Neuromancer1 and Neuromancer2 Regulate Cell Fate Specification in the Developing Embryonic CNS of *Drosophila melanogaster*

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Abstract

T-box genes encode a large family of transcription factors that regulate many developmental processes in vertebrates and invertebrates. In addition to their roles in regulating embryonic heart and epidermal development in *Drosophila*, we provide evidence that the T-box transcription factors *neuromancer1* (*nmr1*) and *neuromancer2* (*nmr2*) play key roles in embryonic CNS development. We verify that *nmr1* and *nmr2* function in a partially redundant manner to regulate neuronal cell fate by inhibiting *even-skipped* (*eve*) expression in specific cells in the CNS. Consistent with their redundant function, *nmr1* and *nmr2* exhibit overlapping yet distinct protein expression profiles within the CNS. Of note, *nmr2* transcript and protein are expressed in identical patterns of segment polarity stripes, defined sets of neuroblasts, many ganglion mother cells and discrete populations of neurons. However, while we observe *nmr1* transcripts in segment polarity stripes and specific neural precursors in early stages of CNS development, we first detect Nmr1 protein in later stages of CNS development where it is restricted to discrete subsets of Nmr2-positive neurons. Expression studies identify nearly all Nmr1/2 co-expressing neurons as interneurons, while a single Eve-positive U/CQ motor neuron weakly co-expresses Nmr2. Lineage studies map a subset of Nmr1/2-positive neurons to neuroblast lineages 2-2, 6-1, and 6-2 while genetic studies reveal that *nmr2* collaborates with *nkx6* to regulate *eve* expression in the CNS. Thus, *nmr1* and *nmr2* appear to act together as members of the combinatorial code of transcription factors that govern neuronal subtype identity in the CNS.

Keywords

CNS development; T-box transcription factors; neuroblast lineage; Neuromancer1/2; Even-skipped

INTRODUCTION

T-box transcription factors play general and conserved roles to regulate the development of all metazoans. For example, T-box genes are known to regulate mesoderm formation,

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morphogenetic movements, cell adhesion, cell migration, tissue patterning, limb patterning, limb bud outgrowth, and organogenesis (King et al., 2006; Fong et al., 2005; Naiche et al., 2005; Showell et al., 2004; Chapman and Papaioannou, 1998; Papaioannou and Silver, 1998; Gibson-Brown et al., 1998). Moreover, an evolutionary conserved role for T-box genes to specify cell fates is supported by studies in simple organisms ranging from sponges, which contain several T-box genes (Larroux et al., 2006; Adell and Muller, 2005), to more complex invertebrate and vertebrate organisms, which can contain up to 21 T-box genes (Naiche et al., 2005; Woollard, 2005). In *Drosophila melanogaster*, eight *T-box* genes from four subfamilies have been identified; these include *Dorsocross-1* (*Doc1*), *Doc2*, *Doc3*, *optomotor blind* (*omb*), *optomotor-blind-related* (*org-1*), *brachyenteron* (*Byn*), *neuromancer1* (*nmr1/H15*), and *neuromancer2* (*nmr2/midline*) (Reim and Frasch, 2005; Reim et al., 2005; Buescher et al., 2004; Griffin et al., 2000; Singer et al., 1996; Poeck et al., 1993). *nmr1* and *nmr2* are members of the Tbx20 gene family and regulate epidermal patterning as well as cardioblast specification in *Drosophila* (Buescher et al., 2006; Reim et al., 2005; Qian et al., 2005, Miskolczi-McCallum et al., 2005; Buescher et al., 2004).

Since mutations in T-box genes are associated with human congenital heart defects (Stennard et al., 2005; Takeuchi et al., 2005; Jerome and Papaioannou, 2001; Basson et al., 1999; Li et al., 1997), a majority of studies have focused on the role of Tbx20 and other T-box genes in regulating vertebrate embryonic heart development. In chick, zebrafish, and frog embryos, *Tbx20* orthologues are expressed in developing cardiovascular structures and regulate the expression of *Tbx5*, a heart morphogenic gene important for the development of the early heart tube as well as chamber formation (Showell et al., 2006; Brown et al., 2005; Plageman and Yutzey, 2004; Szeto et al., 2002; Ahn et al., 2000). Morpholino oligonucleotide knockdown of Zebrafish *Tbx20* (*hrT*) and frog *Tbx20* results in small, malformed hearts and a loss of blood circulation (Brown et al., 2005; Szeto et al., 2002). Likewise, mouse *Tbx20* mutants exhibit hypoplastic hearts with severe looping defects and abnormalities in cardiac chamber differentiation (Takeuchi et al., 2005; Stennard et al., 2005). Consistent with its fundamental role in cardiogenesis, mouse *Tbx20* modulates the expression of *Nkx2-5*, *Gata4* and *Mef2c*, members of the core cardiac transcription factor network, in the developing heart (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005; Stennard et al., 2003).

The *Drosophila* Tbx-20 homologues, *nmr1* and *nmr2*, have been shown to regulate two separable functions during cardiogenesis. Initially, *nmr1* and *nmr2* cooperate with the NK-2 class homeodomain transcription factor *tinman* and the GATA factor *pannier* to specify the fates of cardiac progenitor cells (Reim et al., 2005; Qian et al., 2005; Miskolczi-McCallum et al., 2005). In addition, *nmr2* mutant embryos exhibit increased numbers of *even-skipped*-expressing cardiac progenitors at the expense of *ladybird*-expressing cardiac progenitors (Qian, et al., 2005). After specifying cardiac cell fates, *nmr1* and *nmr2* regulate the epithelial polarity of myocardial cells as repression of *nmr1* and *nmr2* results in the mislocalization of the junctional proteins Discs-large, Dystroglycan, and alpha-Spectrin (Qian et al., 2005). These molecular defects likely lead to the disorganization of the heart tube and misalignment of heart cells consistent with defects in cell adhesion (Fong et al., 2005).

While much research has focused on the function of Tbx20 genes in heart development, a few studies have indicated a likely function for this gene family during CNS development. For example, Tbx20 genes are expressed in cranial motor neurons in zebrafish, mice, and *C. intestinalis* and in dorsal motor neurons in the chick spinal cord (Nordstrom et al., 2006; Song et al., 2006; Dufour et al., 2006; Ahn et al., 2000). Moreover, *mab-9*, the *C. elegans* Tbx20 family member, is expressed in neurons located in specific regions of the ventral nerve cord (Woollard and Hodgkin, 2000). *Drosophila nmr1* and *nmr2* transcripts are also detected in the mature embryonic CNS (Qian et al., 2005) and loss of *nmr1* and *nmr2* results in an increase in Eve-expressing neurons (this study; Buescher et al., 2006).

