



Oral tremor induced by the muscarinic agonist pilocarpine is suppressed by the adenosine A_{2A} antagonists MSX-3 and SCH58261, but not the adenosine A₁ antagonist DPCPX

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ABSTRACT

Tremulous jaw movements in rats, which can be induced by dopamine (DA) antagonists, DA depletion, and cholinomimetics, have served as a useful model for studies of tremor. Although adenosine A_{2A} antagonists can reduce the tremulous jaw movements induced by DA antagonists and DA depletion, there are conflicting reports about the interaction between adenosine antagonists and cholinomimetic drugs. The present studies investigated the ability of adenosine antagonists to reverse the tremorogenic effect of the muscarinic agonist pilocarpine. While the adenosine A_{2A} antagonist MSX-3 was incapable of reversing the tremulous jaw movements induced by the 4.0 mg/kg dose of pilocarpine, both MSX-3 and the adenosine A_{2A} antagonist SCH58261 reversed the tremulous jaw movements elicited by 0.5 mg/kg pilocarpine. Systemic administration of the adenosine A₁ antagonist DPCPX failed to reverse the tremulous jaw movements induced by either an acute 0.5 mg/kg dose of the cholinomimetic pilocarpine or the DA D₂ antagonist pimozide, indicating that the tremorolytic effects of adenosine antagonists may be receptor subtype specific. Behaviorally active doses of MSX-3 and SCH 58261 showed substantial *in vivo* occupancy of A_{2A} receptors, but DPCPX did not. The results of these studies support the use of adenosine A_{2A} antagonists for the treatment of tremor.

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1. Introduction

Although resting tremor is considered to be one of the hallmark symptoms of parkinsonism, relatively little is known about the neural mechanisms underlying tremorogenesis (Bergman and Deuschl, 2002; Deuschl et al., 2001). Depletion of striatal dopamine (DA) is recognized as the primary condition leading to the development of the motor symptoms of idiopathic Parkinson's disease (Hornykiewicz, 1973), and the blockade of DA transmission produced by antipsychotic drugs leads to the development of drug-induced parkinsonism (Marsden et al., 1975; Alkelai et al., 2009). Nevertheless, striatal DA interacts with several other transmitters, including GABA, glutamate, serotonin, adenosine, and acetylcholine (ACh; DeLong, 1990; Hauber, 1998; Obeso et al., 2000; Young and Penney, 1993). For example, the neostriatum contains cholinergic interneurons and expresses several subtypes of muscarinic receptors (Hersch et al., 1994; Ince et al., 1997). Muscarinic antagonists suppress parkinsonian symptoms,

including akinesia and tremor (Aquilonius, 1980; McEvoy, 1983). Cholinomimetic drugs are known to be tremorogenic (Brimblecombe, 1975; Dronfield et al., 2000; Liston et al., 2004; Salamone et al., 2001), and several clinical studies have reported that cholinomimetics can induce or exacerbate parkinsonian symptoms, including tremor, in humans (Aarsland et al., 2003; Arai, 2000; Bourke and Drukenbrod, 1998; Cabeza-Alvarez et al., 1999; Duvoisin, 1967; Gurevich et al., 2006; Iwasaki et al., 1988; Kao et al., 1993; Keltner, 1994; McSwain and Forman, 1995; Ott and Lannon, 1992; Shea et al., 1998; Song et al., 2008).

In addition to studying interactions between DA and ACh, much emphasis has been placed upon characterizing the functional significance of striatal adenosine (Bara-Jimenez et al., 2003; Ferré et al., 1997, 2001, 2004; Mally and Stone, 1996, 1998; Morelli and Pinna, 2002; Salamone et al., 2008b; Simola et al., 2006; Stromberg et al., 2000; Svenningsson et al., 1999). The adenosine A_{2A} receptor subtype is expressed to a high degree in the neostriatum, particularly on the enkephalin-positive striatopallidal neurons that co-express DA D₂ receptors (Fink et al., 1992; Fuxe et al., 2007; Rosin et al., 1998; Schiffman et al., 1991; Svenningsson et al., 1999). Adenosine A_{2A} antagonists have been evaluated in human clinical studies to assess their antiparkinsonian actions (Bara-Jimenez et al., 2003; Jenner, 2005; LeWitt et al., 2008). Furthermore, research with animal models

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has demonstrated that antagonism of adenosine A_{2A} receptors can produce motor effects that are consistent with antiparkinsonian actions (Aoyama et al., 2000; Betz et al., 2009; Correa et al., 2004; Ferré et al., 2001; Ishiwari et al., 2007; LeWitt et al., 2008; Morelli and Pinna 2002; Pinna et al., 2005; Salamone et al., 2008a,b; Schwarzschild et al., 2002; Simola et al., 2004; Tronci et al., 2007). Adenosine A_{2A} antagonists have been shown to reverse the hypolocomotion, catalepsy, and muscle rigidity that are induced by interference with striatal DA transmission (Correa et al., 2004; Hauber et al., 2001; Ishiwari et al., 2007; Salamone et al., 2008a,b; Wardas et al., 2001; Trevitt et al., 2009a).

