Leptin induced inhibition and stimulation of action current firing of nonpreganglionic Edinger-Westphal neurons is dependent on Phosphatidylinositol-3 kinase

Cognitive Neuroscience MSc Thesis

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Date: 07-2010

Leptin induced inhibition and stimulation of action current firing of nonpreganglionic Edinger-Westphal neurons is dependent on Phosphatidylinositol-3 kinase

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Non-preganglionic Edinger-Westphal (npEW) neurons are involved in stress regulation and adaptation, and are the main source of Urocortin1 (Ucn1) in the central nervous system. Urocortin 1, besides its important function in the stress response, is also known for its potent food suppressing actions. Recent evidence suggests an interaction between the feeding circuitry and the stress axis at the level of the npEW. Confirming this role, functional receptors for leptin, the ObR-b, have been found in npEW-Ucn1 neurons. Leptin is a satiety factor produced by adipocytes. It regulates neurons in the central nervous system through activation of ObR-b, inducing multiple intracellular signal transductions pathways controlling gene expression and membrane excitability. Our previous studies have shown that leptin directly inhibits membrane excitability of npEW neurons. The mechanisms by which leptin regulates excitability of these neurons is not known. Therefore, in the present study, using patch-clamp electrophysiology, we tested the hypothesis that leptin regulates npEW neuron excitability via a phosphatidylinositol-3 kinase (PI3-kinase) dependent pathway. Our results show that treatment of acute npEW brain slices with 100 nM leptin reduces the action current firing frequency of the npEW neuron population by 58%, and that the selective PI3-kinase antagonist wortmannin (200 nM) prevents this inhibition. Surprisingly, at the single neuron level leptin induces an excitation in some npEW neurons, which is also PI3-kinase dependent. Confirming the opposing action of PI3-kinase on excitability of npEW, wortmannin inhibits the majority of leptin-nonresponsive npEW neurons, but induces activation in some cases. Finally, treatment with the Katp channel blocker Tolbutamide (200 μM) activates npEW neurons suggesting the presence of functional Katp channels in these neurons. Taken together our results indicate that leptin induces both excitation and inhibition of npEW neurons through activation of PI3-kinase. We suggest that alternative signaling pathways downstream of PI3-kinase determine whether the leptin action on excitability of npEW is stimulatory or inhibitory.

Keywords: nonpreganglionic Edinger-Westphal nucleus, urocortin 1, leptin, PI3-kinase, Katp channels, wortmannin, tolbutamide, action current firing, cell-attached patch-clamp electrophysiology

Introduction

In the Western society obesity is becoming a serious health problem (Bundred et al., 2001; Flegal et al., 1998, 2002; National Audit Office, 2001; Katzmaryk, 2002; Katzmaryk & Ardern, 2004) and (psychological) stress appears to play a role in its development (Dallman et al., 2003; Nieuwenhuizen & Rutters, 2008; Adam & Epel, 2007). Feeding and energy homeostasis belong to the most important functions necessary for survival, especially in times of stress. While it is known that stress and energy balance are tightly associated (Dallman et al., 2003; Nieuwenhuizen & Rutters, 2008; Adam & Epel, 2007), the neuroendocrine factors and neuronal circuitry mediating the interaction, remain to be elucidated. Therefore, it is very important to understand how and by which mechanisms energy balance, metabolism and stress interact. Recent research suggests an interaction between the feeding circuitry and the stress axis at the level of the midbrain. More specifically, the non-preganglionic Edinger-Westphal (npEW) nucleus and the neuropeptide Urocortin 1 seem to be involved in regulation of both stress and feeding (Zalutskaya et al., 2007; Vetter et al., 2002; Kozciz, 2001, 2008; Asakawa, 1999; Spina et al., 1996; Weitemier & Ryabinin, 2006).

The npEW has been reported to contain the largest expression of the neuropeptide Uruocortin 1 (Ucn1) in the mammalian brain (Kozicz, 2007, 1998; Weitemier et al., 2005). The Edinger-Westphal nucleus (EW) is the accessory nucleus of the third oculomotor nerve, which supplies preganglionic parasympathetic input by the ciliary nerve, regulating pupil constriction and lens accommodation (Westphal, 1887; Burde et al., 1982; Kozicz, 2007). Recently, a distinct population of neurons, named the npEW, has been found within the EW that in contrast to EW neurons does not show expression of choline acetyltransferase and lacks projections to the ciliary ganglion, but is positive for urocortin 1 (Weitemier et al., 2005; Ryabinin et al., Gaszner et al., 2007). Urocortin 1 is a member of the corticotropin-releasing factor (CRF) family and binds with high affinity to the CRF1 and CRF2 receptors, whereas CRF only shows high affinity for CRF1 receptors (Kozicz, 2007; Weihong & Kastin, 2008). While stress regulation classically has been known to involve the hypothalamo-pituitary-adrenal (HPA-) axis and the (neuro)-peptides CRF, ACTH and glucocorticoids (Nieuwenhuizen & Rutters, 2008; Adam & Epel, 2007), recent research also suggests a role for the npEW in stress regulation and adaptation. Ucn1 neurons in the npEW have been shown to respond to various acute and chronic stressors. For example, a 3-fold increase of Ucn1 mRNA levels in the npEW has been found after 3 hours of restraint stress (Weninger, 2000). Also, acute pain stress in rats results in elevated Fos immunopositivity and urocortin immunoreactivity within the npEW, peaking 4 hours after the stress administration (Kozicz, 2001). In addition, acute ether stress leads to an activation of npEW-Ucn1 cells, reflected by increased Fos expression, and an increase in Ucn1 mRNA (Gaszner et al., 2004), whereas chronic ether exposure results in a down regulation of Ucn1 mRNA expression without changing Fos expression (Korosi et al., 2005). Furthermore, Ucn1 knockout mice exhibit impaired responses of the HPA-axis to repeated restraint stress and to cold stress (Zalutskaya et al., 2007), while anxiety-associated behavior is enhanced (Vetter et al., 2002). The association between npEW-Ucn1 neurons and stress regulation can also be extended to humans, since in a recent study a substantial increase of Ucn1 mRNA in the npEW has been found in male suicide victims with major depression (Kozicz, 2008).

Besides its involvement in stress regulation and adaption, Ucn1 has also been associated with the regulation of energy metabolism and feeding behavior. Intracerebroventricular administration of Ucn1 increases whole body oxygen consumption and sympathetic nervous system activity (De Fanti & Alfredo Martinez, 2002). In addition, both central and peripheral administration of Ucn1 reduces food intake (Asakawa, 1999; Spina et al., 1996) and Ucn1 has also shown to be more potent in suppressing food intake than

CRF (Spina et al., 1996). It is important to note that the anorexigenic effects of Ucn1 are distinct from and not mediated by the major satiety factor leptin, since obese melanocortin (MC)-4 receptor knockout mice do not respond to leptin, while urocortin can still have anorectic effects in these mice (Marsh et al., 1999). Also the anorexigenic effects of Ucn1 typically occur sooner than that of leptin (Kotz et al., 2002).

Leptin is a negative feedback adiposity signal that acts in the brain to suppress food intake and net catabolic effectors. Leptin is produced by adipocytes and its plasma levels correlate with the number of fat cells and this way serves as a signal about the available energy reserves. Food intake leads to an increase in leptin levels while food deprivation leads to a decrease (Ahima & Flier, 2000). Administration of leptin has been shown to decrease food intake and increase energy expenditure (Ahima & Flier, 2000; Bjorbaek & Kahn, 2004; Reizes et al., 2007). A lack of leptin or leptin receptors in the brain produces severe obesity (Ahima & Flier, 2000; Bjorbaek & Kahn, 2004), signifying the importance of this hormone for the maintenance of energy homeostasis. Leptin signaling is mediated by its receptor ObR encoded by the obesity gene of which 6 isoforms exist, but for which only the long isoform ObR-b contains the intracellular domain necessary for its functional signaling activity (Frübeck, 2006; Sahu, 2004; Bjorbaek & Kahn, 2004). Leptin is thought to exert its anorexigenic effects by acting on specific populations of neurons found in the arcuate nucleus (ARC) of the ventromedial hypothalamus. Leptin stimulates the anorexigenic neurons expressing the peptides Proopiomelanocortin (POMC) and Cocaine- and Amphetamine-regulated transcript (CART) resulting in a reduction of food intake, while inhibiting the orexigenic neurons expressing Neuropeptide Y (NPY) and Agouti-related peptide (AGrP) (Ahima & Flier, 2000; Bjorbaek & Kahn, 2004; Reizes et al., 2007). Although leptin is predominantly associated with the regulation of feeding and energy homeostasis, recent evidence suggests other distinct functions for leptin. For example, ObR-b expression is not limited to the hypothalamus, but has also been found in the hippocampus, brainstem, cortex and midbrain (Caron et al., 2010). In addition, leptin has been shown to modulate hippocampal synaptic plasticity (Harvey et al., 2006), change motivational behavior and food preference (Figlewicz & Benoit, 2009) and has even been implicated in bone remodeling (Yadav et al., 2009).

