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Comparative *In Vivo* Investigation of Intrathecal and Intracerebroventricular Administration with Melanocortin Ligands MTII and AGRP into Mice

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Abstract

Central administration of melanocortin ligands has been used as a critical technique to study energy homeostasis. While intracerebroventricular (ICV) injection is the most commonly used method during these investigations, intrathecal (IT) injection can be equally efficacious for the central delivery of ligands. Importantly, intrathecal administration can optimize exploration of melanocortin receptors in the spinal cord. Herein, we investigate comparative IT and ICV administration of two melanocortin ligands, the synthetic MTII (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH₂) MC4R agonist and agouti-related peptide [AGRP(87-132)] MC4R inverse agonist/antagonist, on the same batch of age-matched mice in TSE metabolic cages undergoing a nocturnal satiated paradigm. To our knowledge, this is the first study to test how central administration of these ligands directly to the spinal cord affects energy homeostasis. Results showed, as expected, that MTII IT administration caused a decrease in food and water intake and an overall negative energy balance without affecting activity. As anticipated, IT administration of AGRP caused weight gain, increase of food/water intake and increase respiratory exchange ratio (RER). Unexpectedly, the prolonged activity of AGRP was notably shorter (2 days) compared to mice given ICV injections of the same concentrations in previous studies (7 days or more)^{1–4}. It appears that IT administration results in a more sensitive response that may be a good approach for testing synthetic compound potency values ranging in nM to high μ M *in vitro* EC₅₀ values. Indeed, our investigation reveals that the spine influences a different melanocortin response compared to the brain for the AGRP ligand. This study indicates that IT administration can be a useful technique for future metabolic studies using melanocortin ligands and highlights the importance of exploring the role of melanocortin receptors in the spinal cord.

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Graphical Abstract

Keywords

IT; ICV; Obesity; MTII; AGRP

Introduction

Intracerebroventricular (ICV) injection is a widely used *in vivo* technique to administer compounds directly into the central nervous system (CNS) through a cannula placed directly into the lateral ventricles of the rodent's brain⁵. This technique is particularly effective to centrally test compounds that may not cross the blood brain barrier (BBB). Throughout recent decades, central administration of melanocortin ligands has been optimized to help investigate energy homeostasis and obesity^{1,6}. Some melanocortin compounds previously investigated using ICV administration include MTII (Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH₂), a melanocortin 1,3,4 and 5 receptor agonist, which has been shown to acutely decrease food and water intake in mice and rats⁷⁻¹⁰. In addition, administration of MTII in mice has been shown to temporarily decrease activity and body temperature but increase energy expenditure¹¹⁻¹³. Intracerebroventricular injection of agouti-related peptide (AGRP), a MC3R antagonist and MC4R inverse agonist/antagonist, into the brain of mice has resulted in a prolonged increase in food intake¹⁻⁴. Further metabolic studies have shown that AGRP increases respiratory exchange ratio (RER) but has no effect on energy expenditure in rodents^{3,4}. Intrathecal administration of AGRP has yet to be used *in vivo* to study metabolism but has been used in previous investigations about chronic pain^{14,15}.

While ICV injection has been demonstrated to be a valid method for exploration of melanocortin ligands in the CNS, it has disadvantages. Between surgical materials and recovery time, preparation for ICV injections *in vivo* is time consuming and has a high monetary cost. Often surgery, recovery and cannula placement validation take up to a month to complete before subjects can be used for experimentation. Fortunately, there is an alternative but underutilized form of central administration namely intrathecal injection (IT) in which compounds are injected straight into CNS through the spinal cord¹⁶. While this technique takes longer to optimize, it becomes less costly and time consuming because it does not require intensive surgery. With this method, subjects are immediately ready for experimentation and no time is wasted with surgery and recovery. It has been speculated that IT administration of melanocortin compounds could be an effective strategy to study obesity related diseases. In particular, it could help investigate the understudied spinal melanocortin receptors and their effect on energy homeostasis. Herein, we perform comparative ICV and

IT administration in the same mice with melanocortin agonist ligand MTII and measure the metabolic differences between these two routes of administration. Mice were housed in TSE metabolic cages configured to measure food intake, water intake, activity, RER and energy expenditure. It is expected that difference in routes of administration may cause small differences in metabolic measures because the mice will be undergoing different environmental factors during injection. Upon validation that IT administration of MTII agonist appears to respond similarly efficaciously as ICV administration, IT injections of AGRP are performed in the same cohort of mice to investigate the metabolic effect of AGRP administered directly to the spinal cord.

Results and Discussion

Intrathecal (IT) administration differentially affects energy homeostasis compared to intracerebroventricular (ICV) administration

Our investigation aimed to test the hypothesis that IT injection of MTII would acutely decrease food intake and to explore the role of melanocortin receptors on the spinal cord. Intrathecal injection is an underutilized technique that injects compounds directly into the CNS through the spinal cord^{16,22}. The benefits of IT administration is that the technique is quick and easily reproducible which is important when using mice because it will reduce environmental stress¹⁶. Intracerebroventricular injection comparatively injects compound into the CNS using a cannula placed into the lateral ventricles⁵. Intracerebroventricular injections are costly in terms of surgical equipment, per diams and personal time. After surgery, recovery and cannula placement validation steps, it can take up to a month for a mouse to be fully prepared for experimental ICV injection. Intrathecal administration is ideal for experimental means because limited preparation, other than the initial IT injection training, is needed to start experimental compound investigation in the CNS. It is important to note that compounds injected into the spinal cord has access to the brain¹⁶. An investigation showed about 5.4% volume of compound injected into the spinal cord can be seen in the brain just after 20 minutes¹⁶. Intrathecal administration was originally discovered to help investigate acute pain but its easy interpretation into a clinical setting makes it optimal to study melanocortin system and obesity related diseases¹⁶.

We injected a single dose of MTII agonist by IT and ICV administration into the same mice to evaluate the behavioral effects of differential receptor pharmacology in the lateral ventricles of the brain compared to the spinal cord. Food intake was significantly greater in mice administered IT saline and MTII at 6 and 8 hours post-injection compared to those administered ICV with saline and MTII (Fig. 2A). At 6 hours post-injection, food was still decreased in mice administered IT with MTII compared to saline IT but interestingly, mice administered saline IT ate significantly more food than mice administered with saline ICV. A similar trend was seen as mice injected with saline and MTII IT ate significantly more than mice administered with saline and MTII ICV at 8 hours post-injection (there was no statistical significant difference in drug at this time point). This is expected because the mice being administered IT undergo different handling conditions than those being administered via ICV.

Mice injected IT with MTII drank significantly less than mice injected with saline IT at 6 hours post-injection. However, mice injected IT with saline drank significantly more than mice injected ICV with saline at 6 hours post-injection (Fig. 2D). Furthermore, at 8 hours post-injection, mice injected with both compounds IT drank significantly more than the mice injected ICV. Overall, IT administration of MTII causes a significant decrease in food and water intake but mice treated IT ate and drank more overall in both control and drug treated groups administered ICV.

Energy expenditure is a measurement that is used to analyze amount of energy consumed in a mouse²⁵. Results showed that there was a significant increase in the energy expenditure in the mice injected IT with saline and MTII at 8, 24, 28 and 32 hours post-injection compared to those administered ICV with saline and MTII. Respiratory exchange ratio (RER) was analyzed between groups administered IT versus ICV. Mode of administration had a significant interaction with time but follow-up tests did not find significance a specific time-points post-injection (Fig. 2C). The data collected from food intake, water intake, RER and energy expenditure indicates not only that there might be differential melanocortin response mediated by the spine compared to the brain, but also that IT administration creates different behavioral environmental factors since the control groups are significantly different.

Locomotor activity was analyzed between modes of administration. It was shown that type of administration did not significantly affect mouse locomotor activity (Fig. 2B). Physical activity is often examined to determine mouse fitness and wellness^{19,28}. Therefore, this data indicates that IT injections are equally healthy for mice compared to the well-established ICV injection method. Overall, our observed results indicate that IT administration is better tolerated in mice compared to ICV administration and could be used for more sensitive investigations of the CNS.

Corresponding intrathecal and intracerebroventricular administration of the MTII agonist causes a decrease in food, water and RER

The MTII agonist has been shown to decrease food and water intake within hours of injection^{1,8-10}. We observed significantly less food intake in mice given single injections of MTII agonist via IT and ICV at 2, 4 and 6 hours post-injection (Fig. 2A). Furthermore, we observed a corresponding decrease in water intake at 6 hours post-injection in mice injected ICV and IT with the MTII agonist compared to saline (Fig. 2D). These results are consistent with the previous literature⁷⁻¹⁰. Our data displays that both IT and ICV administration independently had an immediate effect on food intake within hours of administration (Fig. 2A). While the understanding of how melanocortin specific compounds transferring from the spinal cord to the brain remains unknown, previous literature has shown that compounds (while is small amounts) can enter into the brain within minutes. Hylden and Wilcox has shown that just after 20 minutes of IT injection, 3.6% of a compound can be found in the cerebellum and 1.8% can be found in the forebrain¹⁶. While these percent volumes are relatively small, potent compounds (such as AGRP) can still start having notable effects.

Metabolic measures such as RER, activity and energy expenditure were further examined after mice were administered MTII agonist via IT and ICV administration. When examining RER, it is important to note that carbohydrates and fatty acids produce different levels of

CO₂ and O₂ which helps us identify which fuel sources are being utilized by the mouse. A RER value near 0.7 indicates that fats are being harnessed as a primary fuel source while a RER value of 1.0 indicates carbohydrates are the primary fuel source^{17,24,25}. When mice were given central (IT or ICV) injection of MTII, their RER was significantly lower than mice injected with saline up to 10 hours post-injection (Fig. 2C). This indicates that IT and ICV administration of MTII caused the mouse to use more fats as a primary fuel sources compared to when they were injected with saline.

We did not observe a significant difference in locomotor activity among mice given single injections of IT or ICV with 3nmol of MTII (Fig. 2B). Furthermore, we observed greater energy expenditure in mice injected IT or ICV with MTII during the first 4 hours post-injection (Fig 2E). This is consistent with previous literature that reported an increase in energy expenditure after 4 hours in mice who were injected peripherally with MTII^{12,13}. Overall, IT administration of MTII has similar metabolic effects as ICV administration which supports the hypothesis that IT administration can be a useful and effective tool to study the pharmacology of the melanocortin system.

Intrathecal AGRP increases food intake and causes a positive energy equilibrium

To further examine the *in vivo* comparative effects of the melanocortin receptors in the spinal cord, metabolic data was recorded 5 days post-injection in mice administered intrathecally with 2nmol AGRP. Agouti-related peptide is a MC3R antagonist and a MC4R inverse agonist/antagonist. It has been previously reported to cause a prolonged increase in weight, food intake, and RER but has no change on energy expenditure when administered in rodents via ICV¹⁻⁴. In our study, the total food intake was significantly greater at hours 6 and 8 post-injection (Fig. 3A). Furthermore, total food intake per day remained significantly elevated 1 and 2 days post-injection compared to saline (Figure 4B). In correspondence, there was a significant increase in water intake at hour 6 and on day 1 post-injection in mice administered IT with AGRP compared to those administered IT with saline (Figure 3B and 4C). There was a significant increase in mouse weight 1 to 5 days post-injection in mice administered AGRP IT compared to saline (Fig 4A.). Previous reports for rodents administered ICV with AGRP observed a significant greater food intake 5–7 days post-injection¹⁻⁴. Upon IT treatment, the prolonged action is decreased compared to ICV administration which suggests that the melanocortin receptors accessible via ICV may be responsible for AGRP's 3–7 day prolonged activity.

We further examined other metabolic measures such as RER and energy expenditure. We examined average hourly and daily RER values in mice injected IT with AGRP compared to saline. Results showed that mice injected intrathecally with AGRP had a significant increase in RER during the first 12 hours post-injection (Fig. 3C). Furthermore, this significant increase in RER remained significantly higher 1 to 4 days post-injection (Fig. 5A). Since RER has a biphasic effect because of the mouse circadian rhythm, follow up examination showed that RER was raised significantly increased during the active (dark) and sleep (light) cycles on day one but only during the active cycle on day 2, 3 and 4 post-injection (Fig. 5C). As stated earlier, a RER near 1.0 indicates that carbohydrates are the primary source of fuel which specifies that mice administered with AGRP IT were burning more carbohydrates as a

fuel source compared to mice administered saline IT during their active periods^{24,25}. These mice were burning more carbohydrates because they were eating more chow-related carbohydrates during those first 2 days of the experiment compared to mice injected with saline IT (Fig. 4B). Since the mice injected with AGRP were burning more carbohydrates than fat, this could also explain why we saw such a gain in mouse weight up to 5 days post-injection. Furthermore, as seen in previous studies, there was no difference in energy expenditure between compound groups (Fig. 3D; Fig. 5B).

The question remains as to why AGRP administered intrathecally has a shorter duration of action than when it is injected directly into the brain. It has been noted the AGRP circulates in higher levels in the blood after fasting paradigms indicating that AGRP has a cyclic pathway between the periphery, into the spine and then into the brain²⁹. The long AGRP lifespan and the difference in feeding behavior seen in our investigation shows that melanocortin ligands in the spine may have important contributions to metabolic homeostasis.

Conclusion

Intrathecal administration of melanocortin ligands has similar metabolic effects in mice compared to ICV administration. It is concluded that central administration of melanocortin ligands intrathecally is a valid method to study energy homeostasis. The spinal cord has a limited population of melanocortin receptors but the direct pharmacological effects of these receptors are widely unknown^{30–33}. A more recent study has indicated that MC4Rs in cholinergic (spinal) neurons is linked to obesity-induced hypertension which highlights the importance of further investigation of spinal melanocortin receptors for future therapeutic uses³⁴. There was a significant difference between the two routes of administration. This is not the first time differentiation has been seen between ICV and IT administration for it has also been seen in pain pathways in which some opioid ligands were more potent spinally and did not develop tolerance²³. This suggests that melanocortin receptors in the spine could be targets for new and safe therapeutics. Intrathecal administration is a less costly and time consuming compared to intracerebroventricular administration which makes it a robust technique for studying energy homeostasis.

Methods

Animals

This study was conducted in accordance with the guidelines set up by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota. Wildtype (WT) male mice with a mixed 129/Sv×C57BL/6J background derived from an in house breeding colony were used throughout this experiment as previously reported in literature^{1,8,17,18}. Mice were age-matched at 8 weeks old to initiate the experiment. Each mouse was individually housed to protect cannula assembly in either standard polycarbonate conventional cages provided by the University of Minnesota's Research Animal Resources (RAR) or in TSE PhenoMaster metabolic cages (TSE Systems; Berlin, Germany). Weekly cage changes and TSE metabolic cage maintenance was performed by lab research staff. Mice were housed in a temperature-controlled room (23°–25°C) and maintained on a

reversed 12-h light/dark cycle (lights off at 11:00am). Mice had *ad libitum* access to normal chow (Harlan Teklad 2018 Diet: 18.6% crude protein, 6.2% crude fat, 3.5% crude fiber, with energy density of 3.1kcal/g) and water.

Cannulation surgery and placement validation

Cannulation surgeries were performed as previously reported^{1,12,17–20}. Mice were anesthetized with mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg) given intraperitoneal (IP) injection and placed in a stereotaxic apparatus (David Kopf Instruments). Mice were given an analgesic dose of flunixin meglumine (FluMeglumine, Clipper Distribution Company). A 26-gauge cannula (Cat #8IC315GS4SPC; PlasticsOne, Roanoke, VA) was inserted into the lateral cerebral ventricle at coordinates 1.0 mm lateral and 46 mm posterior to bregma and 2.3 mm ventral to the skull²¹. The cannula was secured using dental cement (C&B Metabond Adhesive Cement Kit #S380) followed by Lang's Jet Dental Repair Kit (Jet Denture Repare Powder, #1220; Jet Liquid, #1403). For three days post-operation, mice were administered 0.5 mL of 0.9% Saline (Hospira, Lake Forrest, IL) S.Q. and flunixin. Mice were allowed to recover 7 days following surgery. Cannula placement was validated using 2.5 µg/2.0 µL human PYY^(3–36) (hPYY) as previously described^{1,17–20}. During cannula placement validation, food intake and mouse weight was measured at 2, 4 and 6 hours post-injection of hPYY. The criterion for proper cannula placement was defined as a mouse consuming 0.8g more food after hPYY ICV administration compared to saline ICV administration at the 4h post-injection time point (94% had successful validations). Mice that did not meet this criterion were excluded from the study. Sixteen male mice with validated cannula placement were then transferred into TSE PhenoMaster metabolic cages and were given 1 week to acclimate.

