The effects of methylmercury on Notch signaling during embryonic neural development in *Drosophila melanogaster*.

**Specific Aims**

Methylmercury (MeHg) is a persistent environmental neurotoxin. While high doses of MeHg are necessary in adults to cause peripheral neuropathy, low dose exposure *in utero* can cause drastic neurodevelopmental insult. Chronic low dose exposure to MeHg is common in the human population due to consumption of dietary fish that are high in mercury, putting a great number of newborns at risk for mercury exposure. Some mechanisms for MeHg induced neurotoxicity have been elucidated, but it is unclear which has the greatest impact during development. Evidence suggests MeHg may alter important cell signaling pathways, including the Notch receptor pathway, during development. Specifically, MeHg has been shown to cause enhanced expression of several Enhancer of Split [E(spl)] complex genes, canonical Notch targets, in neural-derived cell lines *in vitro*. Recent data demonstrates these MeHg effects on E(spl) gene transcription occur independent of the Notch receptor.

We hypothesize that MeHg alters neural development by affecting E(spl) gene expression, and that E(spl) mediated effects of MeHg are specific to neural cell types. We will probe this by investigating the effects of MeHg in *Drosophila* embryos at the genetic, cellular, and organismal level. This will be accomplished with the following specific aims:

**Aim 1. To determine the effects of methylmercury on the Notch targets in the Enhancer of Split complex during embryonic neural development.** We have previously established that MeHg alters the expression of E(spl) genes in neural derived cell lines. We hypothesize that MeHg will similarly affect E(spl) genes in the developing *Drosophila* nervous system during the embryonic and larval stages. We will test this by dosing *Drosophila* embryos and larvae cultured in vitro with MeHg and measuring E(spl) gene expression levels via qPCR.

**Aim 2. To compare neural phenotypes of methylmercury treatment in the developing embryo to phenotypes of Notch pathway alteration.** We hypothesize that phenotypes caused by MeHg treatment of developing embryos will be similar to phenotypes caused by direct manipulation of the Notch pathway. To address this we will analyze alterations in neurogenesis due to MeHg treatment using reporter fly lines to visualize cell fate decisions in a specific neuron lineage and compare the observed phenotypes with known Notch signaling perturbations. We will then attempt to reproduce the observed MeHg phenotypes using genetic manipulation that alters E(spl) gene expression.

**Aim 3. To establish neural specific effects of methylmercury in the embryo.** We hypothesize that MeHg induced E(spl) gene expression affects neural patterning by specifically interfering with neurons and neural precursors. To test this we will express known MeHg resistance genes in neurons and compare the MeHg induced neural phenotypes in these flies. For comparison, the same MeHg resistance genes will be expressed in glia to determine non-autonomous effects on protecting neuronal development.

**Significance:** Understanding the mechanism of action of MeHg is an important step toward developing treatment for MeHg exposure. The potential for MeHg to influence transcription in a specific neurogenic pathway has far reaching implications for deciphering the neurodevelopmental specificity of MeHg toxicity in humans.
Research Strategy

A. Significance:

A1. Methylmercury Sources and Exposures

Methylmercury (MeHg) is a persistent environmental neurotoxin known to specifically target neural development. Because the most common source of exposure to MeHg is seafood, which itself has been shown to have positive effects on development, it is important to refine our understanding of the mechanisms of MeHg toxicity to develop accurate recommendations for MeHg intake.

Mercury is released into the environment by both natural and anthropogenic sources including volcanic activity, mining, industrial processes such as acetaldehyde synthesis, and importantly burning of fossil fuels. Once released some of the mercury travels to wetland and marine environments where it is reduced and methylated by bacteria into MeHg. Biomagnification of the MeHg can then occur, allowing very high concentrations to accumulate in marine mammals and predatory fish. Human ingestion of these animals is the primary route of exposure to MeHg. (Clarkson and Magos 2006)

In very high doses, only encountered after industrial or agricultural accident, MeHg causes specific central neuropathy in adult victims resulting primarily in sensory deficits and eventually death (Ekino, Susa et al. 2007). Two incidents of accidental contamination of food are the sources of much of our data on high dose MeHg exposure. In the 1950s MeHg produced by industry was released as waste into Minamata Bay in Japan; the residents of Minamata and the surrounding region were exposed to this MeHg through fish from the bay (Ekino, Susa et al. 2007). Because of this MeHg poisoning is referred to as Minamata disease. The second large scale poisoning of a population with MeHg occurred in Iraq in the 1970s; in this incident seed grain treated with MeHg as a pesticide was consumed as bread, leading to high dose exposure (Rustam and Hamdi 1974). In both incidents humans exposed in utero showed severe symptoms similar to cerebral palsy at doses which caused few measurable symptoms in their mothers (Rustam and Hamdi 1974; Ekino, Susa et al. 2007).