In *Drosophila nmr1* and *nmr2* negatively regulate Wingless signaling in the ventral epidermis to establish proper segmentation (Buescher et al., 2004). In early stages of CNS development, *nmr2* promotes the formation and specification of several neuroblasts in the CNS (Buescher et al., 2006). However, the roles of *nmr1* and *nmr2* in regulating later steps of CNS development in *Drosophila* remain largely unexplored. Through a forward genetic screen we identified *nmr1* and *nmr2* as regulators of neuronal subtype identity in the *Drosophila* CNS. *nmr1* and *nmr2* function in a partially redundant manner to regulate neuronal specification and in agreement with this, are expressed in overlapping patterns of CNS neurons. Nmr1 and Nmr2 expression appear predominantly confined to interneurons, and lineage tracing studies map subsets of Nmr1-positive neurons to three specific neuroblast lineages. Taken together, these data suggest that *nmr1* and *nmr2* are additional members of the transcriptional regulatory network that specifies neuronal fates in the *Drosophila* embryonic CNS, and that elucidating their function will further our understanding of the mechanisms guiding CNS development.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster strains were maintained at 25°C on cornmeal-yeast-agar media. *white*[11] flies were used as wild-type. We used the following lines in the study: *Df(2L)sc19-4* (Bloomington Stock Center), *Df(2L)x528* (Buescher et al., 2006; Buescher et al., 2004), *nmr1²¹⁰* (Qian et al., 2005), *mid^{GA174}* (Seeger et al., 1993), *mid²* (Nusslein-Vohard et al., 1984), *eve^{ID19}* (Nusslein-Volhard et al., 1985), *hb9^{kk30}* (Broihier and Skeath, 2002), *nkx6^{D25}* (Broihier et al., 2004), and *PZ[lac-Z;ry⁺]H15* ((H15-lacZ); Brook and Cohen (1996)). We generated the double-mutant lines *nmr1²¹⁰;hb9^{kk30}*, *nmr1²¹⁰;nkx6^{D25}*, *mid^{GA174};hb9^{kk30}* (*nmr2;hb9*), and *mid^{GA174};nkx6^{D25}* (*nmr2;nkx6*). We used the *Gal4/UAS* system (Brand and Perrimon, 1993) and *Elav*-Gal4 (Lin and Goodman, 1994) to misexpress the following transgenes in wild-type and specific mutant backgrounds: UAS-*nmr1*, UAS-*nmr2* (Qian et al., 2005) and UAS-*eve* (Landgraf et al., 1999). Due to the observed post-transcriptional regulation of *nmr1* in embryos, we verified that ElavGAL4::UAS-*nmr1* drove generalized Nmr1 protein expression in the CNS via standard immunochemical methods. Embryos used for the lineage analyses were of the following genotype: y w *hsFlp/+*; UAS-*tau-myc*-GFP/+; *flpout*-Gal4/+.

Antibody production, immunofluorescent, and immunohistochemical studies

Amino acids 1-287 of Nmr1 (N-terminal) and 322-557 of Nmr2 (C-terminal) were cloned into pET (Novagen) for protein expression and purification. These protein fragments were used as immunogens to generate antibody responses specific to Nmr1 in rabbits and guinea pigs and to Nmr2 in rabbits at Pocono Rabbit Farm and Laboratory. Both Nmr1 and Nmr2 antibodies are specific as demonstrated by their inability to detect the expression of any other proteins in *Drosophila* homozygous mutant for null alleles of *nmr1* and *nmr2*, respectively. We used the Vector ABC kit for immunocytochemical staining and Alexa 488, Alexa 594, and Alexa 633 with appropriate species specificity for immunofluorescence studies (Molecular Probes). We followed standard protocols published by N.H. Patel (1994) for both immunocytochemical and immunofluorescent staining.

We used the following primary antibodies at the indicated dilutions for this study: rabbit anti-Eve (1:3000; Frasch et al., 1987), mouse anti-Repo (1:100; Halter et al., 1995), rat anti-Nkx6 (1:1000; Broihier et al., 2004), guinea pig anti-Hb9 (1:1000; Broihier and Skeath, 2002), mouse anti-Zfh-1 (1:1000; Lai et al., 1993), rabbit anti-Mef2 (1:3000; Lilly et al., 1995), rabbit anti-Gad (1:1000; a gift of F.R. Jackson; Kulkarni et al., 1994), rabbit anti-Serotonin (1:300; a gift of P. Taghert; Taghert and Goodman, 1988), rabbit anti-DVGLUT (1:10,000; a gift of A. DiAntonio; Daniels et al., 2004), rabbit anti-Dbx (1:1000; J. Skeath), guinea pig or rabbit anti-

Nmr1 (1:3000; this work), and rabbit anti-Nmr2 (1:1000; this work). The following primary antibodies were obtained from the Developmental Studies Hybridoma Bank at The University of Iowa: anti-Dac2-3 (1:10, Mardon et al., 1994), anti-Fasciclin II (1D4, 1:5; Grenningloh et al., 1991), anti-Engrailed/Invected (4D9, 1:10; Patel et al., 1989), and BP-102 anti-Neurotactin (1:20; Hortsch et al., 1990). Rabbit (Jackson Laboratories) and mouse (Promega) β-Galactosidase antibodies were used at a dilution of 1:2000. Rabbit GFP antibody (Torey Pines) was used at a 1:3000 dilution. All secondary antibodies for immunocytochemical staining were diluted at 1:300 (Vector Laboratories).

RNA *in situ* hybridization

In situ hybridization of *Drosophila* embryos was performed essentially as described in Tautz and Pfeiffle (1989). To detect *nmr1* RNA expression we generated two anti-sense digoxygenin RNA probes specific for *nmr1* using the T7 Megascript transcription kit (Ambion). One region corresponded to exons 1 and 2 of *nmr1* (798 bases). This region almost completely overlaps with the fragment of *nmr1* used to generate recombinant Nmr1 protein for antibody generation, which includes exons 1 and 2 and the first 63 bases of exon 3. The other RNA probe was generated from the full-length *nmr1* cDNA (Qian et al., 2005). Both probes detect identical *nmr1* RNA expression patterns in embryos.

Deficiency screening and deficiency mapping

We screened 200 deficiency lines contained within the second and third chromosomal deficiency kits available from the Bloomington Stock Center (IN) for alterations in the CNS expression pattern of Eve. Embryos homozygous for *Df(2L)sc19-4* (25D7;26A9) exhibited ectopic Eve-positive neurons. We then used the following deficiencies to map this Eve CNS phenotype to a smaller genomic interval: *Df(2L)tkv2* (25D2;25E1), *Df(2L)Exel6012* (25D5;25E6), *Df(2L)cl2* (25D2;25F2), *Df(2L)cl-h2* (25D6;25F5), and *Df(2L)cl-h4* (25E1;25E5). The latter deficiency was the most useful for narrowing the genomic interval of interest to 25E1;25E5 within which 16 predicted or known genes reside. We obtained mutant alleles available for 6 of these genes and screened embryos homozygous for each of these mutations for alterations in the Eve CNS expression. Only the *neuromancer2* (*midline*) mutant alleles, *mid^{GA174}* and *mid^2*, recapitulated the *Df(2L)sc19-4* Eve mutant phenotype.

Lineage tracing analyses—We used a modified Flp/FRT (Flipase/Flp Recognition Target) system developed by Harrison and Perrimon (1993) to map the neuroblast lineage and trace axon projection patterns of neurons that express Nmr1. We collected 0–2 hr old embryos and aged them for 2, 4, and 6 hrs at 25°C. Each group of developmentally staged embryos was collected and heat-shocked for seven minutes at 33°C to induce *flp* recombinase, placed at 18° C, and aged until stage 15–17. Embryos were then collected, fixed, and triple-stained with anti-Nmr1, anti-GFP, and anti-Fas II. By varying the time of the Flp expression, we GFP-labeled the whole neuroblast lineage or individual Nmr1-positive neurons. Anti-Fas II labeled the axon connectives. Using confocal microscopy, we obtained approximately 50–60 0.1 μm thick Z-series of each nerve cord containing positive clones and compressed those images into two dimensions using Leica software.

Software programs—The final confocal images were assembled using Adobe Photoshop software. Statistical analyses were performed using Microsoft Excel and the data were presented graphically using Origin software (Microcal Software, Inc.).

