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Awake, long-term intranasal insulin treatment does not affect object memory, odor discrimination, or reversal learning in mice

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Abstract

Intranasal insulin delivery is currently being used in clinical trials to test for improvement in human memory and cognition, and in particular, for lessening memory loss attributed to neurodegenerative diseases. Studies have reported the effects of short-term intranasal insulin treatment on various behaviors, but less have examined long-term effects. The olfactory bulb contains the highest density of insulin receptors in conjunction with the highest level of insulin transport within the brain. Previous research from our laboratory has demonstrated that acute insulin intranasal delivery (IND) enhanced both short- and long-term memory as well as increased two-odor discrimination in a two-choice paradigm. Herein, we investigated the behavioral and physiological effects of chronic insulin IND. Adult, male C57BL6/J mice were intranasally treated with 5 μg/μl of insulin twice daily for 30 and 60 days. Metabolic assessment indicated no change in body weight, caloric intake, or energy expenditure following chronic insulin IND, but an increase in the frequency of meal bouts selectively in the dark cycle. Unlike acute insulin IND, which has been shown to cause enhanced performance in odor habituation/dishabituation and two-odor discrimination tasks in mice, chronic insulin IND did not enhance olfactometry-based odorant discrimination or olfactory reversal learning. In an object memory recognition task, insulin IND-treated mice performed no different from controls regardless of task duration. Biochemical analyses of the olfactory bulb revealed a modest 1.3X increase in IR kinase phosphorylation but no significant increase in Kv1.3 phosphorylation. Substrate phosphorylation of IR Kinase downstream effectors (MAPK/ERK and Akt signaling) proved to be highly variable. These data indicate that chronic administration of insulin IND in mice fails to enhance olfactory ability, object memory recognition, or a majority of systems physiology metabolic factors – as reported to elicit a modulatory effect with acute administration. This leads to two alternative interpretations regarding long-term insulin IND in mice: 1) It causes an initial stage of insulin resistance to dampen the behaviors that would normally be modulated under acute insulin IND, but ability to clear a glucose challenge is still retained, or 2) There is a lack of behavioral

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modulation at high concentration of insulin attributed to the twice daily intervals of hyperinsulinemia caused by insulin IND administration without any insulin resistance, per se.

Keywords

olfactory; intranasal; olfactometry; IR kinase; Kv1.3; meal frequency

1. Introduction

Insulin signaling is well-known to be involved in diverse processes beyond its traditional role in glucose uptake and peripheral metabolism. Within the central nervous system (CNS) insulin can evoke neuromodulatory, neurotrophic, and endocrine effects [1]. Brain insulin signaling can be disrupted across a host of debilitating diseases that are currently on the rise worldwide; including obesity, type II diabetes, Alzheimer's and Parkinson's disease [2, 3] Currently, intranasal insulin is being used in clinical trials to test for improvements in human memory and cognition, and in particular for mitigation of such neurodegenerative diseases [4]. Intranasal delivery (IND) of insulin provides a safe, feasible, and effective route of administration, largely bypassing the blood-brain barrier (BBB) and maximizing distribution to the CNS [5]. Insulin receptor (IR) kinase is located throughout the brain and with high density in the olfactory bulb (OB), piriform cortex, hypothalamus, and hippocampus [6]; brain areas that also happen to have a high binding affinity for insulin [7,8,9]. Peripheral insulin is thought to bypass the BBB, via a saturable, active transport system [10, 11] allowing binding of insulin to IR kinase to elicit dimerization of the receptor followed by autophosphorylation. Once phosphorylated, the receptor is activated and can modulate a variety of behaviors including feeding, reward, whole-body metabolism, and cognition.

IR Kinase is a receptor tyrosine kinase composed of two subunits, IR_{α} and IR_{β}. The IR_{α} subunit is entirely extracellular, while IR_{β} is a transmembrane subunit that possesses a tyrosine-protein-kinase domain [12]. When insulin binds to the α subunit, the catalytic activity of the β subunit phosphorylates or recruits an array of downstream effectors and adaptor proteins, such as the insulin receptor substrate (IRS) family and the Src homology 2/α-collagen-related (SHC) proteins [13]. In studies examining peripheral insulin signaling, it is well documented that there are two major downstream pathways activated following IR Kinase activation, namely the phosphoinositide 3-kinase (PI3K)/AKT and the Ras/mitogen activated protein kinase (MAPK) cascades [14]. Centrally, however, less is known regarding IR signaling cascades. In the hypothalamus, binding of insulin to IR Kinase initiates a signal cascade that leads to the conversion of phosphatidylinositol 3,4-diphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) [1]. In the OB, where IRS is weak to absent, the voltage-dependent potassium channel, Kv1.3, may subserve as the multi-tyrosine phosphorylated protein to allow scaffolding of SH2-containing adaptor proteins [15–18].

The OB contains the highest density of IR Kinase and has the highest level of insulin transport in the brain [8, 19]. Our laboratory has previously demonstrated that IR Kinase can directly phosphorylate the voltage-dependent potassium ion channel, Kv1.3, expressed largely in mitral cells (MCs) [18, 20–22]. These first order neurons of the OB exhibit a

decrease in peak current magnitude when insulin is applied [18]. The MCs become insensitive to insulin-induced modulation following either gene-targeted deletion of the Kv1.3 channel or pharmacological block of the channel vestibule [20, 22–24]. Insulin IND modifies Kv1.3 protein-protein interactions, with enhanced tyrosine phosphorylation and scaffolding with both IR kinase and PSD-95 [16]. Beyond altered Kv1.3 phosphorylation state, insulin IND also induces behavioral modifications, as evident by enhanced object memory recognition and increased performance in the two-odor discrimination task. [16, 25–27]. These studies demonstrate insulin's ability to influence MC activity [18, 20] and therefore olfactory functioning [16, 28].

Many studies in mice (C57BL6/J), including our own, have only investigated the short-term effects of insulin IND [16, 28]. With the increased use of insulin IND in clinical trials for the treatment of neurodegeneration and cognitive disease, examination of long-term insulin IND is needed. Herein, adult mice were given insulin IND twice daily for 30 to 60 days. Following drug delivery, animals were subjected to olfactory and object memory behavioral tests, assessed for changes in metabolic activity and cognition, and then OB's were biochemically examined for changes in insulin signaling.

