NICOTINAMIDE IS A TRPV CHANNEL AGONIST IN *CAENORHABDITIS ELEGANS* AND *DROSOPHILA MELANOGASTER*

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by

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ABSTRACT

Our ability to sense the environment around us and within the body is critical for survival. TRP ion channels are one family of sensory proteins that are implicated in almost every sensory modality i.e., touch, taste, smell, vision, hearing etc. (Islam, 2011). As a result, their dysfunction is implicated in many disease processes making them attractive drug targets (Moran et al., 2011; Nilius and Owsianik, 2010). TRP channels display remarkable diversity during evolution, yet they share many structural and functional similarities among evolutionally diverse species. Model organisms like Caenorhabditis elegans and Drosophila melanogaster provide a tractable system for investigating the complexities of these molecular sensors in a simpler setting. Metabolic regulation of TRP channels is an emerging field (Kahn-kirby et al., 1991; Kumar et al., 2014; Riera et al., 2014). My work reveals role of a form of vitamin B₃, Nicotinamide (NAM) in modulating sensory signaling, behaviors and in some cases eliciting cell death via a TRPV channel in C. elegans and D. melanogaster. My lab has established an excellent C. elegans metabolic mutant model pnc-1 (pyrazinamidase/nicotinamidase-1) to study how metabolism affects biological processes like development, aging and cell death. I began my research by investigating a cell death phenotype in the pnc-1 mutant. Loss of C. elegans
nicotinamidase pnc-1 that is required for NAD salvage synthesis causes metabolic perturbation and increase in its substrate NAM levels (Vrablik et al., 2009). Four cells in the uterus (uterine-vulval 1 cells) of the animal die due to excess NAM in pnc-1 mutant. Here I report that NAM inflicts death on another set of cells found in the head (OLQ neurons) of the animal. I show that the death phenotype is limited to these two cell types because they both express a heteromeric TRPV ion channel made up of OCR-4 and OSM-9 subunits, co-expression of these is necessary for NAM induced cell death. Using electrophysiology in Xenopus oocytes, I determine that NAM is a direct agonist for OCR-4/OSM-9 TRPV ion channel. This is the first successful report of in vitro functional expression any C. elegans TRPV channel. C. elegans TRPV channels are not well characterized and I propose that NAM agonist provides a tool for the same. I further show that NAM agonist activity on TRPV channels is conserved in D. melanogaster. In Drosophila, Nanchung (Nan) and Inactive (Iav) are the OCR-4 and OSM-9 orthologs respectively. Nan/Iav heteromer is also activated by NAM in electrophysiology in Xenopus oocytes. Calcium imaging in Drosophila larvae shows that NAM application induces a calcium spike and over stimulates the chordotonal neurons (the only cells that co-express Nan/Iav heteromer) and renders them inactive. I also show that behaviors mediated by the orthologous
Drosophila channel are responsive to NAM. These results provide an intriguing link between metabolic regulation and TRPV channel activity thus opening avenues for future research.
# TABLE OF CONTENTS

LIST OF FIGURES .............................................................................................................. ix

LIST OF ABBREVIATIONS ................................................................................................. xi

ACKNOWLEDGEMENTS ..................................................................................................... xii

Chapter 1: Background and Introduction ...................................................................... 1
  1.1 Layout of the dissertation ......................................................................................... 1
  1.2 Introduction to C. elegans ......................................................................................... 1
  1.3 C. elegans as a model for cell death ......................................................................... 3
    1.3.1 *pnc-1* mutant: Our model for cell death ......................................................... 4
    1.3.2 Uv1 cells ............................................................................................................ 7
    1.3.3 Uv1 cell specification requires EGF signaling .................................................. 11
    1.3.4 EGF signaling overcomes uv1 cell death in *pnc-1* mutant ......................... 12
    1.3.5 *pnc-1* mutant has perturbed metabolite levels ............................................. 14
    1.3.6 Uv1 cell death is not apoptosis ....................................................................... 15
    1.3.7 Uv1 cell death resembles necrosis .................................................................. 17
    1.3.8 ER calcium signaling may not be required for uv1 cell death ....................... 19
  1.4 Summary of introduction and aims of this study .................................................... 20

Chapter 2: A TRPV channel mediates NAM induced cell death .................................. 22
  2.1 OLQ cells die in *pnc-1* mutants .......................................................................... 22
    2.1.1 An introduction to the OLQ neurons ............................................................... 25
    2.1.2 OLQ cell death in *pnc-1* mutants is a progressive process ....................... 31
    2.1.3 OLQ cell death is less penetrant than uv1 cell death in *pnc-1* mutants ........ 32
    2.1.4 Cell death in response to acute NAM treatment is rapid ........................... 34
    2.1.5 *ocr-4* mutation blocks NAM induced cell death ................................. 36
    2.1.6 An introduction to TRPV channels ............................................................... 37
    2.1.7 C. elegans TRPV homologs ......................................................................... 39
  2.2 Discussion ............................................................................................................... 46
  2.3 Materials and methods ......................................................................................... 48
5.2.1 Loss of PNC-1 causes egg laying (Egl) defect ................................. 90
5.2.2 pnc-1 egg laying defect is caused due to vulva muscle impairment.................................................................................. 90
5.2.3 osm-9 and ocr-4 mediate NAM induced egg laying defect .... 91
5.3 NAM affects OLQ mediated foraging behavior ................................. 92
5.4 Excess NAM causes nose touch defect ............................................. 94
5.5 Discussion ..................................................................................... 95
5.6 Materials and methods .................................................................. 97
  5.6.1 Egg laying assay ......................................................................... 97
  5.6.2 Nose touch assay ....................................................................... 98
  5.6.3 Foraging .................................................................................... 98
  5.6.4 Statistical Analysis .................................................................... 99

Chapter 6: Summary, significance and future directions .................... 100

  6.1 Summary and significance ............................................................. 100
  6.2 Future directions .......................................................................... 105

Supplementary Information .................................................................. 107

References ......................................................................................... 109
LIST OF FIGURES

Figure 1.1: NAD+ biosynthesis pathway in C. elegans ........................................ 5
Figure 1.2: uv1 cells die in pnc-1 mutants .......................................................... 6
Figure 1.3: uv1 cell are located in the uterus of C. elegans. ............................. 7
Figure 1.4: A model for TRPV function in uv1 cells in regulating egg laying........................................................... 10
Figure 1.5: uv1 cell specification requires EGF signaling ................................. 12
Figure 1.6: NAM levels increase in pnc-1 mutants.......................................... 15
Figure 1.7: Uv1 cell death resembles necrotic morphology............................ 16
Figure 1.8: necrotic cell death pathway in C. elegans ..................................... 18
Figure 2.1: OLQ cell death in pnc-1 mutants resembles uv1 cell....................... 24
Figure 2.2: Structure of OLQ cells ...................................................................... 25
Figure 2.3: Neuronal circuit for foraging and head withdrawal ....................... 27
Figure 2.4: Neuronal circuit for gentle nose touch response in C. elegans ...... 30
Figure 2.5: OLQ neurons die by progressive degeneration in the pnc-1 mutant ........................................................... 31
Figure 2.6: Quantification of the cell deaths in pnc-1 null mutants............... 32
Figure 2.7: NAM causes cell death in a dose dependent manner .................... 34
Figure 2.8: Cell death in response to acute NAM treatment is a rapid process........................................................................ 35
Figure 2.9: Structure of TRPV1 channel ............................................................. 37
Figure 2.10: Expression pattern of TRPV subunits in C. elegans ...................... 43
Figure 2.11: OCR-4 and OSM-9 mediate NAM-induced uv1 and OLQ cell death

Figure 3.1: NAM activates the heteromeric OCR-4/OSM-9 channel in *Xenopus* oocytes

Figure 3.2: A schematic of TIRF microscopy principle

Figure 3.3: Stoichiometry of OSM-9/OCR-4 channels

Figure 3.4: Sample images and example analyses from TIRF photobleaching assay for OCR-4::GFP

Figure 4.1: Phylogeny of the TRPV ion channel subfamily

Figure 4.2: Arrangement of abdominal chordotonal organs in the *Drosophila* larvae

Figure 4.3: Structure of a scolopidium

Figure 4.6: NAM diminishes *Drosophila* larva startle response to sound in a dose dependent manner

Figure 4.7: NAM affects Drosophila chordotonal neuron dependent calcium signaling

Figure 4.8: Heteromeric fly TRPV channel Nan/lav is activated by NAM.

Figure 5.1: *pnc-1* mutant has egg laying defect

Figure 5.2: *osm-9* and *ocr-4* mediate NAM-induced Egl phenotype in *C. elegans*

Figure 5.3: NAM induces a foraging defect in *C. elegans*

Figure 5.4: Nose touch defect

Figure S1: The *pnc-1* mutant does not have an osmosensory defect
LIST OF ABBREVIATIONS

NAD+ Nicotinamide adenine dinucleotide

NAM Nicotinamide

NA Nicotinic acid

pnc-1 Pyrazinamidase and nicotinamidase-1 gene

PNC-1 Pyrazinamidase and nicotinamidase-1 protein

OLQ Outer labial quadrant neurons

UV1 Uterine vulval one cells

OSM-9 Osmotic avoidance abnormal

OCR-4 Osm-9 and Capsaicin receptor-Related

TRPV Transient receptor potential channel, vanilloid subfamily

IAV Inactive

NAN Nanchung

TIRF Total internal reflection fluorescence

EC50 Half maximal effective concentration

EGL Egg laying defective

JO Johnston’s organ

JON Johnston’s organ neuron

L1-L4 First to fourth larval stages
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Chapter 1: Background and Introduction

1.1 Layout of the dissertation

This research work begins with investigating the genetic basis of cell death in a metabolic context using the *Caenorhabditis elegans* model organism. In Chapter 1, I review the genetic model *C. elegans* with an emphasis on its use in cell death research and introduce *C. elegans pnc-1* mutant as our model to study cell death. In Chapter 2, I report the discovery of a key role of a TRPV channel in mediating cell death in the *C. elegans pnc-1* mutant that accumulates the metabolite nicotinamide. In Chapters 3 and 4, I proceed to show that nicotinamide is an agonist for a TRPV channel in *C. elegans* (OCR-4/OSM9 channel) and *Drosophila melanogaster* (Nan/Iav channel) by using electrophysiology, TIRF photobleaching and behavior assays. In Chapter 5, I explore the physiological implications of nicotinamide agonist activity in *C. elegans*. Finally, in Chapter 6, the advances made in the field through this research are discussed and future directions are considered.

1.2 Introduction to *C. elegans*

*Caenorhabditis elegans* is a small (~1 mm long), non-parasitic nematode (Brenner, 1974; Riddle et al., 1997). In nature, it lives in microbe rich decaying organic matter such as
rotten fruits, stems, flowers and compost (Félix and Braendle, 2010). It was introduced as a model organism in the 1960’s by biologist Sydney Brenner (Brenner, 1974). In laboratories, C. elegans is grown and maintained at a very low cost on special agar plates (nematode growth media or NGM plates). The worm feeds on a layer of *Escherichia coli* bacteria spotted on the surface of agar on NGM plates. It produces hundreds of progeny every 3-4 days. *C. elegans* was the first multicellular animal to have its genome fully sequenced (*C. elegans* sequencing consortium, 1998). Many of its genes are conserved in higher organisms. *C. elegans* has five pairs of autosomes and one pair of sex chromosomes, and it comes in two sexes, hermaphrodite and male. The sex chromosome in hermaphrodite is XX and in male is XO. Unlike other genetic model organisms, it is effortless to maintain homozygous strain stocks of *C. elegans* because of the availability of the hermaphrodite sex. Strains can be frozen at very low temperatures and then recovered by thawing them, even after a decade (Brenner, 1974). Males naturally occur at a low frequency, but in the laboratory they are very useful as they can be mated with hermaphrodites to make genetic combinations of alleles (Fay, 2006). Various obvious phenotypic markers are available making genetic crosses quick and easy (Fay, 2006). The cuticle of *C. elegans* is transparent, so no sample preparation is required for visualization and a living animal can be directly mounted in buffer (M9) to observe the cells using the
Differential Interference Contrast (DIC) Microscopy. GFP tagging allows identification of specific cells using fluorescence microscope (Chalfie et al., 1994). GFP attached to a calcium sensor molecule allows monitoring of neuronal activity in a live animal (Miyawaki, 2003; Miyawaki et al., 1997). Foreign or ectopic genes can be expressed by producing transgenic strains obtained by injecting DNA into the gonad of the hermaphrodite animal. Injected DNA forms extra-chromosomal arrays that are transferred to some of the progeny producing a transgenic strain (Fire, 1986). Loss of function studies are made facile by RNAi techniques; animals can simply be fed with bacteria that produce dsRNA complementary to the mRNA of the gene we are interested in knocking down (Timmons and Fire, 1998).

1.3 *C. elegans* as a model for cell death

While using *C. elegans* as a genetic model organism has many benefits, it is particularly well suited for studying cell death. In *C. elegans*, when a cell starts to die, its shape, size and refractive properties begin to change. The transparent cuticle of the *C. elegans* allows monitoring of these changes using DIC microscopy to differentiate between an alive and a dying cell. Moreover, the *C. elegans* has an invariant cell lineage (Sulston and Horvitz, 1977; Sulston et al., 1983). Each cell is given a unique name and its location can be tracked at any given larval stage. Every
cell in the animal body follows a specific development plan from its birth to its final developmental fate or death. In fact, it was during the cell lineage studies when researchers first observed that some cells die during development (Sulston, 2002; Nikoletopoulou and Tavernarakis, 2014). So, they could predict the cell death event and monitor it in real time. These efforts lead to the discovery of the genetic pathway for apoptosis, which is conserved in higher eukaryotes (Conradt and Xue, 2005; Ellis et al., 1991; Nikoletopoulou and Tavernarakis, 2014; Sulston, 2002; Sulston and Horvitz, 1977). Later it was found that some cells in *C. elegans* die during the development but their death does not depend on the genetic machinery of apoptosis (Abraham and Shaham, 2004; Abraham et al., 2007). Such cells also differ significantly in their morphology compared to apoptotic cells. Further research lead to discovery of the necrotic pathway of cell death which is centered on ion imbalances and disrupted calcium signaling (Nikoletopoulou and Tavernarakis, 2014; Vlachos and Tavernarakis, 2010).

### 1.3.1 *pnc-1 mutant: Our model for cell death*

From a genetic screen of *C. elegans* mutant animals that are defective in laying eggs, a mutant *pnc-1* was obtained (Huang and Hanna-Rose, 2006; Vrablik et al., 2009). *pnc-1* is a gene that codes for the nicotinamidase enzyme PNC-1 (Pyrazinamidase and
nicotinamidase-1). PNC-1 converts nicotinamide (NAM) to nicotinic acid (NA) (Figure 1.1). NAM and NA are two different chemical forms of vitamin B₃ and both serve as precursors for biosynthesis of β-Nicotinamide adenine dinucleotide (NAD⁺). NAD⁺ is widely used as a coenzyme in many metabolic redox reactions and is a substrate for some NAD⁺ consumer enzymes like poly(ADP-ribose) polymerases (PARPs) and sirtuins. Therefore, NAD⁺ levels impact metabolic pathways and vital processes dependent on PARPs and sirtuins such as gene expression, calcium signaling and cell death (Ying, 2007). Concerted activity of PNC-1 and NAD⁺ consumer enzymes maintain steady state NAD⁺ levels. NAD⁺ consumer enzymes produce NAM as a byproduct of the reactions they catalyze, thus recycling NAM back in the NAD⁺ salvage pathway. NAM levels in turn regulate at least some NAD⁺ consumer activity by feedback inhibition (Bitterman et al., 2002; Landry et al., 2000).