RESULTS

nmr2 and *nmr1* are neuronal fate determinants

To identify genes that regulate neuronal cell fate in the *Drosophila* embryonic CNS, we screened a collection of approximately 200 second and third chromosomal deficiency lines for changes in the CNS expression pattern of *even-skipped* (*eve*). We chose *eve* as a molecular marker because *eve* regulates neuronal cell fates (Doe et al., 1988) and is expressed in a stereotyped pattern of neurons in the CNS (Fig. 1A,B). We focused our attention on *Df(2L) sc19-4* (25D7;26A9) because embryos homozygous for this deficiency exhibit ectopic Eve-positive neurons in the lateral region of the CNS (Fig. 1C) as well as a partial loss of medial Eve-positive aCC/pCC neurons (Fig. 1D).

We used deficiency mapping to localize this genetic function to a smaller interval and identified the T-box genes *neuromancer1* (*nmr1*) and *neuromancer2* (*nmr2*) as likely candidates to regulate *eve* expression in the CNS (see Methods). Since *nmr1* and *nmr2* are known to regulate heart and epidermal development in the *Drosophila* embryo and both are expressed in the CNS (Qian et al., 2005; Buescher et al., 2004), we asked whether loss of function in *nmr1* and/or *nmr2* yielded a phenotype similar to that observed for *Df(2L)sc19-4*. Embryos homozygous for a null allele of *nmr2* (*mid^{GA174}*) display an Eve CNS phenotype similar to but weaker than that observed for embryos homozygous for *Df(2L)sc19-4* (Fig. 1), whereas embryos homozygous for a null allele of *nmr1* (*nmr1^{210}*) exhibit a wild-type Eve expression pattern (data not shown). Thus, *nmr2*, but not *nmr1*, is necessary to regulate *eve* expression in the CNS.

Prior work has shown that *nmr1* and *nmr2* function in a partially redundant manner to control epidermal development in the *Drosophila* embryo with *nmr2* playing a primary role in this process (Buescher et al., 2004). To test whether *nmr1* collaborates with *nmr2* to regulate *eve* expression and to rule out the possibility that other genes uncovered by the deficiency contribute to the mutant phenotype, we removed both copies of *nmr1* and *nmr2* by generating embryos trans-heterozygous for the original deficiency, *Df(2L)sc19-4*, and a different deficiency, *Df(2L)x528*. The region of overlap between these deficiencies specifically removes *nmr1*, *nmr2*, and an annotated gene, CG31647, with unknown function (Buescher et al., 2004). Embryos trans-heterozygous for these two deficiencies yielded a phenotype identical to that observed for embryos homozygous for *Df(2L)sc19-4* (Buescher et al., 2004; data not shown). Thus, we conclude that *nmr1* and *nmr2* function in a partially redundant manner to control *eve* expression within the CNS.

Nmr2 and Nmr1 protein expression during embryogenesis

In order to identify the individual cells that express *nmr1* and/or *nmr2,* and to follow the expression dynamics of these two factors during embryogenesis and neurogenesis we generated Nmr1 and Nmr2 specific antibodies (see Methods). In general, we find that Nmr2 is expressed earlier and more broadly than Nmr1, with Nmr1 expression being restricted to subsets of Nmr2-expressing cells. We also find evidence for temporal regulation of Nmr1 protein translation as we observe *nmr1* transcripts in segment polarity stripes during gastrulation (Buescher et al., 2006; Fig. 2), many hours before Nmr1 protein becomes detectable in a few cells in the CNS (Fig. 2). In contrast, the RNA and protein expression profiles of Nmr2 appear essentially identical (Buescher et al., 2006).

We first detect Nmr2 expression during gastrulation (stage 6/7). At this time and through stage 10 Nmr2 is expressed in segment polarity stripes consistent with its known role in repressing wingless expression during segmentation (Fig. 2A) (Buescher et al., 2006;Buescher et al., 2004). During the remainder of embryogenesis Nmr2 is expressed in a dynamic pattern in many tissues (Fig. 2B–D). In the CNS, Nmr2 is expressed in subsets of neuroblasts, ganglion mother

cells, and neurons and by the end of embryogenesis marks about twenty neurons per hemisegment. In the ectoderm, Nmr2 expression remains in the ventral region of the embryo and is lost most dorsally. By the end of embryogenesis Nmr2 is expressed in ventral stripes of ectodermal cells (white arrows, Fig. 2D) and small clusters of ectodermal cells between these stripes (white arrowheads, Fig. 2D). In the mesoderm, Nmr2 expression is restricted to the heart where Nmr2 is expressed in all cardioblasts as well as their precursors (Fig. 2B,C,H–M).

In contrast to Nmr2, we first detect Nmr1 protein during stage 12 in a few cells in the anterior portion of the CNS (Fig. 2F,G). From this stage forward, the expression pattern of Nmr1 largely parallels that of Nmr2 in the CNS, ectoderm and mesoderm; the only exception being that only a subset of Nmr2-positive neuronal and ectodermal cells express Nmr1 (Fig. 2G and Fig. 3I). The protein expression profile of Nmr1 is noteworthy for two reasons. One, comparisons of the Nmr1 protein and RNA expression patterns indicate that *nmr1* transcript is subject to post-transcriptional regulation during early but not late embryogenesis. For example, while *nmr1* RNA is expressed from stage 7 to stage 12 in a pattern very similar to that observed for *nmr2* (Buescher et al., 2006; Fig. 2E′–H′), Nmr1 protein first appears during late stage 12 in a few cells in the CNS. From late stage 12 forward the Nmr1 protein and RNA patterns appear essentially identical (Fig. 2F–G and F′–G′).

Two, we find that the protein expression profile of Nmr1 is not identical to that driven by the H15-lacZ enhancer trap line, which is inserted roughly 3 Kb upstream of *nmr1,* and has been used as a surrogate for Nmr1 protein expression in embryos (Buescher et al., 2006; Miskolczi-McCallum et al., 2005; Buescher et al., 2004). For example, during late stages of CNS development (stages 13–16) double-label studies indicate that while H15-lacZ labels all Nmr1-positive neurons, many H15-lacZ-positive cells do not express Nmr1 protein (Fig. S1). During earlier stages of development the two patterns do not overlap as H15-lacZ drives expression in segment polarity stripes, many NBs, and ganglion mothers cells, but as noted Nmr1 protein first becomes detectable during stage 12 in a few post-mitotic neurons. The early pattern of H15-lacZ does appear essentially identical to the Nmr2 protein expression profile. However, while more neurons co-express H15-lacZ and Nmr2 than H15-lacZ and Nmr1 during late stages of CNS development, many cells especially those near the midline still express H15-lacZ but not Nmr2 (Fig. S1). Thus, the H15-lacZ expression pattern, likely due to perdurance of β-galactosidase within neuroblasts and neuroblast lineages that transiently express *nmr1/2*, appears to over-estimate the protein expression profiles of Nmr1 and Nmr2 in the mature embryonic CNS (see also below).

Nmr2 and Nmr1 expression in discrete neuronal populations of the CNS

We explored the CNS expression profiles of Nmr1 and Nmr2 in greater detail to gain insight into their possible functions during CNS development. The expression patterns of Nmr2 and Nmr1 are dynamic and complex in the CNS with a number of distinguishing. Nmr2 and Nmr1 are expressed in several discrete clusters of neurons in the CNS per hemisegment. A subset of cells within each cluster of Nmr2-positive neurons expresses Nmr1, and the expression of Nmr1 is delayed relative to that of Nmr2 in these cells. While distinct clusters of neurons reproducibly express Nmr1/Nmr2 at different levels, the relative expression levels of Nmr2 and Nmr1 within any one cluster are similar. The extensive co-expression of Nmr1/2 in the CNS helps explain the partially redundant function of these genes. Moreover, the temporal delay of *nmr1* expression relative to that of *nmr2* in neurons together with the restriction of *nmr1* expression to a subset of *nmr2*-positive neurons suggests the existence of a positive regulatory relationship between *nmr2* and *nmr1*.

To test for a regulatory relationship between *nmr2* and *nmr1* we followed Nmr1 protein expression in embryos that express Nmr2 in all CNS neurons as well as in embryos that lack *nmr2* function. First, we used the *Gal4/UAS* system and the *Elav*-Gal4 driver line to express

nmr2 ectopically in all CNS neurons. This manipulation led to ectopic *nmr1* expression in most, if not all, cells in the CNS (Fig. 4) suggesting that *nmr2* can positively regulate *nmr1* expression in many neurons. However, the pattern and levels of *nmr1* remain essentially normal in embryos homozygous mutant for *nmr2* (data not shown). The pattern of *nmr2* expression remains unchanged in embryos that lack *nmr1* function as well as in those in which *nmr1* is expressed throughout the CNS using the *Gal4/UAS* system (methods; data not shown). These results underscore the complex regulatory relationship between *nmr2* and *nmr1* in the CNS as *nmr2* appears sufficient to activate *nmr1* in most neurons but is not necessary to promote *nmr1* expression in any neurons. Thus, other factors must act in parallel to *nmr2* to activate or to maintain *nmr1* expression in neurons that co-express *nmr1* and *nmr2*, while distinct factors likely repress *nmr1* in cells that only express *nmr2*.

Nmr1 and Nmr2 mark a unique population of interneurons

Next, we sought to determine the specific subtype of cells that express *nmr1* and *nmr2* in the CNS. We first tested whether glia and/or motor neurons express *nmr1* and *nmr2* by looking for co-expression between Nmr1/Nmr2 and molecular markers of the glial (Repo) or motor neuron fate (Eve, Hb9, Zfh-1) (Layden et al., 2006; Lundell et al., 2003; Novotny et al., 2002; Isshiki et al., 2001; Skeath and Doe, 1998). Repo and Nmr1/Nmr2 label largely complementary sets of cells in the CNS with no co-expression observed between Repo and Nmr1 and only one lateral glial cell expressing Nmr2 (Fig. S2). Similarly, Nmr1/Nmr2 exhibit minimal co-expression with Eve and Hb9 (Fig. 9H,I; data not shown for Hb9), which together label most motor neurons (Broihier and Skeath, 2002; Schmid et al., 1999; Landgraf et al., 1997; Sink and Whitington, 1991). No Hb9-positive neurons express Nmr1 or Nmr2, while only one Eve-positive U/CQ neuron weakly expresses Nmr2 (Fig. 9H). Zfh-1 is expressed in most motor neurons and some interneurons in abdominal segments (Skeath and Doe, 1998; Layden et al., 2006). Three Zfh-1 and Nmr2 co-expressing neurons are located ventrally within each hemisegment (Fig. 5A–C) and one dorsal Zfh-1 neuron co-expresses Nmr2 and Nmr1 within each hemisegment (Fig. 5D–F and Fig. 5J–L). Since the vast majority of Zfh-1-positive neurons do not co-express Nmr2 or Nmr1, these analyses indicate that most *nmr1* and *nmr2*-expressing cells in the CNS are interneurons.

To determine if Nmr1/Nmr2-positive neurons identify any well-characterized interneurons, we carried out additional double-labeling studies and assayed for co-expression between Nmr1/Nmr2 and Dachshund (Miguel-Aliaga et al., 2004), Engrailed (Bossing and Brand, 2006), or Apterous (Lundgren et al., 1995), each of which labels discrete sets of interneurons. We find that Nmr1 and Nmr2 are expressed in mutually exclusive sets of interneurons relative to each of these markers. Thus, Nmr1 and Nmr2 identify subsets of interneurons largely distinct from those that have previously been characterized.

Our results contrast with prior work on Nmr1/Nmr2, which indicated that these factors are expressed in the Engrailed-positive ventral and dorsal channel glia as well as in the Engrailed-expressing progeny of the midline neuroblast (Buescher et al., 2006). However, we do not observe expression of Nmr1 or Nmr2 in any Engrailed expressing neurons/glia in the CNS (Fig. S3). Thus, H15-lacZ expression in these cells likely arises due to earlier transient expression of *nmr1/nmr2* within neuroblast 7-4, the progenitor of the channel glia, and the median neuroblast (Buescher et al. 2006). Consistent with *nmr2* acting during early steps of CNS development to regulate the development and differentiation of cells in these two lineages, we confirmed that loss of *nmr2* function leads to the disorganization and occasional loss of the channel glia as well as the Engrailed-positive progeny of the midline neuroblast, as observed previously by Buescher et al. (2006).

Nmr1 and Nmr2 are expressed within interneurons derived from NB lineages 2-2, 6-1, and 6-2

We used lineage tracing to begin to map the neuroblast lineages that give rise to Nmr1-positive neurons. This technique also allowed us to visualize the axon trajectories of Nmr1-positive neurons. Since we demonstrate that Nmr2 labels all Nmr1-positive neurons (Fig. 3I), we are essentially mapping the neuroblast lineages giving rise to subsets of Nmr1 and Nmr2 co-expressing neurons. Briefly, we used a modified version of the FLP/FRT lineage tracing system (Harrison and Perrimon, 1993; see Methods) to create random clones of *tau-myc*-GFP reporter gene expression. We screened lineage clones within the embryonic CNS for those that contained at least one Nmr1-positive neuron, and then identified the parental neuroblast of Nmr1-positive neurons by comparing the location and morphology of our lineage clones to the location and morphology of identified neuroblast lineages as determined by Dil-labeling (Schmid et al., 1999; Schimdt et al., 1997; Bossing et al., 1996). Through this approach we identified the parental neuroblast lineage for three sets of Nmr1-positive neurons (6 neurons) via the analysis of multiple independent clones for each set of neurons (summarized in Fig. 7). For example, two clones in abdominal segments contained the same medially-positioned Nmr1-positive neuron (Fig. 6C). This interneuron resides within a large clone of approximately 12–15 interneurons and 1 motor neuron (Fig. 6A–D). The interneurons project axons across the anterior commissure and then project axons anteriorly and posteriorly along the medial and lateral Fas-II-positive longitudinal fascicles (Fig. 6D). The motor neuron projects its axon out the segmental nerve (Fig. 6D). The morphology of these clones together with their relative location in a segment most closely match those identified for NB 2-2 by DiI labeling studies (Schmid et al., 1999). Thus, we place this medial Nmr1-positive neuron within the 2-2 lineage (Fig. 7).

We used similar criteria to place two other sets of Nmr1-positive neurons in defined lineages. We identified two clones in abdominal segments comprised of ~10 interneurons that contain both a medial and intermediate positioned Nmr1-positive neuron (Fig. 6E,F). The most striking feature of these clones is the cascade of ipsilateral axon branches that extend posteriorly and span at least one segment (Fig. 6F,G). The ventral region of these clones contains two to three large interneurons that project axons across the posterior commissure and then turn anteriorly (Fig. 6G). These morphological features most closely match those defined by DiL labeling for the 6-1 lineage. Thus, two specific Nmr1-positive neurons are derived from this lineage (Fig. 7) (Schmid et al., 1999; Bossing et al., 1996).

Finally we identified four clones each of which labeled the same three Nmr1-positive neurons in abdominal segments. These neurons are located in abdominal segments and their clones contain 12–18 interneurons (Fig. 6H–K) that extend two contralateral axon bundles across the posterior commissure and a small ipsilateral axon branch. In one clone the short ipsilateral axon branch formed a prominent arch peaking towards the anterior end (Fig. 6K, *inset*). This arching phenotype has been described as a distinguishing feature of a small number of NB lineages including NB 6-2. Based on a close match between the morphology and relative location of these clones to those defined for NB 6-2, we place these three Nmr1-positive neurons within the 6-2 lineage (Fig. 7) (Schmid et al., 1999; Bossing et al., 1996).

