The Genetically Obese Mouse's (B6V.*lep* ^{ob}) Response to Thermal Nociception: Effects of Age and Leptin Replacement

by

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Abstract

Two experiments assessed whether age and systemic leptin replacement affected tail-flick latency in male *lep*^{ob} mice, a model of obesity and diabetes. In the first experiment, 15-week- and 23-week-old male *lep* ^{ob} mice's tails were exposed to a 40˚C stimulus until they reflexively flicked from the heat or 10 s, whichever came first. Younger mice displayed shorter latencies than older mice. In the second experiment, 41- to 45-week-old mice received either leptin or vehicle chronic subcutaneous infusions, with tail-flick latencies measured at 7 and 14 days into the infusion. Leptin-treated mice displayed shorter latencies than vehicle-treated mice throughout testing. These results support reports of heightened analgesia in *lep*^{ob} mice. They also suggest that this analgesia increases with age, but can be reduced with leptin administration in these mice.

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Despite its unpleasantness pain is an important sensory experience. Without the ability to feel pain, minor everyday tasks could become life threatening. Imagine stepping into a bathtub full of boiling water - the pain in the tip of one's toe would alert one of the danger and one would not step in. The reaction of an individual with a reduced pain-sensing ability could be drastically different and have tragic results. Differences in pain sensation can occur across human populations (Pradalier, Willer, Boureau, & Dry, 1981), in rats (Roane & Porter, 1986) and in mice (Mogil et al., 1999). Subpopulations experience reduced pain-sensing ability, putting them at risk of tissue damage and decreased quality of life. One subpopulation that has been identified is the genetically obese mouse (B6V-*Lep*^{ob}). Aged (42- to 70-week-old) genetically obese mice exhibit a markedly decreased response on the thermal pain test, tail flick, in comparison with lean mice of the same background strain (Wilson & Rodgers, 2004). This decreased pain response suggests several possible explanations. One explanation is the neural and hormonal differences associated with the mutation in these mice, such as the lack of leptin, changes pain sensation. Another explanation is that age or an interaction of age with the altered physiology of these mice changes pain sensation. Investigating such possibilities requires understanding of the transmission of pain and the physiology of these mice.

Pain is defined as "an unpleasant sensory or emotional experience associated with actual or potential tissue damage" by the International Association for the Study of Pain (1994, ¶ 9). It can result from different types of stimuli including mechanical (intense pressure), chemical (bradykinin or prostaglandin secretion) or thermal (intense heat)

stimuli. Central mediation of pain is stimulus specific. For example, thermal pain is mediated spinally; whereas, mechanical pain is mediated supraspinally. The perception of pain is also referred to as nociception. The pain stimulus is first detected by nociceptors. These are free nerve endings found in skin, muscle, joints and viscera that respond only to intense, potentially damaging stimuli (Willis & Westlund, 1997). The axons of the nociceptors travel to the dorsal horn of the spinal cord. There are two types of axons associated with nociceptors ; they are A-delta fibers and C-fibers. A-delta fibers are thick, lightly myelinated, and transmit at velocities of 2-30m/sec. C-fibers are thin, not myelinated, and transmit at velocities of less than 2 m/sec. A-delta fibers send information on location and intensity of pain; whereas, C-fibers send information that is persistent and poorly localized. Information from the A- delta fibres reaches the brain more quickly than impulses from C-fibers, and is shorter in duration (Willis & Westlund, 1997).

These axons bring information from the nociceptors to the dorsal horn of the spinal cord. The dorsal horn is differentiated into five distinct parallel layers, known as laminae I – V. They display different morphology, neuronal projections and gene expression (Caspary & Anderson, 2003). Laminae I and II (substantia gelatinosa) connect with C-fiber sensory neurons carrying information from nociceptors, which transmit both thermal and noxious (pain) information. Laminae III-V connect with sensory neurons carrying information from mechanoreceptors, which detect touch and information from nociceptors (Caspary & Anderson, 2003).

Neurons entering the dorsal horn interact with interneurons. The interneuronal networks in the dorsal horn are multi-functional. They act to transmit information to the

brain, to modulate incoming information and to pass it to other spinal cord neurons. An example of the modulatory function is seen in the tail-flick response, a spinal reflex. The nociceptive information at the dorsal horn is passed to flexor motoneurons and nociceptive projection neurons (Willis & Westlund, 1997). There are ascending and descending pathways that connect with the interneurons. The ascending pathway travels from the dorsal horn to the thalamus, reticular formation, limbic system and cerebral cortex. The descending pathway consists of neurons originating in the lower and mid brain, particularly the periaqueductal grey, that travel to the dorsal horn where they connect with inhibitory interneurons. When nociception occurs, the descending pathway is activated to inhibit pain (Willis & Westlund, 1997).

Inhibition in the neural circuits of the dorsal horn conveying nociceptive information is also mediated by several neurotransmitters such as gamma-aminobutyric acid (GABA) and glycine and neuropeptides such as the endogenous opioids (Willis & Westlund, 1997). Beta-endorphin (β-endorphin), dynorphin and enkephalins are endogenous opioids that occur naturally and function as analgesics similarly to exogenous opiates such as morphine (Frischkencht, Siegfried, & Waser, 1988). An increase in opiates in an organism's system will attenuate nociception. Together with neurotransmitters and neuropeptides the interneruons help to modulate pain. Therefore, differences in neurotransmitters, neuromodulators, or their receptors in this region may lead to differences in pain response.

In mice, pain responses differ with strain and nociceptive test. Mogil et al. (1999) tested 11 mouse strains on 12 tests of nociception. The tests examined thermal, mechanical, chemical, and neuropathic models of pain. The main result was that

genotype of the mice influenced nociception as measured on all tests. Each strain had a unique response pattern to the nociceptive tests. The C57BL/6J strain was among the most sensitive across all stimulus types and had one of the most distinct nociceptive phenotypes. These mice were particularly sensitive to tests of thermal nociception, such as tail withdrawal, compared with chemical tests such as an injection of formalin. In this regard, the C57BL/6J had a markedly shorter latency (approximately 2.25 s) for tail withdrawal from a hot (49°C) water bath. This suggests an increased sensitivity to thermal pain in C57BL/6J mice. Lariviere et al.'s (2002) study supported Mogil et al.'s findings. They showed that the C57BL/6J strain was more sensitive on thermal nociceptive tests than other strains and especially sensitive to the tail-flick test. The different pain profiles among strains suggest a possible genetic component to pain modulation. The C57BL/6J is the background strain for the genetically obese mouse. Therefore, in examining pain differences, the genetically obese mouse becomes a model of interest. In addition, the genetically obese mouse expresses several phenotypes that have been shown to be linked with changes in pain response in both humans and other rodent models.

The genetically obese mouse (B6V-*Lep* ^{ob}), hereinafter, called *lep* ^{ob}, first appeared in the Jackson Laboratory (JAX) mice in 1949 (Ingalls, Dickie, & Snell, 1950). It resulted from a homozygous recessive mutation in the V stock of JAX mice. Researchers isolated the single gene mutation on mouse Chromosome 6 that was responsible for the physiological and behavioral differences between the *lep*^{ob} mice and the background strain (Zhang, Proenca, Maffei, Barone, Leopold, & Friedman, 1994). The mutation occurred on the OB gene, which codes for the protein hormone, leptin.

Leptin is produced predominately by white adipose tissue (Roberts & Greenberg, 1996; Zhang et al., 1994). However, it is also produced in brown adipose tissue, stomach, placenta, mammary glands, and ovarian follicles (Arora, 2006). In *lep* ^{ob} mice, a nonsense C to T mutation at 105 results in a premature stop codon. As a result the protein produced is truncated and ineffective (Roberts & Greenberg, 1996; Zhang et al., 1994). This stop codon acts as a signal to terminate protein synthesis. The early termination in leptin synthesis results in a lack of leptin produced in the body. Amino residues between 106 and 140 are the crucial region for the biological activity of leptin (Lee, Leinung, & Grasso, in press). The structure of leptin has been revealed in a threading analysis as a cytokine folding pattern (Madej, Boguski, & Bryant, 1995). Leptin contains 4 alpha helices connected by two crossover links and a short loop arranged in a helical bundle (Zhang, Basinski, Beals, Briggs, Churgay, Clawson, et al., 1997), and a disulfide linkage that is vital for its functioning (Zhang et al., 1997).

Leptin is released into circulation in a pulsatile manner from adipocytes, with a half-life of approximately 30 min (Ahren, Baldwin, & Havel, 2000; Arora, 2006). Circulating levels of leptin are elevated at night (Harris et al., 1998), and positively correlated with body fat in humans and rodents (Frederich, Hamann, Anderson, Lollman, Lowell, & Flier, 1995; Maffei, Halaas, Ravussin, Pratley, Lee, Zhang, et al., 1995). Maffei et al. (1995) examined leptin in different mouse models of obesity. Lean control mice had a circulating leptin level of ~ 12.5 ng ml $^{-1}$; whereas, leptin levels were increased tenfold in diabetic obese mice (*db*) and obese yellow agouti mice (A^y). Several studies have found females exhibit higher circulating levels of leptin than males, regardless of body fat differences, in both humans and rodents (Frederich et al., 1995;

Maffei et al., 1995; Saad, Damani, Gingerich, Riad-Gabriel, Khan, Boyadjian, et al., 1997).

The leptin receptor (Ob-R) was first isolated in 1995 and is encoded by the diabetes (*db*) gene on Chromosome 4 (Tartaglia, Dempski, Weng, Deng, Culpepper, Devos et al., 1995). A mutation in this gene creates an abnormal long form of the leptin receptor that produces the diabetic/obese phenotype of the *db/db* mouse (Chen, Charlat, Tartaglia, Woolf, Weng, Ellis, et al., 1996). Leptin receptors belong to the superfamily of cytokine receptors (Tartaglia et al., 1995).

