TITLE OF SENIOR HONORS THESIS:
RNAI KNOCKDOWN OF NITRIC OXIDE SYNTHASE IN MANDUCA SEXTA

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RNAi Knockdown of Nitric Oxide Synthase in *Manduca sexta*

Abstract

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Abstract

Nitric Oxide Synthase (NOS) is believed to play a role in the development of the nervous system in the hawkmoth Manduca sexta. The enzyme produces Nitric Oxide (NO), which is a chemical messenger that triggers both intercellular and intracellular interactions. Specifically within Manduca sexta, NO is believed to help regulate the migration of particular cells during development of the antennal lobe (AL). To test this we will be using RNA interference (RNAi) to knockdown NOS mRNA. Phenotypic changes in axon sorting will be observed by labeling cells within the AL and using confocal immunocytochemistry to image them. Knockdown of mRNA will be measured using Real-Time PCR and Western Blots will be used to determine levels of the NOS protein. Thus far, we have generated three double stranded RNA constructs of NOS, which we hope will knockdown the levels of NOS expression. These constructs have been injected into the developing pupae to look for axon sorting changes during development. Future directions include injecting the NOS constructs within the adult moths and observing olfaction changes.

Introduction

Insect Model

The sensory system used for sensing smells is known as the olfactory system. This system is quite complex and allows for mammals to distinguish between thousands of smells that trigger various emotions. To understand the neural mechanisms behind olfaction it is easier to study insects, because they have simpler nervous systems. For instance, the insect’s olfactory system has far less receptor cells and neurons than the human system. Despite the difference in numbers of receptors, the insect system and
human system share many similar patterns in how they develop. Because of this we choose to study the insect system in an effort to learn more about how the human olfaction system is regulated (Hildebrand and Shepherd, 1997).

**Mechanism of Olfactory System**

In order for an organism to detect odors a series of events takes place as the odor is recognized and processed within the brain. Initially, an odor molecule carrying information is detected by hair like projections called cilia in the olfactory epithelium. In humans this cilia is located in the upper epithelium of the nose, and in insects it is found in the antennae (major olfactory organ of insect). Amongst the cilia are olfactory receptor cells, each of which contains a single type of odorant receptor. Roughly 1,000 different genes are responsible for encoding the different types of olfactory receptor cells within humans (Axel, 1995). These receptors are limited to what types of odorant substances they can detect. The receptor itself is a G protein-coupled receptor and when activated stimulates the formation of cyclic AMP (cAMP). This secondary messenger then activates ion channels triggering an action potential which sends signals to the brain through nerve axons.

In humans, the axons relay the signals that are sensed to glomueruli within the olfactory bulb (OB), the primary olfactory area of the brain. In insects the glomueruli are contained within the antennal lobe (AL). The AL is a structure homologous to the mammalian olfactory bulb and is the primary olfactory center of the animal. The AL receives odorant information coming from the antennae in the moth and processes the odorant information within confined regions known as glomeruli. In the glomeruli, the signals are sorted out and sent to higher regions of the brain (Axel, 1995).
Anatomy of Olfactory Receptors and Structure of Antennal Lobe

In vertebrates the olfactory epithelium contains neuronal stem cells, supporting cells, and olfactory receptors. The supporting cells are dispersed throughout the epithelium and contain microvilli and secretory granules. The neuronal stem cells are able to generate olfactory neurons as needed for an organism throughout its life (Bear et al., 1996).

In contrast the olfactory epithelium of the insect is located within the antennae. The antennae is composed of three segments; scape, pedicel, and flagellum. At the distal segment of the antenna is a flagellum which is subdivided into annuli that contain the olfactory sensilla of the antennae. The two other segments are located at the basal end and contain muscles that allow for movement of the antennae (Kloppenburg et al. 1996).

Neurotransmitters, Nitric Oxide

Our brain cells communicate with each other using a variety of molecules. NO is one such molecule. It is different from the classical neurotransmitters in several aspects. For example, NO does not have a receptor and exerts its effects through other routes. It is a small gaseous molecule allowing it to diffuse across the cell membrane and directly trigger signal events and chemically modify proteins inside the cell.

Within the developing vertebrate olfactory system NO is believed to play an active role in the migration and synchronization of olfactory receptor neuron (ORN) precursor cells. Beyond development NO is also active in the maintenance and regeneration of ORN axons (Roskams et al., 1994).

