CHEMESTHESIS IN THE EARTHWORM, *LUMBRICUS TERRESTRIS*: THE SEARCH FOR TRP CHANNELS

BY

ALBERT H. KIM

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Approved By:

Wayne L. Silver, Ph.D., Advisor

Pat Lord, Ph.D., Chair

Erik Johnson, Ph.D.
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AITC</td>
<td>Allyl isothiocyanate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<tr>
<td>CIN</td>
<td>Cinnamaldehyde</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
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<td>SP</td>
<td>Substance P</td>
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<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPA</td>
<td>TRP Ankyrin family</td>
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<tr>
<td>TRPA1</td>
<td>TRP channel, subfamily A, member 1</td>
</tr>
<tr>
<td>TRPC</td>
<td>TRP Canonical family</td>
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<tr>
<td>TRPM</td>
<td>TRP Melastatin family</td>
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<td>TRPM8</td>
<td>TRP channel, subfamily M, member 8</td>
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<td>TRPML</td>
<td>TRP Mucolipin family</td>
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<td>TRPV</td>
<td>TRP Vanilloid family</td>
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<td>TRP channel, subfamily V, member 1</td>
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ABSTRACT

Chemesthesis is the chemical stimulation of receptors on somatosensory neurons usually by noxious chemicals leading to irritation. Transient receptor potential channels play a major role in detecting chemesthetic stimuli and are found in a wide variety of organisms, from yeast to humans. Currently, there are no reports of TRP channels in earthworms. Earthworms, including *Lumbricus terrestris*, aerate and enrich soil providing a suitable habitat for plants. Hence, chemicals in the soil which repel earthworms could have deleterious effects on plant growth. Allyl isothiocyanate, a prototypical TRPA1 agonist, has been used as an expellant for sampling earthworms, suggesting that earthworms possess TRPA1 channels. We began the search for TRPA1-like channels in earthworms using electrophysiological, immunohistochemical, behavioral and cell dissociation/calium imaging techniques. We obtained responses from segmental nerves to tactile stimulus, 50mM AITC and pH 3.8, 4.0 and 4.2. Some cells in the epidermis displayed positive immunoreactivity for *Drosophila* and human TRPA1 homologs. *L. terrestris* detected and avoided ATIC and cinnamaldehyde (another TRPA1 agonist) but not capsaicin (a TRPV1 agonist) in air and soil T-maze behavioral assays. We were able to dissociate cells from *L. terrestris* epithelium and ventral nerve cord, but could not use them in calcium-imaging experiments.
INTRODUCTION

Chemesthesis

Chemesthesis is the detection of irritants from the environment by receptors found in the skin or mucus membranes. Many organisms possess the ability to detect noxious chemicals from the external environment. Investigation into chemesthesis (it was originally called the common chemical sense) began as early as 1912 when G.H. Parker observed how fish responded to acid and suggested that chemical irritants are detected by free nerve endings in epithelia and mucous membranes (Parker, 1912). We now know that these nerve fibers are not an independent sensory system as Parker suggested, but are part of the general somatosensory system and a subset of temperature and pain detecting fibers (Saunders and Silver, 2016).

Transient Receptor Potential Channel

While a variety of receptor proteins may respond to chemical irritants, Transient Receptor Potential (TRP) channels have been the focus of most of the recent studies of chemesthesis. TRP channels were first discovered in Drosophila but they can be found in all metazoans (Pedersen et al., 2005). TRP channels have now been found in vertebrates, insects, roundworms, ciliates, green algae, and yeast (Chang et al., 2010). TRP channels are six transmembrane ion channels, some of which detect chemesthetic chemicals, pH, temperature, light, and mechanical stimuli (Zheng, 2013). TRP channels are permeable to Ca^{2+} and Mg^{2+} ions and modulate the intracellular concentration of Ca^{2+} by altering the
driving force of Ca$^{2+}$ transmembrane movement and triggering the release of Ca$^{2+}$ from intracellular organelles. There are 28 distinct mammalian TRP channels (Cvetkov, 2011) categorized into seven subfamilies, including the TRP Ankyrin family (TRPA), the TRP Vanilloid family (TRPV), the TRP Melastatin family (TRPM), the TRP Canonical family (TRPC), the TRP Polycystin family (TRPP), the TRP Mucolipin family (TRPML), and the TRP NOMPC family (TRPN) (Pedersen et al., 2005).

**TRPA1**

TRPA1 is a polymodal channel, in that it can be activated by multiple types of stimuli. Chemical stimuli that activate TRPA1 channels include allyl isothiocyanate (active ingredient in wasabi and mustard) (AITC), cinnamaldehyde (active ingredient of cinnamon) (CIN), mechanical stimuli, allicin (active ingredient of garlic), cold, tetrahydrocannabinol (psychoactive ingredient of marijuana), and bradykinin (Pedersen, 2005). In vertebrates, TRPA1 channels are involved in detecting pain and inflammation. This is partly due to TRPA1 being expressed in nociceptor sensory neurons (Cvetkov, 2011).

**Why study chemesthesis in earthworms?**

Different species of earthworms impact soil in a variety of ways, from beneficiary to invasive. These effects on soil by earthworms are magnified and make a substantial contribution to ecosystems due to their significant biomass (Nuzzo, 2009). Earthworms in general have long been known to be beneficial to the soil. Charles Darwin extolling their
impact, wrote a book on earthworms in 1881 called “The Formation of Vegetable Mould through the Action of Worms”.

Water infiltration is a well-known soil improvement that earthworms provide. Vertical burrows are an important means for water to infiltrate soil. Subsoil dwellers (one of three types of earthworm) burrow vertically up to 5 to 6 feet deep in the ground. One of the major members of this group is L. terrestris (Duiker, 2008).

Through burrowing and “turning over” the soil, earthworms provide aeration. This is vital to the development and cultivation of plants and provides necessary oxygen to plant roots (Duiker, 2008). In addition, agricultural production of crops such as rice and maize increase significantly with earthworms present (Derouard et al, 1997).

Before the introduction of L. terrestris, nutrient cycling in northern forests was dependent on decay from fungus. After the introduction of L. terrestris, the nutrient cycle changed due to the worm’s consumption of organic matter in the soil. This change of nutrient cycling altered the composition of the soil which had a negative effect on plants in the northern forest (Bohlen et al., 2004).

Changes in earthworm populations may result in collateral damage to the biodiversity of the northern forest. In a recent study, the effect of L. terrestris as an invasive species was examined (Loss, 2012). There was a correlation between the increase in population of L. terrestris, a non-native species, and the population decline of Ovenbirds (Seiurus aurocapilla) and Hermit Thrushes (Catharus guttatus) (Loss, 2012).

