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Effects of leptin on pedunculopontine nucleus (PPN) neurons

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Abstract

Leptin, a hormone that regulates appetite and energy expenditure, is increased in obese individuals, although these individuals often exhibit leptin resistance. Obesity is characterized by sleep/wake disturbances, such as excessive daytime sleepiness, increased REM sleep, increased nighttime arousals, and decreased percentage of total sleep time. Several studies have shown that short sleep duration is highly correlated with decreased leptin levels in both animal and human models. Arousal and rapid eye movement (REM) sleep are regulated by the cholinergic arm of the reticular activating system, the pedunculopontine nucleus (PPN). The goal of this project was to determine the role of leptin in the PPN, and thus in obesity-related sleep disorders. Whole-cell patch-clamp recordings were conducted on PPN neurons in 9–17 day old rat brainstem slices. Leptin decreased action potential (AP) amplitude, AP frequency, and h-current (I_H). These findings suggest that leptin causes a blockade of Na^+ channels. Therefore, we conducted an experiment to test the effects of leptin on Na^+ conductance. To determine the average voltage dependence of this conductance, results from each cell were equally weighted by expressing conductance as a fraction of the maximum conductance in each cell. I_{Na} amplitude was decreased in a dose-dependent manner, suggesting a direct effect of leptin on these channels. The average decrease in Na^+ conductance by leptin was ~40%. We hypothesize that leptin normally decreases activity in the PPN by reducing I_H and I_{Na} currents, and that in states of leptin dysregulation (i.e., leptin resistance) this effect may be blunted, therefore causing increased arousal and REM sleep drive, and ultimately leading to sleep-related disorders.

Keywords

arousal; hyperpolarization-activated cation current; sodium current

Introduction

Leptin, a hormone that regulates appetite and energy expenditure, is increased in obese individuals, although these individuals often exhibit leptin resistance (Ahima and Flier 2000). Obesity is characterized by sleep/wake disturbances, such as excessive daytime sleepiness *in the absence of sleep-disordered breathing*, increased REM sleep, increased nighttime arousals, increased total wake time, and decreased percentage of total sleep (Dixon

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et al. 2007; Vgontzas et al. 1998). Several studies have shown that short sleep duration is highly correlated with decreased leptin levels in both animal and human models (Aldabal and Bahammam 2011; Spiegel et al. 2005; Taheri et al. 2004). Arousal and REM sleep are regulated by the cholinergic arm of the reticular activating system (RAS), the pedunculo pontine nucleus (PPN) (Shouse and Siegel 1992; Steriade et al. 1990). The aim of this project was to determine the role of leptin in the PPN, and thus the possible link between leptin dysregulation and related sleep disorders.

Leptin has been shown to have widespread effects throughout the brain, including the hippocampus, cerebellum, and neocortex. There is also a wide distribution of leptin receptors throughout the brain, including the parabrachial area in the region of the PPN (Elmqvist et al. 1998). The roles of leptin in the adult *versus* the postnatal rat are different. While leptin is key in the regulation of energy homeostasis in the adult, it is relatively ineffective in these processes in the postnatal rat (Cottrell et al. 2009). Leptin is essential in the development and maturation of neuronal and glial cells in the fetus and neonate, suggesting a role in brain development (Ahima et al. 1999; Udagawa et al. 2007). In the hypothalamus, there is a postnatal leptin surge in both the rat and mouse, which begins around postnatal day 4, peaks at ~day 7 (~500% increase compared to that of baseline levels), and consistently decreases until puberty (Cottrell et al. 2009). Additionally, there is an increase in leptin receptor ObRb (the long, signaling receptor isoform) mRNA expression in the hypothalamus which begins at ~day 12, peaks at ~day 15 (~300% increase compared to levels at day 4), and decreases from ~day 16 to puberty (Cottrell et al. 2009).

This increase in ObRb mRNA expression coincides with the developmental decrease in REM sleep, which occurs from ~days 10 to 30 in the rat (comparable to the decrease in REM sleep in humans which occurs from birth to puberty) (Roffwarg et al. 1966). For over 10 years, our lab has studied the developmental decrease in REM sleep (see review in (Garcia-Rill et al. 2008)). The decrease in REM sleep in the rat is from >80% of sleep time at birth to the adult level of ~15% of sleep time (Jouvet-Mounier et al. 1970). We traced the developmental changes in PPN transmitter regulation (cholinergic, glutamatergic, GABAergic, serotonergic) and found that most transmitter systems undergo marked changes at ~day 15 (Garcia-Rill et al. 2008). Moreover, cholinergic, but not non-cholinergic, PPN neurons hypertrophy to twice their size, peaking in cell area at ~day 15, then shrinking by day 20 (Garcia-Rill et al. 2008). Therefore, there is a developmental peak in leptin receptor (ObRb) expression at 15 days (in the hypothalamus but may also be the case in the brainstem) that coincides with the developmental decrease in REM sleep and major changes in PPN neurotransmitter control. We proposed that, if the developmental decrease in REM sleep does not occur or is blunted, what follows will be a lifetime of increased arousal and REM sleep drive, such as is present in schizophrenia, anxiety disorders like panic attacks, and bipolar depression, all of which have a post-pubertal age of onset (Garcia-Rill 1997; Kobayashi et al. 2004).