Medial Nmr1 and Nmr2 subsets of neurons are GABAergic

To gain insight into the potential function of Nmr1- and Nmr2-positive neurons, we assayed whether Nmr1 and Nmr2 interneurons express specific classes of neurotransmitters. While Nmr1 and Nmr2 neurons do not express serotonin (Taghert and Goodman, 1984) or the vesicular transporter for glutamate (DVGLUT) (Daniels et al., 2004) (data not shown), a single medial Nmr1/Nmr2-positive neuron strongly expresses vesicular glutamic acid decarboxylase (Gad) (Fig. 8). Gad is the biosynthetic enzyme for γ-aminobutyric acid (GABA) (Jackson et

al., 1990), an abundant inhibitory neurotransmitter expressed throughout the embryonic CNS. Thus at least one medial Nmr1/Nmr2-positive interneurons is competent to synthesize GABA and to potentially modulate the activity of neural circuits.

nmr2 negatively regulates *even-skipped* expression

Our loss-of-function studies indicate that *nmr1* and *nmr2* are necessary to repress *eve* expression in a few cells in the CNS (Fig. 1E), thus we focused our attention on exploring the negative regulatory relationship between *nmr2* and *eve.* Specifically, we asked whether *nmr1* and *nmr2* are sufficient to repress *eve* expression. To do this we used the *Gal4/UAS* system and *Elav*-Gal4 to drive *nmr2* or *nmr1* expression in all post-mitotic neurons of the CNS and assayed the effect on *eve* expression (Fig. 9). Ubiquitous misexpression of *nmr2* causes a significant reduction of *eve* expression in all Eve-positive neurons (Fig. 9B). In contrast, misexpression of *nmr1* causes a very mild reduction of Eve expression in the CNS (Fig. 9C). Thus, *nmr2* and to a lesser extent *nmr1* can inhibit *eve* expression in some, but not all, CNS neurons. The mild phenotype observed upon generalized *nmr1* expression is not attributable to low level Nmr1 protein expression, as *ElavGAL4::UAS-nmr1* embryos display high levels of Nmr1 protein throughout the CNS (data not shown).

In a reciprocal manner, we determined whether *eve*, a known transcriptional repressor, can inhibit the expression of *nmr1* and *nmr2*. We misexpressed *eve* in all post-mitotic neurons using the *Gal4/UAS* system and assayed the effect on Nmr1 or Nmr2 expression. Ubiquitous *eve* expression in the CNS causes a dramatic reduction of *nmr2* and *nmr1* expression in the CNS (Fig. 9D–G). However, *eve* appears sufficient but not necessary to inhibit *nmr1* and *nmr2* expression since *eve^{ID19}*, a temperature-sensitive allele of *eve* (Nusslein-Volhard et al., 1985), exhibits normal numbers of Nmr1- and Nmr2-positive neurons (data not shown). As noted, *eve* and *nmr1/nmr2* are expressed in mutually exclusive sets of neurons in the CNS (Fig. 9H,I). Thus, the mutually cross-repressive actions of *nmr2* and *eve* likely help maintain their complementary expression patterns in the CNS. In early embryonic CNS development, we also observed that the loss of *nmr2* resulted in the loss of *eve* expression in the aCC and pCC neurons in predominantly odd-numbered segments (Fig. 1F). Buescher et al. (2006) reported similar observations and attributed this loss of Eve-positive aCC and pCC neurons to the failure of their neuroblast precursor, NB 1-1, to form in odd-numbered segments (Buescher et al., 2006).

nmr1 and *nmr2* interact genetically with *nkx6,* a key regulator of eve

dhb9^{kk30} and *nkx6^{D26}* are two homeodomain transcription factors known to inhibit *eve* expression in the CNS (Broihier et al., 2004; Broihier and Skeath, 2002). Thus, we constructed embryos doubly mutant for *nmr1* or *nmr2* in combination with *dhb9^{kk30}* or *nkx6^{D26}* to determine whether *nmr1* and *nmr2* act with *dhb9^{kk30}* or *nkx6^{D26}* to inhibit eve expression. *nkx6^{D26}* mutants exhibit a wild-type *eve* expression pattern while *dhb9^{kk30}* single mutants exhibit 2–3 ectopic Eve-positive neurons (Broihier and Skeath, 2002). Embryos doubly mutant for *nmr1* or *nmr2* and *dhb9^{kk30}* do not enhance the *eve* CNS phenotype over each single mutant (data not shown). However, embryos doubly mutant for *nkx6^{D26}* and *nmr2* exhibit an increase of ectopic Eve-positive neurons in the lateral CNS relative to either single mutant (Fig. 10A–C). The increase in Eve-positive neurons peaks around stage 12 and diminishes at later stages of development (Fig. 10D). These embryos also exhibit gross defects in the architecture of the CNS, which preclude an ability to assess the exact number of Eve-positive neurons beyond stage 14. We observe co-expression of Nkx6 with Nmr2 (Fig. 9E–G) and Nmr1 (Fig. 10H–J) in a single medial neuron within each hemisegment at later stages of development (stages 13–15). Thus, Nkx6 and Nmr2 are unlikely to act at the same time in the same cells to repress *eve* in a cell autonomous manner. Rather Nkx6 and Nmr2 may act together during earlier stages of development, or sequentially in the same cells or cell lineage, to inhibit *eve* expression;

alternatively these factors may act in the same cells or within the same lineage to initiate specific interneuronal signaling events that inhibit *eve* expression in adjacent cells.

DISCUSSION

The work presented here identifies *nmr1* and *nmr2* as neuronal fate determinants and confirms that *nmr1* and *nmr2* act in a partially redundant manner to repress *eve* expression in a small number of neurons in the CNS (Buescher et al., 2006). In addition, it describes the generation of Nmr1 and Nmr2 specific antibodies, which reveal dynamic and cell-type specific expression profiles of these factors in the CNS, heart and epidermis, while also providing evidence for post-transcriptional regulation of *nmr1*. Through the use of these molecular reagents, we reveal that most Nmr1/Nmr2-positive neurons define a novel class of interneurons and map a subset of these cells to defined CNS lineages. Finally, we show that *nmr1* and *nmr2* exhibit cross-repressive interactions with *eve* in the CNS, and that *nmr2* acts with $nkx6^{D25}$ to repress the CNS expression of *eve*. Below we discuss our results in light of the known genetic requirements for *nmr1* and *nmr2* function in the CNS and embryo, and highlight particular sequence characteristics of these loci that suggest *nmr1* may be in the process of undergoing non-functionalization.

Our detailed studies on the expression of Nmr1 protein and RNA suggest that the *nmr1* transcript is subject to post-transcriptional regulation during early stages of embryogenesis. For example, while the Nmr2 protein pattern faithfully recapitulates its RNA profile, a significant temporal delay exists between the onset of *nmr1* transcription and the initial detection of Nmr1 protein in a few cells in the CNS (Fig. 2). Precedence exists for temporal regulation of mRNA translation in the CNS. For example, the *nerfin* transcript, which codes for a zinc-finger domain containing protein, is expressed in neuroblasts, GMCs and neurons but Nerfin protein is detected only in GMCs and neurons due to the action of multiple microRNAs acting on the *nerfin* 3′UTR (Kuzin et al., 2005,2007). In this context it is interesting to note that the *nmr1* transcript is predicted to contain a short 3′ UTR and a long 538 bp 5′ UTR (Fig. S4). The 5′ UTR contains a number of conserved sequence blocks towards its 5′ end. While these sequences are not target sites for characterized microRNAs (Enright et al., 2003;Stark et al., 2003), they may represent motifs through which RNA-binding proteins or uncharacterized microRNAs mediate the translational silencing of *nmr1*. In the future it will be important to define the relevant sequence motifs in the *nmr1* transcript in order to begin to define the cis- and trans-acting mechanisms responsible for its post-transcriptional regulation.

We should note that it is formally possible that our Nmr1 specific antibodies fail to recognize a protein isoform expressed during early embryogenesis. However, we view this as unlikely due to the design of the antibody and RNA probes. We generated Nmr1 specific antibodies against a protein fragment that contained the first two exons, and a small region of the third exon, of *nmr1* (see Methods), while an anti-sense RNA probe generated from exons 1 and 2 detects the entirety of the *nmr1* transcript pattern. Thus, we believe the simplest interpretation of our data is that *nmr1* is subject to post-transcriptional regulation during early but not late embryogenesis.

Genetic studies reveal a key role for *nmr2* during segmentation, neuroblast formation and specification, heart development and neuronal specification (Buescher et al., 2006; Reim et al., 2005; Qian et al., 2005, Miskolczi-McCallum et al., 2005; Buescher et al., 2004). In contrast, loss of *nmr1* exhibits no phenotype on its own. In fact, *nmr1* has only been shown to contribute to *nmr2* function due to a modest enhancement of the severity of a subset of *nmr2* phenotypes observed in embryos doubly-mutant for *nmr1* and *nmr2* (Buescher et al., 2006; Qian et al., 2005). The clear differences in the genetic requirements for *nmr2* and *nmr1* during embryonic development have been difficult to reconcile with the nearly identical RNA

expression profiles of these factors (Buescher et al., 2006; Qian et al., 2005). In this context, our characterization of the protein expression profiles of Nmr1 and Nmr2 provide a molecular explanation for the functional requirement of *nmr1* during a subset of *nmr2*-dependent events. For example, we observe co-expression of Nmr1 and Nmr2 protein in all cardioblasts and many CNS neurons, consistent with *nmr1* acting in a partly redundant manner to *nmr2* in these tissues. In contrast, we do not observe Nmr1 protein in neuroblasts, consistent with the lack of a genetic requirement for *nmr1* during neuroblast formation and specification. Thus, *nmr1* appears to contribute to *nmr2* function, albeit weakly, in most or all cells that co-express the two proteins.

The genetic requirement for *nmr1* during segmentation remains difficult to explain, as we do not observe Nmr1 protein in segment polarity stripes during early embryogenesis. Nmr1 and Nmr2 proteins are detected in narrow stripes of ventral ectodermal cells during late embryogenesis (Fig. 2). Thus, *nmr2* and *nmr1* function in these cells may help define the final cuticular pattern of the *Drosophila* embryo (Buescher et al., 2006). Consistent with this model, interactions between the Wingless, Notch and EGF signaling pathways in late stage embryos is critical to establish the final cuticular pattern of the *Drosophila* embryo (Walters et al., 2005; Alexandre et al., 1999), and *nmr2* has been shown to mediate its effect on segmentation by negatively regulating *wingless* expression (Buescher et al, 2004). Alternatively, the enhancement of the *nmr2* segmentation phenotype observed in *nmr1/nmr2* double mutant embryos could arise due to heterozygosity for one or more genes, as overlapping deficiencies were used to construct *nmr1/nmr2* double-mutant embryos.

Our work, as well as prior studies, reveals that *nmr1* and *nmr2* act together to control the specification of *eve*-expressing neurons in the CNS (Fig. 1; Beuscher et al., 2006). However, due to the similar RNA expression patterns of *nmr1* and *nmr2* in neuroblasts and neurons, prior studies could not define whether *nmr1* and *nmr2* function in post-mitotic neurons and/or neural precursors to control the development of these cells. Our observation that Nmr1 protein is expressed in post-mitotic neurons, but not in neural precursors, in the CNS provides strong evidence that *nmr1* and *nmr2* act in post-mitotic neurons to regulate *eve* expression. This late function for *nmr1* and *nmr2* does not preclude *nmr2* also acting earlier in the lineage to help mediate this event, a possibility supported by the more severe nature of the *nmr2* phenotype with respect to *eve*-positive neurons. Nonetheless, our work reveals a functional role for *nmr1* and *nmr2* in post-mitotic neurons, and thus places these factors in the combinatorial code of transcription factors known to govern the specification and differentiation of post-mitotic neurons. Moreover, as *nmr1* and *nmr2* are expressed in many neurons, they likely play a much broader role during neuronal differentiation than simply regulating *eve* expression.

nmr1 and *nmr2* are paralogs and arose as the result of a duplication of an ancestral Tbx-20 gene. Thus, *nmr1* and *nmr2* likely exhibited essentially identical gene regulation in the ancestral species in which they first appeared. However, in *Drosophila* the regulation of *nmr1* and *nmr2* display clear differences, with *nmr2* exhibiting significantly higher RNA expression, and *nmr1* being specifically subjected to post-transcriptional regulation. Inspection of the *nmr1* and *nmr2* loci for key regulatory sequences that promote gene transcription and translation suggests greater selective pressure acts on *nmr2* to retain such motifs than on *nmr1*. For example, the *nmr2* locus contains conserved motifs that match the consensus sequences for the Initiator and downstream promoter element (DPE), and for the *Drosophila* Kozak sequence at its translation start site (Kutach and Kadonaga, 2000; Cavener, 1987). In contrast, we were unable to find matches to the consensus sequences for an Initiator, DPE or TATA box at the predicted *nmr1* promoter, while the *nmr1* translation start site contains an imperfect match to the Kozak sequence. We did identify a consensus match to an Initiator motif 62 bp upstream of the predicted start of transcription, after scanning 200 bp upstream and downstream of the predicted promoter (Fig. S4). While this sequence is conserved in *drosophilids*, it is not flanked by a consensus TATA box or DPE. Thus, heightened expression of *nmr2* relative to *nmr1* may

arise, at least in part, due to retention of key regulatory motifs critical for appropriate gene transcription and translation. Together with the paralogous nature of *nmr1* and *nmr2*, these observations indicate that *nmr1* may be undergoing non-functionalization – a process often thought to occur to one of two duplicate genes when neither gene assumes a new function following duplication (Prince and Pickett, 2002).

