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Conditional neural knockout of the adenosine A_{2A} receptor and pharmacological A_{2A} antagonism reduce pilocarpine-induced tremulous jaw movements: Studies with a mouse model of parkinsonian tremor

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Abstract

Tremulous jaw movements are rapid vertical deflections of the lower jaw that resemble chewing but are not directed at any particular stimulus. In rats, tremulous jaw movements can be induced by a number of conditions that parallel those seen in human parkinsonism, including dopamine depletion, dopamine antagonism, and cholinomimetic drugs. Moreover, tremulous jaw movements in rats can be attenuated using antiparkinsonian agents such as L-DOPA, dopamine agonists, muscarinic antagonists, and adenosine A_{2A} antagonists. In the present studies, a mouse model of tremulous jaw movements was established to investigate the effects of adenosine A_{2A} antagonism, and a conditional neuronal knockout of adenosine A_{2A} receptors, on cholinomimetic-induced tremulous jaw movements. The muscarinic agonist pilocarpine significantly induced tremulous jaw movements in a dose-dependent manner (0.25–1.0 mg/kg IP). These movements occurred largely in the 3–7.5 Hz local frequency range. Administration of the adenosine A_{2A} antagonist MSX-3 (2.5–10.0 mg/kg IP) significantly attenuated pilocarpine-induced tremulous jaw movements. Furthermore, adenosine A_{2A} receptor knockout mice showed a significant reduction in pilocarpine-induced tremulous jaw movements compared to littermate controls. These results demonstrate the feasibility of using the tremulous jaw movement model in mice, and indicate that adenosine A_{2A} receptor antagonism and deletion are capable of reducing cholinomimetic-induced tremulous jaw movements in mice. Future

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studies should investigate the effects of additional genetic manipulations using the mouse tremulous jaw movement model.

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1. Introduction

Resting tremor is a cardinal symptom of Parkinson's disease (Deuschl et al., 2001). Moreover, tremor and other parkinsonian symptoms can be induced by various drugs, including dopamine (DA) antagonists (Bezchlibnyk-Butler and Remington, 1994) and cholinomimetics (Song et al., 2008). Adenosine A_{2A} antagonists have emerged as a potential treatment of parkinsonian symptoms, including tremor (Schwarzschild et al., 2006; LeWitt et al., 2008). Adenosine A_{2A} receptors are highly expressed in neostriatum, and A_{2A} antagonists exert effects in animals that are consistent with antiparkinsonian actions (Ferré et al., 2008; Chen et al., 2001; Simola et al., 2004; Salamone et al., 2008; Collins et al., 2010). Clinical reports have indicated that adenosine A_{2A} antagonists significantly improve motor deficits, reduce OFF time, and increase ON time in parkinsonian patients (LeWitt et al., 2008).

One animal test that is useful for assessing the role of adenosine A_{2A} receptors in motor function is tremulous jaw movements (TJMs), an extensively validated rodent model of parkinsonian resting tremor (Simola et al., 2004; Miwa et al., 2009; Collins et al., 2010, 2011; for reviews, see Salamone et al., 1998; Collins-Praino et al., 2011). TJMs are rapid vertical deflections of the lower jaw that are not directed at any stimulus (Salamone et al., 1998), and occur in phasic bursts of repetitive jaw movement activity. TJMs have many of the neurochemical, anatomical, and pharmacological characteristics of parkinsonism, and meet a reasonable set of validation criteria for use as an animal model of parkinsonian tremor (Salamone et al., 1998; Collins-Praino et al., 2011). These movements are induced by conditions associated with parkinsonism, including neurotoxic or pharmacological depletion of striatal DA (Jicha and Salamone, 1991; Salamone et al., 2008), and DA antagonism (Ishiwari et al., 2005; Salamone et al., 2008). TJMs also are induced by cholinomimetic drugs, including muscarinic agonists such as pilocarpine and oxotremorine (Salamone et al., 1986, 1998; Collins et al., 2010), and anticholinesterases (Salamone et al., 1998; Simola et al., 2004; Collins et al., 2011). TJMs occur largely within the 3–7 Hz frequency range that is characteristic of parkinsonian resting tremor (Ishiwari et al., 2005; Collins et al., 2010), and can be attenuated by several classes of antiparkinsonian drugs, including DA agonists and anticholinergics (Salamone et al., 1998, 2005; Betz et al., 2009). Adenosine A_{2A} antagonists attenuate the TJMs induced by DA depletion, DA antagonism and cholinomimetics (Correa et al., 2004; Simola et al., 2004; Salamone et al., 2008; Betz et al., 2009; Collins et al., 2010, 2011; Pinna et al., 2010).