Nitric Oxide Synthase
NO is synthesized from the enzyme nitric oxide synthase (NOS). The enzyme uses arginine and oxygen to produce citrulline and NO. There are three isoforms of NOS found in mammals; iNOS, eNOS, and nNOS. The iNOS isoform functions primarily in the immune system, while the eNOS isoform is predominant in endothelial cells and helps regulate blood pressure. The last isoform, nNOS, stands for neuronal NOS and is expressed primarily in the brain. Its activity is regulated by increases in calcium concentrations which triggers calcium-calmodulin activity to activate the enzyme. This system of regulation is similar to what is found in the NOS enzyme found in insects. Because of this, we are able to study the functions of NOS within insects in comparison to how nNOS may function in the mammalian system.

Levels of NOS expression during the 18 stages of pupal development have been recorded. Analysis of total RNA from the developing brain of *M. sexta* indicated the expression of NOS during development. Peak NOS expression occurred close to stage 5 of development and decreased until it was unexpressed at around stage 12 (Gibson et al., 2000).

Localization of the NOS enzyme has been identified within the AL of developing pupae. NOS antipeptide antiserum (uNOS antibody) used for western blots for stage 5 developing brains expressed NOS protein sizing in 138 kD. Furthermore, nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) hisotchemistry exhibited enzymatic activity within antennal lobe cell bodies (Gibson et al., 2000). Further studies were performed to correlate the enzymatic activity were that of the NOS enzyme.

The best characterized target for NO has been identified as soluble guanylyl cyclase (sGC). Activity of sGC is believed to correlate with the activity of NOS in
nearby locations. The receptor’s activity leads to increase activity of cyclic guanosine monophosphate (cGMP). Recordings of cGMP immunocytochemistry were used as a marker for detection of sGC within the antennal lobe (Collman, 2004). This further supports the activity of NOS within the antennal lobe and subsequent production of NO.

Within the adults moths, NOS and sGC has been further localized within the antennal lobe. Specifically, NOS has been localized within presynaptic ORN axons in all glomeruli and also along the antennal nerve. sGC alpha subunit antiserum used for western blot analysis indicated sGC presence within the postsynaptic neural processes (Collman, 2004).

Within the adult moth, the NOS enzyme has been identified in intrinsic AL neurons where its activity is regulated in part by NO (Gibson et al., 2000). Because of this it is believed that NOS is also present within the antennal lobe and further plays a role not only in development but in the behavior of adults.

**Pharmacological Inhibition of NO**

Inhibition of NO during development using pharmacological drugs has shown disruptions in the normal cellular network. Specifically, patterns for glial cell migration and serotonin-immunoreactive neurons are seen to be altered. The drug N-nitro-L-arginine methyl ester (L-NAME) is a NOS inhibitor that was used to treat animals early in development. The drug acts to bind the NOS active site so that arginine can not bind, therefore inhibiting the synthesis of NO and citrulline (Gibson, 2001). Specifically, the drug was administered to animals that were in mid 2 to early 3 stages of development.

Images of optical sections of the antennal lobe were taken at stage 7 to observe any effects during development. Both the control and experimental animals showed
protoglomeruli formation. However, the experimental animals under treatment of L-NAME showed glial cells that failed to migrate preventing the protoglomeruli to be positioned in their natural location as compared to the control animals. Furthermore, untreated animals show arborization of the serotonin (5-HT) neuron within a confined region of the glomeruli. Experimental animals show arborization of the neuron throughout the entire glomeruli (Gibson, 2001).

Cues for Axon Guidance and Convergence

The olfactory receptor cells within the epithelium are able to sense a particular odor and transmit information down axons to a confined region in the antennal lobe. Axons of newly born ORNs that are specific to a particular odor precisely converge into these confined regions known as the glomeruli. Here the axons make connections with second order neurons in the glomeruli that receive input and send it for processing in higher regions of the brain (Cho et al., 2009). The mechanism by which this order of events takes place is currently being studied.

As neurons are born their axons extend to particular targets which are influenced by intercellular signals that act as attractive or repulsive signals. An example of a repulsive signaling is seen with Eph family of receptor tyrosine kinases. Ephrins play multiple roles, one of these roles is to act as repellants during development to allow for the axon guidance and topographic mapping (O’Leary et al., 1999). As the cells repel one another a fine-level of organization is evident allowing the brain to develop. The repellants prevent scattered cell migration and intermingling between neurons.

In mammals at the surface of growing axons, levels of cAMP/PKA signaling are believed to alter transcription and expression of axon guidance molecules. Experiments
involving the manipulation of cAMP levels within mice indicated reduced expression of genes involved in expression of axon guidance molecules (Cho et al., 2008).

As the populations of axons from various ORNs accumulate within the OB they converge into specific gloernuli. One hypothesis of this sorting is that axons expressing the same receptor show homotypic interactions that signal them to converge. Another hypothesis mentions that axons containing the same families of adhesion molecules are able to fasciculate together based on strength of interactions between the adhesion molecules (Cho et al., 2008).

It is evident that there are multiple mechanisms behind axon guidance during the development of the nervous system. NO is also believed to play a role in the positioning and migration of certain cells that allow for the brain to develop.

Materials and Methods

RNA Interference

Over the past decade, RNA interference (RNAi), has become a widely used tool in studying the activity of genes within organisms. By taking advantage of an organism’s natural defense mechanism, dsRNA is used to knockdown gene expression of targeted RNA. The cellular mechanism involves the introduction of exogenous dsRNA that is complementary to a target gene within an organism. This activates the ribonuclease protein Dicer which cleaves the exogenous dsRNA to 20-25 bp fragments. Next, the RNA-induced silencing complex (RISC) binds to one strand of the short double stranded fragment. The complementary sequence binds to the indigenous mRNA. After this
integration, the RISC complex cleaves the mRNA preventing it from being translated into protein within the organism (Leung, 1996).

In this project, three double stranded fragments of cDNA were constructed using the E-RNAi web application to design primers complementary to *M. sexta* NOS gene. These constructs ranged from 300-500 bp. Along with this, double stranded Green Fluorescent Protein (GFP) cDNA construct was also used as a negative control to measure knockdown within the animals. The primer sequences used for the NOS and GFP fragments used to construct the litmus vectors were as follows:

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Fragment Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS 64 S</td>
<td>5’-CAC CGA AGC GAC ATT ACA GT-3’</td>
<td>474 AS 5’-GGA AAG TCT GAG CGT CTT GC-3’</td>
<td>410 bp</td>
</tr>
<tr>
<td>NOS 2272 S</td>
<td>5’-AGA CCT TCT TCT TGG ACG AA-3’</td>
<td>2589 AS 5’-GGC TCA GTA CAG CGT CAA CA-3’</td>
<td>317 bp</td>
</tr>
<tr>
<td>NOS 2040 S</td>
<td>5’-GCT GGA ACA TCA ACA GCA GA-3’</td>
<td>2589 AS 5’-GGC TCA GTA CAG CGT CAA CA-3’</td>
<td>549 bp</td>
</tr>
<tr>
<td>GFP 542 S</td>
<td>5’-TAT ATC ATG GCC GAC AAG CA-3’</td>
<td>956 AS 5’-TGG TTA CTA GCG TCC CAA GC-3’</td>
<td>414 bp</td>
</tr>
</tbody>
</table>

The PCR fragments were initially amplified from a linear strand of complete NOS and GFP sequence. They were then ligated in PSTBlue-1 vector which allows for a high efficiency for ligation of the fragments into a vector. After this the cDNA was cloned on E. Coli plates and stored for later use. Before going into the actual RNAi experiments the fragments were cut out of the PSTBlue-1 vector and ligated into a Litmus vector. The Litmus vector contains the T7 promoter site that is used for transcription of the DNA. The resultant dsRNA is than used for injection into the pupae.

**Injections**

Pupae at stage 2 were injected with 1.0 ug or 2.0 ug of NOS and GFP dsRNA. Three bugs for each set were placed in a CO2 chamber for 15 minutes and then injected...
with a needle into the head capsule. The injection site was then immediately sealed with hot wax. The bugs were then left alone and allowed to grow.

At stage 5 the pupae were dissected to check for RNA knockdown. This was done by cutting out the antennae of each bug and the brain was simultaneously cut out and stored separate. Before dissection the bugs were placed on ice for 20 minutes. And then a scalpel was used to cut out the exoskeleton which protected the developing antennae and brain. Phosphate buffered saline was then used with a pipet to force out the antennae which was immediately placed in liquid nitrogen. The samples were then placed in a -80°C freezer.

RNA extraction

The mRNA from the antennae was extracted using a Trizol reaction following manufacturer’s instructions. The RNA was then used for qPCR to determine levels of NOS and tubulin.

Real Time PCR

The technique of polymerase chain reaction allows for the exponential amplification of short DNA sequences that are embedded within a longer double stranded molecule of DNA. PCR consists of a denaturation, annealing, and elongation step. Primers that are roughly 20 nucleotides in length and complimentary to the ends of the region being amplified are required for PCR.

Denaturation results in the melting of the DNA template and yields single strands of DNA. Next in the annealing step, the primers bind to the complimentary sequence. In order for elongation to occur DNA polymerase binds to both the primer sequence and template and synthesizes new DNA from the 5’ to 3’ direction.
Real time PCR allows for a quantification measurement to determine if the process of RNAi worked properly, and levels of NOS mRNA are indeed lower. To do this we extract total RNA from the antennal nerve developing pupae at stage 5 of bugs injected with NOS drRNA and control GFP dsRNA. This is when NOS mRNA was seen to be at its peak during the developmental studies conducted by Dr. Gibson and Dr. Nighorn. The RNA is reverse transcribed to DNA and used in Real time-PCR to determine the levels of RNA through fluorescent targeting.

Both the control and experimental bugs are fluorescently tagged using a SYBR green probe and at certain fluorescence the cycle number is recorded. In theory the experimental bugs with NOS levels that are lowered should reach the target fluorescence at a later cycle number than the control.

In order to determine if the knockdown was indeed because of the injection of NOS dsRNA, a standard or reference gene is also measured between the experimental and control. The reference gene should theoretically have the same copy number in both the experimental and control cells. The gene should also be expressed in all cells. The reference gene used for this project is tubulin. The primers used to detect NOS and tubulin levels were as follows:

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Fragment Length</th>
<th>Dissociation Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>*NOS PC30</td>
<td>1128 S 5’-CAC AGC AAC TGC CTT CAA CGG-3’</td>
<td>1412 AS 5’-GCC GGA CAG CCA CCC-3’</td>
<td>284 bp</td>
<td>62°C</td>
</tr>
<tr>
<td>Tubulin</td>
<td>1200 S 5’-TTG TCC AAC ACC ACC GCC A-3’</td>
<td>1455 AS 5’-GGT GCG AGT GCG CCA TAA A-3’</td>
<td>255 bp</td>
<td>62°C</td>
</tr>
</tbody>
</table>

A mathematical model known as the Pfaffl equation is then used to determine if knockdown has occurred and to what extent. The Pfaffl equation takes into account the efficiency of the tubulin and NOS primers used for Real time PCR. The standard values
of the primers were determined using a real time PCR. A serial dilution of linear NOS PC-30 DNA was used as a template and the appropriate primers used for measuring knockdown were used for real-time PCR with the linear NOS. The amplification of the DNA was measured through fluorescence and a linear relationship was seen as the concentrations decreased. The slope of this graph was used to determine efficiency of the primers that could later be used in the Pfaffl equation.

**Results**

Analysis of real-time PCR data indicated which injection of NOS fragments resulted in knockdown. The following amplification plots of Delta Rn (normalized reported signal minus baseline signal) vs cycle number are shown to display the normalized amplification from real-time runs within each cell selected within the plate grid.

The steep rise in Delta Rn is characterized as the geometric phase and indicates a high and constant amplification efficiency. This occurs between the first detectable rise in fluorescence and before the beginning of the leveling of the curve. This region of leveling is known as the linear phase where the slope and amplification decreases steadily. Towards the end of the curve the PCR stops and the Delta Rn remains constant.
Figure 1A: Amplification plot from trial I of animals injected with a 2.0 ug 50:50 mixture of 317 bp and 410 bp cocktail NOS fragments. Plot indicates the Ct values which is the point at which the fluorescence reaches a constant threshold value. Tubulin primers contain Ct values between cycle numbers 15-18. NOS primers contain Ct values between cycle numbers 24-28.

