THE ROLE OF MASH1-E PROTEIN HETERODIMERS IN MASH1 FUNCTION IN THE DEVELOPING NEURAL TUBE

APPROVED BY SUPERVISORY COMMITTEE

Qing Richard Lu, Ph.D. _____

Jane E. Johnson, Ph.D. _____

Leon Avery, Ph.D. _____

DEDICATION

I would like to dedicate this thesis to my boyfriend, David Hawkins, for all his devotion, love and support. I would like to thank my Mom, Ellen W. Collisson, for being such a wonderful role model and friend. Finally, I would like to thank my mentor, Jane, for everything that she has done for me. She is just a great person.

THE ROLE OF MASH1- E PROTEIN HETERODIMERS IN MASH1 FUNCTION IN THE DEVELOPING NEURAL TUBE

by

Tandi Louise Collisson

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Tandi Louise Collisson

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Supervising Professor: Jane E. Johnson, Ph.D.

Neural-specific Class II bHLH transcription factors heterodimerize with ubiquitous Class I bHLH E proteins to form complexes required for neural differentiation. There are four known E proteins, HEB, E12, E47 and E2.2, in the mammalian nervous system, which potentially form heterodimers with Mash1 in the neural tube. To test the relevance of particular Mash1-E protein heterodimer combinations *in vivo*, I constructed tethered Mash1-E protein heterodimers for over-expression in the chick neural tube. By comparing over-expression of Mash1 with over-expression of these Mash1-E protein heterodimers, their abilities to effect neural differentiation and cell-type specification were analyzed. Mash1-E protein heterodimers are interchangeable in the function of driving neurogenesis in the chick neural tube. The effects of Mash1-E protein heterodimers on cell-type specificity were different, suggesting non-redundant functions in effecting dorsal interneuron populations. Furthermore, additional Mash1 heterodimer partners may be required for the cell-type specification function of Mash1.

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Prior Publications

Gowan K, Helms AW, Hunsaker TL, Collisson T, Ebert PJ, Odom R, Johnson JE. Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron. 2001 Aug 2;31(2):219-32.

Xu B, Wilsbacher JL, Collisson T, Cobb MH. The N-terminal ERK-binding site of MEK1 is required for efficient feedback phosphorylation by ERK2 in vitro and ERK activation in vivo. J Biol Chem. 1999 Nov 26;274(48):34029-35.

Nair HP, Collisson T, Gonzalez-Lima F. Postnatal development of cytochrome oxidase activity in fiber tracts of the rat brain. Brain Res Dev Brain Res. 1999 Dec 10;118(1-2):197-203.

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Introduction

Development of the neural tube

During embryonic development three main germ layers are present prior to neurogenesis. They consist of the endoderm, the mesoderm and the overlying ectoderm. The mesoderm produces inductive signals that instruct the ectoderm to become neural plate. The neural plate will ultimately give rise to the entire central and peripheral nervous systems (Figure 1A). The neural plate folds during neurulation and the lateral edges come together to form the neural tube (Figure 1B,C). From the neural tube, neurons and glia of the central nervous system are derived. Along either side of the ventricle of the neural tube, neuroepithelial cells in the ventricular zone divide, exit the cell cycle, migrate laterally and differentiate into neurons and glia. In addition, neural crest cells migrate out of the dorsal neural tube to form parts of the peripheral nervous system (Kandel et al., 2000). Signaling molecules such as Sonic Hedgehog (Shh) and Bone Morphogenic Proteins (BMPs) coordinate the patterning of the neural tube along the dorso-ventral axis . They are secreted from the notochord/floor plate and roof plate, respectively, forming extracellular concentration gradients (Figure 1A-D). This patterning directs the expression of transcription factors such as Basic-Helix-Loop-Helix and Homeodomain proteins, in specific domains along the dorso-ventral axis of the neural tube (Figure 2). Through combinatorial codes this patterned expression results in the precise generation of distinct populations of neurons along the lateral edge of the developing spinal cord, identifiable by expression of

Figure 1: Developmental Stages of Neural Tube Formation

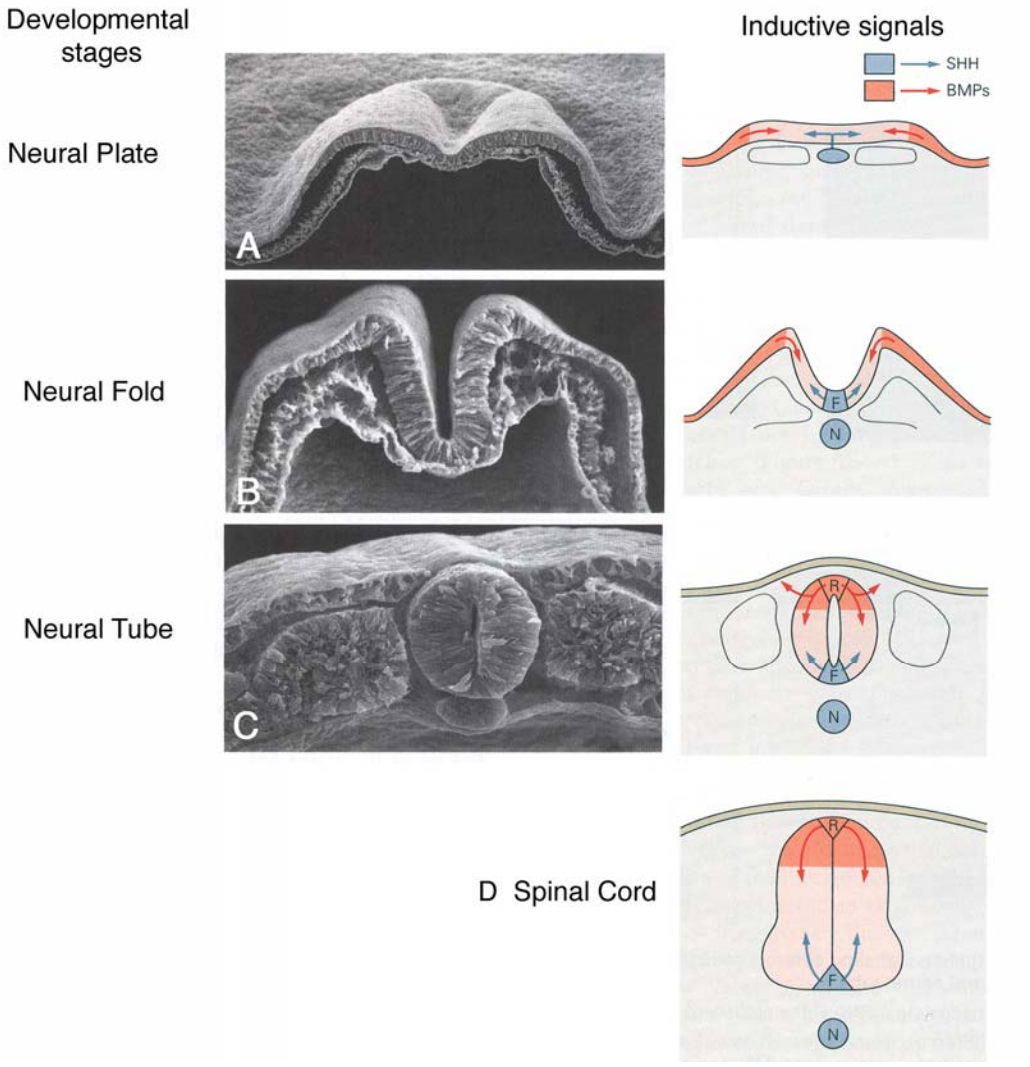


Figure 1: Developmental Stages of Neural Tube Formation

A. Stages in the embryonic development of the spinal cord: The neural plate is formed as a columnar epithelium and is underlaid by axial mesoderm cells of the notochord, and paraxial mesoderm and is flanked by epidermal ectoderm. **B.** During neurulation, the neural plate buckles at its midline to form the neural folds and a floor plate forms at its midline. **C.** The neural tube forms by fusion of the dorsal tips of the neural folds, forming a roof plate at its dorsal midline. **D.** Neuroepithelial cells proliferate and differentiate into neurons located at different dorso-ventral positions. Diagram shows the source of the ventralizing (Sonic Hedgehog, Shh-blue), (F) the floor plate and (N) notochord, and dorsalizing (Bone Morphogenic Protein, BMP-orange), (R) the roof plate, inductive signals at sequential stages of spinal cord development. Adapted from (Kandel et al., 2000)

postmitotic markers such as the Lim homeodomain proteins (Briscoe et al., 2000; Kandel et al., 2000). In addition, transcription factors expressed in the ventricular zone influence the generation of these distinct populations through cross-inhibitory interactions that further define their restricted expression domains in progenitor cells (Briscoe et al., 2000; Gowan et al., 2001; Helms and Johnson, 2003). These highly orchestrated events in the developing neural tube lead to the diverse assortment of cell types and complex organization of the adult spinal cord (Kandel et al., 2000).

HLH family of Transcription Factors

One family of transcription factors, whose restricted expression domains reflect the dorso-ventral patterning of the neural tube early in development, is the Helix-Loop-Helix family of proteins (Helms and Johnson, 2003). Characterized by the structural motif, a helix-loop-helix (HLH) domain, this family of transcriptional regulatory proteins plays a key role in several developmental processes. They are involved in lineage commitment and differentiation during the processes of neurogenesis, hematopoiesis, myogenesis and pancreatic development (Massari and Murre, 2000).

The HLH family can be subdivided into several classes described by their various patterns of expression, dimerization properties, and DNA-binding specificity. Class I HLH proteins, or the E proteins, are broadly expressed and function as either homodimers or heterodimers. This class includes E12, E47, E2-2, and HEB in vertebrates, and Daughterless in *Drosophila*. Class II HLHs are tissue-specific HLH proteins that preferentially heterodimerize with the Class I E proteins (Massari and Murre, 2000). The four main

Figure 2: Expression domains of Homeodomain Proteins and bHLH transcription factors along the dorso-ventral axis of the developing neural tube

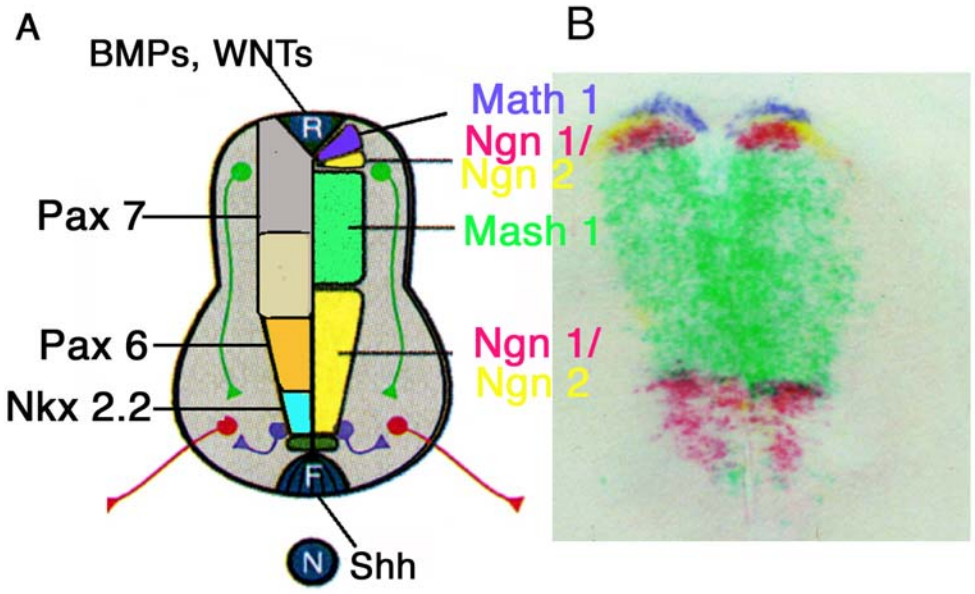


Figure 2: Expression patterns of Homeodomain proteins and bHLH transcription factors along the dorso-ventral axis of the developing neural tube. A. During development of the spinal cord progenitor cells are patterned along the dorsoventral axis. Homeodomain proteins (left side) and bHLH transcription factors (right side) are expressed in the ventricular zone and set up restricted patterns of expression. Math1 (blue), Ngn1 (yellow), Ngn2 (yellow), and Mash1 (green). Adapted from (Tanabe and Jessell, 1996) **B.** Overlapping In Situ Hybridization on serial sections of e11.5 WT embryo. Color was added and overlaid in Photoshop to demonstrate the distinct domains of expression of the bHLH transcription factors Math1(blue), Ngn2 (yellow), Ngn1 (red), and Mash 1 (green).

Class II HLHs that will be discussed here are all expressed early in the developing neural tube around E9.5 to E12.5 in mouse. They include Mash1, Math1, Ngn1 and Ngn2. Both Class I and Class II HLH proteins contain a basic region N-terminal to the HLH motif. This motif is responsible for the ability of these bHLH proteins to bind DNA at the consensus sequence CANNTG, termed the E-box. The basic region of these proteins makes direct contact with the DNA, while the HLH region is the region believed to be involved in protein-protein interactions, such as dimerization with the E proteins (Chavali et al., 2001; Massari and Murre, 2000; Shirakata et al., 1993; Voronova and Baltimore, 1990). Class V HLH proteins, for example Id, lack this basic region of the bHLH motif and are negative regulators of the Class I and Class II bHLH proteins (Massari and Murre, 2000).

Class I HLH proteins; E proteins

The Class I HLH *Drosophila* protein, Daughterless (Da), was first established as essential to sex determination and later, an important role in differentiation of the nervous system was identified (Campos-Ortega, 1998; Massari and Murre, 2000). Daughterless interacts with the Class II bHLH Achaete-scute proneural gene products to form a transcriptional complex that activates downstream genes involved in neuronal differentiation (Jan and Jan, 1990; Modolell, 1997; Ramain et al., 2000).

Mammalian Class I HLH proteins, the E proteins, include E47 and E12, splice variants of the E2A gene, HEB and E2.2. E proteins form homodimers that bind the typical E-box sequence, CANNTG, and these Class I HLH proteins are important in hematopoiesis (Ephrussi et al., 1985; Massari and Murre, 2000). Ubiquitously expressed, the E proteins also

have the ability to form heterodimer complexes with the tissue-specific Class II HLH proteins which are essential to several developmental processes including neurogenesis (Massari and Murre, 2000; Murre et al., 1989). The E proteins have been shown to be expressed in the developing central nervous system (Chiaramello et al., 1995; Neuman T et al., 1993; Soosaar et al., 1994; Suda K et al., 1994; Uittenbogaard M and A., 1999).

These mammalian E proteins have been studied predominantly in the process of hematopoiesis. The E protein knockouts have phenotypes that effect hematopoietic development, predominantly in B cells. The E proteins are all crucial to proper differentiation and specification of B cells leading to their appropriate numbers during hematopoiesis. No B cells are produced in the E2A knockout while both the HEB and E2.2 knockouts have reduced numbers of pro-B-cells. No tissue-specific HLH protein has been identified in B cells, and E47 appears to be the predominant DNA-binding species in B-cell development (Massari and Murre, 2000; Zhuang et al., 1996). E47 homodimers are detected in B-cells and have been shown to bind DNA in vitro, while E12 homodimers do not bind DNA under the same conditions during hematopoiesis (Sun and Baltimore, 1991; Zhuang et al., 1996). Any trans-heterozygous combinations of these knockouts produce postnatal lethalities while each combination generated a more dramatic embryonic phenotype than the single mutants, demonstrated by a reduction in pro-B-cells (Zhuang et al., 1996). Each of the individual E protein knockouts shows no obvious phenotype in the nervous system, suggesting that their functions may be redundant. In addition, the replacement of the E2A gene with HEB fully rescues the E2A knockout, suggesting that they are redundant in

function and there is a dosage requirement for the E proteins during hematopoiesis (Zhuang et al., 1998; Zhuang et al., 1996)

Class II HLH proteins; Sub-class: Neural-specific bHLH transcription factors

Class II HLH transcription factors are made up of HLH proteins that have tissue-restricted patterns of expression. These proteins preferentially heterodimerize with the E proteins of Class I HLH proteins. The heterodimers that form have both typical and atypical E-box specificity. The Class II HLH proteins are essential for differentiation of progenitor cells and lineage commitment (Massari and Murre, 2000; Murre et al., 1989). In *Drosophila*, the Class II bHLH transcription factors *atonal* and *Achaete-scute*, or the proneural genes, form heterodimers with *daughterless* and each of these complexes is required for formation of chordotonal organ/photoreceptor formation and external sensory organs in the nervous system of *Drosophila* , respectively (Chien et al., 1996; Jan and Jan, 1990; Jarman et al., 1993; Jarman et al., 1994). *Achaete-scute* regulates cell number through lateral inhibition mediated by Delta-Notch signaling (Jan and Jan, 1990). The *Achaete-scute* / *Da* complex has been shown to bind to additional co-factors, forming large complexes that act as facilitator factors mediating interactions between enhancer-promoter regions. One such co-factor is the Lim-domain binding protein, Chip, identified in *Drosophila* (Modolell, 1997; Ramain et al., 2000).

