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PYY3-36 Efficacy is Independent of Photoperiod but Dependent on Time of Day

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Abstract:

Approximately 70% of Americans are overweight or obese and studies have shown that poverty-dense American counties are the most prone to obesity (Levine, 2011). Along with diet, the endogenous circadian rhythms have been found to be an important piece of human metabolic health. Circadian disruption in humans, such as jet-lag and shift work, have been linked to metabolic problems such as Type 2 Diabetes Mellitus and Metabolic Syndrome. Since circadian disruption affects many demographics including shift workers and routine business travelers it is imperative to understand the biological mechanism behind the relationship between circadian disruption and weight gain. One potential connection between circadian function and eating behaviors is the Neuropeptide Y (NPY)/Peptide YY (PYY) system which causes satiety after food intake. This study conducted three experiments (i) in a 12:12 light dark cycle (LD) were assigned to four temporal sac groups (ZT 0, ZT 6, ZT 12, or ZT 18) (ii) in a LD mice were assigned to four temporal injection groups (ZT 0, ZT 6, ZT 12, or ZT 18) and paired with either a PYY_{3-36} or vehicle (control) (iii) mice were exposed to LD or constant light (LL) paired with either a PYY_{3-36} or vehicle (control) injection during the onset of their active period, ZT 12. In the first experiment, it was found that PYY_{3-36} is regulated cyclically. In the second experiment, PYY_{3-36} reduced food intake, being most effective during the beginning of the night. PYY_{3-36} injections were also found to be effective in inhibiting food intake regardless of the light cycle. This suggests that photic cues are not essential for PYY_{3-36} active period efficacy.

Introduction:

Circadian Rhythms

Circadian rhythms are endogenous cycles within organisms approximately 24 hours in length which is controlled by a transcriptional-translational feedback loop in mammals. Transcriptional activators CLOCK/BMAL1 upregulate PER1-3/CRY1-2 which throughout the day acquire post-translational modifications which accumulate, localize to the nucleus and inhibit its activators (Huang et al., 2011). This feedback loop occurs within the hypothalamus in a region known as the superchiasmatic nuclei (SCN) which is the master regulator of circadian rhythms within mammals (Fonken and Nelson 2014). Peripheral tissues are then synced to the SCN via neural and hormonal signals; these peripheral tissues also containing core molecular clock mechanisms (Huang et al., 2011). Without the presence of external time cues, referred to as zeitgebers or "time givers", circadian rhythms are free running meaning that their period is slightly greater than 24 hours (Toh, 2008). Circadian rhythms are important in various cycles including sleep/wakefulness, endocrine signaling, body temperature, and feeding/fasting cycles among many others.

Light acts as the most potent zeitgeber by entraining circadian rhythms to the 24 hour day. Light entrains the SCN through excitatory signaling through the retinohypothalamic tract (Plano et al., 2017). External light cues are received through this tract and in turn result in upregulation of clock genes important in resetting the internal clock. The effects of altered lighting conditions in mice can have the following effects (i) dim light at night (DLAN) causes increased day time activity and feeding, blunting of oscillating SCN clock genes and insulin

sensitivity (ii) LL causes arrhythmic activity, loss of feeding rhythm, blunting of oscillating SCN clock genes and insulin sensitivity (iii) simulated jet-lag causes sporadic activity, disrupted feeding behavior, two altered oscillation SCN clock gene rhythms, and blunted insulin sensitivity and all three altered light conditions result in increased body mass (reviewed by Plano et al., 2017). Thus, light conditions play an essential role on circadian rhythms at the behavioral, biochemical, and metabolic level.

Although the SCN syncs and couples to peripheral tissues, there are cases in which peripheral rhythms can be out of sync from the SCN due to other external cues or peripheral tissues adapting slowly in comparison to the SCN (Fonken & Nelson, 2014). For example in simulated-jet lag light exposure, the SCN clock genes have two oscillations: a cycle entrained prior to the jet-lag and a shifted cycle with the peak occurring later with the same amplitude. This rhythm is opposite to the liver tissue clock gene oscillations that have an out of phase cycle (Plano et al., 2017). Opposing rhythms could be influenced by additional external cues such as food availability and locomotor activity (Fonken & Nelson, 2014). Restricted feeding can result in anticipatory wakefulness and increases in locomotor activity and entrains circadian rhythms in the liver (Fonken & Nelson, 2014). This provides evidence on how internal clock mechanisms not only affect internal rhythms but how external cues can in turn affect the internal clock.

Circadian Disruption and Metabolism

In present day, urbanization is leading to increased light pollution that can in turn affect human circadian rhythms and health. With 70% of the U.S. being overweight or obese, it is an interesting question to investigate whether increased amount of light could be contributing to

this multifaceted problem (Levine, 2011). Further, light pollution is not the only form of circadian disruption affecting humans; shift workers, routine business travelers, and the use of technology at night are all forms of altered lighting conditions that can alter circadian rhythms. Natural compared to artificial lighting can also affect endogenous rhythms as it has been shown that humans exposed to natural lighting compared to electrical light had less variable melatonin and sleep rhythms (Fonken & Nelson, 2014). Human studies have found that altered lighting conditions can have adverse impacts on metabolism. Shift workers have been found to be at a higher risk for Metabolic Disorder and have increased body mass index (BMI) (Fonken & Nelson, 2014) and social jetlag has been found to be correlated with metabolic issues and being overweight in individuals with non-communicable chronic diseases (Mota et al. 2017). These studies among many others are a part of a growing body of evidence suggesting circadian disruption from external light cues can be detrimental to human health.