Several animal models have been used to assess motor functions related to parkinsonism (Avila et al., 2009; Castañeda et al., 2005; Pollack and Thomas, 2010), and research employing animal models of tremor also can contribute greatly to our understanding of the neurochemical regulation of tremorogenesis (Miwa 2007; Salamone et al., 1998; Wilms et al., 1999). For this reason, the present studies focused upon the ability of adenosine A_{2A} antagonists to attenuate drug-induced tremulous jaw movements, which are a rodent model of parkinsonian tremor that has been extensively employed (Cenci et al., 2002; Cousins et al., 1998; Ishiwari et al., 2005; Miwa et al., 2008, 2009; Rodriguez Diaz et al., 2001; Salamone et al., 1990, 1998, 2001, 2005, 2008a,b; Simola et al., 2004, 2006; Vanover et al., 2008). These movements are defined as repetitive vertical deflections of the lower jaw that resemble chewing but are not directed at a particular stimulus (Salamone et al., 1998). As shown by studies using videotape analyses or electromyographic methods, these movements occur largely within the 3–7 Hz frequency range that is characteristic of parkinsonian resting tremor (Cousins et al., 1998; Finn et al., 1997; Ishiwari et al., 2005; Mayorga et al., 1997), and can be induced by a number of conditions that parallel the neurochemistry of the pathology of parkinsonism, including striatal DA depletion, DA antagonism, anticholinesterases and muscarinic agonists (Baskin and Salamone, 1993; Betz et al., 2005; Cousins et al., 1998; Finn et al., 1997; Ishiwari et al., 2005; Jicha and Salamone 1991; Mayorga et al., 1997; Rodriguez Diaz et al., 2001; Salamone and Baskin, 1996; Salamone et al., 1990, 1998, 2005, 2008a; Steinpreis et al., 1993; Trevitt et al., 1998). Dopaminergic antiparkinsonian drugs such as apomorphine, L-DOPA, bromocriptine, pergolide, and ropinirole can reduce cholinomimetic-induced tremulous jaw movements (Cousins et al., 1997; Salamone et al., 2005), and their potency for suppressing cholinomimetic-induced tremulous jaw movements is highly correlated ($r=0.88$) with the clinical potency of these drugs for reducing parkinsonian tremor in humans (Salamone et al., 2005). Tremulous jaw movements are sensitive to several other classes of antiparkinsonian drugs, including anticholinergics and adenosine A_{2A} antagonists (Baskin and Salamone 1993; Betz et al., 2007, 2009; Correa et al., 2004; Cousins et al., 1997; Salamone et al., 1998, 2008a; Simola et al., 2004, 2006; Steinpreis et al., 1993; Tronci et al., 2007).

Although it is clear that adenosine A_{2A} antagonists can reduce the tremulous jaw movements induced by DA antagonists and DA depletion (Correa et al., 2004; Salamone et al., 2008a,b; Betz et al., 2009; Trevitt et al., 2009b), there are conflicting reports about the interaction between adenosine antagonists and cholinomimetic drugs. Some studies have demonstrated that adenosine A_{2A} antagonists are capable of reducing the oral tremor induced by the anticholinesterase tacrine (Simola et al., 2004, 2006; Tronci et al., 2007), while a recent paper reported that adenosine antagonists were ineffective at reducing tacrine-induced tremulous jaw movements (Trevitt et al., 2009b). Only one study has examined the ability of an adenosine A_{2A} antagonist to suppress the tremulous jaw movements induced by a muscarinic agonist; in that study Simola et al. (2006) observed that the adenosine A_{2A} antagonist SCH 58261 failed to suppress the tremulous jaw movements induced by 1.0 mg/kg of the muscarinic agonist pilocarpine. Therefore, the present experiments studied the ability of adenosine antagonists to attenuate the tremorogenic effects of the

muscarinic agonist pilocarpine (Finn et al., 1997; Salamone et al., 1986, 1998, 2001; Simola et al., 2006). The first experiment used both behavioral and electromyographic (EMG) methods to study the effects of two doses of pilocarpine; a high dose that has been studied previously (4.0 mg/kg IP), and a low dose (0.5 mg/kg IP) that has not previously been used in studies of tremulous jaw movements. EMG methods were used to assess the local frequency of the jaw movement activity within bursts. The second and third experiments investigated the ability of the adenosine A_{2A} antagonist MSX-3 (2.5–20 mg/kg) to reverse the tremulous jaw movements induced by either the low (0.5 mg/kg) or high (4.0 mg/kg) doses of pilocarpine; it was hypothesized that an adenosine A_{2A} antagonist could more easily reverse the effect of a low dose of pilocarpine than a high dose. The fourth experiment studied the ability of another adenosine A_{2A} antagonist, SCH 58261, to reverse the tremulous jaw movements induced by 0.5 mg/kg pilocarpine. Experiment 5 studied the effects of the adenosine A_1 antagonist DPCPX on the jaw movements induced by 0.5 mg/kg pilocarpine. Because the results indicated that DPCPX did not suppress pilocarpine-induced jaw movements, experiment 6 investigated the effect of DPCPX on pimozone-induced jaw movements. Several recent studies have shown that pimozone-induced jaw movements are attenuated by adenosine A_{2A} antagonists (Salamone et al., 2008a; Betz et al., 2009), but the effect of DPCPX has not been determined. In the final experiment, *in vivo* binding assays were performed to investigate the adenosine A_{2A} receptor occupancy of each of the adenosine antagonists utilized in the present studies.

2. Materials and methods

2.1. Animals

For the behavioral pharmacology experiments, a total of 201 male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) with no prior drug experience were used in the present experiments. The rats weighed 350–450 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 h). These studies were conducted according to University of Connecticut and NIH guidelines for animal care and use. For the *in vivo* occupancy studies, male Sprague–Dawley rats (weighing 180–200) supplied from Charles River (Germany) were used. The animals were housed 3 per cage in Makrolon cages (20 cm × 35 cm) with one plastic house for enrichment. They were kept in an animal room in which temperature (21 ± 2 °C), relative humidity ($55 \pm 5\%$) and a 12-hour light/dark cycle (lights on at 06.00 h) were automatically controlled. Food and water were available *ad libitum*. The rats had a minimum of 5 days adaptation to the animal facility prior to the initiation of experiments and the animals were taken to the experimental room the day before the experiment. Ethical permission for the procedures used in these studies was granted by the animal welfare committee, appointed by the Danish Ministry of Justice and all animal procedures were carried out in compliance with EC Directive 86/609/EEC and with Danish law regulating experiments on animals.

