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Adenosine A₁ Receptors in Mouse Pontine Reticular Formation Modulate Nociception Only in the Presence of Systemic Leptin

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Abstract

Human obesity is associated with increased leptin levels and pain, but the specific brain regions and neurochemical mechanisms underlying this association remain poorly understood. This study used adult male C57BL/6J (B6, $n = 14$) mice and leptin-deficient, obese B6.Cg-Lep^{ob}/J (obese, $n = 10$) mice to evaluate the hypothesis that nociception is altered by systemic leptin levels and by adenosine A₁ receptors in the pontine reticular formation. Nociception was quantified as paw withdrawal latency (PWL) in s after onset of a thermal stimulus. PWL was converted to percent maximum possible effect (%MPE). After obtaining baseline PWL measures, the pontine reticular formation was microinjected with saline (control), three concentrations of the adenosine A₁ receptor agonist N⁶-p-sulfophenyladenosine (SPA), or super-active mouse leptin receptor antagonist (SMLA) followed by SPA 15 min later, and PWL was again quantified. In obese, leptin-deficient mice, nociception was quantified before and during leptin replacement via subcutaneous osmotic pumps. SPA was administered into the pontine reticular formation of leptin-replaced mice and PWL testing was repeated. During baseline (before vehicle or SPA administration), PWL was significantly ($p = 0.0013$) lower in leptin-replaced obese mice than in B6 mice. Microinjecting SPA into the pontine reticular formation of B6 mice caused a significant ($p = 0.0003$) concentration-dependent increase in %MPE. SPA also significantly ($p < 0.05$) increased %MPE in B6 mice and in leptin-replaced obese mice, but not in leptin-deficient obese mice. Microinjection of the mouse super-active leptin antagonist (SMLA) into the pontine reticular formation before SPA did not alter PWL. The results show for the first time that pontine reticular formation administration of the adenosine A₁ receptor agonist SPA produced antinociception only in the presence of systemic leptin. The concentration-response data support the interpretation that adenosine A₁ receptors localized to the pontine reticular formation significantly alter nociception.

Keywords

leptin-deficient; obesity; pain; adenosine A₁ receptors

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Human obesity is associated with increased reports of pain (Hitt et al., 2007, Janke et al., 2007, McVinnie, 2013) and the increasing prevalence of obesity in adults (Swinburn et al., 2011) and children (Cunningham et al., 2014) emphasizes the relevance of this association for pain management (Pizzo and Clark, 2011). The neurochemical mechanisms underlying the association between pain and obesity are complex and poorly understood. The satiety factor leptin, a cytokine secreted by adipocytes (Oswal and Yeo, 2010), is one molecule that has been shown to alter acute (Wang et al., 2009) and neuropathic (Maeda et al., 2009, Li et al., 2013) pain.

Replacing leptin in leptin-deficient, obese mice is a valuable approach for obtaining novel insights regarding the diverse roles of leptin (Lindstrom, 2007). For example, systemic administration of leptin to leptin-deficient mice has revealed that leptin contributes to the control of breathing (Tankersley et al., 1998, O'Donnell et al., 1999, O'Donnell et al., 2000), growth hormone function (Luque et al., 2007), and pain (Wang et al., 2009). Leptin alters the excitability of pedunculo pontine tegmental neurons (Hosoi et al., 2002, Beck et al., 2013a, Beck et al., 2013b) that provide cholinergic input to the pontine reticular formation (Lydic and Baghdoyan, 1993). The ability of leptin to alter the excitability of cholinergic neurons is of interest given the discovery of antinociception caused by administering cholinomimetics into the pontine reticular formation of mouse (Wang et al., 2009).

Previous studies discovered that the adenosine A₁ receptor agonist N⁶-p-sulfophenyladenosine (SPA), delivered to the pontine reticular formation causes antinociception in C57BL/6J (B6) mice that produce leptin, but not in B6.Cg-Lep^{ob}/J (leptin-deficient obese) mice that are leptin-deficient (Wang et al., 2009). The present study sought to determine whether an interaction between leptin and adenosine A₁ receptors (Rice et al., 2000) could be localized to the pontine reticular formation. This study tested the hypotheses that 1) replacing systemic leptin in obese mice that lack endogenous leptin promotes antinociceptive behavior, 2) administering the adenosine A₁ receptor agonist SPA into the pontine reticular formation causes greater antinociceptive behavior in the presence of leptin, and 3) microinjecting a leptin receptor antagonist into the pontine reticular formation blocks antinociception caused by an adenosine A₁ receptor agonist. The results show for the first time that adenosinergic modulation of nociception evoked from the pontine reticular formation is significantly enhanced by the pro-inflammatory cytokine leptin.