As Ucn1 is not only associated with stress regulation but is also implicated in the regulation of feeding and leptin is involved in processes besides energy homeostasis, it is not surprising that npEW-Ucn1 neurons are a site of action for leptin (Caron et al. 2010; Xu, 2009). First, ObR-b mRNA is present in the mouse npEW and is co-localized with Ucn1 (Xu, 2009), indicating the presence of receptors in npEW-Ucn1 neurons for leptin signaling. Second, leptin injection in the mouse npEW induces phosphorylation of STAT3 and increases Ucn1 peptide levels (Xu, L. unpublished data). Leptin binding to the ObR-b receptor exerts chronic effects by induction of gene transcription via Janus Kinase (JAK)-2 and signal transducer and activator of transcription (STAT)-3 (Frübeck, 2006; Sahu, 2004; Bjorbaek & Kahn, 2004). In this signaling pathway STAT3 is an important downstream factor that plays a major role in leptin's control of energy homeostasis. Phosphorylation of STAT3 is an indication of the activation of the JAK2/STAT3 pathway by leptin binding to its ObR-b receptor (Frübeck, 2006; Sahu, 2004; Bjorbaek & Kahn, 2004). Therefore, the finding that leptin injection in the mouse npEW induces STAT3 phosphorylation suggests that the ObR-b receptors in the npEW Ucn1 neurons are functional. This claim is supported by the finding that incubating npEW slices directly with leptin also leads to a phosphorylation of STAT3 (Xu, L. unpublished data). Third, administration of leptin to npEW Ucn1 neurons leads to a direct and reversible inhibition of excitability in npEW neurons, as indicated by a reduction in action current frequency (Scheenen, W., unpublished data). It is interesting to note that long-term treatment of leptin has no effect on the action current frequency of npEW neurons

(Scheenen, W., unpublished data), suggesting that possible activation of JAK2/STAT3 pathway and subsequent altered gene expression by leptin does not affect cellular excitability. Taken together, the presented evidence indicates that the npEW-Ucn1 system indeed is a site of action for leptin and thus there is an interaction between the npEW system and leptin signaling.

Although the JAK/STAT pathway is a primary signaling pathway for leptin, it does not seem to mediate leptin's inhibition of npEW neurons, since the long time course of this signaling pathway does not correspond with the acute reduction in action current frequency observed after leptin application. This acute modulation of cellular activity by leptin has been reported for several brain areas (Cowley et al., 2001;Spanswick et al., 1997; Williams & Smith, 2006; Williams et al. 2006) and suggests that neurons are capable of reacting fast to rapid changes in leptin levels and that these responses do not necessarily require altered gene expression. Recently the phosphatidylinositol 3-kinase (PI3-kinase) pathway has gained attention as a potential signaling pathway mediating rapid effects of leptin on neuronal excitability (Frübeck, 2006; Sahu, 2004; Bjorbaek & Kahn, 2004). For example, leptin induced enhancement of high voltage activated calcium currents in POMC expressing neurons is mediated by an ObRb-JAK2-PI3-kinase pathway (Wang et al., 2008). Also, rapid depolarization of POMC neurons and increased action potential frequency caused by leptin could be prevented by pharmacological inhibition of PI3-kinase (Hill et al., 2008). In NPY neurons PI3-kinase has been shown to be the main regulator of large-conductance Ca^{2+}-activated potassium (BK) currents (Yang et al., 2009), while pharmacological blockage of PI3-kinase prevents leptin-induced hyperpolarization of dorsal motor nucleus of the vagus (Williams et al., 2006). PI3-kinase is thought to be activated by phosphorylated insulin receptor substrate (IRS) proteins through association with PI3-kinase's regulatory subunit (p85) and consequently increasing the activity of the catalytic domain. The stimulation of PI3-kinase allows the enzyme to catalyze the phosphorylation of phosphatidylinositol(4,5)-triphosphate (PIP2) to phosphatidylinositol(3,4,5)-triphosphate (PIP3) (Frübeck, 2006; Plum, 2006b). PIP3 subsequently may, besides recruiting downstream molecules carrying PIP3-binding domains, modulate membrane excitability by disrupting actin filaments, the last known step in enhancing activation of ATP-sensitive potassium channels (Katp) by leptin (Hegyi et al., 2004; Harvey et al. 2000; Frübeck, 2006). This is supported by the finding that application of an actin filament stabilizer prevents activation of Katp channels both by leptin and PIP3 (Harvey et al., 2000). In addition, hypothalamic POMC specific knock out of the PIP3 phosphatase Pten (PPKO) in mice leads to a significant increase in PIP3 levels, a marked hyperpolarization, and a reduction in basal firing rate of POMC neurons (Plum et al., 2006b). Pharmacological blockage of PI3-kinase and Katp restores electrical activity and leptin-induced firing of POMC neurons in these PPKO mice (Plum et al., 2006b), indicating that PI3-kinase and PIP3 are critical regulators of cellular excitability via modulation of Katp channels.

While recent research demonstrates that acute modulation of neuronal activity by leptin in the hypothalamus might utilize PI3-kinase signaling, the role of a leptin-PI3-kinase signaling pathway in the regulation of excitability of npEW-Ucn1 neurons remains to be established. Therefore in the present study we tested the hypothesis that leptin-induced inhibition of npEW-Ucn1 cells is mediated by a PI3-kinase-signaling pathway that ultimately leads to the activation of Katp channels and subsequent inhibition of the cells. We tested our hypothesis by examining the activity of npEW-Ucn1 neurons in the presence of leptin, the selective PI3-kinase antagonist wortmannin, and the Katp channel antagonist tolbutamide.

Materials and Methods

Animals

Male and female C56BL6/J mice (Charles River, Sutzfeld, Germany) of 16-21 days postnatal were used according to a procedure approved by the ethical committee for animal experimentation of Radboud University Nijmegen (DEC-RU), in accordance with the Dutch law for animal experimentation and the Declaration of Helsinki. The mice were housed with their mothers in standard plastic cages with an enriched environment that contained shelters and nesting material. Temperature and humidity were controlled and a 12/12-h light/dark cycles was imposed (lights on: 6:00). Food and water was available *ad libitum*. On the day of the experiment young pups were separated from their mother and decapitated within 15 minutes of separation.

Preparation of acute in vitro brain slices

During the experiment all solutions were continuously carbogenized with a mixture of 5% CO_2-95% O_2. After decapitation brains were rapidly removed from the skull and placed into ice-cold slicing medium, containing: 83.5 mM NaCl, 30.0 mM KCl, 1.3 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 2.6 mM $NaHCO_3$, 1.0 mM HEPES, 2.5 mM glucose and a pH of 7.4. After removing the cortex, olfactory bulb and cerebellum the remaining tissue was glued onto a holding block of a slicing chamber with the periaquaductal grey pointing upwards and flooded with ice-cold slicing medium. Using a vibratome (Leica VT1000S; Leica, Wetzlar, Germany), coronal 300 μm thick brain slices containing the npEW (between Bregma: -3.2 to -3.6 mm) were obtained and transferred to a beaker containing aCSF. The composition of the aCSF was as follows: 120.0 mM NaCl, 3.5 mM KCl, 2.5 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM glucose and a pH of 7.4. Slices were incubated at 35˚C for 30 minutes followed by a 2 hours post-incubation at room temperature.

Drugs applied in the perfusion bath

Leptin (100 nM, Company), wortmannin (200 nM, Tocris, UK) and tolbutamide (200 μM, Sigma-Aldrich, Germany) were added to the aCSF for specific experiments. Leptin was stored as a 100 μM solution in PBS (7.6 pH). Wortmannin was stored as a 200 μM solution in DMSO and added to aCSF to obtain a final DMSO concentration of 0.1%. Tolbutamide was stored as a 200 mM solution in DMSO and added to aCSF to obtain a final DMSO concentration of 0.1%. All bath-applied drugs were stored at -20˚C and dissolved to their final concentrations in aCSF just before bath application. Solution containing one or more drugs was typically perfused for ~2-3 minutes. The following procedure of drug treatment was used for each cell; first, in a control period the slice was perfused with aCSF for 120 seconds. Then, the slice was perfused with aCSF containing leptin for 2-3 minutes. Subsequently, during the washout period the slice was perfused with aCSF. To assess the effect of PI3-kinase on the activity of the cell, after the washout aCSF containing wortmannin was perfused for 2-3 minute, followed by aCSF containing leptin and wortmannin wortmannin and aCSF was perfused. After this, the cell was perfused with wortmannin and finally in a third washout period the cell was perfused with only aCSF. In some recordings the involvement of Katp channels on the activity of the cell was also assessed using the Katp channel blocker tolbutamide. For those recordings, the cell was perfused with aCSF containing tolbutamide after the third washout. After this a solution of leptin and tolbutamide was perfused. Subsequently, the cell was perfused with tolbutamide again and finally in a fourth washout period the cell was perfused with aCSF.

Cell-attached patch-clamp experiments

Recordings were performed at ~32 to 34 ˚C using a HEKA EPC-9 patch-clamp amplifier and PatchMaster software v. 2.20 (HEKA, Lambrecht/Pfaltz, Germany). Filtering of the data was applied using a filter set at 12.9 kHz and recorded at a 20 kHz resolution. Patch pipettes with a resistance between 4 and 6 MΩ were pulled from Wiretrol II glass capillaries (Drummond Scientific Company, Broomall PA, USA) using a Narishige PP-83 pipette puller (Narishige Scientific Instrument Laboratories, Tokyo, Japan). The slices were continuously superfused with aCSF, while temperature was controlled and monitored with an SH-27B inline temperature heater coupled to a TC-324B single channel heater controller (Harvard Apparatus, Holliston, MA). Cell-attached patch clamp recordings in voltage clamp mode with a pipette potential of 0 mV (Yang et al., 2007) were performed and used to measure action currents. In the cell-attached patch-clamp mode action potentials cause biphasic current events (action currents) that are due to the capacitive current and shaped as the first derivative of the action potential (Cornelisse et al., 2002). Using the peakfinding algorithm of Origin software v. 6.1 (OriginLab Corp, Northampton, MA, USA) the interspike interval times (ISI) during every 20-s epoch were determined. For each recording, the averaged ISI from each 20-s epoch was taken as 1 data point. A total of 6-10 data points that represents stable activity levels were taken to calculate the mean ISI and SD during each treatment for each cell. At the end of the experiment the whole-cell configuration was obtained and the cytoplasm was sucked into the pipette to be subjected to a single-cell rt-PCR and qPCR to verify that the recorded cell contained Ucn1 mRNA.