Mouse Metabolic Studies

All experiments consisting of intracerebroventricular (ICV) and intrathecal (IT) injections were of crossover, non-fasting (nocturnal) feeding paradigm. Intrathecal injections were performed as previously described^{16,22,23}. Desired compound or saline control was administered in one single injection via ICV/IT 2 hours before lights out (t=0h). Food intake, water intake, oxygen uptake, carbon dioxide production and locomotor activity were recorded by a TSE PhenoMaster system^{17,19}. Respiratory exchange ratio (RER) and energy expenditure were then calculated from oxygen uptake and carbon dioxide production. The RER was calculated by dividing the volume of CO₂ produced by the mouse by the volume of O₂ consumed as measured by the metabolic cages^{24,25}. Energy expenditure is a measurement that is used to analyze the amount of energy consumed in a mouse and is calculated by number of kilocalories burned per hour which we then normalize to the mouse's weight (kcal/h/kg). In the first cohort, mice were divided into 4 groups (n=3/4 per group, n=15 total) and given a single injection of saline or 3nmol MTII via IT or ICV administration (Fig 1A). In the second cohort, mice were divided into two groups (n=5 and 7 per group, n=12 total) and were either given a single injection of saline IT or 2nmol AGRP IT (Fig 1B). During the first cohort (MTII agonist administration), food intake, water intake and activity were binned in 2 hour increments. Respiratory exchange ratio and energy expenditure were recorded in average every 2 hours. The effects of food intake, water intake and activity from AGRP administration were recorded in two hour increments as well as

totals/averages per day. Respiratory exchange ratio and energy expenditure were average daily and semi-daily (12 hour bins). Mice were monitored daily and given 6–7 days between treatments to re-establish pre-treatment body weight and feeding behavior.

Compounds

A stock solution of hPYY (Cat# H8585; Bachem) was prepared using sterile ddH₂O (final concentration of 10 µg/µL) and was then stored at –20°C. On days of cannula validation experiments, the hPYY was thawed and diluted with sterile 0.9% saline (Hospira, Lake Forrest, IL) to 0.5 µg/µL. Each mouse was then injected with 2.5µg/2µL (0.1mg/kg) hPYY. During cohort 1, a solution of MTII (Cat# H3902; Bachem) was prepared into a stock solution 10nmol/µL using sterile 0.9% saline and stored at –20°C. On days of MTII experiments, the MTII stock was thawed and diluted to sterile 0.9% saline to 1nmol/µL or 3nmol/5µL for ICV or IT administration, respectively. Mice experiencing MTII ICV injections were injected with 3nmol/3µL (3.07µg/3µL). Mice experiencing MTII IT injections were injected with 3nmol/5µL (3.07µg/5µL). For cohort 2, a 2nmol/µL stock of AGRP (Cat#4366s; Peptide Institute; Minoh-shi Osaka, Japan) was prepared using sterile 0.9% saline and was stored at –20°C. Mice experiencing AGRP ICV injections were injected with 2nmol/3µL (10.69µg/3µL) and mice experience AGRP IT injection were injected with 2nmol/5µL (10.69µg/5µL).

Data Analysis

Primary dependent variables were: (i) food intake; (ii) water intake; (iii) locomotor activity; (iv) respiratory exchange ratio (RER); (v) energy expenditure; and (vi) body weight. Metabolic measures were analyzed using a two-factor within-subject Analysis of Variance (ANOVA) with between-sessions variables of compound or mode of administration and the within-subject variable of time. Follow-up independent sample t-tests with Bonferroni correction were used to identify sources significant interactions at specific time-points. Data was graphed using GraphPad Prism. Data was analyzed using Statistical Package for the Social Sciences Software (SPSS) software and was represented as the mean ± error with $P < 0.05$ indicating significance (detailed statistical analysis of these variables is provided in the Supplemental Information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ICV intracerebroventricular

CNS	central nervous system
MTII	melanotan 2
MC4R	melanocortin 4 receptor
MC3R	melanocortin 3 receptor
AGRP	agouti related-peptide
RER	respiratory exchange ratio
IT	intrathecal injection

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Figure 1.