In the mammalian nervous system, homologues of the proneural genes have been identified and are essential to neurogenesis. Four of these bHLH transcription factors, whose functions we study in the context of spinal cord development, are Math1 (Mammalian atonal

homologue), Mash1 (Mammalian *achaete-scute* homologue), Ngn1 (Neurogenin1), and Ngn2 (Neurogenin 2). These four bHLH proteins are expressed in distinct and largely non-overlapping domains in progenitor cells of the neural tube (Akazawa et al., 1995; Gradwohl et al., 1996; Lo et al., 1991; Ma et al., 1996; Ma et al., 1997; Sommer et al., 1996) (Figure 2). Similar to their *Drosophila* counterparts, they play essential roles in the generation of specific cell types. From studying the Mash1 knockout we know that Mash1 is essential to the generation of olfactory neurons, adrenal chromaffin cells, and neurons in the autonomic nervous system (Guillemot et al., 1993). In addition, through over-expression assays, Mash1 has been shown to induce neuronal differentiation. Mash1 over-expression in chick neural tubes leads to cell cycle exit, lateral migration of cells out of the ventricular zone toward the lateral edge of the neural tube, and neuronal differentiation, three aspects of neural differentiation (Figure 3D-F) (Nakada et al., 2003).

Mash1 forms heterodimers with the E proteins, *in vitro*, and subsequently binds the E-box DNA consensus sequence (CANNTG) (Ephrussi et al., 1985). In contrast to E protein homodimers, these heterodimers are not restricted to typical E-box specificity alone (Massari and Murre, 2000; Murre et al., 1989). It is unknown whether the ubiquitously expressed E proteins are all *in vivo* partners to Mash1 or if there is a preferential partner. Finally, it is also unknown whether or not particular E protein partners confer specific activity on the bHLH complex.

To further investigate how neural-specific bHLH transcription factors function, I examined the role of E protein dimerization partners in the activity of Mash1. Similar to hematopoiesis, it is possible that the predicted E protein partners have redundant functions in

Figure 3: Over-expression of Mash1 drives Neurogenesis

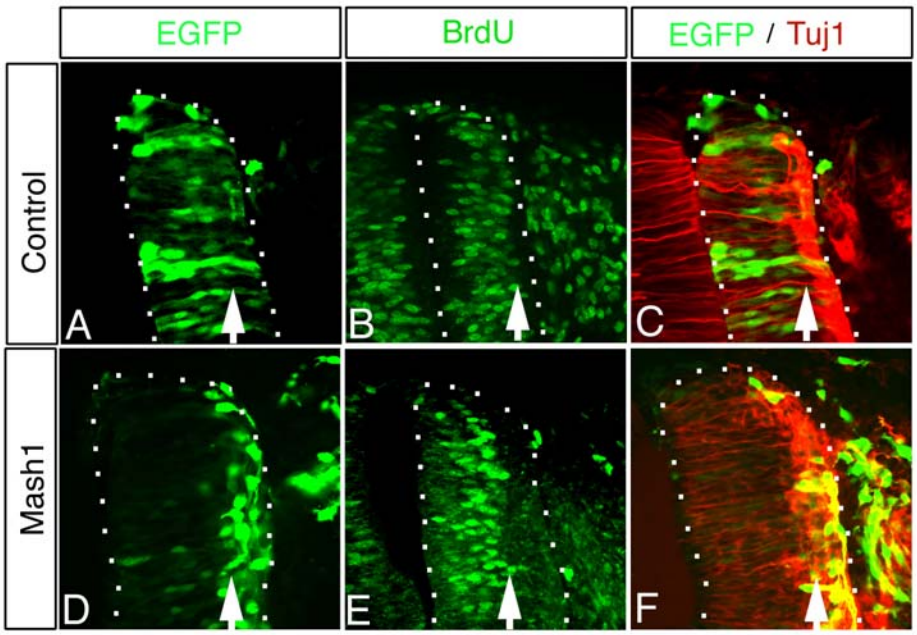


Figure 3: Over-expression of Mash1 drives Neurogenesis. Confocal images of transverse sections from HH24-26 chick neural tubes electroporated at stage HH14-16 with (**A-C)** a control vector (myc-tag only) or Mash1 (**D-E**). Sections were immunostained with anti-BrdU (green) (**B** and **E**), and anti-neuronal B-tubulin (Tuj-1) (red) (**C** and **F**). Panels **A** and **D** show the expression patterns of the co-injected control vector CMV-EGFP (green). Panels **C** and **F** represent an overlay of EGFP (**A** and **D**) with Tuj-1 staining. EGFP labeled cells that were co-electroporated with control plasmid span the neural tube resulting in co-localization with BrdU (**B**) and/or Tuj-1 (**C**) at random. In contrast, Mash1/EGFP electroporated cells move laterally out of the ventricular zone where they co-localize with Tuj-1 positive cells (**D**,**F**) and do not incorporate BrdU (**E**), showing they are post-mitotic. Arrows indicate the lateral regions of the neural tube (mantle layer). Dotted lines outline the neural tube and ventricle. (Nakada et al., 2003)

neurogenesis. In order to address this possibility, the activity of tethered Mash1-E protein heterodimers was compared to the activity of un-tethered Mash1 using the chick electroporation assay in a gain-of-function experiment. Specifically, the effects of putative Mash1-E protein heterodimers on neuronal differentiation and cell-type specification were examined. I evaluated Mash1 and tethered Mash1-E protein heterodimer activities using two phenotypic criteria: the ability of Mash1 to force cells to migrate laterally as it drives the cells out of the cell cycle, and the ability of Mash1 to affect the number and location of specific dorsal interneuron populations of cells identified by the expression of a set of postmitotic markers.

Specifically, I addressed two questions: 1) Does the activity of any of these tethered heterodimers fully recapitulate all aspects of Mash1 activity? 2) Are these E proteins similar enough in structure and function to give the same activity when forced to dimerize with Mash1?

MATERIALS AND METHODS

In Ovo Electroporation Studies

Fertilized White Leghorn eggs were obtained from the Texas A&M Poultry Department (College Station, TX) and incubated at 37°C for 3 days. Solutions of supercoiled plasmid DNA (2 ug/ul) in PBS/0.02% Trypan Blue were injected into the lumen of the closed neural tube and electroporated into epithelial cells on one side of the neural tube by placing gold electrodes on either side of the embryo (Timmer et al., 2001). Square-wave current (five 50-ms pulses of 25 mV) was generated using a BTX (San Diego, CA) T820 electroporator connected to 2-mm gold electrodes. A GFP expression vector (CMV-EGFP) was co-injected as a control to monitor efficiency and extent of electroporation, as well as assay the "lateral phenotype". Injection and electroporation were performed at Hamburger and Hamilton stages 14-15 (Hamburger and Hamilton, 1951), 26-28 hours later (HH 24-26), embryos were harvested, fixed in 4% paraformaldehyde for 1 hour at 4°C, rinsed in PBS at 4°C three times, for thirty minutes each, sunk in 30% sucrose at 4°C overnight, and embedded in OCT (Tissue Tek) for cryosectioning. Embryos were sectioned and processed for immunofluorescence as described below. For each experiment, multiple sections from at least three electroporated embryos were analyzed.

Immunohistochemistry

1. Preparation of tissues for immunohistochemistry

Staged chick embryos collected for thin section analysis were dissected in PBS at 4°C, fixed in 4% paraformaldehyde-PBS (pH 7.2) for 1 hour at 4°C, washed in PBS 3X for 30 minutes each at 4°C, sunk in 30% sucrose in PBS overnight at 4°C, embedded in OCT compound (Tissue Tek), and cryosectioned at 30 μm. All sections shown were taken between the upper and lower limb regions unless otherwise noted.