A2. Low Dose Exposure and Risk Assessment

Because of its neurodevelopmental specificity and sensitivity much research has been directed at determining whether low dose exposure to MeHg, such as from normal dietary intake of fish, causes observable effects in children and fetuses. Unfortunately the data are unclear and studies of MeHg intake show conflicting results. Two studies stand out because of their large sample sizes and methodological rigor: the Faroe Islands and Seychelles Islands studies.

The Faroes population is exposed to MeHg primarily through the consumption of pilot whale blubber. 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, Weihe et al. 1995). At 7 and 14 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, Weihe et al. 1997; Murata, Weihe et al. 1999; Murata, Weihe et al. 2004; Debes, Budtz-Jorgensen et al. 2006).

The Seychelles Islands population is exposed to MeHg via deep sea and reef fish. In the study of this population the 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, Weihe et al. 1995). At 7 and 14 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, Weihe et al. 1997; Murata, Weihe et al. 1999; Murata, Weihe et al. 2004; Debes, Budtz-Jorgensen et al. 2006).

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 food the MeHg was consumed in. 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, Johansson et al. 2008; 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, Johansson et
A3. Methylmercury and the Notch Pathway

The significance of this proposal is best understood in the context of the experiments done to date that implicate the Notch pathway in mechanisms of MeHg toxicity. 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). 13 genes have been identified in the E(spl) complex: \( m\delta, m\gamma, m\beta, m\alpha, m1, m2, m3, m4, m5, m6, m7, m8, \) and groucho (Wurmbach, Wech et al. 1999; Lai, Bodner et al. 2000). Of these, seven \((m\delta, m\gamma, m\beta, m3, m5, m7, m8)\) code for basic helix-loop-helix (bHLH) transcriptional repressors, four \((m\alpha, m2, m4, m6)\) code for Bearded family transcription factors, one \((groucho)\) codes for a transcriptional corepressor, and one \((m1)\) codes for a putative protease inhibitor (Wurmbach, Wech et al. 1999; Lai, Bodner et al. 2000). bHLH E(spl) proteins produced by Notch activation facilitate lateral inhibition during cell differentiation (Jennings, Tyler 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, Tyler 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, Preiss et al. 1994).

Our lab has shown that treatment of neural-derived *Drosophila* cell lines and whole larvae with MeHg induces expression of genes in the E(spl) complex (Bland and Rand 2006; Rand, Bland et al. 2008). This expression was initially attributed to an increase in Notch cleavage and thus increased signaling by the Notch intracellular domain (Bland and Rand 2006). This was believed because knockdown of Notch expression using interfering RNA (RNAi) attenuated the response of the \( m\gamma \) gene to a 16 hour 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, Bland et al. 2008). Looking at RNAi knockdown of either Notch or Su(H) at this time point revealed no effect on MeHg-induced \( m\gamma \) expression (Rand, Bland 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, Bland et al. 2008). This is supported by the finding that the pattern of E(spl) gene expression differs when induced by MeHg versus Notch (Rand, Bland 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, Bland 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 caused upregulation of the \( m3 \) and \( m7 \) genes with modest increases in \( m2, m\gamma, \) and \( m\beta; \) importantly the \( m\delta \) gene showed very little upregulation due to endogenous Notch activity (Rand, Bland et al. 2008). MeHg treatment caused upregulation primarily in the \( m\delta \) gene, with little upregulation of \( m3 \) (Rand, Bland et al. 2008). Were MeHg acting through Notch to cause its upregulation of E(spl) genes it would be expected that the same genes would be upregulated with MeHg and EDTA treatments. Because the \( m\delta \) gene shows little expression due to endogenous Notch activity but is highly upregulated due to MeHg treatment it might prove a valuable target for further study of the effects of MeHg on E(spl) genes.

This 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, Duckworth et al. 2008). This failure to
The effects of methylmercury on Notch signaling during embryonic neural development in *Drosophila melanogaster*.  