2. EXPERIMENTAL PROCEDURES

2.1 Mice and experimental preparation

All experiments and procedures were approved by the University of Michigan Committee on Use and Care of Animals and were conducted in accordance with *Guide for the Care and Use of Laboratory Animals* (The National Academies Press, 8th Ed., Washington, D.C., 2011). Adult male (8 to 10 weeks) B6 (n = 14) and B6.Cg-Lep^{ob}/J (leptin-deficient obese, n = 10) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed with *ad libitum* access to food and water in 24-h illumination to promote free-running circadian rhythms. Because microinjections and pain testing always occurred at the

same time of day (between 13:00 and 17:00 h), this approach minimized the potential for circadian confounds.

Mice were anesthetized with isoflurane (1.5 to 2.2% in 100% O₂, for 1.4 h) and surgically implanted with a guide cannula (part C315GS-4/SPC, Plastics One, Inc., Roanoke, VA, USA) to permit subsequent microinjections into the pontine reticular formation. Stereotaxic coordinates for the microinjection aim sites were 4.24 mm posterior to bregma, 0.8 mm lateral to bregma, and 4.50 mm ventral to the skull surface (Franklin and Paxinos, 2008). During a 1-week recovery period, mice were conditioned for 1 h per day to the Plexiglas chambers of an IITC Model 336T Paw Stimulator Analgesia Meter (IITC Life Science Inc., Woodland Hills, CA, USA). The apparatus was equipped with a heated glass plate (IITC Model 400 Heated Base) set at 30 °C during conditioning and testing.

2.2 Drug administration and nociceptive testing

Nociception to a noxious thermal stimulus was measured using the Hargreaves' method (Hargreaves et al., 1988). On experiment days, mice were placed in the testing chambers and allowed to habituate for 1 h. After the conditioning period, the analgesia meter was used to record baseline (before microinjection) paw withdrawal latency (PWL) for each mouse. For each measurement, the light at standby-level intensity (10% intensity) of the analgesia meter was aligned to the ventral surface of the hind paw. Once alignment was achieved, the timer and testing-level intensity (40% intensity) of the light were started simultaneously by pressing a button. The time it took for the mouse to respond to the higher intensity light was recorded as the PWL (in s). Baseline values for each mouse were determined by averaging 5 measurements that were recorded over a 20-min period. For all paw withdrawal latencies, measurements were alternated between left and right hind paw to prevent either paw from becoming sensitized to the thermal stimulus.

After obtaining baseline measures of nociception, the pontine reticular formation was microinjected with 50 nL of either saline (vehicle control) or the selective adenosine A₁ receptor agonist N⁶-p-sulfophenyladenosine (Jacobson et al., 1992) (SPA; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA). B6 mice (n = 9) received microinjections of 5, 50, and 500 pmol SPA (2.23, 22.27, and 222.69 ng, respectively). Obese mice (n = 10) received 500 pmol of SPA. In an additional set of experiments, the pontine reticular formation of B6 mice (n = 5) was injected with either saline plus 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) or 0.313 pmol of mouse super-active leptin antagonist (SMLA, Protein Laboratories Rehovot Ltd., Rehovot, Israel) plus 0.1% BSA (Gertler, 2006, Shpilman et al., 2011, Chapnik et al., 2013). Pontine administration of SMLA was followed 15 min later by a microinjection of 500 pmol SPA (BSA was used to minimize non-specific binding of SMLA to the microinjection tubing and storage containers per venter advice). Microinjections were made with a manual microdrive equipped with a 1-μL syringe (Hamilton Company, Reno, NV, USA). Injection duration was 60 s. Repeated microinjections in the same mouse were separated by at least 7 days and the order of drug administration was varied. For the concentration-response and leptin-replacement studies, three PWL measurements were obtained, in rapid succession at 10, 20, 30, 60, 90, and 120 min after each microinjection. For the leptin-antagonist study, three PWL measurements

were taken at 10, 20, 30, and 60 min after the SPA microinjection (since previous study indicated peak response to SPA occurred within first 60 min). The three latency measures were averaged for each time point.

