

Repeated Dosing with Oral Allosteric Modulator of Adenosine A1 Receptor Produces Tolerance in Rats with Neuropathic Pain

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Background: The positive allosteric adenosine receptor modulator, T62 (2-amino-3-(4-chlorobenzoyl)-5,6,7,8-tetrahydrobenzothiofene), has been shown to reduce mechanical allodynia in a rat model of neuropathic pain. However, whether chronic oral T62 retains efficacy in this pain model has not been examined. Therefore, the authors studied antiallodynic effects of chronic oral T62 in spinal nerve-ligated rats, as well as motor and sedative behavioral effects.

Methods: Oral T62, 100 mg/kg, or oral oil was applied daily to spinal nerve-ligated rats for 4 weeks, with rat weights examined daily. Sedation, placing and ambulation scores, and withdrawal threshold were observed for 3 h daily for the first 2 weeks and then once a week. At the end of observation, the animals were killed, and the spinal tissues were collected for radioligand binding. In addition, withdrawal thresholds were also observed in rats with 5 days of treatment with 50 mg/kg oral T62. Furthermore, the effects of intrathecal adenosine on rats with oral T62 or oil treatment were compared.

Results: Chronic oral T62, at 100 mg/kg, initially returned the withdrawal threshold to mechanical testing to preinjury levels, with minor or no sedative or motor effects. Tolerance was observed, with a 60% loss of most possible effects in antiallodynia within 5 days of daily administration. Similarly, tolerance also occurred with chronic oral T62 at 50 mg/kg but did not alter the effect of intrathecal adenosine. Furthermore, 4 weeks of exposure to 100 mg/kg T62 resulted in a small reduction in spinal cord A1 receptor number.

Conclusion: The results imply that chronically administered A1 adenosine modulators lose efficacy over time, partly as a result of receptor down-regulation.

CHRONIC pain from various etiologies, including inflammation or neural destruction, is often resistant to treatment with simple analgesics or traditional agents, including opioids.¹ Neuropathic pain, accompanied by hypersensitivity to mechanical or thermal stimuli, is often difficult to treat.² Because of this relative lack of response to current analgesics, neuropathic pain associated with hyperalgesia and allodynia represents an unmet medical need. Among several novel approaches that

seem promising in relief of neuropathic pain, adenosine receptor agonists have attracted considerable attention.^{3,4}

Although adenosine agonists were shown to attenuate pain behaviors in a variety of animal models, adenosine agonists are also implicated in the regulation of many physiologic processes, especially cardiovascular and neurologic, which either reduce the activity of excitable tissues (e.g., by slowing heart rate) or increase the delivery of metabolic substrates (e.g., inducing vasodilation). These multiple physiologic functions controlled by adenosine receptors result in some undesirable side effects from direct adenosine receptor agonists, including headache, nausea,⁵ motor weakness,^{6,7} chest pain, and atrioventricular block,⁸ which limit their clinical application. To avoid these undesirable effects, indirect approaches, such as allosteric adenosine receptor modulation or inhibition of adenosine kinase^{9,10} or adenosine deaminase,¹¹ have been examined and show antinociceptive effects in neuropathic rats. Among these indirect approaches, allosteric adenosine receptor modulation represents a novel drug development direction to potentiate A1 receptor function and agonist binding *via* a conformation change.^{12,13} An adenosine receptor modulator, T62, belonging to a group of thiophene compounds first described a decade ago,¹⁴ has been shown to reduce mechanical allodynia in spinal nerve-injured rats after oral, parenteral, or intrathecal application.^{15,16} However, sustained efficacy and the presence of side effects after chronic oral administration of T62 have not been examined.

The purpose of the current study was to evaluate the effect of chronic oral T62 on mechanical allodynia as well as on sedation and motor behavior in rats after spinal nerve ligation (SNL). In addition, adenosine A1 receptor regulation after chronic oral T62 administration was examined.