**Greg Engel**

Differentiate was correlated with an increase in Notch activity measured by increased cleavage of the Notch intracellular domain (Tamm, Duckworth et al. 2008). Pretreatment with a Notch cleavage inhibitor reversed the repression of neuronal differentiation due to MeHg treatment (Tamm, Duckworth et al. 2008).

Because of its key role in neurodevelopment and the cross-species interaction of it and MeHg, alteration of the Notch pathway may be an important mechanism for toxicity. **We hypothesize that MeHg alters neural development by affecting E(spl) gene expression, and that E(spl) mediated effects of MeHg are specific to neural cell types.**

**Innovation:**

We will utilize the *Drosophila melanogaster* model organism to study MeHg toxicity. This model provides many advantages over traditional mammalian models of toxicity. One such advantage is that it is relatively high throughput. The combination of small size and short generation time make it relatively easy to sample large populations of *Drosophila*, allowing us to utilize qPCR to find trends in gene change for whole groups of animals in a single reaction and look microscopically at MeHg induced phenotypes in large groups of animals under a single slide. These factors also allow breeding of hybrid lines of *Drosophila* quickly and easily, allowing for rapid generation of novel genetic manipulations such as expression of a green fluorescent protein (GFP) reporter or the MeHg target gene *mδ* in embryonic neural precursors.

Another major advantage of the *Drosophila* model is the depth of existing data on developmental pathways and neurogenesis. The Notch pathway was first discovered in *Drosophila* and much of our current understanding of the pathway and its components was found using the *Drosophila* model. Our understanding of neurogenesis in *Drosophila* is also extremely detailed; each neuron and neural precursor in the *Drosophila* embryo has been identified and pathways crucial to its differentiation and final morphology have been discerned.

We have also refined techniques for dosing the *Drosophila* embryo with reliable levels of MeHg *in vitro*. Common practice is to feed fertile adult females food containing MeHg in order to expose the embryos, but any variability in feeding or egg laying behavior in the mothers will cause inconsistent MeHg dosage using this method. In our refined method the embryos are collected after laying, permeabilized by removing the proteinaceous chorion membrane, and partially immersed in buffer containing MeHg. By ensuring contact with both air and buffer the embryos remain hydrated, take up consistent levels of MeHg, and do not drown. Use of this technique will allow us to fully explore the effects of MeHg on embryonic development.

To investigate specific MeHg phenotypes we will make extensive use of the RN2-GFP fly line. This line utilizes a segment of the promoter for the gene *even-skipped (eve)* to drive expression of membrane-tethered green fluorescent protein (GFP) in a small subset of neurons, specifically the ACC and RP2 neurons, which send motor axons along the intersegmental nerve to innervate known muscle targets, and the PCC, an interneuron (Skeath and Doe 1998). Because of the small number of specific neurons expressing the GFP reporter it is possible to detect with great clarity many defects in neuron differentiation and maturation, including duplication, deletion, and failures of axon guidance. Moreover differentiation of the precursor ganglion mother cells is a Notch dependent process and these precursors do not express...
The effects of methylmercury on Notch signaling during embryonic neural development in *Drosophila melanogaster*. Greg Engel

eve (see Figure 1). This allows detailed study of the differentiation and axonogenesis of these neurons, and also allows easy expression of potential rescue genes in the same subset of neurons.

**Approach:**

**Preliminary data:**

Previous publications from our lab show that MeHg treatment of neural-derived cell lines can induce E(spl) gene expression. Knowing this we set out to determine if the effects of MeHg were specific or common to multiple mercurials/stressors. We first needed to assess the levels of toxicity of the mercurials used. To address this we assayed cell viability subsequent to MeHg and HgCl₂ exposures using the MTT cell viability assay. A dose dependent decrease in cell viability was observed with MeHg, which proved more potent than HgCl₂. MeHg exhibited an approximate 20% cell death (80% viability, LC20) at 4 µM and an approximate LC50 of 20 µM. HgCl₂ showed an approximate LC20 of 20 µM and LC50 of 100 µM. (see Figure 2)