In this study, we characterized the direct effects of leptin on intrinsic properties of PPN neurons in brainstem slices from 9–17 day old rat pups using whole-cell patch clamp recordings. Leptin decreased action potential (AP) amplitude, AP frequency, I_H , and sodium ion channel conductance. Therefore, leptin had an overall inhibitory effect on the activity of PPN neurons, which would cause an overall decrease in arousal and REM sleep by directly down-regulating PPN firing, essentially acting as a low pass filter on PPN efferents.

Methods

Slice preparation

Rat pups aged 9–17 days from adult timed-pregnant Sprague-Dawley rats (280–350g) were anesthetized with ketamine (70 mg/kg, I.M.) until the tail pinch reflex was absent. This age range was intended to bracket the developmental peak of leptin receptor expression that occurs around day 15 and coincides with the developmental decrease in REM sleep, and the change in neurotransmitter control: before 9–13; during 14–15; after 16–17 (Cottrell et al. 2009; Jouvet-Mounier et al. 1970). Pups were decapitated and the brain was rapidly removed and cooled in oxygenated sucrose-artificial cerebrospinal fluid (sucrose-aCSF). The sucrose-aCSF consisted of (in mM): 233.7 sucrose, 26 NaHCO₃, 3 KCl, 8 MgCl₂, 0.5 CaCl₂, 20 glucose, 0.4 ascorbic acid, and 2 sodium pyruvate. Sagittal sections (400 μ m) containing the PPN were cut and slices were allowed to equilibrate in normal aCSF at room temperature for 1 hr. The aCSF was composed of (in mM): 117 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 24.9 NaHCO₃ and 11.5 glucose. Slices were recorded at 37°C while perfused (1.5 ml/min) with oxygenated (95% O₂- 5% CO₂) aCSF in an immersion chamber for patch clamp studies. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences, and were in agreement with the National Institutes of Health guidelines for the care and use of laboratory animals.

Whole cell patch clamp recordings

To assess the effect of leptin on PPN cells, we used the whole cell patch clamp method to mimic the natural environment of these cells. Differential interference contrast optics was used to visualize neurons using an upright microscope (Nikon FN-1, Nikon, USA). Whole-cell recordings were performed using borosilicate glass capillaries pulled on a P-97 puller (Sutter Instrument Company, Novato, CA), and filled with either a high-K⁺ intracellular solution, designed to mimic the intracellular electrolyte concentration, of (in mM): 124 K-gluconate, 10 HEPES, 10 phosphocreatine di tris, 0.2 EGTA, 4 Mg₂ATP, 0.3 Na₂GTP; or a high cesium ion (Cs⁺) intracellular solution (in mM): 120 CsMeSO₃, 20 HEPES, 1 EGTA, 10 TEA-Cl, 4 Mg-ATP, 0.4 mM GTP, 10 Phosphocreatine, 2 MgCl₂, designed to block potassium channels from the intracellular side. Osmolarity was adjusted to ~270–290 mOsm and pH to 7.3. Pipettes with a resistance of 2–5 M Ω when filled were used to form of a tight seal (>1 G Ω) in voltage-clamp configuration mode, setting the holding potential at –60 mV (i.e. near the average resting membrane potential of PPN neurons). Whole cell access was accomplished by rupturing the membrane and membrane resistance and capacitance (C_m) was further determined. Capacitance transients were cancelled using computer-controlled circuitry and series resistance was compensated (>35%) in all experiments. Resting membrane potential (RMP) values were not significantly affected by leptin exposure ($n=40$, Paired sample t -test, $p > 0.05$). The RMP values before leptin were 61.5 ± 0.9 mV and 60.9 ± 1.0 mV after leptin.

In current-clamp configuration, AP amplitude and I_H current-mediated membrane potential changes (in mV) were obtained using a voltage-current (V-I) curve protocol, in a CSF extracellular solution containing the synaptic receptor antagonists gabazine (GBZ, GABA_A receptor antagonist, 10 μ M), strychnine (STR, glycine receptor antagonist, 10 μ M), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, AMPA/kainate glutamate receptor antagonist, 10 μ M), and 2-amino-5-phosphonovaleric acid (APV, NMDA receptor antagonist, 20 μ M).

I_H-current-mediated membrane potential (V_m) deflections were measured in current-clamp using 500 ms hyperpolarizing pulses of –180 pA, and direct I_H amplitudes in voltage-clamp were measured using 500 ms hyperpolarizing square pulses of –50 mV.

