Insulin causes hyperthermia by direct inhibition of warm sensitive neurons

Running title: Hyperthermic effect of Insulin

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Objective: Temperature and nutrient homeostasis are two interdependent components of energy balance regulated by distinct sets of hypothalamic neurons. The objective is to examine the role of the metabolic signal insulin in the control of core body temperature (CBT).

Research Design and methods: The effect of preoptic area administration of insulin on CBT in mice was measured by radiotelemetry and respiratory exchange ratio. In vivo 2-[18F]fluoro-2-deoxyglucose (18F-FDG) uptake into brown adipose tissue was measured in rats after insulin treatment by positron emission tomography (PET) combined with X-ray computed tomography (CT) imaging. Insulin receptor-positive neurons were identified by retrograde tracing from the Raphe Pallidus. Insulin was locally applied on hypothalamic slices to determine the direct effects of insulin on intrinsically warm-sensitive neurons by inducing hyperpolarization and reducing firing rates.

Results: Injection of insulin into the preoptic area of the hypothalamus induced a specific and dose dependent elevation of CBT mediated by stimulation of brown adipose tissue thermogenesis as shown by imaging and respiratory ratio measurements. Retrograde tracing indicates that insulin receptor expressing warm sensitive neurons activate brown adipose tissue through projection via the Raphe Pallidus. Insulin applied on hypothalamic slices, acted directly on intrinsically warm-sensitive neurons by inducing hyperpolarization and reducing firing rates. The hyperthermic effects of insulin were blocked by pretreatment with antibodies to insulin or with a PI3kinase inhibitor.

Conclusions: Our findings demonstrate that insulin can directly modulate hypothalamic neurons that regulate thermogenesis and CBT and indicate that insulin plays an important role in coupling metabolism and thermoregulation at the level of anterior hypothalamus.
Core Body temperature (CBT) and nutrient homeostasis are important components of energy balance that can affect each other by mechanisms that are not clearly understood. In homeotherms, both are regulated centrally by distinct hypothalamic nuclei and neurons. The preoptic area (POA) is essential for the regulation of CBT (1). This region contains temperature (warm and cold) sensitive neurons that are considered pivotal in sensing and responding to local- and skin-temperature changes (2; 3). Nutrient homeostasis is regulated by different hypothalamic regions; the paraventricular and the arcuate nucleus, that contain neurons responding to changes in the level of glucose, lipids, ghrelin, leptin and insulin, respectively (4; 5). Intracerebroventricular injection of insulin reduced the unit activity of POA neurons sensitive to peripheral changes in scrotum temperature indicating that this hormone may modulate thermoregulatory responses(6). Since the insulin receptor was detected in the POA (5; 7-9), we hypothesized that insulin could elevate CBT by directly acting on temperature sensitive neurons in the POA. Furthermore our data (in preparation) on single neuron chipping of temperature/warm sensitive neurons in the POA showed the presence of insulin receptor mRNA in these neurons whose firing activity is inversely correlated with the activation of thermogenesis in brown adipose tissue (BAT) (10).

RESEARCH DESIGN AND METHODS

Animals and procedures: All procedures were approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute and were carried out on 3-4 months old male C57BL/6J mice. Animals were maintained on regular chow (Harlan Teklad LM-485 Diet 7012 (65% carbohydrate [kcal], 13% fat, metabolizable energy 3.41 kcal/g)). Access to food and water was ad libitum and the light:dark cycle was 12:12 hrs with lights on at 7AM.

For telemetry studies, male mice were anesthetized with isoflurane (induction 3-5%, maintenance 1-1.5%) and surgically implanted with radio telemetry devices (TA-F20, Data Sciences, Inc.) into the peritoneal cavity for CBT and locomotor activity (LA) measurement. Mice were allowed to recover for two weeks and then submitted for freely moving telemetry recording (each group n=4-6) for 7 days. Mice were individually housed in a Plexiglas cage in a room maintained at 25 ± 0.5°C on a 12:12 h light–dark cycle (lights on at 6:00 a.m.) with ad libitum access to food and water. The cages were positioned onto the receiver plates (RPC-1; Data Sciences) and radio signals from the implanted transmitter were continuously monitored and recorded. CBT and LA (number of horizontal movements) were continuously monitored with a fully automated data acquisition system (Dataquest ART ™, Data Sciences, Inc.). Recordings were made for at least 72 hrs before treatment to ascertain that baseline levels of temperature were stable and that no ongoing febrile response confounds the results.

