought to be the rodent homologue of the primate putamen, which is somatotopically organized in a dorsal-ventral gradient (McGeorge and Faull, 1989). The ventral portion of the primate putamen is responsible for orofacial movement and forelimb motor control (Alexander and Delong, 1985), and the rodent VLS, which receives direct cortical input from head areas of motor cortex (McGeorge and Faull, 1989), also is important for orofacial motor function (Jicha and Salamone, 1991; Salamone et al., 1993).

Chapter 2: Tremulous Jaw Movements Induced by the VMAT2 Inhibitor Tetrabenazine

2.1 Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disorder, after Alzheimer's disease (Nussbaum et al., 2003). Today, 1-2 million people in the United States suffer from idiopathic PD, which is caused by the death of dopamine (DA) producing cell bodies in the substantia nigra pars compacta and subsequent degeneration of nigrostriatal dopamine neurons (Ostrem et al, 2010). Idiopathic PD is just one member of a broader family of movement disorders known as Parkinsonism, the members of which share several cardinal motor symptoms: resting tremor, akinesia, bradykinesia, rigidity, and postural instability. Certain classes of drugs also have been shown to induce Parkinsonism in humans, including DA antagonists (i.e. "typical" antipsychotics, like haloperidol and pimozide) and DA depleting agents (i.e. reserpine and tetrabenazine) (Marsden et al., 1975; McEvoy, 1983; Arbaizar et al., 2008). Cholinomimetics, such as the anticholinesterases used to treat Alzheimer's disease, induce or exacerbate Parkinsonian tremor (Iwasaki et al. 1988; Ott and Lannon, 1992; Kao et al., 1993; Keltner, 1994; Aarsland et al., 2003).

Tetrabenazine (TBZ) is a reversible VMAT2 inhibitor approved by the FDA for treatment of chorea associated with Huntington's Disease (HD). TBZ depletes storage of monoamines, but more selectively targets DA and exhibits the highest binding density in DA-rich areas of the brain including the caudate, putamen, and nucleus accumbens (Pettibone, 1984a, 1984b; Thibaut, 1995). Human research has indicated that TBZ is

effective at treating HD-associated chorea in both the short and long-term, but adverse effects (AEs) often occur (Kenney et al. 2007a, 2007b; Frank, 2009), including drowsiness, depression, and Parkinsonism (Kenney et al. 2007a; Frank, 2009). Since it is known that TBZ depletes DA storage and thereby reduces DA transmission, it is not surprising that Parkinsonism results from TBZ administration. Research on Parkinsonism could thus employ TBZ in animal models.

Parkinsonian resting tremor can be modeled in rodents using the tremulous jaw movement model. TJMs are defined as "rapid vertical deflections of the lower jaw that resemble chewing but are not directed at any particular stimulus" (Salamone et al. 1998). In rodents, TJMs can be induced by the same pharmacological agents that induce Parkinsonian resting tremor in humans, including DA depletion, DA antagonism, and cholinomimetic administration (Baskin and Salamone, 1993; Steinpreis et al., 1993; Baskin et al., 1994; Salamone and Baskin, 1996; Mayorga et al. 1997; Salamone et al., 2008; Betz et al., 2009; Collins et al. 2010a, 2011). Additionally, TJMs have been shown to occur in the 3-7 Hz frequency range, which is the same frequency range as parkinsonian resting tremor (Salamone et al. 1996, 1998; Finn et al., 1997; Mayora et al., 1997; Cousins et al., 1998; Ishiwari et al., 2005; Collins et al., 2011). Furthermore, TJMs are attenuated with anti-parkinsonian drugs including L-Dopa, DA agonists, amantadine, muscarinic antagonists, and adenosine A_{2A} antagonists (Baskin and Salamone, 1993; Steinpreis et al., 1993; Cousins and Salamone, 1996; Cousins et al., 1997; Mayorga et al., 1997; Correa et al., 2004; Salamone et al., 2005, 2008; Betz et al., 2009; Collins et al., 2010a, 2011, 2012; Santerre et al., 2012).

Over the past few years, a large body of research has aimed at identifying the antiparkinsonian properties of adenosine A_{2A} antagonists. Adenosine A_{2A} receptors are highly concentrated in the striatal complex, including the nucleus accumbens and neostriatum, and are colocalized with DA D2 receptors on enkephalin-positive "indirect pathway" striatopallidal medium spiny neurons. Research has shown that these receptor subtypes converge onto the same signal transduction pathway, and are capable of forming heterodimers (Ferre et al., 1997, 2001; Hauber et al., 2001; Fuxe et al., 2003). Since D2 receptors are linked to the g-protein G_i, they inhibit adenylate cyclase, while adenosine A_{2A} receptors are linked to the g-protein G_s/G_{olf} and activate adenylate cyclase. It is this interaction that has lead researchers to investigate adenosine A_{2A} antagonists as possible antiparkinsonian agents. Animal studies have revealed that A_{2A} antagonists are capable of attenuating catalepsy, locomotion suppression, and also suppress the tremulous jaw movements induced by various pro-parkinsonian agents (Kanda et al., 1994; Hauber et al., 2001; Ishiwari et al., 2007; Pinna et al., 2007; Salamone et al., 2008; Collins et al., 2010a). For example, the adenosine A_{2A} antagonist KF17837 counteracted locomotor suppression and significantly reduced tremulous jaw movements induced by the D2 antagonist haloperidol (Correa et al. 2004). In another study, KF17837 attenuated catalepsy induced by haloperidol and the DA-depleting agent reserpine (Kanda et al. 1994). Another adenosine A_{2A} antagonist, MSX-3, has been shown to have antiparkinsonian properties. MSX-3 is a water-soluble phosphate prodrug that is cleaved in vivo by phosphatases into the physiologically active compound MSX-2 (Hockemeyer et al., 2004). MSX-3 has been shown to attenuate catalepsy, locomotion suppression, and the induction of tremulous jaw movements induced by DA antagonism and the DA

depleting agent reserpine (Salamone et al. 2008). Furthermore, MSX-3 significantly reduced TJMs induced by the muscarinic agonist pilocarpine (Collins et al., 2010a; Salamone et al., 2012), as well as the anticholinesterase galantamine (Collins et al., 2012).

Immediate early gene (IEG) protein products, such as c-Fos, can be used to provide a cellular marker neuronal activity. IEGs encode neural proteins involved in signal transduction and are thought to be related to neuronal activity in the CNS (Sagar et al., 1988). Betz et al. (2009) showed an increase in c-Fos expression in the VLS after administration of the DA antagonist pimozide. Additionally, the A_{2A} antagonist KW 6002 significantly attenuated pimozide-induced c-Fos expression. Recently, Santerre et al. (2012) showed that another A_{2A} antagonist, MSX-4, was capable of significantly reducing c-Fos expression in the nucleus accumbens that was induced by the DA D2 antagonist eticlopride. These studies provide further evidence that A_{2A} antagonists blunt the cellular actions of DA D2 antagonists by exerting opposing actions on the same c-AMP related signal transduction pathway.

The present experiments sought to establish an animal model of parkinsonian tremor using the VMAT2 inhibitor TBZ. Experiment 1 examined the effect of various doses of TBZ (0.25, 0.5, 1.0, 2.0 mg/kg IP) on the induction of TJMs. Experiment 2 analyzed the frequency characteristics of the TJMs induced by the peak dose of TBZ (2.0 mg/kg) from experiment 1, to ensure that the TJMs fall into the 3-7 Hz frequency range that is associated with human parkinsonian resting tremor. Experiment 3 investigated the ability of the adenosine A_{2A} antagonist MSX-3 (1.25, 2.5, 5.0, 10.0 mg/kg) to reverse TJMs induced by the peak dose of TBZ (2.0 mg/kg) from experiment 1. Similarly, in

experiment 4 the ability of MSX-3 (2.5, 5.0, 10.0 mg/kg) to attenuate TBZ-induced TJMs was investigated in mice. Finally, experiment 5 sought to provide a cellular marker of TBZ-induced TJMs by examining c-Fos expression in the ventrolateral neostriatum (VLS) after TBZ administration (2.0 mg/kg) and with co-administration of TBZ with MSX-3 (10.0 mg/kg). Together, this group of experiments sought to establish an animal model of Parkinsonian resting tremor that future researchers can employ as a means for studying the neural circuitry involved in tremorogenesis, and as a platform for evaluating possible therapeutic agents.