With the rising importance of genetic manipulations in mice (i.e. transgenic, knockout, knockin, etc.), it is necessary to investigate whether it is possible to extend well-validated behavioral paradigms currently being used in rats to mouse models. Although one previous study showed that

muscarinic M4 receptor knockout mice showed significantly fewer cholinomimetic-induced TJMs than wild-type mice (Salamone et al., 2001), every other study of TJM activity has employed rats. Given the putative antiparkinsonian properties of adenosine A_{2A} receptor antagonists, it is of great interest to determine if adenosine A_{2A} receptor antagonism or genetic deletion reduces levels of TJM activity in mice. In order to investigate this research question, several experiments were necessary. The first experiment studied the ability of the muscarinic agonist pilocarpine to induce TJMs in the specific strain of mice being used for the knockout study (C57/BL6). The second experiment studied the local frequency range of the TJM "bursts" induced by pilocarpine using freeze-frame video analysis. Experiments 3 and 4 investigated the effects of the adenosine A_{2A} antagonist MSX-3 and genetic deletion of the adenosine A_{2A} receptor on pilocarpine-induced TJMs. It was hypothesized that A_{2A} knockout mice would show fewer TJMs than their wild-type littermates.

2. Experimental procedures

2.1. Animals

Male C57BL/6 mice (25; Harlan Laboratories, Indianapolis, IN, USA) were used for the first three studies. For the final study, a total of 24 neuronal A_{2A} receptor conditional knockout mice and their littermate controls (12 *CaMKII α -cre*, *A_{2A} flox/flox* and 12 non-transgenic [no *cre*] *A_{2A} flox/flox* mice) congenic for the C57BL/6 background and with no prior drug experience were obtained from Massachusetts General Hospital (Boston, MA, USA; see Bastia et al., 2005 for details on the generation of these mice). Mice, weighed 15–40 g throughout the course of the experiment, had ad libitum access to lab chow and water, and were group-housed in a colony maintained at 23 °C with a 12-h light/dark cycle (lights on at 0700 h). Studies were conducted according to the University of Connecticut and NIH guidelines for animal care and use.

2.2. Drugs and selection of doses

Pilocarpine (Sigma Aldrich Chemical, St. Louis, MO, USA) was dissolved in 0.9% saline. The adenosine A_{2A} antagonist 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-tetra-hydropurin-3-yl]propyl] ester) was synthesized at the Pharmazeutisches Institut (Universität Bonn; Bonn, Germany), and dissolved in 0.9% saline. MSX-3 is a pro-drug of the active adenosine A_{2A} antagonist, MSX-2. Extensive pilot work was performed to determine doses, and the dose of 1.0 mg/kg pilocarpine used in experiments 2–4 was based upon the results of the first experiment.

2.3. Behavioral procedure: tremulous jaw movements

Observations took place in a 11.5 × 9.5 × 7.5 cm clear glass chamber with a mesh floor, which was elevated 26 cm from the table top. TJMs 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 in mice.

2.4. Experiments

Experiment 1: ability of pilocarpine to induce tremulous jaw movements

Eleven male C57BL/6 mice were used to assess the effect of pilocarpine on TJMs. Mice received IP injections of either 1.0 ml/kg saline or 0.25, 0.5, 0.75, or 1.0 mg/kg pilocarpine in a within-groups design, with all mice receiving all treatments in a randomly varied order (once per week; no treatment sequences were repeated). Five minutes after injection, mice were placed in the observation chamber and allowed 5 min to habituate, after which TJMs were counted for 10 min.

Experiment 2: freeze-frame video analysis of local frequency of the tremulous jaw movements induced by pilocarpine

Three male C57BL/6 mice received an IP injection of 1.0 mg/kg pilocarpine. After five minutes, mice were placed in a flat bottomed mouse restrainer (myNeuroLab.com, Richmond, IL) so that a consistent view of the orofacial area could be achieved. After habituating for 5 min, each mouse was recorded for 15 min using a FlipVideo UltraHD (Cisco Systems, Farmington, CT). The sections of video that allowed for clear observation of the orofacial area were 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 s 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.

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

Eleven male C57BL/6 mice were used to assess the effects of the adenosine A_{2A} antagonist MSX-3 on the TJMs induced by 1.0 mg/kg pilocarpine. 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 were repeated). On the test day each week, each mouse was given an IP injection of either 1.0 ml/kg saline or 2.5, 5.0, or 10.0 mg/kg MSX-3. After Ten minutes, all mice received an IP injection of 1.0 mg/kg pilocarpine. Five minutes after injections, mice were placed in the observation chamber and allowed 5 min to habituate, after which TJMs were counted for 10 min.