Studies in humans show that circadian disruption causes metabolic problems by (i) altering metabolic hormone levels such leptin and thyroid-stimulating hormones (Zimberg, et al 2012); (ii) increasing glucose and insulin levels, conditions commonly found in patients with T2DM (Zimberg, et al., 2012); and (iii) that sleep restrictions lead to increased weight gain and increased caloric intake, especially during evening or “non-active” hours (Spaeth, et al., 2013). To this end in mice, if food availability is constrained to only the active part of the daily cycle, the increased obesity and diabetic phenotypes are negated (Hatori et al., 2012). In addition to circadian disruption affecting metabolism, altered metabolism such as that seen in obese individuals can in turn affect internal hormonal rhythms and body temperature (Fonken & Nelson, 2014).

Mice make great research subjects since they share circadian clock mechanisms and can develop metabolic disorders similar to humans. Hence, mouse-based models have been used to further investigate how circadian disruption leads to metabolic disorders. In mice, varying photoperiods with clear implications in humans are used such as the light cycles previously mentioned: simulated jetlag, DLAN and LL. The latter two simulate human activities from electric light pollution like watching TV and using smartphones throughout the night.

Rodent studies provide models that can further be manipulated to have a greater understanding as to how circadian disruption leads to metabolic disorders and weight gain. It has been found that mice exposed to circadian disruptions such as LL, simulated jetlag, or a 20 hour LD day exhibit increased weight gain and symptoms of T2DM and Metabolic Syndrome (Fonken, et al 2010; Fonken et al., 2013; Karatsoreos et al., 2010). Several factors have been found to potentially contribute to the obesogenic effects of circadian disruption. These factors include disruption of molecular clock rhythms, disruption of hormonal signaling involved in metabolism, alterations in sleep patterns, and alterations in feeding behavior.

Disruption of Molecular Clock Rhythms

Through the use of genetic manipulations core clock genes such as CLOCK and BMAL1 have been altered to study the consequences of mutated molecular clock genes on metabolism. In CLOCK/BMAL1 mutants, it was found that glucose metabolism and insulin signaling have disrupted cycles, reduced insulin secretion and, impaired glucose tolerance (Huang et al., 2011). A conditional CLOCK mutant with no expression in the liver and skeletal muscle had reduced insulin and impaired glucose tolerance as well suggesting peripheral tissues rhythms may play an

important role in energy homeostasis (Kennaway et al., 2007). DLAN was found to cause increased weight gain despite similar food intake suggesting that peripheral tissues internal rhythm are needed for metabolic regulation (Borniger et al., 2014). Interestingly, time restricted sleeping in wildtype mice showed hallmarks of obesity such as increased food intake and leptin resistance however in Per 1/2 double mutants these obesogenic effects were blunted protecting the mice (Husse et al., 2012). Together these findings suggest that core clock genes in both the SCN and peripheral tissues are important in maintaining metabolic rhythms and targeting of particular molecular clock components such as Per 1/2 can protect against obesogenic effects.

Disruption of Hormonal Signaling

Alterations in molecular clock rhythms can dysregulate hormones that rely on this mechanism as a pacemaker. This includes metabolically related hormones such as insulin, leptin, and ghrelin. It has been found that there are alterations of endocrine hormone secretion in clock mutant mice (Fonken & Nelson, 2014). A disrupted 20 hour LD cycle resulted in increased insulin and leptin compared to LD control (Karatsoreos et al., 2011). In BMAL1-knockout mice, insulin secretion loses rhythmicity (Shi et al., 2013). Leptin and Ghrelin rhythms are also affected in BMAL1 mutants (Tsang et al., 2017). Ablation of the SCN causes loss of leptin rhythms showing that circadian rhythms and metabolic signaling are interconnected (Froy, 2010). Previously, it was found that in a LL cycle mice exhibit symptoms of hyperthyroidism in which thyroid stimulating hormone (TSH) was decreased and free thyroid hormone was increased along with abolishment of leptin rhythms (Maroni et al., 2018).

Alterations in Sleep Patterns

Changes to sleep patterns can also contribute to the metabolic problems associated with circadian disruption. In two studies it was found that fragmented sleep can cause increased food intake and glucose intolerance in one study and increased caloric intake and increased leptin in the second (Baud et al., 2012; Wang et al., 2014). Sleep changes seem to be able to occur as a result of obesity as well as it was found that a mouse model of obesity exhibited increase in fragmented sleeping, weakened rhythmicity during REM sleep, and an increase in overall sleep (Laposky et al., 2008).

Alterations in Feeding Behavior

This study is particularly interested in feeding behaviors and how manipulating the temporal point in which they occur could be beneficial against the adverse effects of circadian disruption. Circadian disruption causes altered feeding due to disrupted sleep/wake behaviors that in turn lead to eating at different times. DLAN exposure in mice has been found to cause increased daytime activity in nocturnal mice (Shuboni & Yan, 2010). Circadian disruption leads to higher caloric intake due to consumption during their inactive period (day). This combination of higher caloric intake, plus de-synchrony between overt behavior and internal physiology of an individual, can result in a net positive energy balance and subsequent weight gain (Zimberg, et al 2012). Interestingly, increases in obesity are prevented when mice experiencing circadian misalignment are restricted food during their active phase (nighttime as mice are nocturnal) (Oike, et al 2015). Thus, the temporal point at which food consumption occurs is potentially a significant factor in why circadian disruption leads to problems in overall metabolism.