![Figure 1.1: NAD⁺ biosynthesis pathway in C. elegans](image)

PNC-1 converts NAM to NA, which is then converted into NAD⁺ via a multistep pathway. NAD⁺ consumers use NAD⁺ as a substrate and release NAM as a byproduct.
Loss of PNC-1 enzyme can have many deleterious effects in C. elegans (Vrablik et al., 2009). pnc-1(pk9605) allele carries a nonsense point mutation that codes for a truncated PNC-1 protein. pnc-1 mutants have a highly penetrant cell death phenotype; four cells in the uterus of the animal abnormally die soon after they are born (Figure 1.2).

**Figure 1.2: uv1 cells die in pnc-1 mutants**

a) A worm cartoon showing the location of uterine vulval one (uv1) cells (arrows). b) Alive uv1 (arrows) cells in WT c) Dead uv1 (arrows) cells in pnc-1 mutant. Dying uv1 cells swell to become 3-4 times bigger in size than normal cells, their nucleus (arrow heads) changes morphology and moves to the periphery of the cell. There are four uv1 cells in an animal; only two uv1 cells are visible in this DIC image. The other two dead cells are on a lower focal plane. Scale bar is 10 μm.
1.3.2 Uv1 cells

There are four uterine vulval one (uv1) cells in the uterus of *C. elegans*. They are located on the ventral side of the uterus, sandwiched between uterine seam (utse) cells and vul F cells (Figure 1.3, see below) (Ghosh and Sternberg, 2014).

![Diagram of C. elegans uterus](image)

**Figure 1.3: uv1 cell are located in the uterus of *C. elegans*.**

A schematic of lateral view of fourth larval stage *C. elegans* uterus. Uv1 cells are located between utse cells and vulF cells. Only two uv1 cells are visible in lateral view. Figure is adapted from Developmental Biology, 396(1), Srimoyee Ghosh and Paul W. Sternberg, “Spatial and molecular cues for cell outgrowth during *C. elegans* uterine development”, 121–135, Copyright (2014), with Permission From Elsevier.

Uv1 cells regulate the timing of egg laying (Jose et al., 2007). Egg laying is a complex process regulated by concerted activities of excitatory and inhibitory neurotransmitters (Alkema et al., 2005; Horvitz et al., 1982; Jose et al., 2007; Trent et
al., 1983; Waggoner et al., 1998). Hermaphrodite specific motor neurons (HSN) and the uv1 neuroendocrine cells lie adjacent to each other in *C. elegans* ventral uterus. HSNs produce excitatory neurotransmitters serotonin and acetylcholine that promote egg laying (Croll, 1975; Horvitz et al., 1982; Schafer, 2005; Waggoner et al., 1998) whereas uv1s produce inhibitory neurotransmitter tyramine that inhibits egg laying. Tyramine elicits egg laying inhibition by acting on HSNs to prevent serotonin release (Alkema et al., 2005; Jose et al., 2007). Calcium influx from non-specific TRPV cation channels is proposed to regulate tyramine release from uv1 cells (Jose et al., 2007). A model for egg laying based on genetics and behavior data is as follows: uv1 cells express many homo or heteromeric TRPV ion channels (Figure 1.4). These ion channels function redundantly to bring calcium influx in the cytoplasm thus stimulating tyramine release. Soon after the L4 larva to adult molt occurs, eggs start to accumulate in the uterus of the hermaphrodite. Crowding of the eggs in the uterus stretches the uv1 plasma membrane, which is sensed by mechanosensory TRPV1 channels. Stretching closes the TRPV channels, ceasing the intracellular calcium influx and tyramine release. As the tyramine inhibition is relieved, HSNs start producing serotonin, which activates vulva muscle contraction and egg laying (Jose et al., 2007). Genetically blocking tyramine biosynthesis or inhibiting the TRPV channel function of by incorporating a dominant negative TRPV subunit in the ion channel,
causes premature egg laying (Alkema et al., 2005; Jose et al., 2007). G protein signaling also plays a role in the egg laying process (Jose et al., 2007), however it is beyond the scope of this work therefore it is briefly mentioned in Figure 1.4.
Uv1 cells produce tyramine (and other neurotransmitters) to inhibit egg laying until the eggs fill up the uterus. Crowding of eggs mechanically deforms the uv1 cells. Based on genetic evidence that TRPV and Ga proteins are required for uv1 tyramine release, the following mechanism is proposed: mechanical deformation either directly activates TRPVs (step 1) or directly activates GPCR signaling (step 2), following which TRPV and GPCR signaling either integrate or work in parallel to activate calcium signaling and tyramine release. Homo or heteromers made of various combinations of OCR-1, 2 and 4 (but not OSM-9) TRPV subunits are proposed to be involved in this process. Figure is modified from Genetics. 2007;175(1), Antony M. Jose, I. Amy Bany, Daniel L. Chase, and Michael R. Koelle “A Specific Subset of Transient Receptor Potential Vanilloid-Type Channel Subunits in Caenorhabditis elegans Endocrine Cells Function as Mixed Heteromers to Promote Neurotransmitter Release”, 93-105, Copyright (2007)
1.3.3 Uv1 cell specification requires EGF signaling

Uv1 cells are present in the *C. elegans* hermaphrodite uterus and they regulate the egg-laying process. In the pnc-1 mutant animals however, these cells die before acquiring uv1 cell fate. Therefore, to study why these cells dies prematurely in the in pnc-1 mutant, it is important to review the developmental signaling involved in uv1 cell differentiation. During the *C. elegans* third larval stage, the anchor cell (AC) signals the Pn.p cells (ventral epidermis cells) that lie underneath it to adopt vulval cell fate (Figure 1.5a) (Newman and Sternberg, 1996; Newman et al., 1995). 3-4 hours later, the AC induces ventral uterine intermediate precursor cells to adopt π and ρ cell fate (Newman et al., 1995). π cells then divide asymmetrically to produce a smaller and a larger daughter cell (Figure 1.5a). At the fourth larval stage, a vulva cell called vulF that lies beneath the π progeny cells produces LIN-3 EGF signal. This signal acts on the EGF receptor present on the four outermost π progeny cells to induce uv1 cell fate (Chang et al., 1999; Newman et al., 1995). The rest of the π progeny cells fuse to form a multinucleate utse cell (Figure 1.5b)
1.3.4 EGF signaling overcomes uv1 cell death in pnc-1 mutant

In the pnc-1 mutant, pre-uv1 cells die right before adopting the mature uv1 cell fate (Huang and Hanna-Rose, 2007). Since EGF signaling is required for pre-uv1 to uv1 cell differentiation, previous researchers in my lab investigated the role of EGF signaling pathway in the pnc-1 induced-uv1 death. They found that overactive EGF
signaling achieved by gain-of-function mutation of EGF receptor LET-23/RTK or by overexpression of EGF ligand LIN-3, suppresses uv1 cell death in pnc-1 mutant (Huang and Hanna-Rose, 2006). However, the mechanism of EGF rescue is still not completely understood. Two known downstream effector pathways of EGF signaling in C elegans are the RAS pathway and ITR-1 pathway (Clandinin et al., 1998; Iwasa et al., 2010; Parry and Sundaram, 2014; Sundaram, 2006; Yin et al., 2004; Yu and Driscoll, 2011). Loss of function of LET-60 (RAS) and MPK-1 (ERK) by RNAi partially reverts EGF rescue of the pnc-1 uv1 death phenotype; therefore LET-60 is required in this function. However, the let-60 (n1046) gain-of-function mutant has no effect on the pnc-1 uv1 death phenotype, individually, or with gain-of-function ITR-1 (IP3 receptor). So, LET-60 may not be sufficient for EGF rescue of pnc-1 uv1 death. Other let-60 gain-of-function alleles are reported to have distinct effects in different tissues. Thus, these alleles should also be tested for activity in this assay. Other LET-60 alleles may show a different result than let-60(n1046) in rescue of pnc-1 uv1 death. Gain-of-function or loss-of-function of ITR-1 (IP3 receptor) has no effect on EGF rescue of pnc-1 uv1 death (Matt Crook, submitted). These results are insightful in deciphering the pathways downstream of the insult that triggers uv1 cell death. However, the insult that inflicts uv1 death in the pnc-1 mutant is still unknown and is the main focus of my research.
1.3.5 *pnc-1* mutant has perturbed metabolite levels

To understand why uv1 cells die in the *pnc-1* mutant, previous researchers in my lab explored the differences in wild type animal and the *pnc-1* mutant. Because PNC-1 converts NAM to NA in the salvage pathway for NAD$^+$ biosynthesis, loss of PNC-1 can be expected to block this pathway resulting in the accumulation of its substrate metabolite NAM and depletion of its product NA (Vrablik et al., 2009; Wang et al., 2015). Thus, the defects in *pnc-1* mutants can be attributed to either the lack of NA/NAD$^+$ or to the excess NAM levels or a combination of two. Tracy Vrablik showed that growing wild-type *C. elegans* in NAM supplemented NGM plates induces the uv1 cell death phenotype with a very high penetrance, just like we see in a in *pnc-1* mutant animal (Vrablik et al., 2009). On the other hand, supplementing NA to *pnc-1* mutants does not rescue the uv1 cell death phenotype. This observation lead to the conclusion that the uv1 cell death phenotype of *pnc-1* mutants is caused by accumulation of NAM levels in the worm body and was not due to lack of NA.

Subsequently, LC-MS measurements showed that indeed relative NAM levels are approximately 19 fold higher in *pnc-1* mutants compared to wild-type *C. elegans* (Figure 1.6) (Wang et al., 2015)
1.3.6 Uv1 cell death is not apoptosis

Apoptosis and necrosis are two major types of cell death. In *C. elegans*, the difference between apoptotic and necrotic cell morphology is very distinct and can be identified easily using Nomarski optics. Apoptotic cells shrink in size and have button-like morphology (Figure 1.7a) whereas necrotic cells swell 2-3 times.
in size and look like a vacuole with a nucleus moved to the periphery (Figure 1.7b).

**Figure 1.7: Uv1 cell death resembles necrotic morphology**

a) Seven cells dying by apoptosis in a *C. elegans* embryo are shown by red arrows, they look like raised buttons, scale bar is 5 µm. b) Two touch receptor neurons in the tail of a L2 larva dying by necrosis. Necrotic neurons are swollen and their nucleus has moved to the periphery of the cell pointed by white arrows, scale bar is 100 µm. c) Dead uv1 cells in the uterus of mid L4 stage *C. elegans*. Dead uv1 cells resemble necrotic morphology, they appear swollen and the nucleus has moved to the periphery of the cell pointed by white arrows, scale bar is 10 µm. Figure a) is adapted from bioprotocols, 3(9), Yan Zhang, Haibin Wang and Xiaochen Wang “Quantification of Cell Corpses, Cell Death Occurrence, Cell Corpse Duration”, and Figure b) is adapted from Cell Death and Differentiation, 15, ES Blum, Monica Driscoll and Shai Shaham “Noncanonical cell death programs in the nematode Caenorhabditis elegans”, 1124-1131, Copyright (2008), with Permission From Nature Publishing Group.

The genetic pathway of apoptosis was first reported to exist in *C. elegans* and later found to be conserved in higher mammals (McCall, 2011). Scientists observed that some specific cells in the *C. elegans* embryo invariably die during development. Genetic screens for mutants in which cell death fate was altered lead to the understanding that death by apoptosis is genetically regulated and is
a part of normal development of *C. elegans*. CED-3 and CED-4 caspases (cysteine-aspartate protease) were discovered as the key enzymes of apoptotic cell death pathway (Abraham and Shaham, 2004; Yuan et al., 1993). To test if uv1 cell death is executed by apoptosis, ced-4 caspase was knocked down by RNAi, no effect was observed on the uv1 death phenotype (Huang and Hanna-Rose, 2006). This suggests that uv1 cell death does not depend on the canonical apoptotic pathway.

### 1.3.7 Uv1 cell death resembles necrosis

Unlike apoptosis, necrosis is not part of normal *C. elegans* development. It often occurs due to an internal or external insult overwhelming enough to kill the cells. Studies in various necrosis models have brought to consensus a common underlying theme for execution of necrosis in *C. elegans*. The necrotic pathway can be divided into three steps 1) ion imbalance 2) deregulated calcium signaling 3) activation of degradative proteases (Figure 1.8, see below).
MEC-4 (d) (d stands for degeneration) hyperactive ion channel induced necrosis is a canonical example (Hong and Driscoll, 1994; Syntichaki and Tavernarakis, 2003). The mec-4 gene codes for a pore forming unit of an ion channel expressed in mechanosensory neurons of C. elegans. An amino-acid substitution in mec-4(d) mutant results in a constitutively active ion channel (Bianchi et al., 2004; Brown et al., 2007; Goodman et al., 2002; Hong and Driscoll, 1994). MEC-4 channel

![Figure 1.8: necrotic cell death pathway in C. elegans](image)

Various necrotic signals bring influx of Ca²⁺ in cytosol activating calpains. Calpains enzymatically degrade lysosomal membranes, spilling their contents in the cytosol, which decreases the cytoplasmic pH. Aspartyl proteases get activated in acidic intracellular milieu causing protein degradation and eventually cellular demise. Other parallel necrotic pathways are alleged to exist because genetically eliminating the Ca²⁺-calpain pathway leaves some residual necrotic death in various C. elegans necrotic models. Figure was adapted from Current Biology, 15(13), Popi Syntichaki, Chrysanthi Samara, Nektarios Tavernarakis “The Vacuolar H⁺-ATPase Mediates Intracellular Acidification Required for Neurodegeneration in C. elegans, 1249-1254, Copyright (2005), with Permission From Elsevier.
conducts Na\(^+\) and Ca\(^{2+}\) (Bianchi et al., 2004). An abnormal influx of ions activates calcium-dependent calpain proteases that act on the lysosomal membrane, breaking it open. The lysosomal contents spill out, which decreases the cytosolic pH and activates aspartyl proteases that work in low pH conditions (Syntichaki et al., 2002, 2005). This leads to protein degradation beyond the cell’s ability to repair leading to cellular demise. DEG-1(d) and DEG-3(d) are other examples of aberrant ion channels that induce necrosis (Berger et al., 1998; Blum et al., 2008; Korswagen et al., 1997; Yassin et al., 2001). pnc-1 uv1 death phenotype is partially rescued by the loss of function of calpain proteases clp-1 and aspartyl proteases asp-3 and asp-4 (Huang and Hanna-Rose, 2006). This indicates a role of ion imbalance and in turn, mis-regulation of proteases in the execution of uv1 death downstream of the NAM induced insult.