Single-cell RT-PCR

The cytoplasm, collected in the patch pipette, of each cell was transferred to a reaction site of an AmpliGrid AG480F DNA free microliter reaction slide (Advalytix, Munich, Germany). The cytoplasm on each reaction site was air dried at 37 ˚C using a block heater and the AmpliGrid reaction slide was transferred to an Advalytix slide tray and kept at -80˚C until further processing. An RT-mastermix consisting of 100 U/10 µl reverse transcriptase (Invitrogen, Breda, Netherlands), 5 mU/10 µl random primers (Roche, Almere, Netherlands), 20 U/10 µl Rnasin (Promega, Madison, USA), 10 mM DTT (Invitrogen, Breda, Netherlands) and 0.5 µM dNTPs (Roche, Almere, Netherlands) was used. From the RT-mastermix, 1 µl was applied on each Ampligrid reaction site containing cytoplasm and covered with a 5 µl sealing solution. The AmpliGrid AG480F reaction slide was transferred to an AmpliSpeed ASC2000D slide cycler and a reverse transcriptase reaction was performed. In short, samples were incubated first for 10 minutes at 70˚C, then for 75 minutes at 37˚C and finally for 10 minutes at 95˚C before returning to a steady state temperature of 30˚C. After reverse transcription, 4 µl DEPC-treated Milli-Q was added to each reaction site by pipetting through the sealing solution and mixed well by pipetting up and down. Next, from each reaction site 4.5 µl was taken out and transferred to standard eppendorf tubes. Each 4.5 µl sample was subsequently diluted by adding 16 µl DEPC-treated Milli-Q and distributed among 4 eppendorf tubes, to obtain 5.0 µl c-DNA samples (dilution 1:5). Each 5.0 µl c-DNA sample was brought into a PCR-mastermix (final volume 20 µl) consisting of 12.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom), 1.5 µl DEPC-treated Milli-Q, 3.0 µl primer reverse (5 µM) and 3.0 µl primer forward (5 µM). The final volume of the PCR-mastermix with c-DNA sample was 25 µl. Quantitative polymerase chain reactions were performed according to the following protocol; samples were incubated at 95˚C for 10 min after which a 50 cycle qPCR was performed, each cycle consisting of 30 sec incubation at 95˚C and 60 sec at 60˚C, followed by a dissociation stage consisting of 15 sec incubation at 96˚C, 60 sec at 60˚C and 15 sec at 95˚C. Forward and reverse primers for Ucn1 were 5'-GTAACCCGTTGAACCCCATT and 5'-CCACCGAATCGAATATGATGC

and for 18S RNA 5'-GTAACCCGTTGAACCCCATT and 5'-CCATCCAATCGGTAGTAGCG, leading to PCR products of 342 and 150 bp, respectively. Quantitative PCR samples were separated on gel and a luminescence image taken.

Statistical analysis

Results are expressed as mean and SEM. An analysis at the population level was performed in which the data (mean ISI of each treatment) of all cells was pooled together. Mean ISI values were analyzed by Friedman's test and comparisons between 2 treatments were made by one-tailed Wilcoxon's test ($\alpha = 0.05$). Multiple comparisons were corrected using Bonferroni-Holm method. Analysis at the single cell level was performed by pooling the averaged ISI of each 20s-epoch during a treatment together into one sample and compared with that of other treatments for each cell separately. Comparisons between 2 treatments were made by one-tailed Wilcoxon's test ($\alpha = 0.05$). Multiple comparisons were corrected using Bonferroni-Holm method. For all analyses the statistical package SPSS (Statistical Package for the Social Sciences, version 17.0; Fullerton, CA) was used.

Results

To determine whether PI3-kinase mediates leptin-induced inhibition of npEW-Ucn1 neurons, the action current firing frequency of 16 cells in the cell-attached patch-clamp mode was recorded in *in vitro* slices. In 9 of the 16 analyzed cells the recording could be maintained until after the second wortmannin perfusion period. 16 of the 20 recorded cells could be maintained until after the leptin and wortmannin treatment. The effect of tolbutamide on the firing frequency could be determined in addition for 1 cell. In a separate experiment the isolated effect of wortmannin and tolbutamide on the mean firing rate was assessed for 1 npEW-Ucn1 neurons. Finally 2 npEW-Ucn1 neurons were recorded without any pharmacological treatment, to assess any influence of the perfusion setup on the firing behavior of the neurons and to determine the firing rate characteristics of npEW-Ucn1 neurons when no drug is applied.

PI3-kinase blockade prevents leptin-induced inhibition of npEW neurons.
Application of leptin (100 nM) increased the interspike interval of npEW neurons examined overall by 58% (artificial cerebrospinal fluid [aCSF1]:10910±5195ms; leptin: 17283±101223ms; T=4.0, n=16, p=0.005). Removal of leptin recovered the ISI to a level that was between that of the control ([aCSF1]:10910±5195ms; leptin-washout [aCSF2]: 13276±7163ms; T=7.13, n=16, p=0.298) and leptin perfusion period (leptin: 17283±101223ms; leptin-washout [aCSF2]: 13276±7163ms; T=7.63, n=16, p=0.372). Subsequent application of wortmannin did not alter the activity of npEW neurons significantly (control [aCSF2]: 13276±7163ms; wortmannin: 7354±2571ms; T=7.71, n=16, p=0.248) but prevented the leptin-induced inhibition of npEW neurons (control [aCSF2]: 13276±7163ms; wortmannin and leptin: 9416±5617ms; T=7.50, n=16, p=0.220).

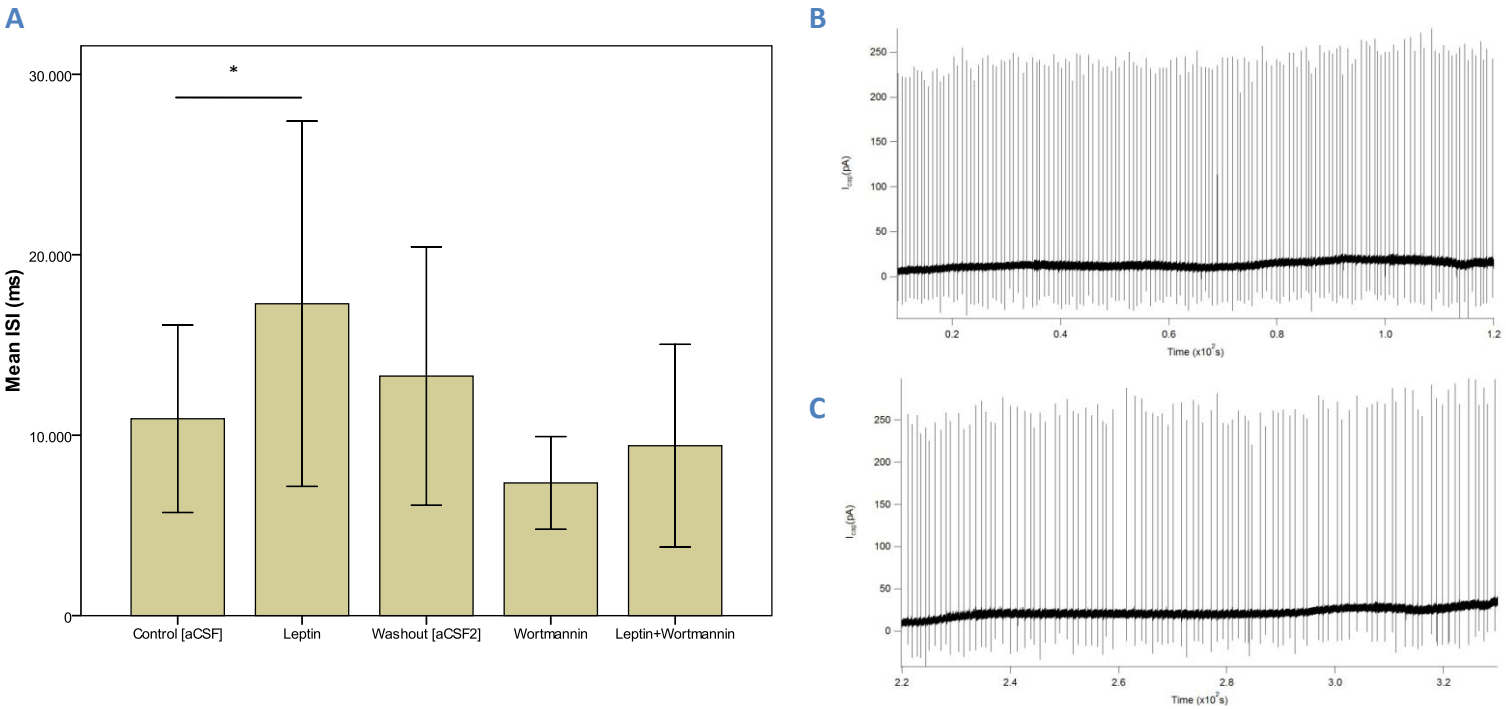
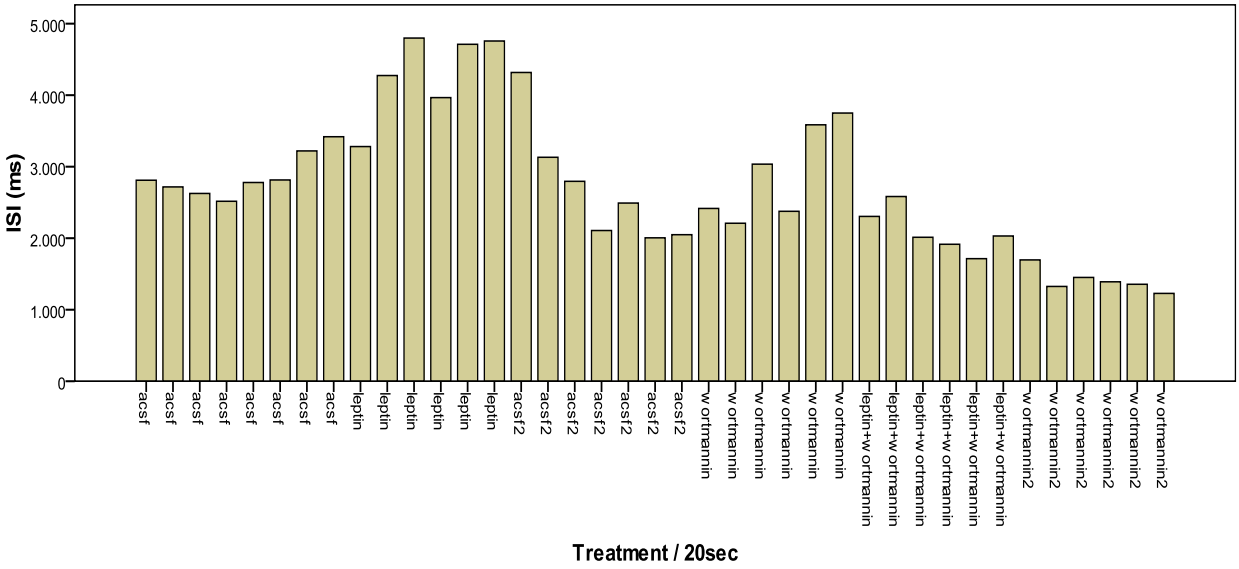