PYY/NPY System

One potential connection between circadian function and eating behaviors is the Neuropeptide Y (NPY)/Peptide YY (PYY) system. PYY exists in two forms, PYY_{1-36} (untruncated form) and PYY_{3-36} (truncated form), which are both secreted from the hind gut; PYY_{1-36} dominates when a mammal is fasted and PYY_{3-36} dominates after feeding (Ballatyne, 2006; De Silva & Bloom, 2012). After secretion, circulating PYY crosses the blood brain barrier and binds to metabotropic receptors in the arcuate nucleus (ARC) of the hypothalamus of which there are four receptor types Y1, Y2, Y4, and Y5 (Ballatyne, 2006). PYY_{1-36} is nondiscriminatory as it binds to all 4 receptor types in the ARC and promotes food intake along with another agonist NPY. During the time of food intake however, PYY_{3-36} secretion increases in addition to the enzyme dipeptidyl peptidase IV (DPP-IV) cleaving circulating PYY_{1-36} to PYY_{3-36} changing its activity to selectively binding to the inhibitory Y2 causing a decrease in appetite (Figure 1) (Michel et al., 2008; Zac-Varghese, et al

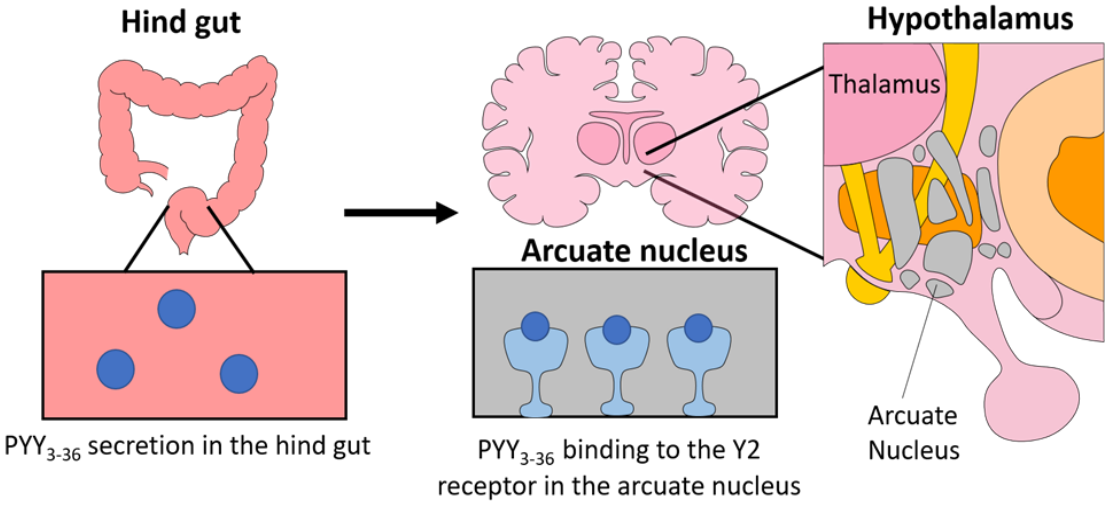

Figure 1. PYY secretion and subsequent binding. Both forms of PYY is secreted from the hindgut and PYY_{1-36} is truncated by DDP-IV which changes it to PYY_{3-36} the form that selectively binds to the Y2 appetite inhibiting receptors in the ARC.

2010). While DPP-IV alters binding of PYY causing decreases in food intake, it inhibits other peptides like glucagon-like peptide-1 (GLP-1) which increases insulin levels to decrease blood sugar levels in response to feeding (Pala et al., 2003).

Previous studies show that inactivation of Y2 receptors in mice led to increased food intake, body mass, and fat deposit (Naveilhan, et al 1999). This implies that, specifically PYY_{3-36} the form specific to the Y2 receptor, seems to be a potent regulator of food consumption and metabolism. Further, when PYY_{3-36} was administered in humans food intake decreased whereas PYY_{1-36} when administered did not have an effect on energy intake and food consumption suggesting that PYY_{3-36} has greater potency in regulating food consumption compared to its full form counterpart (Sloth et al., 2006).

The PYY/NPY system seems to be linked to circadian rhythms as NPY has been found to be a potent cue to reset the circadian clock (Maywood, et al 2002). However, more research is required to understand the relation between PYY and circadian rhythms. Interestingly, the phase advancing characteristics of NPY have been found to be mediated by the Y2 receptor, the same receptor PYY_{3-36} binds (Huhman, et al 1996). Further, nocturnal intraperitoneal injections of PYY_{3-36} caused decreased wakefulness in rats (Akanmu et al., 2006). This implies a potential connection between PYY, food consumption, and circadian rhythm that my research aims to understand.

Experimental Rationale

This study conducted three separate experiments aimed at understanding if either forms of PYY is regulated in a cyclic manner and if PYY_{3-36} injections during the active (i.e., dark) periods could alleviate the negative metabolic symptoms of circadian disruption. Both studies utilized

CD1 mice as this strain has been previously found to have negative metabolic consequences when exposed to circadian disruption. DLAN caused blunting rhythms of the clock gene Per, increased daytime activity and altered light responsiveness; LL caused a decrease in thyroid stimulating hormone (TSH), increased free T4, and abolished day-night variation in leptin indicating that light entrainment is important for physiological rhythms within this strain (Fonken & Nelson 2014; Maroni et al. 2018).

Experiment 1 exposed CD1 mice to 12:12 LD cycle and sacrificed animals at one of the four zeitgeber times ZT 0, ZT 6, ZT 12, and ZT 18. In experiment 2, mice were exposed to LD and injected with vehicle or PYY_{3-36} during one of the four zeitgeber times ZT 0, ZT 6, ZT 12, or ZT 18. Experiment 3 exposed CD1 mice to either a 12:12 LD cycle or LL. Both experiment 2 and experiment 3 groups were paired with either a PYY_{3-36} or vehicle (control) injection during their set injection which varied for experiment 2 but was set at the onset of their active period ZT 12 for experiment 3 (Figure 2). Constant light was used specifically because unlike circadian disruptions such as DLAN and content darkness, constant light results in locomotor arrhythmicity and blunting of body temperature and glucocorticoid rhythms, a metabolically involved hormone (Fonken & Nelson, 2014).