We next examined the transcriptional response of the E(spl) genes in cells exposed to the approximate LC20 and LC50 concentrations determined above for MeHg and HgCl₂. With MeHg treatment the E(spl) genes mδ, mγ and m7 showed the greatest fold-induction of transcripts when assayed via qPCR; mδ and m7 showed more than seven-fold upregulation and mγ showed almost 12-fold upregulation with 20 µM MeHg. E(spl)mβ and m3 showed less substantial increases (less than four-fold) to MeHg treatment, while m2 approached a 6-fold induction. In contrast, when cells were treated with HgCl₂ they showed less than 3-fold response in E(spl) mδ and mγ, and less than 5-fold induction in m7. m2 responded to HgCl₂ treatment with a nearly nine-fold change at 100 µM. Thus, a somewhat differential response of individual genes in the E(spl) locus was observed with MeHg versus HgCl₂. (see Figure 3)

Having observed a unique effect of MeHg on E(spl) gene expression in vitro we examined whether it would have similar effects in vivo. Using our innovative technique for in vitro dosing of live embryos we evaluated the dose-response of embryos to MeHg by assaying gene expression using qPCR. We first examined a dose response to MeHg in this system by monitoring mδ gene expression. The stress response gene Hsp70 Bc was used as an internal control to confirm MeHg entry into the embryo. E(spl) mδ gene expression was seen to increase at every dose of MeHg. At 20 µM MeHg a more than seven-fold increase was seen which appeared to be sustained at 50 µM MeHg. Hsp70 Bc showed a similar dose-responsive increase in gene expression.

![Figure 2. Cell viability of *Drosophila* neural-derived cells after mercurial treatment.](image)

*Drosophila* bg2-c6 cells were treated for three hours with various doses of the organic mercurial MeHg or the inorganic mercurial HgCl₂. The MTT cell viability assay was utilized to approximate LC20 and LC50 for each toxicant.

![Figure 3. E(spl) gene induction in cells by mercurial treatment.](image)

*Drosophila* bg2-c6 cells were treated for three hours with the approximate LC20 or LC50 of MeHg or HgCl₂; E(spl) gene expression was assayed by qRT-PCR and fold induction over controls was calculated. Asterisks indicate significant difference between the Ct values of the indicated gene/treatment and its control; n = 3; * = p<0.05, ** = p<0.01.
indicating that overall gene induction was due to entry of the MeHg. From these data we chose to treat embryos with 50μM MeHg in subsequent analysis to ensure we were above a threshold in effect. (see Figure 4)

To further test the effects of MeHg during neural development RN2-GFP embryos were treated with MeHg and viewed microscopically. Axonal outgrowth appears to be altered in embryos treated with MeHg (Figure 5A-B). This phenotype will be an interesting target for further investigation if it proves to be robust. The RN2-GFP line utilizes the gal4/UAS system to express GFP; this gene/promoter pair adapted from yeast allows expression of target genes in selected subsets of cells. Briefly, the gal4 gene is placed under the control of a tissue specific promoter and the gene of interest inserted under the control of the UAS promoter element; when gal4 binds to the UAS it causes expression of the gene of interest in the specific tissues where gal4 is present (Phelps and Brand 1998). Because this system is already present in the RN2-GFP line it allows for expression of additional genes in the cells we observe our phenotype. To test this RN2-GFP flies were crossed with UAS-lacZ flies. As expected, this cross produces embryos expressing both GFP and β-galactosidase (βgal) in the RN2 subset of neurons (Figure 5C). As described in our specific aims, this will allow us to express genes of interest in these cells and view the effects on our observed MeHg phenotype.
Strategy:
Aim 1.
We hypothesize that MeHg will affect E(spl) genes in the developing Drosophila nervous system during embryogenesis as it does in cultured cells. To test this hypothesis we will look at the effect of MeHg on several E(spl) targets in embryos, to determine whether it shows a specific effect on E(spl) mothers as it does in cultured cells. We will determine the specificity with respect to MeHg by treating embryos with inorganic HgCl2. The strategy best suited to complete these goals is our in vitro treatment of live embryos by soaking. We will also distinguish changes in E(spl) expression due to MeHg from changes in E(spl) expression due to an altered timeline of developmental expression. To explore this we will again employ our soaking strategy but will harvest embryos at different developmental stages.

Aim 2.
We hypothesize that phenotypes caused by MeHg treatment of developing embryos will be similar to phenotypes caused by direct manipulation of the Notch pathway, as we have shown that MeHg treatment alters expression of the E(spl) Notch targets in cells. The first step to test this hypothesis is to compare phenotypes of MeHg treatment to known phenotypes of Notch pathway alteration. Our strategy is to use the RN2-GFP fly line which serves as an unambiguous phenotype in specific neuronal subsets in Drosophila embryos. This will lead to elucidating the MeHg phenotype in embryos where alteration of axon outgrowth/guidance is anticipated from our preliminary results. We will then use a strategy of transgenic expression to mimic the alteration of E(spl) gene expression caused by MeHg treatment to see if the phenotype compares with that of MeHg treatment.