### 1.3.8 ER calcium signaling may not be required for uv1 cell death

Release of Ca\(^{2+}\) from ER is an important step in mec-4(d) induced necrosis (Xu et al., 2001). Influx of extracellular Ca\(^{2+}\) in the cytoplasm through constitutively active MEC-4(d) channel is sensed by ryanodine receptors present on the ER. Ryanodine receptors bind to Ca\(^{2+}\) ions in their proximity and open, releasing more Ca\(^{2+}\) from ER stores, thus increasing the cytoplasmic Ca\(^{2+}\) levels in a
positive feedback loop. Manipulating ER calcium signaling by pharmacology or genetics to reduce or prevent ER calcium release in cytosolic pool partially prevents mec-4(d) induced necrosis (Nagarajan et al., 2014; Xu et al., 2001). EGTA treatment chelates cytoplasmic calcium thus sequestering it (Nagarajan et al., 2014). Dantrolene treatment inhibits ER Ca\(^{2+}\) release by binding and antagonizing ryanodine receptors (Kobayashi et al., 2009). However, treatment with EGTA or dantrolene has no effect on the pnc-1 uv1 cell death phenotype (Matt Crook, unpublished). Similarly, genetically manipulating calcium signaling by loss of function of crt-1 (ER chaperone and Ca\(^{2+}\) binding protein) or cnx-1 (ER membrane bound Ca\(^{2+}\) binding protein) did not affect the pnc-1 uv1 cell death phenotype (Matt Crook, unpublished).

1.4 Summary of introduction and aims of this study

*C. elegans* has proved to be a remarkable genetic model organism for cell death research (McCall, 2011). Therefore, the highly penetrant uv1 cell death phenotype in a NAD\(^+\) salvage pathway mutant *pnc-1* presented an opportunity to investigate cell death in the context of a metabolic mutant. Uv1 cell death is not like the apoptosis observed in *C. elegans* development because it does not depend on caspase proteins (Huang and Hanna-Rose, 2006). The *pnc-1* induced uv1
death phenotype shares two similarities with canonical mec-4(d) induced necrosis; a) the dead uv1 cells have necrotic morphology like mec-4(d) induced necrosis (Figure 1.7) b) like mec-4(d) induced necrosis, uv1 death is partially rescued by knocking down calpain proteases clp-1 and aspartyl proteases asp-3 and asp-4 (Huang and Hanna-Rose, 2006; Syntichaki et al., 2002). However, unlike mec-4(d) induced necrosis, ER calcium signaling may not play a significant role in uv1 death in pnc-1 mutants. pnc-1 mutants accumulate NAM (Wang et al., 2015). Exogenous NAM application to wild type animals recapitulates in pnc-1 induced uv1 cell death, therefore NAM is the cause of uv1 death in pnc-1 mutants (Vrablik et al., 2009). Based on this background information and the premise that necrosis is genetically regulated and hence can be prevented by genetic manipulation, I began my research to seek answers to the following three specific questions:

1) How does excess NAM trigger uv1 cell death in pnc-1 mutant animal?
2) What are the genes involved in uv1 cell death pathway?
3) Why is NAM-induced cell death limited to specific cells?
Chapter 2: A TRPV channel mediates NAM induced cell death

2.1 OLQ cells die in pnc-1 mutants

To investigate which genes mediate NAM induced cell death, I aimed to pursue a genome-wide RNAi screen in search of genes whose knock down would rescue uv1 necrosis in the pnc-1 background. For unambiguous high throughput screening of uv1 cells, I needed to construct a transgenic strain with a GFP marker only in the uv1 cells and nowhere else. In the absence of a uv1 exclusive promoter, I settled upon the ocr-4 promoter which was previously reported to express in the uv1 and in two other locations {in the spermatheca and in outer labial quadrant (OLQ) neurons} (Jose et al., 2007). So, I made the transgenic strain psEx279 (ocr-4p::gfp::ocr-4 3’UTR). In my transgene, the GFP was expressed in uv1 cells in the uterus and OLQ neurons in the head. Meanwhile, my colleague Matt Crook was investigating the role of the EGF pathway in uv1 cell death rescue (reviewed in section 1.3.4). He observed the occasional presence of vacuoles resembling the swollen uv1 corpses in the head of some young larvae (Figure 2.1b). Because of the invariable cell lineage in C. elegans, researchers have successfully delineated the location and identity of each cell in the head/nerve
ring region. Matt took advantage of the neuronal map of *C. elegans* (www.wormatlas.org) and the availability of cell specific GFP markers to identify the dying cell type in the head of *pnc-1* mutants. Intriguingly, among all the cell specific GFP markers chosen by Matt, the only marker that labeled the dying cells in the nerve ring was *ocr-4p::gfp*. As mentioned earlier, *ocr-4* promoter expresses exclusively in the uv1 cells in uterus and OLQ neurons in the head. Thus, Matt concluded that the dying cells in the head are OLQ cells. No other head neurons are affected morphologically in *pnc-1* mutant because there are no corpses that lack the GFP label in the *ocr-4p::gfp; pnc-1* animals (Figure 2.1c and c’).
Figure 2.1: OLQ cell death in \textit{pnc-1} mutants resembles \textit{uv1} cell

a) uterine \textit{uv1} cells and the b) dying cells in the nerve ring in \textit{pnc-1(pk9605)} null mutants (strain HV560) have a similar necrotic morphology. The cell bodies swell to many times normal size (arrows), while the nuclear membrane disintegrates and the nucleus moves to the cell periphery (arrowheads). c) DIC image and c’ corresponding fluorescence image of a \textit{pnc-1} mutant carrying the \textit{pnc-1; psEx279[ocr-4p::GFP]} transgene. The dying cells in the region of the nerve ring (arrows) are marked by GFP. For comparison to swollen cells, multiple cells of normal morphology and size are annotated in a, b and c with an asterisk directly above the nucleus. Scale bars in all panels are 10 µm.
2.1.1 An introduction to the OLQ neurons

*C. elegans* can sense numerous chemical and mechanical stimuli using neurons that reside in the four chemosensory organs of the animal; Amphid, phasmid, inner and outer labial organs. OLQ neurons are ciliated sensory neurons that are part of the outer labial lateral sensilla. They are present at the end of the anterior lobe of the pharynx (Figure 2.2). OLQ neurons function in imparting behaviors like foraging and nose touch response (Chatzigeorgiou and Schafer, 2011; Hart et al., 1995; Kaplan and Horvitz, 1993; Kindt et al., 2007).

Figure 2.2: Structure of OLQ cells

There are four OLQ cells (red) in the head of the worm, only two are seen in the schematic. The pharynx of the worm is shown in green. OLQ cell bodies are located at the end of the anterior lobe of the pharynx. Their dendrites of OLQ cells protrude to reach the nose tip of the animal. Primary cilia present at the distal tip of the dendrites are exposed to the environment. OLQDL=OLQ on Dorsal Left, OLQVL=OLQ on Ventral Left. Figure was adapted from www.wormatlas.org
2.1.1.1 OLQ neurons are required for foraging behavior

Foraging is the process of exploring for food by making small and quick left and right head movements as the animals move on bacterial food lawn (Kindt et al., 2007). When OLQ neurons are removed by laser ablation, these foraging head movements become abnormally slow because the animals start making exaggerated head bends at the nose tip (Hart et al., 1995; Kindt et al., 2007). Another type of neuron called IL1 (inner labial 1) also plays a role in foraging (Figure 2.3) (Hart et al., 1995). OLQ and IL1 sensory neurons synapse with the RMD motor neuron in the foraging neuronal circuit (Figure 2.3) (Hart et al., 1995; Kindt et al., 2007). TRPA-1 ion channel function in the OLQ neurons is required for mediating foraging behavior (Kindt et al., 2007). When animals are touched on the dorsal or ventral side of the anterior tip/nose with an eye lash hair, they withdraw their heads away from the hair. This behavior is called head withdrawal response and is also dependent on OLQ and IL1 sensory neurons (Figure 2.3) (Hart et al., 1995; Kindt et al., 2007). I discuss more about OLQ mediated foraging behavior in Chapter 5.
2.1.1.2 OLQ neurons are required for nose touch mechanosensation

Nose touch response is the behavior observed when the anterior tip/nose of the animal collides gently with an object (like an eyelash hair) in its path; the animal stops and reverses the direction of its movement. By laser ablation of single and multiple neurons at a time, it was discovered that three ciliated sensory neurons
are required for nose touch response behavior; ASH, OLQ and FLP neurons (Kaplan and Horvitz, 1993). Removing ASH cells results in 65% animals defective in nose touch response when compared to 10% animals in untreated WT animals. FLP and OLQ neuron ablation causes 37% and 16% nose touch response defect respectively (Kaplan and Horvitz, 1993). Based on this data, it was concluded that ASH and FLP neurons have major contribution to nose touch response and they sense nose touch autonomously, whereas OLQ neurons have a minor contribution to this response. In 2002, Tobin and colleagues further showed that ASH neurons require OSM-9/OCR-2 TRPV channel for gentle nose touch mechanosensation (Tobin et al., 2002).

However, the nose touch neuronal circuit was recently revised based on in vivo calcium imaging and behavior experiments (Chatzigeorgiou and Schafer, 2011). In the new model, the role of ASH neurons in nose touch sensing remains the same, but the OLQ neurons are shown to have more contribution than previously suggested. Wild-type animals show robust calcium transients in OLQ neurons in response to nose touch, which is completely abolished in OLQs of osm-9 null mutants. OLQ specific OSM-9 expression restores the nose touch evoked calcium transients in the OLQ neurons of osm-9 null mutant animal
Thus OLQs require OSM-9 in a cell autonomous fashion for nose touch evoked calcium transients. FLP neurons also express OSM-9 and require it for nose touch response (Chatzigeorgiou and Schafer, 2011). There is a decrease in nose touch induced FLP calcium transients in osm-9 null mutant. However, cell specific rescue experiments show that OSM-9 expression in OLQ neurons but not in FLP neurons restores nose touch induced FLP calcium transients. This suggests that OSM-9 function in OLQ neurons contributes to the nose touch induced calcium spike in the FLP neuron. OLQ and FLP neurons share gap junctions and perhaps the non-cell autonomous rescue of the FLP nose touch calcium response occurs through electrical signaling between OLQ and FLP neurons. This is supported by the decrease in nose touch evoked FLP neuronal calcium transients in the unc-7 mutant that has defective gap junctions (Chatzigeorgiou and Schafer, 2011). Thus, the new model suggests that OLQ neurons robustly respond to nose touch and are a major contributor to the FLP cell nose touch response. In this revised nose touch sensory circuit, another set of neurons called CEP neurons also contribute to FLPs gentle nose touch response (Figure 2.4) (Chatzigeorgiou and Schafer, 2011). FLP neurons also mediate harsh touch response mediated by DEG/ENaC channel MEC-10 that is beyond the scope of this research and is not
discussed here (Chatzigeorgiou and Schafer, 2011). I will discuss more about OLQ mediated nose touch response in Chapter 5.

Figure 2.4: Neuronal circuit for gentle nose touch response in C. elegans

ASH, FLP and OLQ cells are required for nose touch response. FLP and OLQ neurons share gap junctions through RIH interneuron (dashed lines). ASH functions parallel to OLQs and FLPs in nose touch circuit. ASH and OLQ cells express OSM-9 that mediates nose touch response. Solid lines represent Chemical synapses and triangles signify presynaptic terminals. Figure is adapted from Neuron, 70(2), Marios Chatzigeorgiou and William R. Schafer, “Lateral Facilitation between Primary Mechanosensory Neurons Controls Nose Touch Perception in C. elegans”, 299-309, Copyright (2011).
2.1.2 OLQ cell death in pnc-1 mutants is a progressive process

A healthy OLQ neuron expresses bright ocr-4p::gfp in its cell body and all the way to the distal dendritic tip (Figure 2.5a). Observing OLQ GFP in various larval stages in pnc-1 background reveals that OLQ cell death takes place in a progressive manner. In pnc-1 mutants, the dendrites of OLQ neurons start blebbing towards the end of the L3 stage but before the L4 molt. At L4 stage, the blebbed dendrites eventually fragment. Dots of GFP appear in what used to be a bright linear GFP pattern in an intact dendrite (Figure 2.5b). At L4 and later stages, OLQ cell body begins to swell, loses its characteristic shape and becomes bigger and rounded, a characteristic morphology of mec-4 (d) cell death and uv1 death (Figure 2.5b and Figure 2.1b).

Figure 2.5: OLQ neurons die by progressive degeneration in the pnc-1 mutant

a) The dendrites of the OLQ cell extend to the tip of the nose, visualized in a wild-type animal carrying the ocr-4p::GFP transgene. b,b’) Fluorescence images of the b) late L3 and b’) L4 stage of the same pnc-1; psEx279[ocr-4p::GFP] animal,
demonstrating that death is a degenerative process involving b) first the blebbing of the dendrites b’) followed by their disappearance as the cell bodies round up and begin to disappear. The image in b’ is overexposed relative to b. Scale bars in all panels are 10 µm. (Images taken by Matt Crook).

2.1.3 OLQ cell death is less penetrant than uv1 cell death in pnc-1 mutants

In pnc-1 mutants uv1 cells die with a very high penetrance (Huang and Hanna-Rose, 2006; Vrablik et al., 2009). OLQ cell death penetrance on the other hand, is very low. The absence of ocr-4p::gfp in older animals revealed that 22% of OLQ cells are missing by late larval stages in pnc-1 mutants (Figure 2.6).

![Figure 2.6: Quantification of the cell deaths in pnc-1 null mutants](image)

uv1 cell death (black bars) was scored in late L4 according to the absence of cells with the inls179[ida-1::GFP] marker (strain HV560) and OLQ (gray bars) in late L3 using the psEx279[ocr-4p::GFP] marker (strain HV695). Actual percent missing cells and sample size (number of animals examined) is indicated on each bar. Error bars are 99% confidence intervals (not applicable to 0%). *p <0.001, calculated using Fisher’s exact test. (Data collected by Matt Crook).
Growing wild-type animals on NGM plates supplemented with 25 mM NAM recapitulates uv1 necrosis phenotype of \textit{pnc-1} (Huang and Hanna-Rose, 2006; Vrablik et al., 2009). Therefore, I tested if NAM can induce OLQ cell death phenotype of \textit{pnc-1} mutant in wild-type animals. Indeed OLQ cells die in wild-type animals treated with NAM (Figure 2.7). NAM-induced death is not a response to a change in osmolarity because treatment with 1 M NA (another form of vitamin B\textsubscript{3} and product of PNC-1 enzymatic reaction) has no effect on uv1 or OLQ cells (Figure 2.7). NAM kills uv1 cells more efficiently than it kills OLQ cells at each concentration (Figure 2.7). Treatment of wild type animals with 500mM or higher NAM increases the OLQ cell death penetrance significantly more compared to what is observed in \textit{pnc-1} mutant (22\% OLQ death in \textit{pnc-1} mutant vs 77\% death in 1mM NAM) (Figure 2.6, Figure 2.7). The cuticle of \textit{C. elegans} is highly impermeable, so the effective concentration required to kill the cells could be a lot less than the applied concentration.
2.1.4 Cell death in response to acute NAM treatment is rapid

Previously it was reported that wild-type animals raised on 25 mM NAM for a generation have uv1 cell death (Vrablik et al., 2009). I found that application of a high concentration (50 mM to 1 M) of a drop of NAM induces cell death remarkably rapidly (Figure 2.8). In some animals, I saw the first morphological signs of death; nuclear membrane disintegration, within 50 seconds of NAM
application (Figure 2.8). Compared to uv1 death, OLQ cell death response to acute NAM application is somewhat delayed but still very rapid (Figure 2.8).