It was hypothesized that there are endogenous rhythms of both forms of PYY as they are involved in regulating food intake which is a rhythmic behavior itself. In addition, it was hypothesized that the decreased levels of PYY_{3-36} during the active period would make the ZT 12 and ZT 18 time points have increased efficacy in inhibiting food intake as endogenous levels are lower during these times. In experiment 3, it was hypothesized that the external light output in LL could lead to downstream signaling that dysregulates peripheral tissue rhythms into

outputting hormones as if the mouse is always in its inactive period. Thus causing PYY_{3-36} injections to be less effective at inhibiting food intake in LL due to the external light cues causing an increase in endogenous PYY_{3-36} levels to rise.

Methods:

Experiment 1 – Endogenous Levels of PYY

Twenty-four male CD1 mice were purchased from Charles River Laboratories at (Shrewsbury, MA) approximately 7 weeks of age and were housed individually. After arrival, all animals were placed in a 12:12 LD cycle at 22˚C under 100 lux lighting with water *ad libitum* and Lab Diet 5001 rodent chow. Mice were maintained in these conditions for 1 month and then separated by time of day in which they would be sacrificed (ZT 0, ZT 6, ZT 12, or ZT 18). There were four groups: (1) ZT0 (n=6), (2) ZT 12 (n=6), (3) ZT 12 (n=6), (4) ZT 18 (n=6). Within each group, mice were fasted 12 hours prior to their scheduled sac time and whole blood was collected and allowed to clot. Serum was obtained by centrifugation at 4˚C for 20 minutes at 2000 g, aliquoted into microcentrifuge tubes, and stored in−80˚C.

Statistical analyses

One-way ANOVA (sac time) was used to uncover differences in endogenous PYY_{1-36}, PYY_{3-36}, and fasting glucose.

Experiment 2 – Physiological effects of PYY

Animals and Injections

This study had the approval of Bridgewater State University's Institutional Animal Care and Use Committee. Forty-eight male CD1 mice were purchased from Charles River Laboratories at (Shrewsbury, MA) approximately 7 weeks of age and were housed individually. After arrival, all animals were placed in a 12:12 LD cycle at 22˚C under 100 lux lighting with water *ad libitum* and Lab Diet 5001 rodent chow. Mice were maintained in these conditions for 1 month and then placed into groups either receiving an injection of 1x PBS (control) or a 1 µg/10g PYY_{3-36} (from a 1% PYY_{3-36} solution (w/v) in 1x PBS) intraperitoneal injection after a 12-hour fast and further separated by time of day (ZT 0, ZT 6, ZT 12, or ZT 18), in a 2 × 4 study setup. Thus, there were eight groups: (1) ZT 0/PBS (n = 6), (2) ZT 0/ PYY_{3-36} (n = 6), (3) ZT 6/PBS (n = 6), (4) ZT 6/ PYY_{3-36} (n = 6), (5) ZT 12/PBS (n = 6), (6) ZT 12/ PYY_{3-36} (n = 6), (7) ZT 18/PBS (n = 6), (8) ZT 18/ PYY_{3-36} (n = 6). In this experimental set up animals were only handled for the following experiments during the time point they were assigned; i.e. ZT 0, ZT 6, ZT 12, or ZT 18.

Food Consumption Protocol

Within each injection group, mice were fasted 12 hours prior to their scheduled injection time. Mice were injected at their set ZT group and then give 20g of food. Six hours after their injection time, food was weighed and food consumption was calculated (20g – post 6 hour food weight) (Figure 2).

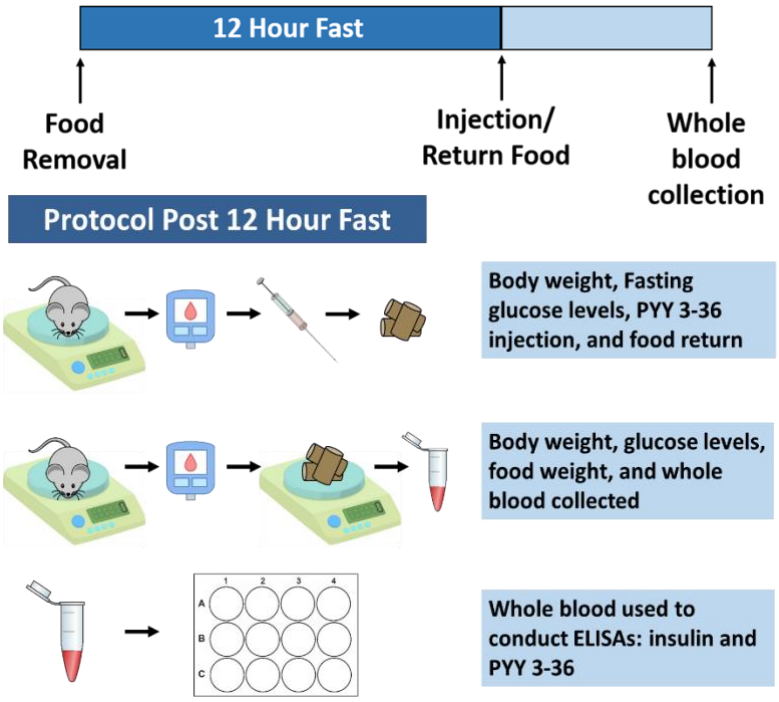

Figure 2. Injection and food consumption protocol. Mice were fasted for 12 hours prior to their ZT injection time, injected at their assigned ZT time, given food, and measured for food consumption 6 hours after and sacrificed.