2. Material and methods

2.1. Animal Care

All mice (C57BL/6J background strain, The Jackson Laboratory, Bar Harbor, ME) were singly-housed in conventional style open cages at the Florida State University (FSU) *vivarium* in accordance with institutional requirements for animal care. Mice were maintained on a reverse 12/12 hour dark/light cycle so that behavioral phenotyping could take place in the dark phase. Rodent chow (5001, Purina, Richmond VA) and water were provided *ad libitum* (unless otherwise specified). Experiments began at adulthood, defined as 4 months of age, and were only performed on male animals. Although sex-specific effects have been reported for rats and humans [57, 59, 80, 81], such has not been found for mice [16] so it was not necessary to utilize both sexes. Because mice also demonstrate a lack of diet-induced obesity (DIO) and associated prediabetic state in females [36], with the intent of applying our study for future application for diabetes, it was pragmatic to hone our current study to male mice. Taking the aforementioned into consideration, we acknowledge the potential study limitations due to the lack of females. All animal experiments were approved by the FSU Institutional Animal Care and Use Committee (IACUC) under protocol #1427 and were conducted in accordance with the American Veterinary Medicine Association (AVMA) and the National Institutes of Health (NIH). For final collection of tissues for biochemistry, mice were sacrificed by cervical dislocation (AVMA Guidelines on Euthanasia, June 2007). Use of the ARRIVE guidelines for reporting animal research was followed in the design of our manuscript [29]

2.2. Awake Intranasal Insulin Administration

For awake insulin IND, adult mice were hand-restrained, placed in a supine position, and given three, 10 μl drops of 5 μg/μl of insulin (11 376 497 001 Roche Diagnostics, Indianapolis, IN) or phosphate buffered saline (PBS) into both nares simultaneously, as

previously described [16]. Treatment was repeated for approximately 30 minutes, or until all mice had received a total of 90 μl of solution; this protocol was administered twice daily at 10:00 AM and 10:00 PM. It was previously shown that intranasally-delivered insulin-like growth factor reaches the OB with an efficiency of 0.11% [82]. Based on those calculations and the equivalent molecular weight of insulin, it is estimated that 0.9 nM (900 pmol/L) insulin is the effective dose reaching the OB. Mice and humans have very comparable levels of insulin and glucose in the plasma. The concentration administered in our study, therefore, represents a physiological range between fasting (90 pmol/L or 0.09 nM) and eating (thirty minutes after glucose/eating, levels of insulin typically rise to about 1600 pmol/L or 1.6 nM). Animals were randomly assigned to one of two cohorts or study groups (Fig. 1A). The first cohort of mice were used for metabolic testing only, and received insulin IND twice daily for a total of 30 days. Behavioral tests for olfactory performance and memory recognition were performed following 30–60 days of insulin IND for the second cohort. Animals remained on twice daily insulin IND regimen throughout behavioral testing, for a total of 60 days. Following either metabolic or behavioral phenotyping, mice were sacrificed two hours after the last insulin IND treatment via cervical dislocation; OB's were quickly dissected and processed for biochemistry (Fig. 1A).

2.3. Olfactometry

An eight channel, liquid dilution, computer-controlled olfactometer (Knosys Olfactometry, Lutz, FL; FSU Mechanical Engineering) was used to measure olfactory threshold, discrimination, and odor reversal learning in the mice [30]. The Knosys Olfactometer utilizes a go no-go operant training paradigm for which trials are initiated upon nose poke [31]. Positive reinforcement was used when pairing an odorant with water reinforcement (called a positive cue or S+). Negative reinforcement was created by systematic punishment of a 10 second (s) time out whereby the mouse was not reinforced with water if it responded to a negative odor cue or diluent (called S−). Prior to a two-week behavioral training period, all mice were water-deprived to 80–85% of their original body weight, by using water restriction of approximately 1.5 to 2 mL of water/day during testing. Subjects were randomly presented ten S+ and ten S− trials, for a total of 20 trials. These 20 trials were summated into one block. An animal was presented a total of ten blocks (200 trials) in a single session lasting under 1 hour (h). The percentage of correct responses per trial was determined by the following formula: % Correct Responses = [(HITs+Correct Rejections)/20] × 100, where a HIT was defined as a criterion response (licking of water spout) in the presence of S+ and a Correct Rejection (CR) was a failure to make a criterion response in the presence of an S−. Each block was only considered successful if the mouse performed at a defined criterion of 80% accuracy or higher.

2.3.1. Odor Threshold—Mice were trained for odorant thresholds using a 5% dilution of ethyl acetate (270989. Sigma-Aldrich; St. Louis, MO) as the S+ and diluent (Milli-Q Water) as the S− odor. Animals were given a maximum of three blocks (or 60 trials) on four sequential dilutions of 5% ethyl acetate. Similar to that formulated by Yoder et al. (2014, 2015) [83,84], odor threshold was recorded as the lowest concentration at which the mouse achieved ≥80% on 1 of 3 consecutive blocks. If a mouse did not reach odor threshold on the four sequential dilutions, it was assigned a threshold of 0.0005% EA. Mice performing with

50% correct responses were defined as performing by chance alone. This performance was similarly observed when no odors were delivered and only the valves of the olfactometer were activated, as previously reported [31]

2.3.2. Two-odor Discrimination—Mice were trained to criteria to discriminate the two odors, 5% ethyl acetate (S+) and 1% acetophenone (S−) (00790. Sigma-Aldrich St. Louis, MO) after a set training of 400 trials. Odorants used for two-odor discrimination were diluted in Milli-Q water.

2.3.3. Odor Reversal Learning—After completion of 400 trials in the two-odor discrimination paradigm, reward contingencies were reversed. The original S+ odor (5% ethyl acetate) was no longer rewarded with water and the original S− odor (1% acetophenone) became the water-rewarded odor. Both the ability and number of blocks required to return to 80% criterion were determined.

2.4. Novel Object Recognition

Mice were tested for short- (1 h) and long-term (24 h) object memory recognition, as previously described [16]. Briefly, two objects were placed in a 26 × 47 × 13.5 cm chamber and mice were given 5 minutes (min) to explore. After 1 h or 24 h, mice were re-tested for memory retention by placing two objects in the chamber at the same position. One object was previously used (familiar object), while the second was not (novel object). Exploratory behavior was assessed for a 5 min interval to score attention to the novel object.