2.3 Leptin replacement in obese mice

After receiving microinjections of saline and SPA (500 pmol), obese mice were anesthetized with isoflurane (2.0% to 2.2% in 100% O₂ for 15 min) and were implanted with a subcutaneous Alzet® (Cupertino, CA, USA) model 1002 osmotic pump set to deliver 15 µg of mouse recombinant leptin/day (National Hormone and Peptide Program, Torrance, CA, USA) for 14 days. During leptin replacement, the microinjection procedures described above were repeated to administer saline and SPA (500 pmol). After each microinjection, nociceptive responses were tested as described above. The osmotic pump was removed after the last PWL experiment.

Serum samples were collected from mice before the osmotic pump was implanted, once during each week of leptin replacement, and 2 days after the pump was removed. Leptin from the serum samples was measured using a mouse leptin enzyme-linked immunosorbent kit (Crystal Chem Inc., Downers Grove, IL, USA). This assay was performed to verify leptin delivery by the subcutaneously implanted osmotic pumps. Body weight before and during leptin replacement also was recorded.

2.4 Histological confirmation of microinjection sites

After the final experiment and the final blood sample was obtained (for obese mice only), mice were deeply anesthetized and decapitated. Brains were removed, frozen, and cut into 40-micron thick coronal sections. Serial sections were mounted on chrom-alum coated glass slides, fixed with 80°C paraformaldehyde vapor, and stained with cresyl violet acetate. Sections containing the microinjection sites were digitized. Stereotaxic coordinates of the microinjection sites were quantified by comparing the stained sections with a mouse brain atlas (Franklin and Paxinos, 2008).

2.5 Analysis of paw withdrawal latency data

PWL measurements in s were converted into percent maximum possible effect (%MPE) with the following equation:

$$\%MPE = ((\text{post microinjection PWL} - \text{baseline PWL}) / (15 - \text{baseline PWL})) \times 100$$
 The denominator value of 15 in the equation represents the cutoff time (in s), which is the time at which the thermal source automatically returns to 10% intensity to prevent tissue damage. The %MPE conversion normalizes the data by taking into account the cutoff time, inter-animal differences, and day-to-day intra-animal variability in baseline PWL.

Statistical analysis of the data was completed in consultation with the University of Michigan Center for Statistical Consultation and Research. Descriptive and inferential statistics were performed using Prism (version 6.0b; GraphPad Software, Inc., La Jolla, CA) and SAS (version 9.3; SAS Institute Inc., Cary, NC). The study was adequately powered to detect a potential difference in nociception as a function of circulating leptin. Inferential

statistics included both one-way and two-way analysis of variance (ANOVA), t-test with a Bonferroni correction, and Šidák's multiple comparisons test.

3. RESULTS

3.1 Systemic leptin replacement increased thermal nociception

To evaluate the effects of leptin alone, baseline PWL values from the pre-microinjection condition were compiled for B6 mice, leptin-deficient obese mice, and leptin-replaced obese mice (Fig. 1). Kruskal-Wallis one-way ANOVA revealed a group-main effect (B6 mice vs. leptin-deficient obese mice vs. leptin-replaced obese mice) on PWL ($H = 9.9$; $df = 2$; $p = 0.0071$). Post hoc t-tests evaluating differences between the three groups revealed that the baseline PWL for the leptin-replaced, obese mice was significantly ($p = 0.0013$) less than the baseline PWL for leptin-deficient mice. Thus, systemic leptin replacement alone increased thermal nociception.

3.2 Microinjection sites were localized to the pontine reticular formation

Histological analysis showed that all microinjections were made into the pontine reticular formation (Fig. 2). The average $\pm$ SEM stereotaxic coordinates of the microinjection sites in the B6 mice that were used for the concentration-response experiments were 4.6 ± 0.1 mm posterior to bregma, 0.9 ± 0.2 mm lateral to midline, and 4.4 ± 0.3 mm ventral to the skull surface. For the obese mice, the average $\pm$ SEM stereotaxic coordinates of the microinjections sites were 4.8 ± 0.2 mm posterior to bregma, 0.7 ± 0.2 mm lateral to midline, and 4.8 ± 0.2 mm ventral to the skull surface. The average $\pm$ SEM stereotaxic coordinates of the microinjection sites in the B6 mice that were used for the leptin-antagonist study were 4.8 ± 0.2 mm posterior to bregma, 0.9 ± 0.2 mm lateral to midline, and 4.3 ± 0.2 mm ventral to the skull surface. ANOVA revealed no differences in posterior, lateral, or ventral stereotaxic coordinates of the microinjection sites between groups of mice.

3.3 SPA caused concentration-dependent antinociception

Before testing the hypothesis that the antinociceptive response to SPA is enhanced by leptin, this study sought to determine the optimal concentration of SPA for microinjection into the pontine reticular formation. Fig. 3A summarizes the effects of SPA on paw withdrawal latency averaged across the 2-h testing period. A significant concentration main-effect on %MPE ($p = 0.0003$) was indicated by non-parametric repeated measures one-way ANOVA. Dunn's multiple comparisons test revealed that 50 pmol and 500 pmol of SPA significantly increased %MPE compared to saline ($p < 0.05$, as indicated by the asterisks).

Fig. 3B illustrates the 2-h time course of SPA on %MPE in B6 mice. Repeated measures two-way ANOVA indicated a concentration main-effect ($F = 16.7$; $df = 3, 24$; $p < 0.0001$), a time main-effect ($F = 10.1$; $df = 5, 40$; $p < 0.0001$) and a time-by-concentration interaction ($F = 2.1$; $df = 15, 120$; $p = 0.0164$). Note that the greatest concentration of SPA (500 pmol/50 nL) increased antinociception for 2 h after the microinjection.

3.4 Leptin replacement partially restored the antinociceptive response to SPA

Having demonstrated that microinjection of SPA into the pontine reticular formation of B6 mice causes concentration-dependent antinociception, this study then tested the hypothesis that replacing systemic leptin in obese mice alters antinociceptive behavior. Fig. 4 summarizes the %MPE on paw withdrawal latency after microinjection of saline and SPA in B6 mice, obese mice prior to leptin replacement (leptin-deficient obese), and obese mice during leptin replacement (leptin-replaced obese). Fig. 4A shows the data collapsed across the 2-h test period. Two-way ANOVA revealed a mouse group main-effect ($F = 4.6$; $df = 2, 26$; $p = 0.0188$), a treatment main-effect ($F = 60.8$; $df = 1, 26$; $p < 0.0001$), and a mouse group-by-treatment interaction ($F = 8.2$; $df = 2, 26$; $p = 0.0018$). Post-hoc tests showed that, compared to saline, SPA caused a significant ($p < 0.05$) increase in %MPE on paw withdrawal latency in B6 mice and in leptin-replaced, obese mice. SPA did not increase %MPE in the leptin-deficient, obese mice. Paw withdrawal latency was not altered by saline administration into the pontine reticular formation of obese mice before or during systemic leptin replacement.

Fig. 4B shows changes in %MPE as a function of time after microinjection of saline and SPA in each of the three groups of mice (B6, leptin-deficient obese, and leptin-replaced obese). A three-way, repeated-measures ANOVA revealed a mouse group main-effect ($F = 4.3$; $df = 2, 295$; $p = 0.0143$), a treatment main-effect ($F = 74.1$; $df = 1, 295$; $p < 0.0001$), a mouse group-by-treatment interaction ($F = 9.4$; $df = 2, 295$; $p = 0.0001$), a time main-effect ($F = 8.3$; $df = 5, 295$; $p < 0.0001$), and a mouse group-by-time interaction ($F = 2.1$; $df = 10, 295$; $p = 0.0245$) for %MPE of SPA on paw withdrawal latency. There was no significant drug-by-time interaction or mouse group-by-drug-by-time interaction. Tukey's post hoc tests revealed that for B6 mice, microinjection of SPA caused a significantly greater %MPE ($p < 0.0167$) on paw withdrawal latency than did saline at all time points (as noted by † in Fig. 4B). Tukey's post hoc test also showed that SPA caused a significantly greater ($p < 0.0167$) %MPE than did saline at 10, 60, and 90 min after microinjection for the leptin-replaced, obese mice (‡ in Fig. 4B). Tukey's post hoc test revealed no effect of SPA on paw withdrawal latency at any time point for the leptin-deficient, obese mice.