2.2. Drugs

Pimozone, pilocarpine, and DPCPX were purchased from Sigma Aldrich Chemical (St. Louis, MO). Pimozone was dissolved in warm 0.3% tartaric acid. Pilocarpine was dissolved in 0.9% saline. Because it is not highly water soluble, DPCPX (8-cyclopentyl-1,3-dipropylxanthine) was dissolved in a 20:80 solution of 100% ethanol:room temperature 0.9% saline (Salamone et al., 2009). 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) was synthesized at the Pharmazeutisches Institut (Universität Bonn; Bonn,

Germany (see Sauer et al., 2000; 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 7.1–7.4). SCH 58261 was purchased from Tocris Chemical (Bristol, England) and dissolved in a 20:80 solution of 100% ethanol:room temperature 0.9% saline.

2.3. Selection of doses and treatment procedures

The subchronic 1.0 mg/kg (IP) pimoizide treatment procedure selected for the present studies was based upon previously published experiments (Salamone et al., 2008a; Ishiwari et al., 2005). The procedure of screening animals by assessing them for tremulous jaw movements the day before the drug challenge day (i.e. on day 7) was the same as that used in a previous study (Salamone et al., 2008a), and was done in order to ensure a robust jaw movement response on the drug challenge day (i.e., day 8). No animals failed to show a substantial jaw movement response to pimoizide (i.e., <15 tremulous jaw movements) on day 7. Pilocarpine-induced tremulous jaw movements have been previously reported to occur after acute administration (Betz et al., 2007; Salamone et al., 2001; Mayorga et al., 1997; Finn et al., 1997; Stewart et al., 1988; Salamone et al., 1986), and the specific high (4.0 mg/kg) and low (0.5 mg/kg) doses used in the present studies were determined based upon these published studies and the first experiment. Doses of MSX-3 used in experiments 2 and 3 were selected based upon pilot data and on previous studies with the tremulous jaw model and with locomotion (Salamone et al., 2008a; Ishiwari et al., 2005). The doses of the adenosine A1 antagonist DPCPX used in experiments 5 and 6 were based upon extensive pilot work in our lab, and are similar to the doses used in another recent study of the effect of DPCPX on food-reinforced tasks (Mott et al., 2009; Salamone et al., 2009). The doses of SCH58261 used in experiment 4 were based upon doses reported in a previously published study that employed the tremulous jaw movement model (Simola et al., 2004). For the *in vivo* binding studies, MSX-3, DPCPX, and SCH58261 were injected IP 20, 30 or 30 min respectively before the animals were sacrificed.

2.4. Behavioral procedures

2.4.1. Tremulous jaw movements

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 $r=0.97$ ($p<0.05$) using these methods.

2.4.2. EMG electrode implantation, recording, and analysis of tremulous jaw movements

Rats were anesthetized and two electrodes of 50.0 μm tungsten wire (California Fine Wire, Grover Beach, CA) were implanted approximately 1.0 mm deep with a 27-gauge needle into each temporalis muscle (for a total of four electrodes). Previous research has demonstrated that the temporalis muscle is the jaw muscle that shows activity most closely related to tremulous jaw movements (Cousins et al., 1998). All electrodes were then attached to a female pin (Omnetics, Minneapolis, MN) secured in a rectangular five by four pin array. Two stainless steel watch screws served as indifferent and ground electrodes. The ensemble was fastened to the skull with two additional screws and cranioplastic cement. Following electrode

implantation, rats were allowed one week to recover. On the test day, rats received an injection of either 4.0 mg/kg pilocarpine or 0.5 mg/kg pilocarpine. Ten minutes later, recordings were performed for 15 min. During the recording session, the animals were connected to the recording apparatus by a multi-wire cable that was attached to a pulley system in the ceiling. All recordings were performed using the Cheetah 16 recording system and Cheetah Data Acquisition Software (Neuralynx, Bozeman, MT). During the recording session, a trained observer recorded tremulous jaw movements. At the conclusion of the recording session, data was examined using the Neuraview program (Neuralynx, Bozeman, MT), which allowed for the simultaneous viewing of EMG traces and tremulous jaw movement event recordings. Traces were then imported into Matlab 7.0.1, bandpass filtered between 300 and 1500 Hz, and plotted graphically.

2.5. Experiments

2.5.1. Experiment 1: Effects of low or high doses of pilocarpine

A group of 5 rats was used to assess the effect of a low dose of pilocarpine (0.5 mg/kg IP) and a high dose of pilocarpine that was used in previous experiments (4.0 mg/kg). All rats received IP injections of 1.0 ml/kg saline, 0.5 mg/kg pilocarpine, or 4.0 mg/kg pilocarpine, once per week, in a randomly varied order. Immediately after IP injection, rats were placed in the Plexiglas observation chamber and allowed to habituate for 10 min. Following habituation, tremulous jaw movements were counted for 15 min, with this observation period being divided into three separate 5-min epochs. In order to characterize the local frequency of jaw movement activity, a small group of animals ($n=2$) was implanted with EMG electrodes into the temporalis muscle, and then allowed one week to recover. On the test day, rats received an injection of either 4.0 mg/kg pilocarpine or 0.5 mg/kg pilocarpine IP. Ten minutes later, recordings were performed for 15 min, as described above.

2.5.2. Experiments 2 and 3: Effect of MSX-3 on tremulous jaw movements induced by the high and low doses of pilocarpine

A group of 30 rats was used to assess the effect of MSX-3 on jaw movement activity induced by a high (4.0 mg/kg) dose of pilocarpine. All rats received IP injections of 4.0 mg/kg pilocarpine and were randomly assigned to receive one of the following doses of MSX-3 or vehicle: saline vehicle control, 2.50 mg/kg, 5.0 mg/kg, 10.0 mg/kg, or 20.0 mg/kg MSX-3 ($n=5$ for each group, total $n=30$). A separate group of 45 rats was used to assess the effect of MSX-3 on jaw movement activity induced by a low (0.5 mg/kg) dose of pilocarpine. All rats received IP injections of 0.5 mg/kg pilocarpine and were randomly assigned to receive one of the following doses of MSX-3 or vehicle: saline vehicle control, 2.50 mg/kg, 5.0 mg/kg, 10.0 mg/kg, or 20.0 mg/kg MSX-3 ($n=9$ for each group, total $n=45$). For both experiments, 10 min following their IP injection of either saline vehicle or MSX-3, rats received their IP injection of pilocarpine and were immediately placed in the Plexiglas observation chamber and allowed to habituate for 10 min. After habituation, tremulous jaw movements were counted for 15 min, with this observation period being divided into three separate 5-min epochs. Jaw movements were recorded for each of the five minute epochs, after which both the total and the average number of jaw movements per 5 min period was calculated.