Earthworm’s effects on ecosystems are highly linked with their activities and movements in the soil (Capowiez et al, 2003). With the crucial impact of earthworms on soil, both
beneficial and invasive, it is important to understand how these species are repelled or attracted to chemical stimuli. Hence, we have chosen to begin examining how repellent chemicals might be detected by the earthworm, *L. terrestris*.

**Possible evidence for Earthworm chemesthesis**

AITC is used as an expellant for experiments which sample earthworms from soil in many eco-toxicological studies (Zaborski, 2003). AITC is the active ingredient in wasabi, horseradish, and mustard gas. This pungent compound is the prototypical chemical detected by TRPA1 (Matsuura et al, 2009).

Leeches are in the same phylum, Annelida, and class, Clitellata, as the earthworms. Recent studies of chemesthesis in medicinal leeches suggest that leeches might have TRPA and TRPV-like channels (Summers et al., 2014, 2015). TRPV-like channels are responsible for detecting capsaicin, the active ingredient in chili peppers. Since TRP channels are thought to be conserved through evolution (Minke, 2010), earthworms are expected to have TRP channels. Given that that AITC is an expellant for earthworms, and that leeches, detect and are repelled by capsaicin and AITC, we hypothesized that earthworms would use TRP channels to detect chemical irritants.
Sensory system of earthworms

The body of *L. terrestris* is segmented and each segment is separated by a body wall. The body wall is bound by columnar epithelial cells covered by a cuticle (Knapp and Mill, 1971). The main nerve cord runs through the center of the ventral side of the body and is generally called the ventral nerve cord (Figure 1). Each ventral nerve cord ganglion gets input from sensory fibers from at least two adjacent segments (Kiszler et al., 2012). Most information to the ventral nerve cord comes from the epidermis as free nerve endings or from sensory cells through segmental nerves (Figure 1) (Knapp and Mill, 1971).

Figure 1. Ventral nerve cord and segmental nerves of *L. terrestris* stained with methylene blue. Figure courtesy of Dr. Wayne Silver.
Multiple ciliated sensory cells in the epidermis of *L. terrestris* form an epidermal sensory organ (Figure 2). The prostomium is the most rostral part of the earthworm, in front of the mouth. It contains numerous epidermal sensory organs. The number of epidermal sensory organs decrease toward the middle segments and increase again in the tail region. (Knapp and Mill, 1971). While epidermal sensory organs are thought to detect sensory stimuli, the type of stimuli detected is unknown.

Figure 2. Scanning Electron Microscope (SEM) image of epidermal sensory organs at the red arrows, on the prostomium of *L. terrestris*. Multiple cilia from many sensory cells can be seen. Figure courtesy of Victoria Elliott and Riley Jay.
The epidermal layer of the earthworm is covered with cuticle which consists of multiple layers of collagen fibers (Figure 3) (Knapp and Mill, 1971). Out of all the cells in the epidermis, only the sensory cells possess cilia that penetrate the cuticle (Knapp and Mill, 1971). Three types of cells that bear cilia are thought to have a sensory function. Hence, these cells with cilia could be responsible for detecting chemical and tactile stimulation.

Multiciliate sensory cells are responsible for forming the epidermal sensory organ. A second type, the uniciliate sensory cells, is situated in some of the epidermal sensory organs. A third type, the isolated multiciliate sensory cells, is structurally different from the other
presumed sensory cells and are found spread out across the epidermis. They are typically not found in groups but rather exist alone and are sometimes called solitary sensory cells (Knapp and Mill, 1971). Although all of these epithelial cells are considered sensory, it is not known what kinds of stimuli activate them.

**Objective**

The objective of the present study was to investigate whether *L. terrestris* possesses TRP-like channels which respond to chemical repellants. Particularly, this study focused on TRPA1 channels. By stimulating the skin of the earthworm with chemicals (i.e. AITC and CIN) that activate TRPA1 channels, a response should be observed in the segmental nerves. Blocking the response with the TRPA1 antagonist HC030031, would suggest that the response came from activation of TRPA1 channels. We used antibodies to *Drosophila* and human TRPA channels to examine whether these channels might be present in earthworm sensory cells. In addition, we used air and soil T-maze behavioral assays to demonstrate whether earthworms can detect and be repelled by various TRP channel agonists such as ATIC, CIN, menthol (detected by TRPM8) and capsaicin. Finally, cells in the *L. terrestris* ventral nerve cord and epithelium were dissociated and prepared for calcium imaging. If cells from the ventral nerve cord and epithelium respond to chemicals that activate TRPA1 channels, blocking the dissociated cells’ response with HC030031, would help confirm that the response resulted from TRPA1-like channel activation.
MATERIALS AND METHODS

Subjects

Earthworms, *L. terrestris* were purchased from a local bait store in Winston-Salem, NC. Earthworms were stored in Styrofoam boxes filled with soil at 6-8°C temperature with no light in a refrigerator. Adult earthworms with a clitellum were used for the experiments.

Stimuli

For electrophysiological and behavioral experiments AITC, cinnamaldehyde, capsaicin and menthol were used. Stock solutions of these stimuli were made up in mineral oil at 10M. The stock solutions were diluted in spring water to 100mM which was further diluted to 75mM, 50mM, 25mM, 10mM and 1mM.

Electrophysiology

The earthworm was first washed with water then dried with paper towels. We then attempted to immobilize the earthworm by immersing it in a solution containing an anesthetizing agent (Table I). When the worm stopped moving, the anterior ten segments were separated from the rest of the body with a razor blade and opened with scissors at the dorsal and ventral border. Tissue was removed with forceps without damaging the ventral nerve cord and its segmental nerves. After the ventral nerve cord and segmental nerves were exposed, the earthworm was pinned on a platform that allowed the segmental nerves
to be submerged in halocarbon oil (which prevented the nerve from desiccation) and the epidermis to be suspended in air. Many different approaches for recording from the segmental nerves (Figure 4) were tested. The preparation shown in Figure 4C was used to obtain the recordings shown in Figure 6. Preparations shown in Figure 4A and 4B demonstrated to be most efficient in stimulating the skin and keeping the segmental nerve moist.

The skin of earthworm was kept moist with earthworm Ringer’s (NaCl, KCl, CaCl2, and NaHCO3 in deionized water, pH= 7.5) during the entire experiment except when the stimulus was being applied. The Ringer’s and stimuli were delivered through a gravity feed system (ValveLink 8.2) to the epidermis of the third to fifth segments. Three different pH levels (4.2, 4.0, and 3.8) of acetic acid and 50mM AITC were used. The acetic acid solutions were diluted with earthworm Ringer’s to get the pH to the desired level. After each stimulus, the epidermis was washed with Ringer’s for 160 seconds. Acetic acid and AITC were applied in 1.0 ml aliquots. For tactile stimulation the skin was touched with a wooden toothpick.