Figure 2.8: Cell death in response to acute NAM treatment is a rapid process. Each dot represents an individual animal and the position on the X-axis represents the time after NAM addition when the first evidence of cell death, nuclear disintegration, was evident. Boxes show the upper and lower quartile values, solid and dashed vertical lines indicate the median and mean of the population distribution, respectively. The dotted rhombus indicates one standard deviation, and error bars indicate the maximum and minimum of the population distribution. Two of the data points for OLQ were statistical outliers and not included in the statistical analysis.
2.1.5 *ocr-4* mutation blocks NAM induced cell death

The expression pattern of *ocr-4*p::gfp caught my attention because it was exclusively limited to the two cell types that die. I wondered whether this is mere co-incidence or perhaps *ocr-4* has a functional significance to the death program. To answer this question, I treated *ocr-4* mutant animals with 1mM NAM (acute treatment) and observed the uv1 and OLQ cell fate. To my surprise, there was no cell death in the NAM treated *ocr-4* mutants (Figure 2.11).

*ocr-4* codes for OCR-4 protein, a TRPV channel subunit. TRPV (Transient Receptor Potential Vanilloid) channels are a member of TRP (Transient Receptor Potential) channel superfamily (Montell, 2005). They consist of subunit proteins with six trans membrane domains that form homo or hetero tetramers. These channels allow mono and/or divalent cations through a pore in the channel lined by the 5\(^{th}\) and 6\(^{th}\) transmembrane domain of each of the four subunits in the tetramer (see Figure 2.9 below ).
A functional TRPV1 (VR1) channel is a tetramer formed by homomeric subunits, shown as red cylinders. Each tetramer consists of six trans membrane (TM) domains, as shown in the inset. TM 5 and 6 of each subunit line the ion-conducting pore. Both N and C terminal of the peptide are intracellular. Various stimuli like temperature (>43 °C), pH (< 5.5), capsaicin, lipids etc can activate TRPV1 channels. Figure was adapted from Chemical Society Reviews, 37(8), S. J. Conway “TRPing the switch on pain: an introduction to the chemistry and biology of capsaicin and TRPV1”, 1530–1545, Copyright (2008), with Permission From Royal Society Of Chemistry

2.1.6 An introduction to TRPV channels

In the year 1997, first TRPV channels were discovered simultaneously in mammalian cell culture and C. elegans by two independent groups (Caterina et
The newly discovered mammalian TRP channel TRPV1 (previously named VR1) was activated by compounds containing vanilloid moiety, like capsaicin and resinferatoxin, so it was named VRI (Vanilloid Receptor 1) and the subfamily was named Transient Receptor Potential Vanilloid (TRPV) after them (Caterina et al., 1997). The *C. elegans* TRPV channel however does not respond to capsaicin. The first discovered *C. elegans* TRPV channel was named OSM-9 because it was found in a screen for mutant animals that were defective in sensing high osmolarity (Colbert and Bargmann, 1995). Later OSM-9 mutants were found to be defective in other sensory modalities like mechanosensation, chemosensation and adaption to certain volatile odors (Colbert and Bargmann, 1995). TRPV channels are polymodal receptors, which means they are activated by a variety of different types of stimuli (Figure 2.9). TRPV1 receptor can be activated by high temperature (>43 °C), certain pH (ranges between 5 and 6), and by a diverse variety of chemicals (Figure 2.9). In fact many of the spicy and pungent flavors from foods and drinks like chilli peppers, black pepper, ginger, vinegar, cloves, alcohol etc are tasted via TRPV1 channels present in our mouth (Simon, 2005; Trevisani and Szallasi, 2011).
2.1.7  *C. elegans* TRPV homologs

There are five homologs of TRPV channels in *C. elegans* namely; OSM-9, OCR-1, OCR-2, OCR-3, OCR-4 (Goodman and Schwarz, 2003). The OCR proteins were found by homology searches after the founding member OSM-9 was discovered. OCR stands for OSM-9/ capsaicin receptor related proteins. Among the five TRPV subunits, OSM-9 is the most widely expressed and has the most attributable functions (Tobin et al., 2002; Xiao and Xu, 2009). TRPV channels function in sensory neurons. *C. elegans* shows a gamut of sensory behaviors. Three of many such behaviors are: a) Chemosensation: animals can smell volatile chemicals and taste water soluble chemicals (Colbert and Bargmann, 1995) b) Osmosensation: when encountered with high a osmolarity solution like 1M glycerol, animals turn their heads away and change their path (Colbert et al., 1997) c) Mechanosensation: when touched lightly on the tip of their nose or on the anterior half of the body, animals reverse and when touched on the posterior half of the body, they accelerate forward (Kaplan and Horvitz, 1993).

Scientists have assigned sensory functions to specific neurons by observing animal behavior after ablating neurons by laser microsurgery (Avery et al., 1989). Here I will discuss three neurons (AWA, AWC, ASH neurons) and their sensory functions in context of OSM-9 TRPV channel.
2.1.7.1 A brief review of OSM-9 TRPV channel functions in *C. elegans*

AWA and AWC neurons are part of amphid chemosensory organ, they are required for sensing some attractant volatile chemicals (Bargmann et al., 1993). AWA neurons sense diacetyl and pyrazine and AWC neurons sense benzaldehyde, butanone, and isoamyl alcohol. ASH neurons, also part of amphid sensilla, are polymodal in nature and sense many different noxious stimuli. They are required for chemosensation (repellent volatile chemicals), mechanosensation (gentle touch to the nose), osmosensation and for sensing acidic pH (Bargmann et al., 1990, 1993; Chatzigeorgiou et al., 2010; Kaplan and Horvitz, 1993; Troemel and Bargmann, 1995). Alleles of *osm-9* were discovered independently in two separate screens of animals defective in osmosensation and mechanosensation (Colbert et al., 1997). *osm-9* alleles are defective in sensing all the behaviors imparted by AWA and ASH neurons (Colbert et al., 1997; Xiao and Xu, 2009). The structure and morphology of neurons are intact in *osm-9* mutants, so the sensory defects in *osm-9* mutants are not due to problems with neuronal development (Bargmann et al., 1993). Noxious stimuli that are sensed by ASH neurons via OSM-9 initiate calcium spike indicating a role of Ca\(^{2+}\) signaling in these behaviors (Hilliard et al., 2005).
Mammalian TRPV4 channel was cloned based on sequence homology to OSM-9 (Xu et al., 2003). TRPV4 is a functional homolog of OSM-9 because its expression in ASH neurons rescues osmosensation and mechanosensation defects (Liedtke et al., 2003). OSM-9 also functions in chemosensory adaptation, which is independent of chemosensation (Colbert and Bargmann, 1995; Tobin et al., 2002). Chemosensory adaption is a complex process where animals pre-exposed to high concentrations of a given chemical show decreased chemotaxis behavior for several hours. Removing the adapted animal away from the given odor for several hours can reverse the adaptation. *osm-9* mutants show defect in adaptation to AWC sensed odorant isoamyl alcohol and butanone. This adaptation defect is not a result of defective osmosensation in *osm-9* mutants because other osmolarity mutant behave normally to isoamyl adaptation (Colbert and Bargmann, 1995).

### 2.1.7.2 OSM-9 TRPV subunit forms heteromeric complexes with OCR subunits

Though OSM-9 occasionally exists as the only TRPV subunit in few cells, majority of the OSM-9 expressing cells also express one or more OCR subunits (Figure 2.10) (Tobin et al., 2002). OSM-9 and OCR-2 share the most functions; they are proposed to form a heteromer involved in chemosensation,
osmosensation and mechanosensation (de Bono et al., 2002; Colbert et al., 1997; 2003a; Kahn-Kirby and Bargmann, 2006; Roayaie et al., 1998; Tobin et al., 2002; Zhang et al., 2004). OSM-9 and OCR-2 localization in cilia is required for their function in ASH neurons are they mutually depend on each other for cilia localization (Tobin et al., 2002). Expression level of ocr-2 is decreased in osm-9 mutant. Low ocr-2 expression in osm-9 mutant is attributed to the instability of the protein in absence of its heteromeric partner (Tobin et al., 2002). In cells where OSM-9 is expressed alone, it is localized in the cell body of the neuron (Colbert et al., 1997; Tobin et al., 2002). Few osm-9 functions are ocr-2 independent and vice versa (Jose et al., 2007; Tobin et al., 2002).

Based on the C. elegans TRPV channel literature, I hypothesized that since most C. elegans TRPV channels function as heteromers, OCR-4 might also have one or more heteromeric partners, and loss of function of the partner subunit/subunits may also block or ameliorate the NAM induced cell death phenotype just like what we see in ocr-4 mutant.
OSM-9, OCR-1 and OCR-2 are co-expressed with OCR-4 in uv1 cells, and OSM-9 is co-expressed with OCR-4 in OLQ (Jose et al., 2007; Tobin et al., 2002). Thus, I predicted that OSM-9 might be required as the heteromeric partner for OCR-4 in NAM-induced necrosis. I subjected osm-9 mutants to acute NAM treatment.

Indeed, like ocr-4, loss of osm-9 also completely prevents NAM-induced death (Figure 2.11). I also examined the other ocr genes and a related TRPA1 gene, trpa-
$I$, for a role in mediating NAM-induced death. However, mutation of these genes had no effect on death of either cell type (Figure 2.11).
Figure 2.11: OCR-4 and OSM-9 mediate NAM-induced uv1 and OLQ cell death

The percentage of animals with dead uv1 cells (black bars) or OLQ cells (gray bars) for each genotype supplemented with 25 mM NAM (+NAM) or in combination with pnc-1(pk9605) is reported. Actual percentages and sample sizes are indicated on each bar. Reported values for NAM supplemented animals are the average of at least 3 experiments and error bars are standard deviation. Error bars for the pnc-1 double mutant strains represent 95% confidence intervals of the proportion of the population (not applicable to values of 0 or 100%). The wild-type strain used is N2. All alleles are loss or reduction of function with the exception of ocr-2(vs29) which is a dominant negative allele. *p <0.001, calculated using Fisher’s exact test.
2.2 Discussion

Uv1 and OLQ cell types die in *pnc-1* mutant due to excess NAM. A fortunate choice of GFP marker led me to the *ocr-4* gene that exclusively expresses in the two cell types (uv1s and OLQs) that die in the *pnc-1* mutants. I discover that the presence of *ocr-4* and its homolog *osm-9* in a cell is required for NAM induced necrosis. The *ocr-4* and *osm-9* genes code for TRPV channel subunits, which are thought to function as heteromers in *C. elegans* (Jose et al., 2007). OLQ cell death morphology resembles that of NAM induced uv1 necrosis and *mec-4(d)* induced necrosis (Figure 2.1 and Figure 1.7). However, there are differences in the penetrance of uv1 and OLQ cell death phenotypes in both *pnc-1* mutants and NAM treated animals (Figure 2.6 and Figure 2.7). It is unclear why the OLQ response is less robust than that of uv1. OSM-9 and OCR-4 have strong expression in both uv1 and OLQ cells (Colbert et al., 1997; Tobin et al., 2002, and my observation). So, the difference in ion channel expression level does not appear to be the cause of differences in the penetrance of death. Uv1 cells express OSM-9, OCR-1, OCR-2, OCR-4 where as OLQ cells express only OSM-9 and OCR-4. It is possible that co-expression of multiple OCR subunits in uv1 cells makes it more susceptible to NAM induced death (Figure 1.4). Or there could be
other cell-type specific factors that need to be investigated to answer this question.

How might OCR-4 and OSM-9 mediate cell death?

Ion imbalance and deregulated Ca\(^{2+}\) signaling can initiate necrosis in *C. elegans* (Nagarajan et al., 2014; Syntichaki et al., 2002). *ocr*-4 and *osm*-9 genes code for TRPV channel subunits that form non specific cation channels. *C. elegans* TRPV subunits are proposed to function as heteromers (Jose et al., 2007; Tobin et al., 2002). Based on this information, I hypothesize that OCR-4 and OSM-9 form a heteromeric cation channel that is directly or indirectly activated by NAM causing influx of ions inside the cell and triggering necrosis. This hypothesis can explain why NAM kills uv1 and OLQ cells exclusively while other cells appear to be unaffected, and it is because OCR-4 and OSM-9 co-express only in uv1 and OLQ cells. Because NAM induced cell death is very rapid, I also hypothesize that most likely NAM directly binds and activates the OCR-4/OSM-9 TRPV channel. In next two chapters, I test this hypothesis using electrophysiology in *Xenopus laevis* oocytes expression system.
2.3 Materials and methods

2.3.1 Caenorhabditis elegans culture and strains

Strains were grown under standard conditions with OP50 E. coli as a food source at 20°C (Brenner, 1974). Strains used in this study are following:

BL5715 inls179[ida-1::GFP] II (Zahn et al., 2001)

HV560 inls179[ida-1::GFP] II; pnc-1(pk9605) IV – null allele (Vrablik et al., 2009)

CX10 osm-9(ky10) IV (Colbert et al., 1997)

LX950 ocr-4(vs137) IV (Jose et al., 2007)

HV720 unc-119(ed3) III; psEx279 (GFP expression present in OLQ and uv1 cells)

HV695 pnc-1(pk9605) IV; psEx279[ocr-4::GFP]

HV784 inls179[ida-1::GFP] II; pnc-1(pk9605) ocr-4(vs137) IV

HV832 inls179[ida-1::GFP] II; pnc-1(pk9605) osm-9(ky10) IV

CX4533 ocr-1(ok132) V (Tobin et al., 2002)

LX671 ocr-2(vs29) IV (Jose et al., 2007)

VM396 ocr-2(ak47) IV (Tobin et al., 2002)

RB1734 ocr-3(ok1559) X (Consortium, 2012)

RB1052 trpa-1(ok999) IV (Kindt et al., 2007)
2.3.2 psEx279 transgene construction

I created an ocr-4p::gfp transcriptional fusion to label the uv1 cells for other purposes and used it in this study as an OLQ neuron marker. I amplified 1015 bp upstream of the start site of ocr-4

(ocr-4 F1 5’GCATGCCACTCAACAACCCATTTGC and
ocr-4 R1 5’ GGATCCTAATACAAGTTAGATTCAGAGAATATTTTACT) and ligated the product to pPD95.69 (Addgene plasmid 1491) using Sph I and Bam HI. I subsequently amplified 570 bp of the ocr-4 3’ UTR

(ocr-4 F2 5’ GAATCTTTTTTTTTACTGTTCATTTCTTCTCCCTAAA and
ocr-4 R2 5’ ACTAGTTTGATAAGATAACATTCCACTCGTTAG) and ligated to the construct above using Eco RI and Spe I. After sequence confirmation, I injected 100 ng/µl plasmid into unc-119(ed3) with 60 ng/µl of unc-119(+) DNA (Maduro and Pilgrim, 1995). The resulting transgene is psEx279.

2.3.3 Phenotypic Analysis

2.3.3.1 Uv1 and OLQ cell death by fluorescence microscopy

inIs179[ida-1::GFP](Zahn et al., 2001) and psEx279[ocr-4p::GFP] mark the four uterine uv1 cells and the four OLQ neurons, respectively. I scored uv1 and OLQ
cell death by examining late L4 (uv1) and late L3 (OLQ) animals for the presence of living (no sign of degeneration) bright GFP+ cells. Data is reported as percent of dead cells = \{(n \times 4)\text{– cells alive}\} \times 100/(n \times 4), where n is the total number of animals scored (Figure 2.6).

### 2.3.3.2 Uv1 and OLQ cell death by DIC microscopy

Late L3 (OLQ) or L4 animals (uv1) were mounted in a drop of 1 M NAM on a 2% agarose pad on a slide and were incubated for at least two minutes. Animals were then counted for presence/absence of cell corpses over twenty minutes. Data is reported as percent of animals with dead cells = (Animals with at least one cell corpse present \(\div n\)) \times 100, where n is the total number of animals scored (Figure 2.7).

### 2.3.4 Time course experiment

One wild-type animal was mounted on a slide with 2% agarose in M9 medium and topped with a cover slip. The animal was observed for the correct stage (L4 and late L3 animals for uv1 and OLQ death, respectively) and healthy cells pre-treatment. The cover slip was then lifted, a drop of 1 M NAM was added (time 0) and the cover slip was replaced. Time was recorded when the first evidence of
cell death (nuclear disintegration) was observed. If no cell death was observed for 20 minutes, the assay was stopped (Figure 2.8).

2.3.5 Statistical Analysis

Statistical tests and sample sizes for each experiment are described in figure legends with relevant $p$ values. The statistical tests were two-tailed. Statistical analyses were performed in GraphPad Software.

Author Contributions

In this section, Matt Crook (Wendy Hanna-Rose lab) discovered and identified OLQ cell death in $pnc-1$ mutants (Figure 2.5, Figure 2.6). I performed rest of the experiments (Figure 2.7, Figure 2.8, Figure 2.11).
Chapter 3: NAM is an agonist for OCR-4/ OSM-9 TRPV channel

3.1 NAM directly binds and activates the OSM-9 / OCR-4 channel

The rapid execution of death upon acute NAM treatment and the striking morphology of the dying cells is reminiscent of excitatory death due to direct membrane depolarization caused by degenerin mutations in C. elegans (Figure 1.7) (Vlachos and Tavernarakis, 2010). Because OSM-9 channels mediate the influx of divalent cations (Lindy et al., 2014), I hypothesized that NAM is an agonist for a channel containing OSM-9 and OCR-4 subunits and that excessive activation of the channel may lead to excitotoxicity. I therefore expressed OSM-9 and OCR-4 individually and in combination in Xenopus oocytes to use electrophysiology to test whether the resulting channel could be activated by NAM (Figure 3.1a). Indeed, NAM activates a large current selectively in oocytes co-expressing OCR-4 and OSM-9 (Figure 3.1b,c). The $K_{1/2}$ value for NAM, derived from a Hill fit of current size, is $63.3 \pm 13.2$ mM (Figure 3.1d). This value suggests that OCR-4/OSM-9 channel activation will be elevated in pnc-1 mutants given the difference in NAM concentrations found in lysates from wild-type (15.7 mM) and pnc-1 mutant (88 mM) animals (Wang et al., 2015). The agonist activity of
NAM is not shared by the related metabolite NA; 100 µM NAM was greater than 100-fold more potent than 130 mM of NA (Figure 3.1e). Therefore, the nicotinamide form, but not the nicotinic acid form, of vitamin B₃ is a potent agonist for a TRPV channel consisting of OSM-9 and OCR-4 subunits.

Figure 3.1: NAM activates the heteromeric OCR-4/OSM-9 channel in *Xenopus* oocytes

A schematic diagram of the two-electrode voltage clamp experiment and voltage ramp protocol. Currents were recorded in response to 2 second voltage ramps from -100 to +100 mV from a -20 mV hold. b,c,d) Large NAM-activated currents were observed in oocytes co-expressing OCR-4 or OSM-9, but not in oocytes expressing each subunit individually. Current size was measured at -100 mV (star), and the half maximal concentration (K₁/₂) for current activation by NAM
was determined from the Hill plot shown in Figure 3.1d. n indicates the Hill coefficient. The data in Figure 3.1d show mean $\pm$ S.E.M ($n$ (sample size) = 5-9 oocytes per NAM concentration), and the red curve is a Hill equation fit. Data from individual oocytes were normalized to the current amplitude observed at 1 mM NAM prior to comparison. e) 5 mM nicotinic acid did not elicit large currents in OSM-9/OCR-4-expressing oocytes (-.154 ± .018 $\mu$A, $n$ = 6 oocytes), but there is a slight increase in current upon exposure to 130 mM NA (-.269 ± .026 $\mu$A, $n$ = 4). Though this is a statistically significant increase (p<.05, two-tailed t-test), 130 mM NA still elicited over 200-fold less current than 100 $\mu$M NAM (-36.5 ± 1.0$\mu$A, $n$ = 4). Experiment was conducted in collaboration with Tim Jegla lab at Penn State University. cRNA were made by Awani Awani and electrophysiology was performed by Tim Jegla and Aditya Pisupati.

### 3.2 The OSM-9/OCR-4 channel is comprised of two of each subunit

The heteromeric structure of *C. elegans* TRPV channels is predicted based on genetics and co-localization data but there is no direct evidence to support it because previous attempts to express *C. elegans* TRPV channels in heterologous systems have failed (Colbert et al., 1997; Jose et al., 2007; Tobin et al., 2002). From electrophysiology experiments we determined that the NAM responsive functional channel is a heteromer made of OSM-9 and OCR-4 subunits. The ability to detect active channels provided us an opportunity to examine the stoichiometry of subunits in the active heteromeric channel, an unanswered question regarding the *C. elegans* TRPV channels. To determine stoichiometry, we collaborated with the Tim Jegla lab (Biology Department at Penn State) and the William Hancock lab (Department of Biomedical Engineering at Penn State).
and used an established TIRF microscopy photobleaching assay that involves counting the number of labeled subunits in channels that successfully traffic to the plasma membrane (Nakajo et al., 2010; Ulbrich and Isacoff, 2007). In this chapter, I will introduce the principle of total internal reflection and the TIRF photobleaching experiment followed by the results.

3.3 Total internal reflection microscopy- Photobleaching

3.3.1.1 Total internal reflection

Glass is more optically dense than water therefore light travels slower in glass than water. A light beam travelling from glass to water bends at the glass-water interface due to change in its speed that takes place at the interface. The angle between the incident beam and the normal (line perpendicular to the surface at the point of incidence) is called the angle of incidence $\theta_i$, and the angle between the refracted beam and the normal is called the angle of refraction $\theta_r$ (Figure 3.2).

If $\theta_i$ is increased gradually, at a certain angle all the light will get reflected back in the denser medium instead of getting refracted in the rarer medium. The angle at which total light is reflected in the denser media is called the critical angle $\theta_c$ and this phenomenon is called total internal reflection (Figure 3.2).
Figure 3.2: A schematic of TIRF microscopy principle

The red line indicates the critical angle $\theta_c$. A) When the angle of incidence $\theta_i$ is below $\theta_c$, some light is refracted into the water at an angle $\theta_r$. This refracted light excites all of the fluorophores throughout the sample. B) When the angle of incidence $\theta_i$ is above critical angle $\theta_c$, the light beam undergoes total internal reflection back into the glass at an angle $\theta_r$ and an evanescent field is generated near the surface of the sample (shown as fading light blue color). The evanescent field can only stimulate the fluorophores within ~100 nm of the glass-water interface before it disappears in space. The fluorophores present in the sample further away from the interface are not excited achieving a high contrast image of the near surface region. Figure is adapted from Cold Spring Harb Protoc, 2010(3), Samara L. Reck-Peterson, Nathan D. Derr, and Nico Stuurman “Imaging single molecules using total internal reflection fluorescence microscopy (TIRFM)”, Copyright (2010), with Permission From Cold Spring
3.3.1.2 TIRF microscopy

TIRF microscopy is based on the principle of total internal reflection and it allows us to image a thin near membrane section of a live-cell at a single molecule resolution. In our TIRF microscopy experiment, the denser media is glass (culture dish), the rarer media is aqueous cytoplasm of a *Xenopus* oocyte placed on the glass dish. The laser light beam used to illuminate the sample hits the glass at an oblique angle greater than the critical angle $\theta_c$ and total internal reflection takes place (Figure 3.2b). While the light does not propagate into the oocyte, an electromagnetic field is produced right above the glass-oocyte membrane interface. This field decays exponentially as the distance from the interface increases; therefore this field is called evanescent (quickly fading) (Figure 3.2b). Because the evanescent field is very thin in depth, only the oocyte membrane and a thin section very close (~100 nm) to it gets illuminated (Figure 3.2b). Thus the whole oocyte is not flooded with light as it happens in an epi-fluorescence microscopy.
3.3.1.3 TIRF microscopy-Photobleaching

Photobleaching is a phenomenon when high intensity light (photons) alters a fluorophore molecule (e.g. GFP) such that it becomes irreversibly non-fluorescent. A GFP molecule bleaches in one discrete step, in an all or none manner. If there are several GFP molecules present in a protein complex, each GFP will photo-bleach independently. This allows us to count the number of GFP tagged subunits that are present in a multimeric ion channel by counting the steps required to completely bleach a single ion channel complex (Yamamura et al., 2015).

Xenopus oocytes were co-injected with OSM-9 and OCR-4. In each oocyte, either OSM-9 or OCR-4 was tagged with GFP. Then using TIRF microscopy, the near membrane region was illuminated to selectively excite the GFP in the membrane while the GFP in the background further inside the cell were not excited. The high intensity laser photobleaches the GFP in the selected membrane region and the photobleaching events were recorded in a movie. Images were processed as explained in section 3.5.2.
The OCR-4 subunit was labeled via fusion of GFP to the C-terminus and OSM-9 subunit was labeled via fusion of GFP to the N-terminus. GFP fusions did not interfere with the assembly or function of channels because NAM induced current was obtained in *Xenopus* electrophysiology of the labeled ion channel complex. Expression of OCR-4::GFP with OSM-9 or GFP::OSM-9 with OCR-4 at 1:1 RNA ratios each resulted in NAM-responsive channels (average currents of 18.50 +/- 2.56 mA, n=5 eggs, and 25.00 +/- 2.86 mA, n=6, in response to 100 mM NAM, respectively). As a control for the TIRF method, we quantified the distribution of channels bleaching in one to four steps using a known homotetrameric potassium channel, Kv2.1, tagged with GFP (Figure 3.3 a). Based on this data, we calculate a GFP detection efficiency of 0.69 in our TIRF system (Figure 3.3 a), a value consistent with other published studies (Kitazawa et al., 2014; Ulbrich and Isacoff, 2007; Yu et al., 2009). Upon injection of OCR-4::GFP or GFP::OSM-9 alone, no validated channels were observed at the plasma membrane (Figure 3.4b). However, when we expressed OCR-4::GFP with OSM-9 or GFP::OSM-9 with OCR-4 at 1:1 RNA ratios, we observed many putative channels at the plasma membrane (Figure 3.4a). Photobleaching of these channels occurred in a predicted stepwise fashion in either one or two steps.
(Figure 3.3b,d and Figure 3.4a), suggesting that a maximum of two of each subunit inserts into the heteromeric channels.
Figure 3.3: Stoichiometry of OSM-9/OCR-4 channels

a) Frequency of the number of bleaching steps observed for 113 channels from oocytes expressing GFP-Kv2.1 homotetramers. The predicted step count
distribution assuming 0.69 GFP detection probability is shown (black symbols) is a best least squares fit of the data. b) Frequency of the number of bleaching steps observed for 71 channels from oocytes expressing GFP::OSM-9/OCR-4. The inset shows example fluorescent traces for two-step (top) and one-step (below) photobleaching. c) Stoichiometry distribution calculated from the observed one and two-step event totals in (b), assuming a 0.69 GFP detection efficiency. d) Frequency of the number of bleaching steps observed for 66 channels from oocytes expressing OSM-9/OCR-4::GFP. e) Stoichiometry distribution calculated from one and two-step events in (d), assuming a 0.69 GFP detection efficiency.

Experiment was done in collaboration with Tim Jegla lab and William Hancock lab at Penn State University. cRNA were made by Awani Awani, oocytes were prepared and injected by Aditya Pisupati (Jegla lab) and TIRF microscopy photobleaching was performed by Keith J. Mickolajczyk (Hancock lab).

We quantified the distribution of channels bleaching in one and two steps (Figure 3.3b,d). Then, using the GFP detection efficiency value (0.69) determined with our control (Figure 3.3a), we calculated that greater than 90% of channels adopt a 2:2 stoichiometry in each of the two reciprocal experiments (Figure 3.3 c, e). We did observe one spot that bleached in three steps for each experiment (Figure 3.3b,d). Thus, we cannot rule out the formation of a small proportion of channels with 3:1 or 1:3 OSM-9:OCR-4 stoichiometry. However, the majority of channels observed in this functional expression experiment are comprised of two of each subunit (Figure 3.3 c, e).
Figure 3.4: Sample images and example analyses from TIRF photobleaching assay for OCR-4::GFP

a) Oocytes injected with OCR-4::GFP and OSM-9 displayed many fluorescent spots on the membrane at the beginning of the observation period (top panel, time 0, representative frame of 300 square pixels from movie). Valid GFP-labeled channels are expected to photobleach in discrete steps. Moreover, photobleaching is permanent and fluorescence from a valid channel spot, which was photobleached, should not reappear. Thus, spots that failed to bleach (none in this field of view), lost fluorescence in a non-stepwise fashion (V, outlined in yellow, with corresponding trace below), even if followed by a distinct bleaching event (III), or behaved erratically by “blinking” or losing and regaining fluorescence (IV) were excluded from the analysis because they either were not channels or represented events in which photobleaching steps could not be reliably counted. Every spot that was excluded from the analysis is indicated with a yellow outline. Every spot that was included as a final data point is
indicated by a blue or green outline. These spots photobleached in one (I, outlined in green with corresponding trace below) or two-step events (II). In contrast, when we ran similar experiments on homotetrameric channels formed by GFP::Kv2.1, spots bleaching in 1, 2, 3, or 4 discrete steps were observed. Most spots were no longer visible at the end of the observation period (bottom panel, time 4 min). b) In contrast to the oocytes injected with both subunits, there are few fluorescent spots on the membrane at the beginning of the observation period (top panel, time 0) in oocytes injected with OCR-4::GFP alone. Those present either failed to photobleach (none in this field of view) or fluoresced erratically over the course of the observation (I, outlined in yellow with corresponding trace below) suggesting that they were not GFP-labeled channels. Keith J. Mickolajczyk (Hancock lab at Penn State University) performed TIRF photobleaching experiments and the analysis.