Figure 1. Mean activity of the population of npEW cells recorded. Leptin (100 nM) decreases npEW neuronal activity via a PI3-kinase dependent manner. (A) Leptin increases mean ISI of all npEW cells by 58% (n=16; p=0.005). Data represents mean ISI and SEM. Asterisk refers to a significant difference. (B-C) Sample traces of cell-attached recording of the same cell during control (aCSF) (B) and leptin (C).

Leptin has a differential effect on individual npEW neurons

Although at the population level leptin has an inhibitory effect on npEW neurons, examining the activity patterns of individual neurons reveals that npEW neurons do not form a homogenous group in their response to leptin. More specifically, application of leptin does not lead to an inhibition in all npEW neurons (Fig. 2&3). In some cells leptin treatment does not increase the ISI, as in a leptin-inhibited cell (Fig. 2A), but reduces the ISI compared to control (aCSF) and thus leads to stimulation (Fig. 2B).

A



B

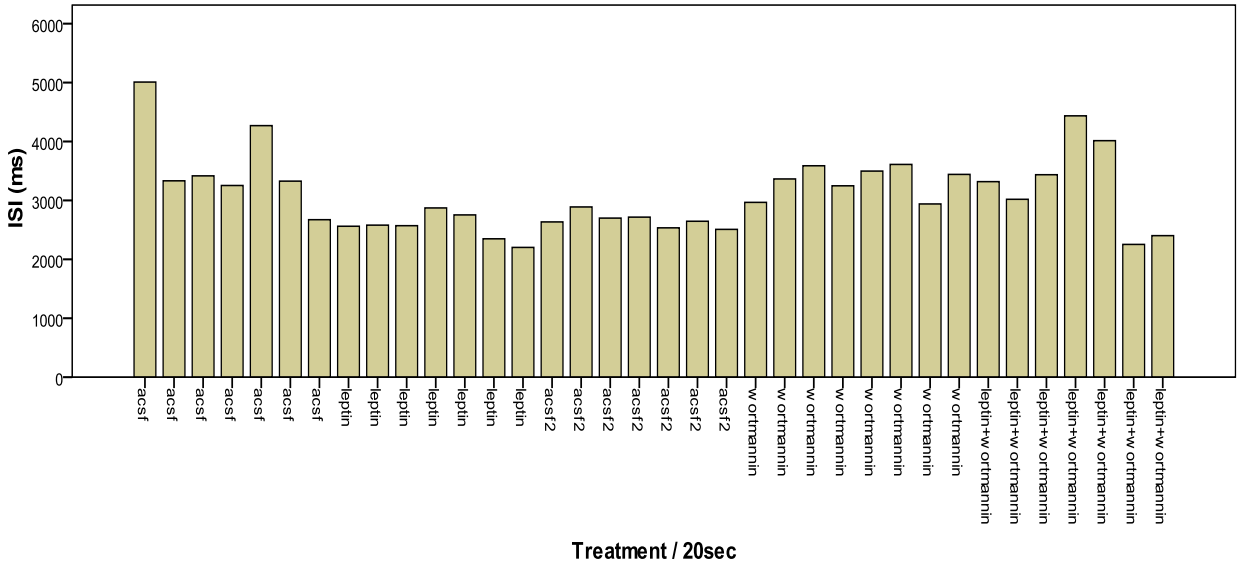


Figure 2. Activity pattern of leptin-inhibited (top) and leptin-excited npEW neuron (bottom). (A) Histogram showing interspike interval of leptin-inhibited npEW neuron (Fig. 3 (right), 4A) organized into 20-s epochs. Note that leptin treatment results in an increased ISI compared to control (aCSF) and washout (aCSF2) (B) Histogram showing interspike inteval of leptin-excited npEW neuron (Fig. 3 (left), 4D) organized into 20-s epochs. Note that leptin treatment results in a reduced ISI compared to control (aCSF). The leptin-induced stimulation is not reversible for this cell as removal of leptin (aCSF2) does not recover the ISI back to control levels (aCSF).