Standard sodium current (I_{Na}) current–voltage (I – V) families were obtained using a high cesium chloride pipette solution to block potassium channels from the intracellular side, and aCSF extracellular solution containing synaptic blockers, tetraethyl ammonium chloride (TEA-Cl 20 mM) to block extracellular potassium channels, cadmium chloride ($CdCl_2$ 0.2 mM) and nickel chloride ($NiCl_2$ 0.2 mM) to block calcium channels, and a low concentration of TTX (3–10 nM) to partially block I_{Na} in order to allow better clamping control of the sodium currents. A series of 5 ms depolarizing test pulses to voltages that ranged from –55 mV to +65 mV in 5 mV increments (the membrane potential of each cell was held at –60 mV) were used to obtain I – V and associated conductance-mediated activation curves. The voltage dependence of inactivation of I_{Na} was measured by applying a double pulse protocol consisting of a 40 ms conditioning potential (–90 mV to 5 mV, 5 mV increments) followed by a fixed test pulse capable of eliciting the maximum current I_{Na} previously observed from I – V curves (–20 mV, 10 ms). Recovery from inactivation was measured by applying a double pulse protocol consisting of two square 10 ms depolarizing pulses to –20 mV, separated by intervals of 2 ms. Current amplitudes (I) were normalized to the maximum control current amplitude (I_{max}). The peak inward current values at each potential was plotted to generate I – V curves. Conductance (G) was determined as $I/(V_m - V_{rev})$, where I is the current, V_m is the potential at which the current was evoked, and V_{rev} is the reversal potential of the current ($E_{Na} = \{RT/zF \ln \{Na^+\}_o / \{Na^+\}_i\}$). The activation curve was fitted using the Boltzmann equation: $G = G_{max} / \{1 + \exp\{(V_{1/2} - V_m)/k\}\}$, where V_m is the test pulse voltage potential at which current was evoked, G_{max} is the calculated maximal conductance, $V_{1/2}$ is the potential of half-activation or inactivation, and k is the slope factor. The normalized inactivation curves were fitted using a Boltzmann distribution equation as follows: $I = I_{max} / \{1 + \exp\{(V_{1/2} - V_m)/k\}\}$, where I_{max} is the peak sodium current elicited after the most hyperpolarized prepulse, V_m is the preconditioning pulse potential, $V_{1/2}$ is the voltage at which the sodium current is half-maximal, and k is the slope factor. Fast compensation was used to maintain the series resistance values below 12 M Ω . Series resistance values of a maximum of 12 M Ω were compensated 30–40%, therefore, series resistance was 8–9 M Ω in all experiments.

All recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) in both current and voltage clamp mode. Analog signals were low-pass filtered at 2 kHz, and digitized at 5 kHz using a Digidata-1440A interface and pClamp10.3 software (Molecular Devices). No significant rundown due to intracellular dialysis of neuronal supra- or sub threshold activity was observed during the recording period for all three types of PPN neurons (up to 40 min).

Drug application

Bath-applied drugs were administered to the slice via a peristaltic pump (Cole-Parmer, Vernon Hills, IL), and a three-way valve system such that solutions reached the slice 1.5 min after the start of application. Perfusion time shown throughout the article considers such a time difference. The sodium channel blocker, TTX, and the synaptic blockers described above were purchased from Sigma (St. Louis, MO). Potassium and calcium channel blockers were purchased from either Peptide International (Pepnet.com) or Alomone labs (Alomone.com). The direct effects of leptin on single cell intrinsic membrane properties were studied using a high-potassium pipette solution, and aCSF extracellular solution containing synaptic receptor antagonists GBZ, STR, CNQX, and APV. Leptin (rat recombinant) was purchased from Sigma (St. Louis, MO). Leptin was dissolved in 20 mM Tris (pH 8) solution. All experiments were conducted with 100 nM leptin, with the exception of the dose-response curve.

Data Analysis

Off-line analyses were performed using Clampfit software (Molecular Devices, Sunnyvale, CA). Differences were considered significant at values of $p \leq 0.05$. All results are presented as mean $\pm$ S.E.M. Comparisons between groups were carried out using a one-way ANOVA, with Bonferroni *post hoc* testing for multiple comparisons, for single cell (action potential frequency, I_H -currents and Na^+ currents) recordings. We used a two-way ANOVA to compare AP amplitude and repeated measures ANOVA with Tukey-Kramer test to compare frequency following depolarizing pulses of different amplitudes. The adjusted p -value for the Tukey-Kramer test is very conservative, so that using the 95% confidence interval we had a more realistic comparison. If the 95% confidence interval at any step contained the value “zero”, it was considered insignificant. If the 95% confidence interval at any step did not contain the value “zero”, it was considered a significant difference.