3.4 Discussion

NAM treatment elicits remarkably rapid death on uv1 and OLQ cells. Loss of OCR-4 or OSM-9 TRPV subunit blocks the NAM induced death. Since TRPVs are nonspecific cation channels that can cause membrane depolarization and ion imbalance is linked to necrosis, I hypothesized that NAM activates TRPV channels to cause depolarization and cell death. I tested this hypothesis to be true in Xenopus oocyte electrophysiology assays. Attempts by other groups to achieve heterologous expression of C. elegans TRPV channel have not succeeded (de Bono et al., 2002; Colbert et al., 1997; Jose et al., 2007; Tobin et al., 2002). OSM-9 and OCR-2 expression alone, or in combination, has not resulted in IP3, thapsigargin, or voltage gated currents (de Bono et al., 2002; Colbert et al., 1997; Jose et al.,
2007; Tobin et al., 2002). Therefore, it was suggested that there might be components necessary for making a functional channel that are yet undiscovered and missing in the *Xenopus* expression system. However, my results suggest that there is no strict requirement for other non-TRPV subunits for trafficking or activity as had been suggested. NAM agonist activity shows that functional heteromeric channels can be obtained in heterologous systems if the appropriate combination and ratio of TRPV subunits are present. Our results also provide insight into NAM interaction with the channel. The maximum value of a Hill coefficient is predictive of the number of binding sites for a ligand on its receptor; therefore it is likely that there are at least two NAM binding sites on the OSM-9:OCR-4 channel. Combined with our stoichiometry experiments, these data suggest that NAM might bind to only one of the subunits (Figure 3.1 and Figure 3.3). The development of this heterologous system will provide an important tool for further structure/function studies of *C. elegans* TRPV channels. I have already used this discovery to show for the first time that functional OSM-9/OCR-4 channels most likely have two of each subunit in the active channel.
3.5 Materials and methods

3.5.1 *Xenopus* oocyte expression and electrophysiology

cRNAs were made from *osm-9* cDNA (in vector pGEMHE, gift of Dr. C. Bargmann, Rockefeller University) and *ocr-4* cDNA (gift of Dr. Y. Kohara, National Institute of Genetics, Japan, transferred to pOX (Jegla and Salkoff, 1997) using mMessage Machine kits (Ambion, TX) and injected at ~50 ng/oocyte. Oocyte preparation, injection and culture have been described (Li et al., 2015a; Martinson et al., 2014). Recordings were made one to three days after injection at room temperature using standard two electrode voltage clamp techniques as described (Li et al., 2015b) in a solution containing (in mM): 98 Na+, 2 K+, 1 Mg2+, 1 Ca2+, 104 Cl−, 5 HEPES (pH 7.5 with NaOH).

3.5.2 Total internal reflection microscopy (TIRF)

Total internal reflection microscopy was performed on a Nikon TE-2000 inverted microscope outfitted with a 60x N.A. 1.45 objective (Nikon). An 80 mW argon ion laser (Spectra Physics) was used for illumination, and an iXon Ultra 888 EMCCD (Andor Technology) camera was used for detection. Movies were acquired at 5 frames per second using Micro-Mananger software (http://www.micro-manager.org). Background-corrected fluorescence intensity signals for single
channels were acquired from raw movies using a custom-built, semi-automated analysis tool coded in MATLAB (Mathworks), which summed counts in a 7 pixel diameter circle drawn around candidate channels, and photobleaching steps were determined using a step-finding algorithm (Chen et al., 2014). Bleach event detection efficiency was calculated from a least squares fit of the step count assuming four GFPs/channels and a binomial distribution of missed events. OCR-4/OSM-9 stoichiometry was reverse-calculated from the aggregate step counts in the same manner using the Kv2.1 detection efficiency value (0.69), a value consistent with other published studies (Kitazawa et al., 2014; Ulbrich and Isacoff, 2007; Yu et al., 2009). Vitelline envelopes were mechanically removed as previously described (Jegla et al., 2012) prior to TIRF, and only stationary spots were analyzed.

### 3.5.3 Statistical Analysis

Statistical tests and sample sizes for each experiment are described in figure legends with relevant $p$ values.
**Statement of Collaboration**

I conducted cloning and cRNA synthesis. Electrophysiology recordings were performed by Aditya Pisupati (Tim Jegla lab). I assisted Aditya in the electrophysiology experiments. Keith J. Mickolajczyk (William Hancock lab) performed the TIRF photobleaching experiments and its analysis.
Research in *Drosophila melanogaster* has contributed vastly to our current understanding of TRP channel function. In fact, it was the observation of a spontaneously generated mutant blind fly in the year 1969, that lead to the discovery of the TRP channel superfamily of proteins (Hardie, 2014). The mutant fly showed a transient (instead of sustained) potential in its photoreceptor cells in response to light, which caused blindness in the fly (Hardie, 2014). The Transient Receptor Potential (TRP) channel superfamily derives its name from this blind mutant fly phenotype.

Both *C. elegans* TRPV subunits OCR-4 and OSM-9 have orthologs in *Drosophila* namely Nanchung (Nan) and Inactive (Iav) respectively (Figure 4.1). Like OCR-4 and OSM-9, Nan and Iav co-localize in the cilia of sensory neurons (Figure 4.3) and their co-expression is mutually required for their proper cilia localization and function (Gong et al., 2004; Tobin et al., 2002). *Drosophila* requires Nan and Iav function in chordotonal neurons for sensing mechanical stimuli like sound, gravity and proprioception (sensing the location of body parts relative to the body).
Figure 4.1: Phylogeny of the TRPV ion channel subfamily

Vertebrate and invertebrate TRPV channels group in separate clusters. *C. elegans* OSM-9 and *Drosophila* Inactive are orthologs and *C. elegans* OCR-4 and *Drosophila* Nanchung are orthologs. Figure adapted from Martinac Boris, “Sensing with ion channels”, ISBN: 978-3-540-72683-8, Copyright (2008), with Permission From Springer –VerlagBerlin/Heidelberg.

### 4.1.1 Chapter outline

In this chapter I aim to examine the effect of NAM on the Nan/Inav channel. For this, I first test the effect of NAM on hearing/mechanosensation in *Drosophila* larvae, a behavior that requires function of the Nan/Inav channel. In the second part, I use the *Xenopus* oocyte expression system to directly test NAM binding on Nan/Inav channel.
I begin with an introduction to the structure and function of chordotonal neurons and the molecules involved in *Drosophila* hearing/mechanosensation system followed by the results and discussion.

### 4.2 *Drosophila* chordotonal neurons: structure and function

Chordotonal organs are mechanosensory organs located all over the body of an adult fly and larva. They sense various mechanosensory stimuli including sound, wind, gravity and proprioception (Eberl, 1999). The fundamental unit of a chordotonal organ is called scolopidium. A chordotonal organ can contain anywhere from one to several hundred scolopidia. Each scolopidium encases one or more chordotonal neurons (Figure 4.3). There are multiple chordotonal organs in every segment of a developing *Drosophila* embryo. In this project, I chose to study abdominal chordotonal organs in *Drosophila* third instar larva (Figure 4.2) because of the available literature and the ease of identification and imaging of these chordotonal organs (Nesterov et al., 2015; Zhang et al., 2013). Eight chordotonal organs (Figure 4.2) occur repeatedly in the lateral or ventral side of each abdominal segment of the *Drosophila* larvae (Orgogozo and Grueber, 2005). The chordotonal organs are named based on their location and the number of scolopidia present in them. (For example, LCh1 = Lateral Chordotonal organ with 1 scolopodium) (Figure
In the calcium imaging experiments, I focused on the lateral chordotonal organ 5 (LCh5) to examine the effect of NAM on chordotonal neurons (Figure 4.2).

**Figure 4.2: Arrangement of abdominal chordotonal organs in the *Drosophila* larvae**

A 10X magnification of an abdominal segment. There are eight chordotonal organs in each abdominal segment. The lateral chordotonal organ 1 (LCh1), ventral chordotonal organs (VChA and VChB) are each made up of a single scolopidia (only VChB is visible here). The lateral chordotonal organ 5 (LCh5) is made up of five scolopidia that lie parallel to each other. The neurons (N) are labeled with the neuronal marker MAb 22C10 (red). The ligament (L), cap (C) and attachment cells (LA, CA) are labeled with anti-αTub-85E (blue). The cap and ligament cells are additionally labeled with a Chordotonal organ specific GFP reporter. Figure is adapted from Halachmi, N., Nachman, A., Salzberg, A. Visualization of Proprioceptors in Drosophila Larvae and Pupae. J. Vis. Exp. (64), (2012).
4.2.1 Ion channels required for hearing in *Drosophila*

In this section, I provide a brief review of *Drosophila* hearing system, thus demonstrating that since Nan/Iav heteromeric channel is required for hearing, it provides a testable phenotype to study the evolutionarily conserved nature of NAM TRPV agonist activity.

For decades there has been a search for the ion channel that transduces the mechanical signal from sound vibrations into neuronal electrical signals. A great deal of our current knowledge about ion channels required for hearing comes from the studies conducted on insect chordotonal neurons especially the largest chordotonal organ of *Drosophila* called Johnston’s organ (JONs). There are TRP proteins from three subfamilies expressed in JONs; TRPA (Painless and Pyrexia), TRPN (NompC) and TRPV (Nan/Iav). NompC (No mechanoreceptor potential C) is localized in the distal part of the dendritic cilia of JONs (Figure 4.3) (Liang et al., 2011; Zhang et al., 2013). Nan and Iav co-localize and heteromerize to form a mechanosensory channel in the proximal part of JONs cilia (Figure 4.3) (Gong et al., 2004; Kernan, 2007). Result from various studies suggest the idea that NompC expressed in the distal cilia of JONs may form the mechano-transducer channel for hearing in *Drosophila* whereas Nan/Iav channel plays a role in amplification and
propagation of the signal (Cheng et al., 2010; Effertz et al., 2011, 2012; Goepfert et al., 2006; Gong et al., 2013; Kamikouchi et al., 2009; Lee et al., 2010; Liang et al., 2011). However, data from a recent study argues against NompC as the mechano-transducer in Drosophila hearing (Gong et al., 2004; Kim et al., 2003; Lehnert et al., 2013). Instead they suggest that Nan/lav may form a transduction complex or at least it is required for the function of an unknown protein that forms the transduction complex (Gong et al., 2004; Kim et al., 2003; Lehnert et al., 2013). In any case, it is accepted that Nan/lav heteromeric channel is required for sensing sound vibrations in Drosophila.
4.2.2 Startle–freeze behavior in response to sound

The ability to sense sound vibrations has two functions critical for survival success of *Drosophila*; 1) mating to reproduce 2) escaping from predators (Greenspan, 2000; Ritchie and Kyriacou, 1994; Zhang et al., 2013). Sensing sound requires JO in adults (Eberl et al., 2000). Larvae can also sense sound by chordotonal neurons (Zhang et
al., 2013). The sound produced by natural predators of *Drosophila* like wasps and yellow jackets is mostly pure tone frequency of range 400-500 Hz. When a larva encounters natural sounds in this range, they display startle-freeze response (Zhang et al., 2013). A characteristic startle-freeze response can be divided into three steps: 1) stopping of a crawling larva immediately in response to the sound, 2) retraction of its mouth hook 3) and excessive side to side turning of the head. Blocking of chordotonal neuronal function by expression of tetanus toxin abolishes the startle response behavior in *Drosophila* larva (Zhang et al., 2013). Startle behavior in response to sound is reduced in NompC mutants and abolished in Iav and Nan mutants (Zhang et al., 2013). 500 Hz/70Db stimulus induces a calcium spike in the larval chordotonal neurons with peak signals at the dendrite tip and axon segment proximal to cell body (Zhang et al., 2013). Larva can respond to frequencies in the range of 100–1,000 Hz with peak responses in the middle frequencies of this range (Zhang et al., 2013).

### 4.3 Results

#### 4.3.1 NAM abolishes startle response in *Drosophila* larvae

Since *Drosophila* TRPV proteins Nan and Iav are orthologous to NAM responsive OCR-4 and OSM-9 proteins respectively, I hypothesized that treatment of *Drosophila*
larva with NAM would either kill the chordotonal neurons that co-express Nan/Iav channels by constitutive activation of the channel, as in *C. elegans* or NAM would over stimulate the channels rendering them inactive for sensing sound vibrations without killing the cells. In both cases, the startle behavior in *Drosophila* larva mediated by Nan/Iav channel in response to sound stimulus would be affected.

**Figure 4.4: NAM diminishes *Drosophila* larva startle response to sound in a dose dependent manner**

3rd instar larva were pretreated with different concentrations of NAM or 1 M NA as a control for 10 minutes then exposed to a 500 Hz tone at 90 dB and scored according to presence or absence of a startle response. Data is presented as percent of animals demonstrating a startle response. Error bars are 95% confidence intervals. Actual percentages and sample sizes are indicated above each bar. **p <0.0001, *p <0.0003 calculated using Fisher’s
Consistent with my hypothesis, treatment of wild-type third instar Drosophila larvae with NAM, but not NA, diminishes their response to sound vibrations (Figure 4.4), similar to loss of either nan or iav function (Gong et al., 2004). To examine whether the diminished response to sound stimulus in larva was due to chordotonal neuronal death or because of their dysfunction, I decided to visualize the chordotonal neurons by using genetically encoded calcium sensor GCaMP6. For this, I collaborated with Matt Shorey from Melissa Rolls’ lab at Penn State University. We observed no sign of cell death in chordotonal neurons even after 30 minutes of treatment with 1 M NAM (n=6)
Figure 4.5: NAM affects Drosophila chordotonal neuron dependent calcium signaling

a) Quantification of fluorescence intensity in resting cells compared to paired NAM-treated or buffer treated controls. Quantification of images taken during stimulation and rest periods indicate that fluorescence intensity of chordotonal neurons expressing GCaMP6 (b) increases in response to mechanical stimulus (b'), increases in response to application of 1 M NAM (b''), but fails to further increase in response to stimulus after application of NAM (b'''). Images are individual frames from a movie. Scale bar is 10 mm. Fiji’s "Fire" Heat map LUT (lookup table) has been applied to images for visualization of intensity differences. c) The change in relative fluorescence intensity in response to mechanical stimulus is plotted for untreated and NAM-treated animals. For c and d, error bars are standard error of the mean. **p < 0.0001, *p < 0.0003
Because the chordotonal neurons failed to die, we were able to examine the effect of NAM on their signaling responses using GCaMP6 fluorescence. We first established that application of NAM to the animals elicits a signaling response; GCaMP6 fluorescence increases in the LCh5 neurons upon application of NAM, indicating an increase in cytosolic calcium (Figure 4.5b, b'' and c). We next tested if NAM influences the ability of the chordotonal neurons to respond directly to the stimuli. We established that a mild mechanical (vibrational) stimulus to 3rd instar larvae results in a rapid and robust calcium influx in the LCh5 neurons, as revealed by a transient increase in GCaMP6 fluorescence (Figure 4.5a, b and b'). However, pre-application of NAM to the cells effectively prevents any subsequent response to mechanical stimuli (Figure 4.5b'', b''' and c).

Finally, once again in collaboration with Tim Jegla lab, we examined the activity of NAM on the Nan/ Iav channel directly using the heterologous Xenopus expression system (Figure 4.6). As with co-expression of OCR-4 and OSM-9, we detect a large current in response to NAM in oocytes co-expressing Nan and Iav (Figure 4.6a,b). The $K_{1/2}$ value for NAM, derived from a Hill fit of current size, is $14.4 \pm 1.0$ mM calculated using a two-tailed t test. This experiment was done in collaboration with Melissa Rolls Lab at Penn State University, in vivo Drosophila calcium imaging experiments were performed by Matthew Shorey.
(Figure 4.6b). Again, the agonist activity of NAM is not shared by the related metabolite nicotinic acid (NA) (Figure 4.6c), and neither Nan (n=6 injections) nor Iav (n=5 injections) alone produced active channels.