2.2 Materials and Methods

Animals

A total of 38 adult male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) with no prior drug experience were used in the present experiments. The rats weighed 350-450g during the course of the experiment and had ad libitum access to lab chow and water. They were pair-housed in a colony that was maintained at approximately 23°C and had a 12-hour light/dark cycle (lights on at 0700 hrs). These studies were conducted according to University of Connecticut and NIH guidelines for animal care and use.

A total of 10 male CD1 mice (Harlan Laboratories, Indianapolis, IN) with no prior drug experience also were used in the present experiments. The mice weighed 35-45 g during the course of the experiment and had ad libitum access to lab chow and water. They were group-housed in a colony that was maintained at approximately 23°C and had a 12-hour light/dark cycle (lights on at 0700 hrs). These studies were conducted according to University of Connecticut and NIH guidelines for animal care and use.

Behavioral Procedures

Tremulous Jaw Movements: Rats. Observations of rats took place in a 30 × 30 × 30 cm clear Plexiglas chamber with a wire mesh floor, which was elevated 42 cm from the table top. This allowed for the viewing of the animal from several angles, including underneath. Tremulous jaw movements were defined as rapid vertical deflections of the lower jaw that resembled chewing but were not directed at any particular stimulus (Salamone et al. 1998). Each individual deflection of the jaw was recorded using a mechanical hand counter by a trained observer, who was blind to the experimental condition of the rat being observed. Separate studies with two observers demonstrated an inter-rater reliability of $r = 0.98$ ($p < 0.001$) using these methods.

Tremulous Jaw Movements: Mice. Observations of mice took place in a 11.5 × 9.5 × 7.5 cm clear glass chamber with a wire mesh floor, which was elevated 26 cm from the table top. As with rats, tremulous jaw movements were defined as rapid vertical deflections of the lower jaw that resembled chewing but were not directed at any particular stimulus (Salamone et al. 1998). Each individual deflection of the jaw was recorded using a mechanical hand counter by a trained observer, who was blind to the experimental condition of the mouse being observed. Separate studies with two observers demonstrated an inter-rater reliability of $r = 0.98$ ($p < 0.001$) using these methods.

Freeze Frame Analysis of Tremulous Jaw Movements: Rats were placed in a clear Plexiglas tube (9 cm in diameter) to maintain their position so that a constant view of the orofacial region could be seen for recording. Each rat was recorded using a (Cannon

PowerShot SX120 digital camera, in video mode) focused on the orofacial region for three 5-minute epochs in order to capture as many tremulous jaw movements as possible. The sections of these video files that allowed for clear observation of the orofacial area were then subjected to a freeze-frame analysis (1 frame = 1/30 s), in which the observer went frame-by-frame through each burst of jaw movements (i.e. each group of at least two jaw movements that were within 1.0 seconds of each other). The observer recorded the inter-movement interval for each pair of jaw movements within bursts, which was defined as the number of frames between each point at which the jaw was fully open during successive jaw movements. This information was used to determine the local frequency within bursts of jaw movements (i.e., the local frequency is the reciprocal of the intermovement time).

c-FOS visualization and quantification: Free floating coronal sections (50µm) were serially cut using a cryostat (Weymouth, MA, USA) and rinsed in 0.01M PBS (pH 7.4). Sections were incubated in 0.3% hydrogen peroxide (H_20_2) for 30 min to block endogenous staining. Sections were then rinsed in PBS (3 x for 5 min) and transferred into the primary antibody, anti- c-Fos at a concentration of 1:5000 (Calbiochem, Germany) for 48 h incubation. Following the primary antibody treatment, sections were rinsed in PBS (3 x for 5 min) and incubated in the secondary antibody, anti-rabbit HRP conjugate, envision plus (DAKO, Carpinteria, CA, USA) 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 (the ventrolateral neostriatum or VLS) were magnified at 20x and captured digitally using SPOT software. Cells that were positively labeled for c-Fos 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 1000x1000μm. For each animal, cell counts were taken bilaterally from at least three sections, and counts were averaged across sides and sections.

Drugs and Selection of Doses

Tetrabenazine (9,10-dimethoxy-3-(2-methylpropyl)-1,3,4,6,7, 11b hexahydrobenzo[a]quinolizin-2-one), the VMAT2 inhibitor, was purchased from Tocris Bioscience (Bristol, UK). Tetrabenazine was dissolved in a vehicle solution of 0.9% saline (80%) and dimethyl sulfoxide (DMSO) (20%). 10 μl hydrochloric acid (HCl)/mL volume was then added to get the drug completely into solution. 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), the adenosine A_{2A} antagonist, was synthesized at the Pharmazeutisches Institut Universität Bonn; Bonn, Germany (see Hockemeyer et al. 2004). MSX-3 was dissolved in 0.9% saline. The pH of the MSX-3 solution was adjusted by adding 1.0 N NaOH until the drug was completely in solution after conversion to its disodium salt (pH 7.1 – 7.7).

The doses of tetrabenazine for experiment 1 (0.25, 0.5, 1.0, and 2.0 mg/kg) were selected based on extensive pilot work done in our laboratory. Doses higher than 2.0 mg/kg (e.g. 3.0 mg/kg, 4.0 mg/kg) induced more profound motor deficits, including severely reduced locomotion and sedation. Therefore, we chose 2.0 mg/kg as our highest dose for experiment 1. A dose of 2.0 mg/kg of tetrabenazine was selected for the subsequent frequency analysis (experiment 2) and MSX-3 reversal experiment (experiment 3) since this dose induced the maximal response and produced results that differed significantly from the vehicle control. The doses of MSX-3 (experiment 3) (1.25, 2.5, 5.0, and 10.0 mg/kg) were selected based on previous work conduced in our laboratory. Collins et al. (2010a) showed doses of 5.0 and 10.0 mg/kg of MSX-3 reduced tremulous jaw movements induced by a dose of 0.5 mg/kg of the muscarinic agonist pilocarpine. Additionally, Collins et al. (2011) found these doses (5.0 and 10.0 mg/kg MSX-3) reduced tremulous jaw movements induced by a dose of 3.0 mg/kg of the anticholinesterase galantamine. The dose of tetrabenazine for experiment 4 (10.0 mg/kg) was selected based on data from López-Cruz et al. (in prep, see appendix 3), which showed 10.0 mg/kg tetrabenazine significantly increases TJMs in CD1 mice compared to vehicle control. The doses of MSX-3 (also experiment 4) (2.5, 5.0, 10.0 mg/kg) were chosen based on previous data from our laboratory. Salamone et al. (2012) showed that 2.5, 5.0, and 10.0 mg/kg doses of MSX-3 significantly attenuated TJMs induced by the muscarinic agonist pilocarpine. For experiment 5, a dose of 2.0 mg/kg tetrabenazine was chosen based on data from experiment 1, showing this dose significantly increases TJMs in rats compared to vehicle control. A dose of 10.0 mg/kg MSX-3 was chosen based on

the data from experiment 3 showing this dose significantly attenuates TJMs induced by 2.0 mg/kg tetrabenazine.