Table 1 shows average serum leptin levels and average percent changes in body weight. Weight loss in obese mice, calculated from the time the osmotic pumps were implanted to the end of the second week of leptin replacement (−15.4%), was significant (paired t test; $p < 0.0001$). As a point of reference, average body weight of B6 mice over the same time interval revealed a non-significant 1.8% increase. Leptin serum levels and decrease in body weight confirmed leptin delivery in each of the 10 obese mice.

3.5 Body weight, not leptin levels, accounted for the majority of variance in nociception

Fig. 5A plots the %MPE on paw withdrawal latency caused by SPA as a function of serum leptin levels for the leptin-deficient obese mice and for the leptin-replaced obese mice. These two functions summarize %MPE before (orange triangles) and during (red diamonds) leptin replacement. The leptin-deficient obese mice showed a large variability in %MPE that extended over a small range of serum leptin levels. This large variability for the leptin-deficient, obese mice is reflected in a 176.0 % coefficient of variation for %MPE. In

contrast, the leptin-replaced, obese mice showed less variability in %MPE over a larger range of serum leptin levels (coefficient of variation = 42.7%). Thus, serum levels of leptin accounted for an insignificant amount of the variance in paw withdrawal latency for leptin-deficient ($R^2 = 0.78\%$) and leptin-replaced ($R^2 = 8.5\%$) obese mice.

Fig. 5B illustrates the relationship between percent change in body weight and %MPE of SPA on paw withdrawal latency. For the leptin-deficient (orange triangles) and leptin-replaced (red diamonds) obese mice, the %MPE was greater (i.e., SPA caused a greater antinociceptive effect) in mice that lost the most weight (abscissa 0 to -30 %) or gained the least weight (abscissa 0 to 10 %). Fig. 5B shows that change in body weight accounted for 68% of the variance in %MPE for the leptin-replaced, obese mice and about 30% of the variance in %MPE for the leptin-deficient, obese mice.

3.6 Pontine administration of a leptin receptor antagonist did not alter nociception

Evidence that leptin alters excitability of brainstem neurons (Hosoi et al., 2002, Beck et al., 2013a, Beck et al., 2013b) encouraged an additional set of studies evaluating the possibility that administering the leptin receptor antagonist SMLA into the pontine reticular formation of B6 mice ($n = 5$) before microinjecting the adenosine A_1 agonist SPA would alter antinociception caused by SPA. These experiments revealed that pretreating the pontine reticular formation with SMLA did not alter SPA-induced antinociception (data not shown).

4. DISCUSSION

The results are discussed relative to the findings that 1) systemic leptin administration was pro-nociceptive in leptin-deficient, obese mice; 2) replacement of systemic leptin in leptin-deficient, obese mice partially restored antinociception caused by administering the adenosine A_1 receptor agonist SPA into the pontine reticular formation; 3) antinociception caused by administering SPA into the pontine reticular formation of B6 mice was concentration-dependent; 4) antinociception varied as a function of body weight; 5) leptin and adenosine may interact to alter nociception, sleep, and obesity; and 6) administering the leptin receptor antagonist SMLA into the pontine reticular formation of B6 mice did not alter SPA-induced antinociception.

4.1 Systemic leptin altered adenosinergic antinociception

Before vehicle or SPA was microinjected into the pontine reticular formation, baseline paw withdrawal latency was measured in B6 mice. Baseline paw withdrawal latency also was measured in leptin-deficient obese mice before and during replacement of systemic leptin (Fig 1). These experiments revealed that latency to paw withdrawal in response to the thermal stimulus was less in the leptin-deficient, obese mice that received leptin replacement (Fig. 1, red bar), than in the leptin-deficient, obese mice without leptin replacement (Fig. 1, orange bar). These results indicate that leptin alone had a pro-nociceptive effect. This finding is consistent with evidence that diet-induced obesity (Rossi et al., 2013) and leptin (Kutlu et al., 2003, Lim et al., 2009, Tian et al., 2011) can promote nociception.

After pontine administration of SPA, the leptin-deficient mice that received leptin replacement (Fig. 4A, red bar) showed antinociception (increased %MPE) that was not

observed in leptin-deficient obese mice without systemic leptin replacement (Fig. 4A, orange bar). Thus, compared to saline (control) the adenosine A₁ receptor agonist SPA microinjected into the pontine reticular formation significantly enhanced antinociception only in the presence of systemic leptin. This effect is also evident in the time-course of all microinjection conditions (Fig 4B). Leptin receptors are expressed in the brainstem, including the rostral (Scott et al., 2009, Patterson et al., 2011) and caudal (Scott et al., 2009) parts of the pontine reticular formation. The present results specify that the antinociceptive response to SPA is mediated, in part, by an interaction between adenosine A₁ receptors localized to the pontine reticular formation and circulating leptin.

4.2 Pontine reticular formation modulation of nociception

All microinjections of the adenosine A₁ receptor agonist SPA were confirmed to be within the rostral and caudal portions of the pontine reticular formation (Fig. 2). The antinociception caused by SPA is consistent with evidence from multiple species indicating that medial regions of the pontine reticular formation modulate nociception (Kshatri et al., 1998, Tanase et al., 2002, Wang et al., 2009, Watson et al., 2010). The finding that the SPA-evoked increase in latency to paw withdrawal varied significantly as a function of SPA concentration (Fig. 3) supports the interpretation that the antinociceptive effects of SPA are mediated, in part by adenosine A₁ receptors within the pontine reticular formation.

4.3 Antinociception varied as a function of body weight

The finding that nociception did not vary as a function of increasing circulating leptin levels (Fig. 5A) may indicate that alterations in nociception depend on leptin levels reaching a certain threshold. This possibility can be evaluated by future studies that chronically administer a wide range of leptin doses while quantifying the effects on both nociception and circulating serum leptin levels. Measures of serum leptin levels during leptin replacement in the present study (1.94 ± 0.35 ng/mL) differed from serum leptin levels (24.50 ± 1.36 ng/mL) reported previously (Wang et al., 2009). These differences likely are a function of the different leptin delivery rates provided by the 1-week osmotic pumps (0.50 μ L/h) used previously (Wang et al., 2009) and the delivery rates of the 2-week osmotic pumps (0.25 μ L/h) used in the present study.

Leptin is a lower-level phenotype that regulates the higher-level phenotype of body weight (Oswal and Yeo, 2010). Figure 5B shows that change in body weight accounted for a greater variance in %MPE caused by SPA than did serum leptin levels. The Fig. 5B data are consistent with evidence that weight loss is an intermediate-level phenotype that can effectively counter the pathological impact for a wide range of disorders (Kuna et al., 2013, Rock et al., 2013, Tyson et al., 2013) and diminish associated pain (Messier et al., 2004, Wai et al., 2008, Pizzo and Clark, 2011, Richette et al., 2011). The Fig. 5 results, and brain imaging data demonstrating an association between higher body mass index and reductions in brain volume (Cole et al., 2013), encourage future efforts to identify brain regions and endogenous molecules mediating the decreases in nociception that are associated with weight loss.

4.4. How might leptin and adenosine function as lower level phenotypes modulating obesity and nociception?

Incomplete knowledge currently precludes a mechanistic answer to the foregoing question. There is evidence, however, suggesting that molecules and brain regions regulating sleep comprise one set of intervening variables linking leptin and adenosine to obesity and pain. There is an association between adenosinergic modulation of sleep, obesity-induced sleep disruption, and the impact of sleep quality and duration on the experience of pain. The present study focused on the pontine brain stem because adenosinergic transmission in the pontine reticular formation contributes to the regulation of sleep (Baghdoyan and Lydic, 2012, Gettys et al., 2013) and nociception (Kshatri et al., 1998, Tanase et al., 2002, Wang et al., 2009, Watson et al., 2010). Chronic sleep restriction facilitates development of obesity (Spiegel et al., 1999, Morselli et al., 2010), increases leptin as a modulator of pro-inflammatory cytokines (reviewed in (Hayes et al., 2011), and — even in healthy volunteers — increases pain (Roehrs et al., 2006, Haack et al., 2007). These human data are paralleled by the finding that obese rats with fragmented sleep have enhanced sensitivity to, and delayed recovery from, neuropathic pain (Muncey et al., 2010). The foregoing data are supported by gain-of-function studies showing that extending sleep duration can reduce human pain sensitivity (Roehrs et al., 2012). Thus, multiple lines of evidence from human and non-human animals encourage continuing efforts that aim to elucidate the mechanisms by which the circulating hormone leptin alters the neurochemical modulation of nociception.