Insulin (I1507, Sigma, St. Louis, MO) was pre-dissolved in saline and subsequently diluted in artificial cerebro-spinal fluid (aCSF). PI3Kinase inhibitor, LY294002 (70920 Cayman Chemical company, Ann Arbor, MI) was pre-dissolved in DMSO and subsequently diluted in aCSF (final concentration of 5% DMSO). Injections of insulin (or vehicle, aCSF), PI3Kinase inhibitor (or vehicle, aCSF with 5% DMSO) or insulin antibodies (I8510, Sigma, St. Louis, MO) (or vehicle aCSF) were administered directly to the POA through the POA implanted cannula using an injector (33 Ga, 10.5 mm length) connected to plastic tubing and a microsyringe (10 µl) in a volume of
0.5ul over a period of 5 mins to allow diffusion, (n= 5 mice per group).

Indirect calorimetry was performed simultaneously in acclimated, singly-housed, standard diet-fed mice using a computer-controlled, open-circuit system (Oxymax System) that is part of an integrated Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). Animals are tested in clear respiratory chambers (20 × 10 × 12.5 cm) with a stainless steel elevated wire floor. Each of these chambers is equipped with a food tray connected to a balance. Room air is passed through chambers at a flow rate of ~0.5 L/min. Exhaust air from each chamber is sampled at 30-min intervals for 1 min. Sample air is sequentially passed through O₂ and CO₂ sensors (Columbus Instruments) for determination of O₂ and CO₂ content, from which measures of oxygen consumption (VO₂) and carbon dioxide production (VCO₂) are estimated. Outdoor air reference values are sampled after every 4 measurements. Gas sensors are calibrated prior to the onset of experiments with primary gas standards containing known concentrations of O₂, CO₂, and N₂ (Airgas Puritan Medical, Ontario, CA). Respiratory exchange ratio (RER) is calculated as the ratio of VCO₂ to VO₂. Energy expenditure measures (VO₂, VCO₂ and heat formation [(3.815 + 1.232*RER)*VO₂ (in liters)]) are corrected for estimated effective metabolic mass per Kleiber’s power function. Mice undergoing indirect calorimetry are acclimated to the respiratory chambers for 3–4 days before the onset of study. Data are recorded under ambient room temperature clamped at 25°C, beginning from the onset of the light cycle 24 hr for three days.

**Positron emission tomography (PET) and computed tomography (CT) imaging:** PET/CT Imaging in this study was carried out with 2-[¹⁸F]fluoro-2-deoxyglucose (¹⁸F-FDG) on 12-wk-old male Sprague Dawley rats (mean body weight, 250 g). Rats are known to have a relatively large amount of BAT in the interscapular region and thus are often used in research for BAT activation (11). Each rat received 18.5 MBq of ¹⁸F-FDG via intraperitoneal and 30 minutes later they were anesthetized with isoflurane (5% induction, 1-2 % maintenance) and imaged with a combined PET/CT scanner (Siemens Medical Solutions) designed for small rodents at baseline, and after microinjection of Insulin into the preoptic area at 15, 30, 60 and 180 minutes. In brief, CT for attenuation correction and 5-min emission PET were performed, followed by thin-slice (1.5 mm-thick) CT. PET and thin-slice CT images were reconstructed as 35-cm field-of-view images, and the PET and CT image sections at the same location were manually fused using image analysis software (Photoshop 6.0; Adobe Systems Inc.). The mild ¹⁸F-FDG uptake in skin, along with the contours of normal organs, were used as landmarks. The PET and CT images were also intrinsically registered because of their acquisition on the dedicated PET/CT scanner. ¹⁸F-FDG uptake in interscapular BAT was evaluated using the PET, CT, and fused PET/CT images. Animal’s body temperature was maintained at 37°C by a heat lamp (Temperature Controller, RET-3 Temperature probe and HL-1, Heat Lamp, Physitemp Instruments, Inc. NJ) in between periods of recording to avoid hypothermia during Isoflurane anesthesia. PET data were analyzed by visual interpretation of coronal, sagittal, and transverse slices alone and in cross-referenced situations. When ¹⁸F-FDG PET uptake increase was observed, two levels were identified in comparison with normal activity: moderate (more or less twice the activity in a reference region) or intense (markedly higher than the reference activity). PET and conventional imaging were interpreted separately and the results were then compared to each other and in certain cases, with the
physiological information on hyperthermia. Bone scans and CT images were read independently by two nuclear medicine physicians and by two radiologists respectively.