Alternate splicing of the *db* gene results in several variants of the receptor. The variants are classified as short, long or soluble (Chen et al., 1996). The short form of the receptor typically contains 30-40 amino acid residues; whereas, the long form contains 302 amino acid residues (Lee, Proenca, Montez, Carroll, Darvishzadeh, Lee, et al., 1996). The short and long forms have two cytokine domains; however, only the long form of the receptor contains the intracellular signaling motifs required for the activation of the JAK-STAT (janus kinases-signal transducers and activators of transcription) signaling pathway (Harvey & Ashford, 2003; Houseknecht, Baile, Matteri, & Spurlock, 1998; Houseknecht & Portocarrero, 1998). Leptin receptors are found in most tissues. However, much of the research on leptin has focused on the role of leptin brain receptors for body weight regulation, and fewer studies have examined the contribution of peripheral leptin receptors.

The long form is found predominately in the brain in areas such as the hypothalamus (arcuate, lateral, ventromedial, and dorsomedial nuclei); whereas, the short form is found in other tissues including adipocytes, liver, pancreas, kidney and muscle

(Harvey & Ashford, 2003; Houseknecht et al., 1998; Houseknecht & Portocarrero, 1998). Short-form receptors are also found in the brain, in particular at the choroid plexus and in the microvessels of the brain, where they may influence leptin transport across the blood-brain barrier (Arora, 2006). In *lep*^{ob} mice gene expression of the leptin receptor has shown an upregulation of leptin receptor (Ob-R^{b}) mRNA in the brain with large increases in the dorsomedial part of the ventral medial hypothalamus (Huang, Lin, & Zhang, 1997). Expression of leptin receptor mRNA for both long and short forms has also been investigated in *lep*^{ob} and *db/db* mice. Baskin, Seeley, Kuijper, Loko, Weigle, Erickson, et al. (1998) studied leptin receptors and effects of leptin replacement. Long form leptin receptor mRNA was 2.3 times greater in both *lep*^{ob} and *db/db* mice's arcuate nuclei in comparison to the lean littermate controls. Systemic administration of leptin in *lep*^{ob} mice resulted in a decrease in long form leptin receptor mRNA in the arcuate nucleus.

The soluble form is different from the other two in that is does not have a transmembrane domain and circulates as a soluble receptor (Huang, Wang, & Li, 2001). The soluble form is found in plasma and is capable of binding to leptin and plays a role in modulating the steady-state level of leptin. Huang et al. (2001) examined the effects of overexpression of the soluble receptor in leptin-replaced *lep*^{ob} mice and found that the soluble receptor protects leptin from degrading.

Leptin plays an important role in many physiological processes including body weight regulation through food intake and metabolism, where it acts as an anorexigenic agent (Arora, 2006). Leptin also acts to regulate blood glucose levels through both appetite and fat storage as well as through glucose storage in the liver independent of energy balance (Bates, Kulkarni, Seifert, & Myers, 2005; Moran & Phillip, 2003). Leptin

also has a role in hormone regulation via processes, such as pro-opiomelanocortin (POMC) production.

Leptin regulates POMC production (Inui, 1999). POMC is the precursor to β-endorphin, an endogenous opioid, and to adrenocorticotropic hormone. Both leptin and POMC have numerous receptors in the hypothalamus. Leptin receptors are found mainly in the arcuate nucleus, paraventricular nucleus, dorsomedial nucleus and lateral region of the hypothalamus (Schwartz, Seeley, Campfield, Burn, & Baskin, 1996). POMC neurons are found in the arcuate nucleus and innervate the paraventricular nucleus (Arora, 2006). Cheung, Clifton, and Steiner (1997), using double label *in situ* hybridization, in rats, to examine the distribution of leptin receptors and POMC neurons, found that 70% of POMC neurons co-express the leptin receptor. This co-expression suggests that POMC neurons are targets for the action of leptin in the hypothalamus.

The physiological importance of leptin is seen in the *lep*^{ob} mouse, where a mutation resulted in the absence of leptin in these mice and several physiological abnormalities.. Some of the physiological changes include impaired thermoregulation, infertility, obesity, Type 2 diabetes (Ingalls et al., 1950), hypercorticoidism (Bray & York, 1979; Garthwaite, Martinson, Tseng, Hagen, & Menahan, 1980) and hyperendorphinemia, an increased level of endogenous opioids (Joosten & van der Kroon, 1974; Khawaja, Bailey, & Green, 1989; Khawaja, Chattopadhyay, & Green, 1991; MacDonald, Smith, & Bailey, 1998; Margules, Moisset, Lewis, Shibuya, & Pert, 1978).

Obesity is the first noticeable abnormality in *lep*^{ob} mice (Ingalls et al., 1950; Joosten & van der Kroon, 1974). They gain weight rapidly after weaning (around 3-4

weeks old) and by 3 months of age are double the size of their lean littermates (Ingalls et al., 1950). Hypertrophy of subcutaneous and visceral fat cells characterizes *lep*^{ob} mice (Bray & York, 1971; 1979).

Obesity has been linked with altered pain profiles in both humans and murine models. For example, Pradalier et al. (1981) found non-diabetic obese women had lower thresholds for painful stimuli (percutaneous stimulation of the sural nerve) than did women of average weight. The researchers speculated that the difference in threshold may result from differences in levels of endogenous opioids. Roane and Porter (1986) linked obesity and pain modulation in female obese Zucker (*fa/fa*) rats. In addition to being obese and diabetic, Zuckers have altered opioid levels. On tests of nociception, Zuckers displayed shorter tail-flick and tail-pinch latencies than did their lean controls. Shortened latencies on nociceptive tests suggest a hyperalgesic effect. Shortened latencies suggest a lowered threshold or exaggerated response to normally painful stimulation. These researchers speculated that the altered endogenous opioid profile of these rats may be responsible for this finding.

Ramzan, Wong, and Corcoran (2003) also implicated endogenous opioids in their study of thermal nociception in dietary-induced obese rats. They found that dietary-induced obesity in non-diabetic rats lengthened their tail-flick latencies to noxious thermal stimulation, and identified a strong, positive correlation between body weight and tail-flick latency. Their results support a relationship between obesity and nociception but instead of the shorter latencies found by Roane and Porter (1986), the dietary-induced obese rats displayed longer latencies. In support of Ramzan et al.'s (2003) inverse relationship between body weight and nociception in obese rats, Roy,

Cheng, Phelan, and Pomeranz (1980) measured jump latencies on a hot-plate test, to test for differences in pain sensitivities between *lep*^{ob} and lean mice. *Lep*^{ob} mice exhibit longer jump latencies from the hot plate at 55 °C than lean mice. However, these results must be interpreted cautiously because (a) jumping is the extreme response on the hot plate test, and (b) jumping as the behavioral indicator confounds differences in response because differences in jumping may reflect differences in motoric ability and not analgesia. Jumping occurs infrequently in adult *lep*^{ob} mice probably because of their decreased musculature and increased fat mass. Thus, the mice may have stayed on the hot plate longer because their motor response was compromised. Although Roy et al.'s results support the link between altered pain sensation and obesity, the results are open to multiple interpretations. In addition, across these several models and species, differences appeared between obese and non-obese controls, but the direction of the difference varied: Obese women (Pradalier et al., 1981) and female Zucker rats (Roane & Porter, 1986) displayed a hyperalgesic response to direct nerve and thermal stimulation, while dietary-induced obese rats (Ramzan et al. 2003) and *lep*^{ob} mice (Roy et al., 1980) displayed an analgesic response to thermal stimulation.

Another characteristic of *lep*^{ob} mice is their diabetic phenotype. *Lep*^{ob} mice have diabetes mellitusType 2. Diabetes mellitus is divided into Type 1 and Type 2. In Type 1 or insulin-dependent diabetes the beta cells of the pancreas fail to produce sufficient levels of insulin. In Type 2 or non-insulin-dependent diabetes insulin receptors display impaired signaling abilities, which cause insulin resistance in the target tissues (Leng, Karlsson, & Zierath, 2004). *Lep*^{ob} mice exhibit two of the main risk factors for Type 2 diabetes: obesity and genetic predisposition. *Lep*^{ob} mice typically develop Type 2

diabetes at weaning. The inability to use the insulin produced promotes elevated circulating levels of glucose and elevated plasma insulin levels in *lep*^{ob} mice (Bray & York, 1971; Joosten & van der Kroon, 1974).

In both types of diabetes mellitus cells are unable to use insulin because of either lack of insulin production or receptor signaling impairment. Pain has been examined in diabetes mellitus and changes in sensations have been found in both Type 1 and Type 2 diabetes.

Kamei, Kawashima, Hitosugi, Misawa, Nagase, and Kasuya (1993) assessed the influence of the central administration of endorphin on pain sensitivity in Type 1 diabetic mice. The subjects used were albino ICR mice, a strain originally bred for cancer research. Diabetes was induced pharmacologically with a 200 μg/kg intraperitoneal injection of streptozotocin (STZ), a compound selectively toxic to insulin-producing beta cells of the pancreas. Diabetic and control mice then received intracerebroventricular (supraspinal) or intrathecal (spinal) administration of β-endorphin. Tail-flick response latency was measured. The results showed a dose-dependent increase in response latency for β-endorphin administration regardless of route. Diabetic mice showed greater increases for intracerebroventricular administration than for intrathecal administration. The results suggest that Type 1 diabetes may affect β-endorphin-induced analgesia differently depending on location. The effects of β-endorphin on analgesia are greater at the supraspinal level than the spinal level in diabetic mice. Chen, Sweigart, Lakoski, & Pan (2002) found that male STZ-induced diabetic rats had fewer functional μ-opioid receptors in the dorsal horn than did normal non-diabetic Sprague-Dawley rats, but they found no differences in functional δ-opioid receptors. Chen and Pan (2003) examined the

effects of intrathecal morphine administration on nociception in STZ-induced diabetic rats. The results show a substantial decrease in the effects of intrathecal morphine in the diabetic rats. Induced-diabetic and non-diabetic rats displayed a similar μ-opioid receptor density and binding affinity. Thus, it is improbable that the reduced potency of morphine on analgesia in induced-diabetic rats is the result of fewer μ-opioid receptors in the spinal cord. The researchers suggest that diabetes may affect μ-opioid receptor –G protein coupling. This study suggests a possible decreased role of the μ-opioid receptors in the analgesic response of *lep*^{ob} mice to thermal stimulation.