Segmental nerve activity was recorded by placing a nerve bundle on a pair of Pt-Ir wire hook electrodes. Neural activity was amplified by a Grass Instruments P511 preamplifier. The amplified neural activity was monitored with a Grass Instruments AM8 audio monitor. An integrator integrated the raw neural activity by taking the absolute value of the running average. The raw and integrated neural activity were digitized by Biopac MP100 data acquisition system and recorded using AcqKnowledge software (Biopac Systems, Goleta, CA).
Figure 4. Electrophysiology preparations. (A) Skin of the earthworm is facing up and the right side of the preparation is raised so that the solutions applied run off due to gravity.
Figure 4 legend continued. (B) A closer look of 4A. (C) Left side of the worm is pinned to the wall on the left and the right side of the worm is pinned to the bottom, making the worm an L shape. (D) The earthworm is sealed with denture adhesive between two petri dishes. The bottom of the petri dish has a running solution constantly touching the earthworm skin. (E) The tip of the delivery system is covered with the earthworm skin. (F) A closer look of 4E. Inside the plastic well, an electrode is hooked to a segmental nerve.

**Earthworm Immobilization**

Because of the difficulty in immobilizing the worms for electrophysiological recording, we tested many different compounds (Table I).

**Immunohistochemistry**

Earthworms were first anesthetized with Alka Seltzer (1/4 tablet in 20 ml of Ringer’s) then cut at the 10th segment. Samples were immersion fixed in a glyceraldehyde (1%)/paraformaldehyde (PFA, 4%)/phosphate-buffered saline (PBS, without calcium and magnesium) fixative agent overnight at 4°C.

Fixed earthworm samples were embedded in freezing medium and were frozen with dry ice on a special block that fits onto the cryostat. Fixed and frozen earthworm tissue was sectioned in the transverse plane at 20 μm. To gain access to the cells, the sections were permeabilized in 0.5% triton X-100 overnight at 4°C. The sections were rinsed once in PBS for 5-10 minutes. Then sections were blocked for nonspecific labeling with 20% fetal bovine serum (FBS) for 30 minutes at room temperature. A second rinse with 0.5% FBS
was done. Primary antibodies (goat polyclonal to human TRPA1 from Santa Cruz Biotechnology diluted in 0.5% FBS (1:500), mouse monoclonal to tubulin from Sigma-Aldrich diluted in 0.5% FBS (1:600), rabbit polyclonal to *Drosophila* dTRPA1 from Novus Biologicals diluted in 0.5% FBS (1:500), rabbit polyclonal to *Drosophila* PYREXIA from Novus Biologicals diluted in 0.5% FBS (1:500), rabbit polyclonal to *Drosophila* PAINLESS from Novus Biologicals diluted in 0.5% FBS (1:500), LK and DH31 obtained from Dr. Erik Johnson’s laboratory) were applied to the tissue overnight at 4°C. The secondary antibodies (Alexa 488 donkey anti-goat from Life technologies diluted in 0.5% FBS (1:600), Alexa 555 donkey anti-rabbit from Life technologies diluted in 0.5% FBS (1:600), Alexa 488 goat anti-mouse from Life technologies diluted in 0.5% FBS (1:600), Alexa 555 goat anti-rabbit from Life technologies diluted in 0.5% FBS (1:600)) were applied overnight at 4°C. Following each application of secondary antibodies, the sections were rinsed three times sequentially with 0.5% FBS for 10 minutes each. Lastly, the sections were rinsed three times with PBS sequentially for 10 minutes each and stored at 4°C.

Fluorescently labeled samples were imaged on a confocal microscope (lsm 710) using Zeiss software. 488nM (human TRPA1, Tubulin) and 555nM (PYREXIA, dTRPA1, PAINLESS, DH31, LK) laser lines were used. The pixel dimensions for the images were 1.75 pixel/ µm (10X), 3.57 pixel µm (20X) and 7.14 pixel/ µm (40X). Images were saved as TIFF files.
Behavioral Assays

Air T-Maze

The air T-maze consisted of a plastic T-tube connected to a plastic funnel (Figure 5A). A 2.54cm by 2.54cm piece of filter paper was soaked with 50µl of stimulus (AITC, cinnamaldehyde, capsaicin, or menthol) or a control (same amount of oil diluted in stimulus but without the stimulus). For AITC, 75mM, 50mM, 25mM, 10mM, and 1mM were tested. For cinnamaldehyde and menthol, 100mM, 75mM, 50mM, 25mM, 10 and 1mM were tested. A coin was tossed to determine which side of the T-maze to place the filter paper with the stimulus. To start a trial, earthworms were rinsed with water, then placed into a funnel connected to a T-tube. A light was suspended above the funnel and the room lights were turned off. Earthworms were then given 3 minutes to choose a side from which to escape the T-maze. When the earthworm escaped the T-maze through the control side, it was recorded as avoiding the stimulus. When the earthworm escaped through the stimulus side, it was recorded that the earthworm did not avoid the stimulus. When the earthworm did not leave the T-tube on either side, no preference was recorded and these data were not included in the analyses.
Soil T-Maze

The soil T-maze consisted of an acrylic tube (length=35.56cm, inside diameter=2.8cm) connected to a funnel (Figure 5B). The funnel was connected at the center of the tube. One half of the tube was filled with 100 ml of garden soil purchased from a local hardware store that was mixed with 20 ml of solution (AITC, cinnamaldehyde, or menthol at different concentrations). The other half was filled with 100 ml of garden soil mixed with 20 ml of mineral oil at the same dilution used in the stimulus solution. A worm was then placed into the funnel with a light shining from above. The earthworm was given up to 30 minutes to enter the tube and burrow to one side or the other. When the earthworm burrowed into the soil on the control side, it was recorded that the earthworm avoided the stimulus. When the worm burrowed into the soil on the stimulus side, it was recorded that
the earthworm did not avoid the stimulus. When the earthworm did not leave the funnel or did not move to either side of the tube, no preference was recorded and these data were not included in the analyses.

Data Analysis

Statistical analysis from data of air and soil T-maze assays was done with IBM SPSS Statistics using chi-square tests. Specifically, we analyzed data from the vehicle (diluted oil in water) versus vehicle and data from the treatment (AITC, cinnamaldehyde, menthol and capsaicin) versus vehicle. Chi-square tests were used because both dependent and independent variables were categorical variables.