**Slice preparation:** The brain was quickly removed from C57Bl/6 mice at 22-35 days old and submerged in ice cold, oxygenated (95% O₂–5% CO₂) normal artificial cerebrospinal fluid (aCSF), composition was (mM): NaCl 126, KCl 3.5, CaCl₂ 2, MgSO₄ 1, NaH₂PO₄ 1.25, NaHCO₃ 26 and glucose 10 (pH 7.4). Coronal slices (350 µm thick) containing the POA were cut by a microslicer (Vibratome).

**Patch clamp recording:** Standard tight seal recordings were performed in current clamp mode (I-fast) with an Axopatch 200B amplifier to record spontaneous action potentials. The external recording solution was aCSF. In some recordings 9mM glucose was replaced by 9mM mannitol. The pipette solution used was (mM): 130 K-gluconate, 10 KCl, 10 HEPES, 2 MgCl₂, 0.5 EGTA, 2 ATP, 1 GTP (pH 7.4). Glass micropipettes were pulled with a horizontal puller (P-87, Sutter Instruments, Novato, CA, USA) using borosilicate glass. The electrode resistance after backfilling was 2–4MΩ. All voltage measurements were corrected for the liquid junction potential (∼-9mV).

**Temperature control:** The temperature of the external solution was controlled with an HCC-100A heating/cooling bath temperature controller (Dagan Corporation, a fast temperature controller equipped with a Peltier element. To prevent changes induced in the electrode reference potential, the ground electrode was thermally isolated in a separate bath connected to the recording bath by a filter paper bridge.

**Data acquisition and analysis:** Recordings were digitized using a Digidata 1320A interface and analyzed using Pclamp9 (Axon Instruments, Union City, CA, USA) software package and stored on the disk of a computer. After establishing whole-cell configuration (or perforated whole-cell) the spontaneous activity of the neuron was recorded for 2-4 min to determine its control behavior at (36-37°C) after which it was tested for temperature sensitivity with a temperature cycle of at least 33-39°C. The firing rate was determined for each 10 s interval and plotted against the temperature using SigmaPlot software. The criteria for classifying a neuron as warm-sensitive were the same as the ones used by previous investigations of temperature-sensitive neurons (Boulant, 2000). Briefly, the thermal coefficient is defined as the slope of the linear regression of the firing rate plotted as a function of temperature. This plot was determined over a temperature range of at least 3°C in which a neuron was most sensitive. A WSN is defined as one with a thermal coefficient of 0.8 impulses s⁻¹°C⁻¹ or higher. Neurons displaying nonreversible firing rate changes during changes in temperature were excluded.

**Immunoblotting:** 90 min after the injection of vehicle (aCSF) or insulin (0.03 IU) (Sigma) into the POA, mice were anaesthetized and POA tissues were obtained and homogenized in freshly prepared ice-cold RIPA buffer (Thermo Scientific, IL) including 1mM Na₃VO₄, 1mM NaF, EDTA-free protease inhibitor and PhosSTOP (Roche, IN). Insoluble material was removed by centrifugation (15000xg, 2 min) at 4 °C. Sample buffer (0.125M Tris pH 6.8, 0.04% glycerol, 0.4 % SDS, 0.01% β-mercaptoethanol, 0.02% Bromophenol Blue) were added and boiled for 5 min before separation in SDS-PAGE using 7.5% Ready Gel Tris-HCl gels (BioRad, CA). Electrotransfer of proteins from the gel to nitrocellulose membrane was performed for 60 min at 100V. The nitrocellulose transfers were probed with anti-insulin receptor beta subunit (IR-β), anti-insulin like growth factor
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1 receptor (IGF1R) and anti-phospho AKT (Ser473) antibodies from Cell Signaling Technology and β-actin (Millipore) for the normalization. Subsequently the blots were incubated in SuperSignal West Pico Chemiluminescent substrates (Thermo Scientific, IL) for 5 min and visualized with autoradiography film (Denville Scientific Inc., NJ). Quantification was performed using the NIH Image J protocol.