2.5.3. Experiments 4 and 5: Effects of SCH 58261 and DPCPX on tremulous jaw movements induced by the low dose of pilocarpine

A group of 40 rats was used to assess the effect of SCH 58261 on jaw movement activity induced by a 0.5 mg/kg dose of pilocarpine. All rats received IP injections of 0.5 mg/kg pilocarpine and were randomly assigned to receive one of the following doses of SCH 58261 or vehicle: 20% ethanol vehicle control, 0.625 mg/kg, 1.25 mg/kg, 2.5 mg/kg, or 5.0 mg/kg SCH58261 ($n=8$ per group, total $n=40$).

Twenty minutes following their IP injection of either saline vehicle or SCH58261, rats received an IP injection of 0.5 mg/kg pilocarpine and were immediately placed in the Plexiglas observation chamber and allowed to habituate for 10 min. After habituation, tremulous jaw movements were tested in the manner described above. An additional group of 40 rats was used to assess the effect of DPCPX on jaw movement activity induced by a 0.5 mg/kg dose of pilocarpine. All rats received IP injections of 0.5 mg/kg pilocarpine and were randomly assigned to receive one of the following doses of DPCPX or vehicle: 20% ethanol vehicle control, 0.375 mg/kg, 0.75 mg/kg, 1.5 mg/kg, or 3.0 mg/kg DPCPX ($n=8$ per group, total $n=40$). Twenty minutes following their IP injection of either 20% ethanol vehicle or DPCPX, rats received an IP injection of 0.5 mg/kg pilocarpine, and were observed in the same manner as described above.

2.5.4. Experiment 6: Effect of DPCPX on pimozone-induced tremulous jaw movements

A group of 39 rats was used to assess the effect of DPCPX on pimozone-induced tremulous jaw movement activity. All rats received IP injections of 1.0 mg/kg pimozone each day for seven days. On day 7 of the subchronic injections, rats were tested for tremulous jaw movements as described above. Only the rats that had >15 jaw movements on day 7 were used for the day 8 drug challenge test. For the day 8 behavioral test, all rats were treated with 1.0 mg/kg pimozone and were randomly assigned to receive one of the following doses of DPCPX or vehicle: 20% ethanol vehicle control, 0.375 mg/kg, 0.75 mg/kg, 1.5 mg/kg, or 3.0 mg/kg DPCPX ($n=7-8$ for each group; total $n=39$). Three hours and 30 min following their daily pimozone injection on day 8, rats received an IP injection of DPCPX, according to the previously assigned doses. Twenty minutes later, rats were placed in the Plexiglas observation chamber and allowed to habituate for 10 min. Following habituation, tremulous jaw movements were tested in the manner described above.

2.5.5. Experiment 7: In vivo binding assay of MSX-3, SCH 58261, and DPCPX

A_{2A} receptor occupancy was measured by *in vivo* binding with [3 H] SCH442416 (Matsuya et al., 2007). Twenty μ Ci radioligand was injected IV in the tail vein. Fifteen minutes after IV injection, the animals were sacrificed and the striatum dissected out. The tissue was homogenized in ice cold buffer (5.0 ml 50 mM K_2PO_4 , pH 7.4). Samples were filtered through Whatman GF/C filters and filters were washed with 2×5 ml ice cold buffer. Filtration was completed 60 s after sacrifice and filters were counted in a scintillation counter. Protein content was determined in all brain samples and used for normalization. Groups of animals were treated with vehicle and radioactivity levels in striatum and cerebellum were used to determine total and non-specific binding. ED_{50} values were calculated using non-linear regression by Prism (Graph Pad).

2.6. Data analyses

The behavioral data for all experiments were analyzed using a between-groups analysis of variance (ANOVA). Average tremulous jaw movements over the three five-min observation epochs were calculated and then used in the ANOVA calculations. A computerized statistical program (SPSS 10.1 for Windows) was used to perform these analyses. 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 total number of comparisons was restricted to the number of treatments minus one (Keppel, 1991). Effect size calculations (R^2 values; Keppel 1991) were performed to assess the magnitude of the treatment effect (i.e., the size of the treatment effect sum of squares expressed as the proportion of total sum of squares, which is a marker of the total variance accounted for by treatment variance; for example $R^2=0.3$ reflects 30% of the variance that is explained by the treatment effect).

3. Results

3.1. Experiment 1: Effects of low or high doses of pilocarpine

Fig. 1A shows the effects of injections of 0.5 and 4.0 mg/kg pilocarpine on tremulous jaw movements. ANOVA revealed that there was a significant overall effect of drug treatment on tremulous jaw movement activity ($F(2,8)=816.6$, $p<0.001$). Planned comparisons showed that both the 0.5 and 4.0 mg/kg pilocarpine treatments differed significantly from saline ($p>0.001$). In addition, EMG recordings during jaw movements were obtained because the low dose of pilocarpine (0.5 mg/kg) had never been assessed with these methods, and it is important to determine if this dose of pilocarpine could induce movements at a local frequency that is within the parkinsonian tremor frequency range. Fig. 1B–C displays temporalis EMG traces from representative animals that received either 0.5 or 4.0 mg/kg

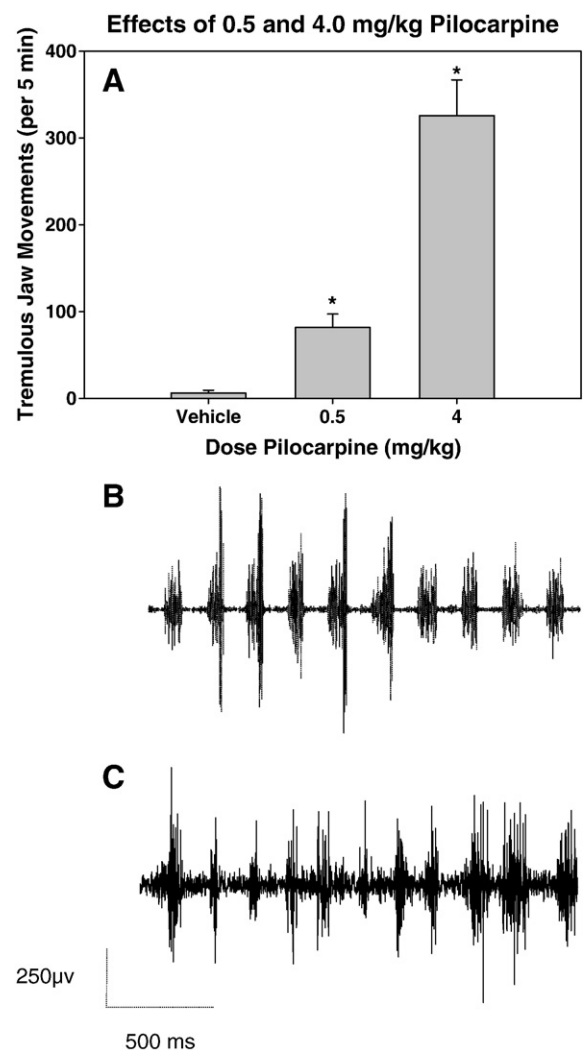


Fig. 1. Effects of 0.5 and 4.0 mg/kg pilocarpine on tremulous jaw movements. A. Mean (\pm SEM) number of jaw movements in rats treated with either saline vehicle or pilocarpine. *significant difference from control ($p<0.001$). B and C. Raw EMG traces (2 s sweeps) from the temporalis muscle of a rat that received 4.0 mg/kg pilocarpine (B), and another rat that received 0.5 mg/kg pilocarpine (C). During these sweeps, each rat showed a burst of multiple jaw movements recorded by the observer. The temporalis muscle is a jaw closing muscle, and these bursts of activity in the 5–6 Hz frequency range corresponded with the jaw closing phase of each jaw movement. Both doses of pilocarpine produced a similar local frequency of EMG activity, indicating that the higher number of jaw movements produced by the 4.0 mg/kg dose of pilocarpine results from a greater amount of time showing tremulous activity, and not from any differences in local frequency.

pilocarpine. It can be seen that both doses of pilocarpine-induced jaw muscle activity in the 5–6 Hz frequency range.

3.2. Experiments 2 and 3: Effect of MSX-3 on tremulous jaw movements induced by high and low doses of pilocarpine

Experiment 2 focused upon the effect of the adenosine A_{2A} antagonist MSX-3 on the tremulous jaw movements induced by a high dose (4.0 mg/kg) of pilocarpine. Co-administration of MSX-3 with pilocarpine was unable to reduce levels of tremulous jaw movement activity (Fig. 2A; $F(4,25) = 0.516$, n.s.; $R^2 = 0.076$). Experiment 3 studied the ability of the adenosine A_{2A} antagonist MSX-3 to attenuate the tremulous jaw movements induced by a low dose (0.5 mg/kg) of pilocarpine (Fig. 2B). There was a significant overall suppressive effect of MSX-3 on the tremulous jaw movements induced by the 0.5 mg/kg dose of pilocarpine ($F(4,40) = 6.54$, $p < 0.001$; $R^2 = 0.395$), with the 5.0, 10.0, and 20.0 mg/kg doses of MSX-3 plus pilocarpine differing significantly from the vehicle plus pilocarpine control ($p < 0.01$).

3.3. Experiment 4: Effect of SCH 58261 on tremulous jaw movements induced by a low dose (0.5 mg/kg) of pilocarpine

Experiment 4 assessed the effect of the adenosine A_{2A} antagonist SCH 58261 on the tremulous jaw movements induced by 0.5 mg/kg pilocarpine, to assess the generality of the effects of adenosine A_{2A} antagonism. Co-administration of SCH58261 with this dose of pilocarpine

resulted in reduced levels of tremulous jaw movement activity compared to pilocarpine plus vehicle (Fig. 3); there was a significant overall effect of SCH58261 on the tremulous jaw movements induced by 0.5 mg/kg pilocarpine ($F(4,35) = 3.059$, $p < 0.05$; $R^2 = .259$), with the 0.625, 2.5 and 5.0 mg/kg doses of SCH 58261 plus pilocarpine differing significantly from the vehicle plus pilocarpine control ($p < 0.01$).

3.4. Experiments 5 and 6: Effect of DPCPX on tremulous jaw movements induced by a low dose (0.5 mg/kg) of pilocarpine and subchronic administration of 1.0 mg/kg pimoizide

Experiment 5 focused upon the effect of the adenosine A_1 antagonist DPCPX on the tremulous jaw movements induced by the low dose (0.5 mg/kg) of pilocarpine. There was no significant effect of DPCPX on pilocarpine-induced tremulous jaw movements ($F(4,35) = 0.478$, $p > 0.05$; $R^2 = 0.052$; Fig. 4A). Experiment 6 studied the effect of DPCPX on the tremulous jaw movements induced by repeated injections of 1.0 mg/kg of the DA D_2 antagonist pimoizide. During the day 8 challenge test, co-administration of DPCPX with pimoizide failed to affect levels of tremulous jaw movement activity (Fig. 4B; $F(4,34) = 0.449$, $p > 0.05$; $R^2 = 0.050$).

3.5. Experiment 7: In vivo binding assay of MSX-3, SCH 58261, and DPCPX

Although these compounds have been studied extensively using *in vitro* binding methods, only SCH 58261 has been studied for its *in vivo* occupancy of adenosine A_{2A} receptors (Matsuya et al., 2007).

The results of the *in vivo* binding study are shown in Fig. 5. MSX-3 and SCH 58261 both showed significant *in vivo* A_{2A} receptor occupancy, with doses higher than 1.0 mg/kg of both drugs producing greater than 50% occupancy of striatal A_{2A} receptors (Fig. 5A–B). In contrast, the doses of DPCPX used in the present experiment did not yield significant dose-related effect, and there was no substantial occupancy of striatal adenosine A_{2A} receptors at any dose (Fig. 5C).

4. Discussion

The present results demonstrate that adenosine A_{2A} antagonists are capable of attenuating the tremulous jaw movements induced by the muscarinic agonist pilocarpine, provided that a relatively low dose (0.5 mg/kg) of pilocarpine is used to induce jaw movement activity. In a previous study that employed a 1.0 mg/kg dose of pilocarpine to induce jaw movements, it was reported that 5.0 mg/kg SCH 58261 did

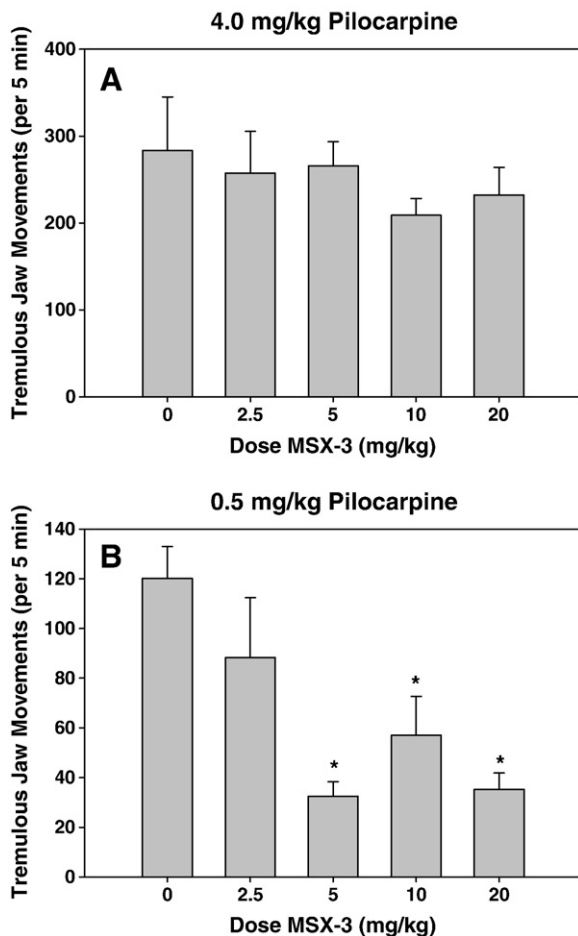


Fig. 2. Effect of the adenosine A_{2A} antagonist MSX-3 on pilocarpine-induced tremulous jaw movements. Mean (\pm SEM) number of jaw movements in rats treated with pilocarpine plus vehicle (0.0), and pilocarpine plus various doses (2.5–20.0 mg/kg) of MSX-3. A. All rats received 4.0 mg/kg pilocarpine. B. All rats received 0.5 mg/kg pilocarpine. *significant difference from control ($p < 0.05$).

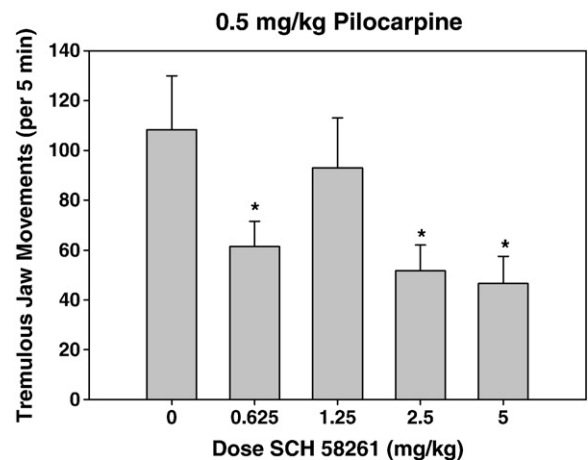


Fig. 3. Effect of the adenosine A_{2A} antagonist SCH 58261 on pilocarpine-induced tremulous jaw movements. Mean (\pm SEM) number of jaw movements in rats treated with pilocarpine plus vehicle (0.0), and pilocarpine plus various doses (0.625–5.0 mg/kg) of SCH 58261. All rats received 0.5 mg/kg pilocarpine. *significant difference from control ($p < 0.05$).

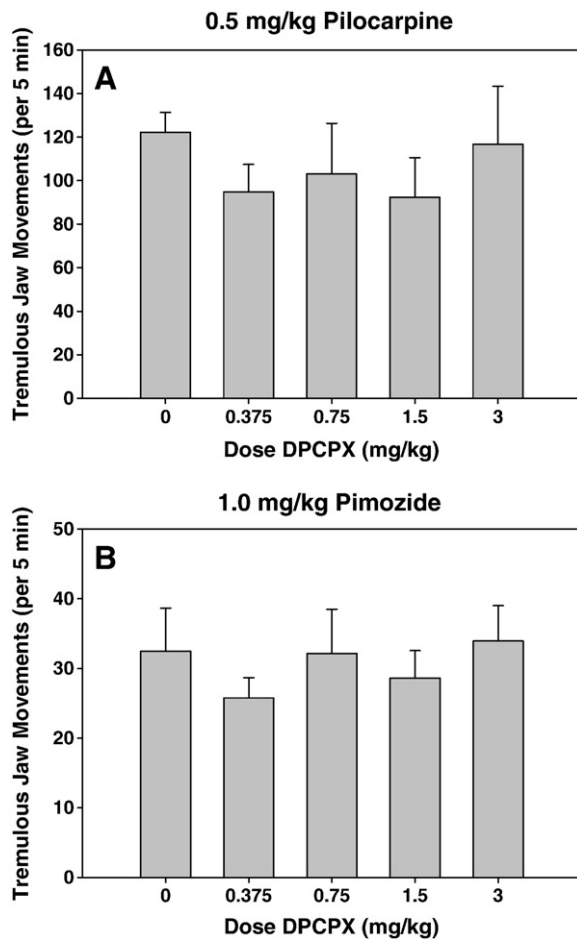


Fig. 4. Effect of the adenosine A_1 antagonist DPCPX on the tremulous jaw movements induced by pilocarpine or pimozide. Mean (\pm SEM) number of jaw movements are shown. A. All rats received 0.5 mg/kg pilocarpine plus various doses of DPCPX (0.375–3.0 mg/kg). B. All rats received 1.0 mg/kg pimozide plus various doses of DPCPX (0.375–3.0 mg/kg).

not suppress tremulous jaw movement activity (Simola et al., 2006). The present experiments demonstrated that MSX-3 and SCH 58261, administered in doses that produce substantial *in vivo* occupancy of striatal adenosine A_{2A} receptors, were able to reduce the tremulous jaw movements induced by 0.5 mg/kg pilocarpine (Figs. 2B and 3). The results of the Simola et al. (2006) study, together with experiments 2–3 above, indicate that the tremulous jaw movements induced by higher doses of pilocarpine (e.g. 1.0–4.0 mg/kg) are relatively insensitive to the effects of adenosine A_{2A} antagonists, while a lower dose of pilocarpine (0.5 mg/kg) produces a jaw movement response that is sensitive to the effects of adenosine A_{2A} antagonists. The observation that the dose of cholinomimetic used is a critical feature of this type of experiment is consistent with previous reports on the effects of the anticholinesterase tacrine. Although Trevitt et al. (2009b) reported that the adenosine A_{2A} antagonist SCH 58261 could not suppress the tremulous jaw movements induced by 5.0 mg/kg tacrine, SCH 58261 and ST1535 have been found to reduce the tremulous jaw movements induced by a lower dose (2.5 mg/kg) of tacrine (Simola et al., 2004, 2006; Tronci et al., 2007). In the present studies, the baseline level of jaw movement activity was considerably lower after injections of 0.5 mg/kg pilocarpine compared to the 4.0 mg/kg dose (Fig. 1A), and it is possible that higher doses of pilocarpine produce a response that is too robust, and not easily suppressed by adenosine antagonism. The fact that 0.5 mg/kg pilocarpine induces a level of jaw movement activity that is readily suppressed by adenosine A_{2A} antagonists, coupled with the observation

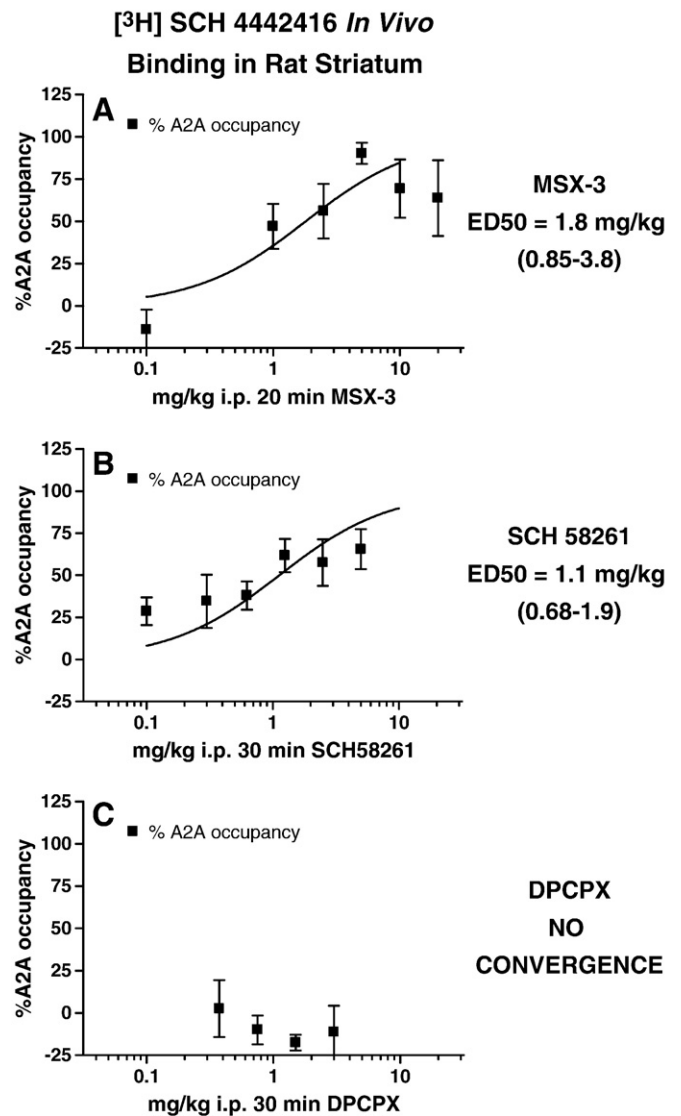


Fig. 5. Adenosine A_{2A} receptor occupancy (expressed as percent occupancy) as measured by *in vivo* binding with [³H]SCH 442416. A. MSX-3. B. SCH 58261. C. DPCPX. Curve-fitting parameters (ED₅₀ and the 95% confidence intervals) are shown to the right of each figure. There was no significant fit (i.e., no convergence) for DPCPX, and no appreciable A_{2A} receptor occupancy.

that the tremulous jaw movements induced by this dose of pilocarpine are characterized by bursts of rhythmic jaw muscle activity that have a local frequency in the 5–6 Hz range (Fig. 1), which is within the local frequency range of parkinsonian resting tremor, suggests that administration of 0.5 mg/kg pilocarpine may be a useful method for inducing tremulous jaw movement activity in future studies. Furthermore, the present studies provide the first report that an adenosine A_{2A} antagonist can reduce the motor effects of a muscarinic agonist.

In contrast to the effects of the adenosine A_{2A} selective antagonists MSX-3 and SCH 58261, the adenosine A_1 selective antagonist DPCPX, given in doses that did not occupy striatal adenosine A_{2A} receptors *in vivo*, was not able to attenuate the tremulous jaw movements induced by 0.5 mg/kg pilocarpine. Furthermore, DPCPX did not suppress the jaw movements induced by repeated administration of the DA D_2 antagonist pimozide. The latter observation is particularly important in view of previous reports indicating that several adenosine A_{2A} antagonists can suppress tremulous jaw movement activity that is induced by interference with DA transmission. The adenosine A_{2A} antagonists KF 17837 and MSX-3 were reported to suppress the tremulous jaw movements induced by the D_2 antagonist haloperidol

(Correa et al., 2004; Salamone et al., 2008a). In addition, both MSX-3 and KW 6002 (istradefylline) were able to suppress the tremulous jaw movements induced by the D₂ antagonist pimozone (Salamone et al., 2008a), using methods that were identical to those used in the present work. MSX-3 also attenuated the tremulous jaw movements induced by the DA depleting agent reserpine (Salamone et al., 2008a). In contrast to these relatively consistent effects of adenosine A_{2A} antagonism, there is conflicting evidence about the role of adenosine A₁ receptors in the regulation of tremor in animal models. Trevitt et al. (2009b) reported that the adenosine A₁ antagonist CPT could attenuate the tremulous jaw movements induced by the DA antagonist haloperidol. However, CPT has a lower selectivity for A₁ receptors relative to A_{2A} receptors compared to DPCPX (Maemoto et al., 1997), which could make it more difficult to interpret the significance of effects produced by CPT. Furthermore, the general role of A₁ receptors in modulating tremor is unclear; a recent study showed that harmaline-induced tremor, which has been used as a model of parkinsonian tremor, could be suppressed by A₁ receptors stimulation, and could actually be enhanced by A₁ receptor antagonism or knockout (Bekar et al., 2008).

The results of the present experiments are consistent with other studies showing differences between the effects of drugs that act upon adenosine A₁ and A_{2A} receptors (Rimondini et al., 1998; Prediger et al., 2005). In recent studies involving instrumental behaviors, including operant conditioning and maze tasks in rats, it was reported that DPCPX was incapable of reversing the behavioral disruptions produced by D₂ antagonism, even though A_{2A} antagonists such as MSX-3 and KW 6002 were effective (Mott et al., 2009; Salamone et al., 2009). Varty et al. (2008) also observed that DPCPX was relatively ineffective at producing antiparkinsonian actions in haloperidol-treated monkeys, in contrast to the effects of the adenosine A_{2A} selective antagonists KW 6002 and SCH 412348. Although the 0.375–3.0 mg/kg dose range that was used for DPCPX in the present study was ineffective at reversing the actions of pilocarpine and pimozone, this dose range of DPCPX has been shown to be effective in studies with rats that observed behaviors related to depression, locomotion, pain perception, memory and other processes (Aubel et al., 2007; Lobato et al., 2008; Maione et al., 2007; Marston et al., 1998; Prediger and Takahashi, 2005). Thus, it appears that DPCPX, despite having other types of behavioral actions, may be relatively weak at producing antiparkinsonian or antitremor effects. This could indicate that adenosine A_{2A} antagonists have stronger tremorolytic effects than highly selective A₁ antagonists, although additional A₁ antagonists need to be assessed.

In view of data indicating that cholinomimetic drugs can induce or exacerbate parkinsonian symptoms in humans (Aarsland et al., 2003; Arai, 2000; Bourke and Drukenbrod 1998; Cabeza-Alvarez et al., 1999; Duvoisin, 1967; Gurevich et al., 2006; McSwain and Forman 1995; Ott and Lannon, 1992; Shea et al., 1998; Song et al., 2008), and that cholinomimetic-induced tremulous jaw movements in rats are sensitive to several classes of antiparkinsonian drugs (Cousins et al., 1997; Salamone et al., 1998, 2005), the present results may have relevance for identifying novel treatments for drug-induced parkinsonism. As suggested previously (Correa et al., 2004; Ishiwari et al., 2007; Salamone et al., 2008a,b; Varty et al., 2008), it is possible that adenosine A_{2A} antagonists could be useful for treating the motor side effects of antipsychotic drugs in humans. Furthermore, the present results, together with those from other laboratories (Simola et al., 2004, 2006; Tronci et al., 2007), suggest that adenosine A_{2A} antagonists could be employed to ameliorate the motor side effects of cholinomimetic drugs that are used to treat Alzheimer's disease or other disorders. Although much research in this area has focused upon DA/adenosine interactions in neostriatum (Ferré et al., 1997, 2001; Fuxe et al., 2007; Svenningsson et al., 1999), the present results indicate that there also are significant interactions between drugs that act upon muscarinic acetylcholine and adenosine A_{2A} receptors. However, it is not clear if these actions are direct or indirect (e.g. Pollack

and Fink 1996). It is possible that the present results are due to direct interactions between adenosine A_{2A} receptors and muscarinic receptors that are localized on the same medium spiny cells in neostriatum. Alternatively, it is possible that tremulous jaw movements are induced by cholinomimetic drugs acting upon muscarinic M4 receptors that are mostly localized on striatonigral neurons, but that adenosine A_{2A} antagonists reduce this jaw movement activity by acting upon another part of the striatal circuitry (i.e., striatopallidal neurons; Betz et al., 2007, 2009). This behavioral research on the effects of adenosine A_{2A} receptor antagonists places additional emphasis on the importance of characterizing the neural mechanisms underlying the interactions between basal ganglia muscarinic and adenosine A_{2A} receptor systems.

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