Cell Dissociation for Calcium Imaging

We attempted to dissociate cells from the epithelium and ventral nerve cord of *L. terrestris* for use in calcium imaging experiments. A worm was immobilized with Alka Seltzer as described above and dissected so that the ventral nerve cord and skin were exposed. The ventral nerve cord was separated from cuticle, epithelium and muscle layers and treated separately. Tissues were transferred into 950 µl of Hank's Balanced Salt Solution (HBSS) and 50 µl of collagenase and incubated for 45 minutes in 37 °C. At the end of a 45 minute incubation period, 50 µl of DNAse and 50 µl of Trypsin I were added and the tissues were incubated for an additional 15 minutes. At the end of the incubation period, the tissues were gently shaken up and down. In a cell culture hood, 4 ml of prewarmed, 37 °C, DRG medium (1 to 1 ratio of Ham's F-12 Nutrient Mixture and Dulbecco's Modified Eagle's Medium
containing 10% fetal bovine serum and 1% Penicillin/Streptomycin) was added to the tissue. The solution containing the tissue was gently triturated and strained through a 100 µm nylon mesh. This was centrifuged at 500 RPM (50xG) for 5 minutes at room temperature. The supernatant was discarded and 5 mL of fresh DRG medium was added and the pellet was gently dispersed by tapping the tube. This was centrifuged and the supernatant was discarded again. The pellet was suspended in 4 ml of DRG medium and this solution was plated into 4 different wells with poly-D-lysine coated coverslips in a 24 well plate. Coverslips were coated with a solution of 400 µl, 1mg/ml, poly-D-lysine diluted in 3.6 ml of PBS. The coverslips were incubated for 15 minutes then washed 3 times with PBS and once with water. Solutions were incubated on coverslips in a 24 well plate for 48 to 72 hours. Coverslips were removed from the 24 well plate and transferred to a petri dish where they were incubated with Fura-2. The coverslips were then examined under a microscope to determine whether cells were sticking.
RESULTS

Electrophysiology

In order to obtain reliable recordings from the segmental nerves it was necessary to immobilize the earthworm for relatively long periods of time (hours). Any movement of the worm causes the electrodes to move, which produces an artifact and makes it impossible to interpret responses to chemical stimuli. Table I lists the compounds which were tested to keep the earthworm from moving. Since earthworm muscles are excited by acetylcholine (ACh) and inhibited by γ-aminobutyric acid (GABA), we tested tubocurarine chloride (0.1mM, 1mM), an ACh receptor blocker, and GABA (0.1mM, 1mM, 10mM, 100mM) and baclofen (0.1mM, 1mM, 10mM), a GABA agonist, as inhibitors of muscle contraction. However, none of these compounds kept the worms immobilized long enough to obtain stable, reliable recordings. Magnesium chloride (10mM, 20mM) was also tested as a paralytic. Both concentrations of MgCl₂ inconsistently stopped muscle movement. However, when the worm was anesthetized with MgCl₂, segmental nerves did not respond to tactile stimuli or 15mM and 20mM AITC. Of the compounds tested (Table I) only Alka Seltzer (CO₂), and only very occasionally, allowed for recording responses from a segmental nerve.

Figure 6 shows integrated neural recordings from a segmental nerve located behind the head around segment 10. This nerve responded to light touch of the skin as well as to 50 mM AITC. Figure 6 also shows responses from a segmental nerve in the 4th segment of a
different worm. It responded to acetic acid at three different pHs. The lowest pH elicited the largest response.

Figure 6. Earthworm’s segmental nerve responded to 50mM AITC, tactile stimulus and acetic acid with different pH levels (pH 4.2- pH 3.8).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Did the earthworm get anesthetized?</th>
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<tr>
<td>Propan-2-ol, 10%, 20%</td>
<td>No</td>
</tr>
<tr>
<td>Ethanol, 15%</td>
<td>No</td>
</tr>
<tr>
<td>GABA, 0.1mM</td>
<td>No</td>
</tr>
<tr>
<td>GABA, 1mM</td>
<td>No</td>
</tr>
<tr>
<td>GABA, 10mM</td>
<td>No</td>
</tr>
<tr>
<td>GABA, 100mM</td>
<td>No</td>
</tr>
<tr>
<td>Baclofen, 0.1mM</td>
<td>No</td>
</tr>
<tr>
<td>Baclofen, 1mM</td>
<td>No</td>
</tr>
<tr>
<td>Baclofen, 10mM</td>
<td>No</td>
</tr>
<tr>
<td>Tubocurarine chloride, 0.1mM, 1mM</td>
<td>No</td>
</tr>
<tr>
<td>MgCl₂, 10mM, 20mM</td>
<td>Inconsistently</td>
</tr>
<tr>
<td>Tricaine-S (AKA MS-222), 2mM</td>
<td>No</td>
</tr>
<tr>
<td>Alka Seltzer (CO₂)</td>
<td>Inconsistently</td>
</tr>
</tbody>
</table>

Table I. Compounds tested for immobilization of *L. terrestris*. 
Immunohistochemistry

Immunohistochemistry was used to help determine whether some epithelial cells in the skin of *L. terrestris* might express TRPA1 channels. Since no antibodies for earthworm TRPA1 channels currently exist, we used antibodies for *Drosophila* and human TRPA1 channels. *Drosophila* possess four TRPA1 homologs, dTRPA1, PAINLESS, PYREXIA, and WATERWITCH. We used primary antibodies against the first three of these channels, which were all made in rabbit. Primary antibodies for human TRPA1 were made in goat. We also tested primary antibodies for DH-31 and LK. These are *Drosophila* proteins which are homologs for mammalian calcitonin gene-related protein (CGRP) and Substance P (SP), respectively. Finally, antibodies against tubulin were used to stain nerve cells and cilia.

As a control for specificity, only the secondary antibodies that contain the fluorescent marker were applied to earthworm epithelial tissue. Secondary antibodies used were Alexa 555 Donkey Anti-Rabbit, Alexa 488 Donkey Anti-Goat, Alexa 555 Goat Anti-Rabbit, and Alexa 488 Goat Anti-Mouse. Earthworm epithelial tissue that was stained with just Alexa 555 Donkey anti-Rabbit or Alexa 488 Donkey Anti-Goat secondary antibodies showed no fluorescence. Only a slight fluorescence was observed in the muscle layer (Figure 7). Epithelial tissue that was stained with Alexa 555 Goat Anti-Rabbit, or Alexa 488 Goat Anti-Mouse also exhibited no fluorescence in the epithelium (Figure 8).

Some earthworm epidermal epithelial cells appear to be positive for dTRPA1 immunoreactivity. dTRPA1 immunoreactivity is primarily localized in pits situated in the epithelium (Figure 9). Among the three *Drosophila* TRPA channel antibodies used, the strongest and clearest immunoreactivity was observed for PYREXIA. PYREXIA immunoreactivity was more punctate in both the epithelium and the muscle layer of the
earthworm compared to dTRPA1. PYREXIA immunoreactivity also seemed to be localized in the muscle layer of the earthworm (Figure 11, 12, and 13). It also vividly followed the pattern of muscle tissues (Figure 11, 12, and 13), especially, if compared to the pattern for tubulin seen in the same Figures. We saw no immunoreactivity for PAINLESS in earthworm tissues, suggesting that PAINLESS is not expressed in earthworm (Figure 14).