Figure 1B: Amplification plot from trial II of animals injected with a 2.0 ug 50:50 mixture of 317 bp and 410 bp cocktail NOS fragments. Plot indicates the Ct values which is the point at which the fluorescence reaches a constant threshold value. Tubulin
primers contain Ct values between cycle numbers 15-17. NOS primers contain Ct values between cycle numbers 24-28.

Figure 1C: Amplification plot from trial of animals injected with 2.0 ug of 317 bp NOS fragments. Plot indicates the Ct values which is the point at which the fluorescence reaches a constant threshold value. Tubulin primers contain Ct values between cycle numbers 14-17. NOS primers contain Ct values between cycle numbers 23-28.

The dissociation graphs shown below display the change in fluorescence as a function of temperatures from a probe (SYBR green) interacting with double stranded DNA (dsDNA). This serves as a quality-control to identify primer-dimers and other products different form the specific amplification products in a quantitative PCR reaction measured with SYBR Green dye.
Figure 2A: Dissociation graph from trial I of animals injected with a 2.0 ug 50:50 mixture of 317 bp and 410 bp cocktail NOS fragments. Melting points of each distinct primer, tubulin primers 1200 S-1455 AS (between 85°C -90°C) and NOS primers 1128 S-1412 AS (between 80°C -85°C) show changes in fluorescence in the relative same region.

Figure 2B: Dissociation graph from trial I of animals injected with a 2.0 ug 50:50 mixture of 317 bp and 410 bp cocktail NOS fragments. Melting points of each distinct primer,
tubulin primers 1200 S- 1455 AS (between 85°C -90°C) and NOS primers 1128 S- 1412 AS (between 80°C -85°C) show changes in fluorescence in the relative same region.

Figure 2C: Dissociation graph from trial I of animals injected with 2.0 ug of 317 bp NOS fragments. Melting points of each distinct primer, tubulin primers 1200 S- 1455 AS (between 85°C-90°C) and NOS primers 1128 S- 1412 AS (between 80°C -85°C) show changes in fluorescence in the relative same region.

Standard values for the efficiency of the primers were determined to be 2.0121 for NOS, and 1.916 for tubulin at 62°C Tm. These were used in the Pfaffl equation to measure knockdown in the various trials. The serial dilution of the NOS primers used for analysis of primer efficiency were as follows:
Figure 3A: The “X” marked cells of the plate grid were not included in determining primer efficiency.

Figure 3B: NOS dissociation curve using NOS primers 1128 S- 1412 AS indicating that primer-dimers and other products different from the specific amplification products in a quantitative PCR reaction were not present.
Figure 3D: Standard Curve analysis of Ct values vs concentration of NOS 1128 S- 1412 AS primers. Slope of line was used for determining primer efficiency.
Figure 4A: Data from serial dilutions of tubulin qPCR using tubulin primers 1200 S-1455 AS. Plot indicates the Ct values which is the point at which the fluorescence reaches a constant threshold value. Cycle numbers are spaced out by roughly 3 cycles as the concentration of the NOS PC-30 was diluted every 10 fold.
Figure 4B: Standard Curve analysis of Ct values vs concentration of tubulin 1200 S- 1455 AS primers. Slope of line was used for determining primer efficiency.

Using the primer efficiency and Ct values of the injection trials the following values of knockdown were measured:
Figure 5A: Graphical representation of trial 1 of NOS mRNA knockdown with a 2.0 ug mixture of 317 bp and 410 bp cocktail (50:50).

Figure 5B: Graphical representation of trial 2 of NOS mRNA knockdown with a 2.0 ug mixture of 317 bp and 410 bp cocktail (50:50).
Figure 5C: Graphical representation of NOS mRNA knockdown with 2.0 ug of 317 bp NOS fragment.

**Conclusion**

Figures 5A and 5B indicate that knockdown occurred relatively consistently and efficiently when using a cocktail of fragments in a 2.0 ug mixture of 317 and 410 bp NOS fragments (1.0 ug of each sample was used in the cocktail). As seen in figure 5C the 317 bp fragment also resulted in knockdown, but was not consistent nor as efficient as the cocktail. The 410 bp fragment alone did not produce any significant knockdown. The 549 bp was the least efficient in knockdown. Data for the 410 bp and 549 bp fragment injected alone resulted in insignificant trials are not shown.
Literature Cited