**Statistics:** Factorial analyses of variance (ANOVA) or Student’s t-tests were used for between-subject comparisons involving >2 or exactly 2 levels, respectively. For analysis of in vivo studies on CBT one way ANOVA with a Tukey post hoc test ($P<0.05$) was used to determine differences in the mean between multiple groups. Area under the curve (AUC) analysis was performed using GraphPad Prism 4 Software. All results are expressed as mean ± SE.

Mice (n=4) were deeply anesthetized with isoflorane (induction 3-5%, maintenance 1-1.5%) and placed in a Kopf stereotactic frame (Kopf Instruments). A 33G injector (SST-33/FT, Plastics One Inc, Roanoak, VA) was connected to a Hamilton syringe with a polyethylene tubing and filled with Texas Red-conjugated Dextran beads (Invitrogen, Carlsbad, CA; 1mg/ml). The injector was inserted into the dorsomedial hypothalamus (DMH) (AP=-1.58 mm, Lat=0.25 mm; V=4.6 mm from the surface of the brain; n=1) or Raphe Pallidus (RPa) (AP=-6.12 mm, Lat=0.0 mm; V=5.8 mm from the surface of the brain; n=3) according to stereotactic coordinates (12) and 1µl of tracer was injected over 1 min. Animals were allowed to recover for 7 days.

**Immunohistochemistry:** Animals were perfused via the ascending aorta with 10ml of 0.9% NaCl followed by 50ml ice-cold 4% (w/v) paraformaldehyde in 0.16M phosphate buffered saline (PBS). Brains were removed and post-fixed for 2 hrs in the same fixative, then transferred to PBS containing 20% (w/v) sucrose and stored over-night at 4°C. Sections from the POA were cut on a cryostat (Leica) at 40µm and transferred to individual wells containing PBS.

For indirect immunohistochemistry free-floating sections were incubated overnight with a rabbit anti-insulin receptor antibody (1:100; Pfizer PP5), rinsed in PBS and incubated with a Donkey anti-rabbit Alexa488 (1:200; Invitrogen) for 1 hr in room temperature. Sections were rinsed in PBS followed by incubation with 0.5µM DAPI (Invitrogen) for 5 min, then rinsed in PBS and mounted onto SuperFrost Plus sides (VWR, Ann Arbor, MI) and mounted with ProLong Gold. Confocal images were captured using a Zeiss Laser Confocal Microscope (LCSM) using Zen 2009 Zeiss software suite (Carl Zeiss Inc., Thornwood, N.Y). All serial optical image sections (0.3µm interval step slices) were imported and spatially re-assembled using Imaris (Bitplane Inc., Saint Paul, MN) to generate a 3-dimesional representation of the tissue and then maximum projected for 2D images (grey scale image panels) in Image Pro Plus (Media Cybernetics Inc, Bethesda, MD).

**RESULTS**

Injection of insulin, into the POA, was followed by elevation of CBT lasting upto six hours , in a dose dependent manner (Fig 1A). Similar to mice injected with a vehicle artificial cerebro spinal fluid (aCSF) animals injected with insulin showed a stress-dependent elevation of CBT at time of treatment. However, CBT returned to baseline in vehicle treated mice but not in animals that received insulin. The extent of CBT elevation was dose dependent: average CBT after injection (after stress-induced peak, from 1-6 hours after injection) was 35.77 ± 0.04°C for vehicle and 36.21 ± 0.07°C, 36.98 ± 0.03°C, 37.11 ± 0.04°C and 37.08 ± 0.11°C (or +0.44, +1.21, +1.34 and +1.31°C, respectively) (p<0.01) for 0.001, 0.015 0.03
and 0.06 IU of insulin, respectively. The hyperthermic effects of insulin were significantly larger in the POA (1.34°C) than in the DMH (0.78°C) (p<0.01) or the RPa (0.57°C) (p<0.01), which are projection areas for the POA warm sensitive neurons (Fig. 1B). Pretreatment of the animals with insulin neutralizing antibody (Fig. 1C) injected into the POA prior to injection of insulin prevented the insulin evoked hyperthermic response. Insulin did not display hyperthermic effects when injected peripherally (ip). The average temperature from 1 to 6 hrs after intraperitoneal injection was 36.28 ± 0.07°C and 36.22 ± 0.10°C for vehicle and insulin (0.03 IU), respectively (Fig. 1D).