LK immunoreactivity appeared more in the basal part of the epithelium, and DH31 immunoreactivity was both prominent in the epithelia and the muscle layer. Immunoreactivity for LK and DH31 was punctate (Figure 15 and 16).
Figure 7. Control with Alexa 555 Donkey Anti-Rabbit, Alexa 488 Donkey Anti-Goat stained, 10x. The tissue was sectioned 80 µm from prostomium. Faint pattern of fluorescence is seen in the circle.
Figure 8. Control with Alexa 555 Goat Anti-Rabbit, and Alexa 488 Goat Anti-Mouse stained, 10x.
Figure 9. Immunoreactivity for dTRPA1 (Red) and Tubulin (Green), 10x. Red arrow points to dTRPA1 showing immunoreactivity in epithelium where sensory cells reside.
Figure 10. Immunoreactivity for Human TRPA1 (Red), and Tubulin (Green), 10x. The tissue was sectioned 60µm from the prostomium. Red arrow is pointing to faint immunoreactivity of Human TRPA1 in the epithelium.
Figure 11. Immunoreactivity for PYREXIA (Red) and Tubulin (Green) 10x. Red arrows show localized PYREXIA in the epithelium.
Figure 12. Immunoreactivity for PYREXIA (Magenta) and Tubulin (Green) 20x. Blue arrow is pointing to PYREXIA in the epithelium.
Figure 13. Immunoreactivity for PYREXIA (Magenta) and Tubulin (Green) 40x. Red arrows are pointing to the PYREXIA immunoreactivity in the muscle and epithelium layer.
Figure 14. Immunoreactivity for PAINLESS (red stain but not present) and Tubulin (Green) 10x.
Figure 15. Immunoreactivity for *Drosophila* LK and Tubulin, 40x.
Figure 16. Immunoreactivity for *Drosophila* DH31 (Magenta), and Tubulin (Green), 40x.
Behavioral Assays

Air T-maze

We used two behavioral assays, the air T-maze and the soil T-maze to assess whether *L. terrestris* can detect and be repelled by AITC and other irritants. We first determined that earthworms had no preference to diluted oil in spring water versus spring water because our stimulus were made up in diluted oil in spring water (Figure 17). Earthworms showed no preference to either diluted oil in spring water or spring water. We also determined that earthworms had no side preference in the air T-maze by putting control stimuli (diluted oil in spring water) in both arms of the T-Maze. Earthworms did not significantly choose one side over the other in these tests (Figure 18). We next determined whether *L. terrestris* could detect and be repelled by different concentrations of TRP channel agonists. Earthworms showed significant avoidance to AITC at 25mM, 50mM, and 75mM (Figure 19) when compared to the control data (Figure 18). However, they did not significantly avoid 10mM or 1mM AITC (Figure 19). Another TRPA1 agonist tested was CIN. Earthworms significantly detected and avoided CIN at concentrations between 25 and 100 mM (Figure 20). The TRPM8 agonist, menthol, was also tested. In the air T-maze earthworms did not significantly detect and avoid menthol at 50mM, 75mM, and 100mM (Figure 21). Earthworms were not repelled by capsaicin at concentrations as high as 2 mM (Figure 22). In fact, the earthworms did not respond to placing a small crystal of capsaicin directly onto the skin.
Figure 17. *Lumbricus terrestris* air T-maze results for diluted oil versus spring water.

Figure 18. *Lumbricus terrestris* air T-maze results for diluted oil versus diluted oil.
Figure 19. *Lumbricus terrestris* air T-maze results for AITC versus control (The equivalent amount of oil that was in each AITC solution).

Figure 20. *Lumbricus terrestris* air T-maze results for cinnamaldehyde versus control (diluted oil).
Figure 21. *Lumbricus terrestris* air T-maze results for menthol versus control (diluted oil).

There were no statistical differences for menthol at any concentration tested.

Figure 22. *Lumbricus terrestris* air T-maze results for capsaicin versus control (diluted oil).

The earthworm could not detect and avoid capsaicin at any concentration.
Soil T-Maze

For the soil T-Maze, we first determined that *L. terrestris* did not have a side preference by putting control (diluted oil in spring water) in both sides of the maze. Earthworms did not show a side preference (Figure 23). Next, we determined whether *L. terrestris* can detect and avoid different concentrations of TRP channel agonists in soil. When compared to the control data, earthworms showed significant avoidance to AITC at 50mM, 25mM, 10mM and 5mM AITC but not at 1mM (Figure 24). Earthworms also showed that they can detect and avoid another TRPA channel agonist, CIN at 5mM, 10mM, 25mM, and 50mM (Figure 26). Additionally, TRPM8 agonist, menthol was detected and avoided at 75mM, 50mM and 10mM (Figure 25).

Figure 23. *Lumbricus terrestris* soil T-maze results for diluted oil versus diluted oil.
Figure 24. *Lumbricus terrestris* soil T-maze results for AITC versus control (diluted oil).

Figure 25. *Lumbricus terrestris* soil T-maze results for menthol versus control (diluted oil).
In order to use calcium imaging to determine whether TRP channel agonists stimulate epithelial cells or cells in the ventral nerve cord it was first necessary to dissociate those cells onto a coverslip in a dish. Once the cells are stuck to coverslips coated with poly-D-lysine, they can be filled with the calcium indicator dye Fura-2 and tested under a fluorescent microscope to see if calcium enters the cell in the presence of AITC or other compounds. If the cells do not stick to the coverslips, they will be washed away when the stimulus is applied. Although we were able to successfully dissociate cells from the earthworm epithelium (Figure 27) and ventral nerve cord (Figure 28), those cells did not stick to coverslips coated with poly-D-lysine and could not be used for calcium imaging.

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Figure 26. *Lumbricus terrestris* soil T-maze results for cinnamaldehyde versus control (diluted oil).
As a control for our technique, cells from the rat dorsal root ganglion (Figure 29) were dissociated and did stick to the cover slip coated with poly-D-lysine.