2.5. Indirect Calorimetry

Metabolic parameters, oxygen consumption (VO_2; ml/kg/hr), carbon dioxide production (VCO_2; ml/kg/hr), respiratory exchange ratio (RER), energy expenditure (EE; kcal/hr), locomotor activity, and caloric and water intake were measured using the Comprehensive Laboratory Animal Monitoring System (CLAMS™) (Columbus Instruments, Columbus, OH). A separate cohort of mice was individually housed in airtight cages for a total of 12 days; a 48 h of acclimation period followed by 10 days of continuous data collection. Mice had *ad libitum* access to food and water in overhead feeders attached to specialized electronic balances that monitored both disturbance and decrease in mass. A threshold of at least 10 s of feeder disturbance and a minimum loss of 0.03 grams (g) of chow was required for a recorded meal bout. VO_2 and VCO_2 were normalized with respect to body weight in kilograms, calculated and averaged over 10 days. RER was calculated as VCO_2/VO_2. EE was calculated using the Lusk Graham equation $(3.815 + 1.232 \times RER) \times VO_2$ [32]. Locomotor activity was continuously measured using optical beams along the X-axis of the cage (Columbus Instruments). Consecutive photo beam breaks were scored as ambulatory movement. Indirect calorimetry data were recorded in intervals using Oxymax software (Columbus Instruments). Each interval measurement represented the average value during a 30 s sampling period per cage.

2.6 Biochemistry

2.6.1. Immunoprecipitation—Tyrosine-phosphorylated proteins were immunoprecipitated as previously [16]. Briefly OBs were individually homogenized using

ice-cold lysis buffer containing protease and phosphatase inhibitors as in [18]. Homogenates were orbitally rotated for 30 min at 4°C, then clarified by centrifugation at 14,000 g (Eppendorf 5414C, Hamburg, Germany) for 30 min at 4°C. Collected supernatants were precleared for 4 h with 3 mg/ml protein A-Sepharose (17-0780-01. GE Healthcare, Pittsburgh, PA). Tyrosine-phosphorylated proteins were immunoprecipitated from the cleared lysates using anti-phosphotyrosine (05-321. Clone 4G10. Millipore, Temecula, CA) in an overnight incubation with 2–4 μg/μl of antibody at 4°C. The immunoprecipitates were washed four times with wash buffer, and stored at −20°C until separated by SDS-PAGE [33].

2.6.2. SDS PAGE/Western Analysis—Nitrocellulose membranes were blocked with 5% nonfat milk (170-6404. Bio-Rad Hercules, CA) or 4% bovine serum albumin (Sigma-Aldrich). Nitrocellulose was incubated overnight at 4°C with the primary antibodies: anti-insulin receptor (β-subunit), clone CT3 at 1:800 concentration (05-1104, Millipore), anti-Akt (pan) at 1:1000 (4685, Cell Signaling Technology, Danvers, MA), anti-p44/42 MAPK (Erk1/2) at 1:2000 (9102, Cell Signaling Technology), and anti-Kv1.3 at 1:1000 concentration (FSU120, Florida State University, Tallahassee, FL)[34]. Membranes were incubated with species-specific horseradish peroxidase (HRP)-conjugated secondary antisera for 90 min at room temperature (rt). Labeled proteins were visualized by enhanced chemiluminesence (ECL) (RPN2106. GE Healthcare) and band pixel density was quantified using scanning densitometry normalized to Beta Tubulin as previously described [17, 35].

2.7. Serum Collection

2.7.1. Glucose Tolerance Test—Mice were fasted overnight and their ability to clear intraperitoneal (I.P.) glucose was assessed over 2 h. Following an overnight (16 h) fast, mice were injected with 1 mg/kg of glucose (G5400. Sigma-Aldrich), whereby blood samples were acquired via tail bleed at time 0, 10, 20, 30, 60, 90, and 120 min post injection. Glucose levels were determined using an Ascensia Contour Blood Glucose Monitoring System (Bayer Healthcare, Mishawaka, IN).

2.7.2. ELISA—An enzyme-linked immunosorbent assay (ELISA) was performed as per manufacturer's protocol using the Ultrasensitive Mouse Insulin ELISA (80-INSMSU-E01, ALPCO Salem, NH) and as previously described [36]. In brief, trunk blood was collected at the time of sacrifice and allowed to coagulate at rt for 10 min. Samples were iced for 30 min, followed by centrifugation at 14,000 g (Eppendorf 5414C) for 10 min at 4 °C. Serum was collected and stored at −20 °C for later examination.

2.8. Statistical Analysis

All statistical analyses were performed using Graph Pad Prism (Version 6). Student's *t*-tests were applied for comparison of normally-distributed, two-sample population studies and paired *t*-tests for repeated measures across the light and dark cycle for metabolic measures. For the analysis of olfactometry and novel object recognition, a two-way, repeated measures (RM) analysis of variance (ANOVA) was applied with treatment and time or object as factors. Post hoc tests for multiple comparisons were performed with a Bonferroni's correction. Statistical significance was determined at the 95% confidence level ($\alpha \leq 0.05$) unless otherwise noted. Values are reported at the mean ± standard error of the mean (SEM).

3. Results

Central insulin plays a crucial role in energy balance, and can work as a feedback regulator of bodyweight and plasma glucose [37]. Given this role and previous findings that demonstrate that block or deletion of Kv1.3 channel decreases bodyweight and increases metabolic rate [38–41], we wanted to observe if there were any alterations to basal metabolic measurements following 30 days of chronic insulin IND. To our surprise, there was no change in bodyweight, caloric or water intake, energy expenditure, or respiratory exchange ratio (RER) following chronic insulin IND (Table 1). Although energy expenditure, RER, oxygen consumption, and locomotor activity were significantly elevated in the dark cycle over that of the light cycle, as would be predicted (Student's *t*-test, $p < 0.05$), there was no treatment effect of insulin IND for any of these metabolic factors within a particular cycle (Student's *t*-test, $p > 0.05$). The change in the level of effect for the locomotor activity (4.0 ± 0.4 fold increase) and oxygen consumption (1.2 ± 0.02 fold increase) upon the switch to the dark cycle, regardless of IND treatment, were most noteworthy. Because ingestive behaviors are known to increase for mice during the dark cycle, we did a more refined analysis of the mean meal size, meal frequency, and meal duration per day and during each of the light and dark cycles, respectively. Mean meal size (0.145 ± 0.07 g = control vs. 0.087 ± 0.04 g = insulin) and meal duration (82.9 ± 18.8 s = control vs. 64.8 ± 13.7 = insulin) were not significantly different (Student's *t*-test, $p > 0.05$) when assessed for four mice in each treatment group measured continuously for 4 days. However, mean meal frequency significantly increased following insulin IND when averaged across the 24 h day (61.3 ± 2.7 bouts = control vs. 82.6 ± 4.5 bouts = insulin; Student's *t*-test, $p = 0.002$) and exhibited a selective and significant enhancement in the number of meal bouts in the dark cycle (Fig. 1B,C; Kolmogorov-Smirnov test, light, $p = 0.3752$; dark, $p = 0.0025$) in just the insulin IND-treated mice. When examining meal size sorted to that occurring in the dark and light cycle there was again no difference in meal size (Kolmogorov-Smirnov test, light (0.057 ± 0.01 g = control vs. 0.053 ± 0.02 g = insulin), $p = 0.6000$; dark (0.076 ± 0.03 g = control vs. 0.054 ± 0.01 g insulin), $p = 0.9999$). These data suggest that meal frequency is significantly increased in the dark cycle following insulin IND but that meal size trends to be decreased but is variable.