Fasting Glucose and Hormonal Assays

Fasting glucose levels were measured after a 12-hour fast and were measured using a One-Touch Ultra-2 Glucose Monitor. Six hours after intraperitoneal injection, glucose levels were collected again and whole blood was collected at the aforementioned circadian time points and allowed to clot. Serum was obtained by centrifugation at 4˚C for 20 minutes at 2000 g, aliquoted into microcentrifuge tubes, and stored in−80˚C. Levels of PYY_{1-36} (EIAM-PTT, Ray Biotech, Norcross, GA), PYY_{3-36} (EK-059-04, Phoenix Pharmaceuticals Inc., Burlingame, CA), and Insulin (90080, Crystal Chem, Elk Grove Village, IL) were measured using their respective ELISA kits.

Statistical analyses

Two-way ANOVAs (injection time and injection type) were utilized to uncover differences in PYY_{3-36} post-injection, insulin, food consumption, and weight change.

Experiment 3 – Effects of constant light on PYY food inhibition

Animals and Injections

Forty-eight male CD1 mice were purchased from Charles River Laboratories (Shrewsbury, MA) approximately 4 weeks of age and were housed individually. Circadian activity in cages were monitored using IR-beams (StarrLife Sciences, Oakmont, PA), as previously described (Nascimento et al. 2016b). After arrival, all animals were placed in a 12:12 LD cycle at 22 ºC under 100 lux lighting with water *ad libitum* and Lab Diet 5001 rodent chow. After 1-week entrainment and acclimation, 24 mice were placed into constant light (LL) for 10 or 20 weeks (varying times are due to receiving 20 mice 10 weeks prior to the remaining 28 mice), while the remaining were kept under the 12:12 LD cycle. Mice were then separated into groups either receiving an injection of 1x PBS (control) or a 1 µg/10g PYY_{3-36} (from a 1% PYY_{3-36} solution (w/v) in 1x PBS) intraperitoneal injection after a 12-hour fast and further separated by light cycle, in a 2 × 2 study setup. Thus, there were four groups: (1) LL/PBS (n = 12), (2) LL/ PYY_{3-36} (n = 12), (3) LD/PBS (n = 12), (4) LD/ PYY_{3-36} (n = 12). We chose to conduct this experiment at ZT 12 due to the finding that it lead to the largest difference in food consumption patterns (see results Experiment 2) and to determine whether LL exposure affects PYY_{3-36} efficacy. The food consumption effects of PYY_{3-36} in LL were conducted in the same manner as in Experiment 2.

Statistical analysis

Two-way ANOVAs (light cycle and injection) were utilized to uncover differences in food consumption.

Results:

This study first wanted to understand the circulating levels of both PYY forms at four different time points throughout a 24-hour day. Endogenous PYY_{1-36} levels did not differ throughout the four times they were measured, ZT 0, ZT 6, ZT 12, or ZT 18 (Figure 3A). Endogenous PYY_{3-36} levels at ZT 6 were increased compared to ZT 12 and ZT 18 (Figure 3B, $p < 0.05$).

Fasting glucose levels in mice prior to a vehicle or PYY_{3-36} injection were significantly higher at ZT 0, the onset of their inactive period, compared to ZT 18, halfway through their active period (Figure 4A, $p < 0.05$). Regardless of injection group, there was a greater difference in glucose levels post-injection in the ZT 18 injected group compared to the ZT 6 and ZT 0 injected groups. Further in ZT 0 injected groups, vehicle injected groups had a greater glucose difference

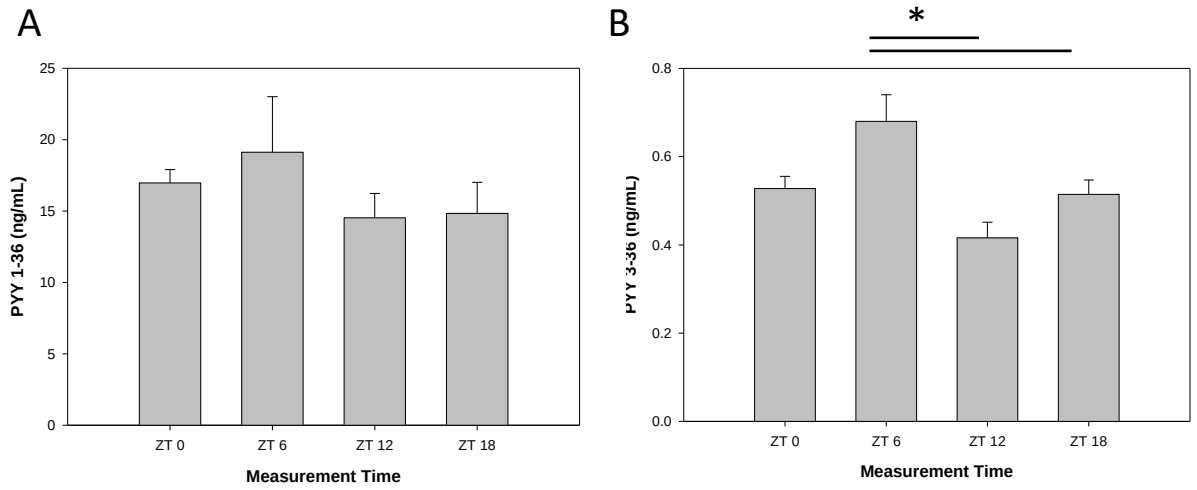

Figure 3. Endogenous PYY_{3-36} levels has day/night variations but PYY_{1-36} does not in CD1 mice. A) PYY_{1-36} levels are not significantly different throughout the day (n = 6). B) PYY_{3-36} levels are increased during their inactive period and are significantly decreased during their active period (n = 6). * indicates $p<0.05$ ZT difference within injection group.