Results

Effect of Leptin on AP amplitude

Whole-cell patch clamp recordings of single PPN neurons were conducted to determine the direct and indirect effects of leptin on PPN neuronal intracellular properties. A total of 123 PPN neurons were studied. First, we determined the effect of leptin on AP amplitude. We measured AP amplitude (from AP threshold to the peak) before and during bath administration of leptin (100 nM) with synaptic blockers (see Methods). We measured AP amplitude at minutes 4, 8, 12, and 15 after leptin administration at current injection levels of 30, 60, and 90 pA. Figure 1A is an example of a single cell current clamp recording before and during administration of leptin showing a decrease in AP amplitude after leptin. The cell was subjected to a 30 pA step at all 3 time points. Note the gradual decrease in AP amplitude at control compared to 4 min and 15 min of leptin exposure; the amplitude of the 1st AP decreased from 57 mV (control) to 49 mV (4 min) and 41 mV (15 min) after leptin exposure. The amplitude of the 2nd AP decreased from 58 mV (control) to 50 mV (4 min) and 43 mV (15 min). Figure 1B shows the percent change in AP amplitude for the 1st and 2nd APs during 30 pA steps ($n=26$, $n=24$ respectively; two-way ANOVA, AP amplitude change $\times$ time: {AP amplitude change}: $F(1,230)=7.8$, $p=0.006$; (time): $F(4,230)=7.1$, $p<0.001$; *post hoc* Tukey's test, $p<0.05$), and 90 pA steps ($n=23$, $n=26$ respectively; two-way ANOVA, $p>0.05$) represented as the percentage of AP amplitude reduction after bath administration of leptin plus synaptic blockers at minutes 4, 8, 12, and 15. These results suggest that leptin decreased AP amplitude when low amplitude steps (30 pA) were used, but the application of higher currents (90 pA steps) was able to overcome this effect by activating sodium channels available for AP generating capacity.

The PPN contains three basic cell types based on *in vitro* intrinsic membrane properties (Kamondi et al. 1992; Leonard and Llinas 1994; Takakusaki and Kitai 1997). Type I PPN neurons display calcium-mediated low threshold spikes (LTS) following a release from hyper polarization. Type II PPN neurons have a hyper polarization activated potassium current (I_A), that delays the return to baseline following a hyperpolarizing current step. Type III PPN neurons have both LTS and I_A currents. The mean percent change in AP amplitude at 30 pA after leptin was $61 \pm 19\%$ for type I cells, $67 \pm 12\%$ for type II cells, and $75 \pm 11\%$ for type III cells. There was no significant difference in the effect of leptin between PPN cell types ($n=27$, Kruskal-Wallis one-way ANOVA on Ranks, $H=0.0776$, $DF=3$, $p=0.994$), therefore, leptin acted similarly on all 3 cell types. The mean percent change in AP amplitude at 30 pA after leptin was $59 \pm 13\%$ for postnatal days 8–13, $81 \pm 11\%$ for days 14–15, and $68 \pm 15\%$ for days 16–17. There was no significant difference in the effect of leptin on AP amplitude between the 3 age groups, therefore, leptin effects did not change with animal age (8–16 days old; $n=27$, Kruskal-Wallis one-way ANOVA on Ranks, $H=1.534$,

DF=3, $p=0.674$). The AP half-width did not change significantly after 15 min of administration of leptin ($n=15$, Kruskal-Wallis non parametric, one-way ANOVA, $p>0.05$).

Effect of leptin on AP frequency

Next, we determined the effect of leptin (100 nM) on AP frequency before and after 15 min of leptin (100 nM) administration. Figures 2A and 2B are examples of single cell recordings demonstrating a decrease in AP frequency during the middle and last (but not the first) segments of the current pulse after leptin exposure. The AP frequency of the cell in 2A decreased during the entire step from 16 Hz to 4 Hz after leptin exposure. The AP frequency of the cell in 2B decreased during the entire step from 18 Hz to 14 Hz after leptin exposure. AP frequency was measured during the initial, middle, and last segments of current injection steps of 30, 60, 90, 120, 150, and 180 pA ($n=9$). The Tukey-Kramer test was used to compare the means of AP frequency before leptin at every step to the means after leptin treatment at every step (Figure 2C). There was no significant decrease in AP frequency after leptin in the initial segment at any current step. However, there was a significant decrease in frequency during the middle segment at all current levels except at 30 pA. There was also a significant decrease in frequency during the last segment of the current step at 90, 120, 150, and 180 pA. The average frequency for the entire current step was significantly decreased at 90, 120, 150, and 180 pA after leptin. Figure 2C illustrates this decrement in frequency by comparing the AP frequency before and after leptin (15 min) at all current injection levels, and during all 3 segments of each current step. Ultimately, the average firing frequency induced before leptin was ~40 Hz, but only ~20 Hz after 15 min of leptin exposure (Figure 2C bottom right).

Effect of leptin on sodium currents in PPN cells

We examined the effects of leptin on the voltage-dependence of I_{Na} activation and inactivation, and the time-dependent recovery of I_{Na} from inactivation. Figure 3A is an example of a single cell recording in voltage clamp showing the inhibitory effect of leptin on both transient (large amplitude peak at the beginning of the current step) and persistent (steady-state level at the end of the current step) sodium currents using voltage injection steps ranging from -55 to 15 mV. Figure 3B shows the average sodium conductance as a fraction of maximum conductance before and after leptin (plus synaptic blockers, Ca^{2+} and K^+ channel blockers, and 3–10 nM TTX) using voltage injection steps ranging from -55 to 15 mV. Leptin reduced the slope of the curve as well as the peak sodium conductance, suggesting a mechanism that explains how higher levels of depolarization are needed to activate these neurons after leptin, so that their maximal responsiveness would be reduced, as reported above. Since different TTX concentrations were used between experiments, leptin-mediated reductions in conductance were normalized to control values. In this way, G_{max} before leptin was 1.00 ± 0.01 ($n=13$) and after leptin was 0.64 ± 0.07 ($n=11$). Therefore, leptin significantly decreased sodium conductance in PPN neurons (DF= 22, $t= 5.1$; two-tailed paired Student's t -test; $p=0.0001$). Leptin did not have an effect on either the voltage ($n=10$; one-way ANOVA, $p>0.05$), or time-dependent inactivation parameters ($n=10$; one-way ANOVA, $p>0.05$).