Next we examined both fasting glucose as well as the ability to clear glucose after a challenge. Following 60 days of insulin IND, control- and insulin-treated mice displayed similar fasting blood glucose (Control = 65.4 ± 4.3 mg/dL, n = 5; Insulin = 69.6 ± 2.4 mg/dL, n = 5; Student's *t*-test, $p > 0.05$). Chronic long-term insulin also did not appear to impede glucose metabolism because there was no observed change in the ability to clear a glucose challenge as determined by an intraperitoneal glucose tolerance test (IPGTT) (Fig 1D). To examine if our design of twice daily IND for 30 and 60 days remained selective to the CNS and did not influence peripheral circulating insulin, we performed an insulin enzyme-linked immunosorbent assay (ELISA) on the trunk blood of mice at sacrifice. Insulin-treated animals displayed an increase in blood plasma insulin levels following 30 days (44.2 ± 8.1 ng/ml, n = 3) and 60 days (50.4 ± 25.2 ng/ml, n = 4) of treatment in comparison to PBS-treated controls that had normal ranges of blood plasma insulin

following 30 days (0.96 ± 0.2 ng/ml, n = 4) and 60 days (1.65 ± 0.5 ng/ml, n = 4) of IND (Student's *t*-test, $p < 0.05$; Fig 1E).

The effect of intranasal insulin on olfactory ability has been well-documented in both human [42–45] and animal [16, 46, 47] studies. Previous research from our laboratory in which insulin IND was administered acutely in mice demonstrated an increased odor discrimination using an odor habituation protocol but only moderate change in odor threshold using a two-choice paradigm [16]. To allow further automation over these manual olfactory behavioral tests as well as good control of concentration and delivery to accurately measure threshold, we examined the effect of chronic insulin IND by use of computerized olfactometry. The determination of olfactory threshold was made using four serial liquid dilutions of 5% ethyl acetate (EA; supplementary data, Figure S1A). Both control- and insulin-treated mice displayed similar performance for odor threshold (Figure S1B, not significantly different, Mann-Whitney U, $p = 0.6$). The mean correct response for detection of EA at each concentration was also similar across treatment groups (Figure S1C; two-way, repeated-measure mixed ANOVA, IND treatment as factor, $p > 0.986$; concentration as factor, $p < 0.0278$). Two-odor discrimination was tested using the odorants 5% EA and 1% acetophenone (AP) (Fig 2A). There was no significant difference in the ability for control vs. insulin IND mice to discriminate between the two odorants (two-way ANOVA, $p > 0.05$). We next tested the ability of these mice to re-learn a reinforced task, when the S+ odor stimulus was switched to the S− odor stimulus [48]. Odor reversal learning examines behavioral flexibility when reward contingencies are reversed [31, 49]. Control and insulin IND mice performed comparable to one-another on a reversal learning task (Fig 2B) (not significantly different, treatment as factor, two-way ANOVA, $p > 0.05$). Both groups demonstrated an increase in performance in their ability to reversal learn (significantly different, time as factor, two-way ANOVA, $p < 0.0001$) and required the same amount of blocks to return to 80% criterion following a reversed award contingency.

Both short- (1h) and long-term (24h) memory tests were performed in control- and insulin-treated mice, after 30 days of IND treatment (Fig 3). Mice in both treatment groups did not favor either of the initially presented objects (object 1 vs. object 2) during the acclimation trial. When mice were re-exposed to object 1 (familiar) vs that of object 3 (novel), exploration of the novel object significantly increased, regardless of IND treatment or duration of memory task (two-way mixed design repeated-measures ANOVA; Fig 3A - short-term ($F(3,32) = 24.65$, $p < 0.0001$), Fig 3B - long-term ($F(3,32) = 23.90$, $p < 0.0001$) (Fig 3B). When exploratory times for the novel object were compared across groups, we observed that the control- and insulin-treated animals had comparable exploratory times in the short-term, $F(1,32) = 0$, $p > 0.05$, and long-term, $F(1,32) = 0$, $p > 0.05$, novel object recognition tests, also suggesting that long-term insulin administration failed to enhance either type of memory-based performance.

Our laboratory has established Kv1.3 to be a substrate for phosphorylation by IR Kinase at tyrosine residues in the N and C terminus of the channel [18, 22] and that a single administration of insulin via intranasal delivery is sufficient to phosphorylate IR kinase and Kv1.3 in the OB [16, 20, 38]. To examine the effect of chronic insulin IND, we immunoprecipitated tyrosine phosphorylated proteins from clarified OB lysates and then

following separation by SDS-PAGE, we probed for IR kinase, Kv1.3 channel, as well as two different downstream signaling cascades by Western analysis (Fig. 4). Insulin treated (I+) animals displayed increased IR kinase phosphorylation (Student's *t*-test, $p < 0.01$, Fig 4A,C) and trended towards increase Kv1.3 phosphorylation that did not reach statistical significance (Student's *t*-test, $p = 0.10$) (Fig 4B,D), when compared to PBS treated controls (C). For these experiments, there were no differences in the results between cohort 1 vs. cohort 2 groups (Fig. 1A), therefore biochemical results were pooled for this analysis. Due to the increased IR kinase activation, we next tested for increased phosphorylation of MAPK signaling and the PI3-Kinase/Akt signaling pathway. Results from these data proved to be highly variable between animals despite equivalence in protein loading standards as visible in Figures 4E–H (not significantly different, Student's *t*-tests, $p > 0.05$). For the data contained in the gels for Fig 4, band pixels were analyzed by quantitative densitometry as normalized for loading standard as visually presented in the histograms below each Western blot (see methods for details).