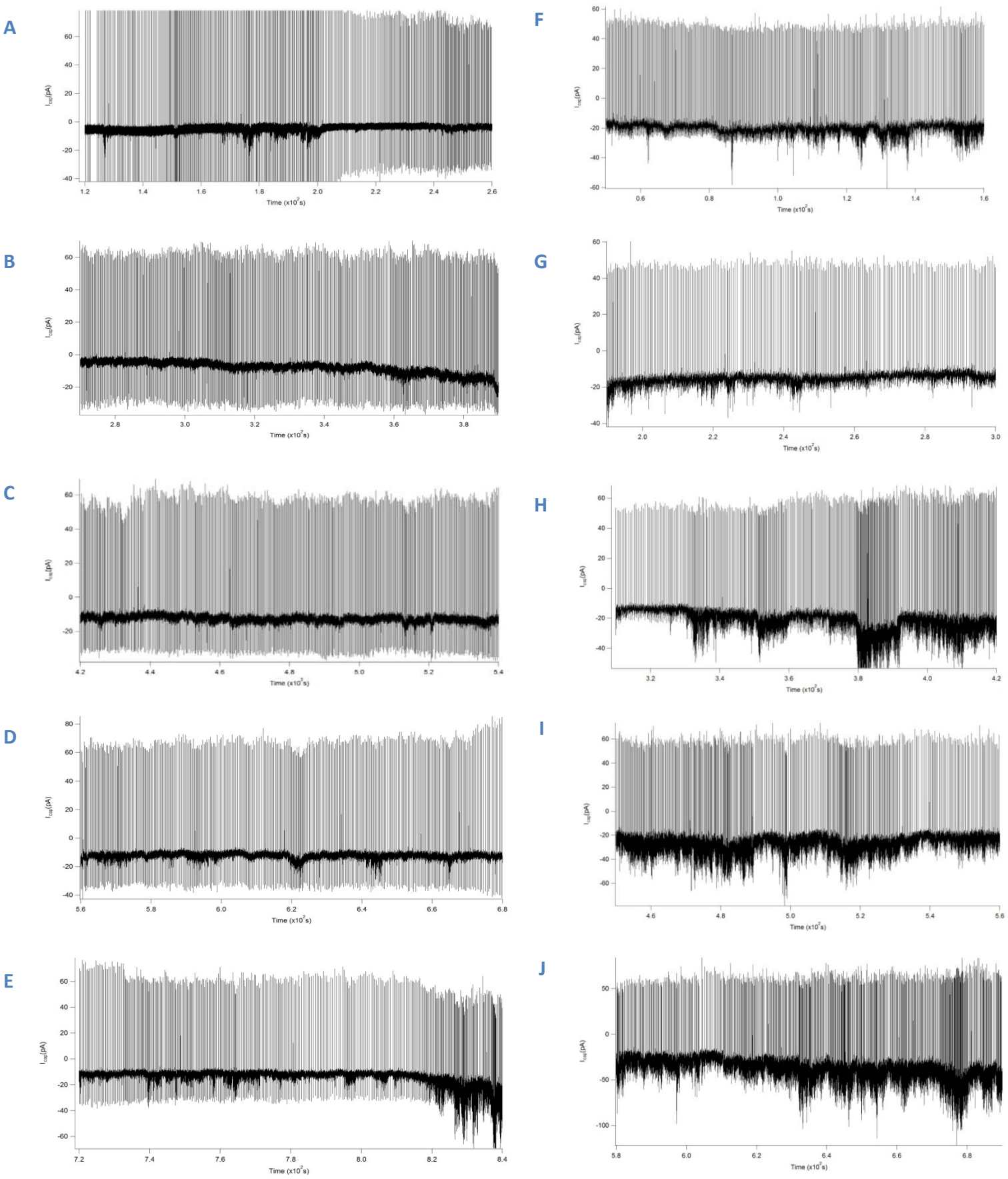


Figure 3. Firing pattern of leptin-inhibited (right) and leptin-excited npEW neuron (left). (A-E) Sample traces of cell-attached recording of leptin-excited cell in control aCSF (A), with leptin (100 nM; B), after wash to control aCSF (C) in the presence of PI3-kinase inhibitor wortmannin (200 nM; D) and in the presence of leptin and wortmannin together (E). (F-J) Sample traces of cell-attached recording of leptin-inhibited cell in control aCSF (F), with leptin (100 nM; G), after wash to control aCSF (H), in the presence of wortmannin (200 nM; I) and in the presence of leptin and wortmannin together (J).

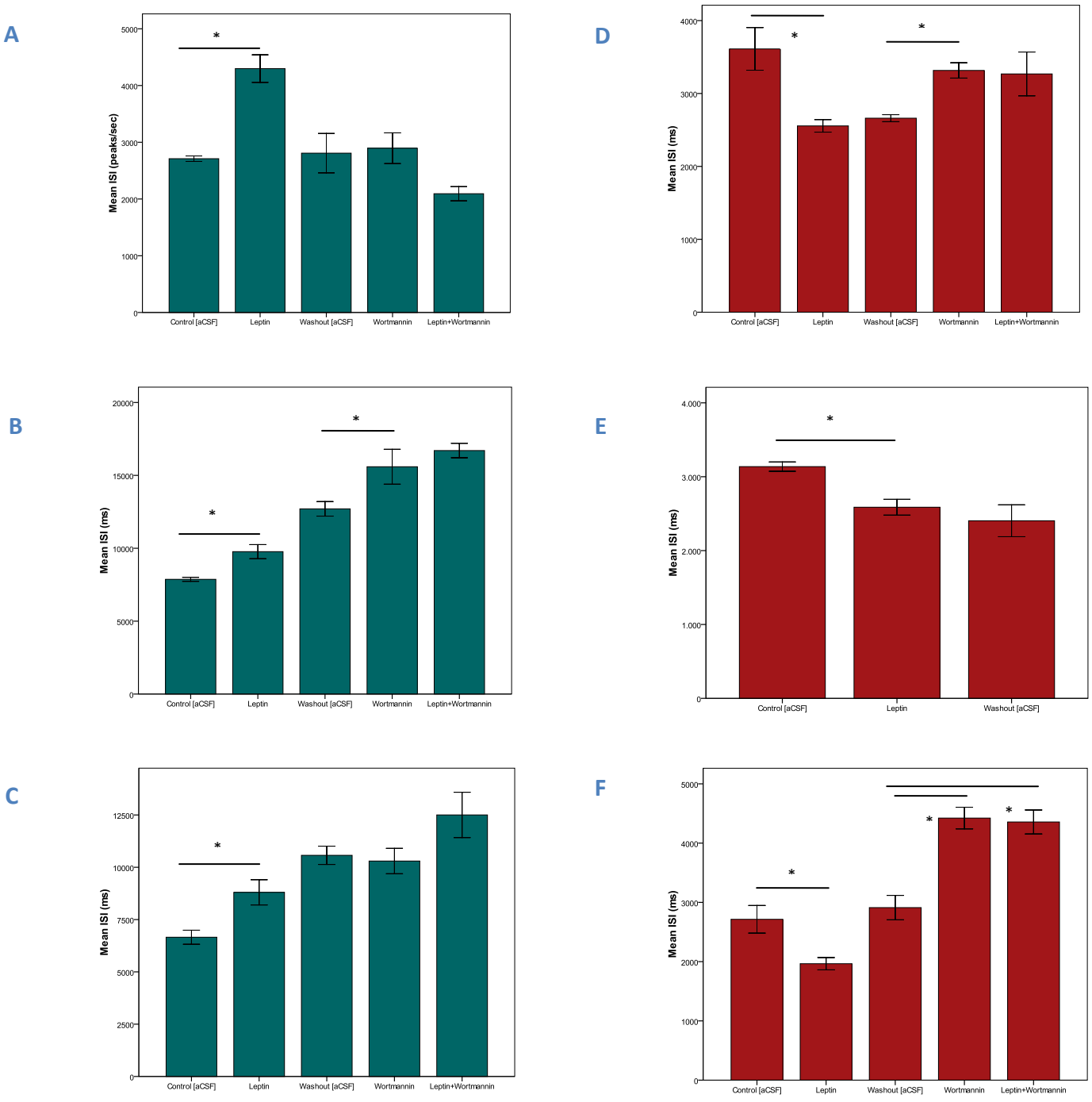


Figure 4. Mean activity of leptin-inhibited (left) and leptin-excited (right) npEW cells. (A-C) Mean ISI during different treatments of the 3 leptin-inhibited cells; (A) Compared to control leptin increased ISI by 50% (n=6, p=0.016). (B) Leptin increased ISI by 32% (n=6, p=0.016), wortmannin increased ISI by 20% (n=10, p=0.001). (C) Leptin increased ISI by 32% (n=7, p=0.023). (D-F) Mean ISI during different treatments of the 3 leptin-excited cells; (D) Leptin decreased ISI by 30% (n=7, p=0.008) and wortmannin increased ISI by 25% (n=7, p=0.008). Note that this cell, indicated by a clear single-cell Ucn1 PCR product of 350 bp, is postive for Ucn1. (E) Leptin decreased ISI by 18% (n=6, p=0.016). (F) Leptin decreased ISI by 24% (n=6, p=0.031) compared to control, wortmannin increased ISI by 58% (n=7, p=0.008) and leptin+wortmannin increased ISI by 47% (n=7, p=0.008) compared to washout. Data represents mean ISI and SEM. Asterisk refers to a significant difference. All comparisons are performed at single cell level using p=0.05 and corrected for multiple comparison by Bonferroni-Holm method.

Leptin significantly reduced the frequency of action currents by 30-50% in 3 of 16 npEW neurons (Fig. 4 A-C), but increased the firing rate significantly in 3 of 16 npEW neurons (Fig. 4 D-F) by 18-30%. In 2 of the 3 leptin-inhibited npEW cells, wortmannin blocked the leptin-induced reduction in action current frequency. In these cells application of leptin during wortmannin treatment did not change the ISI significantly compared to regular aCSF control treatment (Fig. 4A&C; washout vs. wortmannin + leptin). In one of the leptin-inhibited cells (Fig. 4B), wortmannin increased the ISI by 20% and the addition of leptin did not have an additional effect. Interestingly, in the leptin-excited cells wortmannin reduced the firing rate by 25-58% (Fig. 4 D&F). Application of leptin during wortmannin treatment did not result in a leptin-induced activation (Fig. 4 D&F; washout vs. wortmannin + leptin). Wortmannin application not only blocked the leptin-induced activation of the npEW neurons, but actually maintained the inhibition even in the presence of leptin. Together these data suggests that within the npEW both leptin-inhibited (LI) and leptin-excited (LE) neurons are present and that a PI3-kinase-dependent mechanism is required for leptin to exert its actions on npEW neurons.

PI3-kinase blockade differentially affects excitability of npEW neurons

At the individual neuron level, leptin did not significantly alter the ISI of 10 in of 16 npEW neurons. In 6 of these leptin-insensitive npEW neurons, wortmannin treatment reduced the ISI significantly by 25-95% (Table 2, control vs. wortmannin). Leptin application in these cells during wortmannin pretreatment did not significantly affect the firing rate as it did not differ from control aCSF perfusion (Table 2, control vs. wortmannin + leptin).

Cell:	ISI: Control vs. Wortmannin	ISI: Control vs. Wortmannin + Leptin
1	Control: 118690±25421 ms; wortmannin: 5940±878 ms, -95% , p=0.016.	Control: 118690±25421 ms; leptin + wortmannin: 2522±218 ms, -98%, p=0.008.
2	Control:8898±1075 ms; wortmannin: 4429±584 ms, -50%, p=0.008	Control:8898±1075 ms; leptin + wortmannin: 1871±138 ms, -79%, P=0.008
3	Control:3292±461 ms; wortmannin: 1506±173ms, -54%, p=0.008	Control:3292±461 ms; leptin + wortmannin: 889±49ms, -73% p=0.008.
4	Control:4753±663ms; wortmannin: 2270±188 ms, -52%, p=0.008.	Control:4753±663 ms; leptin + wortmannin: 1920±297 ms, -60%, p>0.05.
5	Control:2624±152ms; wortmannin: 1981±110ms, -25%, p=0.019.	Control:2624±152ms; leptin+ wortmannin: 1688±145ms, -35.6%, p=0.001.
6	Control:2697±144 ms; wortmannin: 1894±158 ms, -30%, p=0.002	Control:2697±144 ms; leptin + wortmannin: 1408±71 ms, -48%, p=0.008

Table 2. Wortmannin excites 6 leptin-nonresponsive npEW neurons. All comparisons are performed at single cell level using p=0.05 and corrected for multiple comparison by Bonferroni-Holm method. Note that cell 1 and 5, indicated by a clear single-cell Ucn1 PCR product of 350 bp, are postive for Ucn1.