Examining studies of pain and Type 2 diabetes, Zucker (*fa/fa*) rats, which are both obese and diabetic, display an altered pain response relative to the lean controls (Roane & Porter, 1986). L*ep*^{ob} mice show longer response latencies to noxious thermal stimulation compared with their lean littermates (Roy et al., 1980; Wilson & Rodgers, 2004), suggesting an analgesic response to pain in these mice. These results suggest a link between Type II diabetes and alterations in pain sensation.

Another characteristic of *lep* ^{ob} mice is increased levels of endogenous opioids. Genetic differences in endogenous opioids can occur at three levels - ligand, receptor, and processing of receptor stimulation (Frischkencht et al., 1988). *Lep* ^{ob} mice differ from lean mice at both the ligand and the receptor level.

Margules et al. (1978) found that *lep* ^{ob} mice had increased β-endorphin in their pituitaries. Khawaja, Bailey, and Green (1989) assessed central mu (μ), delta (δ) and kappa (κ) opioid binding sites in *lep*^{ob} mice. Relative to lean mice, *lep*^{ob} mice had a 2.7 fold higher concentration of κ-opioid binding sites in the brain overall. Concentrations of δ-opioid binding sites were also found to be 1.6 fold higher in *lep*^{ob} mice, while there was

a decrease in μ-opioid receptors. However, no group differences occurred in binding affinities of δ- and κ- opioid receptors. Binding affinities for μ-opioid receptors were lower for *lep*^{ob} mice, which is similar to the findings in STZ-induced Type 1 diabetic rats (Chen et al., 2002; Chen & Pan, 2003). The brains of *lep*^{ob} mice also had increased levels of β-endorphin and met-enkephalin, and their pituitaries had particularly high levels of dynorphin. MacDonald, Smith, and Bailey (1998) found both increased β-endorphin and its receptors in the spinal cords of *lep* ^{ob} mice compared to lean mice, with *lep* ^{ob}s displaying a twofold increase in endorphin-sensitive cells in the dorsal horns compared to lean mice. This is particularly interesting because β-endorphins bind to both μ and δ opioid receptors. Furthermore, one notable site for opioid modulation of pain is in the dorsal horn of the spinal cord, where μ and δ receptors are present. Inhibition in the nociceptive circuits of the dorsal horn is mediated by several factors including opioid activity (Willis & Westlund, 1997). This enhanced opioid profile for *lep* ^{ob} mice means that not only are more opioids available but also more opioid receptors are located in areas known to modify nociception.

Lep^{ob} mice also have several additional hypothalamic and pituitary-adrenal abnormalities (Bereiter & Jeanrenaud, 1980; Bray & York, 1979; Garthwaite et al., 1980), which implicate opioids in nociception, and underscore the complexity of neuro-hormonal variables that can influence responsiveness to painful stimuli. In addition to anatomical differences, such as altered dendritic orientation in the ventromedial nucleus and lateral areas of the hypothalamus and decreased neuronal sizes in the dorsolateral and ventrolateral hypothalamus (Bereiter & Jeanrenaud, 1980), *lep*^{ob}s have increased pituitary and adrenal corticosteroid levels, including elevated pituitary

adrenocorticotrophic hormone (ACTH) (Garthwaite et al., 1980), corticotrophin (Bray & York, 1979), and basal serum corticosterone (Dubuc, 1977; Saito & Bray, 1983) levels. These pituitary-adrenal axis abnormalities are linked to endogenous opioids. β-endorphin and ACTH are co-released (Guillemin et al., 1977) and share the common precursor pro-opiomelanocortin (POMC) (Mains, Eipper, & Liing, 1977). The hormones of the hypothalamic-pituitary-adrenal axis are also known as stress hormones and have been associated with attenuation of pain (Jacob, Nicola, Michaud, Vidal, & Prudhomme, 1986).

An alteration in levels of endogenous opioids has been associated with obesity and diabetes and is currently the commonly suggested reason for pain response differences associated with these conditions. The *lep*^{ob} mouse is a good candidate for altered pain sensations. Yet, this has not always been substantiated in research findings. Roy et al. (1980) were among the first to study pain in the *lep*^{ob} mouse and found that they display longer jump latencies on a hotplate than lean mice. This difference suggests an analgesic response to thermal pain in these mice. However, Baxter (2003) assessed tail flick latency in adult (10-15 weeks) *lep*^{ob} and lean mice, another measure of thermal pain and found no group differences. Wilson and Rodgers (2004) assessed tail-flick latency in aged (42-70) *lep*^{ob} and lean mice. Testing occurred over 4 weeks with mice experiencing one of four stimulus temperatures (35, 40, 45, 50°C) a week in a counter-balanced order, and receiving three trials per temperature. Tail-flick testing consisted of the mice being gently restrained and their tails placed over the window of a tail-flick analgesiometer. The heat source to the window was activated; the time from heat source activation until mice reflexively moved their tails from the window was recorded and used as the

measure of tail-flick latency. Overall *lep*^{ob} mice exhibited longer tail-flick latencies than lean mice, and this significantly longer latency occurred at all four temperatures. The mean latency obtained for lean mice at 50°C replicated Mogil et al.'s (1999) observation with younger adult C57BL/6J mice. That result confirms Roy et al. (1980) finding of longer latencies in *lep*^{ob} mice to thermal stimuli and collectively suggests obese mice are analgesic compared to lean mice.

The results show different outcomes, despite both Baxter (2003) and Wilson and Rodgers (2004) using tail-flick as a measure of thermal pain in *lep*^{ob} mice in the same laboratory setting. There are two possible explanations: Age differences and minor procedural differences may have contributed to the different outcomes. While Baxter used 1 tail-flick trial per mouse and located the window for the heat source near the base of the tail, Wilson and Rodgers used 3 trials per mouse and window placement near the tail tip. The greater number of trials allowed for a repeated measures analysis of variance, which has the benefit of reducing the mean squared error term and, therefore, increases the likelihood of a significant result. In rats, window placement contributes to variability in tail-flick latencies, with placements near tail tip yielding shortest latencies (Ness, Jones, & Gebhart, 1987; Yoburn, Morales, Kelly, & Inturrisi, 1984). Furthermore, in a computational analysis of variables influencing outcomes in thermal nociceptive testing with mice, Chesler, Wilson, Lariviere, Rodriguez-Zas, and Mogil (2002) identified *the experimenter* conducting the study to be a more salient factor in determining outcomes than genotype. Their results show that experimenter and other environmental variables accounted for 42% of the variance in the data compared to 27% of the variance attributable to genotype. Hence, minor procedural differences, alone or

combined with age may account for the differences. Nonetheless, Mogil et al.'s (1999) observations in tail-withdrawal thermal analgesia in C57BL/6J were nearly identical to those reported by Wilson and Rodgers (2004). This similarity highlights the robustness and replicablity of the tail-flick phenomenon in C57BL/6J mice despite inter-laboratory contextual differences (Crabbe, Wahlsten, & Dudek, 1999).

Although unclear, the effect of age in pain sensivitiy cannot be discounted in interpreting these results. Observations on pain sensitivity in aging humans and rodents are conflicting. For example, Harkins, Price, and Martelli (1986) assessed six levels of painful thermal stimuli (43-51°C) in young, middle-age, and older adult humans. The results showed a small but significant effect: Older adults were less sensitive to lower intensity stimuli and more sensitive to higher intensities. Middle-aged adults showed the lowest sensitivity overall. Lautenbacher, Kunz, Strate, Nielsen, and Arendt-Nielsen (2005) surveyed pain perception using heat, cold, pressure and vibration in young and older adult humans. Older adults showed increases in threshold for cold and vibration, decreases for pressure pain and no differences in thermal pain threshold. Although the results of studies in humans conflict in terms of thermal pain, they suggest age influences the perception of certain types of painful stimuli.

Pain differences in relation to age have also been found in rodents. Iwata, Fukuoka, Kondo, Tsuboi, Tashiro, et al. (2002) found paw withdrawal latency to a heat stimulus was shorter in aged rats than in young adult rats. Shorter latencies suggest an increase in pain sensation. So, age may be a contributing factor to tail-flick analgesia, although species differences within the family *Rodentia* may be more important or as important in reconciling these differences.

Taken together, these studies suggest that age may influence altered pain sensation in *lep*^{ob} mice. The mechanism responsible for the change in thermal pain sensitivity in the *lep*^{ob} mice is unknown. Changes in neural plasticity associated with aging resulting in a loss of sensation are plausible; however, the lack of change in pain sensation with aging in lean mice suggests that a general aging-plasticity change is not the primary cause.

One probable factor for the altered pain sensation in *lep* ^{ob} mice is the hormone leptin. Deficiency of circulating leptin is the primary effect of the mutation in *lep*^{ob} mice and leptin regulates POMC production and, therefore, indirectly influences β-endorphin and ACTH. Therefore, leptin is a hormone of interest in studying pain sensitivity.