4. Discussion

In 2012, the National Institutes of Health announced the allocation of $7.9 million specifically for clinical trials of intranasal insulin as part of the National Alzheimer's Project Act (NAPA) [50]. Currently, intranasal insulin is in phase II and III of clinical trials, and is expected to become available to patients in 2017. With intranasal insulin approaching the market, it is of importance to determine any long-term effects of chronic intranasal insulin treatment, given that the majority of insulin IND experiments have been undertaken using single or acute treatments in animal models. Herein, our data demonstrate that daily intranasal delivery of insulin in mice has no adverse effects on olfactory threshold, discrimination, or odor-reversal learning. Short- and long-term object memory recognition is not altered. Systems physiology measurements of energy expenditure, RER, body weight, and water and caloric intake are not modified by daily administration of intranasal insulin. Meal-bout frequency is increased selectively in the dark cycle following chronic insulin IND but overall caloric intake and body weight remain equivalent between PBS- and insulin-IND treated mice. Following 30 days insulin IND, plasma insulin levels are elevated following administration but the ability to metabolize glucose is maintained. The extent of insulin phosphorylation, however, is dampened under daily administration of insulin compared to a single administration, and a known substrate for phosphorylation, Kv1.3, and two downstream signaling pathways lack uniform phosphorylation or lack any significant activation; demonstrating high variability of protein modification across subjects.

Previously it has been demonstrated that acute insulin IND enhances odor discrimination and modestly affects odor threshold [16]. Our current data demonstrate no change in odorant discrimination or threshold when insulin is administered in a daily regimen. It is well known that insulin modulates mitral cell (MC) firing frequency when applied acutely [20, 51, 52] as a driver of state-dependent odor processing that could affect olfactory ability during fasting or satiety states [16, 20, 53, 54]. During the postprandial period of insulin release, untreated animals would be expected to respond to such insulin fluctuations, whereas our chronic insulin IND-treated animals with already elevated plasma insulin levels would be predicted to not electrically detect a new insulin baseline by exhibiting altered MC activity. This is

consistent with our findings of the lack of enhanced odorant discrimination following chronic insulin IND. Despite the lack of behavioral change with odorant discrimination, mice do not have any significant complications with metabolism or body weight homeostasis. Intracerebroventricular (ICV) injection of insulin in the hypothalamus elicits a robust cessation of eating and loss of body weight [42], however, such satiation responses are not induced with daily insulin IND administration. In humans, IND suppresses food intake [55] and thermogenesis [56] so it may be that mice do not mimic humans in terms of all anorexigenic effects. In terms of central insulin (ICV) and IND routes, insulin has been reported to induce catabolic effects in both humans and rodent animals [42].

Although we did not observe any changes in bodyweight during the first 30 days of insulin IND treatment, we wanted a more in-depth analysis of energy metabolism after prolonged insulin exposure. Indirect calorimetry measurements were assessed using isolated CLAMS™ metabolic chambers. Previous data in humans have alluded to insulin IND impacting overall metabolism via decreased caloric intake [57–59], increased postprandial thermogenesis, and elevated energy expenditure [56]. We did not observe such changes in energy metabolism in our mice. With intranasal insulin approaching commercial use and the abundance of intranasal insulin clinical studies being conducted on human patients [60–62], it is beneficial to know that use of this therapy would not result in any significant metabolic side effects. We cannot exclude the question of whether mice make an appropriate model for human response to insulin IND, albeit our mice do not appear to exhibit significant modification in system physiology and metabolic factors.

Reports from centrally administered insulin on the effect on olfactory behaviors continue to be variable. Our lab and others have observed increased olfactory performance [16, 26], while others report decreased olfactory performance [63, 64]. An important variable could be duration of treatment given that our own findings demonstrate increase performance and no change in performance dependent upon length of exposure. The design of the olfactory test must also be considered independent of treatment duration. Marks et al. (2009) [16] used a two-choice paradigm and odor habituation/dishabituation test to ascertain threshold and discrimination data. Odor mixtures and single odorant alcohols were diluted 1:100 in either mineral oil or water, applied to a cotton swab, and introduced into the animals' home cage (Marks et al., 2009). These manual tests do not resemble olfactometry-based testing, which provide precise control of stimulus concentration, onset, and termination, allowing for less variability in the acquisition of simple odor-detection and discrimination tasks [30]. In addition, olfactometry is an operant conditioning task, thus motivation of the animal is manipulated and plays a large factor in the results of these types of behavioral experiments, and not when using manual odor behavior tests.

Our long-term administration data do not demonstrate enhanced short- or long-term object memory in insulin IND mice. This contradicts reports of enhanced memory in healthy human subjects after insulin IND [27, 44, 65]. Insulin IND has been shown to be an effective treatment for mild cognitive impairment and Alzheimer's disease (AD) [43, 66]. AD pathology can be attributed to decreased brain insulin signaling, with diabetic patients at an increased risk for developing AD in comparison to healthy controls [67]. With increased incidence of AD in the aging community and with the rise of type II diabetes in the nation,

possibly leading to more AD cases, the further development of non-invasive techniques for therapy such as IND is of great significance. Most insulin IND studies in rodents have examined restoration of memory in models of cognitive impairment [46, 47], while very few examined insulin-induced memory enhancement in normal-healthy animals, such as our study. Moreover, these studies typically investigated spatial memory and not working memory, as was tested here, using a novel object recognition task. Novel object recognition is based on the innate behavior of rodents to explore novelty and is a pure working memory test, free of reference [68] and closely resembles declarative memory tests performed in humans. Intranasal insulin studies performed in healthy human subjects reveal improved memory in both acute- [65] and long-term conditions [45, 69]. While we previously observed enhanced novel object recognition following acute IND insulin [16], we also cannot eliminate age as an additional factor. Marks et al. (2009) [16] used mice that were 2 months in age; while in the current experiment, mice were approximately 5 months old when memory recognition tests began. A recent study investigating long-term ICV administration of insulin reported enhanced memory in young, but not aged rats [25]. Although the “aged” animals from the Adzovic study were considerably older than the animals used in the current study, there could very well be age-dependent factors at play when it comes to intranasal insulin and learning and memory. Physiologically, the present study doesn’t address whether hippocampal IR kinases were activated, nor if the area received too much insulin, inducing IR internalization or resistance [70]. These would be pertinent factors that could modify any learning- and memory-associated behavioral responses.

