Reduction in Expression *of Manduca sexta* Midgut Transport Proteins KAAT1 and CAATCH1 by RNA Interference.

Andrew L. Parmelee and Christopher M. Gillen. Biology Department Kenyon College, Gambier OH

Abstract

Gastrointestinal function is an essential component of maintaining a healthy body weight. Along with factors such as lifestyle choices, improper intestinal absorption can contribute to obesity. Therefore, physiological studies of proteins responsible for the transport of nutrients across intestinal epithelial may offer insights into the cause of obesity. We focused on the genes encoding two amino acid transport proteins, KAAT1 and CAATCH1, in Tobacco Hawkmowth (*Manduca sexta*) larva, an organism which grows 10,000 fold from hatching to pupae. Previously work in our laboratory shows that KAAT1 expression is upregulated in 5th compared to 4th instar animals, while expression of the related gene CAATCH1 is unchanged. To determine the physiological importance of KAAT1, we knocked down its expression by injection a dsRNA matching the KAAT sequence. We hypothesized that reduced KAAT expression would either, slow growth or promote the up-regulation of proteins such as CAATCH1 to compensate for the loss in KAAT1 function. Three rounds of KAAT1 dsRNA injection over a 60 hour period (n=4) elicited a 6-fold reduction of KAAT1 mRNA in the middle mid-gut, and a 10-fold knockdown of CAATCH1 in the posterior mid gut. Hence, different regions of the midgut respond differently to KAAT1 dsRNA and CAATCH1 expression is either directly or indirectly reduced by KAAT1 dsRNA. Injection of KAAT1 dsRNA also inhibited molting from 4th to 5th instar in some larvae. These studies pave the way for further exploration of the role of KAAT1 in larval growth development.

Introduction

Obesity along with its complications kills thousands of Americans each year. Many studies seek understand and remedy the epidemic. Gut nutrient transport proteins may play roles in the development of obesity and other conditions in humans. Transport proteins in the gut carry nutrients from the gut lumen into the bloodstream. The activity of these proteins influences the amount of energy extracted from food. Improper function of amino acid transports within human gut epithelium is known to contribute to blue diaper syndrome and hartnup disorder, but little is known about its link to obesity (Broer, 2008).

Manduca sexta offers a terrific opportunity to study gut transport proteins. The interior of a *M. sexta* larva consists almost completely of midgut. As in humans, localization of transporters is based upon function. We studied the potassium amino acid coupled transporter, KAAT1. Our previous work has determined that KAAT1 expression increases from 4th to 5th instars, possibly contributing to the very rapid growth experienced by the larvae (Yeoh et. al, 2012).

RNA interference allows researchers to knock down mRNA levels of genes. Reduction in mRNA typically leads to reduced protein levels and thus provides an opportunity to probe the physiological importance of certain proteins. Our aim was to decrease mRNA levels of the KAAT1 gene. We hypothesized that *Manduca sexta* would respond to reduced KAAT1 protein levels by eating more, altering rate of midgut transit, growing less, and/or up-regulating related proteins.

Previously, we found that through the administration of KAAT1 dsRNA, mRNA levels of the related protein, cation-anion-activated amino acid co-transporter (CAATCH1), were decreased. Thus we also sought to determine whether KAAT1 dsRNA could be an effective method in eliciting knockdown of this protein. Similar to KAAT1, we hypothesized CAATCH1 loss of function or down regulation would lead to the effects listed above.

Optimization of the dsRNA administration was the immediate goal of our research. We developed a procedure that decreases mRNA levels of both proteins, a result which should help progress the understanding of the function of gut transports. Through our further analysis, research done on the *Manduca sexta* larvae could offer insight into the role of amino acid transport proteins on gut function.

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References

- Broer,S. 2008. Amino Acid Transport Across Mammalian Intestinal and Renal Epithelia. *Physiol Rev.* 88:249-286.
- Reynolds, S. E., Nottingham, S., F. 1985. Effects of temperature on growth and efficiency of food utilization in fifth-instar caterpillars of the tobacco hornworm, *Manduca sexta*. *Journal of Insect Physiology.* 31: 129-134.
- Yeoh, A. J., Davis, K., Vela-Mendoza, A. V., Hartlaub, B. A., Gillen, C. M. 2012. Effect of Body Size on Expression of *Manduca sexta* Midgut Genes. *Journal of Experimental Zoology*. 317:141-151.