4.5 Limitations and conclusions

The results support the conclusion that replacement of circulating leptin in leptin-deficient mice promotes antinociception (Fig. 4) that is mediated by adenosine A₁ receptors (Fig. 3) in the pontine reticular formation (Fig. 2). The results do not specify the mechanisms through which systemic leptin and the adenosine A₁ receptors in the pontine reticular formation interact. The present microinjection of the leptin antagonist SMLA was encouraged by reports that leptin receptors have been localized to pontine regions of the brain stem (Scott et al., 2009, Patterson et al., 2011). Pontine delivery of the leptin receptor antagonist SMLA had no effect on the antinociception evoked by pontine delivery of the adenosine A₁ agonist SPA. These findings, and the pronociceptive effects of systemic leptin, suggest that systemic leptin may alter the excitability of extra-pontine networks projecting to the pontine reticular formation. This possibility encourages future studies that simultaneously administer SPA to the pontine reticular formation while delivering SMLA to additional brain regions. Although the concentrations of SPA used in the present study spanned a limited range, the finding that SPA caused concentration-dependent antinociception (Fig. 3) supports the interpretation of mediation by adenosine A₁ receptors. This conclusion is consistent with the previous discovery that SPA-induced antinociception is blocked by pontine reticular formation injection of the adenosine A₁ receptor antagonist DPCPX (Tanase et al., 2002). Adenosine A₁ receptors are G protein coupled and the present measures of nociception provide functional evidence consistent with biochemical data demonstrating SPA-induced activation of G proteins in the pontine reticular formation (Tanase et al., 2001). Muscarinic cholinergic receptors in the pontine reticular formation are also coupled to G proteins (DeMarco et al., 2003). Therefore, the present results specify for the first time that circulating leptin protein can modulate antinociception via G protein

coupled receptor signaling cascades in the pontine reticular formation that are activated by enhancing cholinergic (Wang et al., 2009) or adenosinergic transmission. Leptin binds to isoforms of the OB receptor which signal via Jak/STAT transduction pathways (Wunderlich et al., 2013). The specific mechanisms by which leptin-activated Jak/STAT signaling and adenosinergic/cholinergic-activated G protein coupled receptor signaling alter downstream modulation of nociception remain to be determined.

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ABBREVIATIONS

%MPE	percent maximum possible effect
ANOVA	analysis of variance
B6	C57BL/6J
BSA	bovine serum albumin
PWL	paw withdrawal latency
SMLA	super-active mouse leptin antagonist
SPA	N ⁶ -p-sulfophenyladenosine

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Highlights

- Pontine administration of an adenosine A₁ agonist caused antinociception in B6 mice.
- The adenosine A₁ agonist did not cause antinociception in obese mice lacking leptin.
- Replacing circulating leptin restored antinociception in obese mice.
- Body weight accounted for the largest amount of variance in antinociceptive behavior.
- Leptin and adenosine modulate the association between obesity, sleep, and nociception.

Fig 1.

Baseline paw withdrawal latency (PWL) measured before microinjections. There were no differences in baseline PWL of B6 mice ($n=9$) and obese mice prior to leptin replacement ($n=10$, leptin-deficient obese). The baseline PWL for obese mice during leptin replacement (leptin-replaced obese) was significantly less ($p < 0.0167$) than the baseline PWL for leptin-deficient, obese mice (asterisk). The same obese mice were used for both the leptin-deficient group and the leptin-replaced group.

Fig 2.

All microinjection sites were confirmed to be within the pontine reticular formation. **A.** Schematic coronal diagrams from a mouse brain atlas (Franklin and Paxinos, 2008) span from 4.36 mm (top section) to 5.02 mm (bottom section) posterior to bregma. These sections have been modified to show the location of each microinjection site for the B6 mice used in the N^6 -*p*-sulfophenyladenosine (SPA) concentration-response study (green dots, $n = 9$), obese mice used in the SPA + leptin-replacement study (orange dots, $n = 10$), and the B6 mice used in the SPA + leptin-antagonist study (gray dots, $n = 5$). **B.** The arrow and digitized image of a representative cresyl violet-stained coronal section identifies a microinjection site in the rostral part of the pontine reticular formation. **C.** Vertical lines on a sagittal diagram (Franklin and Paxinos, 2008) of the mouse brain indicate the anterior-to-posterior range of all the microinjection sites.