![Image of Figure 4.6](image)

**Figure 4.6: Heteromeric fly TRPV channel Nan/Iav is activated by NAM.**

a) An example trace of the effect of NAM on the Nan/Iav channel. Large NAM-activated currents were observed in oocytes co-expressing Nan and Iav, but not in oocytes expressing each subunit individually (not shown). Currents were recorded in response to 2 s voltage ramps from -100 to +100 mV from a -20 mV hold. Current size was measured at -100 mV (star), and the half maximal concentration ($K_{1/2}$) for current activation by NAM was determined from a Hill plot. (n = 5-13 per NAM concentration). b) Nan/Iav channel appears to have a four to five-fold increase in affinity for NAM compared to the OSM9:OCR4 channel (see Figure 3.1 in Chapter 3). c) Current is not induced in Nan/Iav channel by 5 mM nicotinic acid (-.188 ± .020μA, n = 3); There is a slight increase in the conduction of the Nan/Iav channel upon exposure to 130 mM NA (-.422 ± .040μA, n = 4)). Though this is a statistically significant increase (p<0.01, two-tailed t-test), this high (5mM) concentration of NA elicited over 150 fold less current compared to only 100 μM NAM (-.28.7 ± 2.5μA, n = 3). Experiment was conducted in collaboration with Tim Jegla lab at Penn State University. cRNA were made by Awani Awani and electrophysiology was performed by Aditya Pisupati.
4.4 Discussion

In this section I have determine that nicotinamide (NAM) form of vitamin B₃ is an evolutionarily conserved agonist of an invertebrate heteromeric TRPV channel. In *C. elegans*, NAM causes constitutive activation of OCR-4/OSM-9 ion channel leading to death of neurons that express this channel. Similarly, in *Drosophila*, NAM causes dysfunction of chordotonal neurons that express heteromeric TRPV Nan/Iav ion channels by over-stimulating them. Normally, in response to vibration stimulus, LCh5 neurons show a calcium spike in wild-type flies. NAM treatment induces a bright calcium spike after which a vibration stimulus cannot initiate a calcium spike anymore. Over-stimulation caused by NAM renders the chordotonal neuron inactive. The mechanism of action of NAM is reminiscent of the mode of action of insecticide pymetrozine (PM) on chordotonal neurons of *Drosophila* and *locusts* (Jessica Ausborn, Harald Wolf and Kayser, 2005; Nesterov et al., 2015). In next two subsections of this discussion, I will review the use of PM as an insecticide and how NAM has a potential to be used as an insecticide.

4.4.1 Pymetrozine and pyrifluquinazon are agonists for Nan/Iav TRPV channel

PM was known to be an effective target specific insecticide for some decades (Maienfisch et al., 2012). However the reason for its target specificity was
discovered only recently. The discovery that PM acts on chordotonal neurons in insects like *Locust migratoria* and *Drosophila* lead to the search for a receptor for PM in chordotonal neurons (Jessica Ausborn, Harald Wolf and Kayser, 2005).

*Drosophila* chordotonal neurons express two TRPV channel proteins; Nan and Iav. Iav is expressed in JONs in adult antenna and LCh5 in larval abdomen (Nesterov et al., 2015) whereas Nan is more widely expressed. In addition to most chordotonal neurons, Nan is present in hygroreceptors and some multidendritic neurons as well (Nesterov et al., 2015). Nan and Iav co-localize in the proximal end of cilia of JONs in adult antenna and LCh5 in larva (Fig 1)(Kernan, 2007; Nesterov et al., 2015). A recent study showed that bath application of insecticide pymetrozine (PM) and pyrifluquinazon (PFQ) induces Ca\(^{2+}\) spike in wild-type flies but not in *nan*\(^{36a}\) and *iav*\(^{1}\) null mutants (Nesterov et al., 2015). Additionally, otherwise insensitive antennal hygro-receptors that express Nan alone (and lack Iav), were made sensitive to PM and PFQ by ectopically expressing of Nan in them. PM and PFQ induce calcium response in cultured CHO cells when Nan and Iav are co-expressed in them but not when they are expressed alone (Nesterov et al., 2015).

Nan and Iav TRPV proteins are conserved in insect species and not found in birds and mammals (Matsuura et al., 2009). Because birds and mammals lack the receptors for PM and PFQ, they make good candidates for use as an insecticide. PM and PFQ do not activate mammalian TRPV4 channel in cultured CHO cells, again
supporting their specificity to insects TRPV channels (Nesterov et al., 2015).

Treatment with PM over-stimulates chordotonal neurons and renders them inactive, even if PM is rinsed away afterwards (Jessica Ausborn, Harald Wolf and Kayser, 2005; Nesterov et al., 2015). PM exposure makes *Drosophila* and *Locusta migratoria* uncoordinated and inhibits feeding which leads to their death (Jessica Ausborn, Harald Wolf and Kayser, 2005; Nesterov et al., 2015).

### 4.4.2 NAM has a potential to be used as an insecticide

NAM possesses many desired features required in a suitable insecticide. These features are listed below:

- **Target specificity**: Like PM, NAM is an agonist for Iav/Nan TRPV channel. Iav/Nan TRPV channel does not exist in mammals, fish or birds. Therefore NAM is target specific towards insects. However, before NAM’s use as a pesticide can be recommended, it needs to be tested if mammalian TRPV channels are NAM responsive or not in heterologous expression systems.

- **Solubility in water**: NAM will be easy to use as an insecticide because it readily dissolves in water unlike PM and PFQ, which have low water solubility.

- **Safety**: NAM is a form of vitamin B₃ that is part of our daily diet. Mammals can tolerate very high amounts of NAM in their body without any harmful
effect (Gale et al., 2004). NAM is used as food additive and in cosmetics without any known side effects. Therefore, safety of NAM usage is already established. On the other hand, PM is a likely carcinogenic and neurotoxic to mammals and may cause reproductive problems (according to EPA MSDS sheet).

4.5 Materials and methods

4.5.1 Drosophila tone response

3rd instar Drosophila larvae were pretreated with different concentrations of NAM or 1 M NA for ten minutes by soaking. After treatment, larvae were transferred to an NGM plate (no E. coli), placed on a speaker (Big Jambox by Jawbone) and exposed to a 500 Hz tone at 90 decibels (http://soundbible.com/1396-500-Hz-Tone.html). Retraction of the mouth was recorded as a positive startle response (Zhang et al., 2013). Each larva was tested five times with a two to three second recovery between tones to ensure that larvae were moving forward when scoring occurred. Only forward moving larvae were scored. Observers were blind to the treatment condition.
4.5.2 Xenopus oocyte expression and electrophysiology

cDNA of nanchung isoform B in pcDNA3.1 was ordered from GenScript. Inactive cDNA clone (pCG4536) was received from Janghwan Kim lab, South Korea. (Gong et al., 2004). Sequencing of pCG4536 cDNA clone revealed that it contained a missense mutation at 448 amino acid GGT > GCT (Glycine to Alanine), G>C mutation in 1343th bp. pCG4536 cDNA clone also contained the second intron of inactive (iav) ORF. Intron 2 was deleted by PCR splicing and iav cDNA without the intron was subcloned in pCR4-TOPO vector (Renamed as 1A-IavTOPO). iav cDNA was eventually transferred to pOX (Jegla and Salkoff, 1997)

Following primers were used for removing intron 2 by PCR splicing:

InactiveIRESredF: GGctcgaggccaccATGAAGTTCCTCCTGAAGAAATGCC
IavDel3F: CGGAAGGACTAATTGGAATTCCGCCTTG
IavDel2R: CGGAATTCCAATTAGTCCTTCCGTCCGGC
InactiveIRESredR: CCtctagaTCACTTTCTGGCCAGGATCTTGTTGATGTCC

cRNAs were made from cDNAs using mMessage Machine kits (Ambion, TX) and injected at ~50 ng/oocyte. Oocyte preparation, injection and culture have been described (Li et al., 2015a; Martinson et al., 2014). Recordings were made one to three days after injection at room temperature using standard two electrode voltage clamp techniques as described (Li et al., 2015b) in a solution containing (in mM): 98 Na+, 2 K+, 1 Mg2+, 1 Ca2+, 104 Cl−, 5 HEPES (pH 7.5 with NaOH).
4.5.3 In vivo analysis of Drosophila chordotonal neurons

To visualize the activity of chordotonal neurons, flies expressing Gal4 under the pan-neuronal promoter ELAV and UAS driven RFP were crossed to a fly line containing a UAS driven genetically encoded calcium sensor, GCaMP6 (medium kinetics)(Chen et al., 2013). 3rd instar larva were rinsed in PBS and mounted laterally on an agar covered slide for viewing. LCh5 chordotonal neurons in larval abdomen were selected for imaging. Z-stacks through the LCh5 were acquired with a Zeiss LSM510 confocal microscope. Movies were recorded during paired periods of rest and stimulation of the neurons. Stimulation was achieved by gently rubbing the base of the microscope with the ribbed bottom of a glass bottle. After three such paired recordings, we applied solutions of either 1M NAM or 1x PBS to the side of the coverslip, taking care not to touch the slide with the pipet. The solution was drawn to cover the larva via capillary action. After approximately 20 seconds, another series of three pairs of resting/stimulation images were acquired. Image analysis was performed with Fiji (ImageJ). Z-stacks for quantification were flattened with the “z-project” command, and quantified by using the polygon selection tool to tightly select the dendrites, cell bodies and proximal axons of the LCh5 cluster before using the “measure” command. Measurements were exported to Excel for recording and statistical analysis.
4.5.4 Statistical Analysis

Statistical tests and sample sizes for each experiment are described in figure legends with relevant $p$ values. The statistical tests were two-tailed. Statistical analyses were performed in Excel or GraphPad Software.

Statement of Collaboration

- *Drosophila* startle response experiment: I treated the *Drosophila* larva by soaking in NAM or NA and set up the experiment, various members of Wendy Hanna-Rose lab volunteered to assist me in blindly scoring the startle freeze response.

- Electrophysiology: I did cloning and cRNA synthesis. Aditya Pisupati (Tim Jegla lab) performed the electrophysiology recordings while I assisted him.

- *in vivo* calcium imaging of *Drosophila*: I performed the initial experiment to test chordotonal neuron survival in response to NAM in *Drosophila*. All the *in vivo* *Drosophila* calcium-imaging experiments were performed by Matthew Shorey (Melissa Rolls lab).
Chapter 5: Physiological implications of NAM agonist activity

5.1 Chapter outline

So far I have shown that NAM is a direct agonist of *C. elegans* OCR-4/OSM-9 TRPV channel and *D. melanogaster* Nan/Iav channel. I also showed that NAM affects behavior mediated by Nan/Iav channel in *Drosophila*. Does NAM affect any behaviors in *C. elegans* as well? In this chapter, I address this question by examining the effect of NAM on three specific behaviors:

1) Egg laying, a behavior mediated by TRPV channels expressed in uv1 cells
2) Foraging, a behavior mediated by OLQ neurons
3) Nose touch response, a behavior partially mediated by OLQ neurons.

5.2 Egg laying behavior in *C. elegans*

In wild type animals, eggs start accumulating in the uterus shortly after L4 to adult molt (Jose et al., 2007). When the uterus accrues approximately 15 eggs, the vulva muscles contract and the eggs pass through the vulva passage to be deposited in the environment (Schafer, 2005). Egg laying events take place in random short bursts for every 1-2 minutes at approximately 20 minute intervals (Waggoner et al., 1998).
5.2.1 Loss of PNC-1 causes egg laying (Egl) defect

*pnc-1* hermaphrodites cannot lay eggs, instead the eggs hatch inside the mother’s body (Vrablik et al., 2009). The mother dies in the process and the progeny larvae crawl out of the carcass and survive (Figure 5.1).

![pnc-1 mutant](image)

**Figure 5.1: pnc-1 mutant has egg laying defect**

*pnc-1* mutant animal cannot lay eggs. Eggs hatch inside the mother’s body, which kills the mother, but the progeny larvae survive. The progeny larvae coming out of its mother’s carcass can be seen in the picture.

5.2.2 *pnc-1* egg laying defect is caused due to vulva muscle impairment

An egg-laying phenotype could be an effect of defective neuronal or muscle function. Motor neurons release an excitatory neurotransmitter acetylcholine that binds to acetylcholine receptors on vulva muscles thus inducing egg laying.

Application of levamisol, an agonist for acetylcholine receptors, bypassing the need for neurotransmitter release from motor neurons in induction of egg laying. Egg laying is inhibited when wild type animals are placed in M9 medium. Application of levamisol induces egg laying in wild type animals even in M9 medium.
However, treatment with levamisol does not induce egg laying in \textit{pnc-1} mutants soaked in M9 buffer (Vrablik et al., 2011). Poor muscle response to agonist acetylcholine suggests that the Egl phenotype in \textit{pnc-1} mutants is attributable to impaired sex muscle function.

\textbf{5.2.3 \textit{osm-9} and \textit{ocr-4} mediate NAM induced egg laying defect}

The egg-laying defect (Egl phenotype) of \textit{pnc-1} mutants can be recapitulated by culturing wild type animals on exogenous 25 mM NAM (Vrablik et al., 2009). Is the NAM induced Egl phenotype mediated by OCR-4/OSM-9 TRPV channel just like cell death phenotype? I was able to test this hypothesis because neither \textit{osm-9} nor \textit{ocr-4} mutants have an Egl phenotype. I treated \textit{osm-9} or \textit{ocr-4} mutants with 25 mM NAM and observed Egl phenotype in them. Indeed, NAM treatment does not induce an Egl phenotype in the absence of \textit{osm-9}, and loss of \textit{ocr-4} drastically reduces the NAM induced Egl phenotype (Figure 5.2), demonstrating that the TRPV channel subunits must be present for NAM to exert its effect on the egg laying system.
The OLQs are sensory neurons with ciliary endings exposed to the environment through the nose. They play a prominent role in regulating head movements during foraging in response to mechanical sensation of food (please see Chapter 2, section 2.1.1 and Figure 2.3 for more details) (Chatzigeorgiou and Schafer, 2011; Hart et al., 1995; Kindt et al., 2007). Because excess NAM kills OLQ cells, I investigated if excess NAM also impacts the foraging behavior. I found that both pnc-1 mutants and

**Figure 5.2:** *osm-9 and ocr-4 mediate NAM-induced Egl phenotype in *C. elegans*

Actual percentage Egl animals and number of animals examined for each genotype is indicated on each bar. Error bars are 95% confidence intervals. *p* < 0.05, **p** < 0.001, calculated using Fisher’s exact test.

### 5.3 NAM affects OLQ mediated foraging behavior

The OLQs are sensory neurons with ciliary endings exposed to the environment through the nose. They play a prominent role in regulating head movements during foraging in response to mechanical sensation of food (please see Chapter 2, section 2.1.1 and Figure 2.3 for more details) (Chatzigeorgiou and Schafer, 2011; Hart et al., 1995; Kindt et al., 2007). Because excess NAM kills OLQ cells, I investigated if excess NAM also impacts the foraging behavior. I found that both *pnc-1* mutants and
NAM-supplemented wild-type animals display exaggerated head bending during foraging similar to animals with a mutation in *trpa-1*, a gene required in OLQ cells for foraging (Figure 5.3), (Kindt et al., 2007). *osm-9* mutants do not display this phenotype (Figure 5.3). These results suggest that the exaggerated head bending in both NAM supplemented and *pnc-1* mutant animals is a result of TRPV-mediated OLQ cell death.

**Figure 5.3: NAM induces a foraging defect in C. elegans**

a) Images documenting the foraging, exaggerated nose-bending phenotype (brackets). Scale bar is 100 mM. b) *pnc-1* mutants and NAM supplemented...
animals have a foraging phenotype consistent with \textit{trpa-1} mutants. *\( p < 0.05 \), calculated using Fisher’s exact test.

### 5.4 Excess NAM causes nose touch defect

The OSM-9/OCR-2 channel mediates gentle nose touch mechanosensation (see Chapter 2, section 2.1.1) (Hilliard et al., 2005; Tobin et al., 2002). Intriguingly, I found that both \textit{pnc-1} mutants and NAM-supplemented animals have a deficit in responding to gentle nose touch (Figure 5.4). This phenotype mimics that of \textit{osm-9} and \textit{ocr-2} mutants (Tobin et al., 2002). I attempted to test the ability of NAM to activate an OSM-9/ OCR-2 channel in the heterologous expression system, but detected no currents above baseline in co-injected oocytes in the presence of 300 µM to 1 M NAM (n= 5 injections).

I also considered whether the nose-touch phenotype could be a result of death of OLQ cells, which play a minor role in nose touch (Kaplan and Horvitz, 1993). However, the detected phenotype is more penetrant than the 10% reduction reported to occur from ablation of OLQ cells (see section 2.1.1.2) (Kaplan and Horvitz, 1993). A revised model of neural circuit for nose touch suggests that OLQ neurons are electrically coupled with FLP neurons and have a more significant contribution towards nose touch response than previously suggested (Figure
Therefore, it is possible that the NAM induced nose touch phenotype is a result of OLQ cell death.

**Figure 5.4: Nose touch defect**

*pnc-1* mutant and NAM-treated animals have a nose touch defect like that of *osm-9* mutants, but *ocr-4* mutants do not share this phenotype. Ratio relative to wild type control and number of animals examined for each genotype is indicated on each bar. Error bars are 95% confidence intervals. *p <0.05, **p <0.001, calculated using Fisher’s exact test.*

### 5.5 Discussion

In this Chapter, to understand the physiological implications of NAM agonist activity, I investigated the effect of NAM on behaviors mediated by uv1 and OLQ cells.
*pnc-1* mutants are unable to lay eggs due to a vulva muscle defect and they die due to internal hatching of eggs (Vrablik et al., 2009, 2011). Exogenous NAM application in wild type animals recapitulates the Egl phenotype of *pnc-1* mutant. Because previously I discovered that NAM induced cell death phenotype is mediated by OSM-9 and OCR-4, I examined if these TRPV subunits also mediate the NAM induced egg laying defect. Indeed that is the case, *osm-9* and *ocr-4* mutants raised on NAM supplemented media display no and decreased egg laying defect respectively (Figure 5.2). This suggests that OSM-9 and OCR-4 are required for NAM induced Egl defect. This result is particularly interesting because egg laying is a vulva muscle defect (Vrablik et al., 2011) and this result suggests the possibility of OSM-9 and OCR-4 expression in vulva muscles. However, there is no report of expression of OSM-9 and/or OCR-4 in vulva muscle cells (Colbert et al., 1997; Tobin et al., 2002). Sometimes GFP fusion protein expression patterns do not quite represent the true expression pattern of a protein. Therefore further investigation is required to examine if OSM-9 and OCR-4 express and function in vulva muscles. Function of TRPV genes OSM-9 and OCR-4 can be tested by their cell specific expression in vulva muscles in corresponding mutants followed by observation of Egl phenotype restoration upon NAM supplementation.

Next, to further study the impact of NAM on physiology, I examined OLQ neuron mediated behaviors in excess NAM conditions. OLQ neurons are required for the
foraging behavior (see section 2.1.1.1 and Figure 2.3). I found that NAM induces a foraging defect in wild type animals and pnc-1 mutants (Figure 5.3). Because OLQ neurons die in excess NAM conditions, the foraging defect of pnc-1 mutants and NAM supplemented wild type animals is perhaps due to the death of OLQ neurons.

Additionally, excess NAM induces a nose touch defect that is not observed in ocr-4 mutant animals (Figure 5.4). Instead, this defect resembles the nose touch defect of ocr-2 and osm-9 mutants. (Figure 5.4). Therefore, perhaps NAM mediates nose touch defect via a channel other than OCR-4/OSM-9 channel. However, this defect could also be caused due to death of OLQ cells because a recent study reported that OLQ cells have more contribution to nose touch response than what was previously reported (Chatzigeorgiou and Schafer, 2011).

5.6 Materials and methods

5.6.1 Egg laying assay

Each L4 stage hermaphrodite was raised individually and transferred serially to a new plate daily to avoid crowding with progeny. The animal was monitored for egg laying daily for 3 to 4 days. If the embryos hatched inside the mother’s body
instead of being expelled through the vulva, the animal was scored as egg-laying defective (Egl). Data is presented as the percentage of Egl animals.

**5.6.2 Nose touch assay**

I prepared assay plates by placing a small drop of an overnight OP50 culture on an NGM plate and storing plates at 4°C for up to ten days. Plates were moved to room temperature for an hour before use. I placed a single worm on a plate and allowed it to move around for at least 5 minutes. Then I brought a thin eyebrow hair into the path of a forward moving animal to cause it to collide nose on. I scored stopping and changing direction of movement in response to the collision as positive for nose touch response. Each animal was touched 10 times with 10-60 second intervals between the touches. Experimenters were blind to the genotype/condition. Data is presented as the percentage of animals that respond positively to the nose touch.

**5.6.3 Foraging**

Assay plates were identical to nose touch assays. Animals were placed on the plate and their nose tip was closely observed during forward movement for approximately one minute for the presence or absence of exaggerated head bends making the nose tip appear curved (Kindt et al., 2007). Observers were blind to the genotype/condition.
5.6.4 Statistical Analysis

Statistical tests and sample sizes for each experiment are described in figure legends with relevant $p$ values. The statistical tests were two-tailed. Statistical analyses were performed in GraphPad Software.
Chapter 6: Summary, significance and future directions

6.1 Summary and significance

I have demonstrated that nicotinamide, which is a form of vitamin B3 and an endogenous cellular metabolite, is an agonist of a heteromeric *C. elegans* TRPV channel that consists of OCR-4 and OSM-9 subunits. Moreover, agonist activity is evolutionarily conserved as nicotinamide also activates the orthologous *Drosophila* Nan/Iav TRPV channel. I propose that activation of the OSM-9/OCR-4 channels is the trigger mechanism for NAM-induced uv1 and OLQ cell death in *C. elegans*. When NAM levels rise upon loss of the PNC-1 nicotinamidase that metabolizes NAM, the OCR-4 and OSM-9-expressing OLQ and uv1 cells likely undergo excitotoxic death (Figure 2.6). Aberrant activation of TRPV channels in humans has also been associated with cell death (Nilius and Owsianik, 2010; Reilly et al., 2003; Ryskamp et al., 2011), and a gain-of-function mutation in the *C. elegans* TRPN protein TRP-4 causes excitatory cell death of dopaminergic neurons (Nagarajan et al., 2014).
While OLQ and uv1 cells both die in response to NAM, it is unclear why the OLQ response is less robust than that of uv1. Using GFP reporter I establish that OSM-9 and OCR-4 expression levels are similar in the two cell types. So, other cell-intrinsic molecular differences or distinct effective NAM exposure of OLQ and uv1 cells may account for their differential NAM response. Similarly, the multiple OCR proteins that are co-expressed with ocr-4 in uv1 cells could influence the response (Figure 2.10). Thus, I conclude that the insult is same in both the cell types but the response is influenced by cell-type specific factors. In some cells NAM induces cell death while in other cells it does not kill the cell but alters the function of the cell. An example of NAM disrupting the function of TRPV channel-expressing cells without causing their death is seen in Drosophila. In Drosophila, application of NAM prevents the chordotonal neurons from responding further to the applied mechanical stimuli, without causing their death (Figure 4.5).

Sustained presence of agonist is predicted to interfere with the functions of cells that express the TRPV channel in two ways. First, the constitutive opening and likely desensitization of the channel would interfere with any signaling event and behavioral output for which the channel is required. In this scenario prolonged presence of NAM agonist would mimic the genetic loss of the TRPV channel. I found evidence of this type of effect of NAM in the Drosophila sound-response assay (Figure 4.4) and the C. elegans nose-touch phenotypes (Figure 5.4).
Second, constitutive opening of the TRPV channel might be expected to disrupt the physiology of the cell, and perhaps even kill the cell, via ion imbalance or other feedback mechanisms, thereby disrupting other cellular processes in which the TRPV channel has no direct role. In this scenario, the effects of the agonist might actually be blocked by loss of the channel, thus protecting other cellular functions. Again, I found evidence of this effect in the Egl phenotype (Figure 5.2) and the foraging phenotype (Figure 5.3) in *C. elegans* where disruption of egg laying muscle function and the death of the OLQ cells results in phenotypes that aren’t TRPV dependent *per se*.

Results from electrophysiology experiment show that *C. elegans* TRPV channel subunits can assemble to form functional channels in heterologous expression system without requiring accessory proteins as suggested previously (de Bono et al., 2002; Colbert et al., 1997; Jose et al., 2007). By combining the Hill coefficient value obtained in electrophysiology with the stoichiometry data from TIRF photobleaching assay, we interpret that there are two NAM binding sites per channel. NAM may directly bind to only one of the subunits between OCR-4 and OSM-9 or it could bind at the interface of OSM-9-OCR-4 subunits (Figure 3.1 and Figure 3.3). Further development of this OCR-4/OSM-9 heterologous expression system will bring more insights into the structure and biophysical properties of *C. elegans* TRPV channels.
A pictorial summary of the study

**Figure 6.1: Nicotinamide modulates TRPV sensory ion channels in *C. elegans* and *Drosophila***

OSM-9/OCR-4 and Iav/Nan are the orthologous heteromeric TRPV ion channels found in *C. elegans* and *Drosophila melanogaster* respectively. They are required for mediating normal mechanosensory behavior responses like sensing touch/stretch (in *C. elegans*) and sound vibrations (in *Drosophila*). NAM can modulate these ion channels by direct binding and activation. Excess NAM conditions created by exogenous application can cause mechanosensory defects (in *Drosophila* and *C. elegans*) and cell death (in *C. elegans*). NAM is an endogenous metabolite. A block in NAD⁺ pathway, as seen in the *C. elegans* pnc-1 mutant, can lead to NAM accumulation and TRPV channel over-activation causing excitotoxic cell death or sensory defects in the cells expressing these channels.
Does NAM affect other TRPV channels? The effect of NAM is conserved between the *Drosophila* TRPV channel and at least one *C. elegans* TRPV channel. The presence of multiple OCR paralogs in *C. elegans* raises the question of whether other heteromeric combinations of *C. elegans* TRPV channels will respond to NAM. I failed to detect channel activity with or without NAM upon co-expression of OCR-2 and OSM-9, but did not yet test other OSM-9/OCR combinations. Furthermore, the egg laying behavior experiments suggest intriguing differences in how this phenotype responds to mutation of *osm*-9 versus *ocr*-4. This suggests possible involvement of TRPV channels with alternate OCR subunits in NAM-induced Egl phenotype as well as function of OCR-4/OSM-9 in a previously undetected location. Nonetheless, it is clear that not all *osm*-9 mutant phenotypes are mimicked by NAM accumulation; I examined *pnc*-1 mutants but found no indication of an osmo-sensation phenotype (see supplementary information, figure S1). Thus, there are clearly intriguing questions to be addressed regarding the function and NAM sensitivity of the multiple OCR paralogs in *C. elegans*.

Both nicotinic acid and nicotinamide are forms of vitamin B₃ used for biosynthesis of NAD⁺. My results revealed that nicotinic acid is not an agonist of the OSM-9/OCR-4 or Nan/Iav channel (Figure 3.1e). However, the nicotinic acid form of vitamin B₃ is an apparent low-affinity agonist of mammalian TRPV channels (Ma et al., 2014, 2015). Identification of effects of another form of vitamin B₃ as a potent
TRPV agonist raises intriguing possibilities for metabolic regulation of channel activity. Additionally, it would be informative to investigate other physiological or disease conditions where NAM levels in the animal body may rise. For example, Poly (ADP-ribose) polymerase (PARP) enzyme activity produces NAM as a byproduct (Chini et al., 2016). PARPs are activated in response to events like cellular stress, DNA damage, aging etc. It will be insightful to study whether PARP activation and in turn NAM accumulation modulates TRPV channel functions. Furthermore, Identification of a soluble agonist for a C. elegans TRPV combined with recent identification of an insecticide as a Drosophila channel agonist (Nesterov et al., 2015) also paves the way for closer examination of the evolution of TRPV activation mechanisms.

6.2 Future directions

My research has discovered new mechanisms by which metabolic perturbation affects sensory behaviors and cell death. This study lays the groundwork for further investigation of the structure, assembly and the biophysical properties of the C. elegans TRPV channels. Some key questions to address in the future research would be:

1) What is the NAM binding site on OCR-4/ OSM-9 channel
2) What is the identity of cation or cations conducted by OCR-4/OSM-9 channel

3) Do other combinations of TRPV subunits make NAM responsive channels? If yes, are the behaviors mediated by those channels affected by NAM? If no, what is the basis for these differences?

4) Are mammalian TRPV channels NAM responsive?

5) Do OSM-9 and OCR-4 proteins express and function in vulva muscles to mediate NAM induced egg laying phenotype?

6) Are other chordotonal neuron functions like wind and gravity sensing affected by NAM in Drosophila?

7) Are there other physiological or disease conditions where NAM levels rise in the animal body? How is TRPV channels function modulated in such conditions?

8) And finally, conducting experiments to ascertain the specificity of NAM for its potential use as an insecticide

In conclusion, this study reports the discovery of a form of Vitamin B₃, nicotinamide as an evolutionary conserved direct agonist for C. elegans and Drosophila TRPV channels. C. elegans. TRPV subunits OCR-4/OSM-9 form functional channels in heterologous system. Nicotinamide affects biological processes like egg laying, mechanosensation and cell death via its TRPV agonist activity. These results provide an intriguing link between metabolic regulation and TRPV channel activity. Additionally, a potential for using NAM as a pesticide is revealed.
S1. *pnc-1* mutants appear normal for osmosensation

OSM-9 is required for sensing osmolarity in *C. elegans* (see page 40, section 2.1.7.1). Because loss of PNC-1 results in accumulation of NAM and NAM affects OSM-9 TRPV channel, I examined if *pnc-1* mutants have an osmosensory defect like *osm-9* mutant. A wild-type N2 animal reverses and changes its path when it encounters a high osmolarity solution. To assay this behavior, I placed animals (wild-type or mutants) in the center of a 4 M fructose ring and observed them for 20 minutes. Animals defective in osmosensation ignore the 4 M fructose and cross the ring whereas animals that are normal for osmosensation avoid coming close to the 4M fructose ring and stay inside the ring. Data is presented as percentage of animals that stay inside the 4 M fructose ring after 20 minutes (Figure S1).
Figure S1: The pnc-1 mutant does not have an osmosensory defect

**p <0.001, calculated using Fisher’s exact test. n/s= not significant. Error bars are standard deviation calculated from three biological replicates of the experiment.
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