EFFECTS OF METHYLMERCURY ON NOTCH TARGETS AND MOTOR NERVE DEVELOPMENT IN DROSOPHILA

A Dissertation Presented

by

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to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Neuroscience

October, 2012

Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy specializing in Neuroscience.

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ABSTRACT

Methylmercury (MeHg) is a ubiquitous environmental toxin. Exposure to MeHg in humans occurs primarily through the consumption of contaminated seafood. MeHg has been shown to act most strongly during neural development. Epidemiological data on the effect MeHg exposure through seafood has on children and fetuses is conflicted, with large cohort studies showing both presence and absence of MeHg-induced deficits in achieving developmental milestones. Because of this uncertainty in the literature it is important that we come to understand the mechanisms of MeHg toxicity so that we might advise the public more accurately on the risks of MeHg exposure.

Research into the mechanisms of MeHg toxicity has found a number of cellular and molecular effects including disruptions of microtubule formation, Ca^{2+} homeostasis, and glutamate signaling. However, none of these effects of MeHg fully explains its neurodevelopmental specificity. Previous work in Drosophila neural-derived cell lines has shown that MeHg causes upregulation of the canonical Notch response gene *E(spl)mδ*. The Notch pathway is crucial to neural development and perturbation of a Notch target may explain the developmental specificity of MeHg. In this dissertation I describe experiments I performed to test the hypothesis that the observed upregulation of *E(spl)mδ* plays an important role in MeHg toxicity in Drosophila.

I first describe experimental evidence that *E(spl)mδ* is upregulated by MeHg treatment *in vivo* in Drosophila embryos in addition to cells, as has previously been shown. By contrasting the effects of the toxic inorganic mercurial $HgCl_2$ with MeHg I show that the *E(spl)mδ* expression response to MeHg is not simply a stress response and is a likely specific activity of MeHg. I also show that the effect of MeHg on *E(spl)mδ* expression is not simply due to a developmental delay induced by the toxin.

I also identify two neural phenotypes of MeHg toxicity in Drosophila embryos, in the outgrowth of the intersegmental and segmental motor nerves. Genetic manipulation causing overactivity of the Notch pathway in neurons can mimic these phenotypes. However, induced expression of *E(spl)mδ* in neurons does not cause a failure of motor nerve outgrowth. Upon further examination I demonstrate that endogenous expression of *E(spl)mδ* occurs in the muscle. Induced *E(spl)mδ* expression in the muscle causes a segmental nerve phenotype similar to MeHg treatment, indicating a role for *E(spl)mδ* in MeHg toxicity in this system. MeHg treatment and *E(spl)mδ* overexpression in the muscle causes a failure of normal muscle development. Yet, this gross developmental abnormality only partially explains the observed motor nerve phenotype. *E(spl)mδ* is unique among the E(spl) genes in its ability to cause these muscle and motor nerve phenotypes as shown by contrasting genetic manipulation of the closely related *E(spl)mγ*.

Overall my findings support the hypothesis that MeHg toxicity in Drosophila is mediated in part by *E(spl)mδ*. They also suggest that *E(spl)mδ* plays an important role in the formation of the muscle during embryonic development, contributing to the literature describing disparate functions for E(spl) genes despite structural similarities. Finally, my findings suggest that MeHg may be able to impact neural development through toxicity in supporting tissues rather than neurons themselves. This final finding has implications for the study of MeHg toxicity in humans, and is supported by previous findings that describe a role of glia in modulating MeHg neurotoxicity.

CITATION

Material from this dissertation has been published in the following form:

Engel GL, Delwig A, Rand MD (2012) The effects of methylmercury on Notch signaling during embryonic neural development in Drosophila melanogaster. Toxicol In Vitro.

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CHAPTER 1: LITERATURE REVIEW

1.1: Introduction

Methylmercury (MeHg) is a ubiquitous environmental contaminant with well known neurotoxic effects in humans. Acute and chronic high dose exposures due to industrial or agricultural accidents have revealed that MeHg exposure in adults causes a diffuse central neuropathy leading to mental, sensory, and motor deficits. The developing central nervous system has proven even more sensitive to MeHg toxicity; children exposed *in utero* to MeHg in doses that showed subtle signs in the mother have been born with profound neurological deficits including severe mental retardation, seizures, cerebellar ataxia, and sensory deficit. It is hypothesized that even low dose exposure to MeHg *in utero* due to maternal fish consumption can cause subtle developmental deficits, due to the increased sensitivity of the fetal system to MeHg. Because of this the cellular and molecular mechanisms of MeHg toxicity during development are the subjects of intense scrutiny. It has been shown *in vitro* in cell lines derived from the model organisms *Drosophila melanogaster* and *Mus musculus* that the traditional Notch targets in the Enhancer of Split [E(spl)] complex are upregulated after treatment with MeHg (Tamm et al., 2008). Since the Notch pathway is crucial to cell-fate determination during development this perturbation of the E(spl) complex may be a core underlying mechanism in the developmental neurotoxicity of MeHg. This work investigates the effects of MeHg on neural development and interactions with the Notch pathway *in vivo* using the Drosophila embryo model.

1.2: Methylmercury

Pathology and epidemiology of methylmercury poisoning

Much of the information available on the pathology of MeHg poisoning stems from two incidents of accidental mass poisoning of a population. During the 1940-1960's the Chisso Corporation produced MeHg as a byproduct of acetaldehyde synethesis (Ekino et al., 2007). They released the MeHg in chemical waste dumped into rivers flowing into Minamata Bay, a source of dietary fish and shellfish for the inhabitants of the Minamata region of Japan (Ekino et al., 2007). Ingestion of the MeHg contaminated seafood caused acute toxicity in the population (Ekino et al., 2007). Continued exposure to MeHg released into Minamata Bay and the Shiranui Sea as recently as 1968 has caused chronic toxicity (Ekino et al., 2007). Exposure *in utero* to concentrations of MeHg causing only mild toxicity in the mothers has had profound effects on the child, indicating that the developing nervous system is particularly vulnerable (Ekino et al., 2007). In 1956 MeHg poisoning due to contamination of the Minamata Bay was officially recognized and the clinical manifestation of MeHg poisoning was named Minamata disease (MD) (Ekino et al., 2007).

The second mass contamination of a population with MeHg occurred in the 1970's in Iraq. Seed grain given as agricultural aid was treated with MeHg as an antifungal (Myers and Davidson, 2000). Consumption of this grain caused acute poisoning in the population (Myers and Davidson, 2000). Because the source of the poisoning was quickly identified and consumption ceased, exposure was in a large bolus dose, opposed to the Minamata event, in which exposure was to chronic, relatively low doses (Myers and Davidson, 2000). This quick recognition of the MeHg exposure also

allowed immediate study of the results, and reinforced the data indicating MeHg acted most potently on the developing nervous system (Myers and Davidson, 2000).

MD is often classified into three subgroups: acute, chronic, and fetal. Acute MD is found in adults who are exposed to large doses of MeHg. Postmortem analysis of affected individuals indicates diffuse cortical and cerebellar neuropathy (Taber and Hurley, 2008). This results in sensory, motor, and psychiatric deficits. The visual field shows bilateral concentric constriction (Ekino et al., 2007). There is a deficit in speech discrimination (Ekino et al., 2007). Olfaction and gustation show subjective changes (Ekino et al., 2007). There is loss of sensitivity and two point discrimination in the distal extremities (Ekino et al., 2007; Taber and Hurley, 2008). Cerebellar ataxia is common (Ekino et al., 2007; Taber and Hurley, 2008). Changes in personality including lack of volition and apathy are nearly ubiquitous (Ekino et al., 2007; Taber and Hurley, 2008).

Individuals suffering from chronic MD show similar deficits. A stocking and glove pattern loss of sensation in the distal extremities was thought to indicate a peripheral neuropathy. Further research, however, has shown that affected individuals show no reduction in tendon reflexes and have unaltered peripheral conduction velocities, indicating a central neuropathy (Ekino et al., 2007). Cerebellar ataxia has been shown during early exposure to MeHg, but improves over time; chronic MD patients still show deficits in movement and posture, however, due to sensory deficits resulting in sensory ataxia (Ekino et al., 2007). Visual and auditory deficits have also been reported in chronic MD patients (Ekino et al., 2007).

The most severe form of MD affects individuals exposed during development *in utero*. In fetal MD individuals present with mental retardation, epileptic seizures, and

difficulty with most coordinated or involuntary motor tasks including chewing, swallowing, speaking, and walking (Ekino et al., 2007; Taber and Hurley, 2008). Severe cases result in akinetic mutism (Ekino et al., 2007; Taber and Hurley, 2008).

Because of the sensitivity of the developing nervous system to MeHg there has been much concern about the effects of low doses of MeHg on children and fetuses. Mercury can be found in many large bodies of water due to contamination from natural and anthropogenic sources (Myers and Davidson, 2000; Clarkson et al., 2003). Aquatic bacteria can turn elemental mercury into MeHg, which concentrates in large predatory fish due to bioamplification. Fish and other seafood makes up a significant portion of the diet of many populations, and through it they are exposed to MeHg (Myers and Davidson, 2000; Clarkson et al., 2003). Several epidemiological studies have attempted to identify effects of subacute dietary MeHg in developing individuals. Two studies stand out because of their large sample sizes and methodological rigor: the Faroe Islands and Seychelles Islands studies (Myers and Davidson, 2000).

The Faroes population is exposed to MeHg primarily through the consumption of pilot whale blubber (Myers and Davidson, 2000; Castoldi et al., 2008a; Rice, 2008; Taber and Hurley, 2008). In the study of this population MeHg was associated positively with neurodevelopmental milestones in the first year after birth. This can be explained by an association between breastfeeding and MeHg exposure during that timeframe (Grandjean et al., 1995; Myers and Davidson, 2000; Castoldi et al., 2008a; Rice, 2008). At 7 years of age, though, increased MeHg exposure prenatally (as measured by cord blood concentrations) correlated with decreased performance on tests associated with memory,

learning, and attention (Grandjean et al., 1997; Myers and Davidson, 2000; Castoldi et al., 2008a; Rice, 2008).

The Seychelles Islands population is exposed to MeHg via deep sea and reef fish (Myers and Davidson, 2000; Castoldi et al., 2008a; Rice, 2008; Taber and Hurley, 2008; Myers et al., 2009). In the study of this population no negative effects were observed from increased exposure to MeHg, either pre or postnatal. In fact, there were beneficial effects shown to correlate with the amount of fish consumed (Davidson et al., 1995; Myers et al., 1995; Myers and Davidson, 2000; Castoldi et al., 2008a; Rice, 2008; Taber and Hurley, 2008; Myers et al., 2009).

The discordance found between the Faroe and Seychelles island studies has been the topic of much debate. Many factors might have contributed to their disparate results. One of the primary differences is the source of the MeHg. Pilot whale blubber is eaten infrequently on the Faroes, but has a higher concentration of MeHg than fish. It also contains other toxic contaminants such as polychlorinated biphenyls (Myers and Davidson, 2000; Castoldi et al., 2008a; Rice, 2008; Taber and Hurley, 2008). The fish eaten in the Seychelles contains omega-3 fatty acids and selenium, factors that may be beneficial to neurodevelopment, and as such oppose the effects of MeHg (Myers and Davidson, 2000; Castoldi et al., 2008a; Rice, 2008; Taber and Hurley, 2008). There are also some methodological differences, such as a failure to correct for socioeconomic effects in the Faroes study (Rice, 2008). Because of the opposing findings of the studies it is still uncertain what risks the consumption of seafood might pose for fetuses and children. Future studies may be aided by the use of neurophysiological metrics rather than the neuropsychological tests that have predominated thus far. Patients with MD

show alterations in heart rate variability (HRV) indicating parasympathetic hypoactivity and changes in the latency of brainstem auditory evoked potentials (BAEP) correlating with MeHg exposure (Murata et al., 2007). These physiological tests will allow for data that is completely objective and should be comparable across cultures (Murata et al., 2007).

Animal models also allow the study of MeHg toxicity without the confounding effect of diet or culture. Studies on non-human primates have shown similar effects to those in humans, excepting only a relative sparing of the cerebellum in monkeys and ambiguous results on cognitive development (Castoldi et al., 2008b). Rodent models also show similar effects to humans, but must be used with care as the timeframe of neurodevelopment is quite different than humans, with the third trimester in humans roughly corresponding to the first 10 postnatal days in rats (Castoldi et al., 2008b). Despite differences these animal models will continue to be useful for elucidating the effects of MeHg toxicity, since every variable can be more closely controlled and measured.

Methylmercury Biochemistry and Transport

Human exposure to MeHg occurs primarily through contaminated fish and other seafood. In fish MeHg is most commonly found bound to the amino acid cysteine as MeHg-cysteinate (Harris et al., 2003). This compound consists of the MeHg bound to the thiol group of cysteine. MeHg shows a high affinity for thiols, and can rapidly exchange between thiols in solution, allowing it to jump from protein to protein, binding to their cysteine residues (LoPachin and Barber, 2006; Asaduzzaman et al., 2010). While MeHg

is known to bind to DNA the affinity of MeHg for DNA is much lower than that for thiols and no genotoxic effects of MeHg have been observed (Maki and Ott, 1981; Li et al., 2006; Carmona et al., 2008). It has been shown *in vitro* that MeHg-L-cysteinate crosses the plasma membranes of cells in a temperature sensitive process, indicating a protein transporter (Heggland et al., 2009). MeHg-chloride (MeHgCl), however, crosses the plasma membrane in a temperature insensitive process, implying passive diffusion (Heggland et al., 2009). In MeHgCl the mercury-chloride bond is very strong and does not spontaneously dissociate in aqueous solution, however the strong affinity of MeHg for thiol groups allows rapid production of MeHg-cysteinate from MeHgCl upon addition of cysteine (Harris et al., 2003; Heggland et al., 2009; Asaduzzaman et al., 2010).

Once consumed MeHg is taken up in the gut by L-type large amino acid transporters (Clarkson and Magos, 2006; Hoffmeyer et al., 2006; Clarkson et al., 2007; Heggland et al., 2009). MeHg then enters the blood where it accumulates in red blood cells and binds to cysteine residues on hemoglobin and glutathione (Doi and Tagawa, 1983). The MeHg then distributes to tissues throughout the body, leaving the red blood cells with glutathione and entering other tissues after binding free cysteine (Doi and Tagawa, 1983). In this manner it can cross the placental and blood-brain barrier (Clarkson and Magos, 2006; Clarkson et al., 2007). Some MeHg is excreted with glutathione in the bile, but breakdown of the glutathione releases MeHg-cysteine, which can be reabsorbed, forming an enterohepatic cycle (Clarkson and Magos, 2006; Clarkson et al., 2007). A small amount of MeHg in the gut is converted to inorganic mercury by intestinal flora, which is then excreted (Clarkson and Magos, 2006; Clarkson et al., 2007).

Many early studies investigated the distribution of MeHg in the various cell compartments of animals fed or injected with radiolabeled MeHg. Subcellular fractionation of tissues including brain, liver, and kidney showed MeHg accumulation in the lysosome/peroxisome, microsome, and mitochondrial fractions (Norseth and Brendeford, 1971; Syversen, 1974; Mehra and Choi, 1981). Each of the studies also saw MeHg accumulation in the nuclear fraction, though contamination of this fraction by other organelles cast doubt on whether the nuclei themselves contain MeHg (Norseth and Brendeford, 1971; Syversen, 1974; Mehra and Choi, 1981). An investigation using microautoradiographic imaging to determine MeHg distribution in the liver found evidence for MeHg in nuclei, reinforcing the finding from cell fractionation (Sakai, 1975). It is thus possible that MeHg has effects in any cell compartment; specifically, its effects on *E(spl)* gene expression may be due to direct interaction with nuclear proteins.

Cellular and molecular effects of methylmercury

The mechanisms of MeHg toxicity have not yet been fully elucidated. There has been a great effort to characterize the cellular and molecular effects of MeHg in order to begin understanding these mechanisms of toxicity. An important first step has been understanding the deposition and elimination of MeHg in the body.

MeHg is readily absorbed in the gastrointestinal tracts with about 95% of the MeHg consumed being absorbed (Clarkson and Magos, 2006). Within three days most of the MeHg distributes out of the blood into other tissues (Clarkson and Magos, 2006). Brain tissue shows five times more MeHg than the blood and scalp hair shows MeHg at 250 times the concentration found in blood (Clarkson and Magos, 2006).

Recent research has revealed MeHg causes disruption of microtubule formation. By binding to tubulin MeHg has been shown to inhibit polymerization and depolymerize assembled tubules (Castoldi et al., 2001; do Nascimento et al., 2008). This has been observed in many cell lines, including human fibroblasts, neuroblastoma, and glioma cells (Castoldi et al., 2001; do Nascimento et al., 2008). Microtubule formation is critically important to many processes, not the least of which is neural development. This disruption of microtubules is consistent with the effects of MeHg on the brains of infants exposed *in utero* in Iraq; they showed a reduction of brain size and disordering of brain arrangement which could have been caused by lack of microtubule formation (Castoldi et al., 2001; do Nascimento et al., 2008).

MeHg has also been shown to disrupt calcium homeostasis. Calcium ions (Ca^{2+}) have been shown to play a role in central nervous cell death. At low concentrations MeHg has been shown to cause increased intracellular Ca^{2+} concentrations in cerebellar cell culture (Castoldi et al., 2001; do Nascimento et al., 2008). Ca^{2+} channel blockers and the Ca^{2+} chelator BAPTA protected these cells from MeHg-induced cell death (Castoldi et al., 2001; do Nascimento et al., 2008). *In vivo* administration of voltage-dependant Ca^{2+} channel blockers protected rats from neurological disorders due to MeHg treatment (Castoldi et al., 2001; do Nascimento et al., 2008).

Much work has focused on the effects of MeHg on the glutamatergic system and oxidative stress. MeHg has been shown to accumulate in astrocytes where it inhibits uptake and stimulates release of the excitatory neurotransmitter glutamate (Castoldi et al., 2001; Aschner et al., 2007; do Nascimento et al., 2008). This increased concentration of glutamate in the extracellular space can cause excitotoxicity in neurons. Glutamate

activates its receptors, which cause an increase in Na^+ influx, which leads to release of Ca^{2+} from intracellular stores (Castoldi et al., 2001; Aschner et al., 2007; do Nascimento et al., 2008). This in turn leads to the generation of reactive oxygen species (ROS), which accumulate and kill the neurons (Castoldi et al., 2001; Aschner et al., 2007; do Nascimento et al., 2008). The central role of glutamate accumulation in MeHg toxicity has been reinforced by experiments which show that cotreatment with N-methyl-D-aspartate (NMDA) receptor antagonists can attenuate the toxic effects of MeHg in cerebral neuron culture (Castoldi et al., 2001; Aschner et al., 2007; do Nascimento et al., 2008). These findings have led some researchers to postulate that the neurotoxic effects of MeHg are simply repercussions of its effects in astrocytes. Further work highlighting the importance of oxidative stress in MeHg toxicity involves the Nrf2/Keap1 complex. In this complex Keap1 binds to Nrf2, keeping it inactive in the cytosol; when Keap1 is modified by a perturbation of the oxidation state of the cell it releases Nrf2 which enters the nucleus and binds to antioxidant response elements (AREs) that regulate expression of traditional oxidative stress protectors, such as glutamate cysteine ligase (GCL), the rate limiting enzyme in the production of glutathione (Toyama et al., 2007). In human neuroblastoma SH-SY5Y cells treatment with MeHg has been shown to dissociate Nrf2/Keap1, allowing activation of AREs (Toyama et al., 2007).

Other genetic and epigenetic effects have also been observed in response to MeHg exposure. Many metals, including cadmium, arsenic, nickel, and chromium have been shown to influence epigenetic changes. The common cause has been hypothesized to be DNA damage caused by oxidative stress (Baccarelli and Bollati, 2009). Perinatal MeHg exposure has been implicated in the epigenetic alteration of brain derived neurotrophic

factor (BDNF) regulation in the mouse hippocampus. Three major changes were observed in promoter IV of the BDNF gene: an increase in histone H3-K27 trimethylation, an increase in DNA methylation, and a decrease in histone H3 acetylation. Each of these is consistent with the observed decrease in BDNF mRNA levels (Onishchenko et al., 2008).

In Drosophila early work showed that MeHg was toxic at much lower doses than methoxyethyl mercuric compounds (Sorsa and Pfeifer, 1973). It was also found that MeHg altered the puffing pattern of the prepupal salivary chromosomes, a measure indicating changes in gene regulation due to MeHg exposure (Sorsa and Pfeifer, 1973). MeHg has shown no evidence of genotoxicity in Drosophila, though, likely due to lethality by other mechanisms at doses too low to cause genotoxic damage (Carmona et al., 2008).

MeHg has also been shown to cause upregulation of genes in the Notch receptor pathway, specifically genes of the E(spl) complex, in Drosophila (Bland and Rand, 2006). This effect has been shown to occur independent of the Notch receptor itself and its coactivator Suppressor of Hairless Su(H) (Rand et al., 2008). Because of the key role Notch and its targets play in the development of the nervous system, specifically in cell-fate determination, this effect of MeHg on the E(spl) complex genes may prove crucial to the understanding of the neurodevelopmental toxicity of MeHg. To further investigate this effect a detailed understanding of the Notch pathway is important.

1.3: Notch pathway

The Notch cell-to-cell signaling pathway is involved in cell fate determination in many tissues. It is crucial to neural development, and interference with it leads to serious failures in neural differentiation or migration, akin to those seen in fetal MD.