Experimental Procedures

Experiment 1: Ability of tetrabenazine to induce tremulous jaw movements

A group of 10 rats was used to assess the ability of TBZ to induce tremulous jaw movements. All rats received IP injections of either 1.0 ml/kg vehicle solution (80% saline, 20% DMSO) or 0.25, 0.5, 1.0, or 2.0 mg/kg tetrabenazine in a within-groups design, with all rats receiving all drug treatments in a randomly varied order (one treatment per week; no treatment sequences repeated). One hour and 50 minutes after IP injection, the rats were placed in the Plexiglas chamber and allowed to habituate for ten minutes. Following the habituation period, tremulous jaw movements were counted for fifteen minutes, with the observation period divided into three five-minute epochs.

Experiment 2: Freeze frame video analysis of local frequency of the tremulous jaw movements induced by tetrabenazine

One week following the completion of experiment 1, 7 of the rats received an IP injection of 2.0 mg/kg tetrabenazine. After 1 hour and 55 minutes, rats were placed in a clear Plexiglas tube (9 cm in diameter) so that a consistent view of the orofacial area could be achieved. After habituating for 5 minutes, each rat was recorded for 15 minutes using a video camera. The sections of these video files that allowed for clear observation of the orofacial area were then subjected to a freeze-frame analysis (1 frame = 1/30 s), in which the observer went frame-by-frame through each burst of jaw movements (i.e. each

group of at least two jaw movements that were within 1.0 seconds of each other). The observer recorded the inter-movement interval for each pair of jaw movements within bursts, which was defined as the number of frames between each point at which the jaw was fully open during successive jaw movements. This information was used to determine the local frequency within bursts of jaw movements (i.e., the local frequency is the reciprocal of the intermovement time).

Experiment 3: The ability of the adenosine A_{2A} antagonist MSX-3 to attenuate tremulous jaw movements induced by tetrabenazine

A group of 10 rats were used to assess the effects of the adenosine A_{2A} antagonist MSX-3 on tremulous jaw movements induced by 2.0 mg/kg TBZ. A within-groups design was utilized for this study, with all rats receiving all drug treatments in a randomly varied order (one treatment per week; no treatment sequences repeated). On the test day each week, each rat received an IP injection of either 1.0 ml/kg vehicle solution (80% saline, 20% DMSO) or 2.0 mg/kg tetrabenazine. After 1 hour and 40 minutes, rats received an IP injection of either 1.0 ml/kg saline (vehicle) or 1.25, 2.5, 5.0, or 10.0 mg/kg MSX-3. Ten minutes after injections, rats were placed in the Plexiglas observation chamber and allowed to habituate for ten minutes. Tremulous jaw movements were then counted for fifteen minutes, with the observation period divided into three five-minute epochs.

Experiment 4: The ability of the adenosine A_{2A} antagonist MSX-3 to attenuate tremulous jaw movements induced by tetrabenazine in CD1 mice

A group of 10 mice were used to assess the effects of the adenosine A_{2A} antagonist MSX-3 on tremulous jaw movements induced by 10.0 mg/kg tetrabenazine. A within-groups design was utilized for this study, with all mice receiving all drug treatments in a randomly varied order (one treatment per week; no treatment sequences repeated). On the test day each week, each mouse received an IP injection of either 1.0 ml/kg vehicle solution (80% saline, 20% DMSO) or 10.0 mg/kg tetrabenazine. After 1 hour and 35 minutes, mice received an IP injection of either 1.0 ml/kg saline (vehicle) or 2.5, 5.0, or 10.0 mg/kg MSX-3. Fifteen minutes after injections, mice were placed in the glass observation chamber and allowed to habituate for five minutes. Tremulous jaw movements were then counted for twenty minutes, with the observation period divided into four five-minute epochs.

Experiment 5: Ability of MSX-3 to reverse the effects of tetrabenazine on c-Fos immunoreactivity in ventrolateral neostriatum

Experimentally naïve rats (n=18; 6 per group) were randomly assigned to the following IP treatments: 80% saline, 20% DMSO vehicle plus saline vehicle, 2.0 mg/kg tetrabenazine plus saline vehicle, or 2.0 mg/kg tetrabenazine plus 10.0 mg/kg MSX-3. Rats received an injection of 1.0 ml/kg vehicle solution (80% saline, 20% DMSO) or 2.0 mg/kg tetrabenazine. 1 hour and 10 minutes later, rats received an injection of 1.0 ml/kg saline (vehicle) or 10.0 mg/kg MSX-3. After twenty minutes, animals were anesthetized with CO_2 and perfused 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.

Data Analysis

The data for experiments 1-4 were analyzed using a repeated measures analysis of variance (ANOVA). The data for experiment 5 were analyzed using between-groups ANOVA. A computerized statistical program (SPSS 14.0 for Windows) was used to perform the analyses. For experiments 1, 3, and 4 average TJMs over the three five-minute observation periods were calculated and then used in the ANOVA calculations. When there was a significant ANOVA, planned comparisons using the overall error term were used to assess the differences between each dose and the control condition; the number of comparisons was restricted to the number of treatments minus one (Keppel, 1991). The video files from experiment 2 were analyzed using Adobe Premiere CS5.5 for Windows, which allowed freeze-frame analysis in a frame-by-frame sequence (1 frame = 1/30 s).

2.3 Results

Experiment 1: Ability of tetrabenazine to induce tremulous jaw movements

Figure 1 shows the effect of TBZ on tremulous jaw movements. There was a significant overall effect of tetrabenazine on TJMs ($F(4,36) = 15.182$; $p<0.0001$). The 2.0 mg/kg dose tetrabenazine significantly induced TJMs (planned comparisons, $p< 0.05$) compared to vehicle control.

Experiment 2: Freeze frame video analysis of local frequency of the tremulous jaw movements induced by tetrabenazine

Figure 2 shows the results of the freeze frame video analysis. A total of 184 jaw movements were analyzed. Data are shown as the number of inter-movement intervals (i.e. the number of 1/30 s frames that elapsed from one jaw movement to another) from jaw movement bursts, assigned to four frequency bins. To interpret these data in terms of frequencies (i.e. jaw movements per second), the reciprocal of the inter-movement interval was calculated (e.g. 10/30 frames per second corresponds to 3 Hz; 4/30 frames is 7.5 Hz, etc.). Repeated measures ANOVA revealed that the number of inter-movement intervals showed an overall significant difference across the different interval bins ($F(29,174) = 14.464$, $p<.0001$). The vast majority (92%) of the TJMs took place in the 3.0-7.5 Hz frequency range, with a peak from 4.0-6.0 Hz.

Experiment 3: The ability of the adenosine A_{2A} antagonist MSX-3 to attenuate tremulous jaw movements induced by tetrabenazine

Figure 3 shows the ability of the adenosine A_{2A} antagonist MSX-3 attenuate TJMs induced by 2.0 mg/kg TBZ. There was a significant overall effect of MSX-3 treatment on tetrabenazine-induced TJMs ($F(5,45) = 12.181$; $p<0.0001$). 2.0 mg/kg TBZ significantly induced TJMs compared to vehicle control (planned comparisons, $p<0.05$). Additionally, all doses of MSX-3 (1.25, 2.5, 5.0, and 10.0 mg/kg) significantly reduced tetrabenazine-induced TJMs (planned comparisons, $p<0.05$).

Experiment 4: The ability of the adenosine A_{2A} antagonist MSX-3 to attenuate tremulous jaw movements induced by tetrabenazine in CD1 mice

Figure 4 shows the ability of the adenosine A_{2A} antagonist MSX-3 to attenuate TJMs induced by 10.0 mg/kg tetrabenazine. There was a significant overall effect of MSX-3 treatment on tetrabenazine-induced TJMs ($F(4,36) = 9.251$; $p<.0001$). 10.0 mg/kg tetrabenazine significantly induced TJMs compared to vehicle control (planned comparisons, $p<.05$). Additionally, all doses of MSX-3 (2.5, 5.0, and 10.0 mg/kg) significantly reduced tetrabenazine-induced TJMs (planned comparisons, $p<.05$).

