Nitric oxide signaling in the larval salivary glands of the tobacco hornworm, *Manduca sexta*

Lyra R.M. Hall and Harry Itagaki
Dept of Biology, Kenyon College, OH

Abstract:

The protein-secreting portion of the salivary glands of the larval *Manduca sexta* (tobacco hornworm) are not innervated; a molecular signaling system is therefore likely to be essential to cellular communication. The nitric oxide (NO)-cGMP signaling pathway is known to be present in the adult *M. sexta* olfactory system. We have previously found that nitric oxide synthase (NOS) is present in the larval salivary gland as well. However, it is unclear whether the NO-cGMP pathway functions to regulate secretion of saliva. In this study, we performed pharmacological experiments to determine what effect a known NOS inhibitor (L-NAME) had on saliva secretion rates. Results suggest that the NO-cGMP pathway is indeed involved in salivary gland regulation, but further investigation is required.

Introduction:

In recent years, nitric oxide (NO) signaling has been gaining attention as an important signal pathway at the cellular and physiological level. Nitric oxide is a small gaseous molecule, synthesized by the enzyme nitric oxide synthase (NOS) from the substrate L-arginine. Due to its small size, NO can easily diffuse through cell membranes, allowing it to reach a large volume of tissue in a short time (Lamas et al 2007). NO acts by activating the enzyme soluble guanylyl cyclase (sGC), which catalyzes the formation of cyclic guanosine monophosphate (cGMP). cGMP then activates a G-protein, which results in a release of Ca^{+2} inside the cell (Looms et al 2002).

The NO-cGMP pathway regulates many physiological processes in mammals including hypertension, inflammation, long-term potentiation in neurons, and smooth muscle relaxation (Steinert et al 2010). Of particular interest to this project, different research groups have demonstrated conclusively that insects express NOS (nitric oxide synthase) in both the olfactory system (Nighorn et al 1998, Gibson and Nighorn 2000, Wasserman and Itagaki 2003) and in the visual system (Bicker 2001).

Similar to the olfactory and the visual systems, the exocrine system requires close regulation of its internal environment. Exocrine function is defined as secretion of fluid-based mixtures of ions, enzymes, and other proteins. The exocrine system in humans consists of the salivary glands, stomach, intestines, liver, and pancreas. In invertebrates, exocrine function is carried out by the salivary glands, silk glands, malpigian tubules, and the gut (Randall 2002). The adult *Manduca* salivary gland is a simple tubular organ, comprised of five regions: from anterior to posterior, protein secreting region, fluid secreting region, thin duct, bulbous duct, and common duct (Burke 2005, Leslie and Robertson 1973). Intriguingly, the fluid-secreting portion of the adult gland is innervated, but the protein-secreting region is not (Robertson 1974). No similar morphological studies have been made on the larval salivary gland, so we must assume it is structured similarly.

The mechanism (or mechanisms) by which *Manduca* regulate their salivary glands is the focus of this project. We tested the hypothesis that NOS is involved in salivary gland function by inhibiting it with L-NAME, an known NOS inhibitor and measuring saliva secretion.

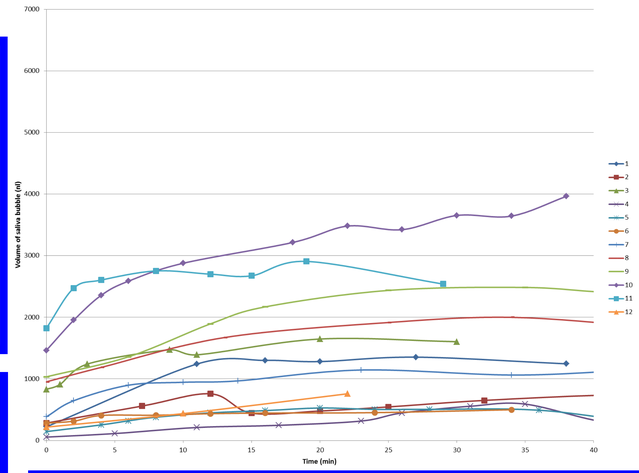

Figure 1. Change in volume of saliva bubble (nl) over time in salivary glands of larval *Manduca sexta*. No agent was added to saline.