Materials and Methods

Spinal Nerve Ligation

After approval by the Animal Care and Use Committee (Wake Forest University School of Medicine, Winston-Salem, North Carolina), male Sprague-Dawley rats (Harran, Indianapolis, IN) weighing 180–200 g underwent SNL under halothane anesthesia as previously described.¹⁷ The left L5 and L6 spinal nerves were isolated adjacent to the vertebral column and tightly ligated with 6-0 silk sutures distal to the dorsal root ganglion. After surgery, animals were housed individually, with free

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access to food and water, and were allowed to recover for at least 2 weeks. Left paw tactile allodynia was confirmed at this time by measuring the hind paw withdrawal threshold in response to application of von Frey filaments, using an up-down method previously described.¹⁸ Withdrawal threshold was determined in all animals 1–3 days before SNL, and this value was considered to be their normal threshold thereafter. Only animals with a withdrawal threshold less than 4 g after SNL surgery were used. Rats showing neurologic deficits were immediately killed by an overdose of pentobarbital. In some rats, an intrathecal catheter was inserted as previously described¹⁹ under halothane anesthesia by insertion of PE-10 tubing through a small hole in the cisterna magnum and directed caudal 8 cm such that the tip lies in the intrathecal space at the lumbar enlargement. Rats showing neurologic deficits were immediately killed by an overdose of pentobarbital; the other animals were allowed to recover for 4 or 5 days before drug testing.

Oral T62 Treatment at 100 mg/kg

Two groups of SNL rats were prepared according to the procedure mentioned in the section Spinal Nerve Ligation. After the development of allodynia from SNL surgery, the first group of 14 rats received 100 mg/kg oral T62 daily through an oral gavage needle for 4 weeks. T62 was dissolved in vegetable oil at a concentration of 25 mg/ml, with an injection volume of 4 ml/kg. The second group of 8 rats received 4 ml/kg oral vegetable oil daily as control for 4 weeks. Withdrawal thresholds were determined before and 1, 2, and 3 h after oral injection daily for the first 5 days and then once a week for 3 more weeks. Rat weights were recorded daily for 4 weeks. Sedation scores (–1/0), reflex placing scores (2/1/0), and ambulation scores (2/1/0) as previously described²⁰ were also recorded before and 1, 2, and 3 h after injection daily for the first 5 days and then once a week for 3 more weeks.

All animals were killed after 4 weeks of treatment with oral T62 or vegetable oil, and spinal cord tissue was collected for [³H]8-cyclopentyl-1,3-dipropylxanthine ([³H]DPCPX) radioligand binding assay. Withdrawal threshold data are presented as mean ± SEM and were analyzed by two-way analysis of variance followed by Fisher protected least significant difference test.²¹ $P < 0.05$ was considered significant.

Oral T62 Treatment at 50 mg/kg

A group of 13 SNL rats was prepared according to the procedure mentioned in the section Spinal Nerve Ligation. Among them, 11 SNL rats received daily treatment with 50 mg/kg oral T62. Withdrawal thresholds were determined daily over a 5-day period.

Intrathecal Adenosine Effects in Oral T62-treated Rats

Two groups of four SNL rats with intrathecal catheters were prepared according to the procedure mentioned in the section of SNL. One group of four rats was treated with 50 mg/kg oral T62 daily for 5 days, and the other group was treated daily with the same volume of oral vegetable oil as control. On the sixth day, both groups of rats were treated with 10 μg intrathecal adenosine in a volume of 10 μl followed by 10 μl saline to clear the catheter dead space. Hind paw withdrawal threshold was recorded before; 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min after; and 20 h after adenosine administration.

[³H]DPCPX Membrane Binding

Examination of A1 receptor density in spinal cord was performed with radioligand binding, using [³H]DPCPX (New England Nuclear Corp., Boston, MA). Preliminary experiments were performed to optimize binding conditions for pH, buffer components, adenosine deaminase concentration, time, temperature, and protein concentration. Spinal cord from rats from each group was quickly collected after euthanasia and stored at –80°C until use. Spinal cords from two or three animals per group were prepared together. All tissue was stored for 2–4 weeks. Tissue was thawed, quickly chopped, and then suspended in Tris buffer (170 mM, pH 7.4). Tissue was homogenized and then disrupted by sonication (5–7 s). The suspension was centrifuged at 3,000 rpm at 4°C for 15 min. The supernatant was then centrifuged at 23,000 rpm for 30–40 min at 4°C. The pellet was resuspended again in the same Tris buffer and disrupted by sonication for 5 s.