Measurement of oxygen consumption, carbon dioxide production and the respiratory exchange ratio (RER) demonstrated that POA injection of insulin was followed by increased oxygen consumption and lower RER compared with vehicle treated mice, indicating a switch from glucose metabolism to elevated fatty acid utilization (Fig. 2A). The lower RER upon POA insulin injection compared with vehicle treated mice was prolonged and still present at 6 hrs post injection. RER for vehicle and 0.03 IU insulin POA was 0.91 ± 0.02 and 0.75 ± 0.01, respectively, (p< 0.01). Locomotor activity did not contribute to CBT increase as it was not affected by either vehicle or insulin injection in the POA (not shown). Positron emission tomography (PET) and computed tomography (CT) imaging carried out on rats demonstrated that insulin injected into the POA activated BAT thermogenesis as the insulin treatment induced up to 5-fold (p<0.05) elevation in BAT glucose, fluorine-18 fluorodeoxyglucose (18F-FDG) uptake (Fig. 2B and C). Since in the arcuate nucleus insulin was shown to activate the phosphatidylinositol 3-kinase (PI3K) pathway (13), the hyperthermic effects of insulin into the POA were also assessed in the presence of a PI3K inhibitor (PI3K-I). Pretreatment with PI3K-I LY294002 (10 nmoles in 0.5 µl) before insulin (0.03 IU), both administered to the POA 20 min apart, showed significant attenuation of the insulin induced hyperthermia (p<0.01) (Fig 3A). Comparison of the area under the curve (AUC) for pretreatment with PI3K-I and followed by either insulin or vehicle did not elicit statistically significant results. The peak hyperthermic response to insulin administered to the POA was observed 2 hrs after the pretreatment injection of vehicle. At this time point pretreatment with PI3K-I reduced the insulin-induced temperature elevation by 1.4°C (from 2.03°C to 0.63°C) (Fig. 3A).

Biochemical analysis of POA tissues confirmed the presence of the insulin receptor in the POA. Comparative analysis demonstrated that insulin (0.03 IU) treatment induced an elevation of the activated, phosphorylated form of AKT (Ser 437): the level of pAKT increased 317 ± 82.7% compared to vehicle 90 min after the injection (Fig. 3B) in line with the involvement of PI3K in the insulin signaling in this hypothalamic area.

The hypothesis, suggested by the presence of the insulin receptor transcript found by single neuron chipping of individual warm sensitive neurons in the POA (in preparation), that insulin might act directly on warm sensitive neurons, was also tested by electrophysiological recording of the effects of insulin on primary warm sensitive PO/anterior hypothalamus (AH) neurons (WSN) in hypothalamic slices. Insulin was locally applied to warm-sensitive PO/AH neurons using a microperfusion tip. WSNs were identified by measuring the change in the firing rate during a temperature ramp; the thermal coefficient of the WSNs was 0.93 impulses s⁻¹°C⁻¹ (Fig. 4A and B) as previously described(14). Insulin (0.3 IU/ml) decreased the firing rate of the WSN from 1.1 Hz to 0.3 Hz (i.e. by 73%) (Fig. 4C). Similar
experiments revealed that the firing rates of 9 out of 19 (43%) warm-sensitive PO/AH neurons tested were reversibly inhibited by insulin (0.3 IU/ml). The decrease in firing rate averaged 54 ± 19% (n=9). The firing rate averaged 4.9 ± 2.6 Hz (n=9) in control and 2.6 ± 2.4 Hz (n=9) in the presence of insulin. This effect was accompanied by a hyperpolarization of 3.2 ± 2.7 mV (n=9). At 0.15 IU/ml insulin reduced the firing rate of warm sensitive PO/AH neurons by 10 ± 7% (n=3). These effects are consistent with the current models of central thermal regulation, that state that the PO/AH sends an inhibitory, GABAergic signal to the thermoregulatory centers DMH and/or RPa and controls thermogenesis induced by local or skin cooling or by binding of pyrogens to their receptors on warm sensitive neurons (10). Thus inhibition of neuronal activity in the PO/AH results in hyperthermia while a decrease in activity results in hypothermia (reviewed in (15)). As predicted by this model, insulin- induced inhibition of the activity of PO/AH warm-sensitive neurons resulted in hyperthermia.