Notch receptor structure and function has been largely elucidated in the Drosophila model. Notch is classified as a neurogenic gene because mutations in it cause an increased number of neuroblasts to form in the neurectoderm, a region in the ventral part of the embryo of *Drosophila melanogaster* (Subiza et al., 1987; Campos-Ortega and Knust, 1990). Other neurogenic genes include Delta and the E(spl) complex, which was originally thought to be a single gene (Subiza et al., 1987; Campos-Ortega and Knust, 1990). Early work established a link between Notch and E(spl), correctly identifying E(spl) as a downstream target of Notch (Subiza et al., 1987; Campos-Ortega and Knust, 1990). It was soon recognized that E(spl) was not a single gene with a large neurogenic effect when mutated, but a complex of several genes which each produced a subtle phenotype (Preiss et al., 1988). 13 genes have been identified in the E(spl) complex: *mδ, mγ, mβ, mα, m1, m2, m3, m4, m5, m6, m7, m8,* and *groucho* (Fig. 1-1) (Wurmbach et al., 1999) (Lai et al., 2000). Of these, seven (*mδ, mγ, mβ, m3, m5, m7, m8*) code for basic helix-loop-helix (bHLH) transcriptional repressors, four (*mα, m2, m4, m6*) code for Bearded family transcription factors, one (*groucho*) codes for a transcriptional corepressor, and one (*m1*) codes for a putative protease inhibitor (Wurmbach et al., 1999; Lai et al., 2000).

The Notch pathway is involved primarily in lateral inhibition during cell fate determination during development. One of the most widely studied examples is

development of the Drosophila peripheral nervous system. In this system sensory organ precursors (SOPs) begin as groups of equipotent cells. The center cell expresses Delta, a ligand of Notch, which causes Notch activation in the surrounding cells, leading to a signal cascade that upregulates expression of E(spl) complex genes (Portin, 2002). Early evidence for the regulation of E(spl) by Notch was the distinct pattern of *E(spl)* expression in the epidermal cells surrounding neuroblasts during nervous system development in Drosophila, which corresponded to known patterns of Notch activation (Jennings et al., 1994).

The Notch pathway has been shown to have a role in the development of many tissue types. The role of Notch in the developing nervous system is well studied. During development of the central nervous system in Drosophila embryos Notch causes lateral inhibition in a population of equipotent cells, allowing only some to differentiate into neuroblasts while the rest become epidermal cells (Portin, 2002). In the peripheral nervous system during embryo development Notch signaling plays a similar role, allowing some cells to become epidermal instead of nervous. Notch signaling is crucial to the proper formation of the sensory bristles during adult development (Portin, 2002). Notch is also involved in the development of the wing; in fact, the name Notch derives from the notched appearance of the wing in certain mutant strains. Notch signaling is involved in the development of wing veins; its ligand Delta is expressed in the region to become a vein, and Notch is expressed in the surrounding area, causing it to remain undifferentiated (Huppert et al., 1997). There is preliminary evidence that indicates Notch plays a role in the development of the Drosophila gut. The E(spl) gene $m\beta$ shows expression throughout the developing larval midgut that drastically increases at

prepuparium (Maeder et al., 2009). Other E(spl) genes, such as *mγ* and *mα* have also been shown to be expressed in the developing midgut (Maeder et al., 2009). During the embryonic development of the muscles Notch has been shown to be involved in the differentiation of muscle progenitors and the maintenance of this cell population until muscle fusion, in which muscle founder cells orchestrate the formation of the final muscle pattern (Fuerstenberg and Giniger, 1998; Maqbool and Jagla, 2007; Vasyutina et al., 2007; Tixier et al., 2010). Notch has been shown to also have a role in oogenesis and development of the appendages and Malpigian tubules of Drosophila (Portin, 2002). Effects of Notch on neurite outgrowth have also been well-established, and will be addressed later.

It has been shown that various E(spl) genes perform certain roles better than others. *mβ* has been shown to be best at wing vein suppression and shows the most extensive expression in the gut (Ligoxygakis et al., 1999; Maeder et al., 2009), *mδ* performs best at lateral inhibition in the eye (Ligoxygakis et al., 1998), *m7* and *m8* have been shown to suppress bristle formation best (Ligoxygakis et al., 1999), and *mγ*has been shown to serve best at activating the gene *cut* during wing formation (Ligoxygakis et al., 1999). Even the lesser studied *Bearded* family E(spl) genes have been shown to have specific effects; *m6* overexpression causes increased bristle formation, but *m2* overexpression causes lack of bristles (Lai et al., 2000). The various E(spl) genes also express in different patterns. In the gut, for example, *mβ* dots the entire midgut while *mγ* is only expressed in two specific bands (Maeder et al., 2009). This differential expression is due to differing sensitivities of the E(spl) genes to proneural genes and other spatially

restricted factors (Nellesen et al., 1999; Wech et al., 1999; Cooper et al., 2000; Maeder et al., 2007). The upstream enhancer regions of the E(spl) genes show patterns of Su(H) and proneural binding sites that are specific to each gene. Paired Su(H) sites and Su(H) sites paired with proneural sites are almost completely conserved among Drosophila species, indicating their importance (Maeder et al., 2007). Recent work has shown that the expression of *m8* is heavily reliant on proneural proteins, but *mγ* shows little *in vivo* reliance on proneural proteins for its expression (Cooper et al., 2000). So it has been shown that the different E(spl) genes show distinct expression patterns and roles in Notch signaling; despite this there is some overlap of function, though, since no tissue expresses only one E(spl) gene and mutation of any one E(spl) shows little discernable phenotype (Wech et al., 1999). In fact, studies of single nucleotide polymorphisms (SNPs) in wild-caught *Drosophila melanogaster* have shown that no significant association can be found between bristle number and E(spl) SNPs; one might expect there to be an association because of the important role of E(spl) in bristle development, but the fact that there isn't reinforces the finding that E(spl) genes have some redundancy (Macdonald et al., 2005).

While much of the data on the Notch pathway has been generated in the Drosophila model its presence has also been verified in other systems. In the murine system four homologues of *Notch* have been identified, *Notch1-4* (Jarriault et al., 1998). Homologues for the Notch ligands Delta and Serrate have also been found; *Delta-like-1* and *Delta-like-3* are murine homologues of *Delta* and *Jagged-2* is the murine homologue of *Serrate* (Jarriault et al., 1998). The *Su(H)* gene also has a murine homologue, named *RBP-J* (Jarriault et al., 1998). The murine homologues of the *E(spl)* genes are the *HES* family genes, with *HES-1* being the most studied (Jarriault et al., 1998). Thus the entire

Notch pathway has been identified in mice, and it works similarly to the Drosophila homologue: Delta-like-1 has been shown to activate Notch-1, which binds to RBP-J and enhances *HES-1* expression (Jarriault et al., 1998). Because this pathway has proved to be highly conserved between Drosophila and mammals it has been identified as a ripe target for the study of toxicological effects; findings in Drosophila are likely to hold at least partially true in mammals (Rand, 2009).

Notch signaling pathway overview

The molecular mechanisms of Notch signaling are well defined in Drosophila. The process progresses from activation of the Notch receptor to a signaling cascade that induces the ultimate effectors of the pathway, the *E(spl)* genes (Fig. 1-2). The E(spl) proteins function in lateral inhibition during tissue development, restricting most equipotent precursors from differentiating and allowing only the appropriate numbers of cells to assume a differentiated fate.

Notch is a transmembrane receptor containing repeated elements of epidermal growth factor (EGF) motifs (Portin, 2002). Two Notch ligands have been identified in Drosophila, Delta and Serrate, which bind the EGF sites and cause Notch activation when expressed on the surface of neighboring cells (Portin, 2002).

Once bound by a ligand the Notch extracellular domain is cleaved from the transmembrane/intracellular domains by ADAM (a disintegrin and metalloprotease) family proteases, such as Kuzbanian (Portin, 2002). After being cleaved from the extracellular domain the transmembrane/intracellular fragment is cleaved again, releasing

the intracellular fragment into the cytosol, in a process mediated by a Presenilin/γ–secretase protein complex (Portin, 2002).

Once free the Notch intracellular domain localizes to the cell nucleus, where it binds Su(H) proteins (Bailey and Posakony, 1995). This Notch binding allows the disassociation of Su(H) from corepressors that cause it to act as a repressor of gene transcription (Bray and Furriols, 2001). In this manner Notch may act as a permissive activator of Su(H), allowing Su(H) to act on its own as a transcriptional enhancer; this is thought to be the case in many Notch activity-dependent processes that do not rely on the *E(spl)* complex, such as proneural enhancement (de Celis et al., 1996; Bray and Furriols, 2001). Notch can also act as an instructive activator of Su(H), recruiting cofactors and facilitating the formation of a protein complex to activate Notch target genes; this is thought to be the case in Notch-dependant activation of *E(spl)* gene expression (Bray and Furriols, 2001).

Once bHLH E(spl) proteins have been produced they facilitate lateral inhibition during cell differentiation (Jennings et al., 1999). By binding to DNA motifs that overlap with binding sites for proneural proteins the bHLH E(spl) proteins directly oppose the effects of the proneurals (Jennings et al., 1999). Since the proneurals are heavily involved in differentiation of cells the effect of the bHLH E(spl) proteins is to stop cells from differentiating due to proneural expression (Jennings et al., 1994). The roles of other E(spl) proteins, those in the Bearded family, groucho, and the putative protease inhibitor m1, are less well understood.

Thus in the peripheral nervous system of *Drosophila melanogaster* during embryonic development the pathway acts as follows. Delta expressed in the center cells

of equipotent groups of ectodermal lineage binds to Notch in the surrounding cells (Portin, 2002). Notch is cleaved twice, once by an ADAM protease and a second time by Presenilin, releasing the intracellular domain. The Notch intracellular domain enters the nucleus where it binds Su(H) and recruits additional cofactors to enhance expression of *E(spl)* genes (Bray and Furriols, 2001). The bHLH E(spl) proteins produced oppose the effects of proneural proteins, which prevents the surrounding cells where Notch was activated from becoming neuronal, while the center cell where Notch was not activated differentiates into a neural precursor (Jennings et al., 1999).

Methylmercury and the Enhancer of Split locus

As early as 1973 it was noticed that MeHg has an effect on the genetics of *Drosophila melanogaster*; treatment with MeHg was observed to alter the puffing pattern of prepupal salivary chromosomes (Sorsa and Pfeifer, 1973). While this method certainly does not have the power of modern techniques it does indicate the ability of MeHg to affect the Drosophila genome. Recent studies have underscored the potential importance of the gene regulatory effects of MeHg on its toxicity.

It has been shown that treatment of neural-derived Drosophila cell lines and whole embryos with MeHg induces expression of genes in the E(spl) complex (Bland and Rand, 2006; Rand et al., 2008). This expression was initially attributed to an increase in Notch cleavage and thus presumed to increase signaling via the Notch intracellular domain (Bland and Rand, 2006). This was supported when knockdown of Notch expression using interfering RNA (RNAi) attenuated the response of the $m\gamma$ gene to MeHg treatment (Bland and Rand, 2006). Further work characterizing the E(spl)

response to MeHg indicated an increase in gene expression in as little as three hours of MeHg treatment (Rand et al., 2008). Looking at RNAi knockdown of Notch and Su(H) at this time point revealed no effect of either on MeHg-induced *mγ* expression (Rand et al., 2008). This conflicting data might indicate that Notch is involved in a secondary effect of MeHg and that it does not play a role until after the initial three hour time point (Rand et al., 2008). This is supported by the finding that the pattern of E(spl) gene expression differs when induced acutely by MeHg versus Notch (Fig. 1-3) (Rand et al., 2008). Treatment of cells with the calcium chelator ethylenediaminetetraacetic acid (EDTA) causes cleavage of Notch expressed on the cell surface, releasing the intracellular domain and stimulating the Notch pathway through endogenous Notch protein (Rand et al., 2008). The effects on the E(spl) locus of treatment with MeHg were compared to endogenous Notch activity stimulated with EDTA using quantitative real-time polymerase chain reaction (qPCR). It was found that Notch primarily causes upregulation of the E(spl) *m3* and *m7* genes with modest increases in *m2*, *mγ*, and *mβ*; importantly the *mδ* gene shows very little upregulation due to endogenous Notch activity (Rand et al., 2008). MeHg treatment caused upregulation primarily in the *mδ* and *mγ* genes, with little upregulation of *m3* (Rand et al., 2008). If MeHg was acting through Notch to cause its upregulation of *E(spl)* genes, it would be expected that MeHg and EDTA treatments would cause upregulation of the same genes. Because the *mδ* gene shows little expression due to endogenous Notch activity but is highly upregulated due to MeHg treatment we hypothesize it plays an important role in the neurotoxicity of MeHg.

The effect of MeHg on E(spl) genes is not exclusive to Drosophila. Studies using rat neural stem cells (NTCs) indicate that MeHg can inhibit NTC differentiation (Tamm et al., 2008). This failure to differentiate was correlated with an increase in Notch activity measured by increased cleavage of the Notch intracellular domain (Tamm et al., 2008). Pretreatment with a Notch cleavage inhibitor reversed the repression of neuronal differentiation due to MeHg treatment (Tamm et al., 2008). While these findings seem to indicate that MeHg acts in a Notch dependant manner in rat NTCs it has yet to be seen if a shorter time course of MeHg treatment relieves the Notch dependence, as has been shown in Drosophila cell lines.

1.4: The Drosophila embryo

Drosophila melanogaster is an important model organism for the study of developmental pathways and processes. It was in Drosophila that the Notch pathway was first described, and Drosophila remains one of the best models in which to study Notch signaling and other fundamental signaling pathways. There are three principle advantages of the Drosophila model. First, it is well studied; the literature contains a wealth of information on the genomics, genetics, transcriptomics, and proteomics that describe an organism. Second, there exist a plethora of tools to utilize in Drosophila. Compared to mammalian models it is easy to create transgenic animals; the gal4/UAS system allows previously created transgenics to be combined into new driver/responder pairs, allowing fast an efficient exploration of gene/tissue interactions (Fig. 1-4). Finally the Drosophila itself is easy to utilize and maintain. Fast generation times and large populations allow for

rapid advancement and quick replication of experiments. Ease of storage removes the need for expensive animal facilities and staff.

Additionally, Drosophila are exceptionally well suited for evaluation of developmental toxicity. The developmental timeline of the embryo is well-established (Fig. 1-5). The size and abundance of embryos allows immunostaining of whole mounts with large samples for each experiment. Exposure of the embryos is also simplified in Drosophila as many toxins, such as MeHg, diffuse readily into embryos that have had the chorion membrane removed (Fig. 1-6).

Motor Nerve Pattern and Guidance

The development and anatomy of motor nerves in the Drosophila embryo has been well studied and is easily accessible using immunofluorescent staining of whole mounted embryos, making it a good model of neurodevelopment. Each abdominal hemisegment of the embryo has two major motor nerves that emerge from the ventral nerve cord, the intersegmental nerve (ISN) and segmental nerve (SN) (Fig. 1-7). The ISN has three branches, the ISNa, ISNb, and ISNd (Kaufmann et al., 1998; Sun et al., 2001). ISNa projects dorsally to innervate muscles in the dorsal field, including the dorsal oblique and dorsal acute muscles (Kaufmann et al., 1998; Sun et al., 2001). The ISNb/d both terminate in the ventral muscle field and innervate muscles including the ventral longitudinal group (Kaufmann et al., 1998; Sun et al., 2001). The SN has two major branches, the SNa and SNc (Kaufmann et al., 1998; Sun et al., 2001). The SNa itself has a characteristic branching pattern, innervating the lateral transverse muscles with its upper branch and the segmental border muscle with its lower branch (Kaufmann et al.,

1998; Sun et al., 2001). The SNc innervates ventral muscle groups (Kaufmann et al., 1998; Sun et al., 2001).

Axon outgrowth in these motor nerves has been well studied. There is evidence that pioneer neurons establish the path for other axons to follow, though there is remarkable resilience to ablation of these pioneers (Lin et al., 1995; Sanchez-Soriano and Prokop, 2005). Fasciculin II (FasII) is the major adhesive molecule that maintains fasciculation in the nerve fiber; higher FasII expression levels in the pioneer neurons have been shown to cause them to grow ahead of other contributing neurons, establishing the path of the nerve (Sanchez-Soriano and Prokop, 2005). Specifically, FasII loss of function in the ISN pioneers, the aCC and RP2 neurons, causes them to lose their ability to influence nerve outgrowth (Sanchez-Soriano and Prokop, 2005). Increasing expression of FasII in follower neurons causes them to exert increased influence on the direction of nerve outgrowth (Sanchez-Soriano and Prokop, 2005).

The guidance cues for motor axon outgrowth have only recently been elucidated. It has been shown that during outgrowth the motor nerves contact glia, sensory nerves, and muscle cells expressing Sidestep (Side), a transmembrane immunoglobulin superfamily protein, in a tightly controlled temporospatial pattern (Fig. 1-8) (Aberle, 2009b, a; Siebert et al., 2009). Each of these tissues expresses Side only while they are in the immediate path of the motor nerves, turning off expression as the growth cone contacts and then passes them (Aberle, 2009b, a; Siebert et al., 2009). In *Side* mutants axons fail to defasciculate, indicating that Side acts in opposition to FasII to cause branching of motor axons (Aberle, 2009b, a; Siebert et al., 2009). Similarly, perturbation of the mesoderm can cause failures in defasciculation of the motor nerves (Landgraf et

al., 1999a). Mesodermal expression of the active Notch intracellular domain causes failure to develop muscle founder cells, which organize the fusion of muscle fibers (Landgraf et al., 1999a). Without muscle fibers to express Side motor axons fail to defasciculate (Landgraf et al., 1999a). The protein Beaten path Ia (Beat) has been shown to be the receptor for Side signals (Aberle, 2009b, a; Siebert et al., 2009). Beat is expressed on motor nerve growth cones and has been shown to interact with Side (Aberle, 2009b, a; Siebert et al., 2009). Beat mutants show motor axon defasciculation failure similar to Side mutants (Aberle, 2009b, a; Siebert et al., 2009). Additionally, unlike wild type axons, Beat mutants fail to follow ectopic Side expression (Aberle, 2009b, a; Siebert et al., 2009). These data indicate that Side/Beat act as the primary attractive cues guiding motor axon outgrowth in embryos.

If Side/Beat are the directors of motor axon outgrowth, actin assembly in the growth cone is the driving force behind it (Kaufmann et al., 1998). Evidence indicates that the Rho-family GTPases Dcdc42 and Drac1 play important roles in organization of the actin cytoskeleton in response to guidance cues (Kaufmann et al., 1998). Neuronal expression of dominantly activated versions of either Dcdc42 or Drac1 causes arrest of motor nerve outgrowth consistent with a disruption of leading edge motility (Kaufmann et al., 1998). Dominant negative expression of the two reveal differences in function, however; Dcdc42 dominant negative expression causes some axons to fail to reach their most distal targets, whereas Drac1 dominant negative expression causes changes in the trajectory of the axons but no change in outgrowth (Kaufmann et al., 1998). Both of these phenotypes can be mimicked pharmacologically utilizing cytochalasin D to block actin assembly (Kaufmann et al., 1998).

Other molecules have also been shown to play important roles in axon outgrowth, though the mechanisms of their action are less well defined. Five receptor-linked protein tyrosine phosphatases, DPTP10D, DLAR, DPTP69D, DPTP99A, and DPTP52F are expressed in embryonic Drosophila axons (Desai et al., 1997; Schindelholz et al., 2001; Sun et al., 2001). Mutation analysis of these proteins indicates that they interact in a variety of cooperative, redundant, or competitive ways depending upon cellular context to contribute to motor nerve outgrowth and guidance (Desai et al., 1997; Schindelholz et al., 2001; Sun et al., 2001). Mutation of these phosphatases, both individually and in combination, can cause a variety of phenotypes in motor nerves including axon stalling, failure to synapse, failure to form branches, and formation of additional branches (Desai et al., 1997; Schindelholz et al., 2001; Sun et al., 2001). What role in axon outgrowth these phosphatases play during normal development is still a matter of speculation.

The Notch pathway has also been shown to play a role in motor axon outgrowth. It has been shown that Notch and Delta expression during outgrowth are necessary for development of the ISN (Giniger et al., 1993; Crowner et al., 2003). Experiments utilizing flies expressing a temperature-sensitive Notch allele have shown that removal of Notch during outgrowth of the ISNb causes the axons to bypass their normal pathway, remaining adhered the ISNa rather than branching (Crowner et al., 2003). Data indicates that during normal axon outgrowth Notch protein expressed on the axon growth cone suppresses activity of the Abl tyrosine kinase in a noncanonical mechanism that does not involve Su(H) or E(spl)s; as the Abl pathway normally causes adhesion of the axons this suppression of Abl by Notch allows the ISNb to defasciculate from the ISNa (Crowner et al., 2003). Research into the role of Notch signaling in growth of longitudinal axons

across segments in the central nervous system have revealed similar Notch/Abl interactions resulting in decreased adhesion of axons to the substratum they grow across, allowing motility (Kuzina et al., 2011). Additional studies have shown that mutation of the Notch pathway regulator Endonuclease GI (EndoGI) causes failure of motor axons to innervate their appropriate muscle targets; this may indicate that EndoGI is active in the Notch/Abl signaling described in other work (O'Keefe et al., 2010). In cultured embryonic mouse neurons Notch has been shown to play the opposite role; increased Notch activity in these cells causes decreased neurite outgrowth (Sestan et al., 1999). The mechanisms for this decrease in outgrowth have not been elucidated. Combined these data indicate a complex, context-dependent role for Notch in neurite outgrowth.

1.5: Summary and significance

MeHg is a potent environmental toxin that strongly influences neural development. Though MeHg has been shown to impact a variety of cellular processes, the mechanism of its neurodevelopmental specificity is not yet fully understood. Experiments have shown that MeHg causes upregulation of canonical Notch-response genes in the E(spl) complex. Because of the crucial role of the Notch pathway in neural development the changes in Notch target expression elicited by MeHg may be an important mediator of the neurodevelopmental specificity of MeHg.

In this dissertation I hypothesize that overexpression of the E(spl) gene *mδ* plays a critical role in MeHg toxicity in Drosophila. To test this I will first establish that MeHg treatment *in vivo* is capable of causing *E(spl)mδ* overexpression, as it is in cell lines. I

will then identify a neural phenotype of MeHg treatment in Drosophila embryos and show that I am able to replicate it using genetic manipulation of *E(spl)mδ*.

The findings of this dissertation will support a role for the Notch pathway target *E(spl)mδ* in MeHg toxicity, suggesting there be further study of the impact of MeHg on E(spl) homologues in mammals. Additionally, my work will show that impacts on muscle development can mediate the effect of MeHg on neural development; supported by the previous work showing a glial impact of MeHg toxicity in mammals this finding suggests that the neurodevelopmental specificity of MeHg may not be due to direct impacts on neurons but instead on supporting tissues. Overall this work suggests new avenues of research to apply these findings toward a greater understanding of MeHg toxicity in humans.

1.6: Figures

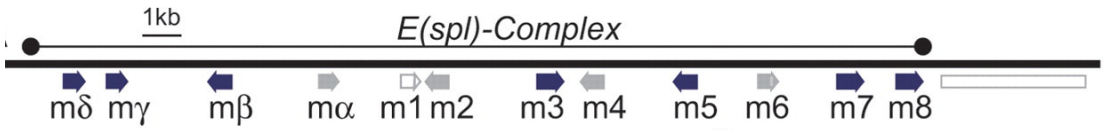

Figure 1 - 1. Schematic of the Enhancer of Split complex.

Adapted from (Krejci and Bray, 2007)

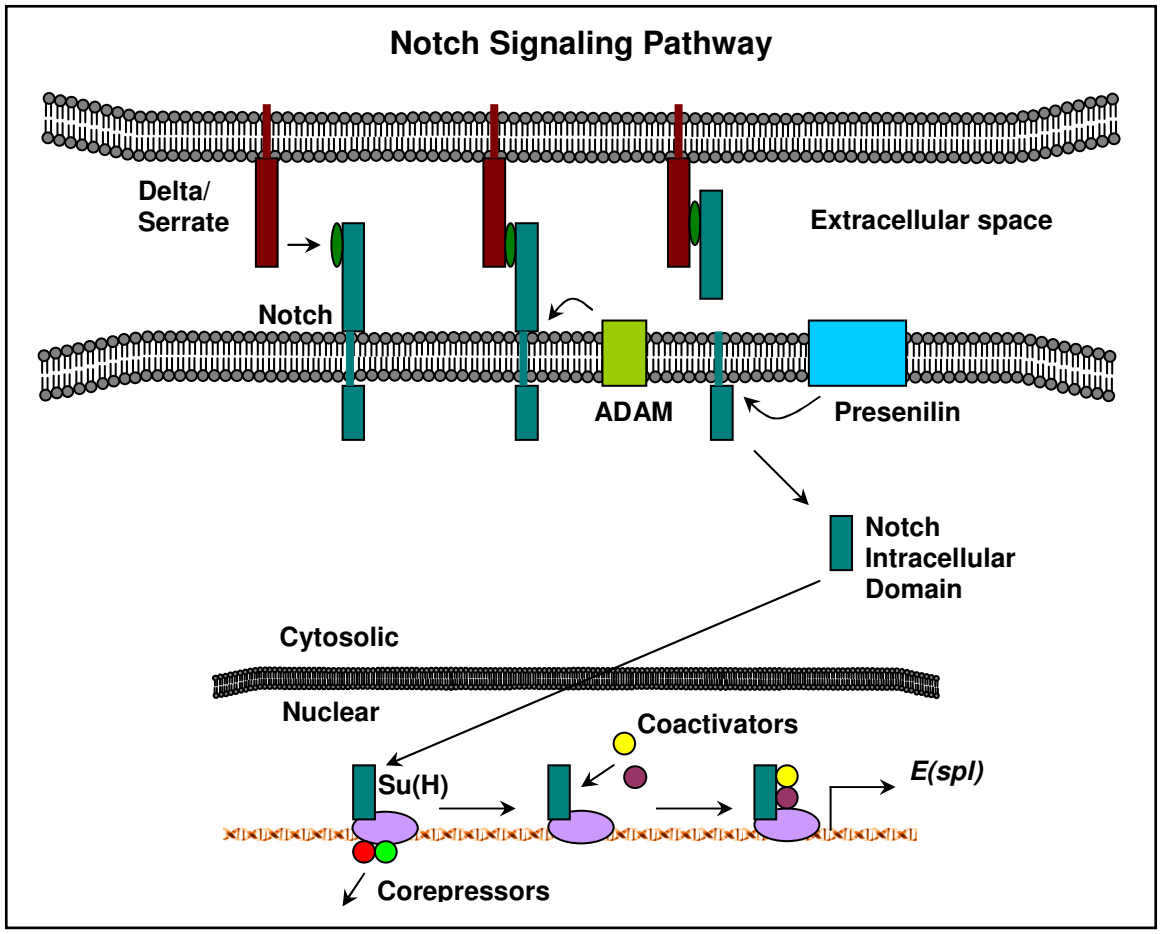

Figure 1 - 2. Schematic of the Notch signaling pathway.

Notch expressed on the cell receiving the signal binds its ligand, expressed on the cell sending the signal. After ligand binding ADAMs cleave the extracellular domain of the Notch protein from the transmembrane/intracellular domains. Presenilin then cleaves the intracellular domain from the transmembrane domain. The free Notch intracellular domain enters the cell nucleus where it binds to Suppressor of Hairless [Su(H)], allowing dissociation from corepressors. Notch then recruits coactivators and the Notch/Su(H) complex drives expression of Notch targets, specifically the *Enhancer of Split* [*E(spl)*] gene complex.

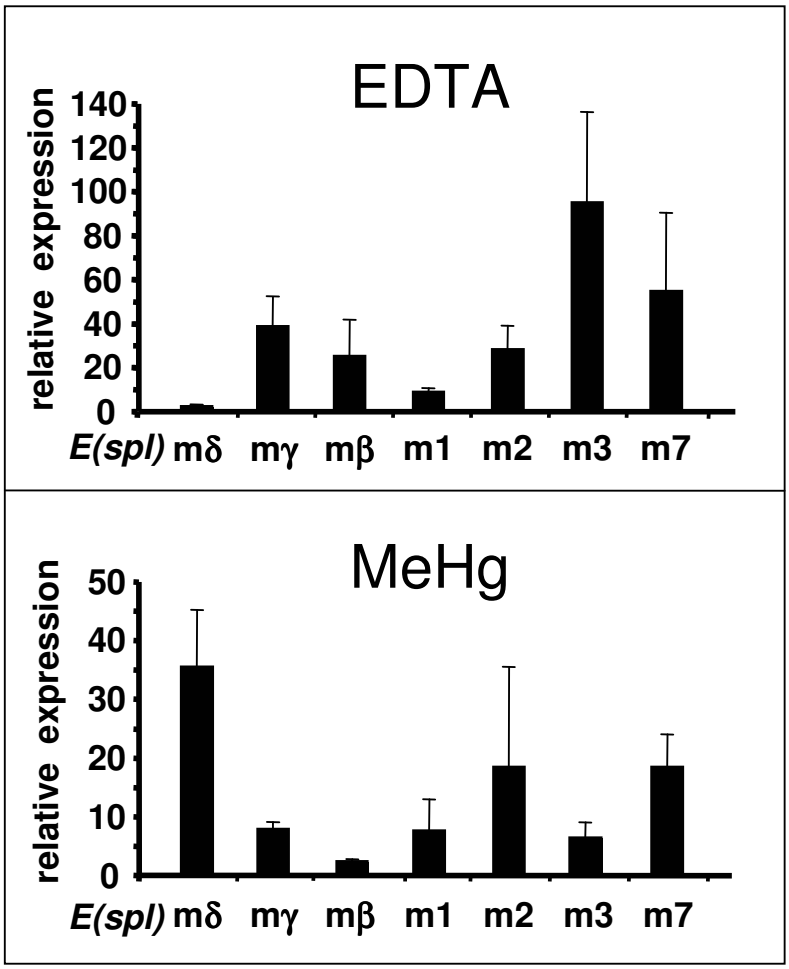

Figure 1 - 3. Methylmercury and Notch signaling show different fingerprints of E(spl) upregulation.

In the Drosophila neural-derived cell line bg2-c6, following MeHg treatment or Notch cleavage through treatment with EDTA the relative expression levels of E(spl) complex genes were assayed via qPCR. The figure shows relative fold change over untreated controls normalized to the RP49 housekeeping gene. Modified from (Rand et al., 2008).

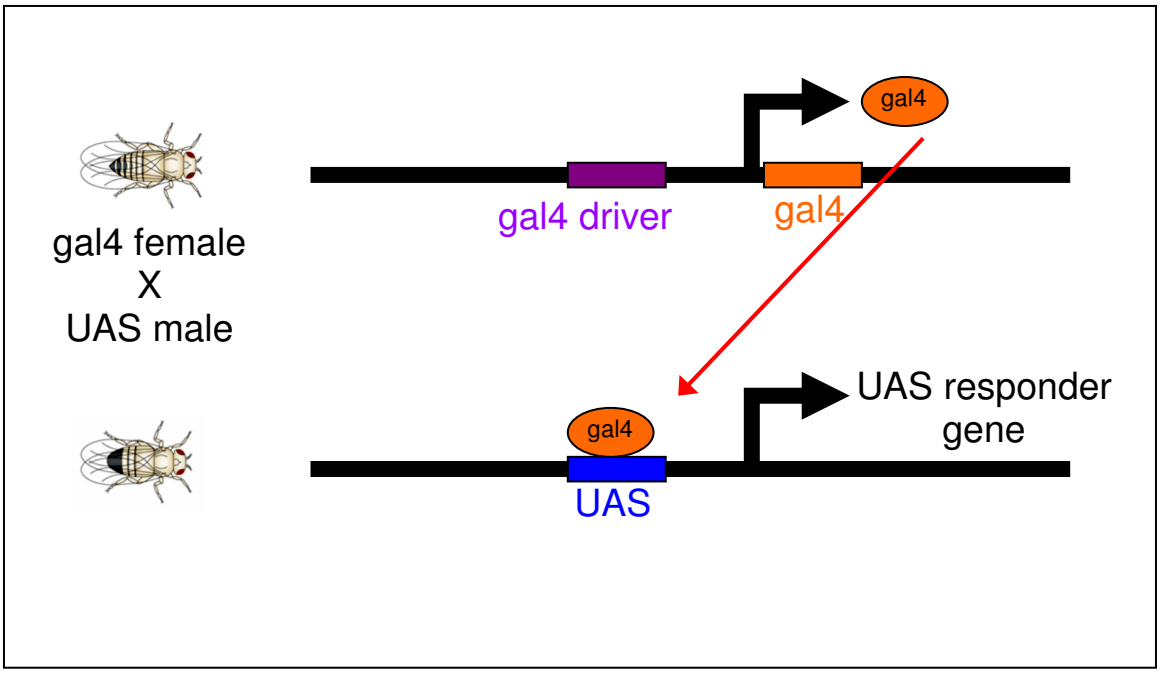

Figure 1 - 4. Schematic of the GAL4/UAS exogenous expression system in Drosophila.

The GAL4/UAS system is widely used in Drosophila to drive expression of genes of interest under the control of a promoter of interest. Female flies carrying a construct in which a driver of interest drives expression of the yeast transcription factor GAL4 are bred with males carring a construct in which the yeast upstream activator sequence (UAS) drives expression of a gene of interest. In cells where GAL4 is expressed it binds to the UAS and drives expression of the gene of interest.

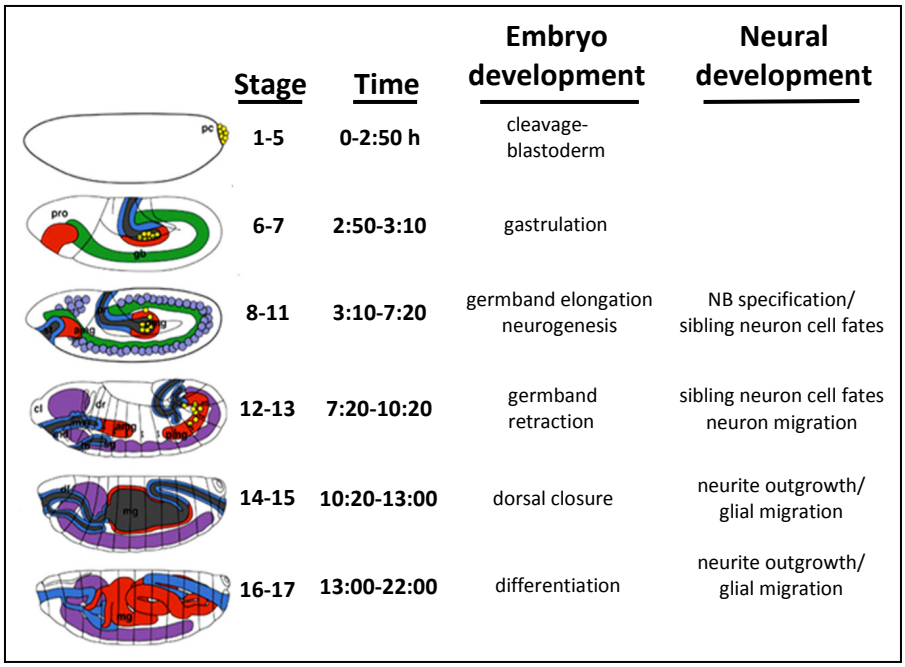

Figure 1 - 5. Stages of Drosophila embryo development.

Anterior is left and dorsal is up. Neuroblasts (light purple, at stage 8-11). Ventral nerve cord (VNC) and brain neurons (purple, at St. 12-17). Endoderm/midgut (red). Mesoderm (green). Foregut/Hindgut (Blue). Adapted from (Weigmann et al., 2003).

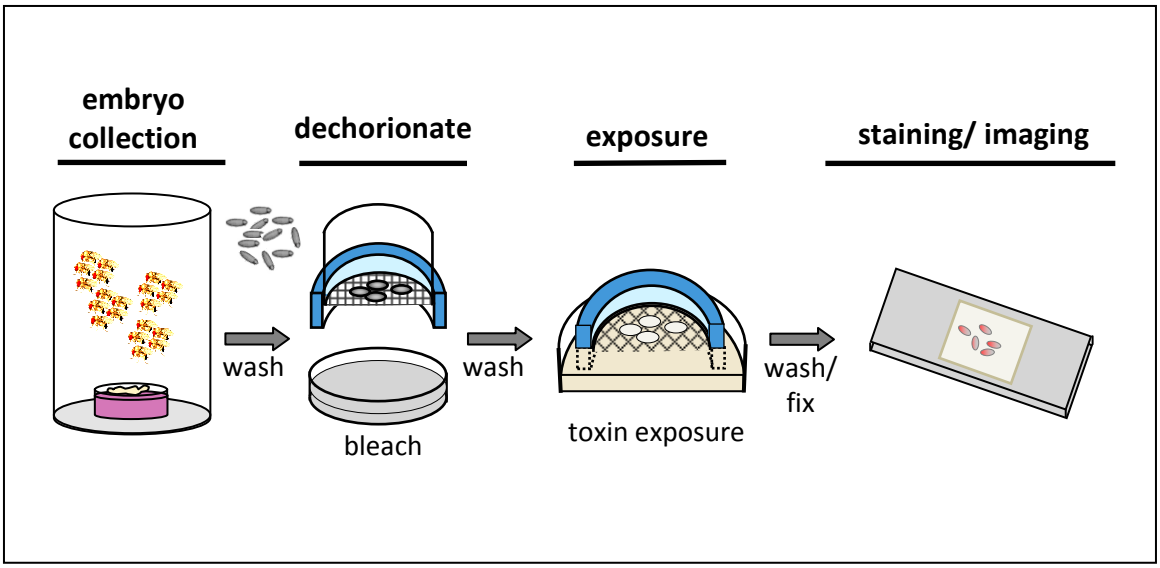

Figure 1 - 6. Methods for in vitro exposure of Drosophila embryos to toxins.

Embryos collected from adults laying on grape-agar plates are dechorionated in dilute bleach. Embryos are then placed in baskets designed to optimize air exposure while being immersed in a solution containing the toxin of interest. After a period of developmental exposure, embryos can then be processed for RNA isolation or for fixation, staining and, imaging. Adapted from (Engel et al., 2012).

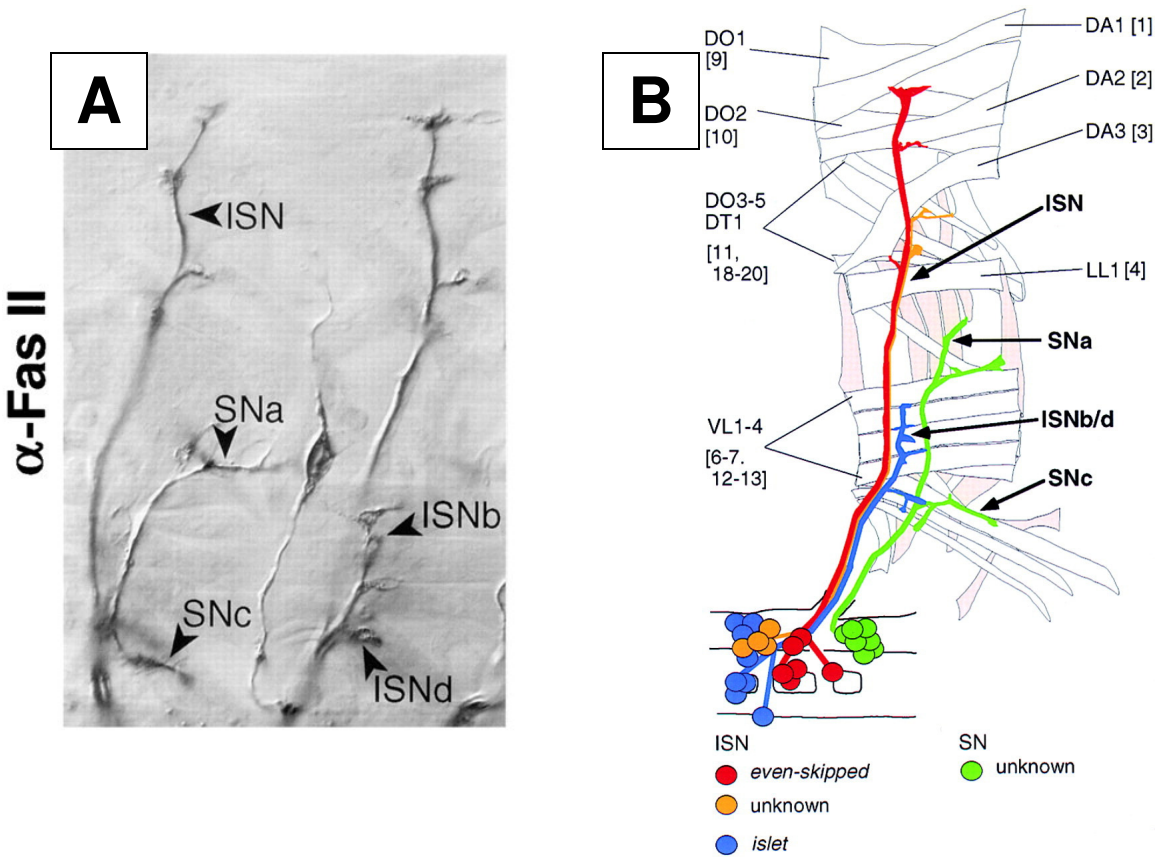

Figure 1 - 7. Image and schematic of motor nerves in the embryo.

A) Immunostaining embryos with αFasII antibody reveals the motor nerves. The intersegmental nerve (ISN) has three main branches, the ISNa (labeled ISN), the ISNb, and the ISNd. The segmental nerve (SN) has two major branches, the SNa and SNc. The SNa itself has a characteristic branch near its terminal so it can innervate both the lateral transverse muscles and segmental border muscle. B) An illustration of the motor nerves and the muscles they innervate in one hemisegment, showing genes specifically expressed in different neuron populations. Adapted from (Landgraf et al., 1999a).

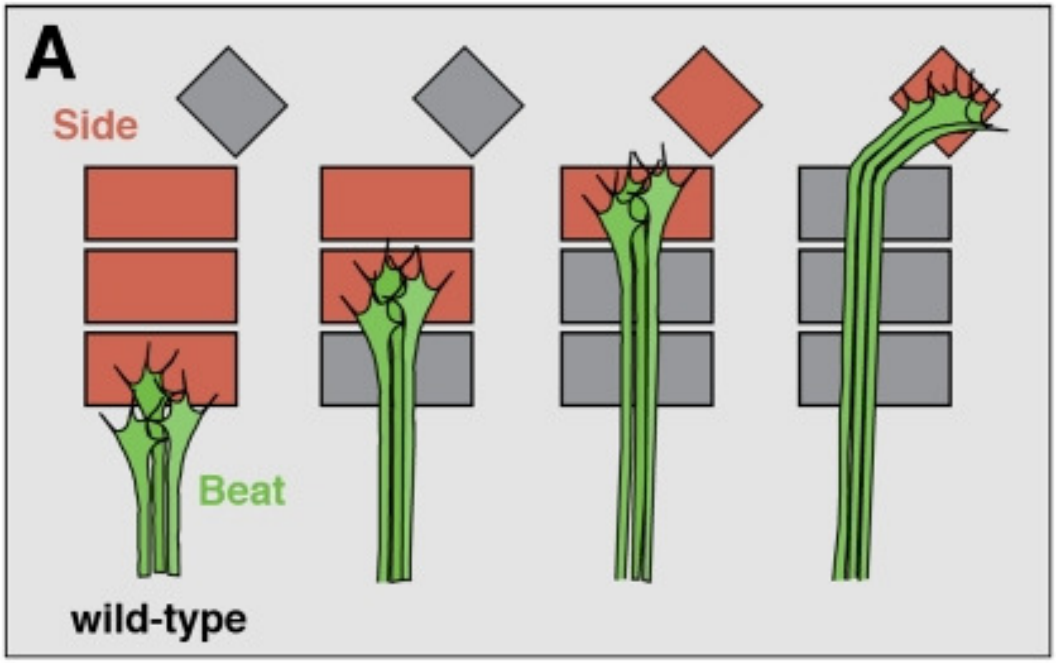

Figure 1 - 8. Temporospatial regulation of Sidestep guides motor axon pathfinding.