Radioligand binding experiments were performed with 250-μl aliquots of tissue in a final incubation volume of 1 ml together with 1 U adenosine deaminase and increasing concentrations of [³H]DPCPX (90 pM–7 nM). Nonspecific binding was defined in the presence of 100 μM adenosine. Tissues were incubated at room temperature for 1 h and then terminated by vacuum filtration over GF/B filters on a cell harvester (Brandel, Gaithersburg, MD) with three washes of 4 ml cold 170 mM Tris buffer. All experiments were performed in duplicate. Protein concentrations were determined by the method of Bradford.²² Radioactivity binding was quantified by immersion of filters in 10 ml scintillation fluid counted in a scintillation counter. Maximum binding (Bmax) and binding dissociation constant (Kd) values were calculated by nonlinear regression using GraphPad Prism (GraphPad, San Diego, CA), and best-fit values were compared with a *t* test.

Materials

T62 was provided by King Pharmaceuticals (Cary, NC). T62 was gradually dissolved in vegetable oil to a final

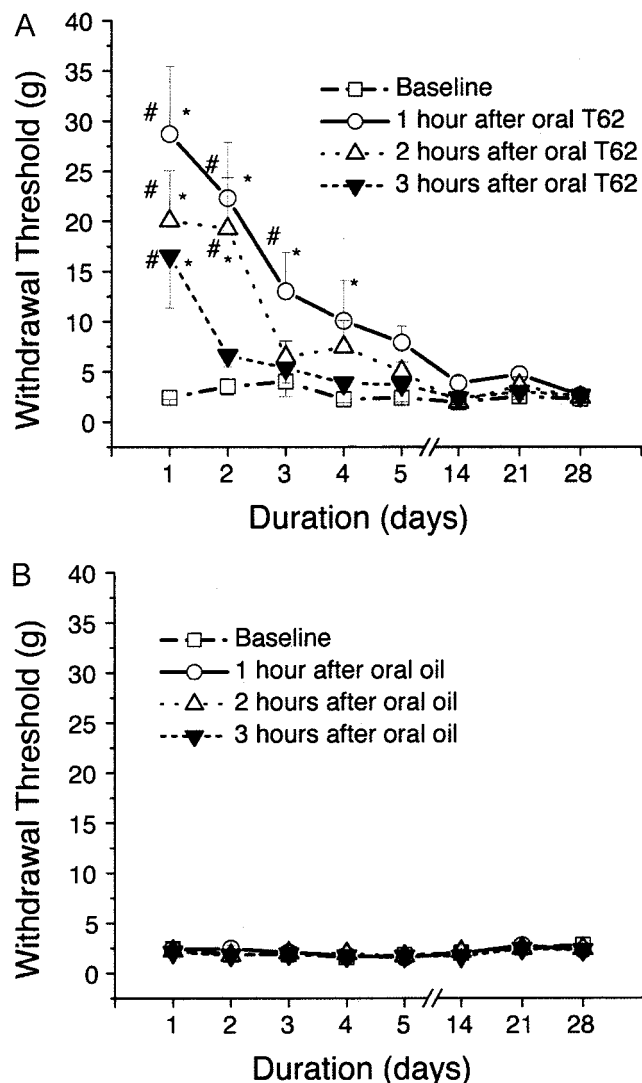


Fig. 1. Time course of hind paw withdrawal threshold over time after administration of (A) chronic oral T62 at 100-mg/kg dose and (B) chronic oral vegetable oil. Each value represents the mean \pm SE. * $P < 0.01$, two-way repeated-measures analysis of variance (Fisher protected least significant difference [PLSD] method) for drug effect against baseline in each study day ($n = 12-14$). # $P < 0.01$, two-way analysis of variance (Fisher PLSD method) for drug effect against other test days ($n = 8$).

concentration of 25 mg/ml as a stock solution for oral injection. Adenosine was obtained from Fujisawa USA, Inc. (Deerfield, IL) at a 3-mg/ml solution. The remaining chemicals were from Sigma Chemical Co. (St. Louis, MO).

Results

Antinociceptive Effects of Chronic Oral T62

Before surgery, the average withdrawal threshold for these animals is around 18.5 g. After SNL surgery, the withdrawal threshold is decreased to an average of 2.5 g. During the 4-week treatment period, oral T62 increased the withdrawal threshold for 3 h after administration on day 1 of treatment, but this effect was lost by 60% in

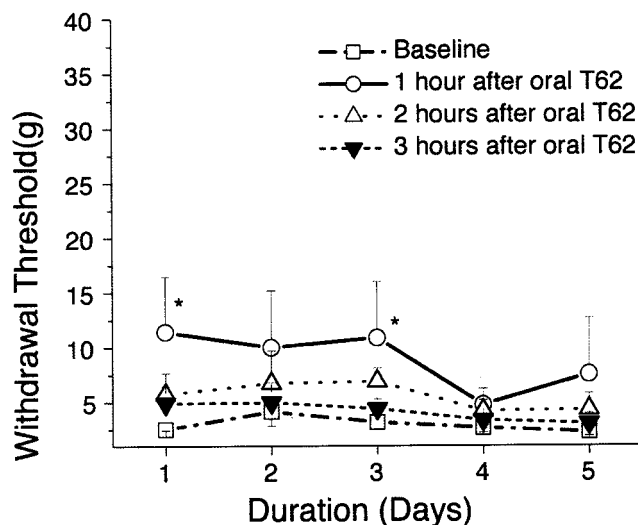


Fig. 2. Time course of hind paw withdrawal threshold over time after administration of chronic oral T62 at 50-mg/kg dose. Each value represents the mean \pm SE of 13 rats. * $P < 0.01$, two-way repeated-measures analysis of variance (Fisher protected least significant difference method) for drug effect against baseline in each study day.

most possible effects (1 h after injection) on the fifth day (fig. 1A). Oral vegetable oil did not affect withdrawal threshold (fig. 1B). A smaller dose of T62 (50 mg/kg) exhibited a small but statistically significant increase in withdrawal threshold 1 h after T62 administration on day 1 of treatment, but tolerance also developed to this dose within 5 days (fig. 2).

General Behavioral Observations on Chronic Oral T62

Animals receiving oral T62 at the 100 mg/kg dose appeared normal, and animals in both the T62 and con-

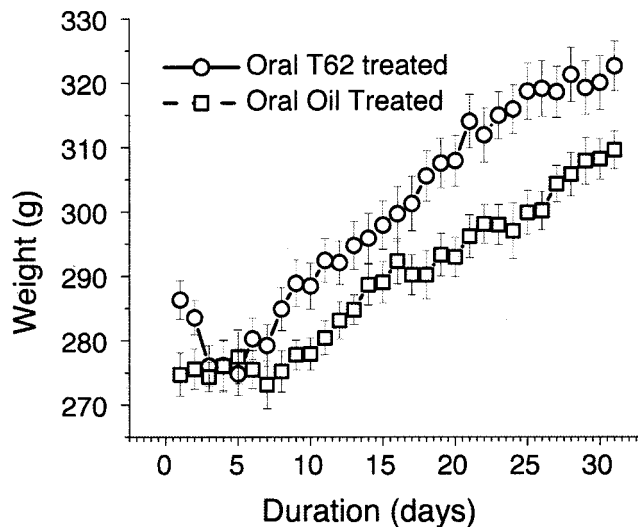


Fig. 3. Time course of weight gain after administration of chronic oral T62 at 100-mg/kg dose or chronic vegetable oil. Each value represents the mean \pm SE ($n = 12$ for oral T62-treated rats, $n = 7$ for oral oil-treated rats).

Table 1. Sedation Scores (–1/0) in: Oral T62 (100 mg/kg)-treated Rats and Oral Oil-treated Rats

Time after Treatment	Day							
	1	2	3	4	5	13	20	27
Oral T62-treated rats								
0	0/14	0/14	0/14	1/13	0/14	0/14	0/12	0/12
1 h	10/4*	12/2*	7/7*	3/11	7/7*	4/10	3/9	3/9
2 h	7/7*	11/3*	4/10	2/12	3/11	2/12	1/11	1/11
3 h	7/7*	8/6*	3/11	1/13	2/12	2/12	2/10	1/11
Oral Oil-treated rats								
0	0/8	0/8	0/8	0/8	0/8	0/8	0/7	0/7
1 h	0/8	0/8	0/8	0/8	0/8	0/8	0/7	0/7
2 h	0/8	0/8	0/8	0/8	0/8	0/8	0/7	0/7
3 h	0/8	0/8	0/8	0/8	0/8	0/8	0/7	0/7

* $P < 0.05$, Fisher exact test for oral T62 treatment against oral oil treatment in the same study period.

tol groups gained weight during the 4-week study in a similar growth pattern (fig. 3). They had a transient weight loss, which may have been because their food intake was affected by the vehicle, vegetable oil.

In addition, oral T62 produced behavioral sedation for 3 h on the first day of the treatment, and this effect was less pronounced with chronic dosing (table 1). In contrast, oral T62 had no effect on reflex placing or ambulation behavior at any observation time (tables 2 and 3). Vegetable oil had no effect on any of these measures.

[³H]DPCPX Radioligand Binding

The effect of chronic T62 treatment on spinal adenosine A1 receptor was demonstrated by [³H]DPCPX ra-

dioligand binding. When rats were treated with 100 mg/kg for 4 weeks, the Bmax for [³H]DPCPX was decreased by a small but statistically significant amount, from 561 ± 20 fmol/mg protein in control rats to 512 ± 11 fmol/mg protein in chronic T62-treated rats ($P < 0.05$; fig. 4).

Antiallodynic Effects of Intrathecal Adenosine in Oral T62-treated Rats

Although 50 mg/kg T62 for 5 days resulted in a loss of its antiallodynic effect, intrathecal adenosine alleviated mechanical allodynia due to the SNL injury similarly in rats treated chronically with oral oil or T62 (fig. 5).

Table 2. Placing Scores (2/1/0) in Oral T62 (100 mg/kg)-treated Rats and Oral Oil-treated Rats

Time after Treatment	Day							
	1	2	3	4	5	13	20	27
Oral T62-treated rats								
0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	12/0/0	12/0/0
1 h	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	12/0/0	12/0/0
2 h	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	12/0/0	12/0/0
3 h	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	12/0/0	12/0/0
Oral oil-treated rats								
0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	7/0/0	7/0/0
1 h	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	7/0/0	7/0/0
2 h	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	7/0/0	7/0/0
3 h	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	7/0/0	7/0/0

Table 3. Ambulation Scores (2/1/0) in Oral T62 (100 mg/kg)-treated rats and Oral Oil-treated Rats

Time after Treatment	Day							
	1	2	3	4	5	13	20	27
Oral T62-treated rats								
0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	12/0/0	12/0/0
1 h	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	12/0/0	12/0/0
2 h	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	12/0/0	12/0/0
3 h	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	14/0/0	12/0/0	12/0/0
Oral oil-treated rats								
0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	7/0/0	7/0/0
1 h	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	7/0/0	7/0/0
2 h	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	7/0/0	7/0/0
3 h	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	8/0/0	7/0/0	7/0/0

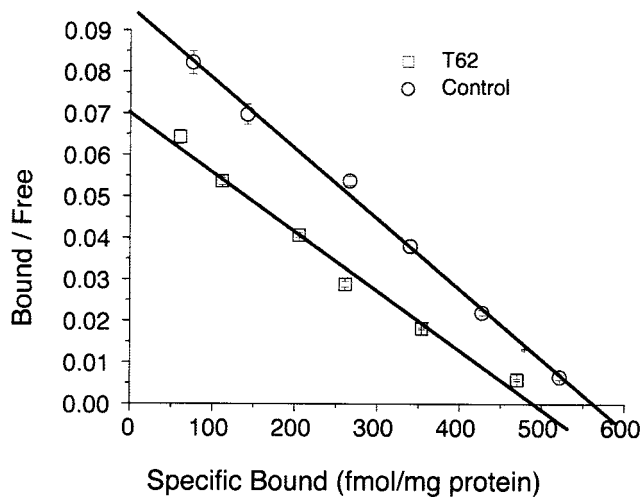


Fig. 4. Spinal cord [3 H]DPCPX membrane binding in spinal nerve-ligated rats receiving chronic treatment with vegetable oil or T62 at 100-mg/kg daily dose for 30 days. In T62-treated rats, maximum binding (B_{max}) is 512 ± 10.93 fmol/mg protein,* and binding dissociation constant (K_d) is 522.1 ± 33.05 pM.* In oral oil-treated rats, B_{max} is 560.5 ± 19.68 fmol/mg protein, and K_d is 380.3 ± 42.71 pM. * $P < 0.05$ compared with controls.

Discussion

The current study shows that oral T62 reduces mechanical allodynia in rats with nerve injury, consistent with our previous observations.^{15,16} There is rapid tolerance to T62 with chronic administration, associated with a decrease in spinal cord adenosine A1 receptor number but, paradoxically, not of the acute effect from intrathecal adenosine.

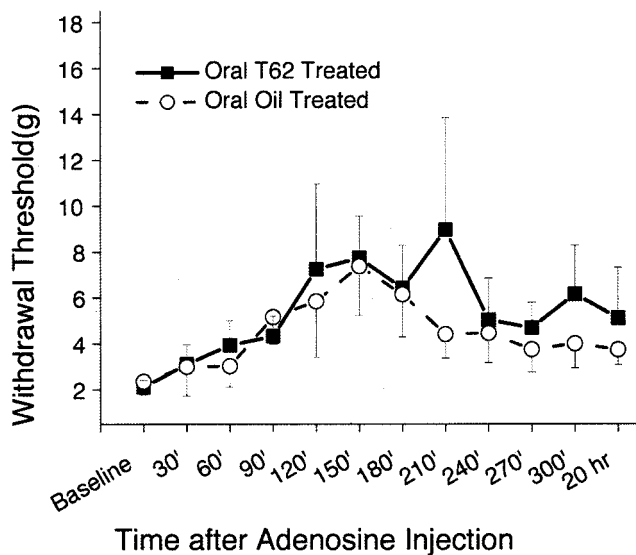


Fig. 5. Time course of hind paw withdrawal threshold over time after administration of intrathecal adenosine in spinal nerve-ligated rats after chronic treatment with T62 at 50-mg/kg dose or vegetable oil. Each value represents the mean \pm SE of four rats in each group. * $P > 0.05$, two-way repeated-measures analysis of variance (Fisher protected least significant difference method) for T62 against oil treatment.

The A1 receptor is primarily coupled to the Gi/o class of G proteins. Chronic activation of A1 receptors has been tested in different pain models. Tolerance does not occur to acute noxious heat stimuli in normal animals²³ or to mechanical stimuli in SNL rats²⁴ after chronic administration of adenosine kinase inhibitors for 5 days, nor does intrathecal adenosine lose its efficacy to treat tactile allodynia in a patient with degenerative neuro-metabolic disorder.²⁵ However, intrathecal R-phenylisopropyl-adenosine loses its efficacy to treat hypersensitivity to mechanical and thermal stimuli in a rat model of central pain after ischemic spinal cord injury²⁶ and to prolong latency to noxious heat in the tail-flick test in normal mice.^{27,28}

Traditionally, drug tolerance is considered to come from either receptor desensitization or receptor down-regulation. A previous study suggested that A1 receptor desensitization might be a factor in the tolerance after chronic exposure to A1 receptor agonists.²⁹ However, the current study indicates that intrathecal adenosine had a similar effect in control and T62-treated rats after 5 days' exposure to T62, suggesting receptor desensitization is unlikely.

Chronic exposure of receptors to their agonists often alters receptor expression, although not always in the same fashion. For example, the receptor number is down-regulated with chronic exposure to opioid agonists³⁰ but is up-regulated with chronic exposure to nicotinic acetylcholine receptor agonists.³¹ Chronic intrathecal R-phenylisopropyl-adenosine exposure does not change A1 receptor expression in the mouse brain.²⁷ The effect of allosteric modulators depends on many factors, including endogenous agonist activity. Long-term exposure of PD 81,723, a well-studied A1 receptor modulator, down-regulates A1 receptors to a small degree.³² Similarly, T62 in the current study down-regulated the A1 receptor number by 10%. This minor down-regulation could result in reduced efficacy of this modulator if endogenously released adenosine remains constant but could allow for a near-full effect of maximum receptor stimulation after exogenous, intrathecal administration of adenosine.

In summary, as with other G protein-coupled receptor analgesics (opioids, α_2 -adrenergic agonists), the current study shows a clear tolerance to the effects of T62 with chronic oral treatment, at least partially due to receptor down-regulation.

References

1. McQuay HJ: Pharmacological treatment of neuralgic and neuropathic pain. *Cancer Surv* 1988; 7:141-59
2. Kingery WS: A critical review of controlled clinical trials for peripheral neuropathic pain and complex regional pain syndromes. *Pain* 1997; 73:123-39
3. Sawynok J: Adenosine receptor activation and nociception. *Eur J Pharmacol* 1998; 347:1-11
4. Belfrage M, Segerdahl M, Arner S, Sollevi A: The safety and efficacy of

- intrathecal adenosine in patients with chronic neuropathic pain. *Anesth Analg* 1999; 89:136-42
5. Biaggioni I, Onrot J, Hollister AS, Robertson D: Cardiovascular effects of adenosine infusion in man and their modulation by dipyrindamole. *Life Sci* 1986; 39:2229-36
 6. Karlsten R, Gordh T Jr, Hartvig P, Post C: Effects of intrathecal injection of the adenosine receptor agonists R-phenylisopropyl-adenosine and N-ethylcarboxamide-adenosine on nociception and motor function in the rat. *Anesth Analg* 1990; 71:60-4
 7. Lee YW, Yaksh TL: Pharmacology of the spinal adenosine receptor which mediates the antiallodynic action of intrathecal adenosine agonists. *J Pharmacol Exp Ther* 1996; 277:1642-8
 8. Sylven C, Beermann B, Jonzon B, Brandt R: Angina pectoris-like pain provoked by intravenous adenosine in healthy volunteers. *BMJ (Clin Res Ed)* 1986; 293:227-30
 9. Keil GJ, DeLander GE: Spinally-mediated antinociception is induced in mice by an adenosine kinase-, but not by an adenosine deaminase-, inhibitor. *Life Sci* 1992; 51:L171-6
 10. Golembiowska K, White TD, Sawynok J: Adenosine kinase inhibitors augment release of adenosine from spinal cord slices. *Eur J Pharmacol* 1996; 307:157-62
 11. Lavand'homme PM, Eisenach JC: Exogenous and endogenous adenosine enhance the spinal antiallodynic effects of morphine in a rat model of neuropathic pain. *Pain* 1999; 80:31-6
 12. Mizumura T, Auchampach JA, Linden J, Bruns RF, Gross GJ: PD 81,723, an allosteric enhancer of the A1 adenosine receptor, lowers the threshold for ischemic preconditioning in dogs. *Circ Res* 1996; 79:415-23
 13. Kourounakis A, Visser C, de Groote M, IJzerman AP: Differential effects of the allosteric enhancer (2-amino-4,5-dimethyl-trienyl)[3-trifluoromethyl] phenylmethanone (PD81,723) on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A1 receptor. *Biochem Pharmacol* 2001; 61:137-44
 14. Bruns RF, Fergus JH: Allosteric enhancement of adenosine A1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol Pharmacol* 1990; 38:939-49
 15. Pan HL, Xu Z, Leung E, Eisenach JC: Allosteric adenosine modulation to reduce allodynia. *ANESTHESIOLOGY* 2001; 95:416-20
 16. Li X, Conklin D, Ma W, Zhu X, Eisenach JC: Spinal noradrenergic activation mediates allodynia reduction from an allosteric adenosine modulator in a rat model of neuropathic pain. *Pain* 2002; 97:117-25
 17. Kim SH, Chung JM: Sympathectomy alleviates mechanical allodynia in an experimental animal model for neuropathy in the rat. *Neurosci Lett* 1991; 134:131-4
 18. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53:55-63
 19. Yaksh TL, Rudy TA: Chronic catheterization of the spinal subarachnoid space. *Physiol Behav* 1976; 7:1032-6
 20. Chiari A, Yaksh TL, Myers RR, Provencher J, Moore L, Lee CS, Eisenach JC: Preclinical toxicity screening of intrathecal adenosine in rats and dogs. *ANESTHESIOLOGY* 1999; 91:824-32
 21. Shapira Y, Lam AM, Eng CC, Laohaprasit V, Michel M: Therapeutic time window and dose response of the beneficial effects of ketamine in experimental head injury. *Stroke* 1994; 25:1637-43
 22. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72:248-54
 23. Kowaluk EA, Kohlhaas KL, Bannon A, Gunther K, Lynch JJ III, Jarvis MF: Characterization of the effects of adenosine kinase inhibitors on acute thermal nociception in mice. *Pharmacol Biochem Behav* 1999; 63:83-91
 24. Jarvis MF, Yu H, McGaraughty S, Wismer CT, Mikusa J, Zhu C, Chu K, Kohlhaas K, Cowart M, Lee CH, Stewart AO, Cox BF, Polakowski J, Kowaluk EA: Analgesic and anti-inflammatory effects of A-286501, a novel orally active adenosine kinase inhibitor. *Pain* 2002; 96:107-18
 25. Gyllenhammar E, Nordfors LO: Systemic adenosine infusions alleviated neuropathic pain. *Pain* 2001; 94:121-2
 26. von Heijne M, Hao JX, Yu W, Sollevi A, Xu XJ, Wiesenfeld-Hallin Z: Reduced anti-allodynic effect of the adenosine A1-receptor agonist R-phenylisopropyladenosine on repeated intrathecal administration and lack of cross-tolerance with morphine in a rat model of central pain. *Anesth Analg* 1998; 87:1367-71
 27. Ahljianian MK, Takemori AE: Cross-tolerance studies between caffeine and (-)-N6-(phenylisopropyl)-adenosine (PIA) in mice. *Life Sci* 1986; 38:577-88
 28. Ahljianian MK, Takemori AE: Effects of (-)-N6-(R-phenylisopropyl)-adenosine (PIA) and caffeine on nociception and morphine-induced analgesia, tolerance and dependence in mice. *Eur J Pharmacol* 1985; 112:171-9
 29. Casati C, Monopoli A, Dionisotti S, Zocchi C, Bonizzoni E, Ongini E: Repeated administration of selective adenosine A1 and A2 receptor agonists in the spontaneously hypertensive rat: Tolerance develops to A1-mediated hemodynamic effects. *J Pharmacol Exp Ther* 1994; 268:1506-11
 30. Sharma SK, Nirenberg M, Klee WA: Morphine receptors as regulators of adenylate cyclase activity. *Proc Natl Acad Sci U S A* 1975; 72:590-4
 31. Buisson B, Bertrand D: Chronic exposure to nicotine upregulates the human (alpha)4(beta)2 nicotinic acetylcholine receptor function. *J Neurosci* 2001; 21:1819-29
 32. Bhattacharya S, Linden J: Effects of long-term treatment with the allosteric enhancer, PD81,723, on Chinese hamster ovary cells expressing recombinant human A1 adenosine receptors. *Mol Pharmacol* 1996; 50:104-11