In 1 of the 10 leptin-nonresponsive npEW neurons only the application of wortmannin and leptin simultaneously induced a significant change as it reduced the ISI by 69% (Fig. 5A, Control: 16686±2420 ms; leptin + wortmannin: 5195±842ms; n=6, p=0.016). Interestingly, in another leptin-insensitive npEW neuron, the application of wortmannin and leptin together induced an increase in ISI by 47% (Fig. 5B, Control: 741±33ms; leptin + wortmannin: 1086±39 ms; n=7, p=0.008). Finally, in one npEW neuron wortmannin treatment resulted in a 26% decreased firing rate (Fig. 5C, Control: 1769±72 ms; wortmannin: 2226±126ms; n=7, p=0.008). Combined with the results from the leptin-sensitive npEW cells, these data indicate that PI3-kinase-blockage has a differential effect on excitability of npEW neurons.

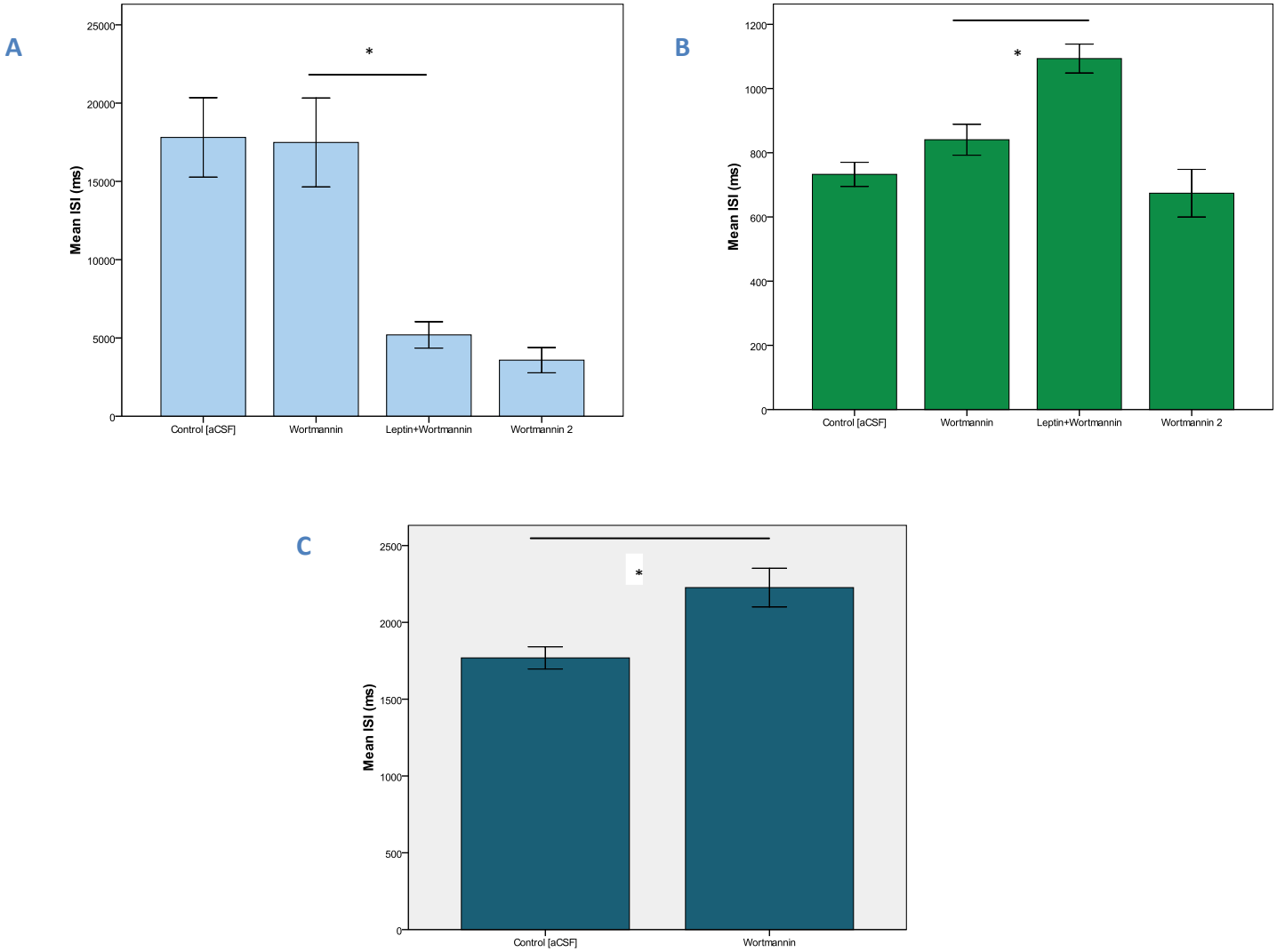


Figure 5. Activity of 3 leptin-nonresponsive npEW cells during wortmannin and leptin+wortmannin treatment. (A) NpEW cell in which only leptin and wortmannin together induced a significant change in mean ISI as it decreased mean ISI by 69% (n=6, p=0.016). Note that this cell, indicated by a clear single-cell Ucn1 PCR product of 350 bp, is postive for Ucn1. (B) NpEW cell in which only leptin and wortmannin together induced a significant change in mean ISI as it increased mean ISI by 47% (n=7, p=0.008). (C) Leptin-nonresponsive npEW cell that is inhibited for 26% by wortmannin (n=7, p=0.008). Data represents mean ISI and SEM. Asterisk refers to a significant difference.

Nonpreganglionic Edinger-Westphal neurons possess functional Katp channels
Application of the selective Katp channel blocker tolbutamide (200 µM) was used to examine the presence of Katp channels in npEW cells. The recording of one of the 16 leptin treated npEW neurons was maintained long enough for treatment with tolbutamide. Application of tolbutamide reduced the firing rate by 72% (Fig. 6, Control: 10311±1909ms; tolbutamide: 2949±160 ms; n=7, p=0.008) in this neuron.

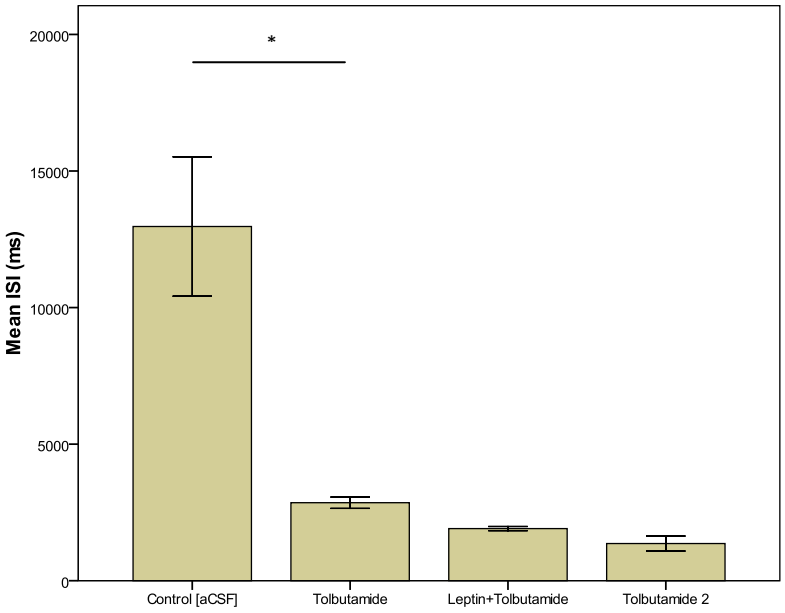


Figure 6. Activity of a leptin-nonresponsive npEW cells during tolbutamide (200 µM) and leptin+tolbutamide treatment. Tolbutamide induced a significant change in mean ISI as it decreased mean ISI by 72% (n=7, p=0.008). Data represents mean ISI and SEM. Asterisk refers to a significant difference.

In a separate experiment the effect of wortmannin and tolbutamide on the firing rate of the cell was assessed without any application of leptin. One npEW neuron was recorded in this fashion and wortmannin significantly reduced the firing rate of this cell by 67% (Fig. 7A, Control: 6905±250ms; Wortmannin: 11521±404ms; n=7, p=0.008). This wortmannin-induced inhibition was reversible as removal of wortmannin recovered the firing rate of this cell back to previous control level (Fig. 7A, Wortmannin: 11521±404ms, Washout: 7049±746ms; n=9 p=0.002). Treatment with tolbutamide on the other hand resulted in non-reversible activation of the npEW neuron by 38% (Fig. 7A, Control: 7049±746 ms; Tolbutamide: 4337±275ms; n=9; p=0.004). In addition, this cell was shown to express urocortin 1, as indicated by a clear single-cell Ucn1 PCR product of 350 bp. Together these data suggests the presence of functional Katp dependent channels within npEW(-Ucn1) neurons.