In 2003, Kutlu, Canpolat, Sandal, Ozcan, Sarsilmaz, and Kelestimur examined the acute effects of exogenous leptin, across time of day, on thermal pain. They administered either a single 25 μg dose of leptin (Mouse Recombinant) or vehicle intraperitoneally to lean male mice (strain unspecified). Half of the subjects received the injection during the day; the remaining half received the injection at night. Thirty min post injection, experimenters performed a hotplate test, whereby mice were placed on a 52°C hot plate and latency until paw lick or jump was measured. The results show that the intraperitoneal leptin decreased response latencies both during the day and at night. Thus, regardless of time of day, leptin increased pain sensitivity. Although the mice used in the study by Kutlu et al. (2003) were not obese the study suggests a role for leptin in heightening pain sensations. One interpretation is that leptin alters pain sensation in the one or more subgroups of the obese, diabetic population. Level of circulating leptin may correlate with degree of pain sensation. *Lep*^{ob} mice have no circulating leptin and display

an analgesic response. Zucker (*fa/fa*) rats have elevated levels of leptin and display a hyperalgesic response to thermal pain, suggesting a possible role for leptin in pain sensation. Alterations in leptin administration in obese/diabetic populations may affect pain sensation. The direction of the effect -- either hyperalgesic or analgesic -- may depend on the particular subgroup.

The question of how age and leptin influences pain launches the main objectives of this study: to assess the role of aging in the altered thermal pain profile of the *lep*^{ob} mice and to examine the effects of leptin hormone replacement on tail-flick analgesia in the *lep*^{ob} mice. Several hypotheses emerge from the current literature and these objectives. First, age is related to tail-flick latency in *lep*^{ob} mice: Older mice have increased tail-flick latencies compared to younger mice. Although Mogil et al.'s and Wilson and Rodgers' data from young and aged adult lean mice, respectively, showed no differences in tail-flick latencies, age at test may render a different result in obese mutant mice where older ages are associated with longer-term exposure to their diabetic, obese, and altered opioid phenotypes. Second, if *lep*^{ob} mice receive leptin hormone-replacement therapy, then their tail-flick latencies will decrease. Because (a) leptin synergizes and synchronizes a range of neurohormonal and neuromodulatory effects, (b) *lep*^{ob} mice have no leptin, and (c) leptin replacement therapy normalizes other aspects of the *ob* phenotype (Harris et al., 1997), chronic leptin replacement may move the behavioral phenotypes of *lep*^{ob} and lean mice closer. Third, when behavioral testing occurs during hormone-replacement therapy, test day should influence tail-flick latency, such that a greater decrease in analgesia will occur the longer the duration of hormone replacement. The effectiveness of leptin replacement in *lep*^{ob} mice typically reveals itself in

progressive weight loss over the course of therapy towards an approximation of the weights of lean mice. Therefore, any changes in analgesia in leptin-treated *lep*^{ob} mice may appear as progressive decreases in tail-flick latencies over the course of their therapy.

Experiment 1

Method

Subjects

Young adult (15 weeks) B6V-*lep*^{ob} (*n = 10*) and adult (23 weeks) B6V-*lep*^{ob} (*n = 10*) male mice (*Mus musculus*), from the Jackson Laboratory (Bar Harbor, ME, USA) were the subjects of this study. On arrival, mice were 8 and 11 weeks old, respectively. The mice were maintained on a 12-hr light – dark cycle (lights on at 0700 hr) in a temperature-controlled environment (23 ± 2 °C, relative humidity of 30-50%). Food (Lab Diet 5P00 Prolab RMH 3000) and water were available in home cages continuously throughout the study. The mice were housed individually in polypropylene cages (18 x 18 x 25 cm) with stainless-steel wire lids. Cages contained woodchip bedding, cardboard tubes and marbles for play opportunities.

Apparatus

Tail-flick testing was done on an Ugo Basile tail flick analgesiometer (Model No. 7360). The analgesiometer (see Figure 1) is a stainless-steel, 42 x 20.3 x 8.5 cm rectangular platform with a digital timer and a flush mounted window that contains a radiant heat source. The radiant heat source is a 50-W bulb of adjustable intensity that is focused by a parabolic mirror through the window (1.0 cm diameter) and onto the mouse's tail (Jones, 2002). Red tape, placed 2 cm from the center of the window, acts as a guide for tail placement. The experimenter places the mouse on the platform with its

tail over the window and depresses the switch that starts the timer and the heat source simultaneously. Movement of the tail off the window stops the timer and inactivates the heat source. The temperature was set at 40°C, according to the settings on the analgesiometer and a thermal spectral analysis (conducted by Biosystems Engineering, University of Manitoba, December, 2002). This temperature was chosen on the basis of previous studies: It is the temperature at which there is room for tail-flick latencies of the *lep*^{ob} mice to either increase or decrease (*e.g.*, Baxter, 2003; Wilson & Rodgers, 2004).

Figure 1. Ugo Basile Tail Flick Analgesiometer

Mouse "havens", adapted from a design by Mogil et al. (1999) and used by Wilson and Rodgers (2004), provided light restraint for the mice during tail-flick testing. The havens are upholstery fabric pockets measuring 14 x 9.5 cm. Each mouse had its

own haven for the duration of adaptation and testing. Havens were stored in individual, sealed plastic bags. Placing a mouse in front of the haven readily stimulates its entry. Applying light manual pressure around the entrance to the haven keeps the mouse in the haven and its tail available for the duration of a test.

Procedure

Pretest handling and adaptation. The mice were separated into two groups based on age. The young adult group entered the study first; whereas, the adult group entered the study a month later, in order to let them age further. Prior to testing all mice were handled for 2 min daily in the mouse colony, for 14 days. Handling helps to reduce stress in the mice, which should reduce any potential stress-induced analgesia during testing. For handling the experimenter covered her forearm with a green towel, then removed the mouse from its cage, and placed the mouse on the towel-covered forearm. The experimenter then stroked the mouse from its head to the tip of its body using two gloved fingers, for a total time of approximately 2 min. Mice were adapted to the test condition prior to testing to reduce stress and introduce the testing situation. Acclimatization to the testing situation occurred on a regular schedule over the 4 days that preceded testing. Adaptation consisted of transporting the mice to a waiting room, where one cage at a time was then taken into the test room. The separation of the mice was necessary to reduce access to distress signaling (both auditory and olfactory) among the mice. The analgesiometer was turned on, and the experimenter placed the mouse in front of the haven, which the mouse would then enter. The experimenter then placed the tail over the window, and timed the trial for 10 s without turning on the heat. The mouse was then returned to its cage where it waited 5-10 min before being re-introduced to the apparatus.

Three 10-s trials constituted the adaptation to the test on each of the 4 days for a total of 12 trials. The analgesiometer was swabbed with a dilute acetic acid solution (0.1% v/v) to remove odor trails from the apparatus during adaptation between different mice.

Tail-flick test. Testing of the mice occurred during mid photo-phase (approximately 1000-1500h) to minimize possible circadian effects in analgesic variability (Kavaliers & Hirst, 1983). The mice were transported to a waiting room; one mouse at a time was taken into the test room. In the test room the experimenter placed the mouse in front of a haven, and the mouse entered with its tail protruding. Then the experimenter placed the mouse's tail over the window on the analgesiometer such that the tip of the tail was at the red tape line, ensuring that the section of the tail 2 cm from the tip was the area exposed to the thermal stimulus. This distance was held constant across all mice. The experimenter depressed a switch that simultaneously activated the radiant heat source beneath the window and a digital timer. Tail flick latency was measured by an electronic timer that began at the time when the switch was depressed and stopped when the mouse reflexively "flicked" its tail away from the window. The latency for the mouse to move its tail from the heat source was recorded to the nearest 0.1 s. To prevent any possible tissue damage, a cutoff latency of 10 s was set as the maximum time for the tail to be exposed to the heat source (Kamei et al., 1993). Between mice the analgesiometer was swabbed with a dilute acetic acid solution (0.1 % v/v) to remove odor trails from the apparatus. Each mouse experienced three separate trials on the test day with 5- to 10-min inter-trial intervals.

Following the conclusion of testing, mice were returned to their home cages in the mouse colony. They remained under standard housing conditions until they aged to, at least, 40 weeks, when they participated in Experiment 2 in this series.

Data analysis. This procedure yielded a 2 X 3 (Age X Trial) mixed design, with Trial as a within-subjects variable. All data were analyzed with SPSS software. The dependent variable--tail-flick latency--was analyzed in 2 X 3 repeated measures analyses of variance (ANOVA). Effect size was assessed using partial eta squared, which represents the proportion of variance in the dependent variable (tail-flick latency) that is accounted for by the independent variable (age). Observed power was calculated in the ANOVA and is important because of the small sample size to ensure confidence in any differences found between groups. Post hoc tests included *t* tests for differences between group means. The alpha level for all statistical tests was set at 0.05.

Results

In Experiment 1, young adult *lep*^{ob} mice had shorter tail-flick latencies (M = 4.6 s, SD = 1.1) than did adult *lep*^{ob} mice (M = 5.8 s, SD = 0.77), F (1, 18) = 8.43, $p < 0.01$), with a partial eta squared of 0.319 and an observed power of 0.784. Examining the within-subjects factor showed no interaction of age and trial. However, a main effect for trial showed that tail-flick latencies became longer over the successive 10-s tests, F (2, 36) = 7.53, $p < 0.01$, with significant linear components, F (1,18) = 15.09, $p < 0.001$, and a partial eta squared of 0.295.

Mean latencies and standard deviations are presented in Table 1, for both Experiment 1 and Wilson and Rodgers (2004) where identical procedures tested tail-flick analgesia in aged *lep*^{ob} and lean mice.

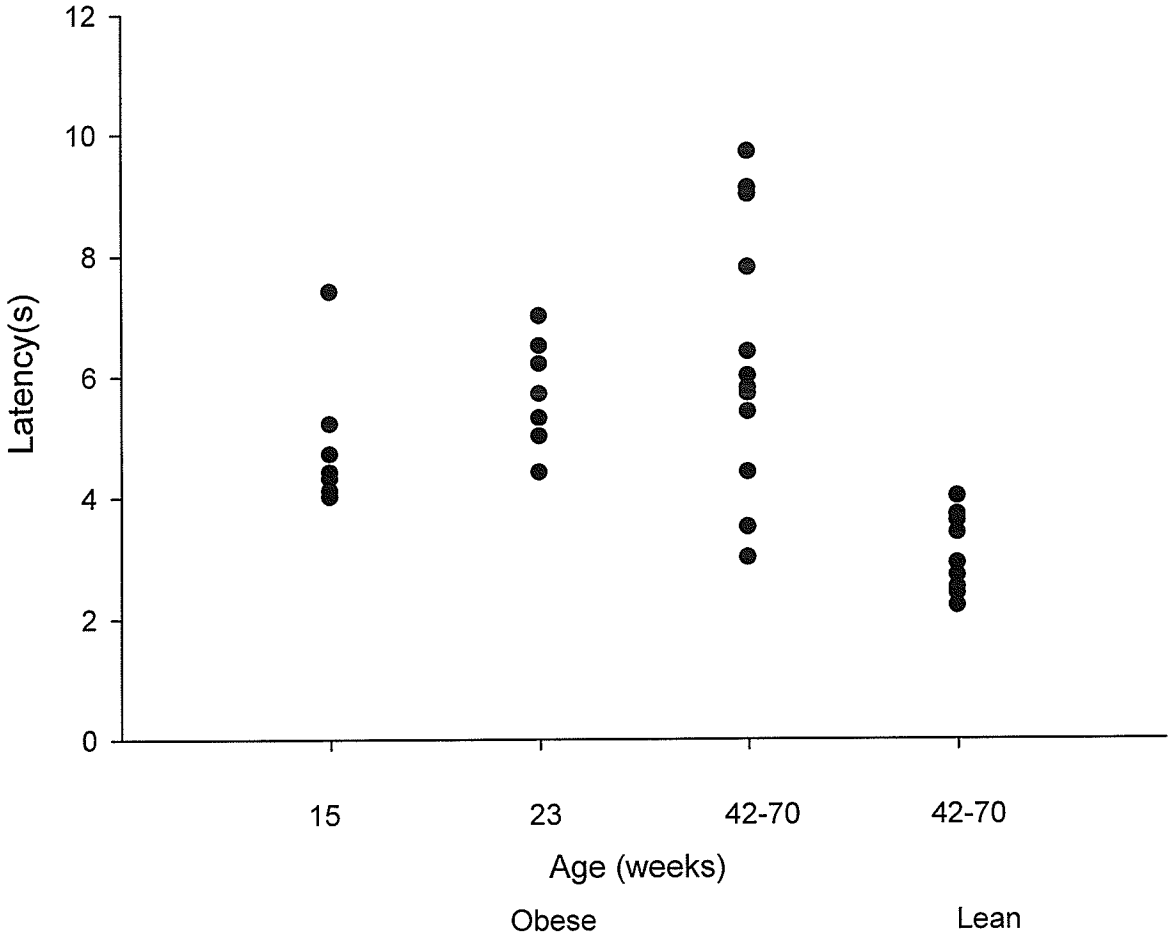
Table 1. Tail-Flick Latency Means and Standard Deviations for *Lep*^{ob} Mice at 3 Ages.

	Trial	Mean	Standard Deviation
Young Adult (15 weeks)	1	3.83	0.89
	2	4.68	1.14
	3	5.27	1.84
	Overall	4.61	1.06
Adult (23 weeks)	1	5.33	1.00
	2	6.09	1.07
	3	5.97	0.83
	Overall	5.80	0.77
Aged (42-70 weeks) (previously collected data)	1	6.24	0.84
	2	6.64	1.20
	3	6.82	0.69
	Overall	6.57	0.61

The individual latencies for both studies are shown in Figure 2.

These data were compared to those previously collected (Wilson & Rodgers, 2004) with a 3 X 3 (Age X Trials) repeated measures analysis of variance. Young adult, adult, and aged mice displayed different tail-flick latencies, $F(2,29) = 5.49, p < 0.01$. Comparisons of group means found that young adult mice had shorter latencies than older adult mice, $t(18) = -2.87, p = 0.01$. Young adult mice also had shorter latencies than aged mice, $t(20) = -2.86, p = 0.01$. Finally, older adult and aged mice had similar latencies, $t(20) = -1.17, p = 0.26$. Examining within-subject effects showed no interaction effect of age and trial, but did show a main effect of trial, $F(2, 58) = 7.76, p < 0.01$, with significant linear components, $F(1,29) = 13.30, p < 0.001$, such that latencies increased on successive trials during tail-flick testing regardless of the age group to which the mice belonged .

Figure 2. Tail-flick latency (s) as a function of age and phenotype



Comparing the *lep*^{ob} mice with the aged lean mice from the Wilson and Rodgers (2004) study showed that *lep*^{ob} mice at all three test ages (young adult, older adult, aged) had longer latencies than aged lean mice, $t(22) = 4.61$, $p < 0.001$; $t(22) = 9.72$, $p < 0.001$; $t(22) = 6.48$, $p < 0.001$, respectively.

Experiment 2

Method

Subjects

Aged adult (aged 41-45 weeks of age) male B6V-*lep*^{ob} (*N = 20*) mice (*Mus musculus*) from Experiment 1 were the subjects of this study. At the time of group assignment five mice had died from natural causes. The remaining mice were assigned to either the hormone replacement group ($n = 8$) or the control group ($n = 7$). The housing and environmental conditions of the mice were identical to those in Experiment 1.

Apparatus and Drugs

Leptin-replacement therapy was carried out using Alzet™ micro-osmotic pumps (Model No.1002). The micro-osmotic pumps are packed as sterile units and have a pumping rate of 0.25 µl/h (+/- 0.05 µl/h) with a duration of 14 days. The dimensions of the pumps are 1.5 cm (length) x 0.6 cm (diameter), with the total empty weight of 0.35 g, and a total volume of 0.5 ml. The pump consists of two components: a flow moderator and a body. The moderator is composed of styrene acrylonitrile (cap) and stainless steel (tube). The materials for the body of the pump consist of cellulose ester blend for the outer membrane and thermoplastic hydrocarbon elastomer for the drug reservoir. The pumps work by osmotic displacement. An empty reservoir that will be filled with the leptin or vehicle solution is in the core of the pump. The high concentration of salt in the chamber surrounding the reservoir causes water to enter the pump through its outer surface, a semi-permeable membrane. The water entering the pump increases volume in the salt chamber, which is separated from the reservoir by an impermeable layer, compresses the flexible reservoir and the leptin or vehicle is then delivered to the mouse

via the exit port. Thus, the hormone solution is continuously delivered at a controlled rate for the pump's duration (14 days).

All mice were weighed via a Mettler PB300 top-loading balance to the nearest 0.1 g. Pump weights were determined via a Mettler (AE166) micro-balance to the nearest 0.0001g. Water intake was measured using 100-ml Wahmann bottles fitted with rubber stoppers and short, straight stainless-steel sipping tubes, which replaced the standard water bottles in each mouse's nesting box following pump implantation. The bottles were graduated in 1-ml units.

Anesthesia for pump implantation was achieved using the E-Z Anesthesia Machine by Euthanex Corporation (Serial # EZ0251). The E-Z Anesthesia Machine consists of an isoflurane vaporizer, induction chamber and a gas delivery mask placed in front of the surgical bed. The machine delivers a precise mixture of isoflurane and oxygen to the plastic induction chamber and to the gas delivery mask. The unit includes air filters to ensure that air released into the room is safe. Heated water (37°C) circulates in both the stainless steel surgical bed and the bed under the induction chamber to help retain the animal's body temperature for the duration of surgery.

Tail-flick testing for this experiment was done using the Ugo Basile tail flick analgesiometer (Model No. 7360) described in Experiment 1. Light restraint for testing was provided by mouse havens also described in Experiment 1. For those mice receiving leptin the top of the haven was folded inwards to create a smaller pocket on Post-implantation Day 14. This was necessary due to the reduction in body mass in these mice.

For the hormone-replacement therapy, leptin from Sigma-Aldrich Canada (L3772) was used in this study. The leptin (mouse recombinant), which comes as a

lyophilized powder, was reconstituted using sterile physiological saline (0.9% w/v NaCl) and frozen at -80° C until it was re-warmed before filling the pumps to deliver 16.62 μg/day. The dosage was chosen based on other work in our lab, and on a leptin-dose response curve of Harris et al. (1998). Sterile physiological saline was used as the vehicle control.

Lurocaine (lidocaine hydrochloride, 20 mg) manufactured by Vetoquinol N.-A Inc. was diluted to a 1-in-3 solution with sterile physiological saline and used as a local subdermal analgesic before pump implantation. Isoflurane, the inhalant anesthetic (Pharmaceutical Partners of Canada) is a halogenated ether that is a non-flammable liquid, which vaporizes easily to induce and maintain anesthesia. Emla cream (2.5% lidocaine and 2.5% prilocaine) manufactured by Astra Zeneca, was used as a post-surgical topical analgesic. The post-surgical antibacterial cream used was Hibitane Veterinary Ointment (chlorhexidine acetate B.P. 1% w/w) manufactured by Ayerst Veterinary Laboratories.

Procedure

Pretest handling and adaptation. Prior to testing all mice were handled for 2 min daily in the mouse colony, for 14 days, and then intermittently for another 14 days using the methods described in Experiment 1. Mice were weighed during the handling period, in order to assign them to either the leptin or vehicle groups. Group assignment was based on weight, using a random, counter-balanced method to ensure that mean weights for each group were comparable at the time of pump implantation. Mice were re-adapted to the test environment and protocol beginning on the second day following

the pump implantation. All mice received 12 trials of adaptation over 4 days in an identical manner to that described in Experiment 1.

Implantation of osmotic pumps. Prior to implantation, the pump and its flow moderator were weighed to calculate initial (empty) pump weight. Then, the osmotic pumps were filled with either the leptin or vehicle solution. Either leptin or vehicle was introduced to the pump reservoir by removing the moderator from the pump and inserting a sterile 27-ga blunt-tipped filling tube attached to a 1.0-ml sterile syringe through the opening. Then the solution was dispensed slowly, to avoid air bubbles, from the syringe into the pump. The flow moderator was reinserted into the pump, the pump was wiped to remove any surface liquid, and the entire unit was reweighed (filled weight). The difference in weight from the initial to the final weighing indicated the net weight of the solution loaded.

In preparation for the surgery, each mouse was retrieved from the colony and weighed. The experimenter then brought the mouse into the surgery suite and placed it in the induction chamber, where it was exposed to isoflurane (3-4%) from the anesthetic vaporizer. Once the mouse succumbed (as assessed by the absence of a pedal reflex), the experimenter removed it from the induction chamber, and clipped the fur from the incision site (between the scapula to the nuchal fold) with Wahl clippers. The experimenter then placed the mouse on a surgical bed and placed its snout in the gas delivery mask to maintain anesthesia (2% isoflurane). Once the mouse was on the surgical bed until it returned to post-operative care, all procedures employed sterile technique. The skin over the implantation site was prepped with Vet Solutions Surgical Scrub (2% chloroxylenol, USP) followed by an alcohol scrub (70% isopropyl). The

experimenter then injected 0 .07 ml of lurocaine subcutaneously at the incision site. After the injection the site was swabbed twice more with the Scrub and alcohol. After the final swabbings, the area was swabbed with betadine solution (7.5% povidone-iodine USP), an antiseptic, to prepare the skin for surgery. The experimenter then made a mid-scapular incision (approximately 0.5 – 1.0 cm), with Mayo scissors, inserted the tips of a hemostatic forceps into the incision, and using blunt dissection, spread the subcutaneous tissue to create a tiny pocket for the pump. In most instances, the incision in this region produced ample subcutaneous space for pump insertion without additional dissection.

The pumps, which were sterile packed and handled with gloved hands during weighing and filling, were sanitized immediately before implantation through successive wipes with alcohol swabs and sterile physiological saline. The pump was then inserted into the subcutaneous pocket, with delivery portal end first. The incision was closed with a single cold-sterilized 9-mm stainless-steel wound clip (Clay Adams). Mice then received applications of Emla cream followed by Hibitaine ointment, and were then removed from anesthesia and monitored until they fully recovered (alert and mobile) and were returned to their home cages, 1 hour following surgery.

Monitoring mouse weights and water intakes. The pumps infused either leptin or vehicle for 14 days during which mice weights (to the nearest 0.1g) and water intake (to the nearest 1.0 ml) were measured daily. This design allowed for the assessment of the relationship between tail-flick latency and other indices of leptin replacement such as weight loss and reduction in lep ^{ob} s' characteristic hyperdipsia. Water intake served as an additional indicator variable of the health of each mouse.

Tail-flick test. Tail-flick testing occurred on Days 7 and 14 of leptin/vehicle infusion, in a similar manner to that described in Experiment 1. These days were chosen to assess whether any effect of leptin on tail-flick analgesia plateaus before the maximal infusion time allowed by the pump's capacity.

Verification of delivery. Mice were euthanized via CO_2 inhalation on post-implantation day 14 after tail-flick testing, and their pumps were removed. Measurement of the residual volume was used to verify that the pump delivered the correct volume for its duration of action. The residual volume is the solution that is left in the reservoir at the end of the infusion period. At the end of the pump's duration the reservoir should still contain some of the solution that was originally loaded into it. To measure residual volume the experimenter used the filling tube and a syringe to aspirate the solution from the reservoir. Based on residual volume, average release rate was calculated by subtracting the residual volume from the initial loading volume and dividing it by the elapsed time. Calculations showed that only one leptin pump retained most of its pre-implantation (filled) volume, and the data from that mouse were excluded from the study.

Data analysis. This procedure yielded a 2 X 2 X 3 (Hormone Treatment X Day X Trial) mixed design, with Test Day and Trial as within-subjects variables for tail-flick latencies and a corresponding mixed factorial analysis of variance. Effect size was assessed using partial eta squared, which represents the proportion of variance in the dependent variable that is accounted for by the independent variable. Observed power was also calculated and was necessary to assess because of small sample size to ensure confidence in any difference found between the groups examined. To assess the overall

effectiveness of leptin hormone-replacement, a repeated measures analysis of variance was done on water intakes (ml), and on body weights (g). The alpha level for all statistical tests was set at 0.05.

Results

Experiment 2 investigated the effects of leptin replacement on pain sensitivity in aged *lep*^{ob} mice. From the original samples, 8 mice received leptin replacement and 7 received vehicle only. One mouse in the vehicle group died prior to collection of data and one pump in the leptin group failed and therefore that mouse's data were excluded resulting in a total of 7 mice in the leptin replacement group and 6 in the vehicle group. The final calculated dosage that the mice in the leptin group received was 16.62 μg/ day. The mice were tested on days 7 and 14 post implantation with 3 trials on each test day. The means and standard deviations for latencies at each trial on post-implantation Day 7 and 14 are shown in Table 2.

Table 2. Mean Tail-Flick Latencies(s) (±Standard Deviations) as a function of Leptin Replacement

Treatment	Trial	Mean (SD)	
		Day 7	Day 14
Leptin	1	3.04 (1.09)	3.30 (0.97)
	2	3.75 (0.67)	3.46 (0.91)
	3	3.79 (0.60)	3.61 (0.65)
Vehicle	1	4.33 (1.57)	4.81 (0.55)
	2	4.91 (0.68)	4.70 (0.61)
	3	5.08 (1.46)	5.85 (0.56)

Mice receiving leptin had shorter latencies ($M = 3.5$ s) than those receiving vehicle only ($M = 5.0$ s), regardless of test day, $F(1, 11) = 28.86$, $p < 0.001$, with partial eta square = 0.724. Observed power for the difference in latency between leptin replacement and vehicle groups was .998. Individual latencies are shown in Figures 3 and 4 for post-implantation Days 7 and 14, respectively. As in Experiment 1, tail-flick latencies increased across successive test trials, $F(1.3, 20.8) = 5.89$, $p < 0.02$, using Greenhouse-Geisser corrected degrees of freedom, and had a partial eta squared = 0.34. This effect of trial showed significant linear components, $F(1, 12) = 25.46$, $p < 0.001$. Day of testing did not affect tail-flick latency, nor were there any interaction effects, $p > 0.05$.

Figure 3. Tail-flick latency (s) on Day 7 as a function of hormone replacement group

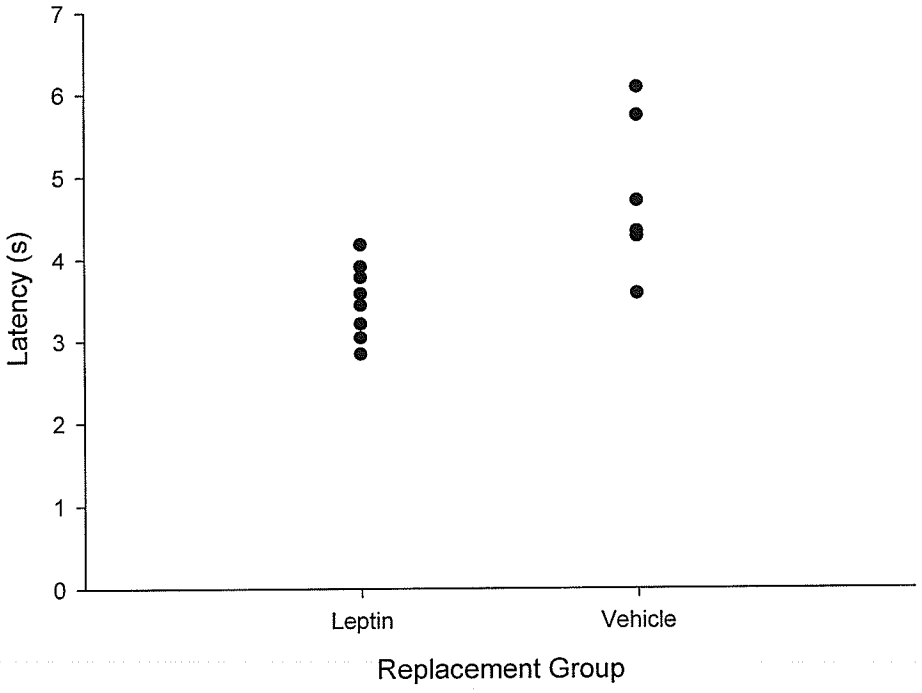
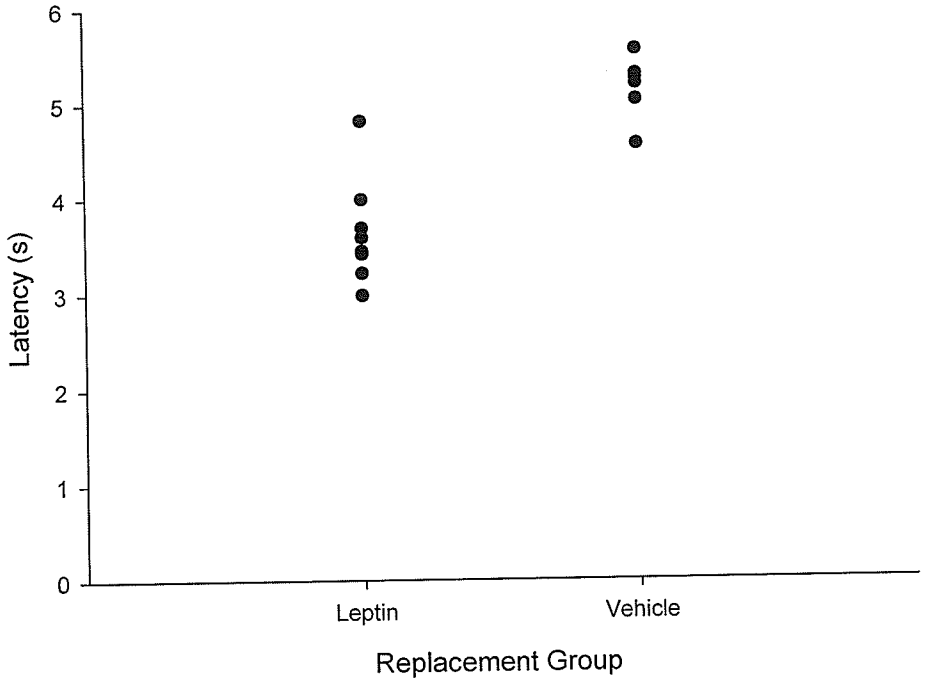
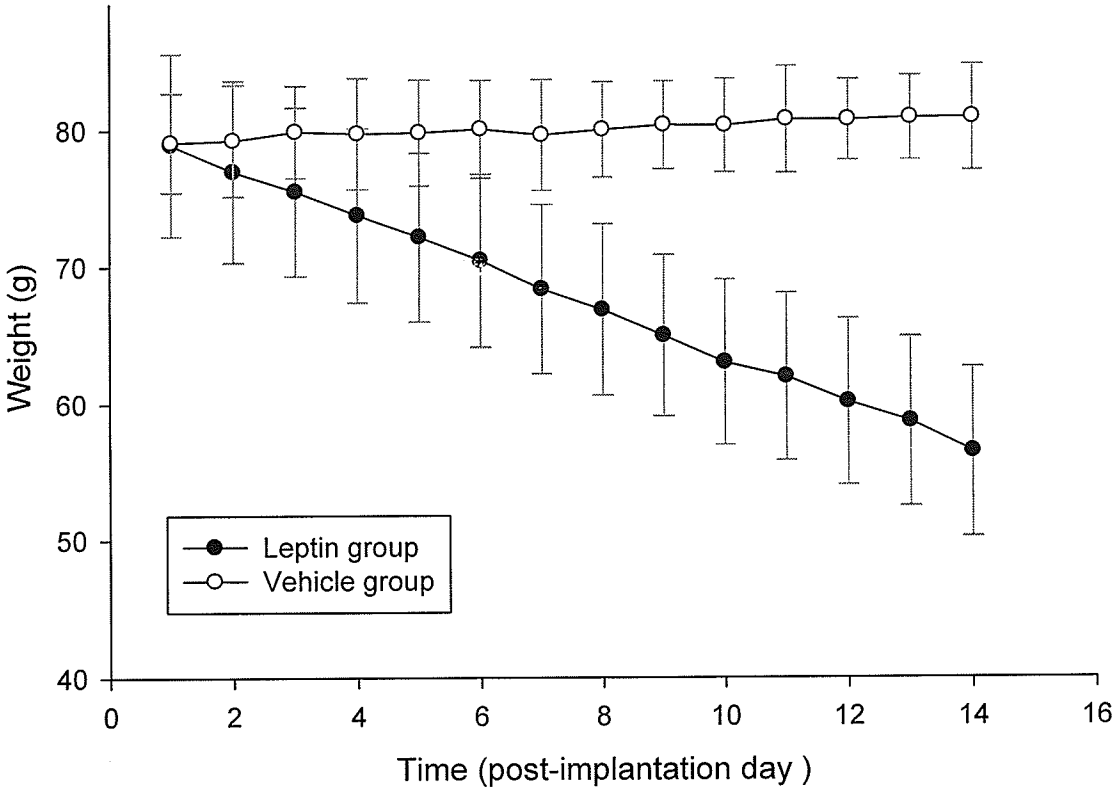


Figure 4. Tail-flick latency (s) on Day 14 as a function of hormone replacement group



The indicator variables showed changes associated with leptin replacement. Body weight measured over 14 days is shown in Figure 5. Mice in the leptin replacement group progressively lost weight over the 14 days of hormone treatment; whereas, the weights of mice receiving vehicle only remained stable, $F = 16.25$, $p < 0.002$, with a partial eta squared of 0.619. A repeated measures analysis of variance showed an interaction of hormone replacement and day, $F\,(13, 130) = 192.56$, $p < 0.001$, with a partial eta squared of 0.951. This interaction effect had significant linear components, $F\,(1, 10) = 267.64$, $p < 0.001$.

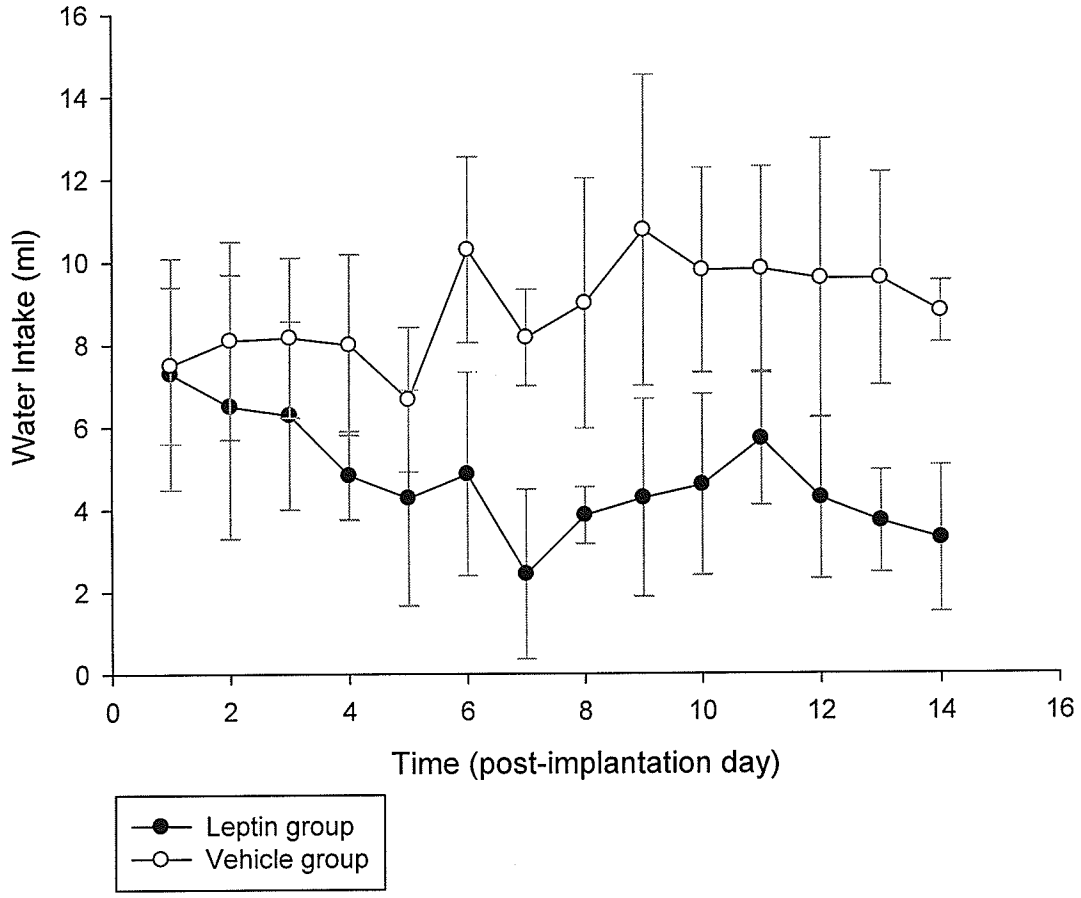
Figure 5. Body weight across 14 days for leptin replacement and vehicle groups



Water intake data for some mice were missing on certain days due to leakage of the bottle stoppers. This represented a total of six missing data points for five different mice. Therefore, missing data were replaced with a value equal to the average intake of other mice in the same group (either leptin or vehicle) on the same day. Water intake over the 14-day hormone replacement period is shown in Figure 6. Water intake in the leptin-replacement group decreased in comparison to the vehicle group over the 14 days, $F(1,11) = 26.48, p < 0.001$, with a partial eta squared of 0.707. A repeated measures analysis of variance showed an interaction of hormone replacement and day, $F(13, 143) = 3.58, p < 0.001$, with a partial eta squared of 0.246. The interaction showed significant linear, $F(1,11) = 46.94, p < 0.001$, and quadratic components, $F(1,11) = 7.45, p < 0.02$.

There was a main effect of day, $F(13,143) = 2.33$, $p < 0.01$, such that water consumption decreased over the 14 days of testing.

Figure 6. Water intake across 14 days for leptin replacement and vehicle groups



Discussion

This study examined tail-flick latency in *lep*^{ob} mice to explore the effects of age (Experiment 1) and leptin replacement (Experiment 2) on pain in an obese/diabetic animal model. The experiments confirmed the hypotheses that age and leptin replacement would affect tail-flick latency. In Experiment 1, tail-flick latencies increased with age and

in Experiment 2 tail-flick latency decreased in the *lep*^{ob} mice receiving leptin replacement.

Lep^{ob} mice are of interest in nociceptive studies because of their many characteristics, such as altered hormonal profile, obesity, and diabetes, that are known to affect nociception. Previous research in our lab on thermal nociception in *lep*^{ob} mice suggested analgesic effects in older mice possibly due to increased opioids, obesity and diabetes (Wilson & Rodgers, 2004). Experiment 1 showed that tail-flick latencies lengthened with increasing age from 15 to 23 weeks. This suggests that age increases the difference in response to noxious stimuli in *lep*^{ob} mice, such that the older the mouse (at least to 23 weeks) the greater the degree of analgesia in response to thermal stimuli. By 15 weeks of age, the physiological characteristics associated with the *ob* phenotype, specifically gross adiposity, diabetes (hyperglycemia, hyperinsulinemia), increased opioids and receptors, have fully developed. Tail-flick latencies at 15 weeks are longer in *lep*^{ob} mice than those reported in the background strain C57 mice (Mogil et al., 1999), signifying that by 15 weeks, the mechanism responsible for the analgesic effect is beginning to play a role in pain modulation in these mice. The increased latency of adult *lep*^{ob} mice at 23 weeks compared to the young adult mice suggests a progressive analgesic effect in obese/diabetic mice. The analgesic effect may result from the aging process or simply a change in sensation based on the length of exposure to an altered internal chemical environment associated with either obesity, diabetes or both.

Comparing the data of Experiment 1 with those previously collected in our lab (Wilson & Rodgers, 2004) allows the evaluation of tail-flick latency across the adult life span of the *lep*^{ob} mouse. This comparison shows group differences between young adult

lep^{ob} mice and both the adult and aged groups of *lep*^{ob} mice, but no differences between adult and aged *lep*^{ob} mice. Differences between 15 and 23 weeks of age, but no differences between mice at 23 and 42 weeks, suggests that once *lep*^{ob} mice reach a certain age in adulthood or a certain stage in their obese/diabetic syndrome the analgesic response to thermal stimuli plateaus. The lack of differences between adult and aged *lep*^{ob} mice tends to support the possibility that age is not causing the difference between young adult and adult *lep*^{ob} mice. Rather, increased latencies are either the direct result of the mutation in these mice that manifests itself over time or of a critical level of exposure to the obese/diabetic phenotype, or both.

Baxter (2003) also tested young adult (10-15 weeks) *lep*^{ob} and lean mice on tail-flick, The mice in her study had a mean tail-flick latency of approximately 3.4 s regardless of phenotype. This latency is comparable to the mean latency of 3.09 s found in the aged lean mice of our previous study (Wilson & Rodgers, 2004). The mean latency of young adult *lep*^{ob} mice in Experiment 1 was 4.61 s, which is different from the mean latency of 3.4 s in Baxter's *lep*^{ob} mice of a similar age. The only major difference between Baxter's study and Experiment 1 is location on the tail for heat placement. Baxter positioned tails over the window of the heat source, 2 cm from their bases. In Experiment 1 tails were positioned 2 cm from the tip over the heat source. Studies in rats have found that site of heating on the tail (either distal or proximal) can affect latency. Ness et al. (1987) compared three studies of tail-flick in rats that used different sections of the tail for thermal stimulation. Despite the studies having different tail-flick protocols, the results were similar: Thermal stimuli to more distal sites resulted in the shortest tail-flick latencies. They suggest that differences in sensitivity along the tail or differences in

processes of nociceptive information along the tail, may be the reason that location of thermal stimuli affects tail-flick latency. The results of Experiment 1 and Baxter (2003) suggest that tail location may affect the pain response. However, in contrast to the studies in rats, it appears that more distal sites for thermal stimuli in *lep*^{ob} mice result in longer tail-flick latencies.

The data from Experiment 1 provide a solid foundation for additional exploration of what neural, or endocrinological variables, or both, are responsible for changed thermal nociceptive responses with age. Experiment 2 explored one such endocrinological variable, the hormone, leptin. The central feature of the OB mutation is the lack of circulating leptin. Leptin becomes a hormone of interest in attempting to understand the reduced pain sensations in *lep*^{ob} mice because (a) its administration increases pain sensation in lean mice (Kutlu et al., 2003) and (b) it is lacking in *lep*^{ob} mice. An acute peripheral injection of leptin in male mice decreased latency on a hotplate, indicating that leptin administration resulted in an increase in pain sensitivity. Kutlu et al. suggested that because leptin administration mimics obesity from a hormonal viewpoint, changes in pain response associated with obesity may be due to either a direct or indirect influence of leptin.

In Experiment 2, effects of leptin replacement, in *lep*^{ob} mice on pain sensation were assessed. The results confirmed the hypothesis that leptin replacement would decrease the analgesic effect found in *lep*^{ob} and produced shorter tail-flick latencies compared to vehicle-treated mice. Although mice were tested at 7 and 14 days after starting leptin replacement, there was no change in latency associated with test day, in either the leptin or vehicle groups. The shorter tail-flick latencies regardless of test day in

the leptin group suggest that leptin is linked to pain modulation; however, length of exposure to leptin does not create a progressive effect. The shorter tail-flick latencies in the leptin group translate to a decrease in analgesic response, bringing the *lep*^{ob} pain response closer to that displayed by lean mice, which have endogenous leptin.

In addition to lacking leptin, *lep*^{ob} mice have several other notable characteristics linked to pain and analgesia. *Lep*^{ob} mice have increased levels of endogenous opioids in both the brain and spinal cord (MacDonald et al., 1998; Margules et al., 1978) as well as increased levels of opioid receptors (Khawaja et al., 1989). Furthermore, *lep*^{ob} mice also have several pituitary-adrenal axis abnormalities including elevated pituitary adrenocorticotropic hormone (Garthwaite et al., 1980), corticotrophin (Bray & York, 1979) and basal serum corticosterone (Dubuc, 1977; Saito & Bray, 1983). Opioids and adrenocorticotropic hormone share a common precursor, POMC, which is regulated by leptin (Inui, 1999; Mains et al., 1977).

The mechanism of leptin action on pain modulation is unknown and literature on leptin and pain is sparse. Several possibilities exist for a mechanism of leptin on pain. Leptin could affect the pain response directly or indirectly through its effects on other physiological processes such as hormones and neurotransmitters, like the endogenous opioids. Leptin plays roles in many processes, such as weight regulation and POMC production and has effects on obesity (Arora, 2006), diabetes (Bates et al., 2005; Moran & Phillip, 2003), glucocorticoids (van Dijk et al., 1997) and endogenous opioids (Cheung et al., 1997). Obesity and diabetes have been linked with altered pain sensations in different species (Pradalier et al., 1981; Roane & Porter, 1986; Roy et al., 1980; Wilson & Rodgers, 2004). Likewise so have glucocorticoids (Filaretov, Bogdanov & Yarushkina,

1996) and endogenous opioids (Frischkencht et al., 1988). One possible method for leptin's action is through POMC. POMC is the precursor to both β-endorphin and adrenocorticotropic hormone and is regulated by leptin (Inui, 1999). POMC neurons co-express the leptin receptor in the brain (Cheung et al., 1997) suggesting that POMC neurons are targets for leptin action. Therefore leptin may mediate nociception through its actions on POMC and its effects on opioids and stress hormones, possibly in key areas such as the dorsal horn of the spinal cord. Further research is needed into leptin and the mechanism through which it mediates the pain response. Studies such as examining leptin receptors in the spinal cord with and without leptin replacement could provide valuable insight into the mechanism of leptin action on pain in *lep*^{ob} mice.

In addition to leptin's effects on pain response in *lep*^{ob} mice, two indicator variables were measured, water intake and weight. The results showed that, over the 14-day period that the mice received leptin, their weights progressively decreased, which replicates findings in other labs (Harris et al., 1997). Water intake also showed a decrease over the course of leptin treatment. The decreases in water and weight show the normalizing effects of leptin in *lep*^{ob} mice. Bringing these phenotypes closer to those of their lean counterparts suggests that the dosage of leptin was enough to have both physiological and behavioral effects in the mouse.

An additional possible explanation for the shorter tail flicks associated with leptin administration is decreased body weight. Leptin caused approximately a 30% decrease in body weight in the *lep*^{ob} mice over the 14 days of administration. Therefore it is possible that it is the weight loss influencing pain responding and not leptin. Support for the leptin theory appears in the lack of a Day X Group interaction. Mice receiving leptin showed a

similar tail-flick response on Day 7 as on Day 14 despite weighing less on Day 14. One way to determine whether it is leptin or leptin-induced weight loss is to restrict the caloric intake to reduce the *lep*^{ob} 's body weight by approximately 30% to mimic the effect of leptin replacement and then test for tail-flick latency.

Leptin is beginning to emerge as an important hormone in the ability to sense thermal pain. In obese/diabetic populations where leptin levels, or receptor activity, or both, are altered, the pain response changes. The results of this study suggest that in the *lep*^{ob} mouse, age (or length of time with altered hormonal environment) results in an analgesic response to a noxious thermal stimulus and that lack of circulating leptin may contribute to this effect. Furthermore, administration of leptin decreases this analgesic response.

This research opens up the possibilities of exploring leptin's effects and mechanism of action on pain not only in *lep*^{ob} mice but also in other models of obesity and diabetes. One possible direction for additional research is to examine leptin replacement across the entire life span of *lep*^{ob} mice from weaning until old age on tail flick. Examining hormone and neuromodulator levels and their receptors at each point in the *lep*^{ob} 's life span would describe the neurophysiological changes that are occurring in these mice in response to leptin replacement. Levels of glucocorticoids and β-endorphin measured in *lep*^{ob} mice receiving leptin replacement would be of particular interest in understanding the role of leptin. The results of such a study would be a first step in understanding the neurophysiological role of leptin in relationship to pain modulation as both stress, which is accompanied by an increase in glucocorticoids, and β-endorphin act to attenuate pain.

Examining receptors for endogenous opioids in the dorsal horn of mice receiving leptin replacement would allow for the assessment of any potential anomalies or changes associated with leptin replacement. For example, MacDonald et al. (1998) highlighted the differences in opioid ligand and receptor neurophysiology between *lep*^{ob} and lean mice's dorsal horns, the first synapse in the ascending pain pathway. If leptin affects tail-flick analgesia at the spinal level in *lep*^{ob} mice, then spinal cord opioid receptors (density, affinity, or both), or ligand levels, or both, should change after leptin replacement to support the decrease in tail-flick latencies.

The results of this study show heightened analgesia in adult and aged mice and demonstrate the potential to at least partially reverse the analgesic response to thermal pain in obese mice. The ability to alter sensations that have been lost in obese mice leads to some exciting possibilities. However, more research is needed to understand the neurophysoiogical role that leptin is playing in modulating pain in this model.

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