Experiment 4: ability of pilocarpine to induce tremulous jaw movements in mice with a knockout of the adenosine A_{2A} receptor

A total of 24 male C57BL/6 mice ($n=12$ postnatal neuronal A_{2A} receptor conditional KO mice ($A_{2A}^{-/-}$); $n=12$ littermate controls ($A_{2A}^{+/+}$)) were used to assess the effect of the knockout of the adenosine A_{2A} receptor on pilocarpine-induced TJMs. For this experiment, only homozygous A_{2A} KO mice and littermate controls were used. All mice received an IP injection of 1.0 mg/kg pilocarpine. Five minutes after IP injection, mice were placed in the observation chamber and allowed 5 min to habituate, after which TJMs were counted for 10 min by an observer blind to the condition of the mouse (i.e. littermate control vs. A_{2A} KO).

2.5. Data analyses

The data for experiments 1 and 3 were analyzed using a repeated measures analysis of variance (ANOVA). Average TJMs over the two five-min observation periods were calculated and then used in the ANOVA calculations (SPSS 12.0 for Windows). 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. The behavioral data from the knockout experiment (Experiment 4) was analyzed using Student's t-test for independent samples.

3. Results

3.1. Experiments 1 and 2: ability of pilocarpine to induce tremulous jaw movements.

There was a significant overall effect of pilocarpine treatment on TJM activity (Fig. 1A; $F(4, 40)=24.46$; $p<0.001$). All doses of pilocarpine significantly induced TJMs (planned comparisons, $p<0.001$) compared to the vehicle condition. Fig. 1B displays the results of the freeze-frame video analyses. A total of 509 jaw movements were analyzed. About 83.69% of these jaw movements took place within "bursts," defined as a group of at least two jaw movements that were within 1.0 s of each other. 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 movements in 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 per second to 7.5 Hz, etc.) The majority (77.60%) of the TJMs took place in the 3.0-7.5 Hz frequency range. There were no jaw movements in the 1-3 Hz or > 10 Hz bins.

3.2. Experiments 3 and 4: ability of adenosine A_{2A} receptor antagonism and knockout attenuate the tremulous jaw movements induced by pilocarpine

The adenosine A_{2A} antagonist MSX-3 attenuated the TJMs induced by 1.0 mg/kg pilocarpine (Fig. 2A). There was a significant overall effect of MSX-3 treatment on pilocarpine-induced TJMs ($F(3,30)=35.88$; $p<0.001$), and the 2.5, 5.0 and 10.0 mg/kg doses of MSX-3 significantly reduced the pilocarpine-induced TJMs (planned comparisons, $p<0.05$). Fig. 2B shows that adenosine A_{2A} receptor neuronal knockout mice ($A_{2A}^{-/-}$) showed significantly fewer pilocarpine-induced TJMs than their littermate controls ($(A_{2A}^{+/+})$; $t=2.45$, $df=22$; $p<0.05$).

4. Discussion

These studies describe the development of a mouse model of TJM activity. Pilocarpine has consistently been shown to induce TJMs in rats (Salamone et al., 1986, 1998; Finn et al., 1997; Betz et al., 2007; Collins et al., 2010), so the first experiment investigated the ability of the pilocarpine to induce TJMs in C57BL/6 mice. Pilocarpine induced TJM