Aim 3.
We hypothesize that MeHg induced E(spl) gene expression affects neural patterning by specifically interfering with neurons and neural precursors. To test this hypothesis we will attempt to rescue MeHg phenotypes by expressing resistance genes in target tissues. This will be contrasted with attempts to rescue the phenotype by driving expression in glia. By contrasting the effects of expression in both, we will discern whether the changes seen in affected neurons are due to cell-autonomous or whole organism effects of MeHg.

Methodology:
Aim 1.
We will first compare the effects of MeHg on E(spl) in embryos to those of HgCl2. CantonS adult flies will be maintained in a cage and allowed to lay for 2 hours on a grape-agar plate at 25C. The plate will then be removed from the cage and incubated at 25C for 2 hours. The embryos will then be removed from the plate and dechorionated in 50% bleach for 3 minutes. A 50μM solution of MeHg and a 1mM solution of HgCl2, which shows similar lethality in experiments on cells, will be made in PBS; these solutions and a third control solution containing no mercurial will be supplemented with DMSO to equal concentrations. Embryos will be immersed in the MeHg solutions in a basket with a nytex mesh bottom and incubated at 18C overnight. Embryos will then be harvested using Trizol reagent to isolate RNA and analyzed via qPCR using Sybr-green dye.

We will next determine whether the effects of MeHg on E(spl) are due to specific genetic interaction or general developmental delay. Flies will be allowed to lay on grape plates and the plates removed after either one or two hours of laying. Embryos will then be aged at 25C for differing periods, dechorionated, and treated for various lengths of time in a solution of either 50μM MeHg or DMSO control in PBS. Final time points for the differently aged embryo groups will be 3-4hr AEL (2hr on MeHg), 6-8hr AEL (4hr on MeHg), 8-10hr AEL (6hr on MeHg), 10-12hr AEL (8hr on MeHg), and 14-16hr AEL (12hr on MeHg). The embryos will then be harvested for RNA extraction with Trizol reagent and analyzed with qPCR.

All PCR results will be normalized to the housekeeping gene RP49. The comparative Ct technique will be used to express data as fold change, allowing us to compare relative abundance of RNA in the control and mercury treated groups. We will also include positive control primers for the known stress responder Hsp70 Bc
Aim 2.

First we will probe for the common neuronal duplication/deletion events associated with Notch pathway perturbation. The RN2-GFP flies will be allowed to lay for 2 hours on a grape-agar plate at 25C. The plate will then be removed and aged an additional 2hr at 25C. Embryos will be removed from the plate and dechorionated. Embryos will be immersed overnight 50µM MeHg or DMSO control in PBS. The embryos will then be fixed for 25 minutes at room temperature in a mixture of 4% paraformaldehyde and n-heptane (to remove the vitelline membrane). After fixation embryos will be suspended in methanol for storage at -20C. Embryos will then be rehydrated in PBS and blocked with 5% normal goat serum/normal donkey serum for 30m. Rabbit-anti-GFP antibody will be added and the embryos rocked overnight at 4C. Embryos will then be rinsed with PBS three times and incubated at room temperature for 1hr with goat-anti-rabbit alexa 488 antibody. Embryos will then be rinsed three times with PBS and suspended in 75% glycerol. Slides will be prepared and the embryos analyzed using fluorescent microscopy to quantify duplication or deletion of the stained ACC, PCC, and RP2 neurons. For consistency in all of our phenotypic analyses only the first five abdominal segments (A1-5) will be considered. Duplication and deletion events will be quantified in both a per embryo and per hemisegment manner, allowing an estimation of penetrance of the phenotype and severity in affected individuals; separate counts will be kept for both duplication and deletion in both the RP2 and ACC/PCC.