Conclusion

nmr1 and *nmr2* are members of the combinatorial code of transcription factors that govern neuronal specification and differentiation. However, extensive expression studies between Nmr1/Nmr2 and many transcription factors known to control motor neuron and interneuron fates revealed little co-expression between these factors (Fig. 5, data not shown). Thus, significant additional work is needed to decipher the functions of *nmr1/nmr2* in neurons, and place their action within the complex genetic regulatory hierarchy that governs neuronal differentiation. In this context, our expression and lineage studies, which identify a set of Nmr1/Nmr2-positive neurons as GABAergic, and map a handful of Nmr1-expressing neurons to defined CNS cell lineages, represent a step towards establishing the descriptive foundation required to dissect in detail the CNS functions of *nmr1* and *nmr2*. Clearly, it will be crucial to identify other transcription factors expressed with Nmr1/Nmr2 in neurons, and to define the target genes through which Nmr2/Nmr1 mediate their effect on neuronal differentiation. Thus, we expect the integration of traditional embryological methods with modern genome-wide searches for transcription factor targets to begin to illuminate how Nmr1/2, and other transcription factors, direct distinct sets of neurons to differentiate from each other.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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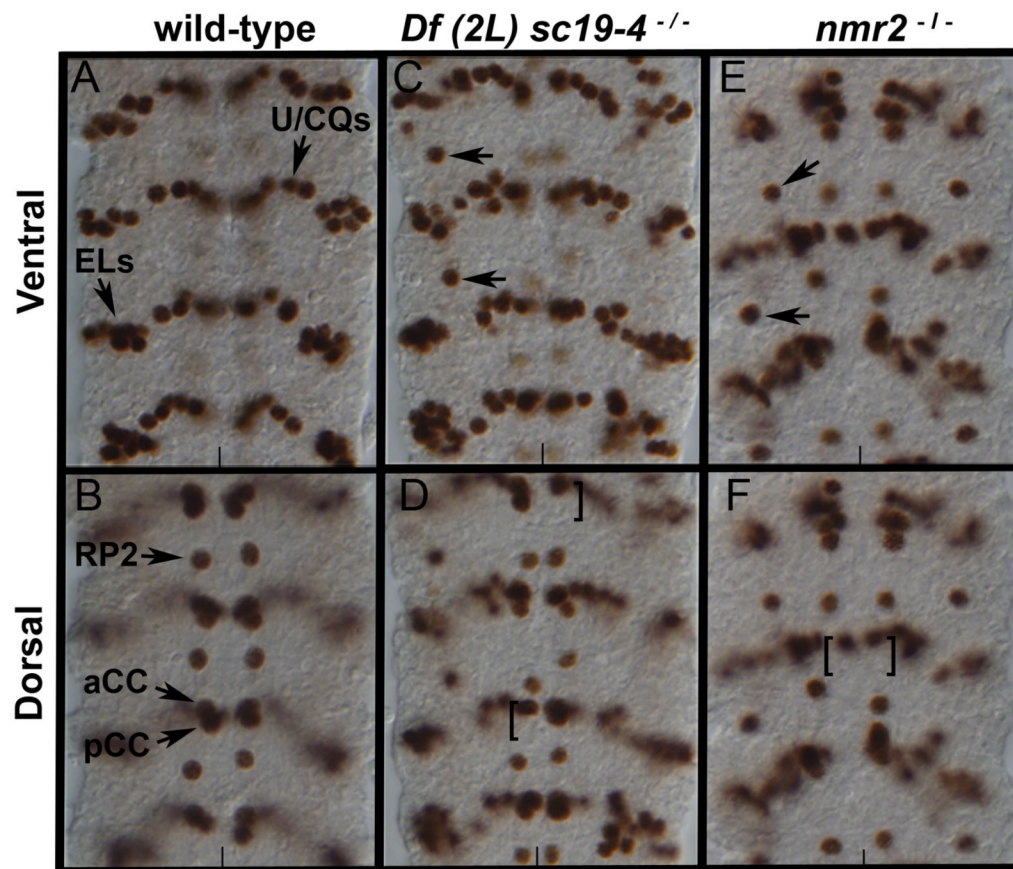
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Figure 1.
nmr2 represses *eve* expression in the *Drosophila* nerve cord.
Stage 15 wild-type (A,B), *Df(2L)sc19-4*$^{-/-}$ (C,D), and *nmr2*$^{-/-}$ (E,F) mutant nerve cords stained for Eve. (A,B) In wild-type nerve cords Eve labels 5–6 U/CQ, 10 EL, RP2, and aCC/pCC neurons as indicated. (C) In *Df(2L)sc19-4*$^{-/-}$ mutant embryos, ectopic Eve-positive neurons arise laterally (*arrows*) and (D) there is a decrease in the presence of Eve-positive aCC/pCC neurons (*brackets*). (E,F) *nmr2*$^{-/-}$ mutant embryos (*mid*^{GA174}) exhibit the same phenotype as that observed in *Df(2L)sc19-4*$^{-/-}$ embryos. Panels A, C, E – ventral views and panels B, D, F – dorsal views of the *Drosophila* nerve cord; the vertical line marks the midline.
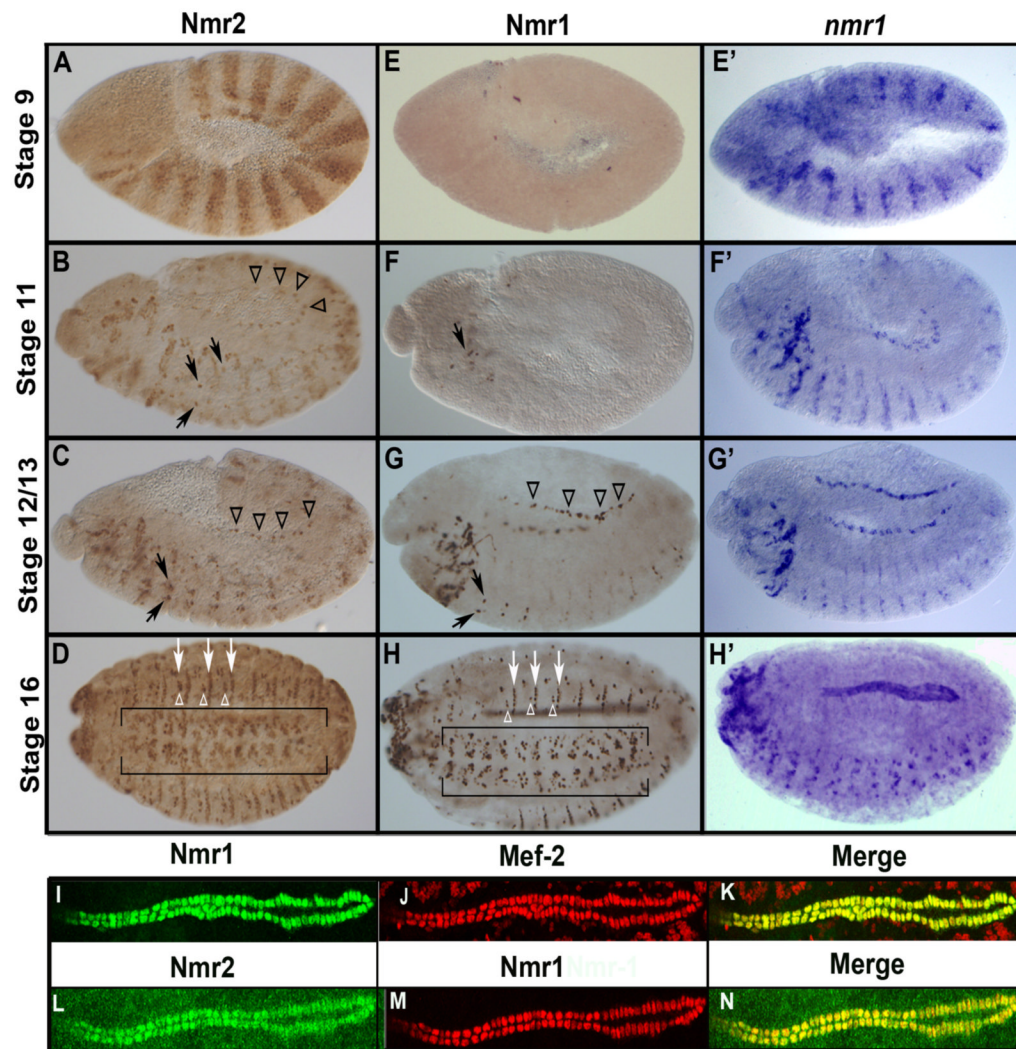
Figure 2.
Nmr1 and Nmr2 protein expression during embryogenesis.
Nmr2 (A–D,L), Nmr1 (E–H,I) and *nmr1* mRNA transcript (E′–H′) expression in wild-type embryos at progressively later stages of development (A) Stage 9 embryos express Nmr2 in segment polarity stripes. (B) During early stage 12, Nmr2 is expressed in subsets of neuroblasts and their progeny in the CNS (*arrows*) as well as in heart precursor cells in the dorsal mesoderm (*arrowheads*). (C) During late stage 12, Nmr2 is expressed prominently in a medial pair of CNS neurons in each segment (*arrows*) and is also expressed in heart precursor cells (*arrowheads*). (D) By stage 16 Nmr2 is expressed in many neurons in the CNS (*brackets*), narrower transverse stripes of ectodermal cells (*white arrows*), and clusters of ectodermal cells (*white arrowheads*) between these stripes. (E) Stage 9 embryos do not express Nmr1 protein while (E′) *nmr1* transcripts are clearly detected in segment polarity stripes. (F) Nmr1 protein is first detected during early stage 12 in a few cells in the cephalic region (*arrow*), (F′) while similarly staged embryos express *nmr1* transcripts in segment polarity stripes, neuronal precursors and neurons. (G, G′) By stage 13, Nmr1 protein and RNA are expressed in identical patterns of CNS neurons (*arrows*) and heart precursors in the mesoderm (*arrowheads*). (H, H′) By stage 16, Nmr1 protein and RNA are co-expressed in many neurons in the CNS (H, *brackets*), all cardioblasts as well as in narrow stripes of ectodermal cells (H, *white arrows*). (I–N) High magnification views of the *Drosophila* heart at stage 16 show that Nmr1 (I,

