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The Notch target *E(spl)mδ* is a muscle-specific gene involved in methylmercury toxicity in motor neuron development

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Abstract

Methylmercury (MeHg) is a ubiquitous environmental toxin that has a selective and potent impact on the nervous system, particularly during neural development yet, the mechanisms for its apparent neurodevelopmental specificity are unknown. The Notch receptor pathway has been implicated as a MeHg target in several studies. Notch signaling mediates cell-cell signals in a number of developmental contexts including neurogenesis and myogenesis, where it fundamentally acts to repress differentiation. Previous work in our lab has shown that MeHg causes preferential upregulation of a canonical Notch response gene, *E(spl)mδ*, in *Drosophila* embryos. In parallel, MeHg is seen to disrupt outgrowth of embryonic intersegmental motor nerves (ISN), which can be mimicked by expression of activated Notch in embryonic neurons. However, overexpression of *E(spl)mδ* in developing neurons fails to elicit motor neuron outgrowth defects, pointing to a non-autonomous role for *E(spl)mδ* in motor axon development. In this study we investigate a role for *E(spl)mδ* in conveying the toxicity of MeHg in the embryo. We find that endogenous expression of the *E(spl)mδ* gene localizes to developing somatic muscles in embryos. Notably, *E(spl)mδ* expression is seen in several muscles that are known synaptic targets for both the ISN and the segmental motor nerve (SN). We also demonstrate that the SN, similar to the ISN, exhibits disrupted axon outgrowth in response to MeHg. *E(spl)mδ* can induce a SN motor neuron phenotype, similar to MeHg treatment; but, only when *E(spl)mδ* expression is targeted to developing muscles. *E(spl)mδ* overexpression in developing muscles also results in aberrant muscle morphology, which is not apparent with expression of the closely related *E(spl)mγ* in developing muscles. Our data point to a role for the Notch target *E(spl)mδ* in mediating MeHg toxicity in embryonic development by disrupting the coordinated targeting of motor neurons to their muscle targets.

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1. Introduction

Methylmercury (MeHg) is an environmental contaminant that causes toxicity in neural development. Chronic low-dose MeHg exposure through the consumption of contaminated seafood is common in many human populations, causing an ongoing concern that necessitates understanding the risks associated with this toxin. It is therefore important that the fundamental mechanisms of MeHg toxicity are elaborated further to formulate evidence-driven strategies to understand and minimize the risk MeHg poses during development.

MeHg causes transcriptional upregulation of the Enhancer of Split [E(spl)] complex gene *m-Delta* (*E(spl)mδ*) in the *Drosophila* embryo model (Bland and Rand 2006; Engel et al. 2012; Rand et al. 2008). The E(spl) locus consists of a group of 13 canonical Notch response genes; *E(spl)mδ* is one of the seven basic helix-loop-helix (bHLH) transcriptional repressors in the locus that are well documented effectors of Notch signaling (reviewed in (Portin 2002)). Notch signaling is central in cell fate determination, most notably in the neurectoderm, suggesting that induction of *E(spl)mδ* by MeHg may contribute to its neurodevelopmental toxicity. Notch signals are also required in other germ layers, including the mesoderm where Notch plays a role in muscle founder cell segregation (Corbin et al. 1991).

Previous work in our lab has characterized deficits in neural development following MeHg treatment of *Drosophila* embryos, including inhibition of neurite outgrowth in both central and peripheral neurons (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 follows a segmentally repeating, easily identifiable pattern (Kaufmann et al. 1998; Landgraf et al. 1999b; Murray et al. 1998; Sanchez-Soriano and Prokop 2005). Two major motor nerves, the intersegmental nerve (ISN) and the segmental nerve (SN), are readily identified by immunostaining for the Fasciclin II (FasII) cell surface antigen (Sanchez-Soriano and Prokop 2005). The ISN produces a characteristic long fiber that emerges from the ventral nerve cord and projects in a straight path producing short branches that synapse with muscles in the internal dorsal and dorsolateral fields. The SN produces a fiber (SNa) that is easily identifiable by its two characteristic branches that innervate the four lateral transverse (LT1-4) muscles and the segment border muscle (SBM), respectively.

The pattern and development of the somatic muscles are similarly well documented (Bate 1990). Myogenesis begins within the muscle competent mesoderm followed by selection of muscle founder cells, which then fuse with a population of nearby fusion competent cells to form multinucleated muscle fibers (Bate 1990; Maqbool and Jagla 2007; Olson et al. 1995; Tixier et al. 2010). Many of these myogenic events occur prior to neural innervation, yet muscle derived signals are known to play a critical role in coordinated targeting and synaptogenesis of motor neurons with muscle partners (Sink et al. 2001). The fact that several neurogenic genes, including Notch, are known to also play a role in myogenesis presents the possibility that aspects of muscle development may be labile to MeHg toxicity, similar to their neural counterparts.

In this study we investigate a role for *E(spl)mδ* in MeHg toxicity in the *Drosophila* embryo. Analysis of motor nerves in MeHg-treated embryos previously demonstrated a failure of the ISN to project dorsally (Engel et al. 2012). Yet, ectopic *E(spl)mδ* expression in neurons does not alter ISN axonal outgrowth (Engel et al. 2012). We now show that *E(spl)mδ* is endogenously expressed in developing somatic muscle cells. Moreover, MeHg is seen to elicit a pronounced muscle phenotype. Targeted overexpression of *E(spl)mδ* in developing muscle also alters muscle morphology and adversely affects SNa branching. In addition, we find that the effects of *E(spl)mδ* on muscle development can not be replicated by ectopic expression of the closely related *E(spl)mγ* gene in developing muscle. Our findings therefore identify *E(spl)mδ* as having a role in muscle, rather than neural, development. Furthermore, upregulated *E(spl)mδ* expression with MeHg in embryos is likely to influence motor neuron development by disrupting coordinated activity between developing muscle targets and their respective motor neurons.

2. Materials and Methods

2.1. Fly stocks and crosses

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-GFP (Bloomington *Drosophila* Stock Center, #4775), UAS-*E(spl)mδ* (Bloomington *Drosophila* Stock Center, #26677), UAS-*E(spl)mδ*RNAi (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.

2.2. Generation of *E(spl)mδ*-GFP reporter strain

The 5kb upstream *E(spl)mδ* promoter region was amplified from Canton S 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 cloned into the *Drosophila* transformation vector pGreen H-Pelican (Barolo et al. 2004) with XbaI and KpnI (New England Biolabs). Bacterial transformation was performed with One Shot TOP10 chemically competent cells (Invitrogen) according to manufacturer's instructions. Ampicillin resistant transformed colonies were grown and the plasmid purified using a Qiagen maxiprep kit (Qiagen). The proper orientation of the *E(spl)mδ* promoter region was verified by DNA sequencing. Transgenic flies were created by P-element mediated germline transformation through a commercial embryo injection service (Rainbow Transgenics, Camarillo, CA). Positive transformant flies were selected by red eye color and verified by GFP expression in living embryos and with immunostaining for GFP as described below. Six independent transformant lines were established, all of which gave an identical pattern in GFP expression in the somatic muscles in living embryos (data not shown).

2.3. Embryo mercury treatments

Embryo treatments were done as previously described (Engel et al. 2012). Briefly, cages of adult flies were set to lay eggs on a grape agar plate with a dab of yeast paste. Embryo collection occurred over a two-hour laying period. Embryos were then aged on grape plates at 25°C for two hours. Embryos were dechorionated in a mesh basket by immersion in 50% bleach for three minutes followed by rinsing under room temperature tap water to remove the bleach. Embryos were transferred to separate mesh baskets for each treatment, which were in turn placed into a 60mm petri dish reservoir containing modified basic insect medium (Engel et al. 2012) with either MeHg (50µM final concentration from 50mM stock solution in DMSO; methylmercury chloride, Aldrich #442534) or DMSO solvent control (0.1% final concentration in all treatments). Basket and reservoir were covered to prevent evaporation and the embryos were allowed to incubate at 25°C for 14-16 hours. Staging of treated embryos was done by evaluating the degree of germband retraction and ventral nerve cord condensation compared to reference images (Ashburner et al. 2005). Rationale for MeHg concentration used in these treatments are based on effects document previously, where dose dependent effects of E(spl)mδ gene induction were characterized (Engel et al. 2012). The 50µM concentration used was selected to ensure we were above a threshold effect that was previously observed occurring near the 20µM MeHg treatment level (Engel et al. 2012).

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 -20°C in methanol until staining. For immunostaining, embryos were permeabilized 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 (Sigma G9023, D9663). Primary antibodies used were: mouse anti-Elav (1:10, 9F8A9), rat anti-FasII (1:10, 1D4) (Developmental Studies Hybridoma Bank, Univ. of Iowa), rabbit anti-GFP (1:1000, Torrey Pines Biolabs, Inc), rabbit anti-βgal (Chemicon International), and rabbit anti-myosin heavy chain (1:1000, gift from Daniel Kiehart, Duke University). Secondary antibodies used were: Alexa488-conjugated goat anti-rat (1:250), Alexa555-conjugated goat anti-mouse (1:400), and Alexa555-conjugated goat anti-rabbit (1:400, 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 or a Nikon AZ100 microscope equipped with an Andor Clara E camera and processed with Nikon Elements software (MVI, Avon, MA). Phenotypes were scored per hemisegment looking exclusively at abdominal segments 1-5. Therefore, a minimum of 20 embryos were scored for each phenotype. Images were assembled in Adobe Photoshop, GIMP, Image J and Microsoft PowerPoint software packages.

3. Results

3.1 Expression of *E(spl)mδ* in developing embryos

In a previous study, we identified a phenotype of MeHg treatment in *Drosophila* embryos affecting outgrowth of the intersegmental motor nerve (ISN) (Engel et al. 2012). This phenotype parallels a selective activation of the Notch target gene *E(spl)mδ* measured by quantitative PCR of total embryonic RNA transcripts. However, ectopic expression of *E(spl)mδ* targeted to neurons fails to elicit ISN defects (Engel et al. 2012) suggesting that *E(spl)mδ* is acting non-autonomously to affect neurite process extension as a consequence of MeHg toxicity. We therefore analyzed endogenous *E(spl)mδ* expression to discern where this Notch target gene might be acting in normal development. We created transgenic flies carrying GFP under the control of a 5kb upstream region of the *E(spl)mδ* gene (*E(spl)mδ*-GFP, see Figure 1A). Care was taken to use the pGreen H-Pelican vector (Barolo et al. 2004), containing insulators surrounding the *E(spl)mδ* upstream sequence, to create a faithful reporter of this gene's expression. Expression of *E(spl)mδ* in Stage 14 embryos is seen in a pattern reminiscent of muscle precursors in thoracic and abdominal segments (Figure 1B). This pattern matches that seen with expression of a GFP reporter under the expression of myocyte enhancer factor-2 (Mef2) (Figure 1C), a specific marker of somatic muscle lineages (Lilly et al. 1994).

To examine the expression of *E(spl)mδ* relative to the timing of motor neuron outgrowth we examined FasII expression, which labels the ISN and other neuronal subsets, in *E(spl)mδ*-GFP embryos. In Stage 12 embryos, *E(spl)mδ* is seen in a region of the mesoderm lying just medial to FasII-positive neurons forming in the ventral nerve cord (Figure 2A, A'). *E(spl)mδ* expression progresses to precursors in the ventral, lateral and dorsal fields in the forming somatic musculature during Stages 14-16 (Figure 2B, C). Differentiated and fused muscle bundles are also seen to express *E(spl)mδ* (Figure 2C). Initial outgrowth of the ISN motor neuron is seen at Stage 14, at time where several *E(spl)mδ*-positive muscle precursors have assumed their position (Figure 2B, B'). The ISN is observed to extend to the dorsal field by Stage 16, when *E(spl)mδ*-positive muscle bundles are detected. In addition, projection of the FasII-positive segmental nerve (SN) is seen to project to *E(spl)mδ* positive muscles in the lateral field and furthermore, occurs subsequent to the ISN reaching the dorsal muscle field. These expression patterns indicate that muscle precursors assume their position just prior to motor neuron outgrowth, which is consistent with what has been previously documented (Bate 1990).

3.2 Analysis of motor neuron innervation of *E(spl)mδ* expressing muscles

The pattern of *E(spl)mδ* expression predicts that the muscle targets of the ISN, SN and potentially other motor neurons are *E(spl)mδ*-positive muscles. The established pattern of muscle innervation by the SNa nerve is illustrated in Figure 3A and visualized with staining for FasII and a myosin heavy chain antibody to reveal the muscle groups (Figure 3B). At Stage 16 SNa synaptic targets are apparent, including the segment border muscle (SBM, solid arrow, Figure 3A, B-B''), that is innervated by the lateral branch of the SNa, and the lateral transverse muscles (LT1-4, open arrow, Figure 3A, B-B''), which are innervated by the dorsal branch of the SNa. Several dorsal muscles are innervated by the ISN (*, Figure

3B-B"). Examination of the *E(spl)mδ*-positive muscles and FasII-positive SNa and ISN motor nerves with confocal microscopy reveals that *E(spl)mδ*-expressing muscles comprise several of the known synaptic targets for these neurons. *E(spl)mδ* expression is apparent in the LT1-4 and SBM muscles, which are innervated by branches of the SNa (Figure 3C-C"). Moreover, several *E(spl)mδ*-positive dorsal muscle groups are innervated by branches of the ISN. These data indicate that endogenous expression of *E(spl)mδ* in late stage embryos occurs in muscles, including those innervated by the SN and ISN.

3.3 Analysis of MeHg and *E(spl)mδ* effects in embryonic motor neurons and muscles

Further characterization of MeHg effects on the motor nerves has lead us to identify a robust phenotype of the failure of branching in the SNa (Figure 4A,B,D) with MeHg exposure. This SNa phenotype is more frequently observed than the previously reported ISN MeHg phenotype, suggesting the SNa axonal outgrowth is even more sensitive to MeHg. We therefore utilized this SNa phenotype to elaborate MeHg effects in this study. With MeHg exposure, failure of one or the other branch of the SNa was commonly seen, which was not observed to favor the development of one branch over the other. Development of the SNa dorsal branch alone, lateral branch alone, and stalling at the branch point were all observed with approximately equal frequency (data not shown).

With insight into the muscle specific expression of *E(spl)mδ* in the embryo, we sought to determine whether the effects of MeHg treatment on SN branching could be influenced by ectopic expression of *E(spl)mδ* in developing muscle. Using the Mef2-GAL4 driver, which drives expression in the somatic musculature, expression of *E(spl)mδ* causes a significant failure in SNa branching (Mef2>mδ, Figure 4C,D). In contrast, overexpression of *E(spl)mδ* in neurons by the Elav-GAL4 driver did not lead to increased SNa branch failure (Elav>mδ, Figure 4D). These data suggest that overexpression of *E(spl)mδ* in muscle targets, but not the nerves themselves, causes failure of proper axonal outgrowth, mimicking that seen with MeHg treatment.

Given that *E(spl)mδ* overexpression in muscle caused a SNa phenotype similar to that of MeHg treatment, we next determined whether either of these treatments grossly impacted muscle development. We stained the muscles of MeHg treated embryos and Mef2>mδ expressing embryos with anti-myosin heavy chain antibody (Figure 5). To assess normal development we evaluated the morphology of LT1-4, known targets of the dorsal branch of the SNa. Among control embryos only 1% of hemisegments analyzed showed a defect in patterning of the LT muscles (Figure 5A,D). MeHg treated embryos showed disorganization of the muscle pattern, with aberrant morphology of the LT1-4 bundles in 15% of hemisegments (Figure 5B,D). Mef2>mδ embryos showed higher rates of muscle pattern failure, demonstrating a disordered muscle phenotype in 58% of hemisegments (Figure 5C,D). These data point to a functional activity for *E(spl)mδ* in muscle formation, which could furthermore impact the elaboration of SNa neurite branches.

With an apparent strong effect of *E(spl)mδ* overexpression in muscles we next investigated the requirement of *E(spl)mδ* for normal muscle development. Knockdown of *E(spl)mδ* expression with an RNAi construct driven by the Mef2-GAL4 driver resulted in complete

lethality of embryos (Figure 4D) indicating a critical role for *E(spl)mδ* in mesoderm and muscle development.

To further elucidate the relationship between the phenotypes in the SN nerve and its muscle targets, we co-immunostained for FasII and myosin heavy chain in MeHg-treated embryos and scored the coincidence of SN branching defects and muscle morphology defects. In MeHg treated embryos over 13% of the hemisegments scored showed a SNa branching phenotype that occurred despite no LT1-4 muscle phenotype, while nearly 12% of hemisegments showed a LT1-4 muscle phenotype but no SNa branching phenotype (Figure 6). Thus, it is apparent that defects in muscle morphology are not entirely co-occurring with the neurite branching defects in the SNa under this MeHg treatment paradigm.

3.4 Assessment of *E(spl)mδ* versus *E(spl)mγ* activity in embryonic muscle and neuronal development

The above results indicated that the MeHg phenotypes observed in both motor nerve outgrowth and muscle formation could largely be correlated with MeHg-induced *E(spl)mδ* expression. *E(spl)mδ* is one of seven bHLH transcriptional repressors in the *E(spl)* locus that are known Notch target genes (Delidakis and Artavanis-Tsakonas 1992). The related structure of these genes prompted us to investigate if the expression pattern and function of *E(spl)mδ* we observed was unique or shared by another *E(spl)* gene. The *E(spl)mγ* gene is closely related and lies immediately adjacent to the *E(spl)mδ* gene (Engel et al. 2012; Wurmbach et al. 1999). We have previously shown that *E(spl)mγ* responds to MeHg treatment of Drosophila cell lines (Rand et al. 2008); however, *E(spl)mγ* does not respond to MeHg treatment in embryos (Engel et al. 2012). Nonetheless, we used *E(spl)mγ* as a tool to assess the specificity of *E(spl)mδ* function in the SNa phenotype. We first confirmed the endogenous expression pattern of *E(spl)mγ* using a lacZ reporter fly stained for βgal and the neuron specific anti-Elav antibody. Expression of *E(spl)mγ* is largely restricted to neural cells in the embryo (Figure 7A-C), consistent with previous reports of *E(spl)mγ* expression by in situ hybridization (Wech et al. 1999). These data indicate that *E(spl)mγ* and *E(spl)mδ* differ considerably in their pattern of expression during late stage embryogenesis. We then tested the functional activity of *E(spl)mγ* by ectopic expression in muscles or neurons, respectively. In contrast to *E(spl)mδ*, expression of *E(spl)mγ* with the Mef2-GAL4 driver showed no increase in the SN phenotype over controls (Figure 7D). Expression of *E(spl)mγ* in neurons with the Elav-Gal4 driver however, resulted in a lethality phenotype at early stages of embryonic development, preempting the ability to assess motor nerve phenotypes, yet demonstrating a robust and distinct activity of *E(spl)mγ* versus *E(spl)mδ*.

4. Discussion

4.1 *E(spl)mδ* expression and activity highlight a potentially novel MeHg mechanism causing developmental toxicity in motor neurons

We demonstrate that *E(spl)mδ*, an established MeHg target gene in Drosophila embryos, has specific localization and activity in developing muscles. Overexpression of *E(spl)mδ* in developing muscle perturbs somatic muscle patterning while knockdown of *E(spl)mδ* causes embryonic lethality. While not completely penetrant, an accompanying phenotype of

defective branching of the SNa motor neuron that innervates *E(spl)mδ*-expressing LT1-4 and SBM muscles is seen with overexpression of *E(spl)mδ* in developing muscles. The most parsimonious conclusion from these results, and our previous characterization of *E(spl)mδ* (Engel et al. 2012), is that MeHg acts to alter *E(spl)mδ* expression in developing muscles that in turn compromises essential signals between the forming muscle and its cognate motor neuron. However, MeHg-induced SNa branching defects are observed in the absence of obvious muscle patterning defects with considerable frequency, suggesting the above mechanism is not exclusive, which we elaborate upon below. Nonetheless, our study has produced an unexpected and fundamental finding of *E(spl)mδ* expression and activity in developing muscle, which may also contribute to understanding the recently described roles for Notch signaling in myogenesis and myoblast fusion (Artero et al. 2003; Gildor et al. 2012).

4.2 MeHg exposure and *E(spl)mδ* overexpression elicit motor neuron and muscle defects

MeHg treatment in embryos causes failure in SNa branches that innervate the LT1-4 and SBM muscles. MeHg can similarly disrupt muscle formation, notably affecting the positioning and morphology of LT1-4 muscle group. Both of these outcomes can be elicited with overexpression of *E(spl)mδ* in the developing muscle. In contrast, *E(spl)mδ* overexpressed in neurons alone does not result in a SNa phenotype (Engel et al. 2012). Furthermore, the SNa and muscle phenotypes are not exclusively occurring in conjunction with each other, and therefore raises the question of which of these events are specifically targeted by MeHg and how these outcomes are potentially linked.

The fact that MeHg specifically upregulates *E(spl)mδ* in the embryo (Engel et al. 2012) and that *E(spl)mδ* expression is apparently restricted to mesodermal myogenic cells suggests that much of the MeHg effect could be mediated via perturbations to muscle cell development. This notion is entirely consistent with the known involvement of Notch, and other neurogenic genes, in establishment of muscle founder cells (Corbin et al. 1991). Importantly, muscle founder cells are reported to produce cues for motor neuron axonal targeting (Landgraf et al. 1999a). Specific signaling mechanisms, involving the Sidestep protein (Side) in embryonic muscle cells, are necessary to engage branching and synaptogenesis of SN and ISN neurons (Sink et al. 2001). These signals are mediated through interactions with the Beaten Path Ia protein (Beat) expressed on the growing motor neuron axon (Siebert et al. 2009). It is therefore possible that MeHg, by altering muscle cell development, could compromise this signaling and, in turn, affect motor axon outgrowth. Preliminary attempts to measure levels of Side and Beat expression levels by qPCR of whole embryo transcripts showed no significant changes in expression with MeHg treatment (data not shown). However, due to the restricted expression of these proteins, this global analysis is likely not sufficient to detect changes in Side and Beat and further investigation of a potential role for this pathway in mediating the MeHg effects is warranted.

4.3 Neuron versus muscle targets in MeHg exposure

Several possible explanations for the incomplete correlation of muscle patterning defects and SNa branching defects seen with this MeHg treatment paradigm exist. In all cases, it is likely that the outcomes of our treatments are sensitive to the timing and concentration of

MeHg exposure. In this regard, it has been shown that penetration of the embryo with small molecules can be variable (Rand et al. 2010). Although MeHg is applied at one concentration (50 μ M), at an early stage (e.g. 4 hours-Stage 8-9), and incubated for 14-16 hours in this study, the rapid course of development of the embryo dictates that variation in MeHg penetration could result in starkly different timing of toxic exposure. Future studies will employ a recently developed protocol in our lab of permeabilizing the embryo eggshell to circumvent this technical difficulty (Rand 2014; Rand et al. 2014). With the above consideration, it is possible that both the motor neuron and the muscle cell are susceptible to the MeHg toxicity, but each has a developmental window of susceptibility that is to some extent non-overlapping. For example, a MeHg insult to the SNa precisely at the time that branching is to occur could result in a failed branch event, while the LT1-4 and SBM muscles are well on their way to assuming their normal morphology. Alternatively, MeHg toxicity during earlier myoblast fusion stages may compromise morphology of the mature LT1-4 and SBM fibers but leave the signaling pathways that engage a normal SNa branching event intact. Future endeavors will delineate the specificity with which MeHg acts on these fundamental cell types by taking steps to control MeHg exposures more precisely.

4.4 Specificity of *E(spl)m δ* activity in neuromuscular development

Contrasting overexpression of *E(spl)m δ* with *E(spl)m γ* provides some additional clues on the observed specificity in the response of *E(spl)m δ* to MeHg treatment. Both *E(spl)m δ* and *E(spl)m γ* are upregulated with MeHg treatment in *Drosophila* cells in culture (Engel et al. 2012). However, only *E(spl)m δ* is upregulated with MeHg in the embryo (Engel et al. 2012). When *E(spl)m γ* is overexpressed in tissues where it is found endogenously (e.g. neurons) it proves lethal to the embryo; when *E(spl)m δ* is overexpressed in a pattern mimicking its endogenous expression in embryos it shows a distinct phenotype, but can develop to late embryonic stages. One possible explanation for this is that *E(spl)m γ* is more lethal when overexpressed in its endogenous setting and is thus more tightly regulated by the embryo than *E(spl)m δ* . In support of this notion, expression of *E(spl)m γ* outside of its normal neural domain (e.g. Mef2-GAL4) likely dissociates it from factors that convey its strong activity. Further evidence supporting this hypothesis could come from overexpressing the other closely related *E(spl)* genes that do not respond to MeHg treatment of embryos, such as *E(spl)m3* or *E(spl)m7* (Engel et al. 2012), and determining lethality in the embryos. Nonetheless, the essential and specific function of *E(spl)m δ* in myogenesis is further reinforced by the lethal outcome of knocking down its expression with RNAi in developing muscle.

4.5 Conclusions

Our data continue to support an important role for *E(spl)m δ* in MeHg toxicity in the *Drosophila* embryo, showing that *E(spl)m δ* specifically, and not the closely related *E(spl)m γ* , can replicate a motor neuron phenotype resulting from MeHg treatment. We have also provided evidence that *E(spl)m δ* is active primarily in the mesoderm and muscle forming cells during embryonic development, rather than the neuroectoderm. Further work investigating MeHg effects on *E(spl)m δ* upregulation in the embryo will likely produce valuable insight into MeHg toxicity mechanisms with respect to neuromuscular development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Artero R, Furlong EE, Beckett K, Scott MP, Baylies M. Notch and Ras signaling pathway effector genes expressed in fusion competent and founder cells during *Drosophila* myogenesis. *Development*. 2003; 130:6257–6272. [PubMed: 14602676]
- Ashburner, M.; Golic, K.; Hawley, R. *Drosophila: A Laboratory Handbook*. Cold Spring Harbor Laboratory Press; New York: 2005.
- Barolo S, Castro B, Posakony JW. New *Drosophila* transgenic reporters: insulated P-element vectors expressing fast-maturing RFP. *BioTechniques*. 2004; 36:436–440. 442. [PubMed: 15038159]
- Bate M. The embryonic development of larval muscles in *Drosophila*. *Development* (Cambridge, England). 1990; 110:791–804.
- Bland CE, Rand MR. Methylmercury induces activation of Notch signaling. *Neurotoxicology*. 2006; 27:982–991. [PubMed: 16757030]
- Cooper MT, Tyler DM, Furriols M, Chalkiadaki A, Delidakis C, Bray S. Spatially Restricted Factors Cooperate with Notch in the Regulation of Enhancer of split Genes. *Developmental biology*. 2000; 221:390–403. [PubMed: 10790334]
- Corbin V, Michelson AM, Abmayr SM, Neel V, Alcamo E, Maniatis T, Young MW. A role for the *Drosophila* neurogenic genes in mesoderm differentiation. *Cell*. 1991; 67:311–323. [PubMed: 1913825]
- Delidakis C, Artavanis-Tsakonas S. The Enhancer of split [E(spl)] locus of *Drosophila* encodes seven independent helix-loop-helix proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 1992; 89:8731–8735. [PubMed: 1528887]
- Engel GL, Delwig A, Rand MD. The effects of methylmercury on Notch signaling during embryonic neural development in *Drosophila melanogaster*. *Toxicol In Vitro*. 2012; 26:485–492. [PubMed: 22230562]
- Gildor B, Schejter ED, Shilo BZ. Bidirectional Notch activation represses fusion competence in swarming adult *Drosophila* myoblasts. *Development*. 2012; 139:4040–4050. [PubMed: 23048185]
- Kaufmann N, Wills ZP, Van Vactor D. *Drosophila* Rac1 controls motor axon guidance. *Development* (Cambridge, England). 1998; 125:453–461.
- Landgraf M, Baylies M, Bate M. Muscle founder cells regulate defasciculation and targeting of motor axons in the *Drosophila* embryo. *Current biology : CB*. 1999a; 9:589–592. [PubMed: 10359699]
- Landgraf M, Roy S, Prokop A, VijayRaghavan K, Bate M. even-skipped determines the dorsal growth of motor axons in *Drosophila*. *Neuron*. 1999b; 22:43–52. [PubMed: 10027288]
- Landgraf M, Thor S. Development of *Drosophila* motoneurons: specification and morphology. *Seminars in cell & developmental biology*. 2006; 17:3–11. [PubMed: 16356739]
- Ligoxygakis P, Bray SJ, Apidianakis Y, Delidakis C. Ectopic expression of individual E(spl) genes has differential effects on different cell fate decisions and underscores the biphasic requirement for Notch activity in wing margin establishment in *Drosophila*. *Development*. 1999; 126:2205–2214. [PubMed: 10207145]
- Lilly B, Galewsky S, Firulli AB, Schulz RA, Olson EN. D-MEF2: a MADS box transcription factor expressed in differentiating mesoderm and muscle cell lineages during *Drosophila* embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91:5662–5666. [PubMed: 8202544]

- Maqbool T, Jagla K. Genetic control of muscle development: learning from *Drosophila*. *J Muscle Res Cell Motil.* 2007; 28:397–407. [PubMed: 18347920]
- Murray MJ, Merritt DJ, Brand AH, Whittington PM. In vivo dynamics of axon pathfinding in the *Drosophila* CNS: a time-lapse study of an identified motoneuron. *Journal of neurobiology.* 1998; 37:607–621. [PubMed: 9858262]
- Olson EN, Perry M, Schulz RA. Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Developmental biology.* 1995; 172:2–14. [PubMed: 7589800]
- Portin P. General outlines of the molecular genetics of the Notch signalling pathway in *Drosophila melanogaster*: a review. *Hereditas.* 2002; 136:89–96. [PubMed: 12369105]
- Rand M. A method of permeabilization of *Drosophila* embryos for assays of small molecule activity. *Journal of Visualized Experiments.* 2014 (in press).
- Rand MD, Bland CE, Bond J. Methylmercury activates enhancer-of-split and bearded complex genes independent of the notch receptor. *Toxicol Sci.* 2008; 104:163–176. [PubMed: 18367466]
- Rand MD, Dao JC, Clason TA. Methylmercury disruption of embryonic neural development in *Drosophila*. *Neurotoxicology.* 2009; 30:794–802. [PubMed: 19409416]
- Rand MD, Kearney AL, Dao J, Clason T. Permeabilization of *Drosophila* embryos for introduction of small molecules. *Insect biochemistry and molecular biology.* 2010; 40:792–804. [PubMed: 20727969]
- Rand MD, Montgomery SL, Prince L, Vorojeina D. Developmental toxicity assays using the *Drosophila* model. *Current Protocols in Toxicology.* 2014 (in press).
- Sanchez-Soriano N, Prokop A. The influence of pioneer neurons on a growing motor nerve in *Drosophila* requires the neural cell adhesion molecule homolog FasciclinII. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2005; 25:78–87. [PubMed: 15634769]
- Siebert M, Banovic D, Goellner B, Aberle H. *Drosophila* motor axons recognize and follow a Sidestep-labeled substrate pathway to reach their target fields. *Genes & development.* 2009; 23:1052–1062. [PubMed: 19369411]
- Sink H, Rehm EJ, Richstone L, Bulls YM, Goodman CS. sidestep encodes a target-derived attractant essential for motor axon guidance in *Drosophila*. *Cell.* 2001; 105:57–67. [PubMed: 11301002]
- Tixier V, Bataille L, Jagla K. Diversification of muscle types: recent insights from *Drosophila*. *Exp Cell Res.* 2010; 316:3019–3027. [PubMed: 20673829]
- Wech I, Bray S, Delidakis C, Preiss A. Distinct expression patterns of different enhancer of split bHLH genes during embryogenesis of *drosophila melanogaster*. *Development genes and evolution.* 1999; 209:370–375. [PubMed: 10370119]
- Wurmbach E, Wech I, Preiss A. The enhancer of split complex of *drosophila melanogaster* harbors three classes of notch responsive genes. *Mechanisms of development.* 1999; 80:171–180. [PubMed: 10072784]

Highlights

- Notch target gene E(spl)m δ shows muscle specific expression in Drosophila.
- Methylmercury perturbs embryonic neuron and muscle development in Drosophila.
- Upregulated E(spl)m δ activity in developing muscle alters muscle development

Figure 1. Expression of *E(spl)mδ* in developing somatic muscles of the embryo

A) Schematic of the reporter construct developed in the pGreen H-Pelican vector (Barolo et al. 2004) to create *E(spl)mδ-GFP* transgenic flies. (P, p-element sequences; I, insulator sequences; *eGFP*, coding sequence for the enhanced green fluorescent protein; *w*, the white gene used for transformant selection). B) GFP expression in Stage 14 *E(spl)mδ-GFP* embryo stained with anti-GFP antibody. C) GFP expression under the control of the *Mef2-GAL4* driver revealing somatic muscle precursors. (Embryo orientation for all figures: anterior left, dorsal up)

Figure 2. Expression of E(spl)mδ relative to motor nerve outgrowth in late stage embryogenesis
 A-C) E(spl)mδ-GFP expression in Stage 12-16 embryos revealed with immunostaining for GFP. Expression in the mesoderm (St 12) resolves to the somatic musculature in later stages (St. 14-16). A'-C') Development and outgrowth of the Intersegmental (ISN) and Segmental nerves (SN) from the ventral nerve cord (VNC) over stages 12-16 is revealed with anti-FasII immunostaining. ISN projection to the dorsal domain is preceded by positioning of E(spl)mδ-expressing muscle precursors at Stage 14 (B') and also occurs prior to outgrowth of the SN nerve (St 16, C').

Figure 3. SNa innervation of E(spl)mδ expressing muscles

A) Schematic of the Segmental Nerve (SN) and a subset of muscles in the lateral and dorsal field (gray and green bars) showing SNa branches (Purple) that innervate the lateral transverse muscle group (LT1-4) and the segment border muscle (SBM, in green) (Adapted from (Landgraf and Thor 2006)). B-B'') Lateral muscle groups revealed with myosin heavy chain immunostaining (Mhc, green) and ISN and SN motor nerves revealed with anti-FasII immunostaining (Purple) in two segments. Innervation of the LT1-4 group by the SNa dorsal branch (open arrow) and the SBM muscle by the lateral SNa branch (filled arrow) is seen in B''. C-C'') Anti-GFP immunostaining (green) and ISN and SN motor nerves revealed with anti-FasII immunostaining (Purple) in an E(spl)mδ-GFP embryo shows the LT1-4 and SBM muscle express E(spl)mδ. Additional E(spl)mδ-positive muscles in the dorsal field are innervated by the ISN nerve (* in B'' and C'').

Figure 4. MeHg treatment and expression of E(spl)mδ in muscle cells affects SNa nerve branching

A, B) Embryos 2-4 hours after egg laying (AEL) were treated for 16 hours in medium containing 50μM MeHg or DMSO solvent control. Embryos were then fixed and stained with anti-FasII. C) Embryos expressing Mef2GAL>UAS-E(spl)mδ (Mef2>mδ) were aged to stage 16 and stained with anti-FasII. Immunostaining reveals the normal branching of the SNa nerve in control embryos (A) and failed branching of SNa in MeHg treated embryos (B) and embryos expressing Mef2>mδ (C). D) SNa branching phenotype quantified for the indicated treatments are shown (n= # of hemisegments scored).

Figure 5. MeHg treatment and expression of E(spl)mδ in muscle cells affects LT1-4 muscle development

Embryos 2-4 hours after egg laying were treated 16 hours in medium containing 50μM MeHg or DMSO solvent control. Embryos were then fixed and stained with anti-myosin heavy chain. A C) Immunostaining for myosin heavy chain reveals a normal pattern of LT1-4 muscle bundle in control embryos (A) and disordered morphology of the bundle in MeHg treated embryos (B) and in embryos expressing Mef2>mδ (C). D) The LT1-4 bundle phenotype quantified for the indicated treatments are shown (n= # of hemisegments scored).

Figure 6. Co-occurrence of nerve and muscle phenotypes with MeHg treatment

Embryos 2-4 hours after egg laying were treated 16 hours in medium containing 50 μ M MeHg. Embryos were fixed and stained for both FasII (white) and myosin heavy chain (Purple). Occurrence of SNa branching (arrows) and/or LT1-4 bundle (bracket) phenotypes is indicated below the respective representative image.

Figure 7. Expression and activity of *E(spl)mγ*

Expression of *E(spl)mγ* in the nervous system is revealed by the expression pattern seen in an *E(spl)mγ-lacZ* embryo immunostained with anti-βgal (A,C red) and the pan-neural anti-Elav (B,C green) antibody. Localization of *E(spl)mγ* to neurons in both the ventral nerve cord (VNC) and peripheral nervous system (PNS) is seen. D) SNa branching phenotype quantified for the indicated crosses are shown (n= # of hemisegments scored).