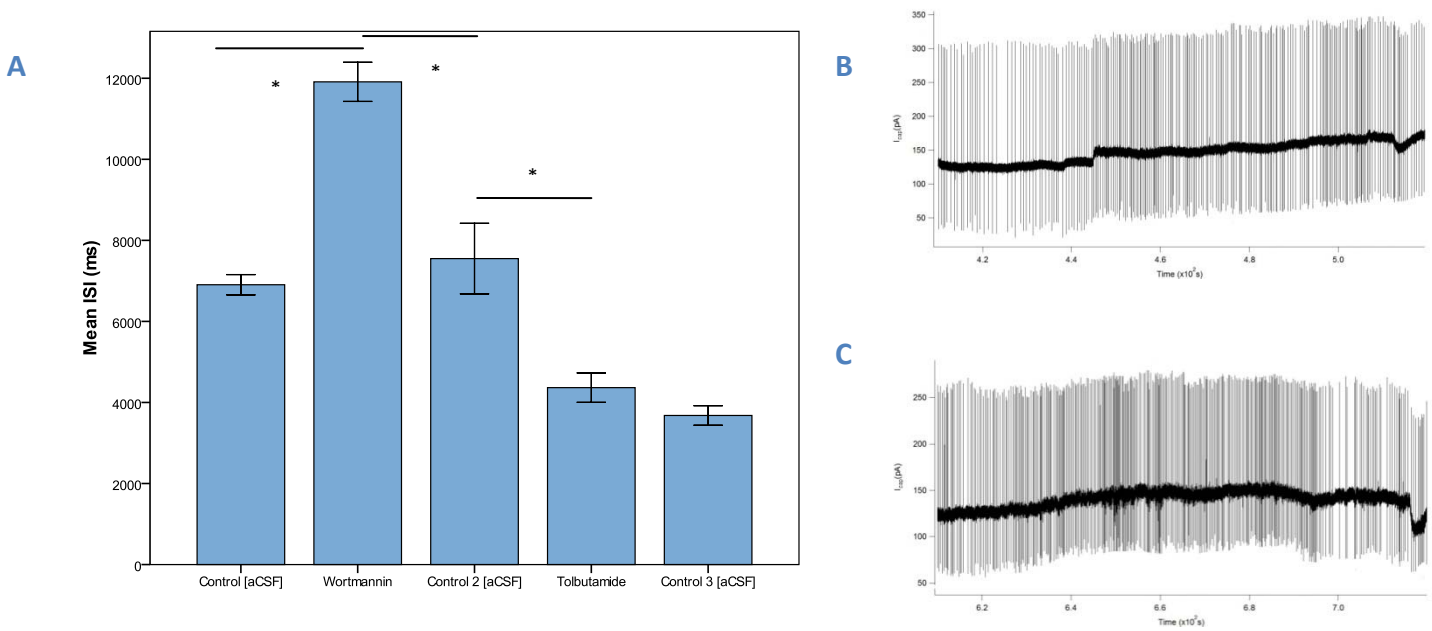


Figure 7. Activity of npEW-Ucn1 neuron recorded without leptin treatment. (A) Wortmannin (200 nM) significantly inhibited this cell by 67% (n=7, p=0.008) and tolbutamide (200 µM) induced a 38% excitation (n=9, p=0.004). Data represents mean ISI and SEM. Asterisk refers to a significant difference. (B-C) Sample traces of cell-attached recording of same cell as in A, during wortmannin (B) and tolbutamide (C).

RT-PCR

8 of the 22 drug treated npEW neurons, indicated by a clear single-cell Ucn1 PCR product of 350 bp (Fig. 8), expressed Ucn1. From the 8 Ucn1 positive neurons, 5 were part of the 16 leptin-treated cells. Leptin treatment increased the firing rate in 1 of 5 npEW-Ucn1 neurons (Fig. 4D). In the remaining 4 npEW-Ucn1 neurons, leptin did not induce a significant change in firing rate, but wortmannin excited 2 (Table 2) and leptin+wortmannin excited 1 (Fig. 5A) of these neurons respectively. Finally, one neuron that was recorded in a separate experiment in which only wortmannin and tolbutamide treatment was applied, also expressed a clear single-cell Ucn1 PCR product. Wortmannin and tolbutamide treatment in this cell resulted in an inhibition and excitation respectively (Fig. 7).

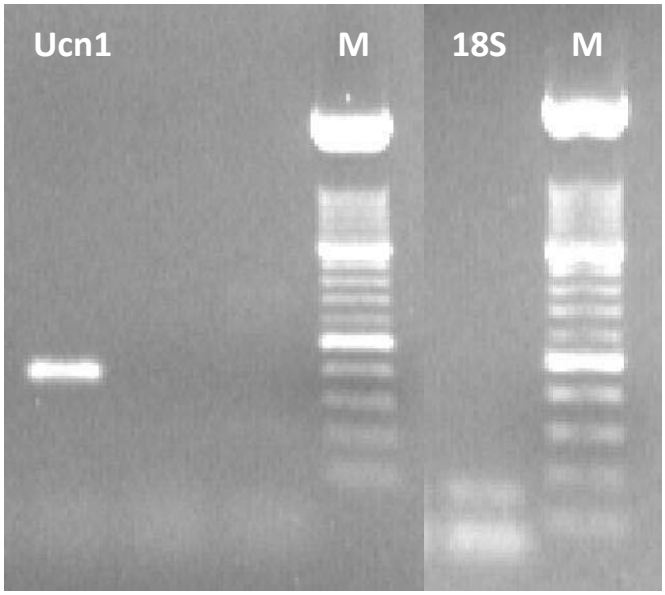


Figure 8. Identification of npEW neuron as Ucn1-positive as seen by a clear single-cell Ucn1 PCR product of 350 bp. M = Marker; 18S = 18S RNA product.

Discussion

Non-preganglionic Edinger-Westphal (npEW) neurons are involved in the regulation of stress and adaptation (Kozicz, 2007; Kozicz et al., 2001; Korosi et al., 2005; Gaszner et al., 2004) and are the dominant source of Urocortin1 in the central nervous system (Kozicz, 2007, 1998; Weitemier et al., 2005). Urocortin 1 (Ucn1), besides its important function in the stress response, is also known to be a potent anorexigenic hormone (Spina et al., 1996; Fekete and Zorilla, 2008). Recent research suggests an interaction between the feeding circuitry and the stress axis at the level of the npEW (Zalutskaya et al., 2007; Vetter et al., 2002; Kozciz, 2001, 2008; Asakawa, 1999; Spina et al., 1996; Weitemier & Ryabinin, 2006). Confirming this role, functional receptors (ObR-b) for one of the main satiety signals, leptin, have been found in npEW-Ucn1 neurons (Caron et al. 2010; Xu, 2009). Importantly, injection of leptin in the mouse npEW induces phosphorylation of STAT3, indicating the activation of the JAK2/STAT3 pathway by leptin binding to its receptor. The functional role of leptin in npEW cells has not been investigated extensively, however, our understanding of leptin sensing by npEW neurons is evolving rapidly as we have shown previously in our lab that leptin directly inhibits npEW neurons.

In the present study we replicated and expanded our previous findings of leptin's effects on npEW neurons. We have shown that leptin reduces the firing rate of the npEW neuronal population by 58%, thus confirming the findings of the previous study in our lab (Scheenen, W., unpublished data). In addition, we have shown for the first time that blockage of PI3-kinase by the selective PI3-kinase antagonist wortmannin prevents this leptin-induced inhibition, suggesting that leptin inhibits npEW neurons directly through a PI3-kinase-dependent mechanism. Furthermore, treatment with the selective Katp channel blocker tolbutamide induces a clear activation of npEW-Ucn1 neurons, suggesting the presence of functional Katp channels that can be the final target in the intracellular leptin-induced signaling pathway changing the cellular excitability of npEW-Ucn1 cells. Recent evidence suggests that leptin-induced inhibition of neurons in several brain regions, like the ventromedial hypothalamus (Irani et al., 2008) and the dorsal motor nucleus of the vagus (Wiliams et al., 2006), is mediated by a PI3-kinase-signaling pathway. Importantly, activation of PI3-kinase has been shown to regulate cell excitability via stimulation of Katp channels in the brain (Plum et al., 2006a; Harvey et al., 2000; Williams et al., 2006). Therefore taken together with the data presented here, we suggest that within the npEW, leptin-inhibition may be mediated via a PI3-kinase-Katp dependent pathway, as npEW-Ucn1 neurons possess Katp channels and an active PI3-kinase system. It is important to note that we did not assess the involvement of Katp channels in leptin-mediated inhibition directly, thus the possibility might exist that PI3-kinase activates a different downstream signaling pathway that does not result in the activation of Katp channels but of other end point effectors. For example, it has been shown that in ARC NPY neurons leptin mediated PI3-kinase activation increases the peak amplitude of large-conductance Ca^{2+}-activated potassium (BK) currents (Yang et al., 2009). Activation of BK channels contributes to cell hyperpolarization and decreased cell excitability and therefore could form the last step in leptin-induced PI3-kinase mediated NPY neuron inhibition. In the present study we have studied one Ucn1 expressing npEW neuron (Fig. 7) that is inhibited by PI3-kinase blockage and stimulated by Katp channel blockage. PI3-kinase activation leads to accumulation of PIP3, which subsequently leads to activation of Katp channels and consecutive cell hyperpolarization (Hegyi et al., 2004; Harvey et al. 2000; Frübeck, 2006). Blockage of PI3-kinase or Katp channels in this pathway results in stimulation of the cell. Since PI3-kinase blockage in the examined neuron did not result in stimulation, but in an inhibition, we conclude that a PI3-kinase-Katp pathway is not utilized in this neuron. It is important to note that as leptin's effect on this cell was not assessed, the

findings from this cell might only reflect cases in which leptin does not inhibit. Thus it is still possible that cells that are inhibited by leptin utilize a PI3-kinase-Katp pathway, while cells in which leptin is not inhibitory this pathway is not used even if it is present in the cell.