Some dissociated cells of *L. terrestris* (in red circles in Figure 27) appear to have cilia, which is a characteristic of sensory cells in the earthworm epithelium.
Figure 27.
Dissociated cells from epithelium of *Lumbricus terrestris* (A, B). 100x was used for A. 400X was used for B. Cells in the red circles resemble sensory cells with cilia from the epithelium of *L. terrestris*. 
Figure 28.
Dissociated cells from ventral nerve cord of *Lumbricus terrestris* (A, B). 100x was used for A. 400X was used for B. The blue arrow is pointing to what appears to be a dissociated cell from ventral nerve cord of *L. terrestris*. 
Figure 29.
Dissociated cells from dorsal root ganglion of Sprague-Dawley (A, B). 100x was used for A. 400X was used for B. The red arrow is pointing to what appears to be an axon and the blue arrow is pointing to what appears to be a dissociated cell of dorsal root ganglion of Spargue-Dawley.
DISCUSSION

Chemesthesis can be defined as the chemical stimulation of receptors on somatosensory neurons, usually by noxious chemicals leading to irritation. The ability to detect noxious and potentially harmful chemicals is found in a wide variety of organisms. In many organisms, TRP channels, which have been found in vertebrates, insects, roundworms, ciliates, green algae, and yeast (Chang et al., 2010) play a role in the detection of these chemesthetic compounds. Earthworms which play an important role in soil management, are clearly able to sense and are repelled by a number of different irritants (Zaborski, 2003, Seamans et al., 2015). However, little is known about how earthworms detect noxious chemicals and no TRP channels have been reported for this group. The current study was initiated to examine chemesthesis in earthworms and whether these animals use TRP channels to sense repellent chemicals.

Electrophysiology

Our initial goal was to develop an electrophysiological preparation that produced consistent and replicable neural responses to chemicals from the segmental nerves in \emph{L. terrestris}. Our plan was to determine whether \emph{L. terrestris} might possess TRPA1 channels. Eliminating responses to AITC, a prototypical TRPA1 channel agonist, with the TRPA1 channel antagonist, HC030031 would have suggested the presence of TRPA1 channels in \emph{L. terrestris}. 

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However, because we were unable to eliminate spontaneous background neural activity and artifacts due to muscle movement, we could not reliably record responses from segmental nerves. Very occasionally a worm would be still enough to obtain records like those see in Figure 6. There are four reports in the literature in which responses to chemicals were recorded from earthworm (including *L. terrestris*) segmental nerves. Prosser (1935) obtained responses to NaCl, HCl, and NaOH and Laverack (1960, 1961) to NaCl, quinine hydrochloride, sucrose, glucose, glycerol and changing pH (using potassium hydrogen phthalate and HCl or NaOH). Mill and Knapp (1968) recorded segmental nerve responses to NaCl. None of these papers reported using any chemical compounds to immobilize the earthworms. In each case, a section containing multiple segments was prepared by opening the worm and simply pinning it to paraffin in a dish. From our experience, we do not see how it would be possible to obtain stable records from earthworm segmental nerves under these conditions.

Both the present study (Figure 6) and Laverack (1961) demonstrated that *L. terrestris* can respond to solutions of different pH. Although there are a number of different receptor proteins which detect changes in pH, including ASIC channels, it is well known that TRP channels (e.g TRPV1 and TRPA1) can detect changes in pH (Pedersen et al., 2005). In Laverack’s experiment (1961), he obtained segmental nerve recordings displaying a pH threshold of 4.3-4.1. In the current study, segmental nerves of *L. terrestris* responded to acetic acid at a pH level between 4.2 and 3.8 (Figure 6), which was a similar to the findings of Laverack (1961).

In the present study, there was a latent period of about three seconds for a solution of pH 4.2 (Figure 6). That is, the response occurred about three seconds after the stimulus was
applied. This is consistent with Laverack’s results (Laverack, 1961). In both the current study and in Laverack’s (1961) decreasing pH resulted in a decreased latent period.

We recorded *L. terrestris* segmental nerve responses to 50mM AITC and tactile stimuli. The response to 50mM AITC grew more slowly compared to the tactile response as seen by the steepness of the slopes of the responses. Laverack (1960) also recorded *L. terrestris*’s responses to tactile stimuli. The tactile response was rapidly adapting, however, Laverack was not sure if the responses to tactile stimuli were actually from proprioceptor/stretch receptors or were muscle responses to touch (Laverack, 1960).

Two recent studies suggest that the medicinal leech *Hirudo verbena*, may possess both TRPV1-like and TRPA1-like channels (Summers *et al.*, 2014, 2015). Leeches are in the same Annelid class (Clitellata) as *L. terrestris*. In electrophysiological experiments, polymodal nociceptive neurons in the segmental ganglia of *H. verbena* were activated by capsaicin and AITC, applied peripherally or centrally. Responses were inhibited by the TRPV1 antagonist SB366791 and the TRPA1 antagonist HC030031, respectively. Interestingly, responses to capsaicin were never seen in our recordings or behavioral assays (Figure 22). However, these findings for the leech support the possibility that earthworms use TRP channels to detect irritant chemicals.
Compounds tested for *Lumbricus terrestris* immobilization

In an attempt to immobilize earthworms for electrophysiological recording, we examined eight different compounds and several combinations (Table I). None of these compounds kept the worms from moving long enough to obtain reliable, stable recordings from the segmental nerves. Eliminating spontaneous muscle activity would have allowed us to avoid recording responses due to muscle movement, activating proprioceptor/touch receptors or causing the electrodes to move, producing an artifact.

Budan et al., (2014), in an attempt to immobilize earthworms (*Eisenia hortensis*) so that they could be examined by magnetic resonance imaging for use in eco-toxicological studies, subjected the worms to twenty five different compounds. These compounds included inhaled anesthetics (e.g. isofluorane), non-depolarizing neuromuscular blockers (e.g. rocuronium), parasympatholytics (e.g. physostigmine), sodium channel blockers (e.g. lidocaine), GABA-agonists (e.g. meprobamate), opiates (e.g. fentanyl), alcohols (e.g. propan-2-ol), ions (e.g. Mg\textsuperscript{2+}), and antihelmintics (e.g. ivermectine).

Budan et al., (2014) reported that only propan-2-ol was successful in immobilizing the earthworms for long periods of time (more than three hours) but only if the worm’s body surface was in constant contact with the propan-2-ol in an airtight box. The duration of the effect of the propanol decreased dramatically from more than 3 hours with the airtight box to just 4 minutes without the airtight box (Budán, 2014). In our experiments, initially, 10% and 20% of propan-2-ol hindered the movement of muscles, but it did not last the entire experiment. Keeping the worm in propan-2-ol for long periods of time was not feasible in our preparations because it would not be possible to stimulate the skin with other chemicals.
D-tubocurarine, GABA and baclofen (a GABA agonist) were other compounds that we tested as earthworm immobilizing agents. ACh is the excitatory neurotransmitter in earthworms. Carbachol, a cholinergic agonist, generated a complete contraction of earthworm muscle, caused by membrane depolarization (Volkov, et al., 2001). This strong depolarization caused by carbachol was not abolished even by well-known nicotinic receptor blockers, d-tubocurarine and a-bungarotoxin. Atropine, a muscarinic receptor blocker also did not terminate depolarization caused by carbachol.