after food consumption whereas PYY_{3-36} injected mice at ZT 0 had nearly no difference (Figure 4B, $p < 0.05$). Insulin levels were decreased in PYY_{3-36} injected mice compared to vehicle injected mice regardless of the time of injection (Figure 4C, $p < 0.05$). Levels of PYY_{3-36} after injections

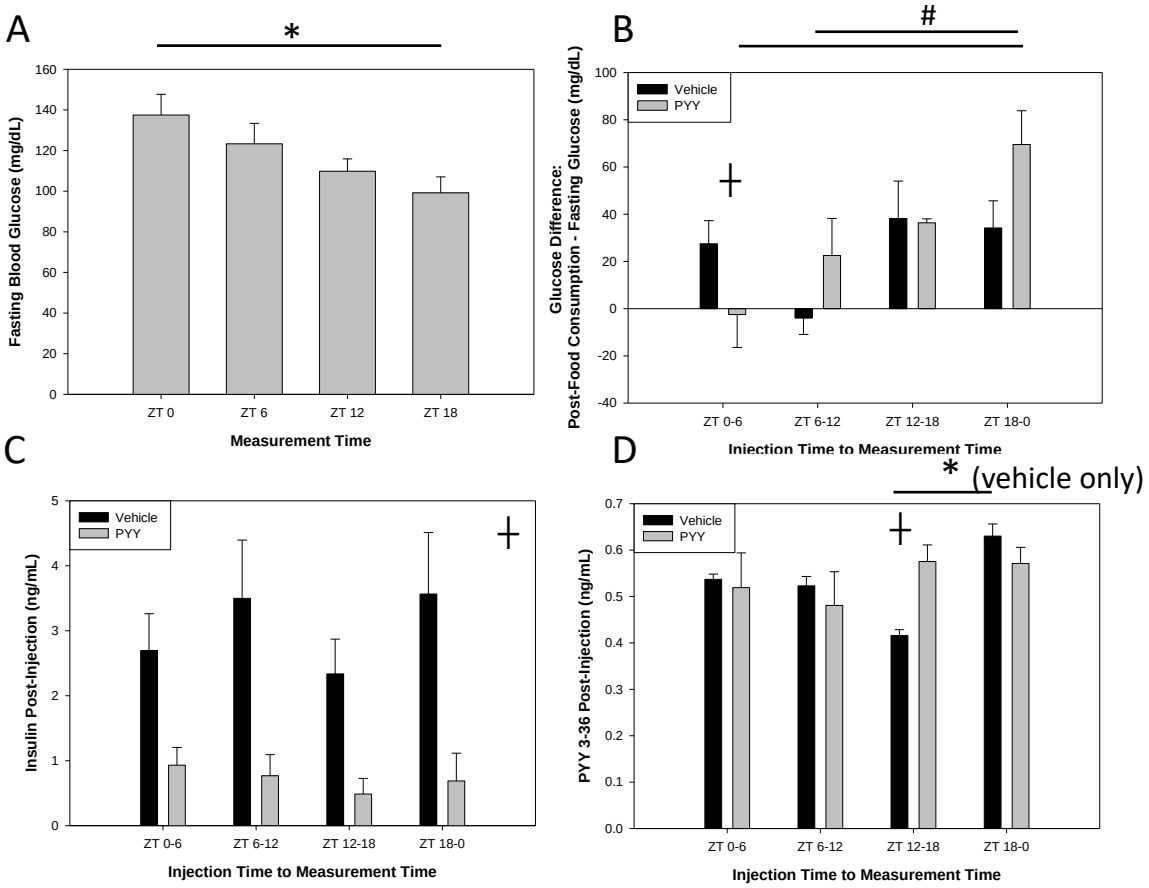

Figure 4. Injections of PYY_{3-36} alters insulin levels and PYY_{3-36} at ZT 12 in CD1 mice. A) Fasting glucose levels in mice with no injections are highest during the onset of their inactive period and they decrease throughout the rest of their cycle (n = 12). B) PYY_{3-36} injected and vehicle mice had higher glucose levels after food consumption during their active period compared to their inactive period (n = 6). C) Insulin levels were significantly decreased in PYY_{3-36} injected mice (n = 6). D) PYY_{3-36} levels were higher during the active period in PYY_{3-36} injected mice compared to vehicle mice (n = 6). * indicates $p<0.05$ ZT difference within injection group. ┼ indicates $p<0.05$ PYY_{3-36} vs. vehicle group. # indicates $p<0.05$ ZT difference regardless of injection group.

were increased in the PYY_{3-36} injected group compared to vehicle only at ZT 12. PYY_{3-36} levels were increased in the vehicle injected animals at ZT 12 compared to ZT 18 (Figure 4D, $p < 0.05$).

PYY_{3-36} injected mice consumed less food than vehicle control mice during all time periods except for the ZT 0 injected group. Additionally, the vehicle injected ZT 12 group consumed more compared to the ZT 0 vehicle injected group (Figure 5A, $p < 0.05$). PYY_{3-36} injected mice had decreased body mass compared to vehicle injected mice during the ZT 18 time point. Vehicle injected mice had increased body mass at ZT 18 compared to the ZT 6 time point (Figure 5B, $p < 0.05$).