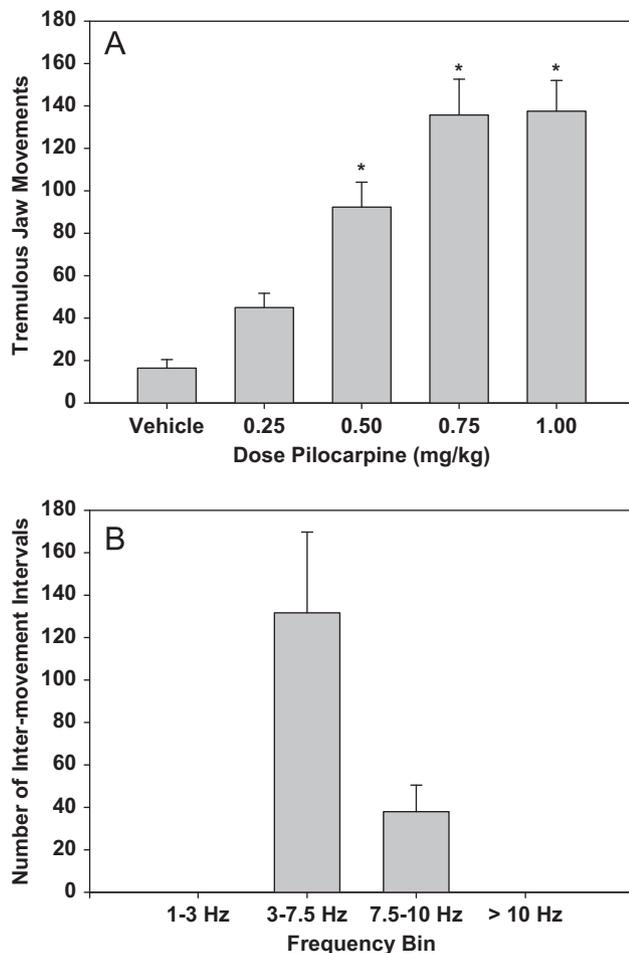


Fig. 1 (A) Effects of different doses of pilocarpine (IP) on tremulous jaw movements. Mean (\pm SEM) number of jaw movements in mice ($n=11$) treated with either saline vehicle or pilocarpine. **Significant difference from vehicle control ($p<0.05$). (B) This figure shows the results of the freeze-frame analysis of inter-movement intervals using the video analysis methods described above. Inter-movement times were determined by freeze-frame analysis of video obtained from three mice treated with 1.0 mg/kg pilocarpine, and were assigned to one of four local frequency bins. Distribution of the mean (\pm SEM) number of inter-movement intervals within each frequency bin is shown.

activity in C57BL/6 mice at all doses tested (i.e. 0.25–1.0 mg/kg). This is consistent with the previous research indicating that the administration of pilocarpine induced TJMs in 129SvEv (50%) \times CF1 (50%) mice (Salamone et al., 2001). Local frequency analysis of the pilocarpine-induced TJMs in mice indicated that pilocarpine-induced TJMs occurred largely in the 3–7.5 Hz frequency range, which is consistent with the findings from previous studies of the local frequency of TJMs induced by DA depletion, D2 antagonism, and administration of cholinomimetic drugs in rats (Ishiwari et al., 2005; Collins et al., 2010; Collins-Praino et al., 2011). Moreover, this 3–7.5 Hz frequency range is similar to that reported during resting tremor in parkinsonian patients (Deuschl et al., 2001). These findings are consistent with the hypothesis that pilocarpine-induced

