

**Characterization of Atonal Class bHLH Transcription Factor Homologs in
the Cnidarian Organism *Nematostella vectensis***

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Specific Aims

The aim of this study is to research the molecular mechanisms involved in neural patterning of the *Nematostella Vectensis* (*N.vectensis*) nervous system. Specifically this will be examined by testing the hypothesis that the *N. Vectensis* bHLH domain containing homologs of the *Drosophila* atonal neural transcription factors are involved in neural patterning during embryonic development. Evaluating the *N.vectensis* atonal class transcription factor homologs in neural patterning of the early embryo will assist in determining how these putative neurogenic factors may have functioned in the neuromorphic gene programs of primordial metazoans during the initial emergence of the nervous system. Since the atonal class transcription factors have been exemplified to be involved in neurogenesis in *Drosophila* comparing their function in *N.vectensis* could help to resolve the ancestral state of neural patterning before the divergence of the Bilateria and Cnidaria.

Background and Significance

What are the molecular mechanisms that induce neuronal development and shape the architectural patterning of the nervous system? In this study we endeavor to further the delineation of the molecular evolution of transcription factors involved in neural patterning. Elucidating the evolution of genetic systems involved in neural patterning is commensurate with engendering a more sated understanding of how those genetic programs, in all their variability, operate to form the nervous systems of many organisms, including humans. Applications of such an understanding can have far reaching implications in various areas, such as therapies for spinal cord regeneration, or indeed reparation of possibly any component of the nervous system.

Many studies of this nature have primarily been executed in the comparison of organisms classified within the bilaterian phyla, such as between the model organism *Drosophila* and vertebrate taxa (Ben-Ari, 1996). While this is certainly valuable and insightful, the perspectives that can be gleaned from comparative studies with an “outgroup” of the bilaterian phyla are necessary for a more comprehensive understanding of the molecular mechanisms of neurogenesis. *Nematostella Vectensis* is classified within the Cnidarian phylum and is therefore a sister clade of the Bilateria (Marlow, 2008). Moreover, *Nematostella Vectensis* is a suitable model organism because it has a fully sequenced and annotated genome, it is readily induced to spawn, and it has rapid embryogenesis. So it is highly amendable for studies of neural patterning and neurogenesis during embryonic development.

Extensive molecular and genetic analysis have been performed on the basic helix-loop-helix (bHLH) protein superfamily that includes many classes of transcription factors, including the atonal class that has been demonstrated to be involved in neurogenesis in *Drosophila* (Simionato, 2007). In order to assess whether or not the atonal homologs are involved in neural patterning of the nervous system in *N.vectensis* it must be determined if they are expressed and if their expression is localized to areas of neural development during embryogenesis. While the *N.vectensis* nervous system is composed of two diffuse neural networks, during development of the neural architecture there are areas of regional localization of specific neural populations (Watanabe, 2009). The achaete-scute proneural homologs have been demonstrated to be expressed in this spatiotemporal pattern (Figure 1, Layden 2010). And our preliminary data shows that the atonal homologs have a similar expression pattern of regionalization throughout the nerve plexus and with discrete expression at the oral pole of the aboral-oral axis (Figure 2). Moreover, it must be demonstrated that these putative neurogenic transcription factors are necessary and sufficient to induce neuronal development.



Figure 1. NvashA expression, showing an expression pattern of regionalization (Layden, 2010)

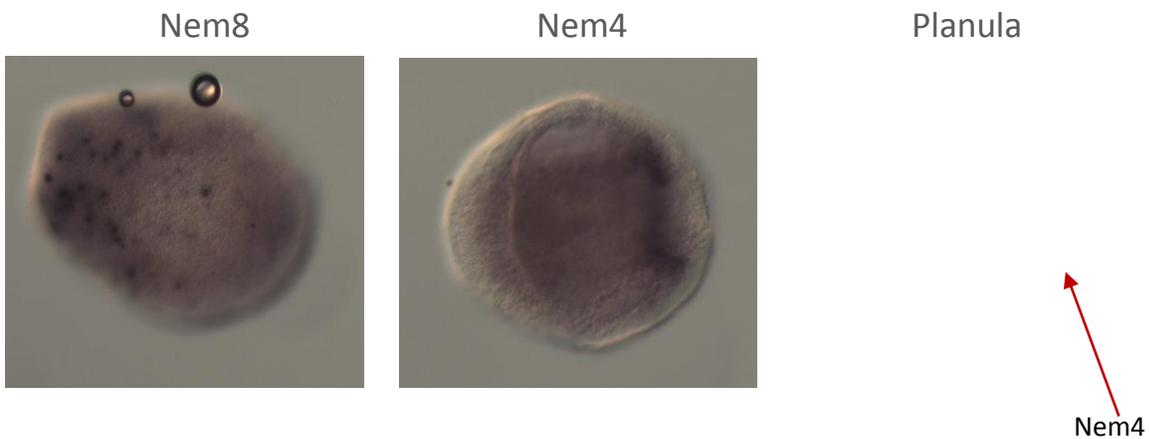


Figure 2. Preliminary data showing the regionally localized expression pattern of the atonal class putative neurogenic bHLH transcription factors Nem8 and Nem4. In later stages of embryonic development expression of nem4 appears around the oral opening.

Research Design and Methods

Aim 1; Demonstrating Expression:

There are 4 homologous proneural genes that will be assessed for functional involvement in neural patterning: Nem4, Nem8, Nem11, and Nem15. To address the first aim of identifying whether or not they are expressed, and if so what their spatiotemporal expression characteristics are, *in situ* mRNA

hybridization assays will be performed. In this technique labeled RNA probes are synthesized from the DNA sequence of the gene of interests, in this case one of the potential proneural genes, from which the template DNA is prepared by PCR amplification of either nuclear or cDNA, and is cloned into plasmid vectors. The Probes are synthesized by PCR amplification of the cloned gene template and are introduced into tissues where they can bind to any complementary mRNA sequences. The probes contain modified uridine bases that are bound with the antigen digoxigenin. If there is a complementary mRNA transcript the probes will hybridize, at which time a phosphatase conjugated digoxigenin antibody is introduced that will bind to the labeled probes. The antibody is stained with alkaline phosphatase, allowing for the visualization of areas where the gene of interest is being transcribed.

If no hybridization is detected, an alternative approach to assessing expression of the putative proneural genes would be to perform RNA extracts of *Nematostella* tissues. The RNA could be isolated using a proprietary RNA extraction kit, such as the Qiagen RNeasy Kit. Extracted RNA will be reverse transcribed using reverse transcriptase PCR (rtPCR). Utilizing primers specific for the atonal class proneural gene homologs PCR amplification will be performed on the cDNA from the previous step. Analysis will be performed to detect any amplicons produced from PCR amplification of the cDNA. Positive results of expression will be demonstrated by detection of PCR product with the appropriate polymer length.

Aim 2; Demonstration of Sufficiency:

Once it is determined that the atonal homologs are being expressed in the appropriate spatiotemporal pattern for a neural regulator, it must be assessed whether or not the putative proneural peptides are necessary and sufficient for regulation of neurogenesis. This will be evaluated by gain of function and loss of function assays. Gain of function assays are performed by mRNA micro-injections, in which functional mRNA transcripts are synthesized and injected into the tissues of viable embryos to see if the mis-expression and ectopic expression of the peptide results in a proliferation of a specific cell-type or tissue, in this case increased neuronal tissue. If there is a proliferation of a specific cell type or cellular function, it supports the supposition that the molecule is sufficient to induce that cell type or function.

If sufficiency is not demonstrated an alternative approach to assessing whether or not the atonal homologs are sufficient for neural induction will be to transfect *Nematostella* with a plasmid vector containing the functional proneural gene. The approach to be utilized will be very similar to that performed by Miljkovic *et al*, in which injection of exogenous DNA is coupled to electroporation (Miljkovic, 2002). Perturbations in normal development, such as aberrant tissue morphology and functionality, will be indicative that the transcription factor(s) are sufficient to direct neurogenesis.

Aim 3; Demonstration of Necessity:

Loss of function assays will be performed using morpholino knockout technology. Morpholino's are short polymers consisting of nucleic acid analogs. Morpholino antisense oligomers are introduced into tissues of viable embryos where they bind to any respective complementary RNA sequence and block access of that transcript by translational machinery. If a cell type is lost or functioning aberrantly, then it is indicative that the molecule is necessary for the proper development and/or function of that cell type or tissue.

If necessity is not demonstrated by morpholino injection then the alternative approach of interfering RNA (RNAi) technology can be utilized. The utilization of RNAi technology for mRNA expression is well documented in many model systems such as *C.elegans*. Under the same conceptual framework RNAi could be introduced into *Nematostella* to selectively mute translation of the atonal homologs. Atypical development or loss of functionality of nervous system components will be indicative that the atonal homologs have a necessary role in neural differentiation.

Overall conclusions and Future directions

If it is demonstrated that the atonal homologs are localized to areas of nerve cell development and have a spatiotemporal expression pattern appropriate for a neural regulator, and are demonstrated to be necessary and sufficient for neuronal development, it will support the hypothesis that atonal class bHLH transcription factor homologs regulate neural development in *Nematostella*, and are therefore proneural genes. The next logical question to propound would be - in what manner do they regulate neural development? Defining their specific functionality within the genetic system of neural differentiation and network architecture will progress our understanding of the molecular mechanisms involved in neurogenesis. This in turn will open deeper avenues of inquiry and possibly enable pragmatic applications in neuronal regeneration therapies.

As a future direction it would also be pertinent to specifically investigate the atonal homologs Nem9 and Nem10. The interrogation of these homologs could be very insightful since phylogenetic analysis have shown them to be the most basal of the putative neurogenes, and as such will be the most facilitative in comparing the function of all the neurogenes. The homologs will be subjected to the same experimental analysis as the other homologs expounded in this report.

Citations

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