2. Conditions for single and double-label immunohistochemistry

Single label immunohistochemistry was performed by incubation with the appropriate dilution of primary antibody (either monoclonal or polyclonal antibody) in PBS/1% goat serum/0.1% Triton X-100, followed by hybridization of either a goat-anti-rabbit or goat-anti-mouse IgG, conjugated to Alexa Fluor 647 (Molecular Probes, Inc.) or Alexa Fluor 594 (Molecular Probes, Inc.). Double label immunofluorescence was performed by simultaneous incubation with antibodies of interest. Primary antibodies used for this study include: mouse anti-Islet1/2 (1:100) (DSHB), rabbit anti-Mash1 (1:500), mouse anti-Mash1 (1:100)(Lo et al., 1991), rabbit anti-LH2A/B (1:8000), mouse anti-BrdU (Becton-Dickinson, Inc) and mouse anti-c-Myc (1:500) (9E10 Santa Cruz Biotechnology), rabbit anti-HEB (1:500) (Santa Cruz Biotechnology), rabbit anti-E2A.E12 (1:500) (Santa Cruz Biotechnology), rabbit anti-Lbx (1:100), mouse anti-Lmx (1:100) (50.5A5 DSHB), rabbit anti-Pax2 (1:100) (Zymed). Mouse monoclonal anti-Tuj-1 was a gift from A. Frankfurter.

3. Fluorescence Microscopy

Immunofluorescence studies were carried out on a Leica DMR microscope equipped for fluorescence. Confocal analysis was carried out on a Bio-Rad MR-1024 confocal microscope. EGFP signal was imaged using the standard FITC filter.

4. Cell Counts

All counts for dorsal interneuron populations were done using confocal images of immunoflourescent labeled transverse sections of experimental chick neural tubes. Each dorsal interneuron population counts included at least three experimental embryos. These population counts were entered into Excel, where graphs were made and the t-tests were done using "Two Sample assuming Unequal Variances". All values that were significant by a p value < .05 are indicated in graphs with *.

Plasmid Description

All gain-of-function studies performed utilize the expression vector pMiwIII, which drives expression through a chick β-actin promoter (Muramatsu et al., 1997). The coding region of rat *Mash1* was cloned into the pMiwIII expression vector using convenient restriction sites. With the exception of rat *Mash1*, all genes inserted into the pMiwIII vector were myc-tagged (5 copies) at the N-terminus. For the tethered construct, full-length Mash1 was first cloned into the pMiwIII-(myc)5 vector with the first half of the tether on the 3' end using PCR, and the restriction sites Nco1 and Xba1. Each E protein was generated by PCR and cut with the appropriate restriction enzymes, XbaI and SpeI for Mash1-E12 and Mash1-E47, and SpeI for Mash1-HEB and Mash1-E2.2. They were then purified and ligated to the linearized pMiwIII-(myc)5 vector containing Mash1 full-length. Each E protein was then subsequently cloned into the Mash1 tether vector with the second half of the tether on the 5' end to give Mash1 with a full-length tether between the E protein of choice. This generated four tethered constructs: Mash1-E12, Mash1-E47, Mash1 -HEB, and Mash1-E2-2. Tethers for the Mash1 -E12 and Mash1 -E47 were made up of the amino acid sequence :

GTSAGPSAAPTSRTPAGSTPRT (Neuhold 2001). Tethers for the Mash1-tether-HEB, and Mash1-tether-E2-2 contained the amino acid sequence GTSAGPSAAPTSSTPAGSTPRT (Neuhold 2001). All PCR-generated constructs were sequenced to make sure no mutations were generated by the PCR reaction.

The E protein coding sequences were obtained from various sources. E12 was from the pCITE-E12 plasmid; HEB was from pBluescript-HEB from Dr. Ray McDonald; E47 was a gift from C. Murre; and E2.2 was a gift from T. Kadesch.

Electrophoretic Mobility Shift Assay (EMSA) Description

1. *In vitro* Transcription/Translations

Mash1-teth-E12 and Mash1 bHLH-teth-E12 bHLH proteins were *in vitro* transcribed and translated from PCR templates using Promega TNT T7 Quick for PCR DNA (Promega, Madison, WI). Mash1 full-length protein was *in vitro* transcribed and translated from plasmid (pCS2-Mash1) using Sp6 TNT Quick Coupled Transcription/Translation Systems (Promega, Madison, WI). E12 full-length protein was *in vitro* transcribed and translated from plasmid (pCITE2-E12) using T7 TNT Quick Coupled Transcription/Translation Systems (Promega, Madison, WI). These protein products were detected and quantitated by incorporating S35-Methionine (Amersham), and visualized using phosphorimager analysis.

2. Annealing and Labeling probe

The WT enhancer B oligo contained a typical E-box site and originates from the Math1 enhancer sequence. The sequence of this oligo was 5'-CACGCGCTGTCAGCTGGTGAGCGCAC-3'. The oligo was generated by annealing equal amounts of complementary DNA oligos. 50ng of the annealed oligo was labeled using 10U

polynucleatide kinase, 10x PNK Buffer (Roche), 5µl γ32P-ATP (5000Ci/ mmol) (Amersham) in a 20 µl reaction. Labeling was done at 37°C for 45 minutes. Probe was then purified using a Biospin 6 (BioRad) spin column and radioactivity was quantitated using the scintillation counter.

3. Binding reactions and analysis

Prior to setting up the binding reactions, lysates were pre-incubated for 30 minutes at 37°C for the reactions Sp6+T7, Mash1+T7, E12+Sp6, and Mash1+E12. Binding reactions contained 4µl 5X bHLH binding buffer (100mM HEPES pH 7.6, 250mM KCl, 5mM DTT, 5mM EDTA, 25% glycerol) (Ebert et al., 2003), 500ng of Poly-dI-dC, 2.5ng labeled probe (or approx. 100,000 cts/µl), 2µl of extract and/or protein extract, and ddH2O to a final volume of 20 µl. Each reaction was incubated at 4°C for 30 minutes. Fifteen µl of each reaction was run on a 4% acrylamide-bisacrylamide/.5X TBE gel (+ 40mgs APS, and 30µl TEMED) in .5X TBE for approx. 45 minutes at 18mA. The gel was fixed in 20% MeOH/10% acetic acid for 2 minutes, dried on Whatman paper for 1 hour and exposed to phosphorimager screen overnight.

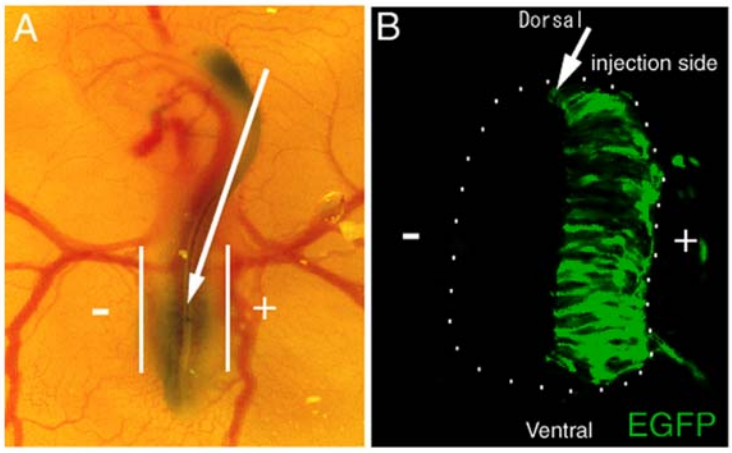
RESULTS

Mash1-E protein heterodimers induce neuronal differentiation in the chick neural tube.