GABA on the other hand, is an inhibitory neurotransmitter at the neuromuscular junction of earthworms (Volkov et al., 2011). We tested 0.1mM, 1mM, 10mM and 100mM GABA. Although the earthworms clearly reacted to GABA by writhing vigorously, they were not immobilized. Movement of earthworm muscles was observed after treatment with 0.1mM, 1mM, 10mM and 100mM of GABA. Volkov et al., (2011) observed that GABA was responsible for hyperpolarizing the resting membrane potential (RMP) of muscle membrane up to 8%. In theory, hyperpolarization of the RMP should hinder muscle action potential firing, and the movement of the muscle. However, since in our hands, GABA did not immobilize the earthworms, it appears that 8% hyperpolarization of RMP is not enough to stop the firing of muscle action potentials and the movement of the muscle in the earthworms.

Baclofen is a powerful GABA agonist which causes an almost 25% increase in the hyperpolarization of the RMP compared to the control condition (Volkov et al., 2011). Yet, from our experiments baclofen was not a good earthworm anesthetic. None of the concentrations that we tested for baclofen, 0.1mM, 1mM and 10mM, resulted in immobilizing earthworms.
In addition, since Ca$^{2+}$ is of obvious importance to muscle contraction and extracellular Mg$^{2+}$ competes with Ca$^{2+}$ (Bara et al., 1993), we thought MgCl$_2$ might immobilize earthworms. Bathing earthworms in MgCl$_2$ resulted in inconsistent immobilization. When MgCl$_2$ did immobilize an earthworm, we were unable to obtain neural responses to either touching the cuticle or to high doses (15mM and 20mM) of AITC.

**Immunohistochemistry**

TRPA channels are found in a wide variety of organisms, from round worms to fruit flies, to humans (Venkatachalam et al, 2014). One way to identify TRPA channels is by determining whether antibodies that recognize these channels bind to tissue of interest. No antibodies specific to earthworm TRPA currently exist. Therefore we employed TRPA antibodies from another invertebrate, *Drosophila*, as well as a vertebrate, human. Fruit flies express four different TRPA channels, dTRPA1, PYREXIA, PAINLESS, and WATERWITCH. We used antibodies against the first three of these *Drosophila* TRPA channels as well as human TRPA1.

Whether or not an antibody from one species (e.g. *Drosophila*) binds with similar target protein in another species (e.g. *Lumbricus*) depends on the sequence similarity between the target proteins from the two species. That is, antibodies to *Drosophila* TRPA channels will only bind to suspected *Lumbricus* TRPA channels if the amino acid sequences for TRPA channels in both species are similar.

In immunohistochemistry experiments, it is not unusual for there to be non-specificity leading to false positives which could result in a conclusion that the target protein is
expressed when is actuality it is not (True, 2008). This can be especially true when antibodies made against target proteins in a different species are used.

However, because there are few antibodies made against earthworm proteins it is necessary to use antibodies made against target proteins in other species. Immunohistochemistry employing antibodies to dopamine (target species not specified) was used to describe the morphology of putative sensory cells in *L. terrestris* (Spörhase-Eichmann et al., 1998). Similar experiments were conducted on *L. terrestris* and the earthworm *Eisenia fetida* using antibodies to GABA, neuropeptide Y, serotonin, tyrosine hydroxylase, histamine, proctolin, and rhodopsin which were derived from rabbit (Csoknya et al., 2005). They concluded that solitary sensory cells in earthworms express serotonin, tyrosine hydroxylase, histamine, GABA, proctolin or rhodopsin.

In the current study, cuticle, epithelia, and muscle layers of *L. terrestris* were stained with *Drosophila* or human antibodies to TRPA channels. In addition, tissue stained with *Drosophila* antibodies to DH-31 and LK and mouse antibodies to tubulin was also examined. DH-31 is a homolog to mammalian CGRP and LK is a homolog to mammalian SP. In fruit flies DH-31 and LK appear to be expressed in cells containing TRPA channels (W.L. Silver, personal communication) and in vertebrates CGRP and SP are colocalized in cells expressing TRPA1 (Nilius et al., 2012). Tubulin is often used to stain cilia and nerve cells (Jockusch et al., 1979).

Out of the three *Drosophila* TRPA channel antibodies used, the ones for PYREXIA provided the most consistent and punctate patterns in the earthworm. PYREXIA was prominent in the muscle but was also localized in the epithelium (Figure 11, 12, and 13). dTRPA1 appeared in discrete points in the epithelium layer (Figure 9). Human TRPA1 was
more localized on the cuticle with a small amount in the epithelium (Figure 10). There was no staining to PAINLESS suggesting that it is not present in the earthworm (Figure 14).

Localization of TRPA channels in the earthworm epithelium is consistent with findings from *Drosophila* larvae where they are found in nociceptive cells in the body wall (Johnson and Carder, 2012), from vertebrates where they are found in nociceptive free nerve endings in epithelia (Koivisto et al., 2014) as well as in the plasma membrane of sensory neuronal cells (Vilceanu and Stucky, 2010).

The finding that TRPA channels may be expressed in the earthworm epithelium does not mean that they are chemoreceptors. All three of the *Drosophila* TRPA channel homologs tested are known to detect temperature and TRPA1 is reported to detect mechanical stress (Venkatachalam and Montel, 2007). dTRPA1 channels are activated by mild heat (≥24-27°C) and PYREXIA by noxious heat (≥ 40°C) (Venkatachalam and Montel, 2007).

Two neuropeptides known to be involved in pain signaling, SP and CGRP, were also tested for immunoreactivity in *L. terrestris* because they colocalize with TRPA1 channels (Pedersen et al., 2005). In a radioimmunoassay study, SP-like peptide was observed in the ventral nerve cord, intestine and body wall of *L. terrestris* (Kaloustian et al., 1986). This is inconsistent with what we found, in that immunoreactivity of LK was localized to the basal part of the epithelium (Figure 15). Curry et al. (1989) demonstrated that *L. terrestris* epidermal cells are immunoreactive to CGRP. This was consistent with our results that DH31 showed immunoreactivity in both the epithelium and muscle layer (Figure 16). Interestingly, no immunoreactivity to SP was found in that study (Curry et al., 1989).
Behavioral Assays

AITC (or mustard powder) is the preferred expellant for sampling earthworms in studies where knowing how many worms are present in a given amount of soil is important (Pelosi et al., 2014). For example, ecotoxicology and biomonitoring studies employ AITC to assess the effects of chemical pollutants on the number of earthworms in a given area of soil (Velki et al., 2012). Zaborski (2003) tested eight different concentrations of AITC and reported that 1 mM was the most effective concentration for expelling earthworm. Clearly, earthworms are able to detect AITC, the prototypical TRPA1 agonist.

We modified a T-maze assay initially used for examining whether earthworms could learn to follow chemical (pheromonal) clues in order to avoid being shocked (Rosenkoetter and Boice, 1975). We first used an air T-maze in which the worms were placed into an open-air T-connector with a piece of filter paper on one side soaked in the stimulus. A piece of filter paper soaked in the vehicle on the other side served as the control. In the air T-maze, earthworms significantly detected and avoided AITC at concentrations above 25 mM (Figure 19). They also avoided cinnamaldehyde, but not menthol or capsaicin.