Guidepost cells in the direct path of the developing embryonic motor axon express Sidestep (Side), an attractive cue. The axon growth cones express Beaten path Ia (Beat), the receptor for Side. It is the carefully timed expression of Side in the cells the axon must next grow towards that guides motor nerve pathfinding in Drosophila. Adapted from (Siebert et al., 2009).

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CHAPTER 2: THE EFFECTS OF METHYLMERCURY ON NOTCH SIGNALING DURING EMBRYONIC NEURAL DEVELOPMENT IN DROSOPHILA MELANOGASTER

Material from this chapter has been published in the following form:

Engel GL, Delwig A, Rand MD (2012) The effects of methylmercury on Notch signaling during embryonic neural development in Drosophila melanogaster. Toxicol In Vitro.

2.1: Abstract

Methylmercury (MeHg) is a ubiquitous toxicant that targets the developing fetal nervous system. MeHg interacts with the Notch signaling pathway, a highly-conserved intercellular signaling mechanism required for normal development. Notch signaling is conveyed by activation of the genes in the Enhancer of Split (E(spl)) locus in *Drosophila*. We have previously shown that acute high doses of MeHg upregulate several E(spl) genes in *Drosophila* neural-derived C6 cells. Furthermore, MeHg induction of E(spl) can occur independent of the Notch receptor itself. We now show that MeHg, unlike inorganic mercury ($HgCl_2$), preferentially upregulates *E(spl)mδ* and *E(spl)mγ* in *Drosophila* C6 cells. This is distinct from Delta ligand-induced Notch signaling in which no induction of *E(spl)mδ* is seen. MeHg is also seen to specifically upregulate *E(spl)mδ* in *Drosophila* embryos where $HgCl_2$ showed no such effect. Additionally, treatment of embryos with MeHg caused a consistent failure in axonal outgrowth of the intersegmental nerve (ISN). This ISN phenotype was partially replicated by genetic activation of the Notch pathway, but was not replicated by increasing expression of *E(spl)mδ*. These data suggest a role for Notch signaling and the *E(spl)mδ* target gene in MeHg toxicity, however, the site of action for *E(spl)mδ* in this system remains to be elucidated.

2.2: Introduction

Methylmercury (MeHg) is a ubiquitous environmental toxin that preferentially targets the developing nervous system. Because of its apparent specificity for neural tissue, signaling pathways in neural development may be important targets in MeHg

toxicity. Several studies in both mammalian and invertebrate systems now support the hypothesis that the Notch pathway is a potential target for MeHg. Notch is a fundamental cell-cell signaling pathway that directs cell fate decisions during neurogenesis. Being first elucidated in *Drosophila*, it is now well understood that signals through Notch receptors cause activation of downstream effectors; those of the Enhancer of Split [E(spl)] gene locus in flies and the Hairy/Enhancer of Split (HES) genes in mammals (de-la-Concha et al., 1988; Preiss et al., 1988; Jarriault et al., 1998). The E(spl) locus in flies consists of 11 genes in a single 50kb locus. Seven of these E(spl) genes, *E(spl)mδ, E(spl)mγ, E(spl)mβ, E(spl)m3, E(spl)m5, E(spl)m7,* and *E(spl)m8*, are basic helix-loop-helix transcriptional repressors. While different E(spl) genes are known to be preferentially expressed in various developing tissues, manipulations in *Drosophila* demonstrate that all the E(spl) genes are capable of responding to Notch signals (Jennings et al., 1999; Nellesen et al., 1999; Wech et al., 1999; Wurmbach et al., 1999). Signals at the level of the Notch receptor are propagated by cleavage and activation by members of the ADAM family of metalloproteases. The potential for MeHg to stimulate ADAM activity initially led to the hypothesis that MeHg could ultimately induce Notch signals (Bland and Rand, 2006). This was supported by evidence that *E(spl)mγ* and *E(spl)mβ* show a dose-dependent increase in transcription with MeHg applied to *Drosophila* neural cells in culture (Bland and Rand, 2006). In subsequent studies we have shown that stimulation of E(spl) genes in *Drosophila* cells by MeHg can occur despite knockdown of Notch receptor expression (Rand et al., 2008). These observations suggest MeHg can act through a more direct mechanism, bypassing the receptor to stimulate transcription of Notch targets.

In this study, using MeHg exposures to *Drosophila* C6 neural derived cells in culture in addition to exposures of the whole animal at various developmental stages, we confirm a specific action of MeHg toward the *E(spl)mδ* gene. We also demonstrate that *E(spl)mδ*, in stark contrast to the other E(spl)s, is not responsive to Notch signals propagated by its cognate ligand, Delta, in the C6 neural cell line, allowing us to elucidate the MeHg specific action on this gene target. A specific effect of MeHg relative to inorganic mercury ($HgCl_2$) in *E(spl)mδ* activation is observed in C6 cells and in embryos, however, mercury induction of *E(spl)mδ* was not seen at later developmental stages. MeHg treated embryos exhibit an overt defect in formation of the intersegmental nerve (ISN). Increasing Notch pathway activity in neurons by driving expression of the Notch intracellular domain (N_{ICD}) under the control of the pan-neural elav promoter causes a similar defect in ISN outgrowth, however driving expression of *E(spl)mδ* in neurons did not elicit an ISN phenotype.

Our findings indicate that MeHg specifically effects *E(spl)mδ in vitro* and *in vivo* during *Drosophila* embryogenesis. Our data shows specificity in gene activation by MeHg compared to other stressors, such as $HgCl_2$, highlighting the potential for *E(spl)mδ* to mediate some MeHg-induced changes in developmental signaling in the embryo. However, neuron-specific expression of *E(spl)mδ* did not replicate a characteristic ISN MeHg phenotype, which could be partially replicated with neuron-specific Notch activation. These results point to novel non-canonical Notch pathway mechanisms that contribute to MeHg toxicity in the embryonic nervous system.

2.3: Methods

Cell Culture:

Drosophila bg2-c6 cells (C6 cells), a neural cell line obtained from the Drosophila Genomics Resource Center (Ui et al., 1994) were cultured in Shields and Sang M3 medium with added bactopeptone and yeastolate (BPYE), supplemented with bovine serum, insulin, and penicillin/streptomycin at 25°C in a humidified incubator.

Cell culture mercury treatments:

Stock solutions of methylmercury (MeHg chloride, Aldrich 442534) and mercury chloride ($HgCl_2$, Sigma-Aldrich 215465) were prepared in dimethyl sulfoxide (DMSO) at 50mM. Mercury treatments were made at concentration ranging from 0-100µM. DMSO concentration was adjusted to be equivalent across control and mercury treatments and never exceeded 0.1% DMSO. Cells were plated at 80% confluence in standard medium and allowed to adhere and recover for one hour. They were then washed three times with M3 medium lacking serum or antibiotics (M3-) and the medium was replaced with M3- with added mercury or DMSO control. Cells were treated for three hours, after which the medium was removed and cells were harvested for either RNA extraction with Trizol reagent (Invitrogen) or viability assays were performed.

Cell viability assay:

Cell viability was determined by dual staining with calcein and ethidium using the LIVE/DEAD Viability/Cytotoxicity Kit for mammals (Invitrogen) as per product instructions. Briefly, cells were treated with various concentrations of MeHg or $HgCl_2$ for

3hr, then treated with 2μM calcein AM and ethidium homodimer and incubated for 30min. Cells were washed, then plated and counted for green and red cells. Breakdown of calcein AM by esterases causes green fluorescence and marks living cells, while disruption of cell membranes in death allows permeability to ethidium homodimer causing nuclear red fluorescence. The ratio of green to red cells, normalized to control cell treatments and treatments eliciting 100% cell death, were used to determine the measure of cell viability. (n>150 cells per single treatment across seven concentrations).

Fly stocks and crosses:

Unless otherwise stated, fly stocks were maintained on standard cornmeal-yeast-molasses food at 25°C. Lines used include Canton S, elav-GAL4 (Bloomington Drosophila Stock Center, #458), UAS-Notch$_{intra}$ (gift from Cedric Wesley, University of Wisconsin Laboratory of Genetics), and UAS-E(spl)mδ (Bloomington Drosophila Stock Center, #26677). Standard crosses were performed between virgin female Gal4 driver lines and male UAS responder lines to generate F1 progeny to be tested.

Embryo mercury treatments:

Cages of adult flies were allowed to lay eggs on a grape agar plate with yeast paste smeared on the center. (See Fig. 2-4) Unless stated otherwise the embryo collection occurred over a two hour laying period. Embryos were then aged on the grape plates at 25°C; standard aging time was two hours. Embryos were dechorionated using a standard protocol. Briefly, embryos were washed from the plate using tap water and a brush into a nytex basket, then rinsed to remove yeast; baskets were transferred to 50% bleach (~3.8%

sodium hypochlorite) for three minutes, then rinsed in tap water to remove bleach. Embryos were transferred to separate nytex baskets for each treatment, which were in turn placed into petri dishes containing phosphate buffered saline (PBS) with added mercury or DMSO control. These dishes were covered to prevent evaporation and the embryos were allowed to incubate for 16-18 hours or various times where indicated. A schematic of this novel *in vitro* method to dose embryos with toxins can be found in Figure 2-4.

Immunostaining:

Immunostaining was performed as previously described (Rand et al., 2009). Treated embryos were fixed in 500 µL of a 50:50 mix of 8% paraformaldehyde in 0.1 M PIPES, 2 mM EGTA, 1 mM MgSO4, pH 6.9 (PEM) with heptane by rocking for 25 min. Vitelline membranes were subsequently removed by discarding the lower PEM layer and adding 750 mL MeOH, then vortexing for 30 s. Settled embryos were collected, washed and stored at -20C in methanol until staining. For immunostaining, embryos were permeablized in phosphate buffered saline (PBS) with 1% BSA, 0.1% triton X-100 (PBT). Subsequent blocking, primary and secondary antibody incubations were done in PBT with 5% each of donkey and goat serum. Primary antibodies used were: mouse anti-elav (9F8A9), mouse anti-notch (9C6), and rat anti-FasII (1D4) (Obtained from the Developmental Studies Hybridoma Bank, Univ. of Iowa). Secondary antibodies used were: Alexa488-conjugated goat anti-rat and Alexa555-conjugated goat anti-mouse (Jackson ImmunoResearch). Embryos were visualized by fluorescence microscopy on a Leitz Orthoplan 2 microscope equipped with a Spot One digital camera and associated

acquisition software (MVI, Avon, MA). Images were assembled in Adobe Photoshop, GIMP, and Microsoft PowerPoint.

RNA Extraction and quantitative PCR:

After mercury treatments, embryos were transferred with a paintbrush into microcentrifuge tubes containing PBS supplemented with 0.1% Triton-X and 1% bovine serum albumin (PBT). The PBT was removed with a pipette and replaced with Trizol reagent (Invitrogen). Embryos were homogenized in Trizol and processed for RNA isolation. RNA samples were treated with Turbo DNAse (Ambion) and reversed transcribed using SSII reverse transcriptase (Invitrogen). RNA was subsequently removed with RNAse H (USB**).** cDNA samples were assayed for gene expression via qRT-PCR using SybrGreen dye (Bio-Rad). Data were normalized to the ribosomal protein RP49 housekeeping gene and analyzed using the comparative Ct method (Livak and Schmittgen, 2001). Primer sequences for *E(spl)mδ, E(spl)mγ, E(spl)mβ, E(spl)m2, E(spl)m3, E(spl)m7, Notch,* and the *RP49* control were taken from (Rand et al., 2008). The sequences used for *Hsp70Ab* were Forward: TGAGAGTGATAAGAATGTTTCGAT and Reverse: AGTCTACAAAACATTAAATGACCAAGTT. For *Hsp70Bc* the sequences were Forward: ATCAGCAGGGAGCGGGAGCA and Reverse: TCACTTTTAAAAACTTAAGCCGAAA.

Larval and adult mercury treatments:

Embryos collected from an overnight laying in a population cage were transferred to bottles of standard cornmeal-molasses food supplemented with 15 μM MeHg or DMSO (control). Embryos were allowed to develop until they reached the late third instar wandering stage (approximately 4 days). At this stage they were harvested and disrupted for RNA isolation using an RNeasy mini kit (Qiagen).

Adult flies were cultured for three days on food containing concentrations of MeHg up to 100μM. The flies were then frozen using liquid nitrogen and broken apart using a vortexer. Heads were collected using a sieve and subsequently homogenized in Trizol reagent. RNA transcript levels were assayed by qRT-PCR as described above.

2.4: Results

We have previously demonstrated an ability of MeHg to induce E(spl) gene expression in cultured *Drosophila* C6 cells (Rand et al., 2008). In this previous study EDTA was used as a proxy to invoke Notch signaling in C6 cells for comparative effects on E(spl) expression. Since notable differences between EDTA-induced and MeHg-induced E(spl) activation was observed, we wished to further validate these effects with respect to Notch signaling. To examine Notch signaling explicitly we co-cultured the C6 cells on fixed preparations of cells expressing Delta, the endogenous ligand of the Notch receptor, in a previously established assay in our laboratory (Delwig and Rand, 2008). We then analyzed E(spl) expression exclusively in the Notch expressing cells via qRT-PCR (Fig. 2-1). This treatment caused the greatest induction of *E(spl)mβ* and *E(spl)m3* (>20-fold), and a nearly 15-fold induction of *E(spl)mγ*. In contrast, *E(spl)mδ* was not

upregulated by Delta induced Notch signaling. Overall, Delta induced Notch signaling under these conditions gives a similar pattern of E(spl) induction as was seen with EDTA earlier (Rand et al., 2008) indicating this profile is representative of Notch signaling in this cell line.

We next sought to compare MeHg effects with inorganic mercury to determine if the E(spl) activation profile of MeHg is unique or shares properties with other mercurials. We first determined levels of toxicity of MeHg and $HgCl_2$ toward C6 cells. Cell viability subsequent to MeHg and $HgCl_2$ exposures was determined using dual calcein/ethidium staining (Fig. 2-2). A dose dependent decrease in cell viability was observed with MeHg, which proved more potent than $HgCl_2$. MeHg exhibited an approximate 20% cell death (80% viability) at 4μM and an approximate 50% reduction in viability at 20μM. $HgCl_2$ proved weaker showing an approximate 20% reduced viability at 20μM and 50% reduced viability with 100μM $HgCl_2$. Similar results were obtained using alternative determinations with Trypan Blue reagent (Data not shown). These doses were implemented in subsequent assays of acute exposure effects on E(spl) activation.

With MeHg treatment of C6 cells *E(spl)mδ*, *E(spl)mγ*, and *E(spl)m7* showed the greatest fold-induction (7 to 12-fold, Fig. 2-3) of six representative E(spl) genes spanning the E(spl) locus. *E(spl)mβ* and *E(spl)m3* showed less substantial increases (less than 4-fold) with MeHg treatment, while *E(spl)m2* approached a 6-fold induction. In contrast, cells treated with $HgCl_2$ showed less than 3-fold response in *E(spl)mδ* and *E(spl)mγ*, and less than 5-fold induction in *E(spl)m7* (Fig. 2-3). *E(spl)m2* responded to $HgCl_2$ treatment

with a nearly 9-fold change with 100μM MeHg (Fig. 2-3). These data show a differential response of individual genes in the E(spl) locus with MeHg versus $HgCl_2$ exposure.

The apparent unique effect of MeHg on E(spl) gene expression *in vitro* prompted us to investigate similar effects *in vivo*. We have previously established an ability to dose *Drosophila* embryos with MeHg cultured in vitro (Rand et al., 2009). This methodology is summarized in Figure 2-4. The method takes advantage of the unique property that fly embryos denuded of their outer chorion layers of the eggshell are permeable to MeHg and are also able to continue development suspended in a defined culture media (see methods and Figure 2-4). Using this technique we evaluated the dose-response of embryos to MeHg by assaying gene expression using qRT-PCR and monitoring the response of a ubiquitous stress response gene, *Hsp70*. The *Hsp70 Bc* gene showed a robust increase in expression with increasing levels of MeHg, confirming the entry of the MeHg in embryonic tissues (Fig. 2-5). In parallel we probed *E(spl)mδ* gene expression which was seen to increase across all concentrations of MeHg. A more than seven-fold increase in *E(spl)mδ* was seen at 20μM MeHg, which appeared to be sustained at 50μM MeHg. From these data we chose to treat embryos with 50μM MeHg in subsequent analyses to ensure we were above a threshold in effect.

To assess the level of specificity with which MeHg acts on embryonic tissues we again compared E(spl) expression response to MeHg versus $HgCl_2$ treatment (Fig. 2-6). After MeHg, treatment (50μM) *E(spl)mδ* consistently showed greater than three-fold upregulation in embryos across several trials. In contrast, none of the other E(spl) genes assayed showed a response to MeHg in embryos treated *in vitro*. In addition, *Notch* showed no change in expression in response to MeHg, indicating that increases in

E(spl)mδ could not stem from increased receptor expression. An induction of two *Hsp70* genes *Hsp70Ab* and *Hsp70Bc* was observed for MeHg, again confirming entry of the toxicant into the embryos. Treatment of embryos with 1mM $HgCl_2$ did not cause any increase in E(spl) gene expression. In contrast, a modest decrease was seen in levels across all the E(spl) genes and *Notch* after $HgCl_2$ treatment. Upregulation of *Hsp70* genes after $HgCl_2$ treatment indicated that the dose of $HgCl_2$ used showed a similar degree of entry and overall toxic insult to that of MeHg. These data indicate that MeHg acts selectively on *E(spl)mδ* transcription in *Drosophila* embryos.

E(spl) gene expression is known to change over the course of embryogenesis (Tweedie et al., 2009). Recent data from gene expression arrays performed within the large scale ModEncode project (Tweedie et al., 2009) and publically available on Flybase (Graveley et al.) permit a comprehensive analysis of developmental expression of the E(spl) gene during normal embryogenesis. Transcript levels of *E(spl)mδ*, *E(spl)mγ,* and *E(spl)m7* show a similar profile as Notch, which shows a peak of expression at 6-8 hours of development after egg laying (Fig. 2-7). In contrast, *E(spl)m3* shows peak expression discernibly later, peaking at 8-10 hours AEL. The bell-shaped expression of the E(spl) genes prompted us to test whether the effect of MeHg on increasing *E(spl)mδ* in embryos was simply due to a developmental delay and shift in peak of gene expression versus an ectopic induction of gene expression. To achieve this we incubated batches of developmentally staged embryos with or without MeHg for various treatment intervals, and compared E(spl) expression via qRT-PCR. In untreated embryos the overall profile of E(spl) expression showed the characteristic increase followed by a decrease over the

course of embryogenesis. For *E(spl)mδ*, *E(spl)mγ*, and *E(spl)m7* a peak of expression between 6-8 hours after egg laying (AEL) was observed (Fig. 2-8). For *E(spl)m3* this peak was seen between 8-10 hours AEL. *Notch* expression showed a gradual decline over the course of embryogenesis. With MeHg treatment, *E(spl)mδ* showed higher expression at each time point after 6hr AEL compared to untreated embryos, with more than 2-fold higher expression at the 8-10hr interval. Peak expression remained at the 6-8 hour AEL interval with MeHg, indicating the developmental delay was not substantial at this time point. Higher expression due to MeHg was not consistently observed for *E(spl)mγ* and *E(spl)m3*, while *E(spl)m7* did show modest increases at time points after 8hrs AEL with MeHg treatment. *Notch* showed no consistent difference in expression due to MeHg treatment over these developmental periods (Fig. 2-8). *Hsp70 Bc*, like *E(spl)mδ*, showed increased expression due to MeHg treatment at every time point, confirming the access of MeHg to embryonic tissues at all stages.

Observing that *E(spl)mδ* is upregulated in embryos after MeHg treatment we investigated whether or not the MeHg effect was penetrant at later developmental stages. First instar (L1) larvae were cultured on food containing 15μM MeHg and harvested for qRT-PCR after reaching the wandering third instar (L3) stage. Previous analyses have demonstrated that treatment of larvae with 15μM MeHg shows similar toxicity to 50μM treatments of embryos (Rand et al., 2009; Mahapatra et al.). We then examined global transcript levels of E(spl)s and *Notch* from whole larval extracts using qRT-PCR (Fig. 2-9). No change in expression due to MeHg in *E(spl)mδ* or any E(spl) in the larvae was

observed. These data suggest that *Drosophila* tissues at later developmental stages than the embryo are refractory to MeHg induced expression of E(spl).

To further test whether the effect of MeHg on *E(spl)mδ* expression is specific to the embryo we tested E(spl) expression responses to MeHg in adult flies. Adult *Drosophila* were cultured for three days on food containing various concentrations of MeHg up to 100μM. RNA transcript levels in extracts prepared from isolated heads were assayed by qRT-PCR. We opted to examine this tissue since Notch activity and E(spl) expression is a strong determinant in neural tissues and the fly head is rich in brain tissue. We determined that no consistent changes in *E(spl)mδ*, or any of the E(spl) genes examined, were seen with MeHg treatments (data not shown). Altogether, the data from larval and adult assays indicate that the global effect of MeHg on *E(spl)mδ* expression is specific to embryos.

We next turned to examining the embryonic nervous system for phenotypes that characteristically reflected MeHg insult to development. Our previous studies have identified several features in the embryo CNS and PNS that reflect compromised development with MeHg exposure (Rand et al., 2009). Notably, neurite outgrowth of CNS and PNS axons visualized in the lateral field of the late stage embryo has been seen to be compromised (Rand et al., 2009). Using an antibody specific to the ISN, a bundle of four axons of central motor neurons, we observed that these axons frequently failed to develop properly in MeHg treated embryos (Fig. 2-10). This phenotype of MeHg exposure presents as stunted or misguided ISNs in treated embryos (Fig. 2-10 D-F) and is easily scored by the growth of axons relative to the position of elav-positive PNS neurons

in the lateral field (Fig. 2-10 F). Interestingly, previous studies have identified a role for Notch signaling in the appropriate projection of the ISN neuron (Giniger et al., 1993).

We therefore set out to test whether genetic manipulations of Notch in general, and *E(spl)mδ* in particular, could mirror effects of MeHg on ISN development. We first examined the effect of ubiquitous activation of Notch in post mitotic neurons by driving expression of N_{ICD} under control of the pan-neural elav promoter using the GAL4/UAS system (see methods). Ectopic expression of N_{ICD} in neurons could be detected with an antibody to the N_{ICD} (Fig. 2-11 A-C) and by qRT-PCR. In general, the potent activity of Notch was evident by an overall failure of a substantial number of embryos to develop to late stages. Of those that were able to develop to late stage a common feature was seen in a stunted outgrowth of the ISN (Fig. 2-11 D-F) akin to that seen with MeHg treatment. As *E(spl)mδ* was the most consistent E(spl) responder to MeHg we next determined if *E(spl)mδ* overexpression by the elav promoter could elicit an analogous MeHg-like ISN phenotype. Despite unambiguous overexpression of *E(spl)mδ* in elav-GAL4>UAS-*E(Spl)mδ* embryos (as determined by qRT-PCR, as no *E(spl)mδ* antibodies are available) no apparent phenotype in ISN development was observed (Fig. 2-11 G-I).

2.5: Discussion

In this study we have shown that MeHg causes an upregulation of *E(spl)mδ* in *Drosophila* neural cells and, importantly, in embryos treated *in vitro*. This effect is specific to MeHg and does not occur with inorganic mercury. This *E(spl)mδ* specific response is starkly different from E(spl) activation profiles resulting from Notch receptor

activation by the Delta ligand in the C6 neural cell line. MeHg elicits a characteristic failure in ISN axon outgrowth that, to some extent, can be induced with neural overexpression of Notch activity. However, targeted E(spl)*mδ* expression in neurons has no effect on ISN development. A role for E(spl)*mδ* in mediating MeHg effects via non-neuronal cells remains to be investigated. Altogether, these data highlight a novel and specific action for MeHg to target induced expression of a neurogenic signaling gene in conjunction with elicited neural developmental phenotypes.

E(spl) genes, are basic helix-loop-helix (bHLH) transcriptional repressors. These bHLH genes are the main effectors of Notch signaling in the nervous system, acting to repress transcription of proneural genes and generally preventing a default neuronal differentiation program from occurring. While various E(spl) genes have different expression patterns there is enough redundancy in expression and function to make discerning phenotypes difficult if only one E(spl) gene is perturbed. Nonetheless, *E(spl)mδ* siRNA injection in *Drosophila* embryos results a neurogenic phenotype, albeit with low frequency, consistent with a perturbation of Notch signaling (Nagel et al., 2004). The low penetrance of phenotypic effects of *E(spl)mδ* perturbation predict that its contribution to MeHg effects in the embryo will be subtle. It is thus not surprising that genetic manipulation of *E(spl)mδ* exclusively in neurons is not sufficient to replicate the MeHg-induced ISN phenotype. That genetic manipulation of the Notch pathway can replicate this phenotype suggests that there may be a central role for the Notch pathway in MeHg toxicity, potentially through the additive effects on *E(spl)mδ* and other E(spl) genes. Further study of MeHg in this system may not only elucidate the mechanisms of

MeHg toxicity but also reveal unique mechanisms for the differential expression of E(spl) genes.

Our findings suggest that induction of *E(spl)mδ* by MeHg *in vivo* is restricted to the embryonic stage. This observation is consistent with the notion that cellular defense mechanisms (e.g. glutathione pathway) are not fully developed in early embryogenesis, as compared with the differentiated larval and adult tissues. This outcome grossly mimics the preferential toxicity of MeHg for the fetus versus adult in higher organisms. Alternatively, differences may stem from our method of administration of the MeHg, which differ between our embryonic and larval/adult treatments: larvae and adult flies consume MeHg with the food, and may be able to mitigate toxicity via the gut. In contrast, embryos are soaked in MeHg medium allowing for more direct contact with embryonic cells. Yet, at the doses used, larvae show similar lethality as embryos (Mahapatra et al.). Assuming that embryo-specific induction of E(spl)*mδ* is not related to the route of MeHg administration it may help elucidate the mechanism for the specific neurodevelopmental toxicity in mice and humans.

In summary, we demonstrate that MeHg causes increased expression of *E(spl)mδ* *in vivo* in *Drosophila* embryos. We also demonstrated that while *E(spl)mδ* transcript upregulation correlates with MeHg neuronal phenotypes in embryos, *E(spl)mδ* overexpression restricted to neurons is not sufficient to replicate this characteristic axon outgrowth phenotype, pointing to a non-neuronal activity of *E(spl)mδ* in this toxicity model. These findings set the stage for investigating novel mechanisms of MeHg toxicity via non-canonical pathways of Notch signaling.

2.6: Acknowledgements

The Elav-9F8A9 antibody developed by G.M. Rubin, the C17.9c6 antibody developed by S. Artavanis-Tsakonas, and the 1D4 antibody developed by C. Goodman were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

We are grateful to Julie Dao, Cecon Mahapatra, Amanda Burton, Ben Moody and other members of the Rand lab for technical help and discussion of data.

This work was supported by NIEHS R01-ES015550 awarded to M.D.R. and the UVM Environmental Pathology Training Grant NIEHS T32-ES07122.

2.7: Figures

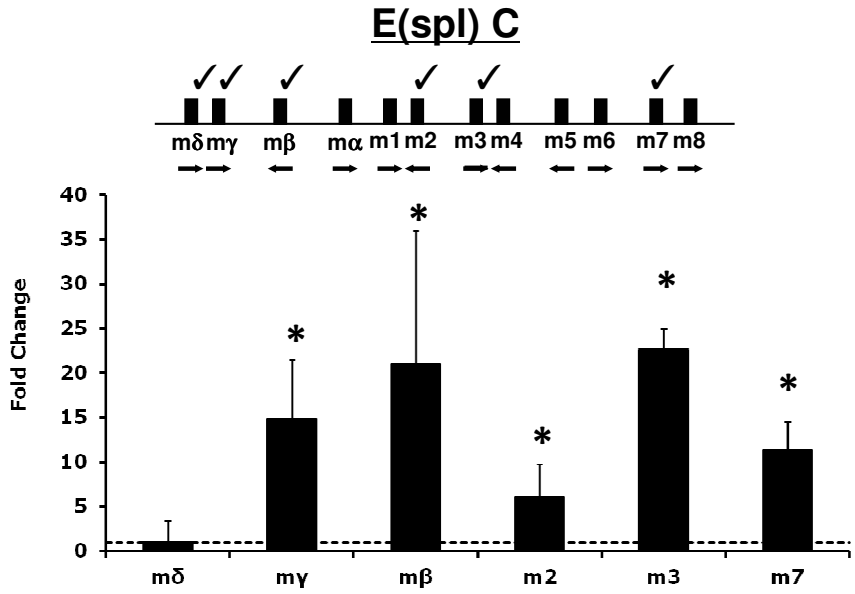

Figure 2 - 1. E(spl) gene expression in Delta ligand induced Notch signaling.

Organization of the Enhancer of split (E(spl)C) locus is diagramed and select genes analyzed in this study are indicated with a "check mark". Delta induced E(spl) expression was determined with a previously described co-culture assay (Delwig and Rand, 2008). Briefly, S2 cells stably expressing the Notch ligand Delta (Dl-S2 cells) or control S2 parent cells lacking the ligand were cultured as a monolayer then stabilized through brief fixation. C6 cells, which express Notch endogenously, but not the Delta ligand, were then co-cultured with either the fixed Dl-S2 cells or control S2 cells. E(spl) gene expression was measured via qRT-PCR. Data represent the fold change in E(spl) expression in C6 cells due to Delta exposure, Error bars indicate standard deviation. (n=6 (m2, m3, m7, mδ) to 12 (mβ and mγ) independent experimental determinations, * = $p<0.01$ by Student's t-test).

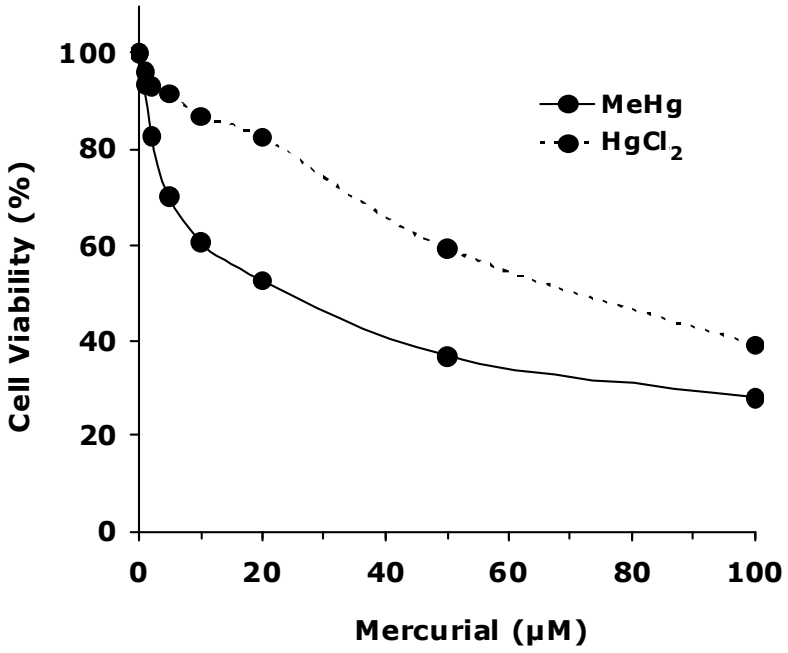

Figure 2 - 2. Cell viability of Drosophila neural-derived cells after mercurial treatment.

Drosophila C6 cells were treated for three hours with various doses of MeHg or $HgCl_2$. Viability was determined with Calcein AM hydrolysis and ethidium homodimer permeability (see methods).

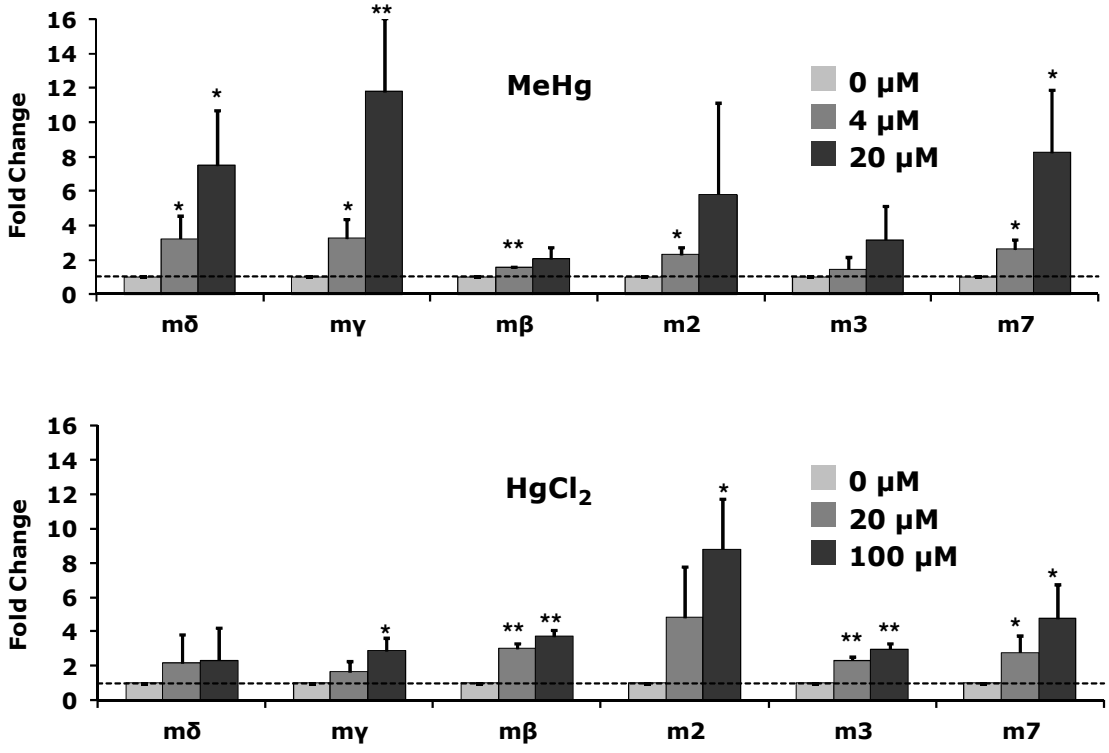

Figure 2 - 3. E(spl) gene induction by mercurial treatment.

Drosophila C6 cells were treated for three hours with the indicated concentrations of MeHg or $HgCl_2$. E(spl) gene expression determined by qRT-PCR is expressed in fold change over control treatments. (n = 3 independent experiments; * = $p<0.05$, ** = $p<0.01$ by Student's t-test).

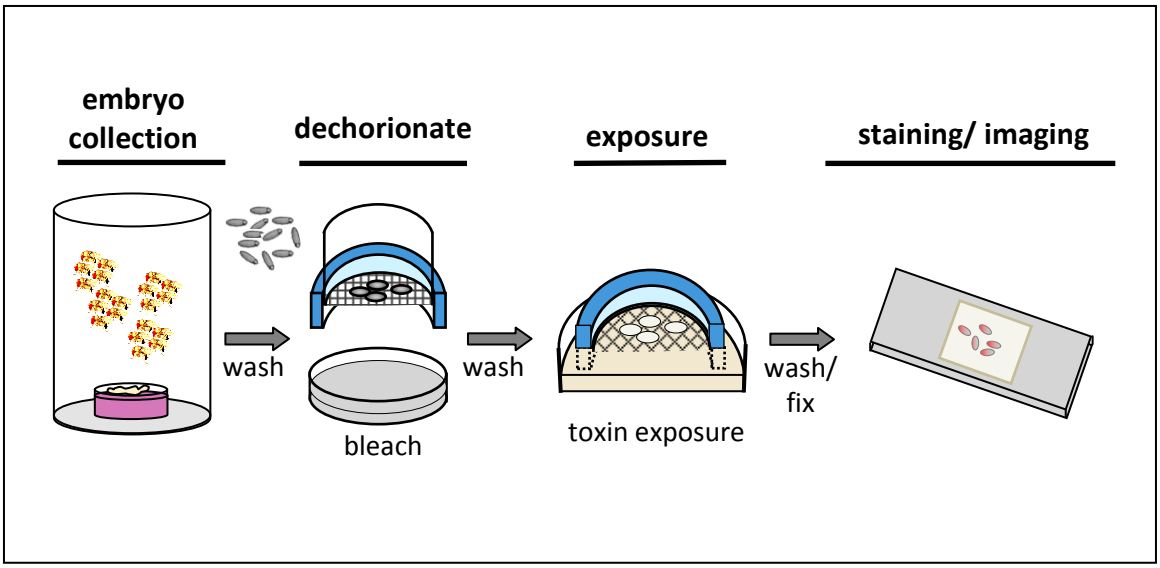

Figure 2 - 4. A schematic representation of in vitro dosing of Drosophila embryos with toxins.

Embryos collected from adults laying on grape-agar plates are dechorionated in dilute bleach. Embryos are then placed in baskets designed to optimize air exposure while being immersed in a solution containing the toxin of interest. After a period of developmental exposure, embryos can then be processed for RNA isolation or for fixation, staining and, imaging.

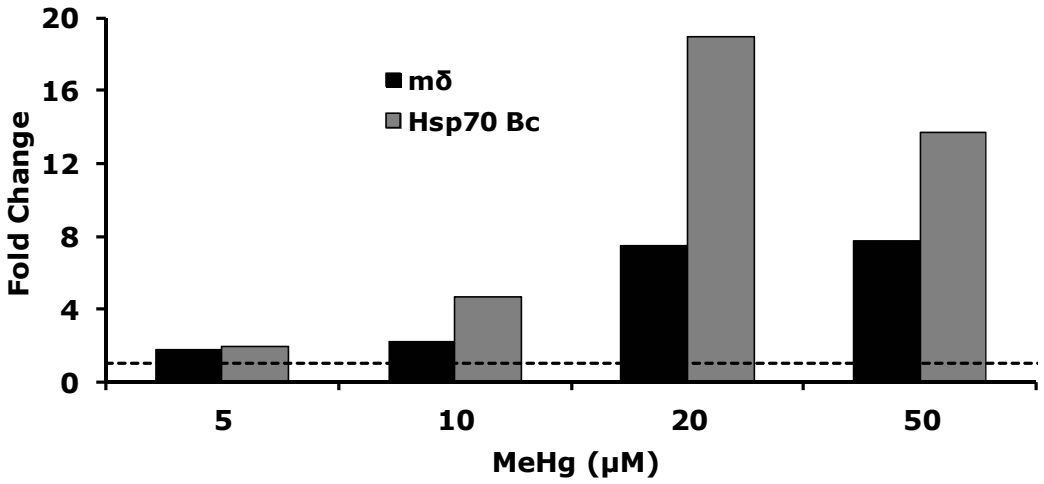

Figure 2 - 5. Dose response of E(spl)mδ in Drosophila embryos after MeHg treatment.

Drosophila embryos (2-4 hours after egg laying) were dechorionated and soaked in buffer containing indicated concentration of MeHg for 16 hours. Gene expression for *E(spl)mδ* and the stress-response gene *Hsp70 Bc* determined by qRT-PCR is expressed in fold change over 0 μM MeHg treatments. (n>300 pooled embryos per each treatment)

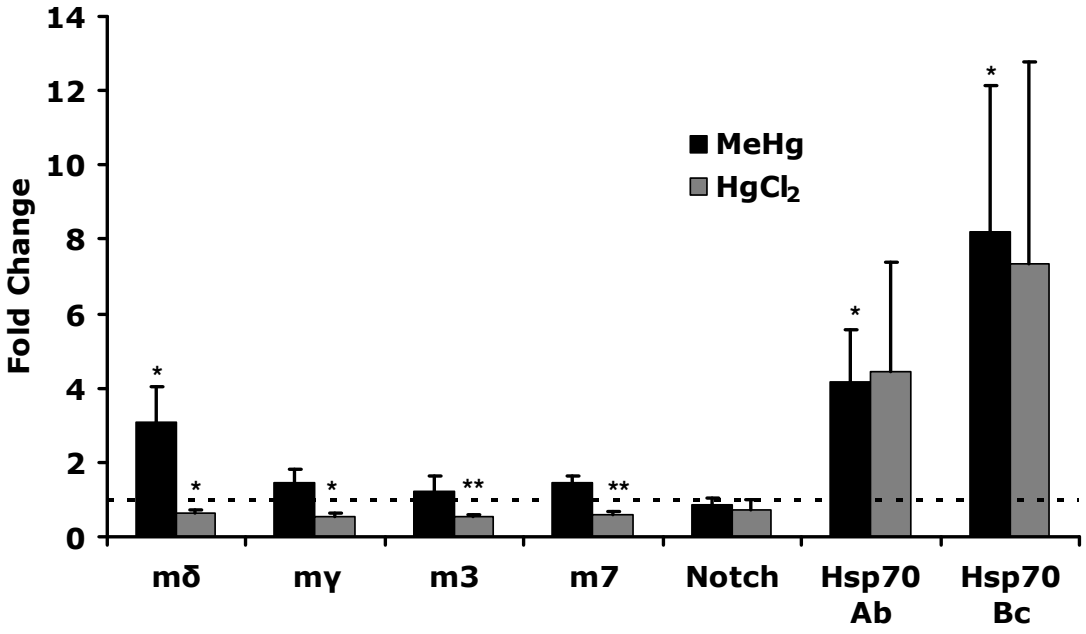

Figure 2 - 6. E(spl) gene induction in Drosophila embryos after mercurial treatment.

Drosophila embryos 2-4 hours after egg laying were soaked overnight in buffer containing 50µM MeHg or 1mM $HgCl_2$. Gene expression determined by qRT-PCR is expressed in fold change over control treatments. Values are the mean (and SEM) of three independent treatments of embryo batches (n = 3 independent experiments; * = $p<0.05$, ** = $p<0.01$ by Student's t-test).

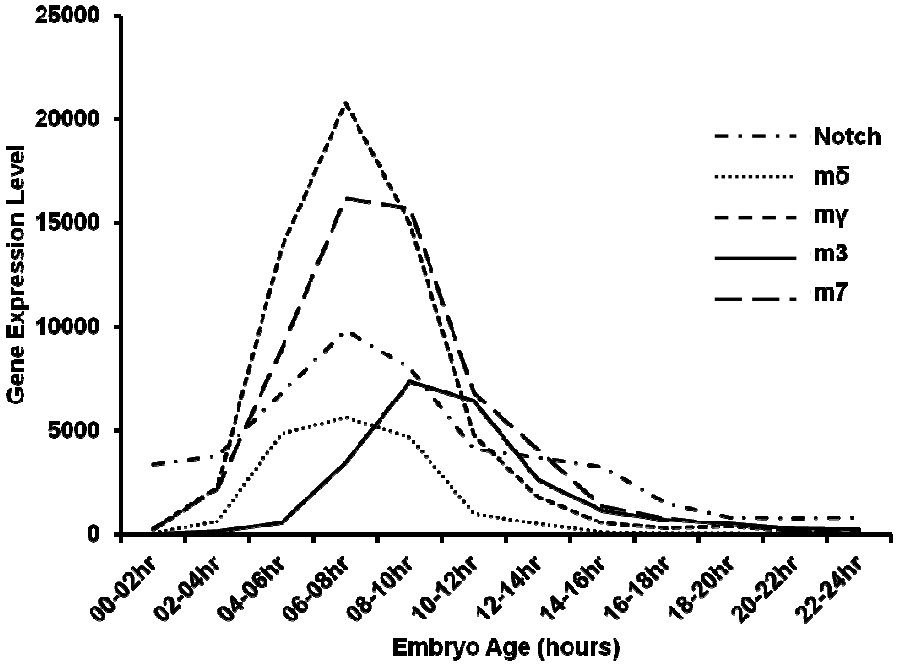

Figure 2 - 7. Relative gene expression levels of select E(spl) genes and Notch during embryonic development.

Relative gene expression levels determined through the Mod Encode project (Graveley et al.) were adapted from FlyBase (Tweedie et al., 2009) and expressed graphically to allow comparison of peak expression timing. *E(spl)mδ*, *E(spl)mγ*, and *E(spl)m7* coincide with *Notch* peak expression at 6-8 hours after egg laying. In contrast, *E(spl)m3* expression occurs later peaking around 8-10 hours after egg laying.

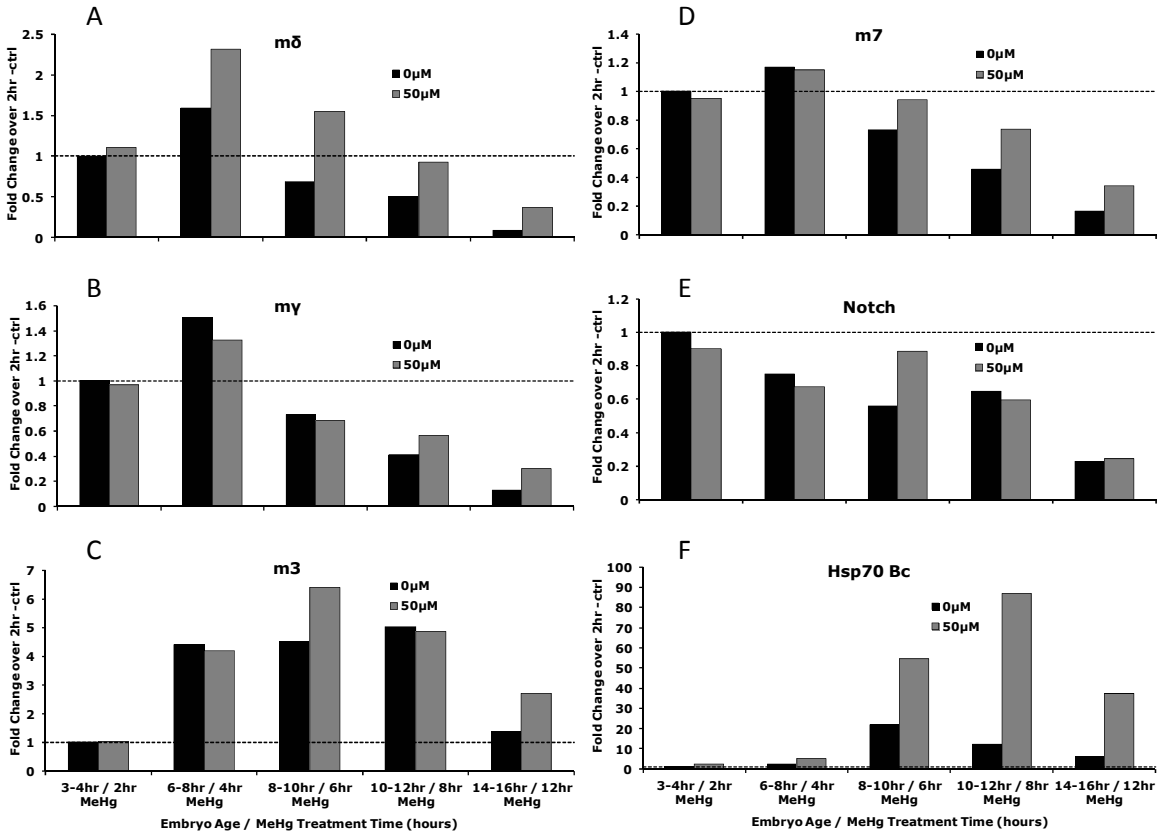

Figure 2 - 8. Response of E(spl) genes in Drosophila embryos after MeHg treatment over the course of development.

Drosophila embryos were dechorionated and soaked in buffer containing 50μM MeHg for indicated lengths of time. Gene expression determined by qRT-PCR is expressed in fold change over control treatments for A) *E(spl)mδ*, B) *E(spl)mγ*, C) *E(spl)m3*, D) *E(spl)m7*, E) *Notch*, and F) *HSP70 Bc*. (Each data point is derived from >300 pooled embryos from a treatment sampled at the indicated developmental time points).

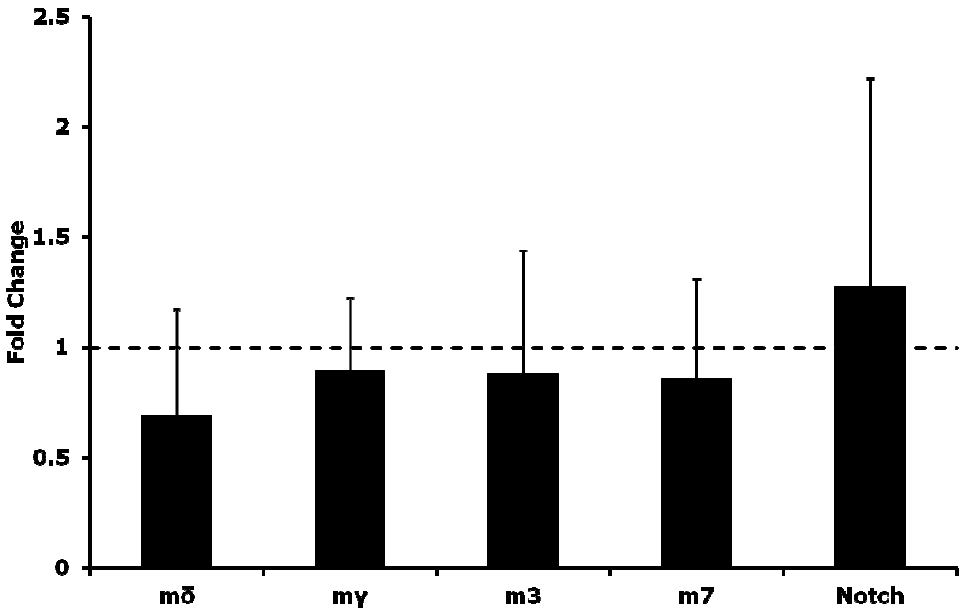

Figure 2 - 9. E(spl) gene induction in Drosophila larvae after MeHg treatment.

Drosophila embryos were placed on standard cornmeal-molasses food containing 15µM MeHg or DMSO vehicle control. Development was allowed to progress to the late third instar larvae stage. Larvae were then homogenized, RNA extracted and gene expression determined by qRT-PCR, expressed in fold change over control treatments. (Error bars indicate standard deviation of three experimental replicates. No significant changes were found using Student's t-test).

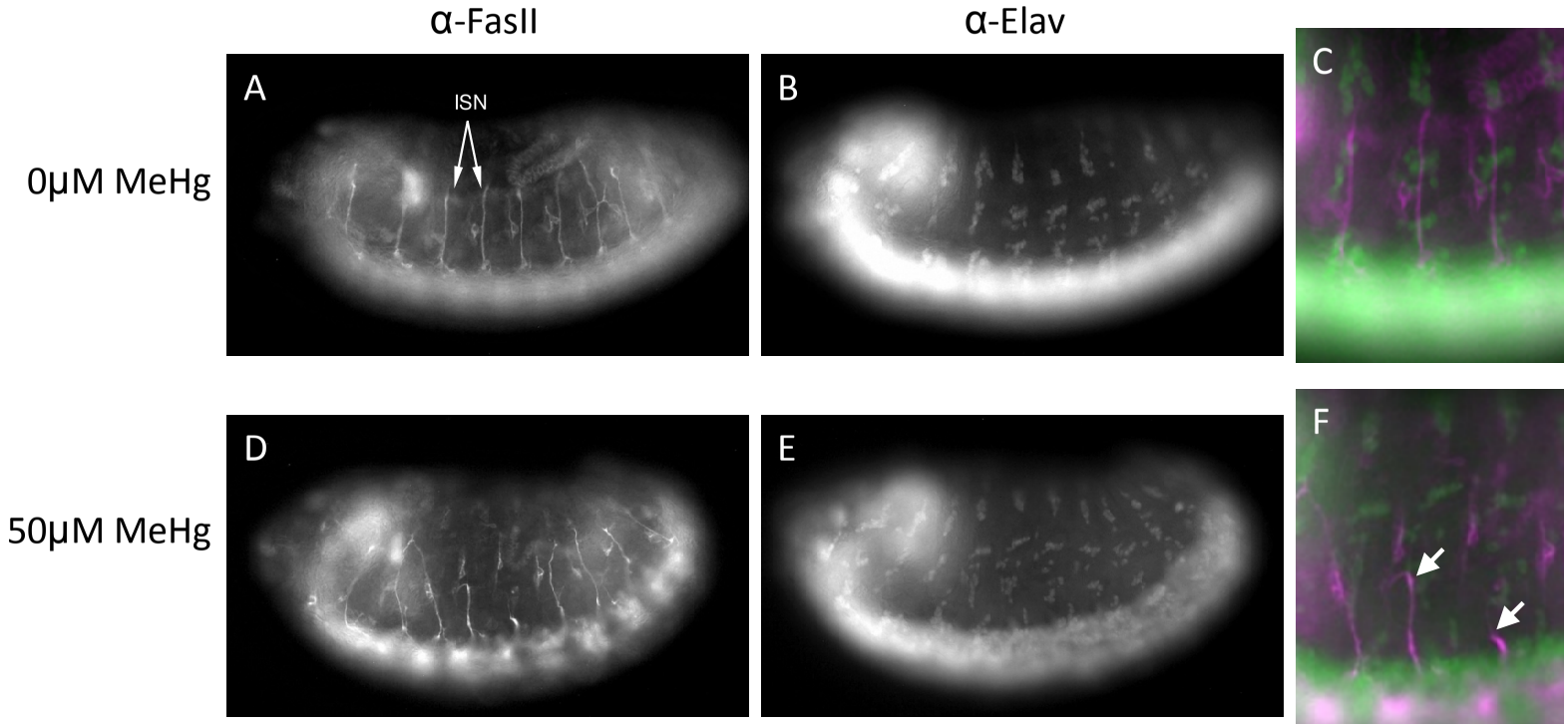

Figure 2 - 10. MeHg treatment causes axonal disruption in embryos.

A-C) Control embryos at developmental stage 14 were co-stained to show the intersegmental nerve (ISN) and all neuronal nuclei using antibodies for FasII (A) and elav (B), respectively. D-F) Embryos were treated with MeHg (50μM) and stained for FasII (D) and elav (E); disruption of ISN outgrowth is indicated by arrowheads.

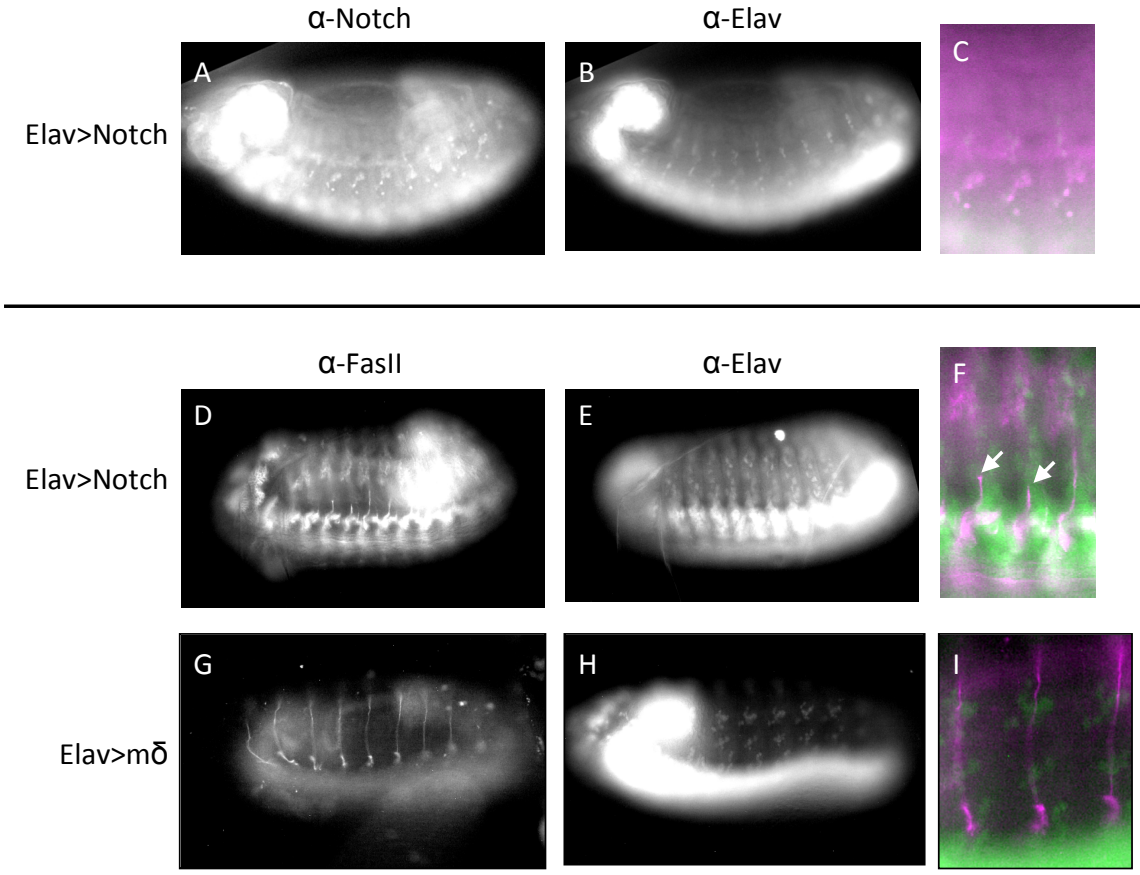

Figure 2 - 11. Notch pathway activation in neurons disrupts nerve outgrowth in embryos that is not induced with E(spl)mδ overexpression.

A-C) Embryos driving expression of the Notch intracellular domain (N_{ICD}), the active component of the Notch receptor, under control of elav show notch immunoreactivity in neurons. D-F) elav> N_{ICD} embryos stained for FasII (D) and elav (E) demonstrate stunted outgrowth of the ISN (arrow head). G-I) Embryos with targeted expression of the *E(spl)mδ* gene in neurons stained for FasII (G) and elav (H) show normal ISN outgrowth morphology.

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CHAPTER 3: THE EFFECTS OF METHYLMERCURY ON THE NOTCH SIGNALING PATHWAY AND MOTOR NERVE FORMATION DURING DROSOPHILA EMBRYONIC DEVELOPMENT

3.1: Abstract

Methylmercury (MeHg) is a ubiquitous environmental toxin. It is known that MeHg has a potent impact on neural development, but the mechanisms for this neurodevelopmental specificity are unknown. Previous work in our lab has shown that in Drosophila embryos MeHg causes upregulation of a canonical Notch response gene *E(spl)mδ*. In this study we hypothesize that this change in *E(spl)mδ* contributes to the toxicity of MeHg in the embryo. We test this by exogenously overexpressing *E(spl)mδ* in the embryo in an attempt to replicate a phenotype observed in the motor nerves following MeHg treatment. Our experiments show that *E(spl)mδ*, but not the closely related gene *E(spl)mγ*, can mimic the MeHg phenotype; however, the MeHg-like phenotype is only elicited when *E(spl)mδ* is overexpressed in developing muscles and not neurons as we originally predicted. Interestingly, we show that *E(spl)mδ* is endogenously expressed in developing muscle. This work shows not only that *E(spl)mδ* plays an important role in MeHg toxicity in the Drosophila embryo, but also that MeHg is capable of causing developmental anomalies in the nervous system via non-autonomous effects on supporting tissues.

3.2: Introduction

Methylmercury (MeHg) is an environmental contaminant that causes toxicity during human neural development. Though large exposures can be prevented, low dose MeHg exposure through the consumption of contaminated seafood is unavoidable for

many populations. Contemporary epidemiological studies into the effects of this exposure on children and fetuses have shown mixed results (Grandjean et al., 1995; Myers et al., 1995; Rice, 2004). As such, it is important that we investigate the mechanisms of MeHg toxicity so that we can formulate evidence-driven strategies to minimize the risk MeHg poses to human health.

Previous work in our lab has shown that MeHg causes transcriptional upregulation of the Enhancer of Split [E(spl)] complex gene *E(spl)mδ* in the embryonic Drosophila model organism (Bland and Rand, 2006; Rand et al., 2008; Engel et al., 2012). The E(spl) complex is a group of 13 canonical Notch response genes; *E(spl)mδ* is one of several basic helix-loop-helix (bHLH) transcriptional repressors best known as effectors of Notch signaling (Portin, 2002). Because Notch signaling is central in cell fate determination in the neurectoderm we hypothesized that this induction of *E(spl)mδ* by MeHg is important in MeHg toxicity and may contribute to its neurodevelopmental specificity.

To investigate this possibility we sought a readily quantifiable neural phenotype of MeHg toxicity in the embryos. Previous work in our lab had shown some deficits in neural patterning and migration following MeHg treatment of embryos (Rand et al., 2009). In this study we evaluate discernable defects in embryonic motor neuron axon outgrowth that result from MeHg treatment. Motor nerve development in the Drosophila embryo has been well characterized in the literature and follows a segmentally repeating easily indentified pattern (Kaufmann et al., 1998; Murray et al., 1998; Landgraf et al., 1999b; Sun et al., 2001; Sanchez-Soriano and Prokop, 2005). There are two major motor nerves in the system, the dorsally projecting intersegmental nerve (ISN) and the laterally

projecting segmental nerve (SN). The ISN is characterized by a single long fiber that emerges from the ventral nerve cord and travels in a predominantly straight path to the dorsal muscle field, synapsing with muscles along its path. The SN emerges from the ventral nerve cord and immediately curves toward the lateral transverse (LT) muscle group, forming a characteristic branch in order to innervate both the LT muscles and the segmental border muscle (SBM). The pattern and development of muscles are similarly well studied. All muscles in the Drosophila embryo begin with the selection of muscle founder cells, which then fuse with a population of nearby fusion competent cells to form multinucleated muscle fibers (Olson et al., 1995; Maqbool and Jagla, 2007; Tixier et al., 2010). This recruitment and fusion by the muscle founder cells forms a segmentally repeated muscle pattern. Among the field of several muscle groups the pattern of the four LT muscles can be clearly observed as a cluster of parallel fibers.

In this study we maintain our hypothesis that *E(spl)mδ* plays an important role in MeHg neurotoxicity. Staining for motor nerves in MeHg-treated embryos using antibodies specific to the fasciculin II (FasII) adhesion molecule we observed a failure of the ISN to project past lateral sensory neurons stained with a neural specific marker (Elav) (Engel et al., 2012). We attempted to replicate this phenotype by overexpressing *E(spl)mδ* in neurons, but did not observe any changes in ISN axon projection (Engel et al., 2012)., We subsequently found *E(spl)mδ* is endogenously expressed in developing muscle cells. By manipulating *E(spl)mδ* in developing muscle we observe a significant impact on motor nerve outgrowth. Additionally, we demonstrate a specificity of *E(spl)mδ* as a possible effector of MeHg toxicity by comparing it to the closely related E(spl) gene *E(spl)mγ* which has no effect when ectopically expressed in developing muscle. Our

findings show that MeHg is likely to influence nervous system development via gene expression in supporting tissues and, furthermore, that *E(spl)mδ* plays a crucial role in muscle, rather than neural, development.

3.3: Materials and Methods

Fly stocks and crosses:

Unless otherwise stated, fly stocks were maintained on standard cornmeal-yeast-molasses food at 25°C. Fly strains used include Canton S, elav-GAL4 (Bloomington Drosophila Stock Center, #458), Mef2-gal4 (gift from Jim Vigoreaux, University of Vermont), UAS-nuclearGFP (Bloomington Drosophila Stock Center, #4775), UAS-E(spl)mδ (Bloomington Drosophila Stock Center, #26677), UAS-E(spl)mδRNAi (Vienna Drosophila RNAi Center, #13077), E(spl)mγ-lacZ (gift from Sarah Bray, University of Cambridge (Cooper et al., 2000)), and UAS-E(spl)mγ (gift from Christos Delidakis, University of Crete (Ligoxygakis et al., 1999)). Standard crosses were performed between virgin female Gal4 driver lines and male UAS responder lines to generate F1 progeny to be tested.

Generation of E(spl)mδ-GFP reporter strain:

The 5kb upstream E(spl)mδ promoter region was amplified from genomic DNA via PCR using Phusion DNA polymerase (New England Biolabs) to include restriction endonuclease sites at each end (XbaI at 5', KpnI at 3') with the following primers: Forward: gaTCTAGAgtaaattacagccacttgaag, Reverse: gaGGTACCgtagctgctggtgccgtac. The product was then inserted into the Drosophila transformation vector pGreen H-

Pelican (Barolo et al., 2000) by cleaving both with XbaI and KpnI (New England Biolabs) and ligating with T4 DNA ligase (New England Biolabs) (Fig. 3-2). Bacterial transformation was performed with One Shot TOP10 chemically competent cells (Invitrogen) according to manufacturer's instructions. Bacteria were then plated on LB agar plates containing ampicillin to select for transformants. Transformed colonies were grown and the plasmid purified using a Qiagen maxiprep kit. The plasmid was verified to ensure proper construction by DNA sequencing (Vermont Cancer Center DNA Analysis Core Facility). Transgenic flies were created by P-element mediated germline transformation (BestGene Inc).

Embryo mercury treatments:

Cages of adult flies were allowed to lay eggs on a grape agar plate with yeast paste smeared on the center. Embryo collection occurred over a two hour laying period. Embryos were then aged on the grape plates at 25°C for two hours. Embryos were dechorionated using a standard protocol. Briefly, embryos were washed from the plate using tap water and a brush into a nytex basket, then rinsed to remove yeast; baskets were transferred to 50% bleach (~3.8% sodium hypochlorite) for three minutes, then rinsed in tap water to remove bleach. Embryos were transferred to separate nytex baskets for each treatment, which were in turn placed into petri dishes containing modified basic insect medium with added mercury or DMSO control. These dishes were covered to prevent evaporation and the embryos were allowed to incubate for 16-18 hours or various times where indicated.

Immunostaining:

Immunostaining was performed as previously described (Rand et al., 2009). Treated embryos were fixed in 500 µL of a 50:50 mix of 8% paraformaldehyde in 0.1 M PIPES, 2 mM EGTA, 1 mM MgSO4, pH 6.9 (PEM) with heptane by rocking for 25 min. Vitelline membranes were subsequently removed by discarding the lower PEM layer and adding 750 mL MeOH, then vortexing for 30 s. Settled embryos were collected, washed and stored at -20C in methanol until staining. For immunostaining, embryos were permeablized in phosphate buffered saline (PBS) with 1% BSA, 0.1% triton X-100 (PBT). Subsequent blocking, primary and secondary antibody incubations were done in PBT with 5% each of donkey and goat serum. Primary antibodies used were: mouse anti-elav (9F8A9), rat anti-FasII (1D4) (Developmental Studies Hybridoma Bank, Univ. of Iowa), rabbit anti-GFP (Torrey Pines Biolabs, Inc), rabbit anti-βgal (Chemicon International), and rabbit anti-myosin (gift from Daniel Kiehart, Duke University). Secondary antibodies used were: Alexa488-conjugated goat anti-rat, Alexa555-conjugated goat anti-mouse, and Alexa555-conjugated goat anti-rabbit (Jackson ImmunoResearch). Embryos were visualized by fluorescence microscopy on a Leitz Orthoplan 2 microscope equipped with a Spot One digital camera and associated acquisition software (MVI, Avon, MA). Phenotypes were scored per hemisegment looking exclusively at abdominal segments 1-5. Images were assembled in Adobe Photoshop, GIMP, and Microsoft PowerPoint.

RNA extraction and quantitative PCR:

After mercury treatments, embryos were transferred with a paintbrush into microcentrifuge tubes containing PBS supplemented with 0.1% Triton-X and 1% bovine serum albumin (PBT). The PBT was removed with a pipette and replaced with Trizol reagent (Invitrogen). Embryos were homogenized in Trizol and processed for RNA isolation. RNA samples were treated with Turbo DNAse (Ambion) and reversed transcribed using SSII reverse transcriptase (Invitrogen). RNA was subsequently removed with RNAse H (USB). cDNA samples were assayed for gene expression via qRT-PCR using SybrGreen dye (Bio-Rad). Data were normalized to the ribosomal protein RP49 housekeeping gene and analyzed using the comparative Ct method (Livak and Schmittgen, 2001). Primer sequences for E(spl)mδ and the RP49 control were taken from (Rand et al., 2008). The sequences used for Sidestep were Forward: GCGGCGGATATTAGGGCACGG and Reverse: CGGGTCGTTTAGGCTGGGCT. For Beaten path Ia the sequences were Forward: CCGGTTCGTCCAGTTCCCGC and Reverse: GCTTTGTTGCGCTGACCCGC.

3.4: Results

In a previous study we identified a phenotype of MeHg treatment in embryos in the ISN (Engel et al., 2012). Additional observations identified a second phenotype of MeHg treatment in embryonic motor nerves visualized with α-FasII antibodies; MeHg treated embryos show a significant failure of branching in the SN (Fig. 3-1). This branch failure was not observed to favor the development of one branch over the other; development of the upper branch alone, lower branch alone, and stalling at the branch

point were all observed with approximately equal frequency (data not shown). This SN phenotype is more readily observed than the ISN phenotype, as it requires staining of only the nerve itself and no additional marker. It is also easily quantified as individual segments can be clearly identified as branching or failing to branch. We therefore utilized this SN phenotype to elaborate MeHg effects in this study.

We have previously shown that overexpressing *E(spl)mδ* in neurons fails to cause any changes in ISN outgrowth (Engel et al., 2012). We therefore analyzed endogenous *E(spl)mδ* expression to discern where it might be acting in normal development. Transgenic flies expressing an *E(spl)mδ*-GFP reporter construct show a similar pattern to expression of GFP under control of the muscle-specific Mef2-gal4 driver, indicating that endogenous expression of *E(spl)mδ* in embryos is in the muscle (Fig. 3-2).

We endeavored to determine whether we could replicate the effects of MeHg treatment on SN branching by overexpression of *E(spl)mδ* in embryos. When overexpressed in muscle, where it is endogenously expressed, using the Mef2-gal4 driver *E(spl)mδ* causes a significant failure in SN branching (Fig. 3-3). Overexpression of *E(spl)mδ* in neurons by Elav-gal4, however, did not cause increased SN branch failure (Fig. 3-3). These data suggest that overexpression of *E(spl)mδ* in the substrata on which the nerves elaborate, but not the nerves themselves, causes failure of proper axonal outgrowth, mimicking that seen with MeHg treatment.

Seeing that *E(spl)mδ* overexpression in muscle caused a SN phenotype similar to MeHg we wanted to determine whether either of these treatments grossly impacted muscle development. We stained the muscles of MeHg treated, Mef2>mδ, and control embryos using an α-myosin antibody and visualized them with immunofluorescence (Fig.

3-4). To assess normal development we evaluated the morphology of the lateral transverse muscles 1-4 (LT 1-4), known targets of the SN. Among control embryos only 1% of hemisegments observed showed a defect by the inability to identify each of the four (Fig. 3-4 A). MeHg treated embryos showed disorganization of the muscle pattern, with unidentifiable LT 1-4 in 15% of hemisegments (Fig. 3-4 B). Mef2>mδ embryos showed even higher rates of muscle pattern failure, showing a disordered muscle phenotype in 58% of hemisegments (Fig. 3-4 C). These data correlate with the SN phenotype, which is more prevalent in Mef2>mδ embryos than those treated with MeHg (Fig. 3-1, 3-3).

We wished to further investigate the relationship between the phenotypes we observed in the SN and muscle. We co-stained MeHg treated and Mef2> mδ embryos for FasII and myosin and looked at the coincidence of SN and muscle failure. Though muscle phenotype was a significant predictor of SN phenotype it predicted only 84.4% of the SN phenotype data correctly (binary logistic regression; $n = 225$; $p < 0.001$). In MeHg treated embryos over 13% of the hemisegments quantified showed a SN phenotype but no muscle phenotype, while nearly 12% showed a muscle phenotype but no SN phenotype (Fig. 3-5). This indicates that factors other than gross muscle pattern play a role in SN failure in our experiments.

It has been shown that interaction between *Side* expressed in the path of the developing SN and *Beat* expressed in the axon growth cones plays an important role in SN guidance during development (Siebert et al., 2009). We hypothesized that changes in expression of these signaling molecules due to MeHg treatment or overexpression of *E(spl)mδ* may contribute to the formation of the SN phenotype. To assay this we assessed

expression levels of these signaling factors using qPCR on RNA extracted from whole embryos treated with MeHg or overexpressing *E(spl)mδ* by Mef2-gal. This analysis showed no significant change in expression of either gene in either treatment compared to controls (Fig. 3-6).

In addition to looking at potential causes of the SN phenotype we observed after MeHg treatment and *E(spl)mδ* upregulation in muscle we endeavored to determine whether this effect was specific to *E(spl)mδ* or a common effect to other E(spl) genes. We have previously shown that *E(spl)mγ* responds to MeHg treatment of Drosophila cell lines (Rand et al., 2008). Though it does not respond to MeHg treatment in embryos, *E(spl)mγ* is closely related to *E(spl)mδ*, and lies immediately downstream on the chromosome (Wurmbach et al., 1999; Engel et al., 2012). In order to use *E(spl)mγ* as a tool to look at the specificity of *E(spl)mδ* in the SN phenotype we first wanted to confirm its endogenous expression pattern. mγ-lacZ flies stained for βgal and Elav show that expression of *E(spl)mγ* largely restricted to neural cells in the embryo (Fig. 3-7).

In order to test whether *E(spl)mγ* could replicate the SN phenotype caused by *E(spl)mδ* overexpression in muscle we drove expression of *E(spl)mγ* using the same Mef2-gal4 driver line; no increase in the SN phenotype over controls was seen (Table 1). Additionally, we attempted to drive expression of *E(spl)mγ* in neurons, where it is endogenously expressed, to see what effect this might have on motor nerve development. However, this cross proved lethal at early stages of embryonic development, preempting the ability to assess effects on motor nerve phenotype.

3.5: Discussion

We have shown that MeHg treatment in embryos causes failure of axon outgrowth in the SN. When *E(spl)mδ* is overexpressed in muscle, where it is endogenously expressed, but not the neurons themselves we see similar SN phenotypes. In both MeHg treated and *E(spl)mδ* overexpressing embryos we see disruption of muscle development, but we were unable to measure changes in expression of guidance cues known to be expressed by the muscle. These phenotypes do not occur when another E(spl), *E(spl)mγ* is overexpressed.

The combined results of these experiments lead us to a new model of how MeHg interferes with embryonic development to impact the nervous system (Fig. 3-8). In this model MeHg enters the cells of the embryo and through an as-of-yet unknown mechanism causes the upregulation of *E(spl)mδ*. Though this upregulation may occur in several tissue types it is in muscle progenitors that it has the greatest effect, causing failure of the muscle to form normal patterns. *E(spl)mδ* overexpression in neurons does not produce an observable phenotype, but perturbation of muscles by *E(spl)mδ* upregulation influences the outgrowth of motor neurons. *E(spl)mδ* may also impact muscle cells in other ways, such as disruption of the precise timing, but not overall expression level, of *Side* that is necessary for motor nerve guidance.

This model proposes that MeHg-induced *E(spl)mδ* expression is not influencing nervous system development by directly affecting neurons, but instead affecting supporting cells required for the normal development of those neurons. Other labs have found evidence that MeHg toxicity may be mediated by the effect of MeHg on glia (Shanker et al., 2001). Further experiments on the effects of MeHg on neural

development should analyze whether MeHg acts directly on neurons or rather on supporting cells in the system studied.

The earliest and best studied role of the Notch pathway is in cell fate determination of the neurectoderm. Like many highly conserved signaling pathways, though, Notch is involved in many different processes. It has been shown that components of the Notch pathway influence mesoderm development (Vasyutina et al., 2007). Our work reinforces this literature by demonstrating a role for *E(spl)mδ* in the formation of muscle patterning. Additional evidence for the important role of *E(spl)mδ* in mesoderm development comes from experiments where we attempted to knockdown *E(spl)mδ* expression using an RNAi strain driven by Mef2-gal4. This cross proved lethal early in embryonic development [data not shown], which combined with the knowledge that *E(spl)mδ* is endogenously expressed in the muscle and that overexpressing *E(spl)mδ* causes disorganization of the muscle pattern indicates a critical role for *E(spl)mδ* in mesoderm development.

Contrasting overexpression of *E(spl)mδ* with *E(spl)mγ* provides some additional clues on the observed specificity in the response of *E(spl)mδ* to MeHg treatment. When *E(spl)mγ* is overexpressed in tissues where it is found endogenously it proves lethal to the embryo; when *E(spl)mδ* is overexpressed in a pattern mimicking its endogenous expression embryos show a distinct phenotype, but develop to late embryonic stages and, in fact, hatch into larvae at levels comparable to controls [data not shown]. One possible explanation for this is that MeHg causes increased expression of both *E(spl)mδ* and *E(spl)mγ* at variable levels in different embryos, and our observation that only *E(spl)mδ* is upregulated in embryos following MeHg treatment is due to bias caused by the early

lethality of embryos in which *E(spl)mγ* is upregulated in neurons. This seems unlikely, however, as qPCR is a relatively sensitive measure of RNA transcript level, and *E(spl)mγ* transcript will necessarily increase before the *E(spl)mγ* protein that presumably causes lethality. A more interesting explanation for why *E(spl)mδ* and not *E(spl)mγ* is upregulated is that because it is more lethal when overexpressed *E(spl)mγ* is more tightly regulated by the embryo than *E(spl)mδ*. Evidence supporting this hypothesis could be obtained by overexpressing other closely related E(spl) genes that do not respond to MeHg treatment of embryos, such as *E(spl)m3* or *E(spl)m7*, and measuring lethality in the embryos; should these other E(spl) genes prove lethal when overexpressed it may indicate that *E(spl)mδ* is uniquely benign among E(spl) genes, allowing it to be less tightly regulated than other E(spl)s and thus more responsive to MeHg.

3.6: Conclusion

MeHg is characterized in the literature as a toxin that most potently impacts neural development. In our Drosophila embryo model we observed effects of MeHg on neural development, specifically in the outgrowth of motor axons. However, further investigation into the observed motor nerve phenotype revealed that it is MeHg toxicity affecting the muscles that causes the failure in nerves. Further experiments suggested by this finding include investigating the precise interaction between muscle and nerve that is impacted by MeHg treatment. This finding should also inform future research into MeHg toxicity in general; where possible steps should be taken to analyze the effect of MeHg on all tissues likely to impact the system being studied. Though MeHg toxicity in mammals has been shown to impact neural development most severely, we should

continue to investigate the impacts of MeHg on other tissues and the contributions that might have to the observed neural phenotypes.

Our data continue to support an important role for *E(spl)mδ* in MeHg toxicity in the Drosophila embryo, showing that *E(spl)mδ* specifically, and not the closely related *E(spl)mγ*, can replicate a neural phenotype of MeHg treatment. We have also provided evidence that *E(spl)mδ* is active primarily in the mesoderm during embryonic development, rather than the neuroectoderm where Notch targets play their canonical role. Further work investigating the molecular mechanisms by which MeHg effects *E(spl)mδ* upregulation may provide valuable insight into MeHg toxicity, but also the differential regulation of E(spl) genes in response to their endogenous activator, Notch.

3.7: Tables and Figures

Table 3 - 1. Overexpression of E(spl)mγ does not cause branch failure in the SN.

Mef2-gal4 female flies were crossed with UAS-mγ males, producing offspring that overexpress E(spl)mγ in mesoderm (Mef2>mγ). Mef2>mγ embryos at stage 14/15 were fixed and stained with αFasII and compared to control embryos (Mef2>GFP). SN failure rate was the same in both groups. UAS-mγ was also crossed with Elav-gal4, producing Elav>mγ overexpressing E(spl)mγ in neurons. This cross proved lethal, and embryos did not develop beyond early stages. Each n is a single abdominal segment, A1-5, in which the segmental nerve was seen to develop normally (Normal) or failed to branch (Failed). (n = 100 segments per treatment)

	Normal %	**Failed %**
Mef2>GFP (100)	**93**	**7**
Mef2>mγ (100)	**93**	**7**
Elav>mγ	**Lethal**	

Normal	Failed axon
Control	(n = 300)
99.0%	1.0%
MeHg*	(n = 300)
81.0%	19.0%

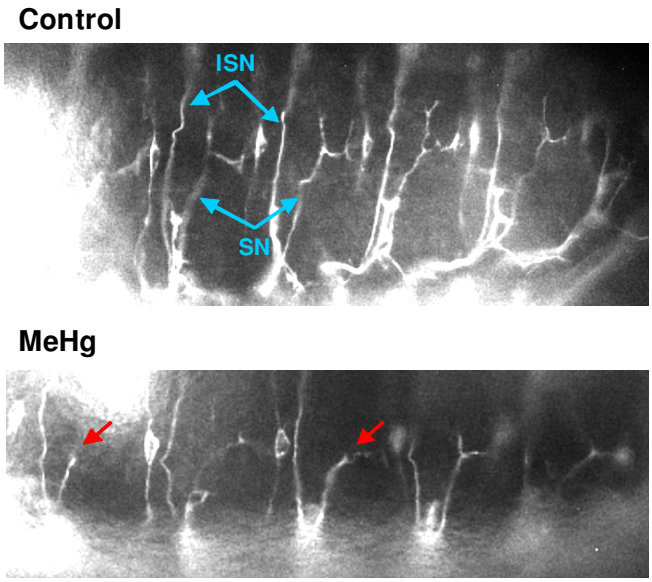


Figure 3 - 1. MeHg treatment significantly alters SN branching.

Drosophila embryos 2-4 hours AEL were soaked overnight in buffer containing 50µM MeHg or DMSO control. They were then fixed and stained with αFasII. Only embryos that developed to stage 14/15 were used for quantification. Each n is a single abdominal segment, A1-5, in which the segmental nerve was seen to develop normally (Normal) or failed to branch (Failed). Red arrows indicate SN branch failure. (n = 300 segments per treatment; * = $p<0.0001$ by Fisher's exact test for the treatment indicated)

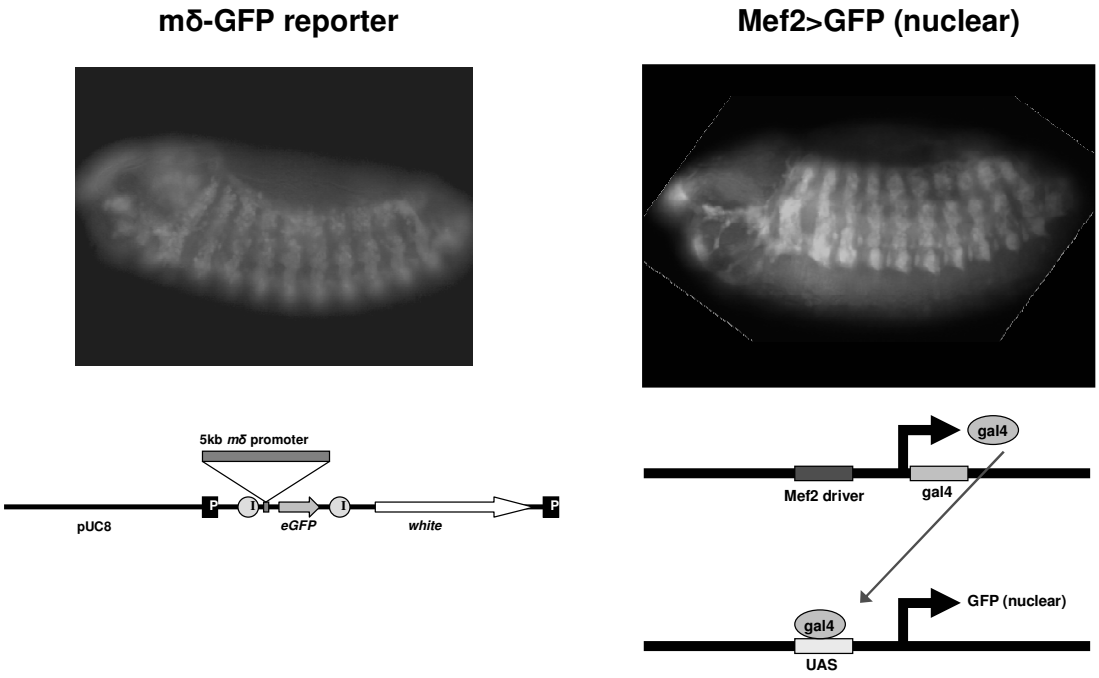
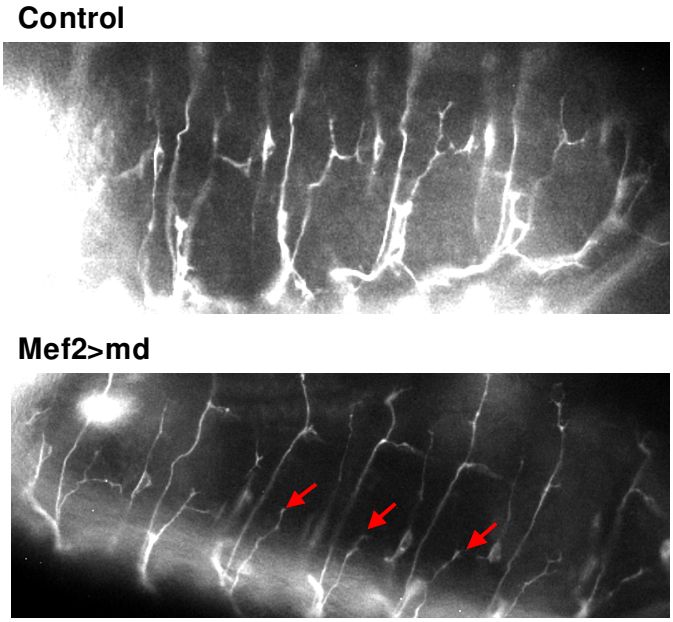


Figure 3 - 2. E(spl)mδ is endogenously expressed in mesodermal tissues in the Drosophila embryo.

Transgenic mδ-GFP reporter flies were created with the plasmid shown (see methods). Expression of the green fluorescent protein (GFP) reporter in the transgenic flies shows a pattern similar to GFP expressed under the control of a known mesodermal driver (Mef2) using the GAL4/UAS expression system.

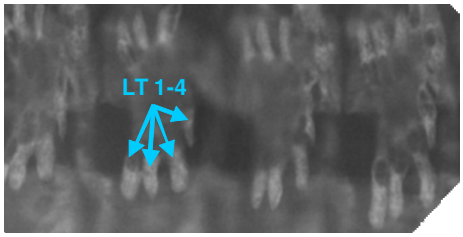


Normal	Failed axon
Mef2>GFP	(n = 100)
93.0%	7.0%
Mef2>mδ*	(n = 200)
76.5%	23.5%
Elav-gal4	(n = 100)
94.0%	6.0%
Elav>mδ	(n = 100)
94.0%	6.0%

Figure 3 - 3. Overexpression of E(spl)mδ in mesoderm significantly alters SN branching, replicating the MeHg induced phenotype.

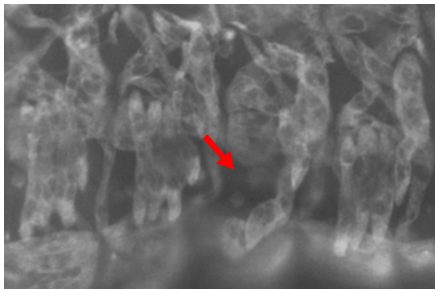
Mef2-gal4 female flies were crossed with UAS-mδ males, producing offspring that overexpress *E(spl)mδ* in mesoderm (Mef2>mδ). Mef2>mδ embryos at stage 14/15 were fixed and stained with αFasII and compared to control embryos (Mef2>GFP). UAS-mδ was also crossed with Elav-gal4, producing Elav>mδ overexpressing *E(spl)mδ* in neurons. These were compared with the Elav-gal4 driver line. Each n is a single abdominal segment, A1-5, in which the segmental nerve was seen to develop normally (Normal) or failed to branch (Failed). Red arrows indicate SN branch failure. (n = 200 (Mef2>mδ) or 100 (Mef2>GFP, Elav>mδ, Elav-gal4) segments per treatment; * = $p<0.001$ by Fisher's exact test for the treatment indicated)

A)



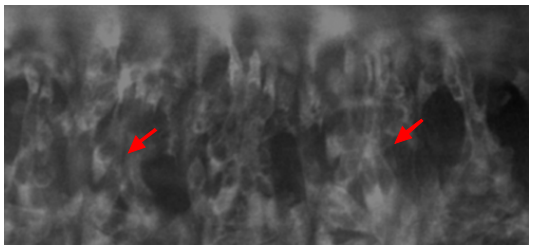
Control

B)



50µM MeHg

C)



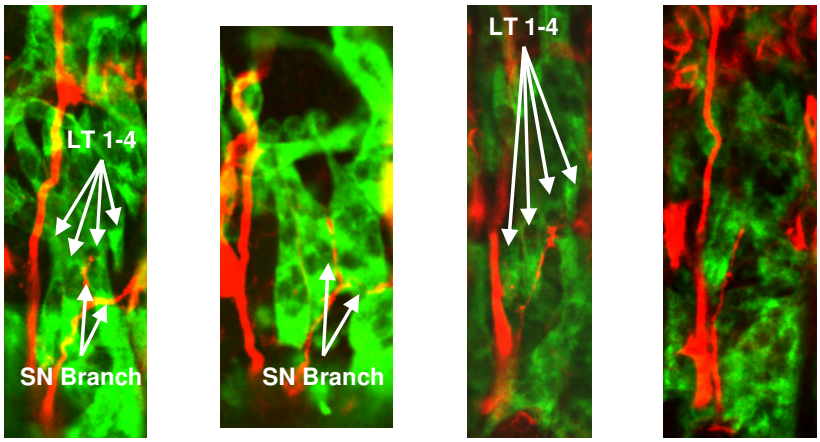
Mef2>mδ

D)

Treatment:	% unidentifiable muscle
CS 0 (n = 100)	1.0%
CS 50* (n = 100)	15.0%
Mef2>mδ** (n = 100)	58.0%

Figure 3 - 4. MeHg treated and Mef2>mδ embryos show a disorganized muscle pattern.

Drosophila embryos 2-4 hours AEL were soaked overnight in buffer containing 50µM MeHg or DMSO control (50µM MeHg and Control). These were compared to similarly aged untreated embryos overexpressing *E(spl)mδ* in muscles (Mef2>mδ). Embryos were fixed and stained with αmyosin. Only embryos that developed to stage 14/15 were used for quantification. Each n is a single abdominal segment, A1-5, in which the lateral transverse muscles 1-4 were observed to determine if they had developed sufficiently to be identifiable or were so disorganized as to be unidentifiable. Red arrows indicate unidentifiable muscles. (n = 100 segments per treatment; * = $p<0.001$ by Fisher's exact test for the treatment indicated, ** = $p<0.0001$ by Fisher's exact test for the treatment indicated)



SN Phenotype:	-	-	+	+
Muscle Phenotype:	-	+	-	+
% in MeHg Treated Embryos	69.7%	11.8%	13.2%	5.3%

Figure 3 - 5. SN and muscle phenotypes do not always co-occur.

Drosophila embryos 2-4 hours AEL were soaked overnight in buffer containing 50μM MeHg or DMSO control. They were then fixed and stained with αFasII (red) and αmyosin (green). Only embryos that developed to stage 14/15 are shown. Images were obtained from either MeHg treated or control embryos and show that each combination of SN and muscle phenotypes occurred. MeHg treated embryos were scored by a blinded scorer to record the presence of SN and/or muscle phenotypes for each abdominal hemisegment, A1-5. Data show the percentage of scored hemisegments showing each phenotype combination. (n = 75)

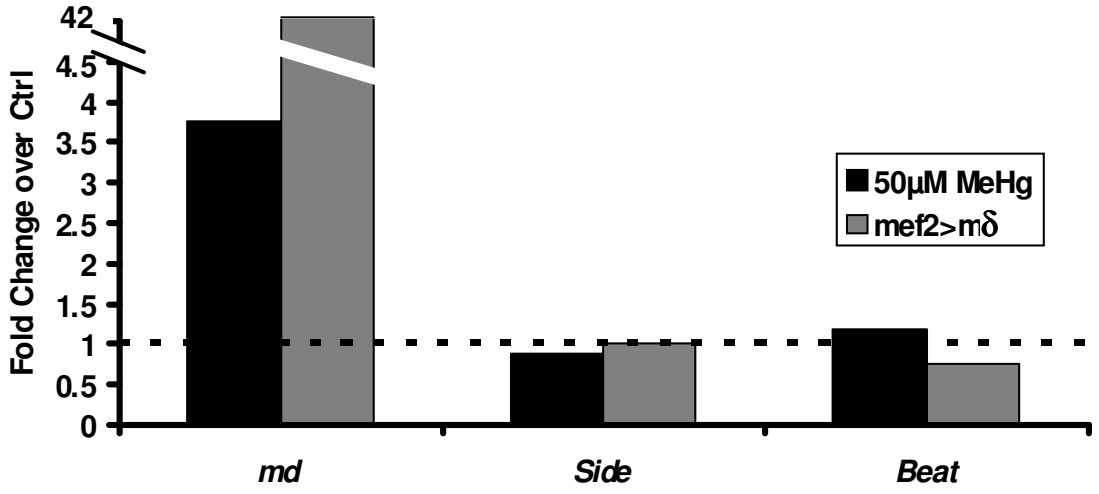


Figure 3 - 6. Expression of known guidance cues is not altered by MeHg treatment or E(spl)mδ overexpression.

RNA was harvested from groups of whole Drosophila embryos treated with 50µM MeHg and compared to DMSO treated controls or overexpressing *E(spl)mδ* in muscles (Mef2>mδ) and compared to Mef2>GFP embryos. Data shown are the fold induction of gene expression over appropriate controls for the treated embryos, normalized to the unresponsive RP49 housekeeping gene.

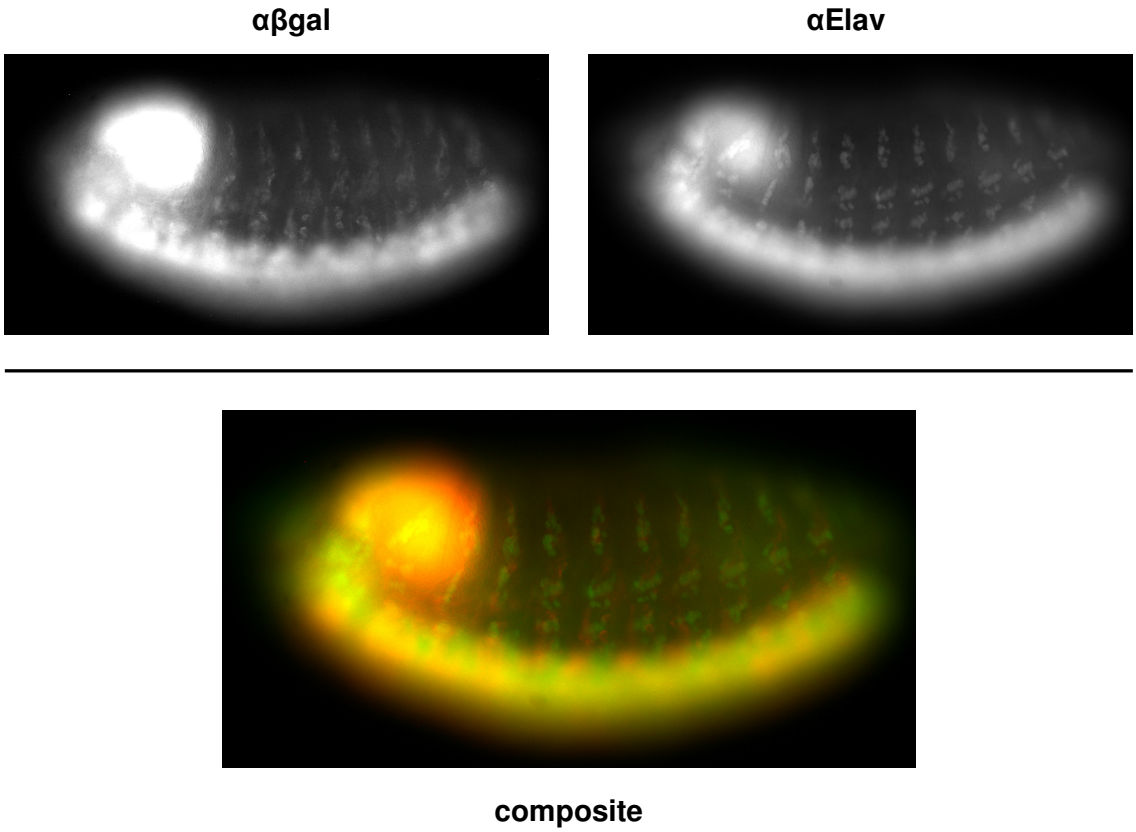


Figure 3 - 7. E(spl)mγ is expressed in the nervous system.

Embryos from an E(spl)mγ-lacZ fly strain were double stained for βgal (red), which should express where *E(spl)mγ* is, and Elav (green), which is expressed in neurons.

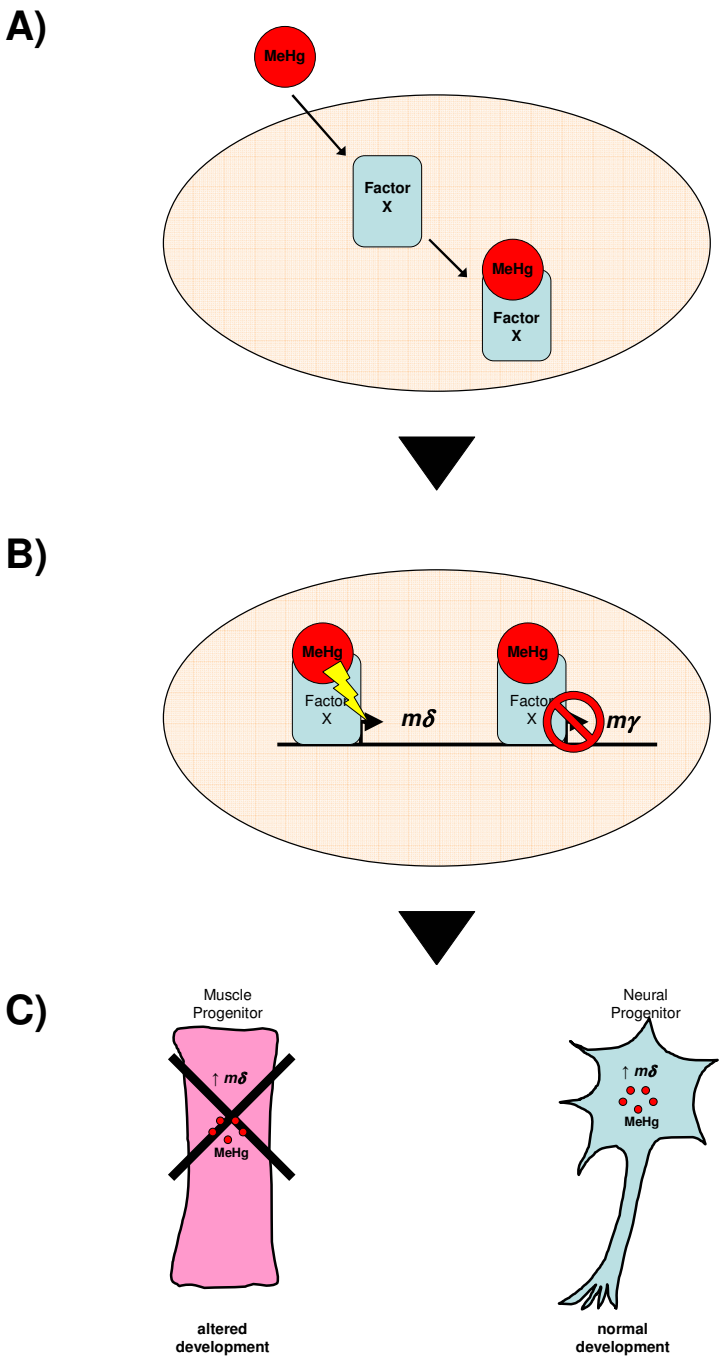


Figure 3 - 8. Working model of MeHg toxicity in Drosophila embryos.

Our current model for how MeHg affects Drosophila embryos to produce phenotypes in motor nerves. A) MeHg enters cells in the embryo and binds to an unknown factor, X. B) The MeHg-Factor X complex causes upregulation of *E(spl)mδ*, but is unable to cause upregulation of *E(spl)mγ* or other E(spl) genes. C) In neurons this upregulation does not cause pathological changes, but in muscles overexpression of *E(spl)mδ* causes failures that lead to improper/incomplete development. These changes in muscle ultimately lead to an inability of motor axons, which normally receive important guidance cues from the muscles, to properly follow their normal path.

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CHAPTER 4: SUMMARY AND CONCLUSIONS

MeHg is a potent environmental neurotoxin that shows particular toxicity during neural development. Exposure to low doses of MeHg through fish and other seafood has shown variability in effects on fetuses and children in a number of population studies, inconsistently resulting in subtle behavioral and developmental deficits (Rice, 2004). Due to the importance of seafood in the diet of many peoples and the near ubiquitous contamination by MeHg, exposure to this toxin is inevitable. It is therefore crucial to improve our understanding of the mechanisms involved in MeHg toxicity, so that we might find ways to advise the public on ways to avoid any adverse effects of MeHg exposure.

The studies in this dissertation advance the understanding of MeHg toxicity primarily through dissecting its interaction with the neurogenic Notch signaling pathway. The Notch pathway is known to be involved with development and differentiation of many tissues, but plays its most prominent role in neural development (Portin, 2002). It has been previously shown that MeHg treatment in neural-derived Drosophila cell lines causes upregulation of the gene *E(spl)mδ*, a canonical Notch target, independent of Notch receptor protein itself (Rand et al., 2008). Because the Notch pathway is so highly involved in development of the nervous system I hypothesized that this change in *E(spl)mδ* expression plays an important role in the neurodevelopmental specificity of MeHg toxicity, and as such plays a role in MeHg toxicity in general.

Data from my studies show that MeHg treatment *in vivo* in embryos causes the same characteristic upregulation of *E(spl)mδ* as it does in cultured cell lines. This is crucial to establishing that the upregulation of *E(spl)mδ* observed in cells is not an artifact

of the system being studied, and is not mitigated by the resilience of a whole organism. My findings also show that this effect on *E(spl)mδ* is specific to MeHg, and can not be replicated by the inorganic mercurial $HgCl_2$ at doses showing similar toxicity. Establishing the specificity of MeHg to induce this effect is crucial in showing it is not a more generic response to stress or toxicity, which would indicate *E(spl)mδ* does not play a role in defining the neurodevelopmental specificity of MeHg. Further, my studies establish that this effect of MeHg on *E(spl)mδ* is not due to overall developmental delay in the embryos. Though gross developmental delay is a plausible mechanism for MeHg to cause toxicity it would have indicated that *E(spl)mδ* was simply a read-out of MeHg exposure, and not an important effector of MeHg toxicity. (Engel et al., 2012)

Though my experiments provide evidence for a specific upregulation of *E(spl)mδ* by MeHg, they do not indicate the mechanism that causes this effect. In my working model of MeHg toxicity I suggest that this upregulation is caused by binding of MeHg to an unidentified factor, X, with the MeHg/X complex driving expression of *E(spl)mδ*. I believe that Factor X belongs to one of two classes of proteins, traditional transcription factors or epigenetic regulators. If X is a traditional transcription factor it would have to be one that binds the promoter of *E(spl)mδ* but not other E(spl) genes, as the other E(spl)s are not upregulated by MeHg treatment *in vivo*. One plausible mechanism is that binding of MeHg might alter the activity of the factor X, either in preventing normal activity of a repressor or stimulating binding of an enhancer. A more thorough analysis of regulators of E(spl) gene transcription might then reveal the identity of X. Factor X might also be an epigenetic regulator of gene expression, such as a histone or histone deacetylase. If this is the case then binding of MeHg may prevent normal function of the protein, ultimately

affecting access of the regulatory sequences controlling expression of *E(spl)mδ*. As in the case of X being a traditional transcription factor if X is an epigenetic regulator it will necessarily interact with *E(spl)mδ* but not other E(spl) genes. As such identification of X could be accomplished using techniques such as chromatin immunoprecipitation and comparing the factors that associate with different E(spl)s to identify one that interacts only with *E(spl)mδ*.

After establishing that *E(spl)mδ* is affected by MeHg *in vivo* I sought to identify an appropriate phenotype of MeHg neural toxicity to determine whether *E(spl)mδ* is involved. Utilizing immunofluorescence and referring to the abundant literature on Drosophila development I identified phenotypes of MeHg treatment in the outgrowth and pathfinding of motor nerves in the embryo. This finding provided an accessible model of MeHg neural toxicity, which I probed to determine if genetic manipulation of *E(spl)mδ* replicates MeHg-induced phenotypes. Unexpectedly, driving expression of *E(spl)mδ* in neurons showed no discernable phenotype. I was then guided by the endogenous expression of *E(spl)mδ,* which showed expression localized to muscle during embryonic development. Overexpression of *E(spl)mδ* in muscle does cause a MeHg-like phenotype in motor nerve pathfinding. This finding was criticial, as it provides strong evidence for an important role of *E(spl)mδ* in MeHg toxicity; it also implies that the effects of MeHg in the system studied are not due to direct toxicity in neurons but instead due to effects on the substrate and targets for axon pathfinding.

By visualizing the muscles I was able to discern that both MeHg treatment and *E(spl)mδ* overexpression cause gross developmental anomalies in muscle patterning. This finding further supports the evidence indicating the phenotype I observed is due to

toxicity in the muscle rather than neurons. Further experiments looking at the co-occurrence of the nerve and muscle phenotypes implicated factors beyond the gross alterations in muscle pattern are impacting the success or failure of neural development. Though I attempted to elucidate changes in signaling molecules known to impact motor nerve pathfinding I was unable to observe any.

My experiments were unable to fully elucidate the mechanism by which *E(spl)mδ* overexpression in the muscle was able to cause failure of SN outgrowth. There are several possible mechanisms by which this may occur. First, the position of the muscles may be compromised; if the muscles are not in the correct position the nerve might not be able to find its target and thus stall. This could happen if the muscles are even subtly misplaced, such that I could not discern a muscle phenotype but the nerve still failed to branch. Failure of the muscles to occupy their proper position could also cause them to directly block the normal path of the axon, preventing it from growing normally. The other likely explanation for alteration of gene expression in muscles impacting nerve development is an alteration in cell-to-cell signaling. This could occur because of a failure of the normal attractive cues to be expressed. While I saw no global changes in *Sidestep* expression in Mef2>mδ embryos there might be alteration in the tight temporospatial regulation of the signal, which is crucial to the appropriate development of the motor nerves (Siebert et al., 2009). More sensitive analysis of *Sidestep* expression, perhaps using sidestep specific antibodies, would be necessary to determine exactly how *E(spl)mδ* overexpression alters sidestep expression. Alternatively, induced expression of *E(spl)mδ* in muscles could also cause inappropriate expression of a repulsive cue. This would cause failure of motor nerve outgrowth just as surely as lack of attractive cues, and

could be assayed simply in a first pass approach using qPCR as I have done here for *Side* and *Beat*.

In this dissertation I also performed experiments to assess *E(spl)mδ* specificity in its ability to induce the changes in muscle and motor nerve phenotype. Using a parallel approach I investigated *E(spl)mγ* activity and found that overexpression in muscle does not replicate the phenotypes seen with MeHg or *E(spl)mδ.* This indicates that *E(spl)mδ* is unique among E(spl)s in its ability to impact muscle development, and contributes to the literature showing that E(spl) genes act in discrete tissues during development (Jennings et al., 1999).

The findings of this work suggest further experiments that would address the impact of MeHg on human health. There has already been some study of the effect of MeHg on the Notch pathway in rat neural stem cells, but given my findings on the important role *E(spl)mδ* plays in Drosophila it will be worthwhile to more carefully study the impact of MeHg on E(spl) homologues, the HES genes, in mammals (Jarriault et al., 1998; Tamm et al., 2008). My studies show that only one E(spl) gene responds to MeHg consistently in the Drosophila embryo. As such it will be important to look at each HES gene individually to assess MeHg response. Should one or more HES genes prove to be responsive to MeHg experiments that replicate my approach in Drosophila would be appropriate; a neural phenotype of MeHg treatment could be identified in a mouse embryo and steps taken to attempt to reproduce the phenotype using genetic manipulations of HES genes. An additional line of inquiry applicable to human health suggested by my results is looking at non-neural contributions to apparently neural phenotypes of MeHg toxicity. Some work already suggests a role for glia in MeHg

toxicity in mammals (Castoldi et al., 2001; Aschner et al., 2007; do Nascimento et al., 2008). My work supports looking deeper into this connection and investigating the role of other tissues, such as the circulatory system, that may interact non-autonomously to potentiate MeHg neural toxicity.

The knowledge gained from my experiments reinforces the link between MeHg toxicity and the Notch signaling pathway. Moreover, this work suggests several new avenues of study that will continue to refine our understanding of MeHg. With further experiments we may be able to make more accurate assessment of the susceptibility or tolerance of a developing organism to MeHg, which will eventually inform recommendations on seafood intake and lead to ways to ameliorate MeHg toxicity, thereby reducing the threat of this global toxin.

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