The second experiment in this aim will quantify the axonal phenotype of MeHg treatment observed in the preliminary data. RN2 embryos will be collected as in experiment 1, with 2 hours of laying and then 2 hour of aging. They will be dechorionated and treated overnight with MeHg or DMSO control. The will then be fixed and stained as previously described, including rabbit-anti-GFP and rat-anti-elav primary antibodies and goat-anti-rabbit alexa 488 and goat-anti-rat alexa 555 secondary antibodies. Slides will be prepared and axon outgrowth defects described and quantified. When quantifying axon outgrowth we will use the median cell cluster revealed by anti-elav staining as a landmark, scoring axons based upon whether they reach the cluster or not. As with the duplication/deletion analysis we will quantify both per embryo and per segment, again allowing information on penetrance and severity. Data will be analyzed statistically using Fisher's exact test with a standard p<0.05 threshold.

The third experiment in this aim will use genetic manipulation to specifically upregulate the primary E(spl) target of MeHg seen in cells, mō, in order to compare observable structural phenotypes to those of MeHg treatment. Male flies of the UAS-mō line, which contains an inducible copy of the mō gene, will be crossed with RN2-GFP virgin females, which already utilize the gal4/UAS system to express GFP. The mated flies will be allowed to lay on grape-agar plates and resulting embryos will be collected as described previously, resulting in 2-4 hour AEL embryos. These embryos will then be allowed to age overnight at 18C to bring them to the same developmental stage as those in previous experiments. The embryos will be fixed and stained as in previous experiments with rabbit-anti-GFP and rat-anti-elav primary antibodies and goat-anti-rabbit alexa 488 and goat-anti-rat alexa 555 secondary antibodies. Slides will be prepared and neuronal phenotypes analyzed. For mō overexpression experiments gene expression in the cross will be confirmed using qPCR before phenotypic observations are made. Embryos will be observed for phenotypes and if appropriate counts of cell duplication/deletion and axon outgrowth will be made as with RN2 embryos. These counts will be compared to MeHg treatment counts and analyzed using Fisher's exact test.

Aim 3.

First, we will drive expression of the known MeHg resistance gene glutamate-cysteine ligase, catalytic subunit (gclc) in neurons. It has previously been shown that increased levels of the thiol-rich antioxidant glutathione can protect organisms from toxicity due to MeHg exposure. We have shown that overexpression of the rate-limiting enzyme in glutathione synthesis, gclc, in Drosophila larvae increases eclosion into adults after MeHg treatment. As such we will attempt rescue of the embryonic neural phenotype of MeHg treatment by expressing gclc in the nervous system using the elav neural promoter, showing that it is direct effect on neurons
or neural precursors that causes the neural patterning phenotypes of MeHg treatment, rather than gross developmental alterations. Elav-gal4 virgin females will be mated with UAS-gclc males. 2-4 hour AEL embryos will be collected as previously described in aim1, dechorionated, and treated overnight with 50μM MeHg or DMSO control at 18C. The embryos will be fixed, and stained with mouse-anti-22C10 and goat-anti-mouse 488 antibodies. Slides will be made and using confocal microscopy the embryos will be analyzed and quantified for the phenotypes found most prominent in aim2 to determine if there is significantly reduced in frequency.

The second experiment we propose will see if glial expression of the resistance gene gclc alters MeHg induced neuronal phenotypes. Repo-gal4 virgin females will be mated with UAS-gclc males. 2-4 hour AEL embryos will be collected, dechorionated, and treated overnight with MeHg or DMSO control at 18C. The embryos will be fixed, and stained with mouse-anti-22C10 and goat-anti-mouse 488 antibodies. As with the elav>gclc cross the embryos will be analyzed using confocal microscopy to discern MeHg induced phenotypes.

Analysis:

Aim 1.

Because of natural variation in gene expression levels only two-fold or higher changes in expression will be considered "real" changes in any of our qPCR experiments. One possible outcome for our first experiment, treating embryos with MeHg and HgCl₂, is that MeHg will cause upregulation of primarily mδ and mγ while the effect of HgCl₂ will instead be upregulation of m2. This would be consistent with the data generated in cell lines, and would indicate that MeHg and HgCl₂ act through different mechanisms to affect E(spl) expression. Another possibility is that no difference in E(spl) gene expression between MeHg and HgCl₂ treated groups will be observed. This would indicate a common mechanism for the effects of the two mercurials in embryos.

Our second experiment also utilizes qPCR; as with the first experiment only two-fold or higher changes in gene expression will be considered. One likely outcome for the experiment is that the effects of MeHg on E(spl) gene expression will be consistent across all time points. If this is the case we will conclude that it is not general developmental delay that leads to these effects, but instead a more specific interaction. Another outcome is that a subset of the E(spl) genes will show consistent effects at all time points while others do not. Should this prove to be the case the genes that show consistency in their response to MeHg will be considered the principle E(spl) targets of MeHg.

Aim 2.

In our first experiment the results of a Fisher's exact test comparing duplication/deletion of neurons in MeHg versus control groups must be significant at the p<0.05 level for a difference to be considered. One possible outcome is that the MeHg treated embryos will show significantly higher numbers of duplication/deletion events than the controls. In this case it is likely that the effect of MeHg on E(spl) expression in the embryos is sufficient to cause a Notch phenotype. If there is no such significant difference, however, it will indicate that the E(spl) expression changes are not causing changes in the fate of these neurons. In this case if the E(spl) gene changes are contributing to the neurodevelopmental toxicity of MeHg they are not doing it through canonical E(spl) mechanisms.

As with our first experiment, the results of our second experiment will be considered significant if they reach the p<0.05 threshold in a Fisher's exact test comparing axon development failure in MeHg and control groups. Two outcomes are likely; MeHg and control groups will show significantly different numbers of axon development failure or no significant difference will be present. If there is a significant difference, which preliminary data indicates is likely, then MeHg causes failure of axon development in embryos and this phenotype can be further studied. If no significant difference is found then axon outgrowth is not significantly impaired by MeHg treatment in embryos and alternative phenotypes will need to be explored.

In order for an RN2>mδ cross to be considered successful qPCR analysis of RNA expression level must show at least two-fold upregulation of mδ over control flies. Any phenotypes observed in the embryos will need to reach statistical significance at the p<0.05 level on Fisher's exact test when compared to controls in order to be considered. It is possible that despite successful upregulation of mδ in the flies no phenotype will be
The effects of methylmercury on Notch signaling during embryonic neural development in *Drosophila melanogaster*.

Greg Engel

observable, or that a phenotype will be observed that is not present in control embryos treated with MeHg. In this case we will conclude that it is not the upregulation of mδ by MeHg that is responsible for the phenotypic changes observed after MeHg treatment. It is also possible that a phenotype will be observed that is common between RN2> mδ and MeHg treated embryos. In this case it is probable that it is the overexpression of mδ that causes the phenotype in MeHg treated embryos.

**Aim 3.**

As in aim2 success of fly crosses will be assessed with qPCR to ensure at least two-fold upregulation of gclc before further experiments are carried out. Two outcomes are likely, either RN2-driven or Repo-driven expression of gclc will significantly reduce the frequency of MeHg-induced phenotypes. Should RN2-driven expression reduce the phenotype frequency it will indicate that MeHg is acting specifically to disrupt signaling intracellularly, leading to developmental failure of affected cells. If Repo-driven expression reduces the phenotype frequency it will indicate that MeHg is more prominently active in extracellular developmental signals.

**Alternative Strategies:**

**Aim 1.**

A potential pitfall of this aim is that no effects on E(spl) gene expression will be consistent across time points. In this case it will be important to distinguish whether the effects observed indicate a general developmental delay due to MeHg or sensitive windows during development in which MeHg is particularly potent. Analysis of the trend of expression changes due to MeHg treatment compared to endogenous gene change due to development will allow this distinction to be made.

**Aim 2.**

The most prominent potential problem with this aim is that none of the phenotypes assessed will prove significantly different from the control. This is unlikely, as preliminary data indicates axonal phenotypes are common, but should it occur alternative read-outs of MeHg toxicity can be used for experiments requiring MeHg phenotypes. One such alternative that we have used successfully in our lab is measuring the rate of larval hatching, counting the number of animals that survive the transition from embryo to larva.

Another potential problem might occur because crossing the RN2-GFP line with another reduces number of copies per cell of the GFP gene. The reduction of GFP expression this causes may reduce signal strength sufficiently to preclude accurate visualization of axon outgrowth. In this case anti-22C10 antibody can be utilized instead of anti-GFP to allow visualization of the intersegmental nerve.

**Aim 3.**

The problem most likely to be encountered with this aim is failure of the Repo-gal4 line to drive expression of gclc. Should this be the case alternate gal4 lines can be used to drive gclc in the fat body (a liver analogue).
The effects of methylmercury on Notch signaling during embryonic neural development in *Drosophila melanogaster.*

Greg Engel

Works Cited:


