Research Report

The role of nucleus accumbens adenosine–opioid interaction in mediating palatable food intake

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ABSTRACT
Nucleus accumbens $\mu$-opioid stimulation leads to robust increases in the intake of highly palatable foods, such as a high-fat diet. While interactions between opioids and certain striatal neurotransmitters underlying this phenomenon have been explored, many potential interactions have not. Striatal adenosine has been shown to have a significant influence on striatal neurotransmission and locomotor activity behavior, however the interaction between opioids and adenosine on feeding behaviors has received less attention. The present study explored this interaction within the context of opioid-driven consumption of a high-fat diet. Specifically, intra-accumbens administration of selective A1 and A2$\textsubscript{A}$ adenosine receptor ligands, with or without concurrent administration of the $\mu$-opioid agonist d-Ala$^2$,N-Me-Phe$^4$,Gly-$\text{ol}^\text{2}$-enkephalin (DAMGO), on high-fat consumption and associated locomotor activity was examined. The A1 receptor agonist 2-Chloro-N6-cyclopentyladenosine (CCPA) had no effect on either baseline or DAMGO-induced locomotor or consumption behaviors associated with the high-fat diet. However, the A2$\textsubscript{A}$ receptor agonist 2-p-(2 carboxyethyl)-phenethylamino-5$'$-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680) and the prodrug of the A2$\textsubscript{A}$ receptor antagonist MSX-2, 3-(3-hydroxypropyl)-8-(m-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3) produced the expected decrease and increase in locomotor activity, respectively. CGS 21680 had no effect on baseline or DAMGO-driven consumption of the high-fat diet. MSX-3 had no effect on DAMGO-induced locomotor activity but increased DAMGO-induced consumption. Lastly, the increased activity and consumption produced by MSX-3 alone was blocked by prior administration of the opioid antagonist naltrexone. In summary, these results suggest a potential role of striatal adenosine A2$\textsubscript{A}$ receptors in mediating baseline and striatal opioid-mediated intake of a high-fat diet.

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1. Introduction
It is well established that activation of $\mu$-opioid receptors within the nucleus accumbens increases the motivation to seek and consume food, preferentially highly palatable foods (Baldo and Kelley, 2007; Barbano and Cador, 2007; Kelley et al., 2005). In particular, intra-accumbens administration of the $\mu$-opioid receptor agonist d-Ala$^2$,N-Me-Phe$^4$,Gly-$\text{ol}^\text{2}$-enkephalin...
(DAMGO) elicits a powerful and consistent feeding response to palatable foods such as those high in fat and sugar (see Kelley et al., 2005 for review). While interactions between opioids and certain striatal neurotransmitters underlying this phenomenon have been explored (Kelley et al., 2000; MacDonald et al., 2004; Will et al., 2006), many potential interactions have not. Striatal adenosine has been shown to have a significant influence on neurotransmission and associated locomotor activity behaviors within the striatum (Ferré, 1997), however the role of adenosine in mediating feeding behaviors, including opioid-mediated feeding behaviors, has received less attention.

The role of striatal adenosine in opiate addiction has been widely studied (Coupar and Tran, 2002; Kaplan and Sears, 1996; Salem and Hope, 1997), while less is known of its impact on other opioid-mediated processes, such as the consumption of palatable food. There is evidence that striatal adenosine may be involved in mediating some aspects of consumption yet its precise role remains unclear (Burnstock, 2007). Adenosine is a major neuromodulator within the striatum, altering both neurotransmitter release and intra-cellular functioning (Dunwiddie, 1985; Schiffmann et al., 2007). The A1 and A2\_ receptor subtypes are distributed throughout the striatum with A2\_ receptors particularly abundant (Ferré, 2007; Ongini and Fredholm, 1996, Schiffmann et al., 2007). Within the striatum, adenosine receptors are localized upon enkephalinergic GABAergic neurons where they interact with the striatal opioid system (Ammon-Treiber and Höllt, 2005; Brundge and Williams, 2002; Franco et al., 2007; Halimi et al., 2000; Kaplan and Coyle, 1998; Schiffmann et al., 1991).

The ability of adenosine to alter opioid driven consumption of chow has been investigated, although receptor specific effects and site of action have not been determined. Systemically administered adenosine reduced the feeding induced by systemic opioid administration (Wager-Srdar et al., 1984). However, non-specific activation of adenosine receptors alone has produced both increases and decreases in chow consumption (Levine and Morley, 1983; Levine et al., 1989). This discrepancy is likely due to the degree to which A1 and A2\_ receptors are activated, as they produce opposing effects on neurotransmitter release (Franco et al., 2007). Recently, intra-accumbens administration of 3-(3-hydroxypropyl)-8-(m-methoxystyril)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3), prodrug of the A2\_ receptor antagonist MSX-2, was shown to decrease chow consumption in food-restricted rats (Nagel et al., 2003). While this implies intra-accumbens adenosine is involved in feeding driven by energy deficit, the influence of specific striatal adenosine receptors on reward-driven feeding in a sated state, such as that modeled by opioid-driven consumption of a high-fat diet, has not been explored. Given the implications from past research, the concentrated localization of adenosine receptors within the accumbens and their interaction with the opioid system, it can be posited that adenosine receptors may be involved in mediating reward-driven feeding.

The purpose of the present study was to explore the role of striatal A1 and A2\_ adenosine receptors in mediating intra-accumbens opioid-mediated binge feeding of a high-fat diet. The adenosine A1 agonist 2-Chloro-N6-cyclopentyladenosine (CCPA), the A2\_ agonist 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamido adenosine hydrochloride (CGS 21680), and 3-(3-hydroxypropyl)-8-(m-methoxystyril)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3), prodrug of the A2\_ receptor antagonist MSX-2 were each examined for their influence on baseline and DAMGO-induced high fat consumption and associated locomotor activity (Fig. 1).

### 2. Results

#### 2.1. Experiment 1. Intra-accumbens CCPA effect on baseline and DAMGO-induced feeding behaviors

An overall ANOVA revealed there was no effect of CCPA for locomotor activity ($F_{3,20}=0.314, \text{ns}$; Fig. 2A) and no effect of CCPA for high fat intake ($F_{3,20}=1.464, \text{ns}$) (Fig. 2B). Post-hoc analysis revealed that the highest dose of the A1 agonist CCPA (1 \text{\mu}M/0.25 \text{\mu}l/side) produced the greatest decrease in feeding ($p=0.055$), therefore that dose was chosen for the second part of Experiment 1.

In the second part of Experiment 1 in which a near threshold dose of DAMGO (0.025 \text{\mu}g/0.25 \text{\mu}l/side) was infused following infusion of CCPA (1 \text{\mu}M/0.25 \text{\mu}l/side) or saline, an overall ANOVA on locomotor activity data revealed a significant effect of treatment ($F_{2,15}=4.208, p < 0.05$) (Fig. 3A). Bilateral intra-accumbens administration of 0.025 \text{\mu}g DAMGO significantly increased activity compared to saline ($p<0.05$) while infusion of 1 \text{\mu} M CCPA had no effect on the DAMGO induced increase ($p=0.747$). An overall ANOVA also revealed a significant effect of treatment for high fat intake ($F_{2,15}=4.652, p<0.05$) (Fig. 3B). Bilateral intra-accumbens administration of DAMGO significantly increased high fat intake compared to saline alone ($p<0.05$), while the 1 \text{\mu} M dose of CCPA had no effect on the feeding induced by DAMGO ($p=0.570$).
2.2. Experiment 2. Intra-accumbens CGS 21680 effects on baseline and DAMGO-induced behaviors

An ANOVA conducted on locomotor activity revealed a significant effect of treatment ($F_{(7,56)}=2.209$, $p<0.05$) (Fig. 4A). While DAMGO produced no significant changes in feeding associated locomotor activity, bilateral administration of CGS 21680 decreased locomotor activity in a dose-dependent manner. The low dose of CGS 21680 had no effect on locomotor activity ($p=0.083$), while the middle ($p<0.05$) and high dose ($p<0.01$) of CGS 21680 attenuated normal activity levels.

An overall ANOVA also revealed a significant main effect of treatment ($F_{(7,56)}=8.3738$, $p<0.001$) (Fig. 4B). Bilateral administration of the near-threshold-dose of DAMGO (0.025 µg/0.25 µl/side) increased baseline high fat intake ($p<0.001$). While the low dose of MSX-3 (10 mM/0.25 µl/side) had no effect on feeding alone ($p=0.625$) and did not alter DAMGO-induced feeding ($p=0.553$). The high dose of MSX-3 (20 mM/0.25 µl/side) increased baseline high fat intake ($p<0.01$) and facilitated DAMGO-induced intake ($p<0.05$).

A separate analysis conducted on the interaction between naltrexone and the high dose of MSX-3 (20 mM/0.25 µl/side) revealed a significant effect of treatment for locomotor activity ($F_{(3,28)}=7.674$, $p<0.001$) (Fig. 5A). Bilateral infusion of naltrexone alone did not decrease locomotor activity ($p=0.315$) but did block the increase in locomotor activity observed following MSX-3 infusion ($p<0.01$). There was also a significant effect of treatment for high fat intake ($F_{(3,28)}=8.193$, $p<0.001$) (Fig. 5B). Bilateral administration of naltrexone alone did not decrease

2.3. Experiment 3. Intra-accumbens MSX-3 effects on baseline and DAMGO-induced behaviors

An overall ANOVA conducted on locomotor activity revealed a significant effect of treatment ($F_{(3,28)}=8.3738$, $p<0.001$) (Fig. 4B). Intra-accumbens administration of the A2A agonist CGS 21680 had no effect on the intake of high fat diet at the low dose (500 nM/0.25 µl/side) ($p=0.441$), middle dose (2.5 µM/0.25 µl/side) ($p=0.890$) or high dose (5.0 µM/0.25 µl/side) ($p=0.994$). A near-threshold dose of DAMGO (0.025 µg/0.25 µl/side) significantly increased high fat intake ($p<0.01$), as observed in previous studies (Zhang et al., 1998). However, pre-treatment of CGS 21680 had no effect on DAMGO-induced feeding at the low ($p=0.214$), middle ($p=0.133$) or high ($p=0.054$) dose.

An overall ANOVA conducted on high fat intake also revealed a significant main effect of treatment ($F_{(7,56)}=12.940$, $p<0.001$) (Fig. 5B). Bilateral administration of the near-threshold-dose of DAMGO increased baseline high fat intake ($p<0.01$). The low dose of MSX-3 (10 mM/0.25 µl/side) had no effect on feeding alone ($p=0.625$) and did not alter DAMGO-induced feeding ($p=0.553$). The high dose of MSX-3 (20 mM/0.25 µl/side) increased baseline high fat intake ($p<0.01$) and facilitated DAMGO-induced intake ($p<0.05$).
The A1 receptor agonist CCPA had no effect on high fat consumption and did not alter the behavioral response to μ-opioid system activation by intra-accumbens DAMGO. Alone, administration of CCPA into the accumbens produced no change in high fat intake yet produced a trend in decreasing locomotor activity, in line with previous research (Schwienbacher et al., 2002). Furthermore, CCPA had no effect on DAMGO-induced consumption or locomotor activity, suggesting a minimal role for striatal A1 receptors in mediating these well-established effects caused by opioid activation of the striatum.

Intra-accumbens administration of the A2A agonist CGS 21680 produced a dose-dependent decrease in baseline locomotor activity, in line with previous findings using intracranial (Barraco et al., 1993; Hauber and Münkle, 1997) or systemic administration (Antoniou et al., 2005; Cabeza de Vaca et al., 2007; Karcz-Kubicha et al., 2003). However, intra-accumbens infusion of the A2A agonist CGS 21680 had no effect on baseline or DAMGO-induced consumption of the high-fat diet. On the other hand, administration of the A2A antagonist MSX-3 produced significant behavioral changes.

### Figure 4
Effects of intra-accumbens adenosine A2A agonist CGS 21680 on locomotor activity (A) and high fat intake (B) prior to administration of saline or DAMGO (0.025 μg/0.25 μl/side). +p < 0.05, ++p < 0.01, compared to 0 nM CGS 21680-saline; *p < 0.05, compared to saline-0 nM CGS 21680. Error bars represent one SEM.

### Figure 5
Effects of intra-accumbens adenosine A2A antagonist MSX-3 on locomotor activity (A) and high fat intake (B) prior to administration of saline or DAMGO (0.025 μg/0.25 μl/side) and effects of intra-accumbens saline or the opioid antagonist naltrexone (20 μg/0.25 μl/side) on the high-dose (20 mM) of MSX-3-induced increases of locomotor activity (A) and high fat intake (B). +p < 0.05, ++p < 0.01, +++p < 0.001, compared to 0 mM MSX-3-DAMGO; *p < 0.05, compared to saline-0 mM MSX-3-MSX; ##p < 0.05, compared to saline-20 mM MSX.

**3. Discussion**

The current study examined the role of adenosine within the nucleus accumbens in mediating consumption and locomotor activity associated with a palatable high-fat diet. No change in locomotor activity was observed in response to A1 receptor activation while A2A receptor activation suppressed and A2A receptor blockade enhanced locomotor activity. Administration of A1 or A2A receptor agonists appeared to have very little influence on baseline or intra-accumbens opioid mediated consumption of a high-fat diet. However, blockade of adenosine A2A receptors with MSX-3 administration significantly increased the baseline consumption of a high-fat diet, an effect completely blocked by prior administration of the opioid antagonist naltrexone. Intra-accumbens administration of MSX-3 also enhanced DAMGO-induced consumption at the highest dose. The present study complements previous research on A2A receptor-mediated changes in locomotor activity and adds new evidence demonstrating striatal A2A receptors alter consumption of a high-fat diet.

Baseline intake (p=0.154) but did block the increase in high fat intake induced by MSX-3 (p<0.01).

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Intra-accumbens administration of MSX-3 increased baseline locomotor activity, in agreement with previous reports (Nagel et al., 2003), yet had no influence on the activity produced by DAMGO. Interestingly, administration of MSX-3 produced a dose-dependent increase in high fat consumption on its own, and when concurrently administered with DAMGO, consumption was significantly increased above levels produced by either drug independently. Moreover, naltrexone completely blocked the MSX-3-induced increases in both high fat intake and locomotor activity, indicating these increases may be partially regulated through opioid receptor activation. Striatal administration of naltrexone has been consistently shown to decrease palatable food consumption and block opiate-induced feeding (Zhang et al., 1998; Woolley et al., 2006; Will et al., 2006), possibly by decreasing the hedonic value of tasty foods.

A functional interaction between A2A and opioid has been previously reported, as A2A antagonists have been shown to alter opiate administration (Yao et al., 2006), opiate withdrawal syndrome (Kaplan and Sears, 1996; Salem and Hope, 1997) and opioid nociception (Ferré et al., 2007), while chronic opiate treatment has been shown to upregulate or sensitize adenosine receptors (Ammon-Treiber and Höllt, 2005; Brundege and Williams, 2002). However, the present findings are the first to suggest a role for striatal A2A receptors in mediating the consumption of a high-fat diet, an effect shown to be dependent on opioid receptor activation. Palatability driven feeding is considered a model of natural reward and DAMGO-dependent on opioid receptor activation. Palatability driven feeding under very different conditions, palatable food intake under energy-deficit, the present findings demonstrate enhanced feeding driven by striatal opioids or the palatable nature of food could prove beneficial in understanding potential targets for treating obesity and other disorders related to excessive consumption of palatable foods.

4. Experimental procedures

4.1. Subjects

Subjects were 22 male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 to 300 g. Rats were housed in Plexiglas cages, 2 per cage, in a temperature and humidity controlled room at 22 °C and maintained on a 12/12 light:dark cycle (lights on at 0700 h) with all experiments being conducted during the light phase (1100-1400 h). Throughout the experiment, animals were allowed ad libitum access to water and standard laboratory chow (Purina LabDiets, St. Louis, MO) in their home cages. Experimental procedures used were in accordance with

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the University of Missouri Institutional Animal Care and Use Committee guidelines and approved protocols.

4.2. Surgical placement of cannula

Animals were anesthetized with a ketamine/xylazine mixture of 90 mg/kg ketamine/9 mg/kg xylazine (Sigma, St. Louis, MO) and stereotaxically implanted with bilateral guide cannulae (23 gauge, 10 mm) aimed at the nucleus accumbens using the following coordinates, from bregma: +1.4 AP, ±2.0 ML, –7.8 DV (Paxinos and Watson, 1998). Following standard flat skull procedures, the guide cannula were secured to the skull using stainless steel screws and jet acrylic (Lang Dental Mfg. Co. Inc., Wheeling, IL). Following surgeries and throughout experiments, wire stylets (10.5 mm) were kept in guide cannula to prevent blockage. Animals were allowed 1 week for recovery prior to treatment.

4.3. Specialized diet

The high fat diet (HFD) was obtained from Teklad Diets (Madison, WI) and contained 278.3 g/kg vitamin free casein, 4.2 g/kg DL-methionine, 100.0 g/kg sucrose, 441.2 g/kg hydrogenated vegetable shortening, 77.7 g/kg linoleic safflower oil, 26.3 g/kg cellulose, 53.3 g/kg AIN-76 mineral mix, 15.2 g/kg AIN-76A vitamin mix, and 3.8 g/kg choline chloride. The diet consisted of 6.2 kilocalories/gram; 16.5% kcal from protein, 7.8% kcal from carbohydrates, and 75.6% of kcal from fat.

4.4. Apparatus and behavioral assessment of feeding behavior

Testing took place in a room separate from the colony room in eight Plexiglas (30.5 cm × 24.1 cm × 21.0 cm) feeding chambers (Med Associates, St. Albans, VT). Feeding chambers were equipped with four infra-red photobeams at intervals of 6 cm and positioned 4.3 cm above the bar floor to measure feeding associated locomotor activity across the chamber, an automated weigh scale for the food hopper to continuously monitor the weight of the hopper while automatically correcting for spillage, and a water bottle. The feeding hopper and water bottle were located on opposite corners of the same side of the chamber wall and a removable waste tray was located beneath the bar floor. Measurements taken included locomotor activity (number of horizontal beam breaks) and amount consumed (grams of diet consumed). Manual weights of the high fat diet were taken at the end of the session in addition to the automated measurements by the software to ensure accuracy. Testing periods consisted of 1 h of continuous behavioral monitoring in the feeding chambers by the monitoring software, Med-PC Version IV (Med Associates, St. Albans, VT). Following standard flat skull procedures, the guide cannula were secured to the skull using stainless steel screws and jet acrylic (Lang Dental Mfg. Co. Inc., Wheeling, IL). Following surgeries and throughout experiments, wire stylets (10.5 mm) were kept in guide cannula to prevent blockage. Animals were allowed 1 week for recovery prior to treatment.

4.5. Intra-accumbens infusion procedure

Animals were gently hand-held during the injection procedure. Drugs or control vehicle were administered through thirty-three gauge, 12.5 mm injectors with the tip extending 2.5 mm beyond the end of the cannula. Infusions were administered using a microdrive pump (Harvard Apparatus, South Natick, MA) connected via polyethylene tubing (PE-10). The injection rate was 0.16 µl/min for 93 s, with an additional 60 s to allow for diffusion. Injectors were removed and stylets replaced following infusion.

4.6. General procedure

Animals had ad libitum access to water and high fat diet (approximately 35 g) in the feeding chambers during all testing sessions. Subjects were placed in the feeding chambers for 1 h daily until stable food consumption across 3 days was obtained, which was usually in 6 days. During the last 2 days of this habituation, animals were acclimated to the injection procedure. On the first day of the acclimation procedure, a 10.0 mm injector was inserted and left in place for 2 min, though no volume was administered. On the second day, animals received an injection of saline into the accumbens with a 12.5-mm injector. Animals then received drug and vehicle treatments in a within-subjects, counter-balanced design. Immediately following each drug treatment, the animal was placed in the feeding chamber for 1 h of individual computer automated behavioral monitoring. At the end of the 1 h session, animals were returned to their home cages and placed in the colony room. There was a minimum of one day occurred between treatment sessions. One hour of monitoring was chosen as behavioral effects following intra-accumbens and i.c.v. CGS 21680 (Cabeza de Vaca et al., 2007; Font et al., 2008) and MSX-3 (Ishiwari et al., 2007; Nagel et al., 2003) have all been observed in 1 h or less. Furthermore, the consumption effects of intra-accumbens DAMGO are most pronounced in the first hour (Will et al., 2009) so examination of consumption and associated locomotor behaviors beyond 1 h may not accurately reflect peak responses to the drugs.

4.7. Experiment 1: intra-accumbens CCPA dose effect experiment

After baseline and acclimation procedures were completed, all animals (n=6) received intra-accumbens injection of saline or CCPA (10 nM, 100 nM, and 1 µM/0.25 µl/side). Doses were assigned in a counterbalanced order and were chosen based on the range of doses used previously (Khan et al., 2001; Okada et al., 1996). Considering the dose–response effect of CCPA, the most behaviorally active dose of CCPA was used to test the CCPA-DAMGO interaction. Animals then received counter-balanced combinations of a pretreatment of saline or CCPA (1 µM/0.25 µl/side) prior to administration of a near threshold dose of DAMGO (0.025 µg/0.25 µl/side). DAMGO doses from this and subsequent experiments were chosen based on studies within our laboratory and previous literature investigating feeding behaviors in the nucleus accumbens (Zhang et al., 1998; Will et al., 2006).

4.8. Experiment 2: intra-accumbens CGS 21680 experiment

After baseline and acclimation procedures were completed, animals (n=8) were given intra-accumbens injection of saline or CGS 21680 (500 nM, 2.5 µM and 5.0 µM /0.25 µl/side), prior
to saline or DAMGO (0.025 µg/0.25 µl/side) administration, in a counterbalanced order. Doses were chosen based on previously effective intra-accumbens doses in the literature (Quarta et al., 2004). Each dose was used to test the interaction between CGS 21680 and DAMGO. All animals received a pretreatment of saline or CGS 21680 prior to administration of DAMGO (0.025 µg/0.25 µl/side) in a counterbalanced order.

4.9. Experiment 3: intra-accumbens MSX-3 experiment

After baseline and acclimation procedures were completed, animals (n = 8) were given intra-accumbens injection of saline or MSX-3 (10 mM and 20 mM/0.25 µl/side), prior to saline or DAMGO (0.025 µg/0.25 µl/side) administration. The high dose of MSX-3 was also administered following treatment of saline or naltrexone (20 µg/0.25 µl/side). Treatment order was counter-balanced. The dose range of MSX-3 was chosen based on previously determined effective intra-accumbens doses in the literature investigating feeding behaviors (Ishiwari et al., 2007; Nagel et al., 2003). The naltrexone dose was chosen as this dose has no influence on baseline feeding of this particular diet, yet completely blocks DAMGO-induced feeding (Will et al., 2006).

4.10. Drugs

The adenosine A1 agonist 2-Chloro-N6-cyclopentyladenosine (CCPA), the A2A prodrug of the antagonist MSX-2, 3-(3-hydroxypropyl)-8-(m-methoxyt Gentriyl)-7-methyl-1-propargylyxanthine phosphate disodium salt (MSX-3), the µ-opioid receptor agonist (D)-Ala²,NMe-Phe⁴,Gly⁰-ol⁵-epsilon-Enkaphalin (DAMGO), and the opioid antagonist naltrexone hydrochloride were all obtained from Sigma Chemical Company (St. Louis, MO). The A2A agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido adenosine hydrochloride (CGS 21680) was obtained from Tocris Bioscience, UK. The vehicle for all drugs was sterile 0.9% saline.

4.11. Histology

At the conclusion of the experiment, animals were overdosed with sodium pentobarbital and perfused transcardially using 200 ml heparinized saline followed by 10% buffered formalin solution (500 ml). Brains were extracted and kept in 20% sucrose and 10% formalin mixture. Frozen serial sections (40 µm slices) of the injection site were collected and mounted on slides, stained with Cresyl violet and cover slipped. Cannulae placements of all animals were assessed with a light microscopy and photographed and no animals were excluded based on criteria of injector placement. A schematic representing the accepted region for the tip of the injector track is represented in Fig. 1.

4.12. Statistical analysis

Data was analyzed using SPSS (SPSS, Inc.). The data across various treatment conditions was analyzed using a one-way repeated measures analysis of variance (ANOVA), followed by post-hoc orthogonal contrasts of means when appropriate.
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