We then tested whether insulin caused hyperpolarization and reduction in firing rate involved the activation of ATP-sensitive K+ channels (K<sub>ATP</sub> channels) as previously described on insulin effects in the arcuate neurons (5). The K<sub>ATP</sub> channel blocker glybenclamide (20 µM) was applied to the bath for 5 min prior to local insulin application. The channel blocker increased the firing rate of all neurons tested by 21 ± 15% (n=6) (Fig. 4D and E). The presence of glybenclamide blocked the previously observed inhibitory effect of insulin (0.3 IU/ml) on the firing rate in warm sensitive POA/AH neurons (Fig. 4D and E). The PI3K-I LY249002 (5 µM in the bath) inhibits the in vivo hyperthermic effects of insulin (Fig. 1C) also prevents the insulin induced inhibition of the firing rate of the WSNs in the POA. The inhibitory effect of LY249002 on insulin’s action (fig 4F and G) suggests that at least some of the effects of insulin in the POA are exerted at the level of regulating the firing rate of WSNs and through this mechanism the level of BAT activation and thermogenesis. No effects of leptin (0.1 µM) and hypoglycemia (glucose from 10 to 1 mM) on the activity of WSN were observed (data not shown), although in other hypothalamic nuclei there are interactions between glucose, leptin, and insulin signaling.

Retrograde tracing from the RPa (Fig. 5A) or DMH (not shown) show labeled neurons in the POA. Immunohistochemical detection of the insulin receptor shows co-localization with retrogradely traced neurons projecting to the RPa (Fig. 5A-E), as well as adjacent insulin receptor-positive, only neurons (Fig. 5E).

DISCUSSION

Injection of insulin into the preoptic area of the hypothalamus induced a specific and dose dependent elevation of CBT mediated by stimulation of BAT thermogenesis as shown by imaging and RER measurement. These data show how insulin, primarily known for its peripheral action as regulator of glucose uptake, may act as central stimulator of fatty acid oxidation in BAT. Retrograde tracing indicates that insulin receptor expressing warm sensitive neurons activate brown adipose tissue through projection via the Raphe Pallidus. WSNs are functional components of the neuronal circuitry that regulates temperature homeostasis. These neurons are known to respond to local changes in temperature and to pyrogens during infection or diseases. Insulin applied on hypothalamic slices, acted directly on intrinsically warm-sensitive neurons by inducing hyperpolarization and reducing firing rates. It should be noted that the POA insulin doses employed give rise to POA insulin concentrations that are in the range of circulating plasma concentrations of
insulin and are unlikely to exert the BAT effects via leakage to other brain areas or to the periphery. However, not all WSN tested responded to insulin and none responded to leptin or changes in glucose concentrations (not shown) indicating that WSNs constitute a heterogeneous population of neurons and that insulin inhibits only a subset of these POA neurons, however this subset represents a potent enough input to the BAT regulation to cause a hyperthermic response. The insulin sensitive WSNs may mediate actions important for the energy balance and may participate in the thermic effects of food, a phenomenon inversely correlated to the degree of insulin resistance and of body fat and proposed to be important in the development of obesity (16). Our data shows how insulin can affect BAT activity via central action on warm sensitive neurons. This is important in light of recent findings on the role of BAT activity in diabetes and in obesity (17; 18). The demonstration that WSNs in the POA respond directly to insulin with increased thermogenesis provides an important link between thermoregulation and energy homeostasis.

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Figure 1. Effects of central or peripheral insulin injections on CBT. A. Profile of the dose dependent effects of local injection of four different doses of insulin (0.001= diamonds, 0.015= circles, 0.03= squares and 0.06 IU=triangle) (n=8 animals per group). B. Graph showing the differential effects on CBT of the injection of 0.03IU of insulin in the POA, the DMH and the RPa (POA = circles; DHH=triangles; RPa=diamonds, white = insulin; black = control). C. Neutralizing antibody to insulin injected POA prior to insulin blocks the hyperthermic effects of insulin. (insulin antibody = triangles; vehicle = circles, white = insulin; black = vehicle). D. Graph showing the effects on CBT of intraperitoneal injection of 0.03 IU of insulin or vehicle. All graphs show CBT profile for the first 6 hr after injection. Arrow indicates time of injection, the CBT increase observed in all conditions during the first hour is due to stress associated with injection. Data are presented as mean ± SE.