Fig 3.

The adenosine A₁ receptor agonist N⁶-*p*-sulfophenyladenosine (SPA) microinjected into the pontine reticular formation caused a concentration-dependent increase in percent maximum possible effect (%MPE) in B6 mice. A. For each concentration of SPA, the %MPE was averaged across all measures obtained during the 2-h experiment. Asterisks (*) indicate a significant increase in %MPE compared to control (0 pmol SPA). Antinociception was produced by 50 and 500 pmol SPA. B. Time-course plot shows that SPA increased antinociception in a time-dependent and concentration-dependent manner. The time points at which %MPE was significantly ($p < 0.05$) greater than control (0 pmol) are indicated by asterisks (*) for 50 pmol of SPA and by daggers (†) for 500 pmol of SPA.

Fig 4.

Percent maximum possible effect (%MPE) on thermal nociception caused by microinjecting saline and SPA into the pontine reticular formation. Data are plotted for B6 mice, obese mice prior to leptin replacement (leptin-deficient obese), and obese mice during leptin replacement (leptin-replaced obese). **A.** %MPE values were averaged over the 2-h experiments. Asterisks (*) indicate a significant ($p < 0.05$) increase in %MPE compared to control (saline). Pound sign (#) indicates that the %MPE of SPA in the leptin-deficient, obese mice was significantly less than the %MPE in B6 mice. **B.** The 2-h time course of the %MPE values after microinjection of saline and SPA (500 pmol) for B6, leptin-deficient obese, and leptin-replaced, obese mice. Time points when the %MPE of SPA was significantly ($p < 0.05$) greater than that of saline are noted by † for the B6 mice and by ‡ for the leptin-replaced, obese mice.

Fig 5.

The relationship between %MPE of SPA on paw withdrawal latency versus serum leptin concentration (**A**), and percent change in body weight (**B**). Data from the leptin-deficient, obese mice are represented by the orange triangles. The red diamonds plot data from the leptin-replaced, obese mice. The lines matching the colored data points indicate the linear correlations associated with each group of data. Serum leptin levels for the leptin-deficient, obese mice are from blood samples taken prior to pump implantation. Serum leptin levels for leptin-replaced, obese mice are an average from samples taken during week 1 and week 2 of leptin replacement. Weight changes for the leptin-deficient, obese mice are the difference between the weight at the second week of testing prior to leptin replacement and surgery for guide tube implantation. For the leptin-replaced, obese mice, the changes are the

differences between the weight at the second week of testing during leptin replacement and the weight at the time of pump implantation.

Table 1**Leptin Levels and Change in Body Weight**

Serum leptin levels and changes in body weight during the testing period. Measurements of leptin levels for the leptin-deficient, obese mice (leptin-deficient obese) are from blood samples that were taken just prior to implantation of subcutaneous osmotic pumps. Serum measurements from obese mice receiving leptin (leptin-replaced obese) are from blood samples that were taken during leptin replacement at the end of week 1 and week 2, and then averaged. Weight changes for B6 and leptin-deficient, obese mice were calculated from the date of intracranial guide tube implantation to the date of the second microinjection (2-week time period). Weight changes for the leptin-replaced obese were calculated from weight at the date of pump implantation to weight at the end of week 2 of leptin replacement. Increases in serum leptin levels and decreases in body weight are consistent with successful leptin replacement. Serum leptin values for B6 mice are from (Wang et al., 2009).

	Average Serum Leptin Level (ng/mL $\pm$ SEM)	Average Change in Body Weight (% $\pm$ SEM)
B6	5.24 $\pm$ 0.50	1.8 $\pm$ 1.9
Leptin-Deficient Obese	0.17 $\pm$ 0.06	3.2 $\pm$ 1.2
Leptin-Replaced Obese	1.94 $\pm$ 0.35	-15.4 $\pm$ 2.2