Methods

- ***Formation of dsRNA***: We amplified a 435 b.p KAAT1 cDNA sequence from 5A plasmid with T7KAAT1 primer set using Platinum Blue protocol (Life Technologies, Aaron Yeoh). The T7KAAT1 primers add the T7 sequence to the ends of the amplicon. DNA template was extracted by gel purification of PCR product. dsRNA was produced using the MegaScript RNAi kit (Ambion) with T7 primers. We also produced dsRNA from a 500 b.p. control template as a negative control.
- ***Animal care and rearing***: *Manduca sexta* eggs were obtained from Carolina Biological Supply Company and reared until early 4th instar. Organisms were fed each day with Tobacco Hornworm Medium Bulk food (Carolina Biological Supply Company). Sixteen newly molted 4th instar larvae were selected based upon good health.
- ***dsRNA microinjections:*** 20µL injections consisting of 800ng of dsRNA were administered to each larvae three separate times. The three injections took place at 24hr intervals. The dsRNA was administered through an injection into the hemolymph by laterally piercing the organism on the dorsal side in between body segments. We took care not to puncture the midgut in this process. 12 hours following the last injection, standard midgut dissections were performed. Posterior, middle and anterior midgut samples were collected, and frass was collected. Body mass and developmental stage was also tracked over the experimental 60 hrs.
- ***RT-PCR***: Tissue samples were homogenized and RNA extracted using the TRIzol reagent (Life technologies.) Genomic DNA was removed from RNA samples using Turbo-DNAse (Ambion). The RNA was then used as a template for cDNA synthesis which was done with both positive and negative controls. Quantitative PCR was conducted using the cDNA. 18s ribosomal protein primer sets were used as the endogenous control, while KAAT1 and CAATCH1 primer sets were experimentally analyzed.

Results

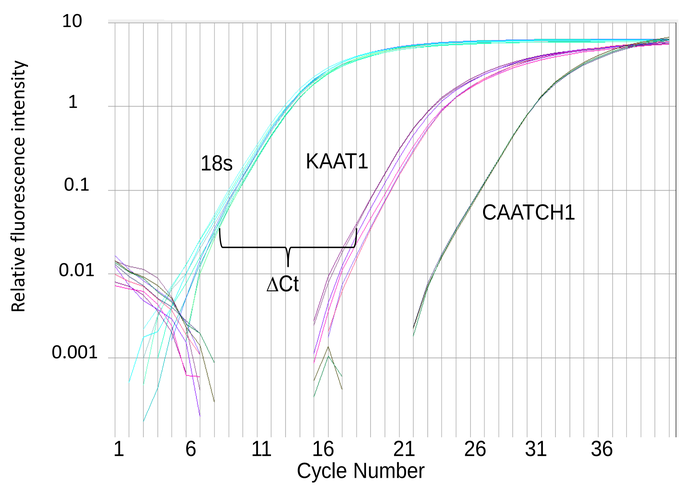

Figure 1. Quantitative-PCR results of the mRNA levels of the Posterior Midgut after KAAT1 dsRNA administration. Description of how ΔCt is calculated.

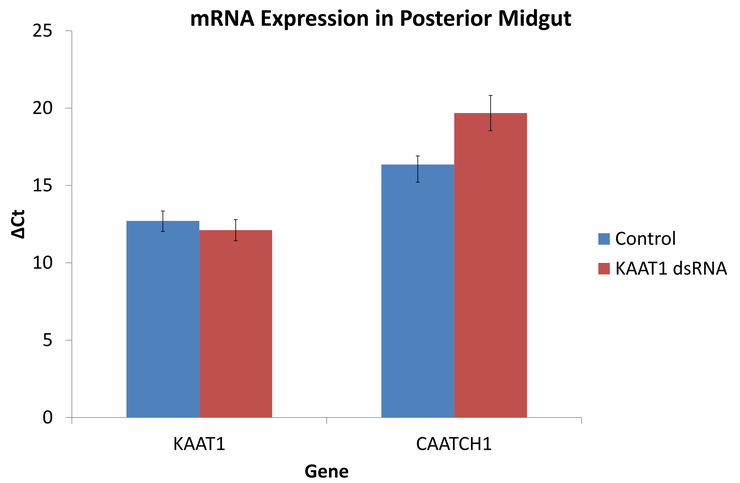

Figure 2. mRNA expression levels after 3 days of treatment with 800ng KAAT1 dsRNA in *M. sexta*. ΔCt levels were determined using the endogenous RNA protein 18s. CAATCH1 showed a 9.8 fold decrease in expression while KAAT1 mRNA expression showed no change over same injection sequence. Higher ΔCT values indicate lower expression. (Two Sample-T for CAATCH1: t= 2.71, df=4, p=.054) (Two Sample- T for KAAT1: T= -0.28, df=4, P-Value= 0.788)