In order to force Mash1 to heterodimerize with a specific E protein partner, I designed expression constructs that encoded Mash1 tethered with 22 amino acids to each of the four known E proteins, E12, E47, HEB, and E2-2. This strategy was adapted from Neuhold *et al*. where the E protein, E47 was tethered to the myogenic bHLH MyoD, and it was shown that the activity of the resulting protein was resistant to inhibition by Id, an HLH inhibitor (Neuhold and Wold, 1993). Each Mash1-E protein tether was cloned into a chick expression vector, pMIWIIImyc5. Using *in ovo* chick electroporation, these constructs were over-expressed in the neural tube, and the activities of these putative forced heterodimers were compared with the activity of un-tethered Mash1. The Mash1-E protein heterodimer constructs were electroporated into the chick neural tube at stage HH14-16 and embryos were harvested at HH24- 26 (26-28 hours later) (Figure 4 A-B). Mash1 (positive control) and Mash1 AQ (negative control) were tested in parallel with the Mash1-E protein tethers. Protein expression of each tethered construct was verified by using Mash1, E2A, HEB and/or myc antibodies (data not shown).

Over-expression of the DNA-binding mutant, Mash1 AQ, along with EGFP, exhibits an indiscriminate distribution of electroporated cells throughout the neural tube in both the ventricular zone and lateral regions (Figure 5 A,B). These GFP-expressing cells do not preferentially co-localize with the early pan-neural marker β-III-tubulin, (Tuj-1) (Figure 5B).

Figure 4: *In Ovo* Electroporation of DNA into the Developing Chick Neural Tube



Inject stage HH14-16 Harvest stage HH24-26

Figure 4. *In Ovo* Electroporation of DNA into the Developing Chick Neural Tube. Fertilized White Leghorn eggs were obtained from the Texas A&M Poultry Department (College Station, TX) and incubated at 39°C for 3 days. **A.** Solutions of supercoiled plasmid DNA (2 ug/ul) in PBS/0.02% Trypan Blue were microinjected into the lumen of the closed neural tube and electroporated into epithelial cells on one side of the neural tube by placing electrodes on either side of the embryo (Timmer et al., 2001). **B.** A GFP expression vector (CMV-EGFP) was co-injected as a control to monitor efficiency and extent of electroporation. Injection and electroporation were performed at Hamburger and Hamilton stages 14-16 (Hamburger and Hamilton, 1951). Control on left side of transverse sections and experimental side on right side of transverse sections.

Figure 5: Over-expression of Mash1-E protein heterodimers induces neuronal differeniation in the chick neural tube.

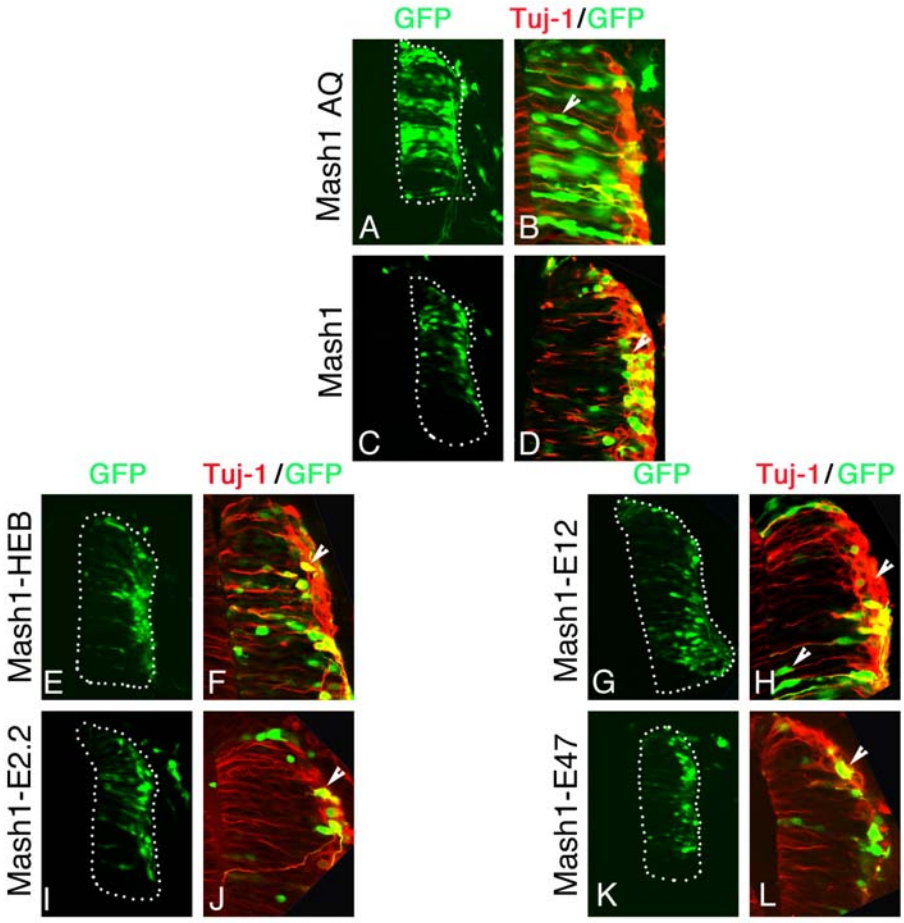


Figure 5: Over-expression of Mash1-E protein heterodimers induces neuronal differentiation in the chick neural tube.

Confocal images of transverse sections of chick neural tube after electroporation at HH14-16 and harvest at HH24-26. Each construct was co-injected with CMV-EGFP to visualize electroporation. White dotted lines outline the right, electroporated half of the neural tube. Tethered Mash1-E protein heterodimers induced neurogenesis comparable to that of Mash1 electroporations. Electroporated cells moved laterally toward the mantle layer of the neural tube (**C**,**E**,**G**,**K**,**I**) and co-expressed Tuj-1 (red) (**D**, **F**,**H**,**L**,**J**). This was in contrast to the Mash1 AQ control where cells were randomly dispersed along the medio-lateral axis and randomly expressed Tuj-1 (**A**,**B**).

In contrast, over-expression of Mash1 causes electroporated, GFP-expressing cells to preferentially migrate to positions along the lateral edge of the neural tube. Mash1 induces progenitor cells to undergo neuronal differentiation as assayed by this lateral position and their co-expression of Tuj-1 (Figure 5C,D). Over-expression of each of the tethered Mash1-E protein heterodimers induced neuronal differentiation comparable to that seen with the over-expression of Mash1 alone. They induced lateral migration of GFP-expressing cells and co-expression of Tuj-1 (Figure 5E-L; arrowheads). The un-tethered E protein E47 had no activity in this assay (data not shown). These results suggest that, *in vivo*, Mash1 likely heterodimerizes with E proteins, and this complex is important in the neuronal differentiation activity seen with Mash1. Furthermore, the E proteins appear interchangeable in this activity since each Mash1-E protein heterodimer appears to function similarly.

Mash1-E protein heterodimers have consequences for neuronal cell-type Specification

In *Drosophila*, the proneural genes *achaete-scute* and *atonal* have been shown to be both necessary and sufficient for the formation of specific neuronal populations. *Achaete-scute* is required for external sensory organs, while *atonal* is required for chordotonal organs (Chien et al., 1996). This suggests that bHLH transcription factors provide some cell-type specific information during the process of driving neurogenesis.

In vertebrates, the neural tube is patterned along the dorso-ventral axis during embryonic development. Recent studies have divided the dorsal half of the neural tube into six distinct progenitor domains that give rise to dorsal interneuron populations in the developing neural tube (Figure 6). These dorsal interneuron populations have been defined according to the characteristic expression of transcription factors, mostly of the

homeodomain family (Helms and Johnson, 2003). The Johnson lab has demonstrated that over-expression of Math1 increases the dI1 population, over-expression of Ngn1 increases the dI2 population, and over-expression of Mash1 increases the dI3 population (Figure 7). Thus, although these neural bHLH factors share the function of inducing neurogenesis, they have distinct activities in neuronal cell-type specification. To examine whether the Mash1-E protein heterodimers functioned similarly to Mash1 in this cell-type specification assay, I examined dorsal populations of dI1-dI3 and dI4/dI6 in the electroporated neural tubes.

When Mash1 is over-expressed in the chick electroporation assay, there is a significant increase in Islet1 positive cells, indicative of an increase in the dI3 population (Figure 8K). Inversely, there is a decrease in Lhx 2/9 positive neurons, dI1, in Lhx1/5 positive neurons, dI2, and in Pax2 positive neurons, dI4 and/or dI6 (Figure8A,F) See Figure 8P-S for quantitation of this data. In contrast to the similar functions in neural differentiation, over-expression of the tethered Mash1-E protein heterodimers revealed differences in activity of the different putative heterodimers. Only the Mash1-HEB heterodimer produced an increase of the dI3, Islet1 positive population of cells, similar to un-tethered Mash1 (Figure 8K-O, R). The over-expression of Mash1 gave a more dramatic increase than that of the Mash1-HEB heterodimer (Figure 8R). In both cases, these ectopic cells were found in a similar dorsally confined area of the neural tube (Figure 8K-L). In contrast, the over-expression of Mash1-E12 and Mash1-E47 heterodimers led rather to decreases in the number of cells of the dI3, Islet1 positive

Figure 6: Dorsal Progenitor and Dorsal Interneuron Populations in the developing neural tube.

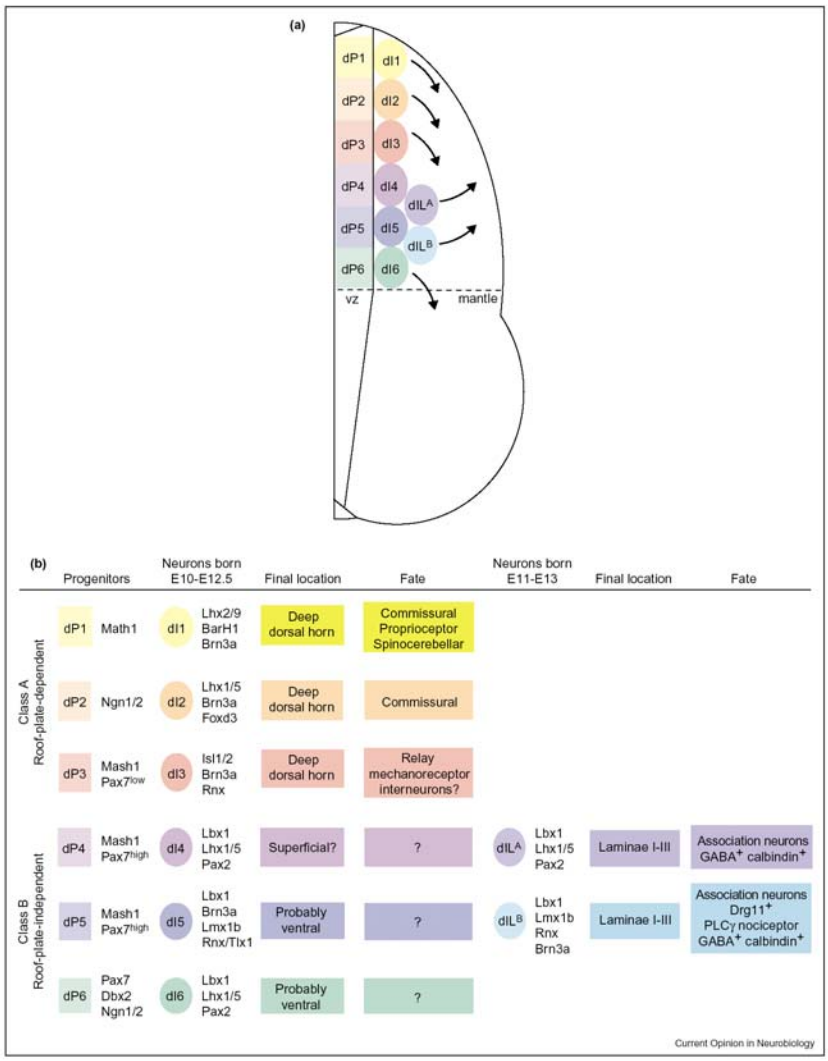


Figure 6: Dorsal Progenitor and Dorsal Interneuron Populations defined in the developing neural tube.

(a) Diagram of a neural tube showing the organization of the dorsal progenitor and interneuron populations and their initial migrations. Details are shown in (b). (b) Class A and Class B neurons are distinguished by their dependence and independence, respectively, on roof plate signals. Progenitor populations are defined by expression of bHLH and HD factors, and are partially inferred from their location relative to distinct interneuron populations. Eight populations of interneurons can be identified by the combination of HD factors they express, their immediate migratory behavior, their birth date, and their requirement for roof plate signals. vz, ventricular zone
(HELMS AND JOHNSON, 2003).

Figure 7: Over-expression of bHLH transcription factors alters dorsal Interneuron cell-fates.

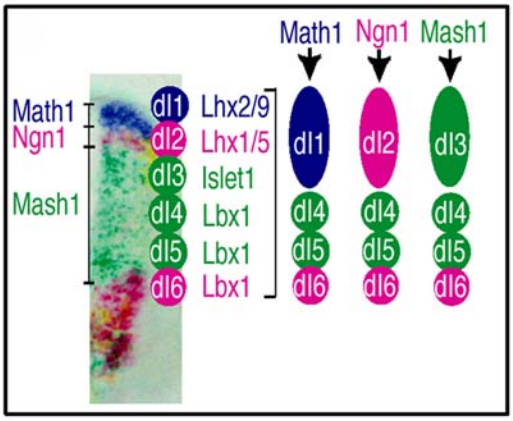


Figure 7: Over-expression of bHLH transcription factors alters dorsal Interneuron cell-fates.

On the left, is overlapping *In situ* showing distinct expression domains of Math1, Ngn1 and Mash1 in the dorsal half of the neural tube. Over-expression of any one of these biases dorsal Interneuron fates. Math1 over-expression increases the dI1 population at the expense of dI2 and dI3. Ngn1 over-expression increases the dI2 population at the expense of dI1 and dI3. Finally, Mash1 over-expression increases the dI3 population at the expense of both dI1 and dI2 populations.

population of neurons (Figure 8; M-N). The Mash1-E2.2 heterodimer generally decreased this population, however, it was not statistically significant (Figure 8O).

Taken together, for the neuronal cell-type specific activity of Mash1, defined by the increase in the dI3 population, HEB may be the relevant *in vivo* partner. Importantly, this assay suggests that there are non-redundant functions for the different Mash1-E protein heterodimers.

The conclusion that HEB may be the relevant *in vivo* partner for Mash1 is complicated by the fact that the changes in the dI1 and dI2 populations with the different tethered constructs do not fit this model. Normally, the dI1 and dI2 populations, derived from the Math1 and Ngn1 expressing progenitors, are decreased when Mash1 is over-expressed (Figure 7). So, based on the resulting increase of the dI3 interneurons, Mash1-HEB would be predicted to mimic the Mash1 phenotype and result in a loss of dI1 and dI2 interneurons. This was true for the dI1 population but not the dI2 (Figure 8B,G).

Three of the four Mash1-E protein heterodimers decrease the dI1 populations in the neural tube, identified by Lhx2/9 expression (Figure 8B,D,E , P) similar to Mash1. The exception is Mash1-E12, that had no significant effect on this population (Figure 8C, P). The heterodimers, Mash1-HEB, Mash1-E47, and Mash1-E2.2 all decreased the dI1 population as dramatically as the decrease seen with over-expression of un-tethered Mash1.

In the case of the dI2 population of neurons, the Lhx1/5 positive, Pax2 negative interneurons, Mash1-HEB did not phenocopy Mash1 alone (Figure 8F,G, Q). Rather, it had the opposite effect and the number of dI2 positive cells shows a significant increase. This increase of the dI2 population indicates that the Mash1-HEB heterodimer is not

Figure 8: Mash1-E protein heterodimers have different effects on cell-type specification in the dorsal neural tube.

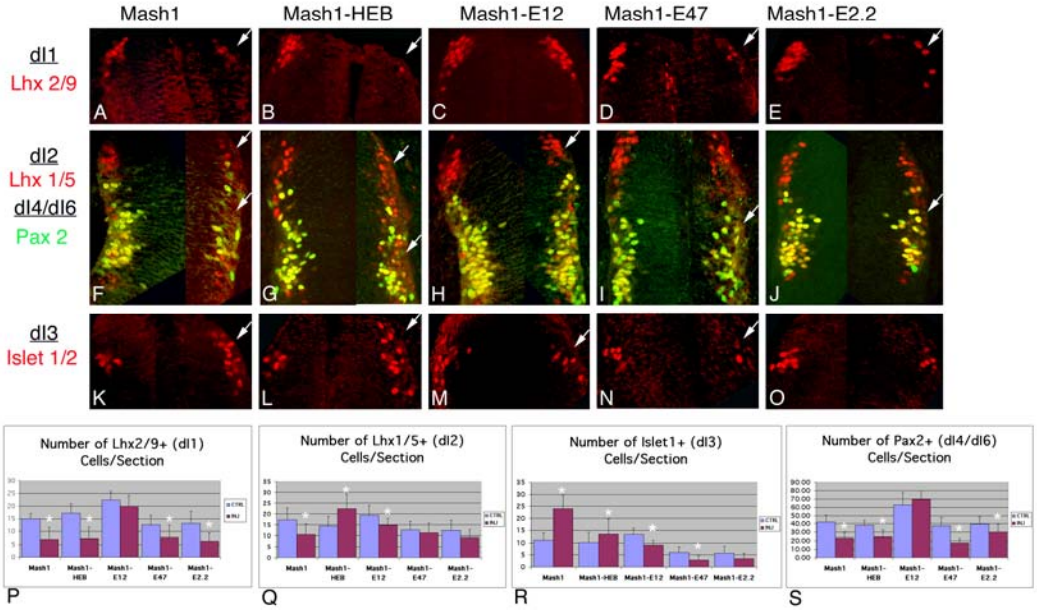


Figure 8: Mash1-E protein heterodimers have different effects on cell-type specification in the dorsal neural tube.
All constructs were electroporated and harvested as previously described. Right side is electroporated side, left side is internal control side. Transverse sections of chick neural tube were labeled by immunofluorescence to indicate specific dorsal interneuron population and imaged using confocal microscope. (A-E,P) Anti-Lhx2/9 antibody was used to visualize the dI1 population (red). dI2 population was identified by anti-Lim1/2 positive cells (red) that were negative for labeling by anti-Pax2 (green/yellow), which marked the dI4/dI6 populations (F-J,Q,S). dI3 population was labeled using anti-Islet1/2 (red) antibody (K-O,R). * indicates significant difference with a p value < .05. Arrows point to dorsal interneuron populations changed on the electroporated side of the neural tube.

completely recapitulating the phenotype of the over-expression of Mash1 alone. In contrast, Mash1-E12 phenocopies Mash1 activity, since in this case the decrease in dI2 was comparable to Mash1 (Figure 8F,H,Q). Mash1-E2.2 and Mash1-E47 had no significant effect on this population (Figure 8I,J). Finally, analysis of the Pax2 positive interneurons, labeling dI4 and dI6 populations, revealed that Mash1-HEB, Mash1-E47, and Mash1-E2.2 all induced decreases comparable to that of Mash1 over-expression (Figure 8F,G,I,J,S). Mash1-E12 over-expression had no significant effect on these populations of dorsal interneurons, dI4 and/or dI6 (Figure 8H,R).

Taken together, each Mash1-E protein tether can mimic at least one of the Mash1 over-expression phenotypes in specifying the dorsal interneuron populations (Table 1). However, no single putative heterodimer can mimic the complete repertoire of Mash1 activities. It is also interesting to note that although over-expression of Mash1-E12, Mash1-E47, and Mash1-E2.2 all appear to induce neurogenesis, we have not identified a specific population that increases. Finally, although Mash1 increases the Islet1 dI3 population it is important to note that Mash1 is not required for this Islet1 population to form since it is still present in the Mash1 mutant (A.W. Helms, communication).

Tethered Mash1-E12 forced heterodimer binds DNA.

In these experiments, the assumption is made that an intra-molecular "heterodimer" is formed, and it is the activity of this putative protein complex that is being tested. To gain support for proper folding of this tethered protein, Mash1-E12 was tested for its ability to bind DNA in an eletrophoretic mobility shift assay (EMSA). It has been previously shown that Mash1 without E12 does not efficiently bind DNA in EMSA (Helms et al., 2000).

Table 1: Summary of effects of tethered Mash1-E protein heterodimers compared with Mash1.

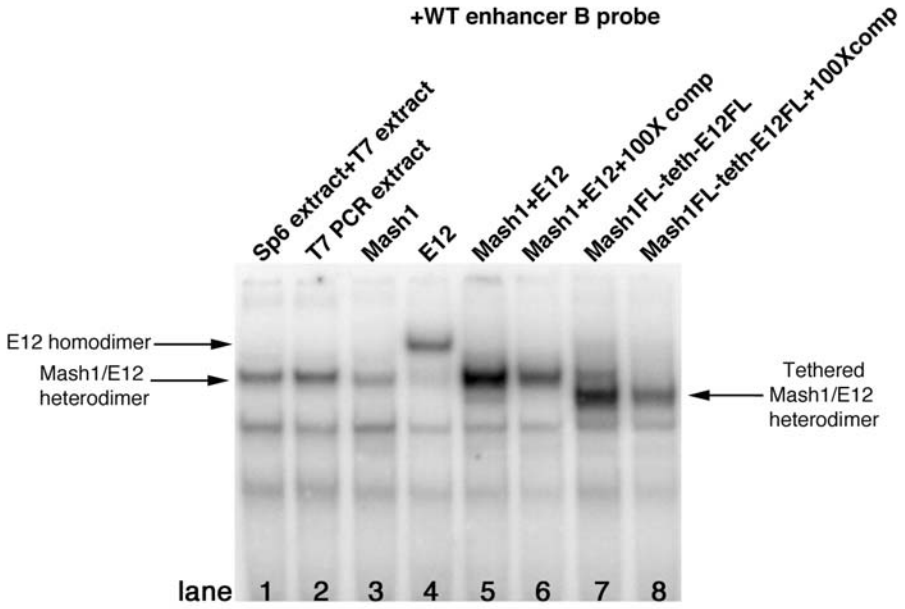
Expression Construct	Neurogenic Activity	dI1	dI2	dI3	dI4/dI6
Mash1	+	↓	↓	↑	↓
Mash1-HEB	+	↓	↑	↑	↓
Mash1-E12	+	NC	↓	↓	NC
Mash1-E47	+	↓	NC	↓	↓
Mash1-E2.2	+	↓	NC	NC	↓

Table 1: Summary of effects of tethered Mash1-E protein heterodimers compared with Mash1.

Headings indicate effects including Neurogenic activity and the effects on the dorsal interneuron populations dI1-dI3,and dI4/dI6. Arrows up indicate a significant increase in the dI population, arrows down indicate a significant decrease in the dI population. NC indicates that there was no statistically significant change.

In the gel shift assay, E12 alone binds DNA (Figure 9, lane4), while Mash1 alone does not bind E-box DNA under the same conditions (Figure 9, lane3). However, when Mash1 protein and E12 protein are added together in the same binding reaction, they form a heterodimeric complex that binds the E-box DNA (Figure 9, lane 5). Note, reticulocyte lysate has E-box binding activity as previously noted (Helms et al., 2000). The Mash1-E12 tethered protein, in the absence of added E12 protein, binds the E-box DNA, as well (Figure 9, lane 7). The Mash1-E12 tethered protein runs slightly faster than that from the Mash1 plus E12 heterodimer possibly reflecting a constraint on what normally would be a "free end" but is now forced into a tighter conformation. The ability of the Mash1-E12 tethered proteins to bind E-box DNA supports the premise that this tethered protein is capable of folding properly and functioning (at least by binding DNA).

Figure 9: Tethered Mash1-E12 heterodimer binds DNA



WT enhancer B probe:
5'-CACGCGCTGTCAGCTGGTGAGCGCAC-3'

Figure 9: Tethered Mash1-E12 heterodimer binds DNA.

Electrophoretic Mobility Shift assay with reticulocyte lysates alone (**lane 1&2**), with Mash1 alone (**lane 3**), with E12 alone (**lane 4**), with Mash1 plus E12 (**lane 5**), with Mash1, E12, plus 100X competitor (**lane 6**), with tethered Mash1-E12 heterodimer alone (**lane 7**), with tethered Mash1-E12 heterodimer plus 100X competitor (**lane 8**). The probe and competitor is 25 bp oligonucleotide containing the E-box (shown in red). Arrows indicate respective bHLH/E box complexes.

DISCUSSION

Mash1-E protein heterodimers possess neurogenic activity.

The over-expression of Mash1 alone in the chick electroporation assay causes cells to exit the cell cycle, migrate laterally and express a pan-neuronal marker. This neurogenic activity is recapitulated by the over-expression of Mash1-E protein heterodimers in this same assay. In regards to this activity, different E proteins seem to be redundant. In addition, these results are consistent with previous studies suggesting that the neural Class II bHLH transcription factors heterodimerize with the Class I E proteins to form a complex involved in the process of neurogenesis. The over-expression of many of the neural-specific Class II bHLH transcription factors, including Ngn1, Ngn2, Math1 and Mash1 in the chick neural tube all lead to neurogenesis. Based on these results, all of these Class II bHLH proteins may also be utilizing an E protein partner when performing the neurogenic activity.

Mash1-E protein heterodimers do not fully recapitulate the phenotype of un-tethered Mash1 in dorsal Interneuron populations.

The effects of over-expression of Mash1-E protein heterodimers on sub-populations of dorsal interneurons suggest that the E proteins are not redundant when it comes to their function in cell-type specification. None of the Mash1-E protein heterodimers were able to completely recapitulate the phenotype of the over-expression of Mash1 alone (Table 1). There are several possibilities to explain these results.

The first possibility is that Mash1 can heterodimerize with any of the E proteins and bind target sequences, *in vivo*, and that all four putative heterodimers confer a specific aspect

of Mash1 function. Another possibility is that for the cell-type specification function, Mash1 may dimerize with other bHLH factors, such as Ngn2. The over-expression of Mash1 alone would allow Mash1 to bind to these additional proteins, other than just the E proteins, where it can subsequently demonstrate additional activity in the assay, such as generation of additional cell-types. In the dorsal neural tube of the developing mouse embryo at e11.5, Ngn2 protein and Mash1 protein are co-localized in a subset of cells (Figure 10; arrowheads) as demonstrated by double immunofluorescence labeling. In addition, Ngn2 was first identified out of a yeast-two-hybrid screen as a protein that interacted with Mash1 (Gradwohl et al., 1996). This evidence suggests that it is possible that Mash1 and Ngn2 could interact in certain cells to contribute additional functions in cell-type specification.

In addition, it is possible that Mash1 could form homodimers *in vivo*, that have activity contributing to its function. Homodimers of Mash1 have been demonstrated to form *in vitro* under specific conditions. Although their binding to an E-box sequence is not as efficient as heterodimer binding, there still remains the possibility that they actually do form *in vivo*, and perhaps possess different affinities for E-box binding than heterodimers (Kunne and Allemann, 1997). Of course, another possibility could be that the deficient activity of these tethered constructs is due to a defective structure of the tethered construct. The structure could be disrupted either by way of mis-folding or possibly obstruction of an important protein-interaction domain from the presence of the tether itself.

Figure 10: Mash1 and Ngn2 proteins are co-localized in a subset of cells of the dorsal neural tube in mouse e11.5.

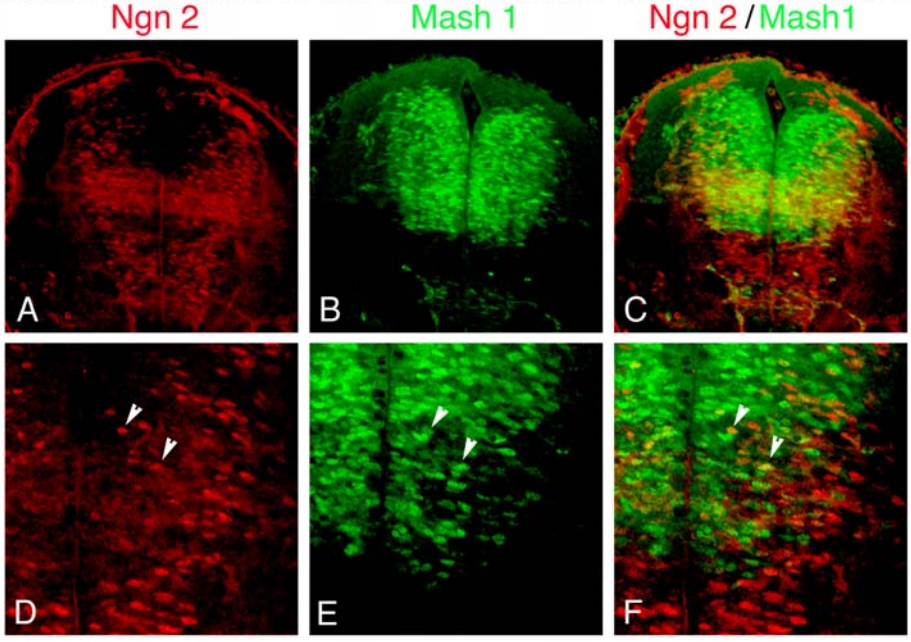


Figure 10: Mash1 and Ngn 2 proteins are co-localized in a subset of cells of the dorsal neural tube in mouse e11.5.

Confocal images of transverse section of WT e11.5 mouse neural tube double-labeled with anti-Ngn2 antibody (red) and anti-Mash1 (green). **A**-**C** are low magnification pictures from the 20X objective showing overlapping expression domains of Ngn 2 and Mash1. **D**-**F** shows higher magnification images (40X) of Ngn2 and Mash1 protein in single cells and **F** shows that their proteins co-localize in some cells, arrowheads.

One important observation is that the over-expression of these four heterodimers appear to have different effects on the dorsal interneuron populations in comparison to one another. These differential effects could reflect their differing specificities for binding cofactors involved in the production of these particular dorsal interneuron subtypes. It may also reflect the distinction between functions for activating sub-type programs versus repressing the sub-type program. Again, these differences in specific functional activities for the tethered heterodimers could be due to differences in specificities *in vivo*, or could simply reflect defects of the heterodimer constructs themselves.

The differences seen in the effects on dorsal interneuron populations by the over-expression of Mash1- E protein heterodimers could reflect functions in cross-inhibition of transcription factors. The particular combination of Mash1-E protein partners may be specific for inhibiting other bHLH proteins. It is known that over-expression of Ngn1 in chick neural tubes causes decreases in Cath1 expression and subsequently in the population of dI1 neurons. Conversely, the over-expression of Math1 causes a decrease in cNgn1 expression and a subsequent decrease in the population of dI2 neurons. Similarly, over-expression of Mash1 represses both Cath1 and cNgn1, decreasing dI1 and dI2 populations. These effects demonstrate cross-inhibitory influences of the bHLH transcription factors on one another in the neural tube. Although the mechanism is unknown, these effects are believed to be involved in establishing and maintaining the discrete domains of expression of these bHLH factors (Gowan et al., 2001).

An important insight gained from this study is that the characteristic features of the phenotype of the over-expression of Mash1 are distinct. The over-expression of Mash1 leads

to neural differentiation, an increase in dI3 neurons, and a decrease in dI1 and dI2 neurons. Though these results can be considered one overall phenotype, the use of these tethered heterodimer proteins demonstrates that these effects can be uncoupled from one another. The neural differentiation phenotype is distinguishable from the effects on cell-type specification and is a common activity of each Mash1-E protein heterodimer. The increase of the dI3 interneuron population is distinguishable and can be uncoupled from the decrease of the dI1 and dI2 populations. It is still not clear, however, which E protein is the *in vivo* partner to Mash1. Since all of these E proteins are expressed in the neural tube at some level, Mash1 could partner with all of them to confer neurogenic activity. Subsequently, there could be competition between each of the E proteins or other bHLH factors, such as Ngn2, to effect dorsal interneuron populations.

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VITAE

Tandi Louise Collisson was born in Birmingham, Alabama on November 16, 1973. She has lived in Texas since the age of 11 and graduated from Bryan High School in 1992. She attended the University of Texas at Austin, graduating with a Bachelor of Arts from the School of Liberal Arts with a degree in Humanities, concentration in Biology and Psychology. After graduating from college, she worked as a waitress and traveled across Europe in the summer of 1998, visiting nine countries. Upon returning to the U.S. she moved to Dallas, TX and was employed as a technician in the lab of Dr. Melanie Cobb in the Pharmacology Department at UT Southwestern. After a year of employment, she started as a graduate student in the Division of Cellular and Molecular Biology in the Fall of 1999. After her first year, she joined the Neuroscience graduate program. She began pursuing her research in the lab of Dr. Jane E. Johnson and studying the role of bHLH transcription factors in neural tube development.

Permanent Address: 11385 Goldenmist

College Station, TX 77845