Experiment 5: Ability of MSX-3 to reverse the effects of tetrabenazine on c-Fos immunoreactivity in ventrolateral striatum

The results of experiment 5 are shown in Figure 5. There was an overall effect of drug treatment on the number of c-Fos positive cells ($F(2,17) = 14.731$, $p < 0.0001$). TBZ increased c-Fos positive cell counts relative to vehicle alone (Tukey test, $p < 0.0001$), and the combination of MSX-3 plus tetrabenazine differed significantly from tetrabenazine (Tukey test, $p < 0.05$), demonstrating that MSX-3 significantly reduced TBZ-induced c-Fos expression.

2.4 Discussion

The present studies were conducted to establish an animal model of Parkinsonian resting tremor using the VMAT2 inhibitor tetrabenazine (TBZ). By blocking vesicular uptake, TBZ reversibly inhibits the storage of catecholamines into synaptic vesicles, and is known to deplete DA. When administered to rats, TBZ induced TJMs in a dose-

dependent manner. These TJMs occurred primarily in the 3-7.5 Hz frequency range, and were attenuated with coadministration of the adenosine A_{2A} antagonist MSX-3. MSX-3 was also capable of reducing TBZ-induced TJMs in mice. It is hypothesized that this attenuation occurred because of the colocalization of DA D2 and adenosine A_{2A} receptors on enkephalin-positive "indirect-pathway" striatopallidal medium spiny neurons. Research examining the actions of these drugs on the cellular level lends support to this hypothesis. Administration of 2.0 mg/kg TBZ significantly increases the number of c-Fos positive cells in the ventrolateral neostriatum (VLS) compared to vehicle control. Additionally, coadministration of 10.0 mg/kg MSX-3 significantly reduced the number of c-Fos positive cells.

Results from experiment 1 showed that TBZ administration increased TJMs in a dose dependent manner, with a dose of 2.0 mg/kg significantly increasing the number of TJMs compared to vehicle control. Pilot studies indicated that higher doses (e.g. 4.0 mg/kg) produced more severe motor impairments, akinesia, catalepsy and profound sedation, but variable effects on TJMs. The ability of TBZ to induce TJMs is consistent with previous studies showing another VMAT inhibitor, reserpine, also can induce TJMs (Baskin and Salamone 1993; Salamone and Baskin 1996). Baskin and Salamone (1993) reported that systemic administration of 2.5 mg/kg and 5.0 mg/kg of reserpine significantly induced TJMs compared to vehicle control. In addition, DA depletions produced by intrastriatal injections of the neurotoxic agent 6-OHDA also were shown to induce TJMs (Jicha and Salamone, 1991; Finn et al., 1998). DA antagonists such as haloperidol and pimozide, which are "typical" antipsychotics in humans, also have been shown to induce TJMs (Steinpreis et al. 1993; Ishiwari et al., 2005; Salamone et al. 2008;

Betz et al. 2009). Taken together, these results indicate that interference with striatal DA transmission, either with DA antagonism, neurotoxic agents, or pharmacological depletion of DA, leads to the production of TJMs in rodents. Furthermore, the ability of TBZ to induce TJMs in rats is consistent with reports indicating that TBZ can produce Parkinsonian symptoms in humans (Kenney et al., 2007a; Kenney et al., 2007b; Frank, 2009).

In experiment 2, a local frequency analysis of the TBZ-induced TJMs revealed that they primarily occurred in bursts with an average frequency within bursts of 3.0-7.5 Hz, with the peak occurring between 4.0-6.0 Hz. This frequency range is consistent with the general finding that TJMs induced by DA depletion, DA antagonism, and cholinomimetic administration occur in the 3-7 Hz frequency range (Salamone and Baskin 1996; Mayora et al. 1997; Cousins et al. 1998; Ishiwari et al. 2005; Collins et al. 2010; Collins et al. 2011; Salamone et al. 2012). Importantly, this frequency range is characteristic of parkinsonian resting tremor and distinct from dyskinesias (1-2 Hz) and postural tremor (8-12 Hz) (Findley et al. 1981; Deuschl et al. 2000). Previous research has employed EMG methods to further characterize the local frequency of TJMs. Cousins et al. (1998) showed that the temporalis muscle is the major contributor to the muscle activity shown during TJMs, as indicated by consistent rhythmic bursts of EMG activity recorded during bursts of tacrine-induced TJMs. EMG activity induced by tacrine in that study occurred in the frequency range 3.0-5.0 Hz, again consistent with that observed in parkinsonian resting tremor (Cousins et al., 1998). Consistent with this early report, later research using EMG recording methods showed that bursts of TJMs induced by the anticholinesterase galantamine were accompanied by temporalis jaw muscle activity in

the 5.0-6.0 Hz frequency range (Collins et al., 2011). Future research should use EMG recording methods to further characterize the local frequency of TBZ-induced TJMs.

In experiments 3 and 4, the adenosine A_{2A} antagonist MSX-3 significantly attenuated TBZ-induced TJMs in both rats and mice. DA D2 receptors are colocalized with adenosine A_{2A} receptors on enkephalin-positive "indirect pathway" medium spiny neurons (Ferre et al., 1997, 2001, 2008) and these two receptors have been shown to form heterodimers (Fuxe et al., 2003). DA D2 receptors are linked to the g-protein G_i, and therefore inhibit adenylate cyclase, while adenosine A_{2A} receptors are linked to the g-proteins G_s/G_{olf} and activate adenylate cyclase (Ferre et al., 2008). Thus, in addition to forming heteromeric complexes, there is evidence that these receptors interact via convergence onto the same c-AMP related signal transduction pathway, at which they exert opposite effects (Ferre et al., 2001, 2008; Hauber et al., 2001). This opposing interaction between receptor subtypes has led researchers to investigate adenosine A_{2A} antagonists as possible antiparkinsonian agents. Salamone et al. (2008) showed that administration of MSX-3 significantly attenuated the induction of TJMs, as well as the suppression of locomotion and the display of catalepsy, which were induced by reserpine administration in rats. Adenosine A_{2A} antagonists have also been shown to reverse TJMs induced by DA antagonists and cholinomimetics (Correa et al., 2004; Salamone et al., 2008; Betz et al., 2009; Collins et al., 2010, 2011, 2012; Santerre et al., 2012). Therefore, results from experiment 3 are consistent with these previous studies, and lend further support to the use of A_{2A} antagonists as a treatment for Parkinsonian tremor.

Recently, the TJM model has been extended to mice. This is extremely valuable given the rising importance of genetic manipulations in mice (i.e. transgenic, knockin,

knockout). Initial studies have shown that the muscarinic agonist pilocarpine (Salamone et al., 2012) and the anticholinesterase galantamine (Podurgiel et al., in prep; see Appendix 1) can induce TJMs in mice in a dose dependent manner. As is seen with rats, MSX-3 is capable of attenuating TJMs induced by these cholinomimetics (Salamone et al. 2012; Podurgiel et al., in prep). Additionally, adenosine A_{2A} receptor knockout (KO) mice show significantly fewer pilocarpine-induced TJMs compared to wild-type (wt) controls. This same pattern of behavior is seen with administration of tetrabenazine. TBZ administration in mice induces TJMs in a dose-dependent manner and A_{2A} KO mice show significantly less TBZ-induced TJMs compared to wild-type mice (López-Cruz et al., in prep, see appendix 3). Results from experiment 4 are therefore consistent with these previous studies with mice, but extend them by showing that pharmacological depletion of DA can induce TJMs in mice, and that pharmacological antagonism of the A_{2A} receptor is capable of attenuating TBZ-induced TJMs in mice.