The highest binding affinity of insulin and IR kinase expression of the brain is reported for the OB [71]. As such, we have mapped the distribution and co-localization of IR kinase with the Kv1.3 ion channel [16, 22, 72] to predominately the MC layer and determined by site-directed mutagenesis that IR kinase phosphorylates Kv1.3 in the N- and C-terminal aspects of the channel to decrease Kv1.3 conductance [18, 22]. Acute IND insulin elicits a 2.5× enhancement of Kv1.3 phosphorylation [16] but following chronic IND insulin administration for 30 days, the channel protein was not significantly phosphorylated over that of PBS controls. Likewise, the 4× IR kinase phosphorylation observed acutely [16] was reduced to 1.3× under chronic delivery. We did not attempt to wash out insulin with PBS following the 30 day chronic delivery so it is unknown if this type of developed resistance might be reversible. Interestingly, following diet-induced obesity in wild-type mice, we did observe a similar resistance or reduction of Kv1.3 phosphorylation in response to IND insulin treatment [16]. This reduction in IR kinase activity and phosphorylation of a well-characterized substrate (Kv1.3) and downstream effectors (MAPK/ERK and AKT signaling) would be indicative of early stages of hyperinsulinemia given our elevated plasma insulin levels, but not yet failure in glucose metabolism.

Peripheral block of Kv1.3 channel conductance (by either pharmacological means or via acute phosphorylation by IR kinase) or whole-body deletion of the channel {104, 105, 106, 142} does not have identical effects as IND long-term insulin administration in terms of alteration of body weight, metabolism, or ingestive behavior. For example, Schwartz (2000) [76] and Hallshmid et al., (2004) [59] report a reduction in body weight that is sex-selective based upon dosage in response to intracerebroventricular (ICV) injection of insulin. With a 7

day IND insulin administration, IND-treated mice have a slight reduction in body weight (5%) that does not reach statistical significance [16]. Following 30 day IND treatment, however, we observed no significant change in bodyweight. This is in contrast to an 11% reduction in body weight reported in Kv1.3−/− mice {105, 106, 142} compared with their wildtype counterparts or a reduction of 13% when Kv1.3 channel blockers are delivered intravenously [73]. Block of Kv1.3 conductance through targeted deletion of the channel protein is also well characterized to increase mass-specific metabolism {105, 106} with a large enhancement of the locomotor activity in the dark cycle [24]. While chronic insulin IND did not alter the total daily mass specific metabolism, the magnitude change in metabolism from the light to the dark cycle in the insulin-treated mice had a greater level of significance compared to that of control-treated mice (Table 1). This was also true for locomotor activity, where the change in locomotor activity from the light to the dark cycle had a greater level of significant difference compared with that of control-treated mice. Sartorious et al. (2016) [77] recently reported an enhancement in locomotor activity for both insulin and insulin detemir, as well as elevated cortical activity when administered by ICV. Interestingly, Kv1.3-/- mice are reported to eat in frequent and smaller meal sizes regardless of the light cycle [24]. This is in contrast to what we observed with chronic insulin IND whereby mice elicited no change in meal size or overall daily meal frequency, but exhibited a significant increase in meal frequency selectively in the dark cycle. Because Kv1.3 is known to scaffold to a variety of signaling adaptor proteins via protein-protein interactions, some of which are phosphorylation dependent, eliminating the conductance of the channel through genetic deletion or pore occlusion may not be expected to trigger the same series of physiological events as those resulting in IR kinase phosphorylation. Secondarily, our data demonstrate a dampening in IR kinase phosphorylation compared with that of acute insulin IND, an elevation in plasma insulin following insulin IND, but retention of the ability of treated animals to clear glucose.

5. Conclusions

While insulin IND is currently being used to therapeutically treat a broad range of cognitive disorders, as well as other disorders like type II diabetes and Parkinson’s disease [4, 43, 67, 78, 79], long-term use could potentially produce unintentional off-target effects on olfactory ability, cognition, and peripheral metabolism. We found that chronic, long-term administration of insulin IND did not affect olfactory behaviors, energy expenditure, or object memory recognition. The change in locomotor activity and oxygen consumption that typically is accentuated in the dark cycle, appears to be modestly enhanced following chronic insulin IND, whereas the number of meal bouts or frequency of ingestion is significantly increased selectively in the dark cycle. These changes in ingestive behavior and locomotion do not appear to be prominent enough to modify bodyweight, long-term energy expenditure, or modify total caloric intake. Physiologically, we observed a dampened phosphorylation of IR kinase in comparison to an acute administration, and lack of consistent phosphorylation at the level of the Kv1.3 ion channel or along IR signaling pathways in the OB. These biochemical findings may indicate the onset of insulin resistance with still the retention of the ability to clear a glucose challenge. Collectively our data suggest a need to systematically compare the duration of intranasal delivery as an

experimental variable, as well as further comparisons between human/mouse models to appreciate effects of long-term central insulin administration in both disease and non-disease models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Chronic intranasal insulin increases the change in metabolic and locomotor activity between the light and dark cycle
- Chronic intranasal insulin significantly increases the meal frequency during the dark cycle
- Odorant ability and object memory are not affected with long-term insulin intranasal administration
- Insulin phosphorylation of IR kinase in the olfactory bulb is dampened by long-term stimulation
- There is variability in activation of insulin signaling following long-term insulin stimulation

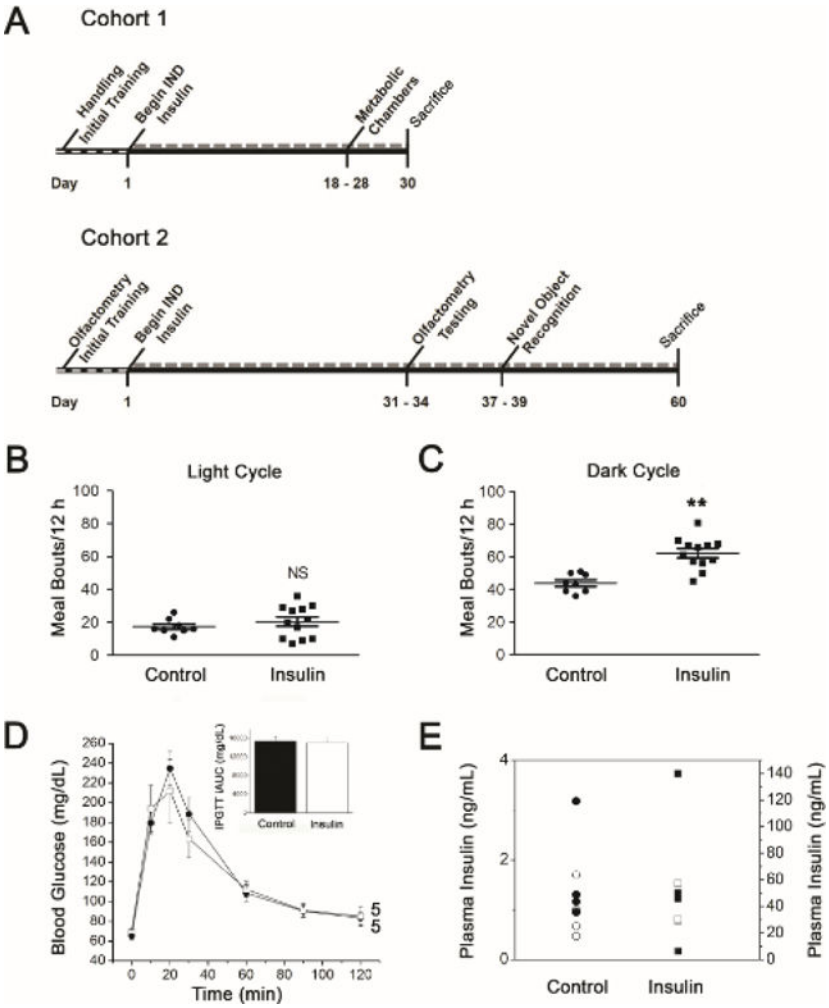

Figure 1.

Ingestive behavior and glucose clearance in mice following chronic insulin intranasal delivery (IND) along two time schedules. (A) Timeline of behavioral experiments for Animal Cohorts 1 and 2. (B,C) Scatter plot demonstrating the number of meal bouts per 12 h interval during the (B) light or (C) dark cycle for mice in Cohort 1 (Metabolic Chambers). Each data point represents cumulative number of meals for an individual mouse collected continuously during specified time interval. (D) Line graph of plasma glucose concentration over time for an intraperitoneal glucose tolerance test (IPGTT) for mice in Cohort 2 following final IND treatment (Sacrifice). Inset, bar graph of the mean integration of the area under the curve (iAUC). (E) Scatter plot of plasma insulin levels for 8 mice in Cohort 1 (open symbols) and 7 mice in Cohort 2 (closed symbols) following final IND treatment (Sacrifice). Squares = PBS (Control), Circles = insulin. B, C – Kolmogorov-Smirnov test, $^{**}p < 0.0025$. D - Not-significantly different (NS) means, Student *t*-test, $p > 0.05$. Dark bars or circles = Control, Light bars or light squares = Insulin. E – Student's t-test, $p < 0.05$. All values represent mean ± SEM in this and subsequent figures. N = Number of mice as indicated.

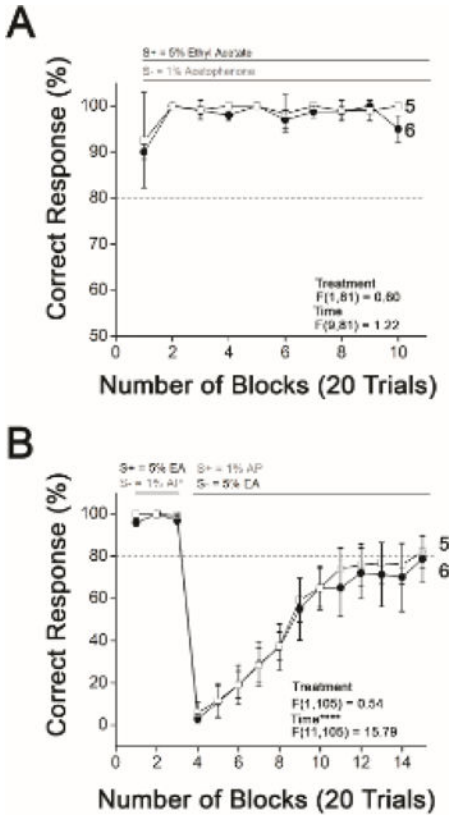

Figure 2.

Long-term intranasal insulin administration does not alter olfactory ability. (A) Line graph of the correct responses for mice in Cohort 2 trained in a two-odor discrimination task (Olfactometry Testing; Figure 1) that were operant trained to discriminate between the odorants 5% ethyl acetate (EA) and 1% acetophenone (AP). NS, two-way RM ANOVA, using treatment and time as factors, $p > 0.05$. (B) Line graph of the correct responses for the same mice trained to perform an odor reversal-learning paradigm. S+/S− reversed at bar break. Two-way RM ANOVA using treatment ($p > 0.05$) and time (***$p < 0.001$) as factors. Control (dark circle) vs. Insulin (open square). Dashed line = 80% correct responses (criteria). N = Number of mice as indicated.

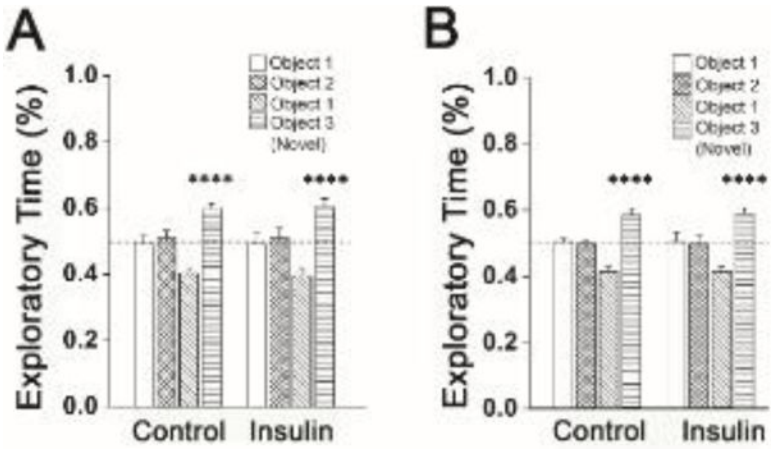

Figure 3.

Long-term intranasal insulin does not enhance short- or long-term memory in a novel object recognition task. Bar graphs of the exploratory time to explore two objects during a familiarization phase (Object 1, Object 2) followed by a recognition phase (Object 1, Object 3) where a novel object is presented (Object 3). Time between familiarization and recognition phase is set for (A) short-term memory, or 5 min, or (B) long-term memory, or 24 h. Dashed line = 50% exploration of Object 1, or equal exploration across objects. Two-way, repeated-measure mixed ANOVA, Bonferroni's post-hoc test, ****$p < 0.0001$. N = 5 animals/treatment group, Cohort 2 (Figure 1; Novel Object Recognition).