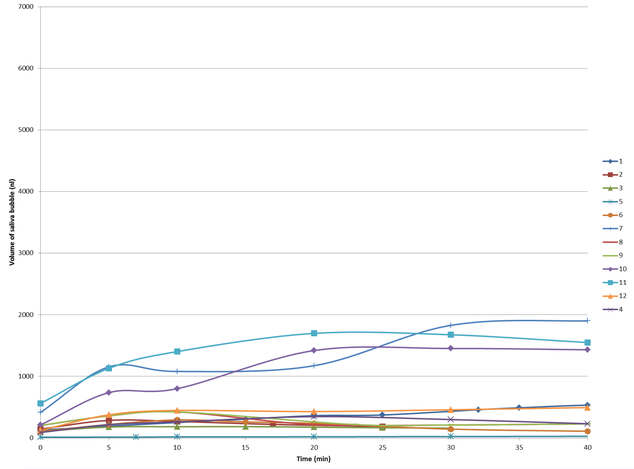

Figure 2. Change in volume of saliva bubble (nl) over time in salivary glands in larval *Manduca sexta*. L-NAME (1 µM) was added to saline dish at 10 min.

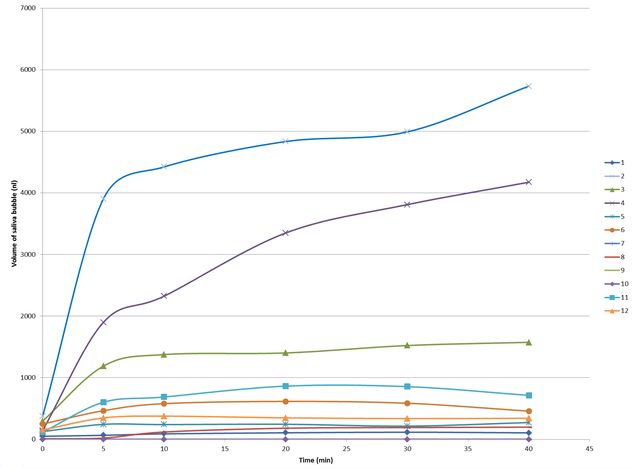

Figure 3. Change in volume of saliva bubble (nl) over time in salivary glands in larval *Manduca sexta*. D-NAME (1 µM) was added to saline dish at 10 min.

Materials and Methods:

Manduca sexta (Carolina Biological Supply) were raised in growth chambers at 27˚ C on a 16L:8D photocycle. Larvae were fed an artificial diet (Carolina Biological Supply). Salivary glands were dissected in physiological saline (pH~6.8, 6.5mM NaCl, 34mM KCl, 16mM $MgCl_2$, 172mM dextrose, 1.25mM $NaHC0_3$, 1.25mM NaH_2PO_4, 14mM $CaCl_2$).

L-NAME (N-nitro L-arginine methylester) and D-NAME (N-nitro D-arginine methylester) (Sigma Co.) were made in a 10 mM stock solution and added at 10 minutes post-dissection for a final dilution of 1 µM. L-NAME is known to inhibit NOS, while D-NAME is its inactive enantiomer and serves as a control.

Rate of saliva secretion was measured volumetrically. The gland was cut in the anterior region and the posterior end was placed in a pool of mineral oil in a Vaseline well. Pictures were taken with a Canon Powershot A620 on auto mode at zoom 6x through a dissecting scope with magnification 1.0. The time of each picture was recorded. Saliva bubbles were treated as ellipsoids. Volume change was calculated with the formula $V = 4/3*\pi*a*b*c$, where a, b, and c are the radii of the ellipsoid in the x, y, and z planes. Due to the impossibility of estimating height from a two-dimensional picture, the z radius was assumed to be equal to the shorter of the x and y radii. Preps where no saliva flow was observed were not included in the analysis.

Statistical analysis was performed using Minitab and data was analyzed and graphed using Microsoft Excel.