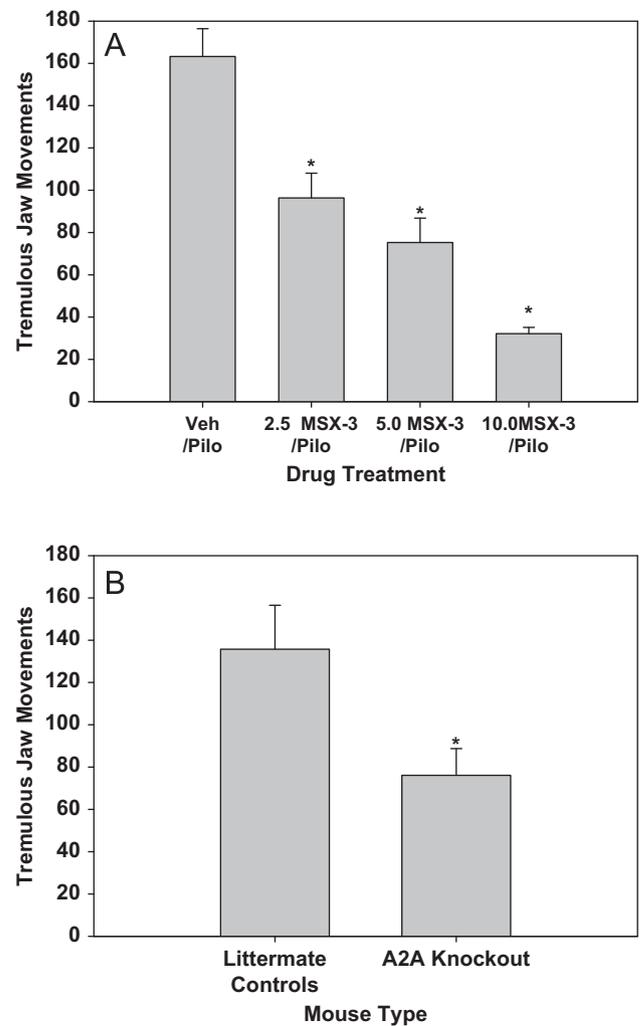


Fig. 2 (A) Effect of the adenosine A_{2A} antagonist MSX-3 on the tremulous jaw movements induced by 1.0 mg/kg pilocarpine. Mean (\pm SEM) number of jaw movements in mice ($n=11$) treated with pilocarpine plus vehicle (Veh/Pilo), and pilocarpine (Pilo) plus various doses (2.5, 5.0 and 10.0 mg/kg IP) of MSX-3. *Significant difference from pilocarpine plus vehicle control ($p<0.05$). (B) Effect of neuronal adenosine A_{2A} receptor knockout on the tremulous jaw movements induced by 1.0 mg/kg pilocarpine. Mean (\pm SEM) number of jaw movements in knockout mice ($n=12$) and littermate controls ($n=12$) treated with pilocarpine. *Significant difference from littermate controls ($p<0.05$).

TJMs pilocarpine are potentially a useful mouse model of parkinsonian resting tremor. Also, the finding that pilocarpine is capable of significantly inducing TJMs in mice highlights the role that ACh plays in striatal motor functions related to parkinsonism. Cholinomimetic drugs, such as muscarinic agonists and anticholinesterases used for the treatment of Alzheimer's disease, have been shown to induce or exacerbate parkinsonian symptoms, including tremor, in humans (Song et al., 2008; Collins-Praino et al., 2011). Furthermore, muscarinic receptor antagonists have been used as treatments for the motor symptoms of parkinsonism (Bezchlibnyk-Butler and Remington, 1994).

Adenosine A_{2A} antagonists have emerged as a potential treatment of parkinsonian motor impairments. One clinical report suggested that tremor was particularly sensitive to the effects of adenosine A_{2A} antagonism (Bara-Jimenez et al., 2003). Adenosine A_{2A} receptors are highly expressed in neostriatum, and A_{2A} antagonists exert motor effects in rodents and primates that are consistent with antiparkinsonian actions (Ferré et al., 2008; Chen et al., 2001; Salamone et al., 2008; Collins et al., 2010). For that reason, the final two experiments investigated the ability of adenosine A_{2A} receptor antagonism or genetic deletion to attenuate pilocarpine-induced TJMs. The adenosine A_{2A} antagonist MSX-3 significantly attenuated pilocarpine-induced TJMs in mice, which is consistent with previous findings in rats (Correa et al., 2004; Simola et al., 2004; Salamone et al., 2008; Pinna et al., 2010; Collins et al., 2010, 2011). Furthermore, deletion of the adenosine A_{2A} receptor also resulted in significantly lower levels of pilocarpine-induced TJMs compared to wild-type mice. This is consistent with previous research showing that knockout of the adenosine A_{2A} receptor is capable of reversing the catalepsy induced by the DA D1 antagonist SCH 23390, the D2 antagonist haloperidol, and the muscarinic agonist pilocarpine (El Yacoubi et al., 2001). Moreover, genetic deletion of the adenosine A_{2A} receptor in mice has been shown to alter the locomotor response to adenosine antagonists (Yu et al., 2008), and to affect amphetamine sensitization (Chen et al., 2003), self-administration of cocaine and MDMA (Ruiz-Medina et al., 2011), aspects of cognition (Wei et al., 2011), and effort-related choice behavior (Pardo et al., 2012). Furthermore, mice lacking striatal adenosine A_{2A} receptors showed an absence of motor stimulation in response to adenosine A_{2A} antagonists (Yu et al., 2008; Wei et al., 2011).

The present results demonstrate the feasibility of using the TJM model in mice, and indicate that adenosine A_{2A} receptor antagonism and deletion are capable of reducing cholinomimetic-induced TJMs in mice. These findings add to growing evidence demonstrating that adenosine A_{2A} function is involved in regulating motor functions in animals that are potentially related to parkinsonism. Additional studies should further characterize the effects of adenosine A_{2A} receptor deletion on motor function, and should investigate the effects regionally-specific knockout of A_{2A} receptors (e.g. Lazarus et al., 2011).

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Contributors

All authors contributed significantly to this manuscript. The work is part of the Ph.D. dissertations of L. Collins and M. Pardo. L. Collins, M. Pardo, S. Podurgiel and M. Correa performed the behavioral studies. Y. Baqi and C.E. Müller provided the MSX-3, and M.

Schwarzschild provided the knockout mice. J. Salamone and M. Correa supervised the entire project.

Conflict of interest

There are no conflicts of interest connected to this work. In addition to the income received from my primary employer, compensation has been received from Merck-Serono and Pfizer within the last 3 years. There are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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