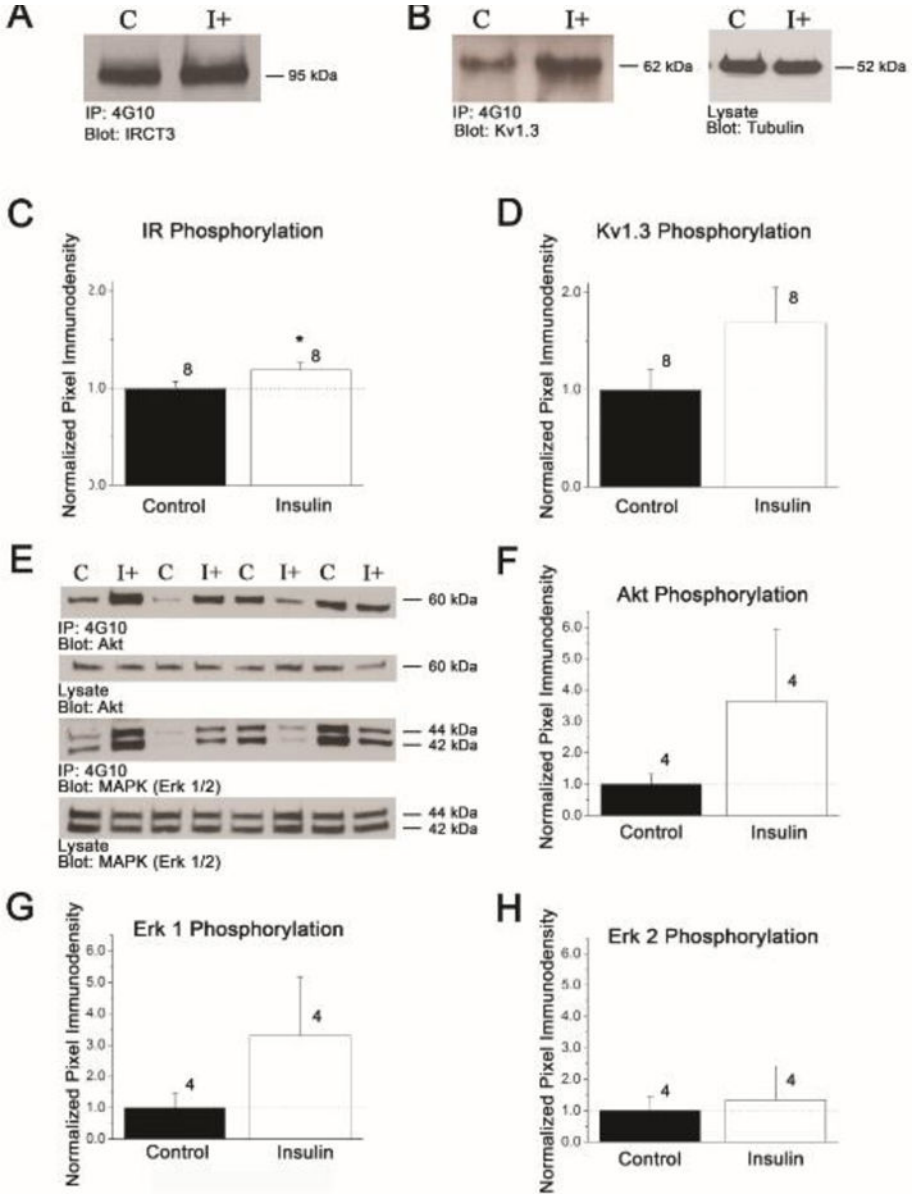

Figure 4.

Long-term Intranasal insulin increases insulin receptor (IR) kinase phosphorylation within the olfactory bulb but does not uniformly phosphorylate downstream substrates. Immunoprecipitated (IP) tyrosine phosphorylated proteins (4G10) were separated by SDS-PAGE and blotted (Blot) with antisera against insulin (IRCT3) or Kv1.3 channel (Kv1.3). Western analysis and associated bar graph of normalized immunodensity values for (A,C) IR kinase phosphorylation and (B,D) Kv1.3 phosphorylation for animals pooled across Cohorts 1 and 2 (Figure 1, Sacrifice). Also shown is input lysate for A–D, blotted with the loading control, beta-tubulin III (Tubulin). (E–H) Same as (A–D) for animals from Cohort 1 (Figure 1, Sacrifice), but tyrosine phosphorylated proteins (4G10) were blotted for AKT, MAPK (Erk1/2). Input lysates were blotted with Akt and MAPK (Erk 1/2), respectively. M_r in kDa

as specified. Dashed line = ratio 1.0 for insulin/control. C = Control, +I = Insulin. N = number of animals (both olfactory bulbs per sample). Student's *t*-test, *$p < 0.05$.

Table 1

Comparison of whole-animal physiological properties in long-term chronic insulin or control IND.

Property	Control IND *n = 4*	Insulin IND *n = 4*
Bodyweight (g)	26.3 ± 0.9	27.1 ± 0.6
Water Intake (g)	4.0 ± 0.1	3.6 ± 0.1
Caloric Intake (kcal)	13.9 ± 0.9	13.9 ± 1.0
Energy Expenditure (kcal/h)		
12 h dark	0.42 ± 0.002****	0.42 ± 0.007****
12 h light	0.33 ± 0.005	0.34 ± 0.004
Respiratory Exchange Ratio		
12 h dark	0.94 ± 0.008**	0.96 ± 0.015**
12 h light	0.88 ± 0.008	0.88 ± 0.009
Normalized VO_2 (ml/kg/h)		
12 h dark	3198.7 ± 92.0**	3089.0 ± 33.7***
12 h light	2580.7 ± 90.0	2532.0 ± 65.4
Locomotor Activity (beam breaks)		
12 h dark	197.8 ± 26.4**	220.7 ± 19.7***
12 h light	50.1 ± 3.7	57.0 ± 8.5

Values represent mean ± SEM for mice in Cohort 2 (see Figure 1). Bodyweight (BW) was acquired once daily over the 10 days mice were in the metabolic chamber. Ingestive and locomotor activity were acquired from continuous data collection for 10 days. All other measurements were acquired from 30 second binned intervals (collected ~every 18 minutes/cage) for 10 days. There were no significant differences found for any physiological parameter as a result of insulin (Student's *t*-test, $p > 0.05$). Parameters were significantly different when compared between dark and light cycle within either control- or insulin-treated mice (Paired *t*-test, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).