PYY_{3-36} injected mice consumed less food than the vehicle injected mice within their respective photoperiods (Figure 6, $p < 0.05$). Further, it was found that food consumption was

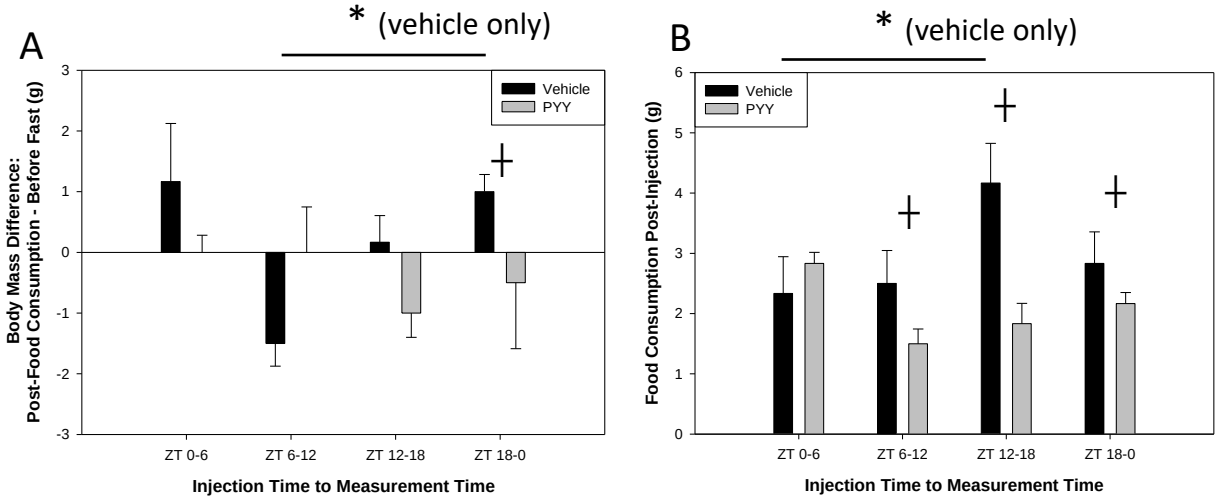

Figure 5. PYY_{3-36} is Efficacious at Reducing Food consumption and body mass at ZT 12 in CD1 mice. A) PYY_{3-36} injected mice consumed less than vehicle during all time periods except the ZT 0 injected group. The vehicle mice consumed more during their active period (n = 6). B) PYY_{3-36} injected mice lost the most body mass during their active period at ZT 18 whereas vehicle injected mice lost the most body mass during their inactive period at ZT 6. PYY_{3-36} injected mice lost significantly more during ZT 18 compared to vehicle (n = 6). * indicates $p<0.05$ ZT difference within injection group. ┼ indicates $p<0.05$ PYY_{3-36} vs. vehicle group.

higher in the LD photoperiod compared to the LL in vehicle injected mice. There was no body mass increase in mice in LL (data not shown).

Discussion:

PYY_{3-36} has an Endogenous Rhythm

Due to the lack of variation found in levels in PYY_{1-36}, it suggests that the untruncated form does not have endogenous rhythmicity (Figure 3A). This is opposite of the truncated PYY_{3-36} as it was found to be increased halfway through the mice inactive period compared to both active period temporal points (Figure 3B). PYY_{1-36} shows high binding affinity to all four Y receptors found within the ARC whereas PYY_{3-36} shows high affinity specifically in the Y2 receptor (Ballantyne, 2006). This finding along with previous studies that have shown that NPY has already been found to bind to and act as a resetting cue through the Y2 receptor suggests that this

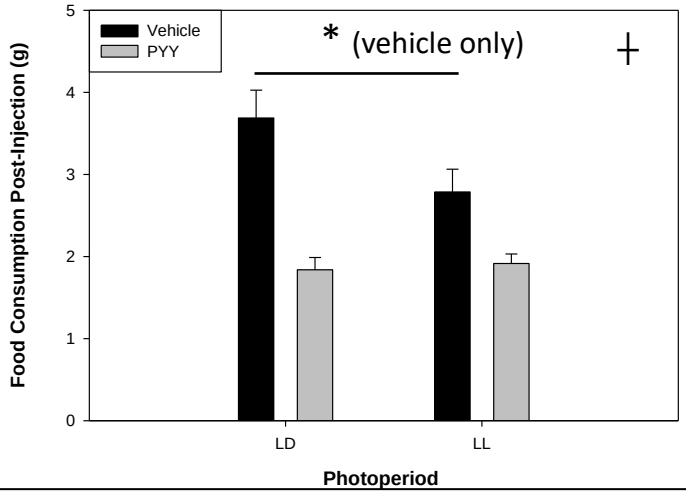

Figure 6. Food consumption is altered by photoperiod in vehicle injected but not PYY_{3-36} injected mice. PYY_{3-36} injected consumed less than vehicle within its respective light of LD or LL. Vehicle LD consumed more than Vehicle LL (n = 12).* indicates $p<0.05$ photoperiod difference within injection group. ┼ indicates $p<0.05$ PYY_{3-36} vs. vehicle group.

receptor is specifically important in PYY/NPY rhythmicity. Further this endogenous rhythm of PYY_{3-36}, the form that has been found to reduce food intake, follows with the logic that feeding should be occurring during active periods. If this rhythm were to be disrupted and feeding occurred during the inactive period this could lead to the weight gain correlated with circadian disruption.

Exogenous PYY_{3-36} Alters Insulin Production, Glucose, and PYY_{3-36} Levels

Fasting glucose levels were increased during the onset of the inactive period ZT 0 compared to halfway through the active period ZT18 (Figure 4A). Insulin is normally secreted in response to increased circulating glucose levels which occurs after food intake and food intake generally occurs during the active phase (Night ZT 12/ZT 18). Despite a lack of food intake, this difference in glucose levels could be attributed to insulin sustaining its day/night variation. This variation results in processing of glucose during the active period of ZT 18 but a lack of processing during the inactive period ZT 0 resulting in the difference in glucose levels.