In the soil T-maze, an acrylic tube was filled with garden soil. The soil in one half was mixed with the stimulus, while the soil in the other half was mixed with the vehicle and served as the control. Earthworms avoided AITC in the soil T-maze with a threshold concentration around 5 mM. Earthworms also avoided cinnamaldehyde and menthol. Capsaicin was not tested in the soil T-maze, but worms did not respond to the placement of capsaicin crystals directly on their skin.
Although results from both T-maze assay showed that earthworms can avoid AITC, threshold concentrations were higher for the air T-maze (25mM to 5mM). No significant detection of menthol was seen in the air T-maze assay, but in the soil T-maze assay earthworms were repelled by concentrations as low as 10mM. Clearly the soil T-maze is a more sensitive assay than the air T-maze, presumably because it more closely mimics the earthworm’s natural habitat. The concentration of the irritant was uniform on the stimulus side of the soil T-maze. It would be interesting to determine whether earthworms can detect chemical gradients in soil.

The concentrations detected in both behavioral assays were higher than the preferred concentration for AITC used as an expellant in the field (1mM) (Zaborski, 2003). This could be because the AITC used in the field was diluted with isopropanol, which perhaps could be an expellant to earthworms by itself. In addition, 20 liters of 1mM AITC was delivered to the 0.5 m² test arenas (Zaborski). Interestingly no controls were used. In our experiments, AITC was diluted in mineral oil. The same percentages of oil used in making the AITC solutions were tested against spring water to make sure that mineral oil itself was not an expellant for L. terrestris (Figure 17).

Recently, behavioral tests for the detection of AITC and capsaicin have been reported for the medicinal leech (Summers et al., 2014, 2015). Leeches exhibited a withdrawal response when 0.1mM AITC was placed directly on their sucker and a swimming-like behavior when the AITC concentration was increased to 0.25mM. Both behaviors were significantly attenuated when the AITC antagonist, HC030031 was mixed with the AITC. The concentrations of AITC which elicited responses in the leech were 2 orders of magnitude lower than the concentration of AITC detected by earthworms in the soil T-maze assay.
This could be due to the difference in the application of the AITC, directly onto the exposed sucker of the leech versus in the soil surrounding the earthworm, or to the possibility that leeches are more sensitive to TRPA1 agonists. Interestingly, in the leech study, the vehicle for the AITC was dimethyl sulfoxide (DMSO). There were no behavioral responses to DMSO by itself. In our study, however, DMSO was a highly effective irritant when placed directly on the earthworm (data not shown). It was so effective, that we switched to mineral oil (which elicited no responses from earthworms) as the vehicle in all of our experiments.

Leeches also exhibited sucker withdrawal responses and locomotion to capsaicin, a TRPV1 agonist, at concentrations as low as 0.01mM. These responses were attenuated in the presence of SB 366791, a TRPV1 antagonist. This is very different then what was seen in the earthworm, which was completely insensitive to capsaicin, even when a capsaicin crystal was place directly on its head.
Cell Dissociation for Calcium Imaging

Our hypothesis is that sensory cells in the earthworm skin and ventral nerve cord express TRP channels which are activated by irritant chemicals. Three types of ciliated sensory epithelial cells have been described in the earthworm (Langdon, 1895) although the stimuli which activate these cells are unknown. Our plan was to dissociate epidermal cells, fill them with a calcium indicator, and assess whether they were activated by TRP channels agonists before and after application of TRP channel blockers. This approach has been successful, for example, in looking for TRP channels in trigeminal ganglion cells (Lübbert et al., 2013).

Cells in the epithelium and ventral nerve cord of *L. terrestris* were successfully dissociated. However, the dissociated cells would not stick to a coverslip, which is a necessary step for calcium imaging, since control fluid as well as the stimulus are flowed over the cells and nonsticking cells just wash away. We tried plastic and glass coverslips, coated with poly-D-lysine. Dead or unhealthy cells are less likely to stick to a coverslip. Therefore, we tried dissociating the cells without chemicals (e.g. trypsin and DNAse) that can damage the cells. However, getting rid of these chemicals did not help the cells stick to coverslips. We have also tried incubating the cells in a lower temperature, 21°C, since the natural environment that earthworms live in is colder than that of mammals, but this also did not help the dissociated cells stick to coverslips. We believe it will take experimenting with incubation times and temperatures as well as enzymes and other reagents used for dissociation to obtain the proper combination of variables that will keep the cells alive and healthy and allow them to stick to coverslips.
Some of the dissociated cells we observed resembled cells that were previously described (Knapp and Mill, 1971). These cells (Figure 29) display cilia and have a cell body that is similar to that described by Knapp and Mill (1971).

**Conclusion**

The results of this study suggests that *L. terrestris* possesses sensory receptors which detect TRP channel agonists. Earthworms were able to detect TRPA1 agonists such as AITC, cinnamaldehyde and the TRPM8 agonist menthol. On the other hand, earthworms could not detect the TRPV1 agonist capsaicin. It was clear that earthworms can better detect chemicals in settings that mimic their natural soil habitat. The immunoreactivity of earthworms to TRPA1 channels, suggests that TRPA1 channel occur in the skin epithelium. However, more work is necessary to conclude that earthworms detect TRP agonists using TRP channels. Using pharmacological techniques to block the detection of the TRP agonists with TRP blockers would strengthen the case for TRP channels being responsible for earthworm’s detection of TRP agonists. If the response to the agonists is eliminated, it would suggest that earthworms possess and use TRP channels to detect irritants. Pharmacological TRP channel blockers could be applied in electrophysiological, cell dissociation/calcium imaging, and soil T-maze experiments.
REFERENCES


SCHOLASTIC VITA

Albert Hue-Soo Kim

BORN: February 1989, Columbus, Ohio

UNDERGRADUATE STUDY: The Ohio State University
Columbus, Ohio
B.S. Pharmaceutical Sciences, 2012

GRADUATE STUDY: Wake Forest University
Winston-Salem, North Carolina
M.S. Biology, 2016

HONORS AND AWARD:
National Buckeye Scholarship, 2008 – 2012

SCHOLASTIC AND PROFESSIONAL EXPERIENCE:
Graduate Teaching Assistant, Comparative Physiology (2 semesters), Cell biology (1 semester), Microscopy (1 semester), Wake Forest University, Winston-Salem, North Carolina, 2014 – 2016

Postbaccalaureate Intramural Research Training Award (IRTA) recipient at National Institutes of Health, National Institute on Drug Abuse, March 2013 - May 2014

ABSTRACTS AND PUBLICATIONS:


