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Leptin Replacement Restores Supraspinal Cholinergic Antinociception in Leptin-Deficient Obese Mice

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Abstract: A single gene deletion causes lack of leptin and obesity in B6.V-Lep^{ob} (obese; ob) mice compared with wild-type C57BL/6J (B6) mice. This study compared the phenotype of nociception and supraspinal antinociception in obese and B6 mice by testing 2 hypotheses: (1) microinjection of cholinomimetics or an adenosine receptor agonist, but not morphine, into the pontine reticular formation (PRF) is antinociceptive in B6 but not obese mice, and (2) leptin replacement in obese mice attenuates differences in nociceptive responses between obese and B6 mice. Adult male mice (n = 22) were implanted with microinjection guide tubes aimed for the PRF. The PRF was injected with neostigmine, carbachol, nicotine, N^6-*p*-sulfophenyladenosine (SPA), morphine, or saline (control), and latency to paw withdrawal (PWL) from a thermal stimulus was recorded. B6 and ob mice did not differ in PWL after saline microinjection into the PRF. Neostigmine, carbachol, and SPA caused PWL to increase significantly in B6 but not obese mice. An additional 15 obese mice were implanted with osmotic pumps that delivered leptin for 7 days. Leptin replacement in obese mice restored the analgesic effect of PRF neostigmine to the level displayed by B6 mice. The results show for the first time that leptin significantly alters supraspinal cholinergic antinociception.

***Perspective:** This study specifies a brain region (the pontine reticular formation), cholinergic neurotransmission, and a protein (leptin) modulating thermal nociception. The results are relevant for efforts to understand the association between obesity, disordered sleep, and hyperalgesia.*

***Key words:** Acetylcholine, adenosine, morphine, obesity, sleep, pain.*

B6.V-Lep^{ob} (obese; ob) mice differ from wild-type C57BL/6 J (B6) mice by a single nonsense mutation at the *ob* gene.[22,53] This mutation results in the inability of obese mice to produce the protein leptin. Obese mice exhibit many phenotypic differences from congenic B6 mice including altered metabolic function, respiratory abnormalities[34] and disrupted sleep.[14,25] Human obesity is associated with increased complaints of pain.[21,30] This association encouraged us to determine whether the nociceptive phenotype was differentially expressed in B6 and obese mice.

The brain regions and molecules that link obesity and nociception are complex and poorly understood. Administration of opioid, cholinergic, and adenosinergic drugs to the pontine reticular formation (PRF) alters nociceptive responses of cat[23,44] and rat.[50] Therefore, the present study was designed to determine whether PRF administration of opioid, cholinergic, and adenosinergic drugs alters latency of paw withdrawal from a thermal stimulus as a function of mouse genotype and leptin replacement in obese mice. Portions of these results have been presented in abstract form.[47-49]

Methods

Surgery, Recovery, and Behavioral Conditioning

All experiments and procedures were approved by the University of Michigan Committee on Use and Care of Animals. Adult male B6 (n = 13) and obese (n = 9) mice from Jackson Laboratory (Bar Harbor, ME) were anesthetized with 2% to 3% isoflurane (Abbott Laboratories, North Chicago, IL) in 100% oxygen and placed in a David Kopf (Tujunga, CA) model 926 stereotaxic frame with model 923 mouse head holder and anesthesia mask. Isoflurane levels were reduced to 1.3% to 1.8% and maintained throughout the surgery. To enable access to the PRF, mice were

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implanted with 1 26-gauge stainless steel guide tube (Plastics One, Roanoke, VA) aimed for coordinates 4.72 mm posterior, 0.65 mm lateral, and 5.60 mm ventral to bregma.[36] Dental acrylic was applied to the guide tube and 2 stainless steel screws were placed in the skull to provide an anchor surface for the acrylic. Additional obese mice (n = 15) also were each implanted with an Alzet (Cupertino, CA) model 1007D osmotic pump set to deliver leptin at 15 μg/d for 7 days. All mice were allowed to recover for 7 days after surgery, during which time they were handled and conditioned daily to being placed in a Plexiglas chamber. Animals were housed individually in a constantly illuminated and temperature regulated room with free access to food, water, and bedding.

Quantification of Nociception

Thermal nociceptive threshold was measured using an IITC Life Sciences Model 336 Plantar Simulator Analgesia Meter (Woodland Hills, CA) after a Hargreaves paw withdrawal protocol.[19] Mice were placed into individual Plexiglas chambers on a raised, tempered glass surface. A light box beneath the glass surface provided a radiant heat source as a 4 $\times$ 6 mm light beam. The adjustable heat source was set at 40% active intensity and 10% idle intensity (to allow aiming of the beam when inactivated). To test for thermal nociceptive threshold, the light was focused on the plantar surface of one hind paw. The light and timer were activated simultaneously and stopped when the mouse reacted by moving its paw away from the thermal stimulus. The time to the nearest 0.01 second between turning on the light and the reaction of the mouse was recorded as the paw withdrawal latency (PWL). A cutoff time of 15 second for the light beam was maintained throughout all experiments to ensure no injury to the paw. Measurements were taken at least 30 seconds apart and alternated between right and left hind paws to prevent habituation to the stimulus. PWL measurements were expressed as a percent change from premicroinjection baseline values in the form of percent maximum potential effect (%MPE)[4] using the following equation:

$$\%MPE = \frac{PWL - baseline}{cutoff - baseline} \times 100\%$$

Percent MPE provides a quantitative index of nociception while accounting for individual differences between mice, as well as stimulus cutoff time.

Experimental Procedure

At the beginning of each experiment, mice were allowed 1 hour to habituate to the chambers before 5 baseline PWL measurements were taken, each 5 minutes apart. The PRF was injected with 50 nL of either 0.9% saline (vehicle control) or drug using a 31-gauge microinjector (Plastics One, Roanoke, VA) connected to a Hamilton syringe via polyethylene tubing (PE 20). Three PWL measurements were taken at each of 6 time points, occurring at 10, 20, 30, 60, 90, and 120 minutes after injection. Drug solutions (10 mM) microinjected included the adenosine A_1 receptor agonist N^6-*p*-sulfophenyladenosine (SPA; 222.7 ng), the acetylcholinesterase inhibitor neostigmine bromide (151.6 ng), the cholinergic receptor agonist carbachol (91.4 ng), the μ-opioid agonist morphine sulfate (379.4 ng), and the nicotinic acetylcholine receptor agonist nicotine base (81.1 ng). Repeated microinjections into the same mouse were separated by at least 5 days. Obese mice used for the leptin replacement portion of the study received only 1 microinjection of either saline or neostigmine on the 7th day of leptin replacement. After PWL testing, obese mice were anesthetized and the osmotic pump was removed. Blood was collected from a tail snip, and the serum was separated and frozen for later verification of leptin delivery.

Histological Confirmation of Microinjection Sites

Mice were deeply anesthetized and decapitated 2 to 5 days after the final microinjection experiment. For the group of obese mice that received leptin, a final blood collection was made immediately after decapitation. Serum was separated and frozen for a subsequent leptin assay. Brains were quickly removed, frozen, and sliced in 40-μm coronal sections. Sections were mounted serially onto glass slides, fixed with hot (80 °C) paraformaldehyde vapor, and stained with cresyl violet. Stereotaxic coordinates of each microinjection site were obtained by comparing the stained sections with those in a mouse brain atlas.[36]

Assay for Leptin

Delivery of leptin to obese mice implanted with osmotic pumps was verified using a leptin ELISA kit (Crystal Chem Inc, Downers Grove, IL). Blood collected on day 7, the last day of leptin treatment, was used to verify presence of leptin. Blood collected on day 9, 2 days after pump removal, was used to verify degradation of leptin. Positive and negative control samples from B6 and obese mice, respectively, were also tested in each assay.

Statistical Analyses

All %MPE data were evaluated by 2-way analysis of variance (ANOVA; drug by time and strain by time) for repeated or random measures. Additional inferential statistics included Student *t* test and Tukey Kramer post hoc multiple comparisons test. All tests used $P < .05$ as an indication of significance. Statistical tests were performed in consultation with the University of Michigan Center for Statistical Consultation and Research using Statistical Analysis System v9.1.3 (SAS Institute, Inc, Cary, NC) and GBStat (Dynamic Microsystems, Inc, Silver Spring, MD). Data are reported as mean $\pm$ standard error of the mean (SEM).

Results

All Microinjection Sites Were Localized to the PRF

Fig 1 shows that all microinjection sites were localized to the PRF. Average $\pm$SEM stereotaxic coordinates of the

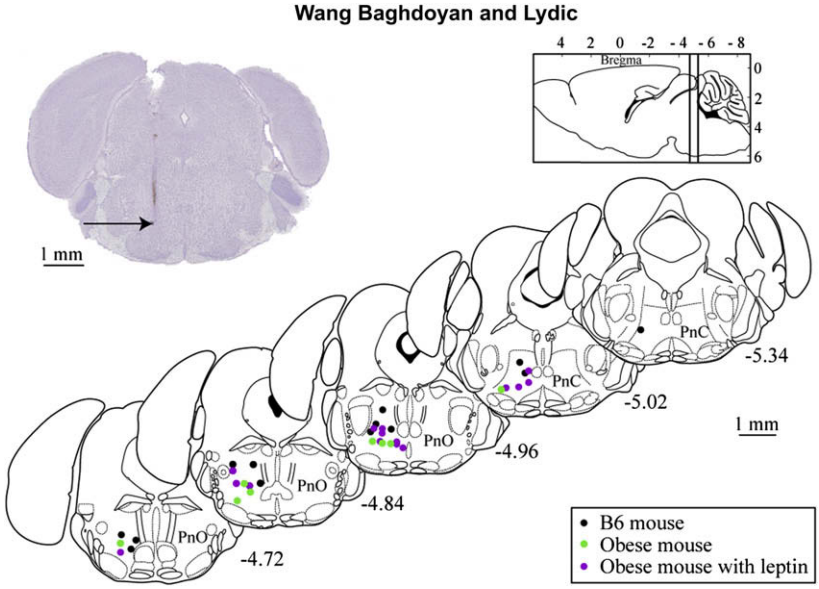

Figure 1. All microinjection sites were localized to pontine reticular formation regions comprised of the oral (PnO) and caudal (PnC) pontine reticular nucleus.[36] Microinjection sites for B6 mice (black dots, n = 13), obese mice (green dots, n = 8), and obese mice that received leptin (purple dots, n = 15) are shown on 5 coronal drawings of the mouse brainstem. Numbers at the lower right of each drawing indicate distance (mm) posterior to bregma. The top left figure shows a typical cresyl violet–stained tissue section with an arrow pointing to the microinjection site. The top right figure represents a sagittal view of the mouse brain with vertical lines indicating the anterior to posterior range of the microinjection sites. Brain drawings were modified from the Paxinos and Franklin mouse brain atlas.[36]

microinjection sites were 4.92 ± 0.05 mm posterior, 0.75 ± 0.07 mm lateral, and 4.61 ± 0.08 mm ventral to bregma for B6 mice. Microinjection coordinates for obese mice that did not undergo leptin treatment were 4.85 ± 0.04 mm posterior, 0.87 ± 0.07 mm lateral, and 4.99 ± 0.08 mm ventral to bregma. Microinjection coordinates for obese mice that received leptin replacement were 4.94 ± 0.02 mm posterior, 0.73 ± 0.08 mm lateral, and 4.94 ± 0.07 mm ventral to bregma.

Cholinomimetics and SPA Produced an Antinociceptive Response in B6 Mice

Fig 2 (left column, A through E) shows %MPE as a function of time after microinjection of 5 drugs into the PRF. Consistent with previous findings in cat,[23] 2-way ANOVA revealed no significant change in %MPE after microinjection of morphine (Fig 2A). Nicotine administration also did not significantly alter %MPE in B6 mice (Fig 2B). ANOVA revealed that %MPE was significantly altered as a function of drug after microinjection of SPA (Fig 2C; $F = 5.53$; df = 1,119; $P < .05$), carbachol (Fig 2D; $F = 7.54$; df = 1,119; $P < .05$), and neostigmine (Fig 2E; $F = 18.37$; df = 1,107; $P < .001$). Microinjection of SPA (Fig 2C; $F = 4.04$; df = 5, 119; $P < .01$) and carbachol (Fig 2D; $F = 2.74$; df = 5,119; $P < .05$) produced a significant time-dependent change in %MPE. ANOVA also revealed a significant time by drug interaction after microinjection of SPA (Fig 2C; $F = 4.04$; df = 5,119; $P < .01$). These interactions derive from the drug effects dissipating over time. Post hoc Tukey Kramer test comparing drug and saline %MPE values revealed that SPA (Fig 2C) significantly ($P < .05$) increased %MPE values at 20 and 30 minutes after injection and that carbachol (Fig 2D) significantly ($P < .05$) increased %MPE values at 10, 60, and 90 minutes after injection. Tukey Kramer test also showed that neostigmine (Fig 2E) increased %MPE values at all time points except 90 minutes after injection. Consistent with hyperalgesia reported for some obese humans[21,30] and rats,[17] mean PWL in seconds was significantly ($P < .01$) less for obese (5.31 seconds) than for B6 (7.0 seconds) mice after PRF microinjection of saline.

Morphine, Cholinomimetics, and SPA Had No Effect on Thermal Nociception in Obese Mice

Fig 2 (right column, F through J) reports %MPE as a function of time and drug for obese mice. As observed in the B6 mice, microinjection of morphine (Fig 2F) produced no significant change in %MPE. Obese mice also showed no significant increase in %MPE caused by microinjection of nicotine (Fig 2G), SPA (Fig 2H), or carbachol (Fig 2I). After neostigmine administration (Fig 2J) there was a significant time by drug effect ($F = 3.97$; df = 5,107; $P < .01$). Post hoc Tukey Kramer analysis revealed that %MPE was significantly ($P < .05$) increased by neostigmine at 10 minutes after injection.

Leptin Replacement in Obese Mice Restored the Antinociceptive Effect of PRF Neostigmine Administration

To determine whether leptin replacement restores the antinociceptive response to neostigmine, another group of obese mice (n = 15) was given continuous leptin for 1 week via osmotic pumps and the PRF was then microinjected with either neostigmine or saline. Fig 3 shows the %MPE (mean + SEM) after PRF neostigmine administration to 9 B6 mice and 11 obese mice that received leptin replacement. There was no significant

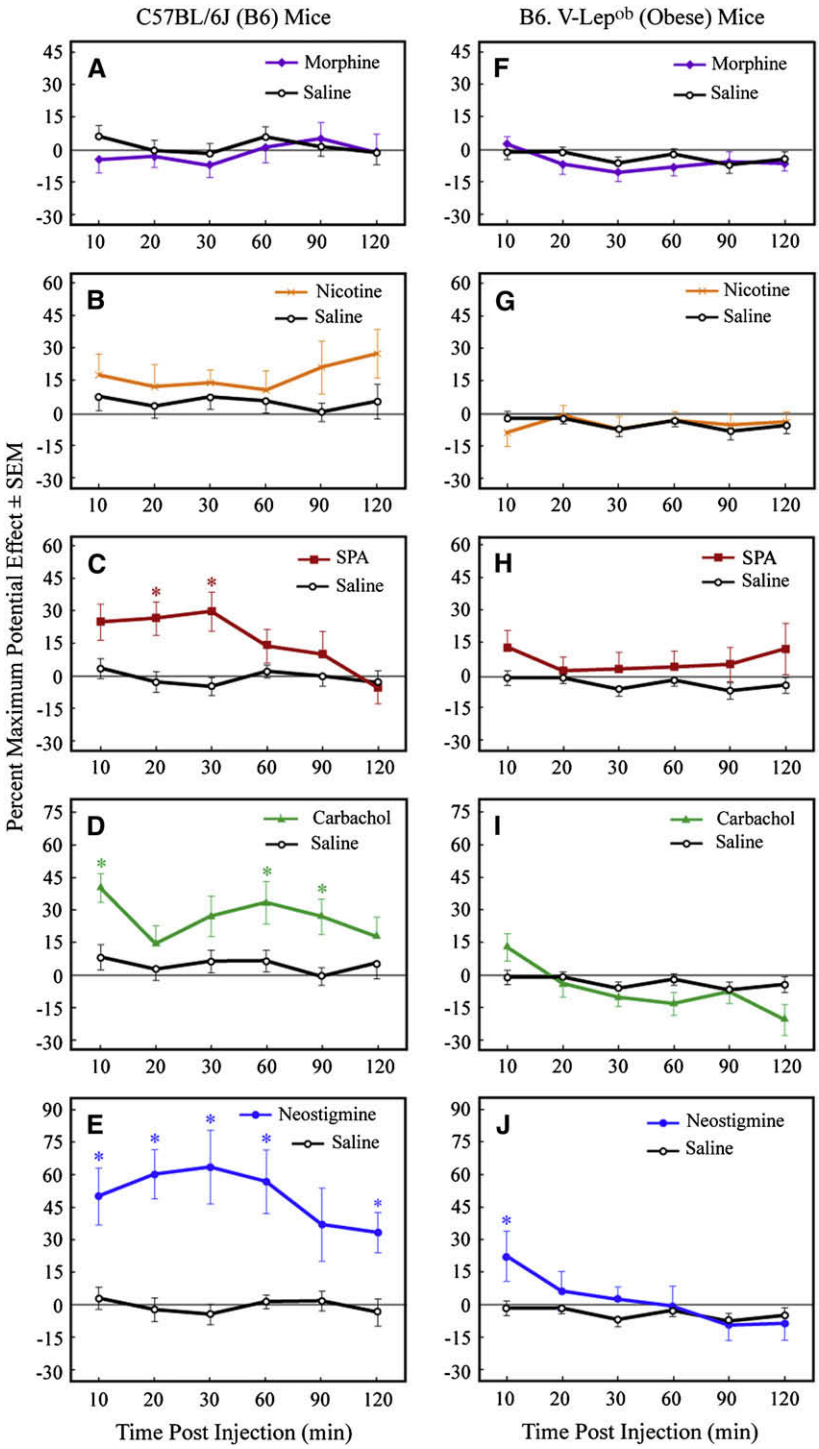

Figure 2. Percent maximum potential effect (%MPE) values after microinjections into the pontine reticular formation of B6 and B6.V-Lep^{ob} (obese) mice. Graphs showing data from B6 and obese mice for each drug are presented side by side to facilitate comparison. The %MPE is a measurement of change from baseline (no injection) values. Higher %MPE values indicate a longer delay in moving the paw away from the stimulus, consistent with decreased nociception. Saline microinjections (vehicle control) produced %MPE values around 0, indicating little change from baseline (no injection) values. Asterisks indicate a significant difference from saline at designated time points.

difference (t = 0.29; P > .05) for B6 mice (49.85 + 11.3) compared with obese mice that received leptin (46.33 + 3.9). Thus, leptin replacement in obese mice restored %MPE to levels displayed by B6 mice. Seven days of leptin replacement also reduced the body weight of obese mice by approximately 21% compared with preleptin treatment weight (Table 1). Measures of leptin and body weight confirmed leptin delivery for all 15 obese mice (Table 1).

Discussion

The results show for the first time that microinjection of cholinomimetics and an adenosine A_1 receptor

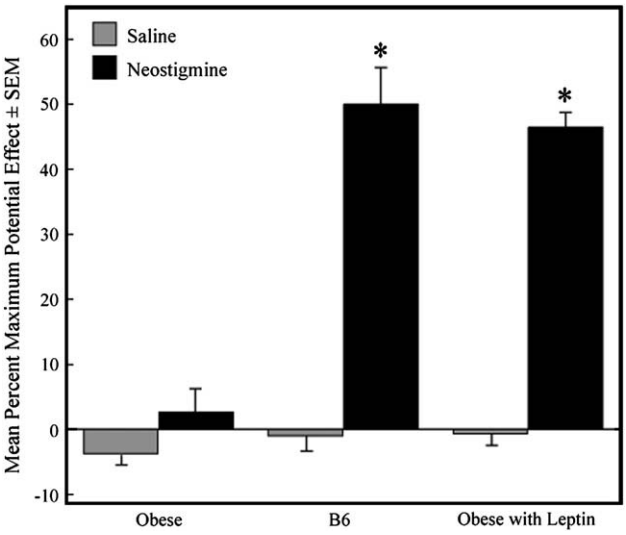

Figure 3. Leptin replacement restored the antinociceptive effect of pontine reticular formation neostigmine in obese mice. Graph shows %MPE values averaged over the course of 2 hours after microinjection of saline or neostigmine for obese mice, B6 mice, and obese mice that received leptin replacement. Asterisks indicate a significant difference ($P < .05$) between response to neostigmine and saline.

agonist, but not morphine, into the PRF of B6 mouse modulates thermal antinociception. Cholinomimetics and the adenosine A_1 receptor agonist did not produce a significant antinociceptive response in obese mice. These differences in nociceptive phenotype are of interest relative to the fact that B6.V-Lep^{ob} and B6 mice differ by lack of the leptin-producing *ob* gene. The finding that leptin replacement in obese mice restored the thermal antinociceptive response to neostigmine supports the interpretation that the protein leptin can modulate supraspinal cholinergic antinociception. The results are discussed in relation to the emerging understanding of a significant interaction between obesity, sleep disruption, and pain perception.

Supraspinal Cholinergic Antinociception

For B6 mice, time course measures of %MPE showed that PRF microinjection of SPA, carbachol, and neostigmine caused significant antinociceptive responses (Fig 2). PRF administration of morphine and nicotine did not alter the time course of %MPE. These results are similar to the effects of morphine, cholinomimetics,[23] and SPA[44] microinjected into homologous regions of cat PRF. Similar drug effects observed in mouse, cat, and ongoing studies of rat,[50] support the interpretation that the present thermal antinociceptive effects of SPA, carbachol, and neostigmine are not species-specific.

Neostigmine prevents the breakdown of the endogenous neurotransmitter acetylcholine (Ach). This mechanism of action makes the antinociceptive effects of neostigmine particularly relevant for understanding the role of the PRF in thermal nociception. Spinally administered neostigmine has been used in patients with promising antinociceptive results.[11] Intranasal nicotine also has been shown to reduce postoperative pain in women recovering from uterine surgery.[15] In contrast to B6 mice, the congenic line of obese mice showed no significant enhancement of antinociceptive behavior after microinjection of cholinomimetics or SPA (Fig 2). These results are in line with data showing that obese mice have a differential sleep response to PRF neostigmine administration compared with B6 mice.[14]

Table 1. Leptin Replacement in Obese Mice

	Leptin ± SEM (ng/mL)	% Decrease in Body Weight ± SEM
B6 control	5.24 ± 0.50	—
Obese control	0.03 ± 0.01	—
Obese leptin day 7	24.50 ± 1.36	20.82 ± 2.05
Obese leptin day 9	0.02 ± 0.01	21.89 ± 2.11

NOTE. Leptin replacement was verified using an ELISA. Leptin day 7 values indicate leptin concentrations (ng/mL) on the last day of leptin treatment. Leptin day 9 values were tested to verify elimination of leptin 2 days after removal of the osmotic pump. Leptin replacement also decreased body weight in obese mice, as expected.

Microinjection of morphine in obese and B6 mice (Fig 2, A and F) produced no change in nociceptive response when compared with microinjection of saline. This finding is consistent with previous data from cat[23] and suggests that the PRF is not a brain region that contributes to the pain-relieving effect of morphine. Morphine administered to the PRF has been shown to decrease acetylcholine release in the PRF[29] and to inhibit the rapid eye movement (REM) phase of sleep.[27]

The PRF is comprised of the pontine reticular nucleus, oral part (PnO), and caudal part (PnC).[36] The present focus on the PRF as a brain region modulating supraspinal thermal antinociception derives, in part, from the fact that pain and sleep are inversely related and that both cholinergic[28,45] and adenosinergic[10] neurotransmission in the PRF regulates sleep. Sleep disruption is a leading complaint of patients experiencing pain and pain patients frequently exhibit disrupted sleep.[8,24] Depriving healthy subjects of 1 night of REM sleep causes a hyperalgesic response to nociceptive stimuli.[37] The foregoing points are clinically relevant because opioids disrupt the sleep/wake cycle and decrease the amount of REM sleep.[7,27]

Obese humans have disordered sleep,[5,20,39,40,46] and diet-induced obese mice show altered sleep patterns that are reversed with weight loss.[18] Rat models of metabolic syndrome[52] are hyperalgesic for acute[17] and chronic[32] pain and have disrupted sleep.[33] The foregoing evidence and the present results support the view that the PRF is 1 brain region modulating the association between obesity, disordered sleep, and nociception.

The Role of Leptin in Antinociception

Leptin was discovered in 1994[53] and has been widely reported to function as a satiety factor involved in metabolic regulation of energy expenditure and energy input.[16] Leptin receptors are localized to the arcuate nucleus[38] and ventromedial hypothalamus,[12] further demonstrating that leptin regulates feeding and energy

balance.[16] Studies of obese mice have provided insight into the roles of leptin. Obese mice have deficiencies in brain development,[1] immunology,[26] breathing,[34] and endothelial function[51] and show disrupted sleep.[14,25] Replacement of leptin has been found to restore partial or complete function associated with leptin deficiencies. Leptin levels have also been shown to be influenced by the duration of sleep.[41] In particular, there exists a correlation in obese humans between short sleep duration and decreased levels of leptin.[43] The present results show that leptin replacement for 7 days restored the antinociceptive effect of PRF neostigmine such that the %MPE response of obese mice to neostigmine was no different from the response of B6 mice (Fig 3). Leptin replacement for 7 days was modeled after previous studies showing that leptin normalized breathing in leptin deficient mice.[34] The Table 1 data show that leptin replacement produced serum leptin levels in ob mice that were about 5 times greater than levels of leptin in B6 mice. The mechanisms contributing to this 5-fold increase in leptin are not known. These data encourage future studies to determine whether leptin delivered to normal B6 mice can enhance thermal antinociceptive responses.

The mechanisms by which leptin altered supraspinal cholinergic antinociception are unknown. Administering neostigmine into the PRF decreases the degradation of ACh released from the terminals within the PRF. These ACh-releasing terminals originate from cholinergic neurons localized to the laterodorsal and pedunculopontine tegmental nuclei.[42] Leptin can modulate cholinergic function by altering expression of choline acetyltransferase,[13] the enzyme responsible for synthesis of ACh. It remains to be determined whether replacing leptin in obese mice increases the production of acetylcholine.

Limitations and Conclusions

Publication of the mouse genome documents a 99% homology with the human genome,[31] indicating that genotypic and phenotypic studies of mice can provide novel insights into human disorders.[6] One limitation for studies using the B6.V-Lep^{ob} mouse is that the mutation causing the obese mouse phenotype is rarely present in humans. Some forms of human obesity are associated with reduced leptin receptor affinity for leptin and elevated serum levels of leptin.[35] Studies quantifying the relationship between serum and cerebrospinal fluid levels of leptin show that defective transport of leptin across the blood-brain barrier can be more important than decreased leptin receptor availability or affinity.[2,3] Thus, it should be clear that the link between obesity and nociception is multifactorial and cannot be attributed to leptin alone. Despite these limitations, loss of the influence of leptin may account for some of the phenotypic similarities between obese mice and humans.[43,46] The results encourage future studies designed to examine nociceptive responses in the db/db, mouse that has a mutation in the *lepr* gene that encodes for the leptin receptor.[9]

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