Decreased insulin levels in PYY_{3-36} injected mice aligns with previous studies in which it was found that PYY_{3-36} injections result in decreased insulin levels (Figure 4C; Böttcher et al., 1989). It has also been found that insulin levels are increased in PYY-knockout mice (Boey et al., 2006). Since PYY_{3-36} reduces food intake, circulating glucose levels will begin to reduce as well having there be less need for circulating insulin to process available glucose. Further, DPP-IV inactivates GLP-1 decreasing insulin secretion while activating appetite suppressing PYY_{3-36} (Pala et al., 2003). Therefore, these results follow with the expected outcomes in the current metabolic point the mice are at.

Despite all temporal points being injected with PYY_{3-36} there was only a significant difference in the PYY_{3-36} injected versus control during the ZT 18 measurement time (Figure 4D). This could be due to the low PYY_{3-36} levels endogenously expressed at ZT 18 (Figure 3B) however, PYY_{3-36} levels were also low during ZT 12 in which no differences in PYY_{3-36} injected versus control were found. The absence of difference in the other three temporal points ZT 0, ZT 6, and ZT 12 is likely because PYY_{3-36} only has a half-life of 8 minutes so the increase in exogenous PYY_{3-36} likely tapers off to basal levels by 6 hours post injection (De Silva & Bloom, 2012).

The Timing of PYY Injections Affects its Efficacy

Surprisingly, the only body mass difference between PYY_{3-36} versus control was at the ZT 18 injection group which was not the injection time with the greatest food consumption difference in PYY_{3-36} injected versus control however, there was still a significant difference in food consumption in the PYY_{3-36} compared to the vehicle control at this time (Figure 5A, 5B). As expected, PYY_{3-36} reduced food intake, but our results also indicate that it is most effective during the beginning of the night, the same time that food restriction is efficacious in alleviating obesity and T2DM symptoms. A study in monkeys found a similar result in which PYY_{3-36} injections were only able to inhibit food intake during the day time (active period) but not the night time (inactive period) (Koegler et al., 2005). This alteration in efficacy could be due to the lower levels during an animal's active period. These lower circulating levels of PYY_{3-36} could mean that injections can have a greater effect compared to when levels are high during the animals' inactive period. PYY_{3-36} may competitively bind to the Y2 receptor causing anorexigenic signaling compared to other Y2 agonists NPY and PYY_{1-36}. Further, vehicle injected animals consumed significantly more during

their active period ZT 18 compared to the onset of their inactive period ZT 0 which follows with the fasting glucose data previously found (Figure 4A).

PYY Efficacy is not Affected by LL

PYY_{3-36} reduced food intake regardless of the cycle suggesting that photic cues did not result in an increased secretion of PYY_{3-36} due to the constant external photic cues (Figure 6). However, this cannot be confirmed until concentration of PYY_{3-36} is quantified. Surprisingly in this study, there were no significant differences in body mass (data not shown). A study previously found that CD1 strain exhibits hyper locomotion when exposed to altered light which could explain the lack of body mass increase in LL mice and additionally LL conditions can cause symptoms of hyper thyroidism which results in weight loss (McGowan & Coogan, 2013; Maroni et al. 2018). As explained previously, LL affects activity and various metabolic hormones. So this raises the question as to how PYY_{3-36} was still able to effectively inhibit food intake when the internal rhythms of both the SCN and peripheral tissues are likely out of phase. One explanation could have to do with the PYY_{3-36} levels being blunted overall. In experiment 2, it was found that PYY_{3-36} injections were able to significantly reduce food intake at ZT 6, ZT 12, and ZT 18 being most effective when the endogenous levels were the lowest. This finding paired with experiment 3 could mean that instead of the external photic cues causing increased PYY_{3-36} secretion or out of phase rhythms it could be causing blunting of the PYY_{3-36} making it effective at all time points. To answer this hypothesis, PYY_{3-36} injection efficacy would need to be tested at all time points like in experiment 2.

The finding that there was increased food consumption in vehicle injected mice during LD compared to LL is of interest. This finding supports previous studies where circadian disruption can alter feeding however it did not increase food intake. The decreased food consumption during ZT 12 in the LL vehicle injected mice could be indicating that there may be increased anorexigenic signals like PYY_{3-36} and leptin however this does not align with the the finding that PYY_{3-36} was effective at reducing food intake at this time. The decreased feeding in the vehicle LL mice could mean that feeding times are shifted to a different ZT as well and due to their unpredictable arrhythmic behavior the vehicle mice happened to be tested at a time point that they were inactive and not eating as much. Monitoring the feeding behaviors of CD1 mice exposed to constant light would be needed to understand if testing them at an inactive time caused the reduced food intake in vehicle LL mice.

Conclusions

Overall, this study provides evidence that PYY_{3-36} has an endogenous rhythm and although it reduces food intake when injected at ZT 6, ZT 12, and ZT 18 it is most efficacious at ZT 12. The effectiveness of PYY_{3-36} in LL could mean that it could potentially be used to inhibit food intake and reduce weight gain in circadian disrupted individuals. Endogenous PYY_{3-36} and insulin levels in experiment 3 are still needed to have a better understanding of the cause behind different food consumption in the vehicle LD versus vehicle LL but not the PYY_{3-36} LD versus PYY_{3-36} LL. This research underscores the importance of an individual's circadian clock and how circadian disruption relates to food consumption. This will also give a better understanding of PYY_{3-36} and the timing of food consumption and their potential to alleviate metabolic problems caused by poor diet and circadian misalignment.

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