Figure 2. POA injection of insulin increased fatty acid utilization and brown adipose tissue activity
A. 6 hr profile of respiratory exchange ratio of mice treated with vehicle of 0.03 IU insulin in the POA, demonstrating that insulin injection decreased RER, indicating an elevation of fatty acid utilization. B. 3 hr profile of PET/CT of 18F-FDG uptake in rats injected with vehicle or 0.03 IU insulin in the POA. The top panel shows the representative PET/CT, squared in dotted lines is the area investigated following treatment with insulin or vehicle shown below. The anatomical position of BAT is indicated with an arrow. C. Graph showing the 3 hr profile quantification of 18F-FDG uptake into BAT following insulin treatment as indicated. Data are presented as mean ± SE.

Figure 3. The hyperthermic effects of POA injection of insulin are inhibited by the PI3K inhibitor and induce an elevation of the activated, phosphorylated form of AKT.
A. 6h profile of the effects of PI3K-I treatment on insulin-induced elevation of CBT. Animals were pretreated with 10 nmoles of PI3K-I in 0.5 µl 20 min before injection of 0.03 IU of insulin, arrows indicate time of injections (squares = PI3KI; circles = vehicle pretreatment; white = insulin; black = vehicle). Data are presented as mean ± SE. B. Western blot analysis of POA tissues confirmed the presence of the insulin receptor in the POA: the level of pAKT increased 317 ± 82.7% compared to vehicle 90 min after the injection of insulin to the POA.

Figure 4. Effects of temperature and insulin on the firing rate activity of a PO/AH warm-sensitive neuron and the role of K<sub>ATP</sub> channels and PI3K in the inhibitory effects of insulin on warm-sensitive PO/AH neurons
A. Spontaneous firing activity of a PO/AH neuron at three different temperatures. B. Firing rate vs. temperature plot. The slope of the linear regression, indicated by the solid line, is 0.93 impulses s<sup>-1</sup> °C<sup>-1</sup>. C. Application of insulin (0.3 IU/ml) induced a small hyperpolarization (2mV) and decreased the firing rate of the neuron from 1.1Hz to 0.3Hz. D. Bath application of the K<sub>ATP</sub> channel blocker glybenclamide (20µM) reduced the firing rate of WSNs by 43%. In the presence of the K<sub>ATP</sub> blocker insulin (0.3 IU/ml) did not affect the firing rate of WSNs. E. Bar chart summarizing the effect of insulin (0.3 IU/ml, n=9; 0.1 IU/ml; n=5), glybenclamide (20µM; n=6) and insulin (0.3 I.U./ml, n=6) in the presence of the blocker. F and G. Bath application of the PI3K inhibitor LY294002 (5µM) prevents the insulin (0.3 IU/ml; n=6) caused inhibition of firing rate (F). Data are presented as mean ± SE.

Figure 5. Insulin receptor-positive neurons identified by retrograde tracing from the Raphe Pallidus.
A-D. Texas Red tracer (A, red in D,E) is co-localized with the Insulin receptor (B, green in D,E) and the nucleus stained with DAPI (C, blue in D,E) (E) A three-dimensional reconstruction of the same neuron as seen in (A-D) showing a insulin receptor-positive traced neuron (arrow in D and E) and an insulin receptor only positive neuron (arrowhead). All images were captured with a 63x objective.
Figure 1

A

B

C

D

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Figure 2

(A) RER (VCO₂/VO₂) vs. Time (h)

(B) PET/CT images showing differences between Insulin and Vehicle groups.

(C) Graph depicting % increase in FDG uptake over time (min).
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Figure 3

A

![Graph showing temperature over time with different treatments: vehicle + insulin 0.03 IU, PI3K (10 nmol) + insulin 0.03 IU, PI3K (10 nmol) + vehicle, vehicle.](image)

B

![Diagram showing POA with insulin - and + treatments.](image)
Figure 4

A

B

C

D

E

F

G
Figure 5

(a) 

(b) 

(c) 

(d) 

(e)