Illustration of the cross-over paradigms used in this study. (A) In Cohort 1, mice were divided into 4 segments labeled as Group 1 (n=4), Group 2 (n=4), Group 3 (n=4) and Group 4 (n=3). Mice were injected with either with a single injection of 3nmol MTII agonist via ICV administration, a single injection of 3nmol MTII agonist via IT administration, a single injection of saline control via ICV administration or a single injection of saline control via a single injection IT administration on given experimental day. The mice were then subject to a wash period of 1 week to ensure normal body homeostasis as measured by a return to normal body weight and food intake levels. (B) In Cohort 2, mice were divided into 2 segments labeled as Group 1 (n=7) and Group 2 (n=5). Mice were injected with a single injection of 2nmol AGRP via IT administration or saline control via IT administration. The mice were then subject to a wash period of 2 weeks. The same cohort of mice were used for both MTII and AGRP experiments.

Figure 2.

Summary of energy homeostasis in WT male mice in TSE metabolic cages comparing IT administration (circle) to ICV administration (square) when injected with 3nmol MTII (open shape) or saline (closed shape). Total food intake (A) was significantly decreased at 2h, 4h, and 6h post-injection in mice administered with MTII compared to saline. Mice that underwent IT administration ate significantly more at 6h and 8h post-injection compared to mice injected ICV. Total water intake (B) was significantly decreased in mice injected with MTII compared to saline at 6h post-injection. Mice injected via IT drank significantly more water compared to mice injected via ICV at 6h and 8h post-injection. (D) Locomotor (beam-break) activity did not significantly differ among all groups. Administration of 3nmol MTII resulted in a decrease in RER (C) for the first 10 hours post-injection, regardless of the route of administration. Mice administered with MTII had an acute increase in energy expenditure (E) at 4 hours post-injection. Mice that underwent IT administration had an increase in energy expenditure at 8h, 24h and 28h post-injection. ** $p < 0.01$, *** $p < 0.001$ for MTII compared to saline. ## $p < 0.01$ ### $p < 0.001$ for IT administration compared to ICV administration.

Figure 3.

Summary of acute food intake, water intake, RER and energy expenditure of WT male mice in TSE metabolic cages comparing 2nmol AGRP administration (open square, blue line) to saline (closed square, black line). Food intake (A) significantly higher in mice injected IT with AGRP for 6 and 8 hours post-injection compared to mice injected IT with saline. Water intake (B) was significantly greater 6 hours post-injection in mice administered IT with AGRP compared to saline. Mice injected IT with AGRP had a significantly higher RER (C) all 12 hours post-injection. Energy expenditure (D) did not differ between groups. ** $p < 0.01$, *** $p < 0.001$ for AGRP compared to saline.

Figure 4.

Summary of prolonged body weight, food intake and water intake of WT mice 1 to 5 days post-injection in TSE metabolic cages comparing 2nmol AGRP administration (open square, blue line) to saline (closed square, black line). Mouse weight (A) significantly increased in mice injected with AGRP for 5 days post-injection compared to mice injected with saline. Total food intake (B) was significantly increased for the first two days post-injection in mice administered with AGRP compared to saline. Total water intake (C) was significantly increased in mice injected with AGRP compared to saline on day 1 post-injection. ** $p < 0.01$, *** $p < 0.001$ for AGRP compared to saline.

Figure 5.

Summary of prolonged RER and energy expenditure of WT mice 1 to 5 days post-injection in TSE metabolic cages comparing 2nmol AGRP administration (open square and blue line; gray bar) to saline (closed square and black line; black bar). Average RER was significantly increased for the first four days post-injection in mice administered with AGRP compared to saline. Follow-up analysis (C) of the first 4 days of experimentation shows that RER was significantly increased during the dark cycles on days 1 thru 4 and during the light cycle on day 1. ** $p < 0.01$, *** $p < 0.001$ for AGRP compared to saline. Energy expenditure (B) did not significantly differ 5 days post-injection.