In order to provide a cellular marker of the ability of MSX-3 to attenuate the cellular actions of TBZ, we used c-Fos immunoreactivity. The results of experiment 5 demonstrated that a behaviorally active dose of MSX-3 was able to reduce TBZ-induced increases in c-Fos expression. c-Fos provides a cellular marker related to signal transduction processes (Segovia et al., 2012), and previous research has shown that DA D2 receptor antagonism induces c-Fos expression in striatal areas (Pinna et al., 1999; Betz et al., 2009; Farrar et al., 2010; Santerre et al., 2012). Similarly, earlier work by Pollack and Angerer (2005) showed an increase in Fos-like immunoreactivity in the rat striatum after administration of reserpine. Systemic injections of A_{2A} antagonists have been shown to attenuate striatal c-Fos expression induced by DA antagonists, including

haloperidol and pimozide (Pinna et al., 1999; Betz et al., 2009; Farrar et al., 2010; Santerre et al., 2012). In experiment 5, MSX-3 reversed TBZ-induced c-Fos expression in a neostriatal subregion known as the VLS. The VLS was chosen as the region of interest because this is the neostriatal region most closely related to the production of TJMs induced by DA depletions (Jicha and Salamone, 1991; Finn et al., 1998) and cholinomimetics (Salamone et al., 1990; Cousins et al., 1998). Furthermore, Salamone et al. (2008) demonstrated that pimozide induced TJMs could be attenuated by local injections of MSX-3 into the VLS. These data are consistent with previous research by Betz et al. (2009) who showed that the A_{2A} antagonist KW 6002 attenuated pimozide-induced c-Fos expression in the VLS. The results from experiment 5 therefore suggest that MSX-3 attenuated TBZ-induced TJMs because it blunted the basic cellular effects caused by a reduction in DA transmission at D2 receptors. Since A_{2A} and D2 receptors are colocalized, and MSX-3 was capable of attenuating the increase in c-Fos positive cells induced by TBZ, this cellular interaction is likely to be taking place in the same set of neurons. Therefore, even though TBZ reduces DA transmission in both the striatonigral and striatopallidal pathway by reducing stimulation of D1 and D2 receptors respectively, our data suggests that the increase in c-Fos expression is due to actions on striatopallidal pathway neurons. This conclusion is in accordance with previous research showing that D2 receptor blockade consistently induces c-Fos expression (a marker of c-AMP-related signal transduction pathways) in the neostriatum (Dragunow et al., 1990; MacGibbon et al., 1994; Pinna et al., 1997, 1999; Atkins et al., 1999; Young et al. 1999; Betz et al., 2009; Farrar et al., 2010; Santerre et al., 2012), and that D2 and A_{2A} receptors are co-localized on enkephalin-positive “indirect pathway” striatopallidal medium spiny

neurons and converge on the same signal transduction mechanisms (Ferre et al., 2001, 2008).

Currently in our laboratory, we use the DA depleting agent reserpine and the DA antagonists haloperidol and pimozide as DA-related models for the induction of TJMs (Salamone et al., 2008; Betz et al., 2009; Collins et al., 2012). Yet despite the utility of these drugs, our results indicate that TBZ has several advantages over reserpine, pimozide or haloperidol as a method for inducing TJM activity. As previously discussed, reserpine has a similar mechanism of action as TBZ (blockade of vesicular storage through VMAT inhibition), and thus can be used as a DA depleting agent for the induction of TJMs (Steinpreis and Salamone, 1993; Salamone et al., 2008). Nevertheless, there also are some differences between these two drugs; reserpine is nonselective for VMAT1 and VMAT2, and in contrast to TBZ, reserpine is irreversible. When used in the TJM model, reserpine-induced motor impairments were shown to sensitize over repeated injections (unpublished observations). This meant that in a repeated measures design with multiple injections, reserpine-treated animals eventually developed severe akinesia and catalepsy, as well sedation, and these effects interfered with the ability to display TJMs (unpublished observations). In contrast, our results showed a consistent response with TBZ throughout the course of the experiments, and we never recorded the emergence of these gross impairments after repeated weekly injections of TBZ. Furthermore, although haloperidol and pimozide can induce TJMs after acute injection, we have found that a robust jaw movement response with haloperidol and pimozide requires subchronic administration to prime the behavioral response. In these models, TJMs are typically observed on day 14 of haloperidol treatment and on day 7 of pimozide

treatment (e.g. Salamone et al., 2008; Betz et al., 2009; Collins et al., 2012). In contrast, TBZ administration induced consistent TJMs after acute administration. For all these reasons, TBZ appears to be a more useful tool for the induction of TJM activity than many other substances that have previously been employed.

TBZ is also currently being employed in behavioral paradigms that evaluate effort-related choice processes. Nunes et al. (in prep) have found that systemic administration of lower doses of TBZ (i.e., 0.75 mg/kg-1.0 mg/kg) alter effort-related decision making as measured by the concurrent fixed ratio (FR 5)/chow feeding choice task. Furthermore, Yohn et al. (in prep) demonstrated that 0.75 mg/kg TBZ altered arm selection in a T-maze barrier choice task, which also provides a measure of effort related choice behavior. In both instances, administration of TBZ decreased selection of a high cost/high reinforcement option, and increased selection of a low cost/low reinforcement option. Furthermore, in both cases, coadministration of MSX-3 (0.5, 1.0, 2.0 mg/kg) was able to attenuate these shifts in effort-related choice behavior. Additionally, administration of 0.75 mg/kg TBZ induces an increase in c-Fos and pDARPP-32(Thr^{34}) expression in the nucleus accumbens core, a critical area for $D2/A_{2A}$ interactions regulating effort-related choice behavior (Nunes et al., in prep). These results are consistent with the behavioral data presented in experiments 1-4, as well as the immunocytochemisty presented in experiment 5. Although TJMs are related to neostriatal function, and represent an animal model of Parkinsonian tremor, tests of effort-based choice behavior are thought to be useful as animal models of the effort-related motivational symptoms of depression. Thus, recent animal work with TBZ indicates that two of the major side effects of this drug in humans, depression-related symptoms and

Parkinsonism, can be studied in rodent models, provided that the right doses and behavioral tasks are used.

Future research should continue to develop and characterize the TBZ model of Parkinsonian resting tremor. As previously discussed, EMG recordings from the temporalis muscle should be obtained to further characterize the local frequency of TJMs and pattern of muscle activity induced by TBZ. Additionally, future studies should study the induction of DARPP-32 immunoreactivity. DARPP-32 is another useful marker of DA-related signal transduction activity (Segovia et al., 2012). Therefore, future research should assess the expression of phosphorylated DARPP32 at the threonine 34 site (pDARPP32 Thr^{34}), and phosphorylated DARPP32 at the threonine 75 site (pDARPP32 Thr^{75}), in the VLS after administration of TBZ. The histological technique of double labeling immunofluorescence should be used in order to determine which subset of neurons preferentially express pDARPP32 Thr^{34} and pDARPP32 Thr^{75} after systemic injection of TBZ. Using this technique, researchers will be able to visualize two antigens (e.g. DARPP32 and Enkephalin) in the same neuron. Based upon previous studies of DARPP-32 phosphorylation (Bateup et al., 2008), TBZ administration should increase pDARPP32 Thr^{75} expression in substance-P containing neurons, and pDARPP32 Thr^{34} expression should occur mainly in enkephalin-positive neurons. Future research could also employ this animal model of Parkinsonian resting tremor as a platform for evaluating possible antiparkinsonian agents. Using this animal model of Parkinsonian tremor, our results from experiments 3 and 4 suggest that adenosine A_{2A} antagonists may alleviate the Parkinsonian side effects seen in HD patients taking TBZ for treatment of chorea.

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Figures:

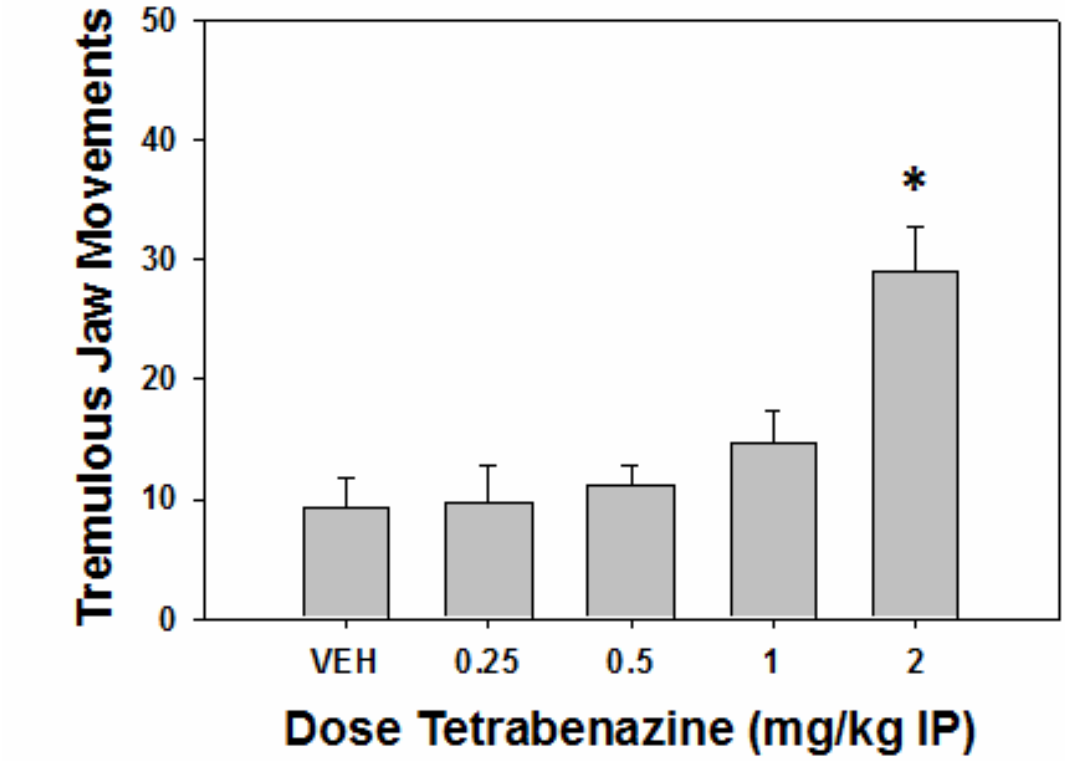


Figure 1: The VMAT2 inhibitor tetrabenazine (2.0 mg/kg) significantly induces tremulous jaw movements compared to vehicle control. * = $p < .05$

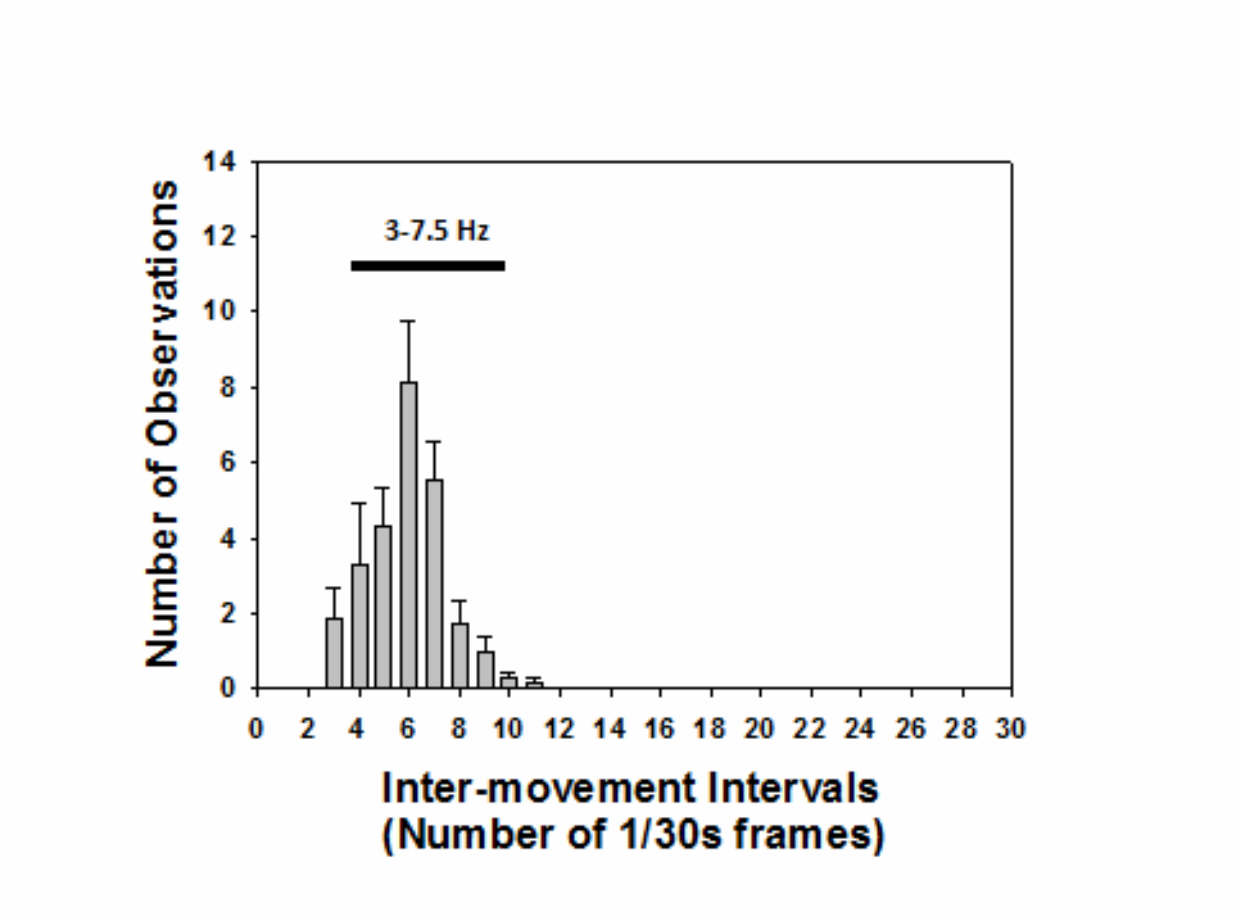


Figure 2: The vast majority (92%) of the tremulous jaw movements induced by 2.0 mg/kg tetrabenazine takes place in the 3.0-7.5 Hz frequency range, with a peak from 4.0-6.0 Hz. The number of inter-movement intervals showed an overall significant difference across the different interval bins ($F(29,174) = 14.464$, $p<.0001$)

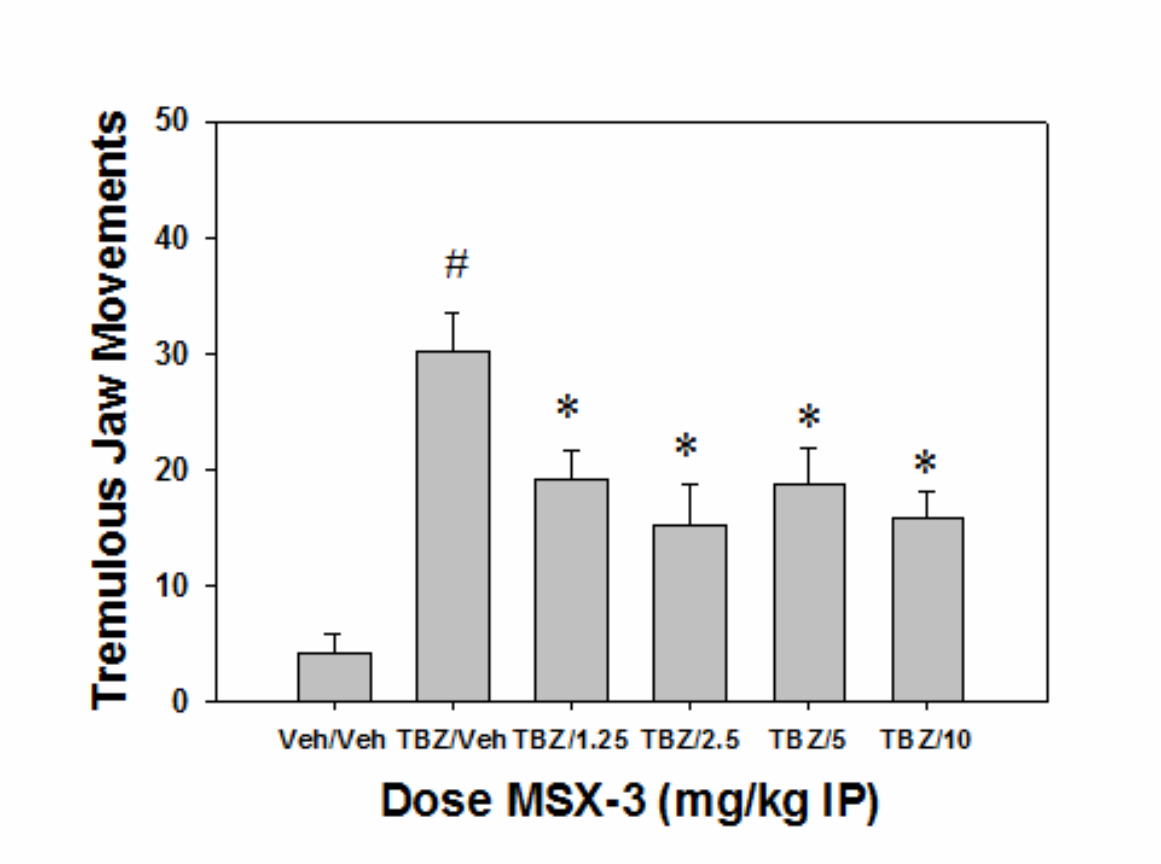


Figure 3: 2.0 mg/kg tetrabenazine significantly induces tremulous jaw movements compared to vehicle control in rats, # = $p < .05$. The adenosine A_{2A} antagonist MSX-3 attenuates tremulous jaw movements induced by 2.0 mg/kg tetrabenazine in rats, * = $p < .05$.

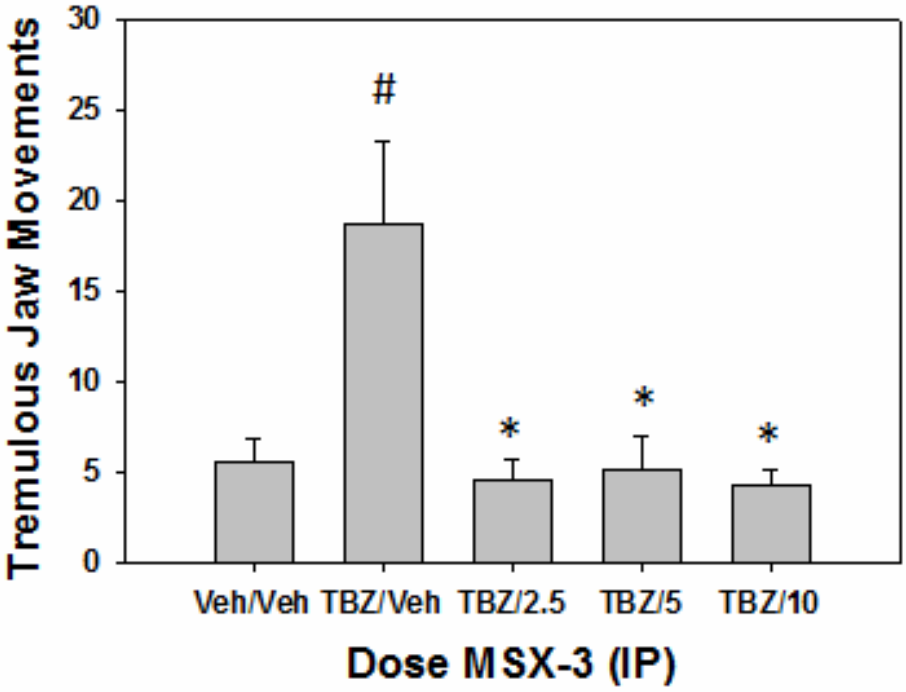


Figure 4: 10.0 mg/kg tetrabenazine significantly induces tremulous jaw movements compared to vehicle control in mice, # = $p < .05$. The adenosine A_{2A} antagonist MSX-3 attenuates tremulous jaw movements induced by 10.0 mg/kg tetrabenazine in mice, * = $p < .05$.