Results

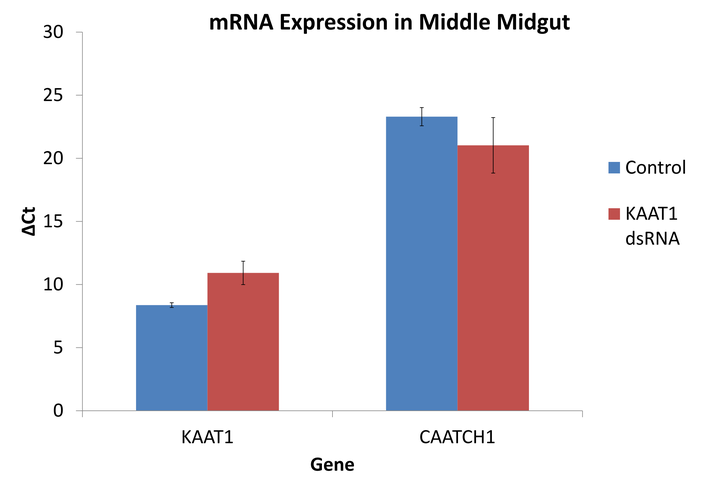

Figure 3. mRNA expression levels after 3 days of treatment with 800ng KAAT1 dsRNA in *M.sexta*. ΔCt levels were determined using the endogenous RNA protein 18s. KAAT1 displayed a 6 fold decrease in expression while CAATCH1 showed no knockdown in expression. (Two sample-T for KAAT1: t=2.95, df=3, p=.06) (Two sample-T for CAATCH1 T= -0.98, df=3, p= 0.397)

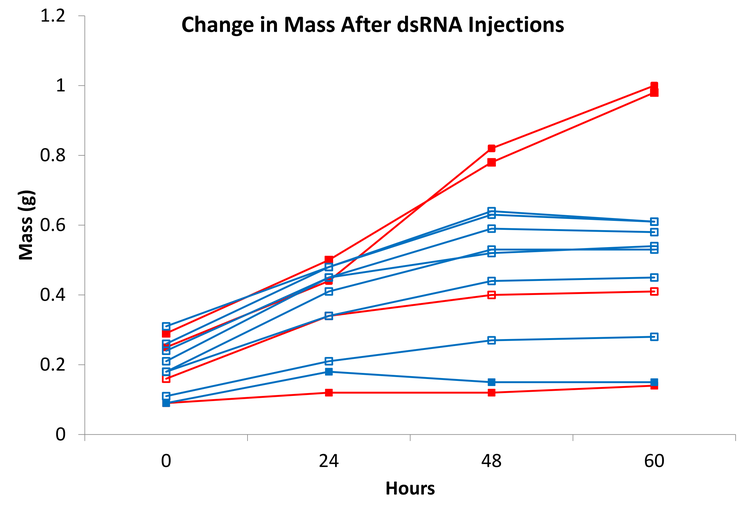

Figure 4. Change in mass over the 60 hour experimental time period in larvae injected with KAAT1 dsRNA (red) and controls (blue). Larvae that were intermolt 4th instar at 60 hours have filled symbols, larvae preparing to molt or in 5th instar have open symbols. The largest organisms were 4th instar larvae in the experimental group. This result is unexpected because 4th instar animals typically molt to 5th instar before reaching 0.8g (Safranek et. al).

Summary

- CAATCH1 mRNA levels decreased by 9.8 fold in the posterior midgut of the larvae. This result correlates with the previous results of Aaron Yeoh '12, who also found slight knockdown of CAATCH1 mRNA when KAAT1 dsRNA was administered.
- KAAT1 mRNA levels decreased by 6 fold in the middle midgut. This finding is new, as our previous studies did not achieve consistent knockdown of KAAT1 gene expression.
- CAATCH1 mRNA levels decreased by 4 fold in the posterior midgut of the larvae after one dsRNA administration 24 hours before dissection (data not shown).
- The increased mass of two experimental 4th instar organisms is interesting. Often late instar molting is caused by malnutrition or environmental temperature (Reynolds and Nottingham, 1985)
- The mechanism by which KAAT1 specific dsRNA hinders the gene expression of CAATCH1 is an area for further study.