Figure 3C is a dose-response curve showing the percent of I_{Na} block/reduction using a -20 mV step after 15 min exposure to five different leptin concentrations (0.0001, 0.001, 0.01, 0.1 and 1 M) compared to vehicle control. The dose-response curve was fitted to a sigmoidal equation ($R^2>0.7$), showing an IC_{50} for leptin of 21 nM and the slope of the curve of 14. The most significant block/reduction was seen with the highest concentration, 1 μ M. Percentage of block at 0.0001 μ M leptin was significantly different to the observed block at 0.001 μ M (df= 11, $t=2.4$; two-tailed paired Student's t -test; $p=0.03$), at 0.1 μ M (df= 12, $t=2.3$; two-tailed paired Student's t -test; $p=0.04$), and at 1 M (df= 9, $t= 4.2$; two-tailed paired

Student's t-test; $p=0.002$). However, blocking effects observed at either $0.0001\ \mu\text{M}$ or $0.01\ \mu\text{M}$ were not significantly different from each other ($df=10$, $t=1.1$; two-tailed paired Student's t-test; $p=0.27$).

We also studied the effects of leptin on high threshold calcium channel-mediated gamma oscillations using 2 sec depolarizing ramps as previously described (Kezunovic et al. 2011). Gamma band oscillations before leptin were $35.6\pm 1.78\ \text{Hz}$ and $38.1\pm 2.6\ \text{Hz}$ after leptin. No significant change in gamma band oscillation frequency was observed after leptin compared to control ($n=9$, Paired Student's t-test, $p=0.09$). These results suggest that high threshold voltage-dependent calcium channel function was not affected by leptin.

Effect of leptin on hyper polarization-activated cation current (I_H)

Leptin was found to also decrease I_H in PPN neurons. I_H is a hyper polarization-activated depolarizing cation current, mediated by HCN channels, that plays a role in regulating resting membrane potential (RMP), neuronal responses to hyper polarization, and pacemaker potentials such as the rate of rhythmic oscillations (Luthi and McCormick 1998). Figure 4A shows a single cell recording in current clamp, illustrating the decrease in I_H -mediated membrane potential change (V_m amplitude in mV) at minutes 4 and 15 of leptin administration compared to control. Figure 4B shows the mean percent decrease in I_H mediated amplitude (mV) in current clamp mode at minutes 4, 8, 12, and 15 of leptin compared to control, showing a significant reduction ($n=39$; Kruskal-Wallis one-way ANOVA on Ranks $\{H=150.4; DF=34; p<0.001\}$; post-hoc Tukey test, $\{q=17.1; p<0.05\}$). Figure 4C shows a single cell recording in voltage clamp that demonstrates a decrease in I_H amplitude (in pA) after 15 min of leptin compared to control. Figure 4D shows a comparison of the mean percent change in I_H amplitude (in pA) before and after 15 min of leptin ($n=6$; two-tailed Student's t-test, $t=2.26$; $DF=10$; $p<0.05$). The mean percent change in I_H amplitude at $-150\ \text{pA}$ was $57\pm 8\%$ for postnatal days 8–13, $43\pm 8\%$ for days 14–15, and $50\pm 15\%$ for days 16–17. There was no significant difference in the effect of leptin on I_H amplitude between the 3 age groups ($n=20$, one-way ANOVA, $F(3,36)=0.33$; $p=0.82$). These results suggest that leptin directly decreased I_H current amplitude across all age groups. Also, the effects of leptin on I_H did not depend on PPN cell type. By decreasing the amplitude of I_H , leptin would decrease the resulting depolarization/rebound at the end of an inhibitory input. That is, I_H normally leads to a rebound excitation at the end of the inhibition (Figure 4A left arrow), but by decreasing I_H , leptin diminished the rebound (Figure 4A right arrow).

Discussion

The results from this study show that leptin decreased PPN cell activity by partially decreasing AP firing rate and amplitude, which is a consequence of decreasing sodium currents, and also by partially decreasing I_H . Our findings suggest that AP amplitude was decreased by 20–30%, and that such an effect can be overcome by higher amplitude stimulation (Figure 1). That is, leptin has a partial effect that can be overcome by activating additional channels by using higher amplitude current steps.