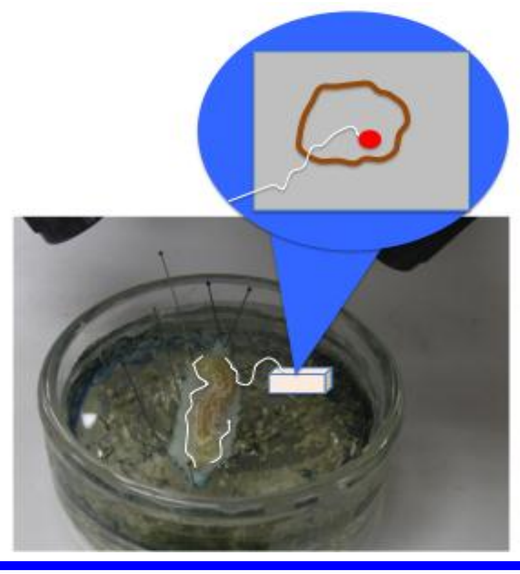

Figure 4. Diagram of dissection dish with *M. sexta* gland partially dissected.

Table 1. Difference between 0-10 min and 20-40 min secretory rates of *M. sexta* salivary glands.

	Difference in rate of flow (nl/min)	Sample size (n)
Control	57.5 ± 13.4[3]	12
D-NAME	56.7 ± 16.5	12
L-NAME	1.84 ± 3.39	12

[3] Values reported as mean ± SEM. P = 0.004 for ANOVA with Tukey's test: Control and D-NAME are grouped together, L-NAME is grouped separately.

Table 2. Ratio of 0-10 min secretory rates to 20-40 min secretory rates of *M. sexta* salivary glands.

	Ratio (before:after) in rate of flow (nl/min)	Sample size (n)
Control	0.206 ± 0.17[3]	12
D-NAME	-0.012 ± 0.117	12
L-NAME	0.067 ± 0.113	12

[3] Values reported as mean ± SEM. P = 0.532 for ANOVA

Summary:

Results are inconclusive. The L-NAME treatment exhibited a smaller difference between the 0-10 min and 20-40 min rates than either the control or D-NAME treatments, which would suggest that NOS inhibition does not decrease the rate of saliva flow. This is the opposite of the trend found previously in the lab. Attempts were made to normalize rates to individual samples by comparing via ratio, but due to variability between individuals, no significant trends were exhibited. Methodology should be refined to cut down on individual variation in future.

References:

Bicker G. (2001). Sources and targets of nitric oxide signaling in insect nervous systems. Cell Tissue Res 303: 137-146.

Burke, KA. (2005). Nitric oxide as a signaling molecule in the salivary gland of larval Manduca sexta. Honors Thesis, Dept of Chemistry, Kenyon College. Unpublished.

Gibson NJ and Nighorn A. (2000). Expression of nitric oxide synthase and soluble guanylyl cyclase in the developing olfactory system of Manduca sexta. J Comp Neurol 422: 191-205.

Just F and Walz B. (1996). The effects of serotonin and dopamine on salivary secretion by isolated cockroach salivary glands. J Exp Biol 199: 407-413.

Lamas S, Lowenstein CJ, and Michel T. (2007). Nitric oxide signaling comes of age: 20 years and thriving. Cardiovascular Research 75: 207–209.

Leslie RA and Robertson HA. (1973). The structure of the salivary gland of the moth (Manduca sexta). Z Zellforsch 146: 553-564.

Looms D, Tritsaris K, Pederson AM, Nauntofte B, and Dissing S. (2002). Nitric oxide signaling in salivary glands. J Oral Pathol Med 31: 569-584.

Nighorn A, Gibson NJ, Rivers DM, Hildebrand JG, and Morton DB. (1998). The nitric-oxide-cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of Manduca sexta. J Neurosci 18: 7244-7255.

Robertson HA. (1974). The innervation of the salivary gland of the moth Manduca sexta. Cell Tiss Res 148: 237-245.

Wasserman SL, Itagaki H. (2003). The olfactory responses of the antenna and maxillary palp of the fleshfly, Neobellieria bullata (Diptera: Sarchophagidae), and their sensitivity to blockage of nitric oxide synthase. Journal of Insect Physiology 49: 271-280.

Acknowledgements:

I would like to profusely thank Dr. Itagaki for his wise and patient mentorship over the course of the past two years. I would also like to thank all my fellow Summer Science Scholars, as well as all the professors in the Chemistry and Biology Departments who kept us company over the summer.

This work was supported by the NSF Interdisciplinary Training for Undergraduates in Biological and Mathematical Sciences Program (UBM Grant #0827208) and the Kenyon College Summer Science Program.