Whereas leptin generally reduces action current frequency in the npEW, our results also suggest the existence of npEW-Ucn1 neurons that are stimulated by leptin. These results are consistent with previous studies that show that leptin differentially regulates neuronal activity of neurons in the ventromedial (VMN) and arcuate hypothalamic nuclei (ARC) (Irani et al., 2008). Interesting in this respect is the fact that leptin has opposing effects on neurons from the same nucleus. For example 20% of the VMN neurons are inhibited by leptin, while 24% are excited (Irani et al., 2008). A different distribution is shown in the ARC, where 40% of the neurons are leptin excited and only 10% are leptin inhibited (Irani et al. 2008). Here we show for the first time that similar to the VMN and ARC, the npEW also does not form a homogenous population in its response to leptin, as several cells are stimulated by leptin. Thus, similar to the VMN and ARC, leptin's effect in the npEW is not uniform. Surprisingly, blockage of PI3-kinase in the leptin-excited cells of the npEW not only abrogates the leptin-induced activation but also results in a reduction of activity. This suggests, that leptin excitation of npEW cells also utilizes a PI3-kinase dependent mechanism. Confirming the opposing action of PI3-kinase on the neuronal activity of different npEW neurons, the data presented here indicate that although the majority of the npEW neurons show stimulation during PI3-kinase blockage, in a subset of the cells, an inhibition is observed. Thus, PI3-kinase is involved both in stimulation and inhibition of npEW neurons and therefore alternative signaling pathways downstream of PI3-kinase must be present that determine the direction of excitability of npEW neurons. Whether leptin stimulates or inhibits an npEW neuron depends on which signaling pathway downstream of PI3-kinase is activated. It is important to note that since blind cell-attached recordings have been performed in the present study, we cannot be certain about whether all the recorded npEW cells are Ucn1 positive. The differential effect of PI3-kinase blockage on the excitability of npEW neurons could be explained by the possibility that Ucn1-expressing npEW neurons differ in their response to PI3-kinase blockage from non Ucn1 npEW neurons. Our single-cell rt-PCR findings though show that the variability in the effect of PI3-kinase blockage is also observed in Ucn1 expressing npEW neurons. Therefore, we conclude that PI3-kinase activates alternative downstream pathways to stimulate and inhibit npEW-Ucn1 neurons.

The question as to how leptin can inhibit and excite neurons from the same nucleus, via an identical intracellular signaling molecule is an intriguing one. Previous studies have indicated for different brain regions that leptin-induced PI3-kinase mediated inhibition utilizes Katp channels (Plum et al., 2006b; Harvey et al., 2000; Williams et al., 2006). Given the data presented here a similar mechanism is likely to be utilized for leptin-induced PI3-kinase mediated inhibition of npEW neurons. The difficult issue is how PI3-kinase is also utilized by leptin to induce stimulatory effects in the same type of neurons. The involvement of PI3-kinase in mediating leptin-induced activation has been reported in several occasions (Wang et al., 2008, Hill et al., 2008). For example in POMC neurons leptin causes a rapid depolarization and increases the action potential frequency via a PI3-kinase-dependent pathway. Despite the reports of leptin-induced PI3-kinase mediated excitation, not much is known about the mechanisms downstream of PI3-kinase. Recently it has been shown that leptin activates TRPC channels through a Jak2-PI3k-PLCγ pathway and TRPC channels appear to be the key channels mediating the PI3-kinase dependent depolarizing effects of leptin in POMC neurons (Qui et al., 2010). Since TRPC channels, with the exception of TRPC2, are widely distributed in the mammalian brain (Venkatachalam and Montell, 2007) and given that these channels are activated by stimulation of PI3-kinase (Qiu et al., 2010), this mechanism could explain how leptin-induced activation of PI3-kinase ultimately leads to a

stimulation of npEW cells. Based on our findings and other published data we hypothesize, that leptin-induced inhibition and stimulation of npEW neurons is dependent on a PI3-kinase-Katp and PI3-kinase-TRPC signaling pathway, respectively. Additional studies are needed to understand the precise mechanisms by which PI3-kinase mediates both inhibitory and excitatory effects of leptin in npEW neurons. Specifically, the importance of Katp channels and TRPC channels for leptin-inhibited and leptin-excited npEW neurons respectively needs to be determined.

While our studies have shed light on the mechanisms by which leptin affects npEW neurons, little is known about the functional significance of leptin's action on the npEW-Ucn1 system. The data presented here show a divergent role for leptin and complicate the issue even more. As leptin and urocortin 1 are both implicated in the regulation of feeding, this suggests a role for the npEW system in the control of feeding. While the arcuate nucleus POMC and NPY neurons are classically viewed as the 'first order' neurons in sensing changes in levels of circulating insulin and leptin and regulate feeding and energy balance (Ahima & Flier, 2000; Bjorbaek & Kahn, 2004; Reizes et al., 2007), new evidence modifies this picture. Recently it has been shown that through cell-specific gene deletion of the rate-limiting enzyme of serotonin production (tryptophan hydroxylase 2) or the leptin receptor, serotonin enhances appetite and decreases energy expenditure and that leptin regulates these functions by inhibiting serotonin synthesis (Yadav et al., 2009). This indicates that the brainstem serotonergic neurons are the initial target of leptin and not the hypothalamic feeding centers. Interestingly, injection of urocortin 1 into the dorsal raphe significantly reduces food consumption and weight gain, suggesting that the dorsal raphe is a neuroanatomical substrate of urocortin 1-induced reductions in feedings through modulation of the serotonergic activity of this nucleus (Weitemier & Ryabinin, 2006). Since leptin directly modulates the excitability of npEW-Ucn1 neurons, and given the extensive interactions between the npEW and the serotonergic dorsal Raphe nucleus (Kozicz, 2010; Neufeld-Cohen et al., 2010; Kozicz, 2007), this suggests a key role for the npEW in mediating leptin's actions on the midbrain circuitry and regulating energy homeostasis. Thus, leptin might regulate feeding by acting directly or indirectly on npEW and DR neurons. In addition, as these midbrain areas play a crucial role in controlling the stress-response (Adell, 1988; Naughton, 2000; Chaouloff, 2000; Korosi et al, 2005; Kozicz, 2004; Gaszner, 2004; Kozicz, 2007; Kozicz, 2008), leptin's actions in these brain regions might also have consequences for the regulation of stress and mood.

Distinct from its role as a satiety factor leptin has also been implicated in various other processes like synaptic plasticity (Harvey et al, 2006) and bone remodeling (Yadav et al., 2009). Given that the npEW system is known for its involvement in the regulation of stress, the functional implication of leptin's action on the npEW might extend beyond the control of energy homeostasis. Evidence suggests that leptin and its receptor contribute to brain development in mouse fetuses and neonates, independent of their role in adults (Bouret et al., 2004; Pinto et al., 2004; Cottrell et al., 2009; Bouret & Simerly, 2004; Louis & Myers Jr., 2007; Gupta et al., 2009, Udagawa et al., 2007). Important in this regard is that leptin acts specifically on ObR-b-expressing neurons to promote the establishment of projections to downstream nuclei. Interestingly, the npEW and DR contain ObR-b receptors during the crucial postnatal leptin surge (Caron et al., 2010) suggesting a possible role for leptin and its receptor in the development of the npEW and DR. A consequence of this hypothesis is that alteration of the timing or amplitude of the postnatal leptin surge, like in fetal or perinatal nutritional restriction or excess, could alter the development of these midbrain circuits. As mounting evidence suggests that the coordinated interaction of urocortins with the DR-serotonerggic system plays an important role in regulating homeostatic equilibrium under stress and mood (Kozicz, 2010; Neufeld-Cohen et al., 2010; Kozicz et al., 2008), altered

perinatal programming of these brain centers would subsequently result in a dysregulated functioning and consequently confer risk for stress-related anxiety- or depressive-like behavior.

In summary, the data presented here show that leptin reduces the firing rate of the npEW neuronal population through a PI3-kinase dependent pathway. In some npEW neurons leptin induces a stimulation that is also PI3-kinase dependent. Moreover, confirming the opposing action of PI3-kinase on the neuronal activity of npEW neurons, blockage of PI3-kinase reduces the firing rate of the majority of npEW neurons, while in some neurons it results in activation. Finally, treatment with Katp channel blockers activates npEW neurons, suggesting the presence of functional Katp channels that can be the final target in the intracellular signaling pathway of leptin-induced inhibition of urocortinergic npEW cells. As a leptin-PI3-kinase pathway is also utilized in the activation of npEW-Ucn1 neurons, alternative signaling pathways downstream of PI3-kinase must be present in leptin-excited cells. It remains to be determined whether indeed different pathways mediate leptin induced inhibition and excitation of npEW cells respectively.

Acknowledgements
I would like to thank both my supervisors, Wim J.J.M. Scheenen and Eric W. Roubos, for their excellent theoretical and practical support during the course of this project. In addition, I am thankful to Tamas Kozicz for his additional input. Furthermore, I would like to thank the participants of the Cellular Animal Physiology department meetings for their suggestions and remarks.

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