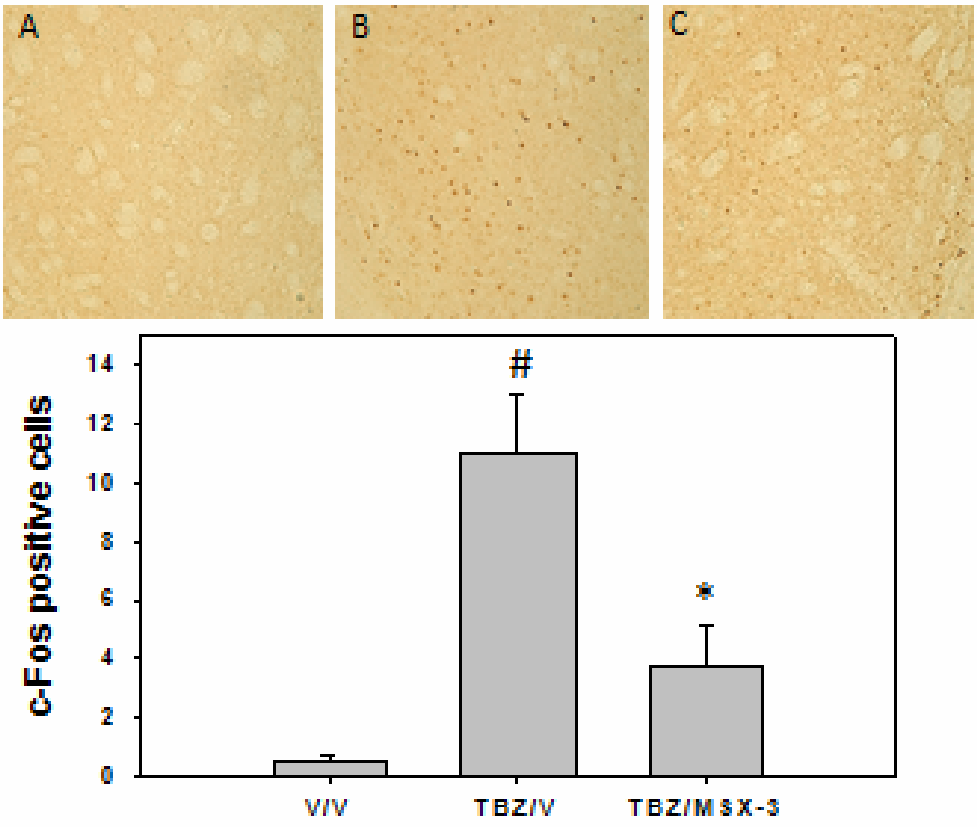
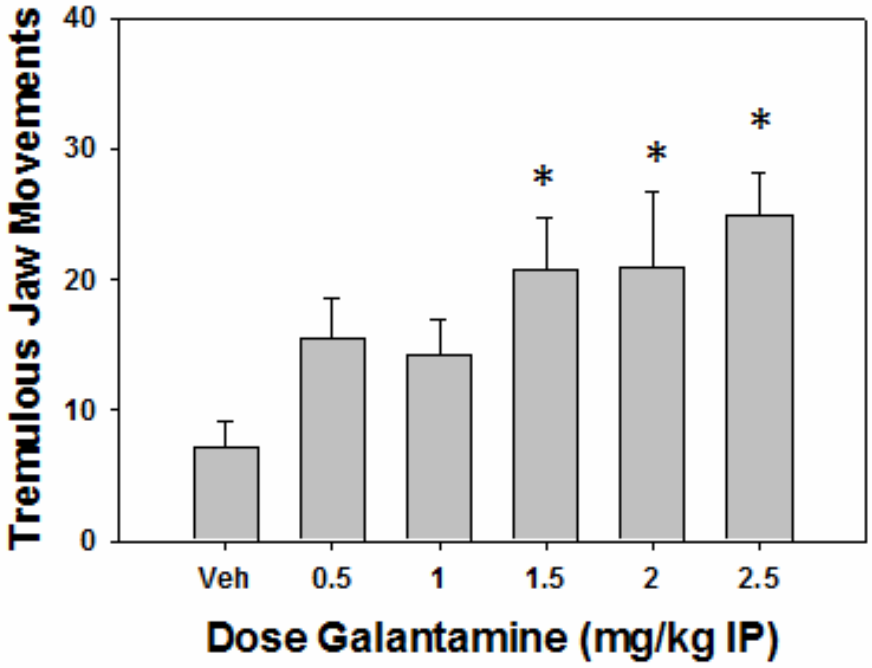
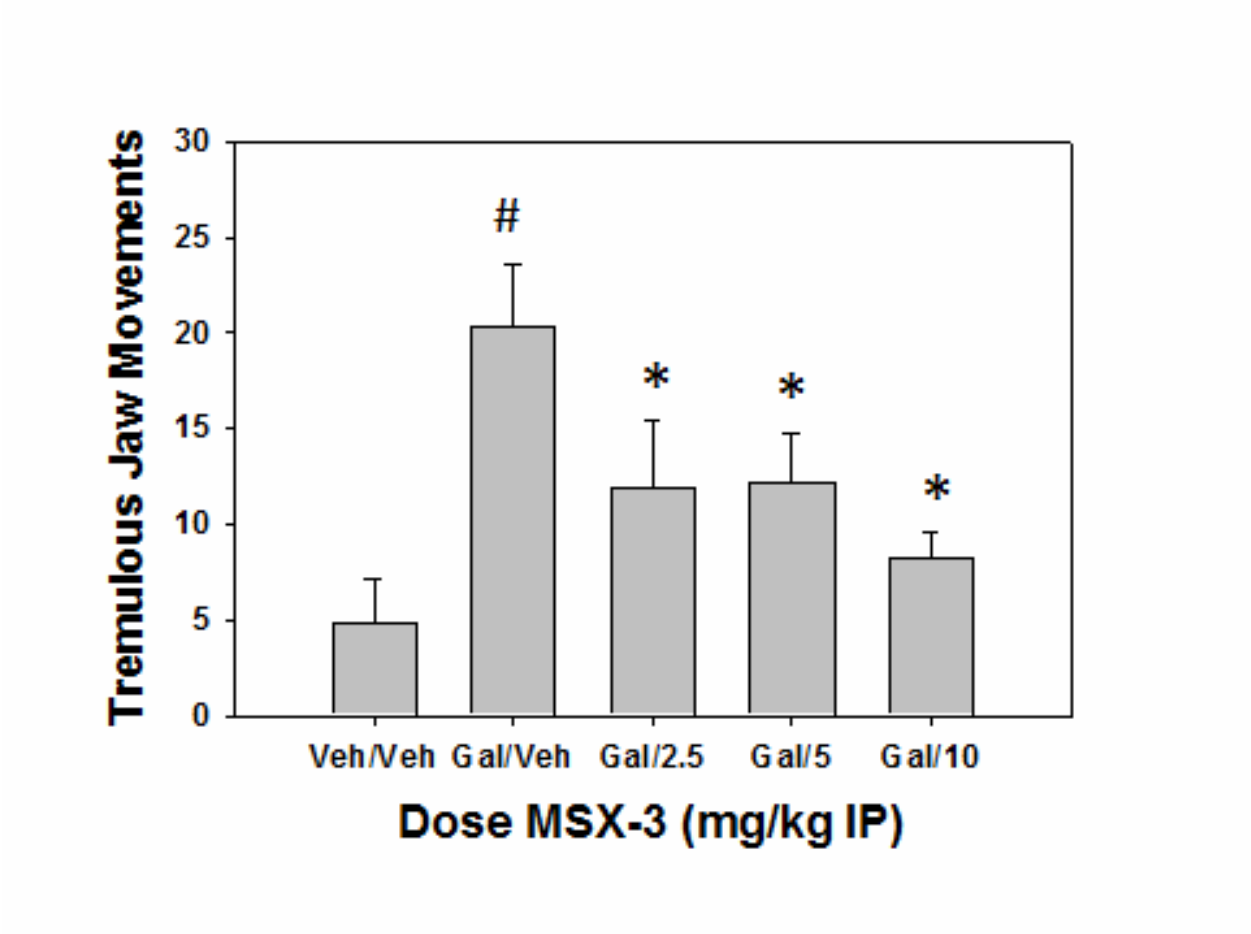


Figure 5: 2.0 mg/kg tetrabenazine significantly increases c-Fos expression in the VLS compared to vehicle control. # = $p < .05$ The adenosine A_{2A} antagonist MSX-3 attenuates c-Fos expression in the VLS induced by 2.0 mg/kg tetravenazine * = $p < .05$. Panel A: V/V; Panel B: TBZ/V; Panel C: TBZ/MSX-3

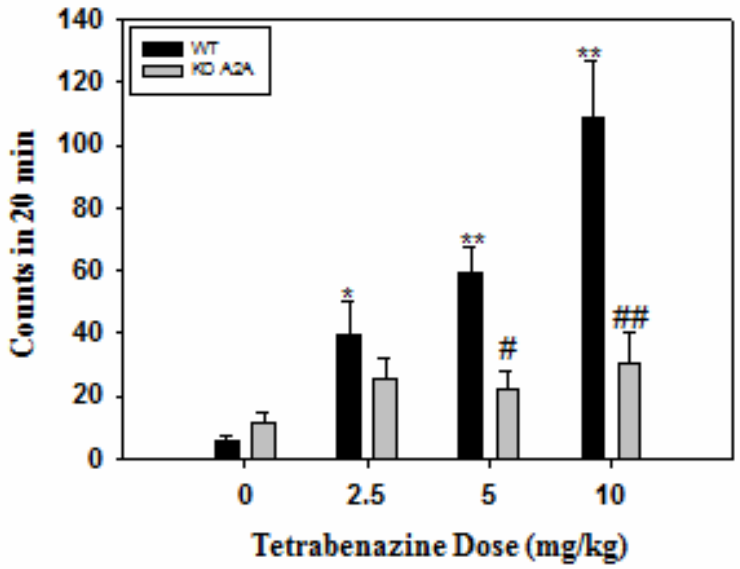
Appendix:



Appendix 1: The anticholinesterase galantamine induces tremulous jaw movements in mice.
* = $p < .05$



Appendix 2: 2.5 mg/kg tetrabenazine significantly induces tremulous jaw movements compared to vehicle control in mice, # = $p < .05$. The adenosine A_{2A} antagonist MSX-3 attenuates tremulous jaw movements induced by 2.5 mg/kg galantamine in mice, * = $p < .05$



Strain effect: $F(1,16)=20.72$, $p<0.01$
Dose effect $F(3,48)=16.75$, $p<0.01$
Interaction $F(3,48)= 8.58$, $p<0.01$

Appendix 3: Tetrabenazine induces tremulous jaw movements in wild type (WT) mice. A_{2A} KO mice show significantly less tetrabenazine-induced tremulous jaw movements than WT mice in response to 5.0 and 10.0 mg/kg tetrabenazine.