A similar effect was exerted on AP frequency by leptin, decreasing the maximal frequency of activation during current steps from $\sim 40\ \text{Hz}$ to $\sim 20\ \text{Hz}$ (Figure 2). Such an effect is critical during arousal states. We recently demonstrated that PPN neurons fire at gamma band frequencies when maximally activated, and such frequencies are capped at 40–60 Hz (Simon et al. 2010). We identified the mechanism behind this property as P/Q-type voltage-sensitive calcium channels that are found on all PPN neurons regardless of electrophysiological type (I, II, or III) or transmitter type (cholinergic, GABAergic, or glutamatergic) (Kezunovic et al. 2011). In fact, the PPN has been likened to a “gamma

making machine”(Urbano et al. 2012). Continuously circulating levels of leptin (that would mimic the super fusion used in these studies) would probably decrease the maximal AP firing frequency of PPN neurons from the gamma (~40 Hz) to the beta (~20 Hz) range or lower, markedly altering its arousal and REM sleep modulation. Furthermore, results presented here suggest that the effects of leptin on AP frequency are related to a direct blocking effect on sodium channels (i.e., reducing I_{Na} amplitude) rather than a direct effect on calcium channel-dependent sub threshold oscillations.

We identified a potential mechanism behind the decrease in AP amplitude and frequency as a ~40% decrement in sodium currents in all PPN cells tested (Figure 3). There are two main types of gating for Na^+ channels, so that channels regulating AP generation express transient openings, and channels regulating AP threshold are persistent and are characterized by prolonged open periods (Taylor 1993). Persistent Na^+ currents potentiate depolarization after excitatory events, facilitate AP generation, decrease inter-AP intervals, and promote bursting (Crill 1996). Sodium channels are, of course, present in all PPN neurons and appear to be depressed by leptin in a dose-dependent manner. Interestingly, a similar phenomenon has been seen with the thyrotropin-releasing hormone (TRH) analog, NP-647, in the hippocampus. NP-647 was shown to inhibit epileptiform activity by inhibiting sodium channels, resulting in a decrease in AP frequency in hippocampal neurons (Sah et al. 2011). It is not known if leptin has a similar effect in the PPN, but it would be a very interesting avenue to explore. In addition, sleep deprivation has been shown to decrease I_{NaP} and I_H resulting in a reduction in neuronal excitability, firing frequency, and rebound excitability in hippocampal neurons (Yang et al. 2010b). And of course, in the most extreme case of Na^+ current blockade, the inhibition of presynaptic sodium channels by volatile anesthetics is involved in mediating their effects, which include amnesia, lack of consciousness, and immobility (Herold and Hemmings 2012). The effects of leptin on the PPN do not appear as dramatic as anesthetics, but they are significant in reducing firing (Figure 2).

Our analysis of the dose-response curve for leptin on PPN cells showed a steady-state minimum blocking effect at 30% even at very low concentrations such as 0.1 nM. This suggests that a combination of intracellular cascades might be interacting in PPN neurons. Such mechanisms might be part of a wider activation/inactivation effect mediated by leptin receptors that could be moved towards net inhibition of I_{Na} at higher concentrations of leptin. Leptin has been shown to act via several intracellular pathways, including the JAK/STAT, MAPK, PI3K, and mTOR pathways. Leptin receptors are class I cytokine receptors that signal via janus tyrosine kinases (JAKs) (Durakoglugil et al. 2005). Activated JAKs activate several pathways, including PI 3-kinases (Shanley et al. 2002a; Shanley et al. 2002b; Niswender et al. 2001), and the JAK2-STA3 pathway of leptin signaling is also under the negative feedback control of suppressor of cytokine signaling-3 (SOCS-3) (Krebs and Hilton 2000). Another target is the adaptor protein, SH2-containing tyrosine phosphatase (SHP-2), which initiates the Grb2-Ras-RafMAPK (mitogen-activated protein kinase) cascade in neurons (Shanley et al. 2002a). It has also been shown that the anorexigenic effects of leptin on the Ventral Tegmental Area were blocked by U0126, an inhibitor of ERK1/2 phosphorylation. It has been found that increased ERK1/2 signaling in the PPN is associated with maintenance of sleep via suppression of wakefulness (Desarnaud et al. 2011), and that activation of intracellular PKA in the PPN contributes to REM sleep recovery following REM sleep deprivation (Datta and Desarnaud 2010). Future experiments will involve determining which signaling pathways in the PPN are affected by leptin. These further experiments will include testing the percent block of I_{Na} at different time points during the 15 minute leptin administration to determine if there is a higher amplitude block with early leptin exposure compared to the block after 15 minutes of leptin. If the results show that there is a higher block earlier in leptin exposure, it would be interesting to explore possible mechanisms of a “bi-phasic” compensatory response to leptin by PPN neurons. The

investigation of these intracellular mechanisms is very important in order to determine the action of leptin and possible origins of leptin resistance in the PPN.

Another possibility for this effect is the potential action of leptin on BK channels. BK channels are calcium-activated potassium channels which drive AP repolarization and fast after hyper polarization (AHP) (Storm 1987). Leptin has been shown to increase BK currents in hypothalamic neurons, an effect blocked by PI3-K inhibitors (Yang et al. 2010a). Leptin has also been shown to inhibit epileptiform activity in hippocampal neurons via PI3K-driven activation of BK channels (O'Malley et al. 2005; Shanley et al. 2002b). Therefore, another possible explanation for this effect is leptin-induced activation of BK channels leading to increased AP repolarization and AHP. Future studies involve the investigation of leptin's effect on potassium channels.

Finally, leptin decreased the amplitude of I_H , which is present in approximately 40% of PPN cells. This current regulates RMP, responses to hyperpolarization, and pacemaker potentials such as the rate of rhythmic oscillations (Luthi and McCormick 1998). The effect of leptin on this current would decrease the ability of cells to recover from inhibition, decreasing the rebound from inhibition (as shown in Figure 4), as well as decreasing coherence, which is critical to the modulation of sleep-wake states by the PPN (Urbano et al. 2012).

These blocking effects by leptin were seen in all three electrophysiological cell types within the PPN; therefore, all PPN cell types appear to bear leptin receptors and are similarly affected. Moreover, since type I cells are GABAergic or glutamatergic, type II cells are 2/3 cholinergic and the rest either GABAergic or glutamatergic, and type III cells are 1/3 cholinergic and the rest GABAergic or glutamatergic, the results suggest that all transmitter types in the PPN are similarly affected by leptin. Therefore, leptin appears to have an overall down-regulating effect on the PPN, which would ultimately lead to a decrease in arousal and REM sleep, two high frequency EEG states modulated by the PPN (Shouse and Siegel 1992; Steriade et al. 1990). These studies demonstrate the action of leptin in the non-obese, leptin-sensitive state. We propose that leptin normally acts directly on RAS neurons to alter activity, helping decrease arousal and REM sleep, so that when leptin resistance arises, that suppression is diminished, resulting in increased arousals and REM sleep drive during sleep.

Clinical implications

There has been a worldwide increase in the prevalence of obesity over the last several decades. This increase is paralleled by a trend in reduced sleep duration in both adults and children. Both longitudinal and prospective studies have shown that chronic partial sleep loss is associated with an increase in obesity (Beccuti and Pannain 2011). Obesity is characterized by excessive daytime sleepiness (in the absence of sleep-disordered breathing), decreased total sleep time, and decreased total percentage of sleep (Vgontzas et al. 1998). It has also been shown that there is an inverse relationship between body mass index (BMI) and REM sleep duration (Rutters et al. 2012). Therefore, it is clinically imperative that the association between reduced sleep and obesity be revealed. The factors that lead to shortened sleep time are frequent arousals and increased REM sleep drive during sleep. These are precisely the main states that the PPN modulates.

Waking and sleep are regulated by a distributed system of centers in the basal forebrain, hypothalamus and midbrain. The cholinergic arm of the RAS, the PPN, modulates both waking and REM sleep, states characterized by high frequency EEG activity (Garcia-Rill 2009). The basal forebrain and hypothalamus, while important in the whole animal, were shown years ago in *cerveauisole*' (precollicular transection) studies to be unable to drive high frequency activity in the cortex when disconnected from the brainstem RAS (Lindsley et al. 1949; Moruzzi and Magoun 1995). Stimulation of hypothalamic orexin neurons in

optogenetic studies elicited waking after ~20 sec (Carter et al. 2012), but stimulation of the RAS induced arousal in one-tenth the latency (<2 sec) (Garcia-Rill 2009; Moruzzi and Magoun 1995). Moreover, recent studies showed that excitation of orexin neurons failed to awaken the animal if RAS neurons were inhibited (Adamantidis et al. 2010), suggesting that *the lateral hypothalamic system acts through the RAS (both locus coeruleus-LC and PPN) to elicit arousal*. However, the LC is not involved in REM sleep at all. Thus, the RAS, in particular the PPN, is the final common pathway for arousal and REM sleep. *The direct effects of leptin on the PPN, therefore, are critical to understanding its role in the sleep dysregulation (increased arousal and REM sleep) of obese, leptin-resistant individuals.*

A possible mechanism for sleep dysregulation in obese individuals is leptin resistance within the PPN. Further studies are needed to investigate this hypothesis to gain knowledge of the mechanisms of action of leptin in the PPN, which will allow us to design strategies to compensate for the consequences of leptin resistance. Such studies include investigating the intracellular mechanism of leptin action within the PPN. Whole cell patch-clamp recordings of PPN neurons will need to be performed to investigate the effects of leptin on specific intracellular messengers and transcription factors such as cAMP, protein kinase A, and SOCS-3 (all of which play a role in the normal intracellular effects of leptin signaling in hypothalamic cells) (Sahu 2011). Other studies needed include cell-specific deletion of leptin receptors, which could involve using an ObRb gene knockout rodent model and applying an exogenous source of Cre-recombinase via an adenovirus to the PPN (Gan et al. 2010). These future studies will help us understand the mechanism by which leptin down regulates the PPN, and give us insights into the role of leptin in obesity and associated sleep disorders.

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Figure 1. Leptin reduced AP amplitude in PPN cells

A. Whole-cell patch-clamp recording in the presence of synaptic blockers (APV, CNQX, STR, GBZ, see methods) in the same PPN neuron before (left), after 4 min of leptin (100 nM) exposure (middle), and after 15 min of leptin exposure (right). This PPN neuron was subjected to a 30 pA depolarizing current step at all 3 time points. The 1st and 2nd APs are highlighted in red from AP threshold to AP peak amplitude. Note the gradual decrease in AP amplitude during the time course. The amplitude of the 1st AP decreased from 57 mV (control) to 49 mV (4 min) and 41 mV (15 min) after leptin exposure. The amplitude of the 2nd AP decreased from 58 mV (control) to 50 mV (4 min) and 43 mV (15 min) after leptin

exposure. **B.** The average and SEM of percent change in AP amplitude for the 1st and 2nd APs during 30 pA current steps (n= 26, n=24 respectively; two-way ANOVA, AP amplitude change $\times$ time: {AP amplitude change}: $F(1,230)=7.8$, $p=0.006$; (time): $F(4,230)=7.1$, $p<0.001$; *posthoc* Tukey's test, $p<0.05$), and 90 pA current steps (n=23, n=26 respectively; two-way ANOVA, $p>0.05$) represented as the percentage of AP amplitude reduction after bath administration of leptin (100 nM) *plus synaptic blockers* at minutes 4, 8, 12, and 15.

Figure 2. Leptin reduced AP frequency in PPN neurons

A. Whole-cell patch clamp recording in the same PPN neuron before (black record) and after 15 min of leptin exposure (red record). The neuron was subjected to a 90 pA current step in both records. AP frequency decreased during the entire step from 16 Hz to 4 Hz after leptin exposure. **B.** Whole-cell patch clamp recording in the same PPN neuron before (black record) and after 15 min of leptin exposure (red record). The neuron was subjected to a 120 pA current step in both records. AP frequency decreased during the entire step from 18 Hz to 14 Hz after leptin exposure. **C.** Summary plots of the average AP frequency before (black squares) and after 15 min leptin exposure in PPN neurons ($n=10$). PPN cells were subjected to current steps of 30, 60, 90, 120, 150, and 180 pA before and after leptin. AP frequency was measured during the initial, middle, and last segments of each current step. The Tukey-Kramer test was used to compare the means of AP frequency before leptin at

every step to the means after leptin treatment at every step. If the confidence interval at any step contained the value “zero”, it was considered insignificant. If the confidence interval at any step did not contain the value “zero”, it was considered significant. Top left: average AP frequency during the initial segment. There was no significant decrease in AP frequency after leptin at any of the current levels. Top right: average AP frequency during the middle segment. There was a significant decrease in frequency at 60, 90, 120, 150, and 180 pA after leptin. Bottom left: average AP frequency during the last segment. There was a significant decrease in frequency at 90, 120, 150, and 180 pA after leptin. Bottom right: average AP frequency during the entire step. There was a significant decrease in frequency at 90, 120, 150, and 180 pA steps

Figure 3. Leptin decreased sodium current conductance in PPN cells

A. Whole-cell patch clamp recording in the same PPN neuron in the presence of synaptic blockers, 0.2 mM CdCl₂, 0.2 mM NiCl₂, 20 mM TEA-Cl, and 3–10 nM TTX before (black records) and after 15 min of leptin exposure (100 nM; red records). The neuron was subjected to voltage steps from -60 mV to +15 mV. **B.** Average sodium conductance as a fraction of maximum conductance before (black curve) and after 15 min of leptin exposure (red curve) in the presence of synaptic blockers, 0.2 mM CdCl₂, 0.2 mM NiCl₂, 20 mM TEA-Cl, and 3–10 nM TTX (n=10). **C.** Dose-response curve showing the percent of I_{Na} block using a -20 mV step after 15 min of leptin exposure compared to control. I_{Na} block by

leptin was measured at leptin concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 μ M. The number of neurons tested for each concentration is shown in parentheses.

Figure 4. Leptin reduced I_H current in PPN neurons

A. Whole-cell patch clamp recording in the same PPN neuron before (left), after 4 min of leptin (100 nM) exposure (middle), and after 15 min of leptin exposure (right). The neuron was subjected to a -150 pA hyperpolarizing current step in all 3 records. I_H amplitude (represented by grey triangles) decreased from 10 mV (before leptin), to 5 mV (4 min leptin), and 2 mV (15 min leptin). The arrows at the top of each record demonstrate a reduction in the I_H rebound depolarization after leptin. **B.** Summary graph of the average percent reduction and SEM of I_H amplitude after 4, 8, 12, and 15 min of leptin exposure in PPN neurons. I_H amplitude was significantly decreased at all time points ($n=39$; Kruskal-

Wallis one-way ANOVA on Ranks $\{H=150.4; DF=34; p<0.001\}$; *posthoc* Tukey test, $\{q=17.1; *p<0.05\}$). **C.** Wholecell patch clamp recording in the same PPN neuron before (black record) and after 15 min of leptin exposure (grey record). The neuron was subjected to a -50mV voltage step in both records. I_H amplitude decreased from 68 pA (black vertical bar) to 39 pA (grey vertical bar) after leptin. Horizontal arrows indicate the peak current and the current at the end of the voltage step, i.e. the points at which I_H amplitude was measured for control (solid arrows) and after leptin (dashed arrows). **D.** Summary graph of the average percent change in amplitude (%) and SEM of I_H before (black column) and after (red column) leptin (100 nM). There was a significant reduction in I_H amplitude after exposure to leptin ($n=6$, two-tailed Student's t -test, $t=2.26$; $DF=10$; $p<0.05$)