green; M, *red*) and Nmr2 (L, *green*) are co-expressed with Mef-2 (J, *red*) in all cardioblasts (K,N). Whole mounts in the lateral orientation; Anterior, *left*; Posterior, *right*; Dorsal, *up*; Ventral, *down*.
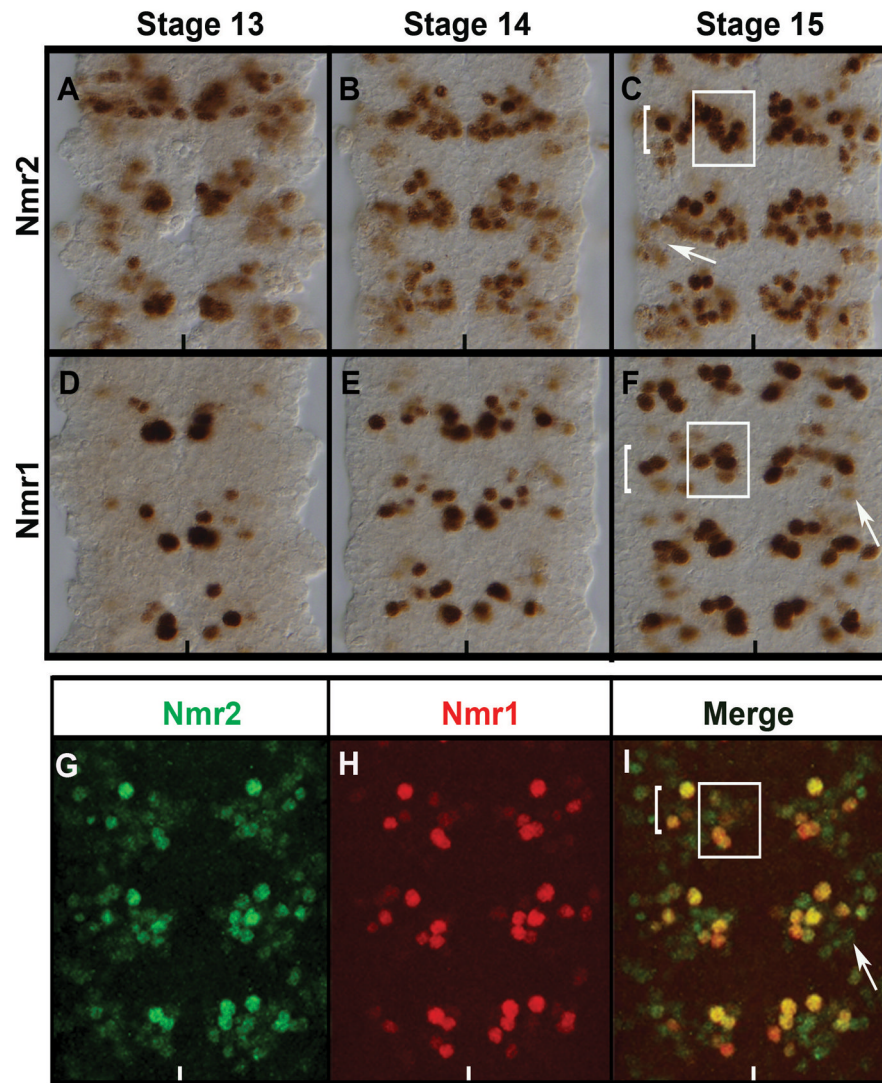
Figure 3.
Co-expression of Nmr2 and Nmr1 in the *Drosophila* CNS.
Stage 13 (A,D,) stage 14 (B,E), and late stage 15 (C,F,G–I) wild-type embryos labeled for
Nmr2 (A–C, G, I), Nmr1 (D–F, H, I) and co-labeled for Nmr1 and Nmr2 (I). (A) During stage
13 Nmr2 is expressed strongly in a cluster of medial neurons and weakly in a group of lateral
neurons. (B) By stage 14, Nmr2 is expressed strongly in a cluster of approximately 10–15 cells
per hemisegment with enhanced expression in the medial and intermediate regions of the nerve
cord and diminished expression laterally. (C) This pattern of expression is prominently defined
in stage 15 where Nmr2 is expressed strongly in a cluster of approximately 8 medial neurons
(*box*) and 8 lateral neurons (*bracket*). Nmr2 is also expressed weakly in about 5–6 neurons
within the lateral cluster (*arrow*). (D) During stage 13 Nmr1 is expressed strongly in a small
number of medial neurons and expressed weakly in a few neurons in the intermediate region.
(E) By stage 14, Nmr1 expression remains high in medial neurons and expression is now
observed in additional neurons in the intermediate regions of the CNS. (F) By stage 15, Nmr1
is expressed in many discrete sets of neurons in the CNS: a medial cluster of approximately
3–5 neurons (*box*), a lateral cluster of 7–8 neurons (*bracket*), and a few dispersed lateral neurons

(*arrow*). (G–I) Double-labeling for Nmr2 (*green*) and Nmr1 (*red*) in stage 15 embryos indicates that a subset of Nmr2-positive neurons express Nmr1 (*merge*).
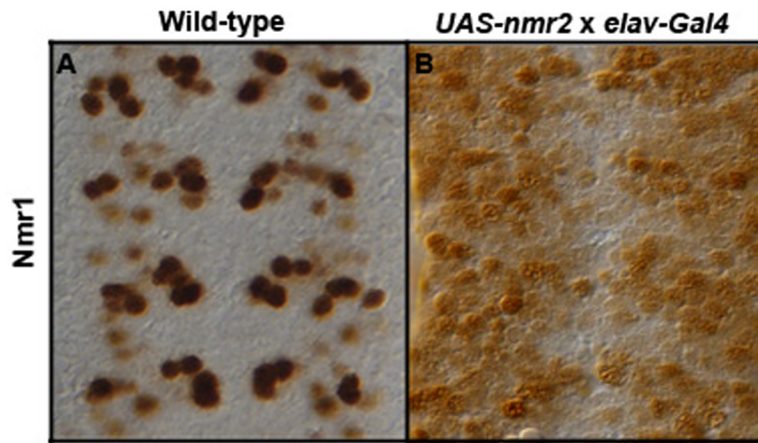
Figure 4.
Nmr2 enhances Nmr1 expression.
Stage 15 embryonic nerve cords stained for Nmr1. (A) Wild-type expression pattern of Nmr1 in the ventral nerve cord. (B) Misexpression of Nmr2 in all post-mitotic neurons (*ElavGAL4::UAS-nmr2*) significantly enhances Nmr1 expression throughout the nerve cord.
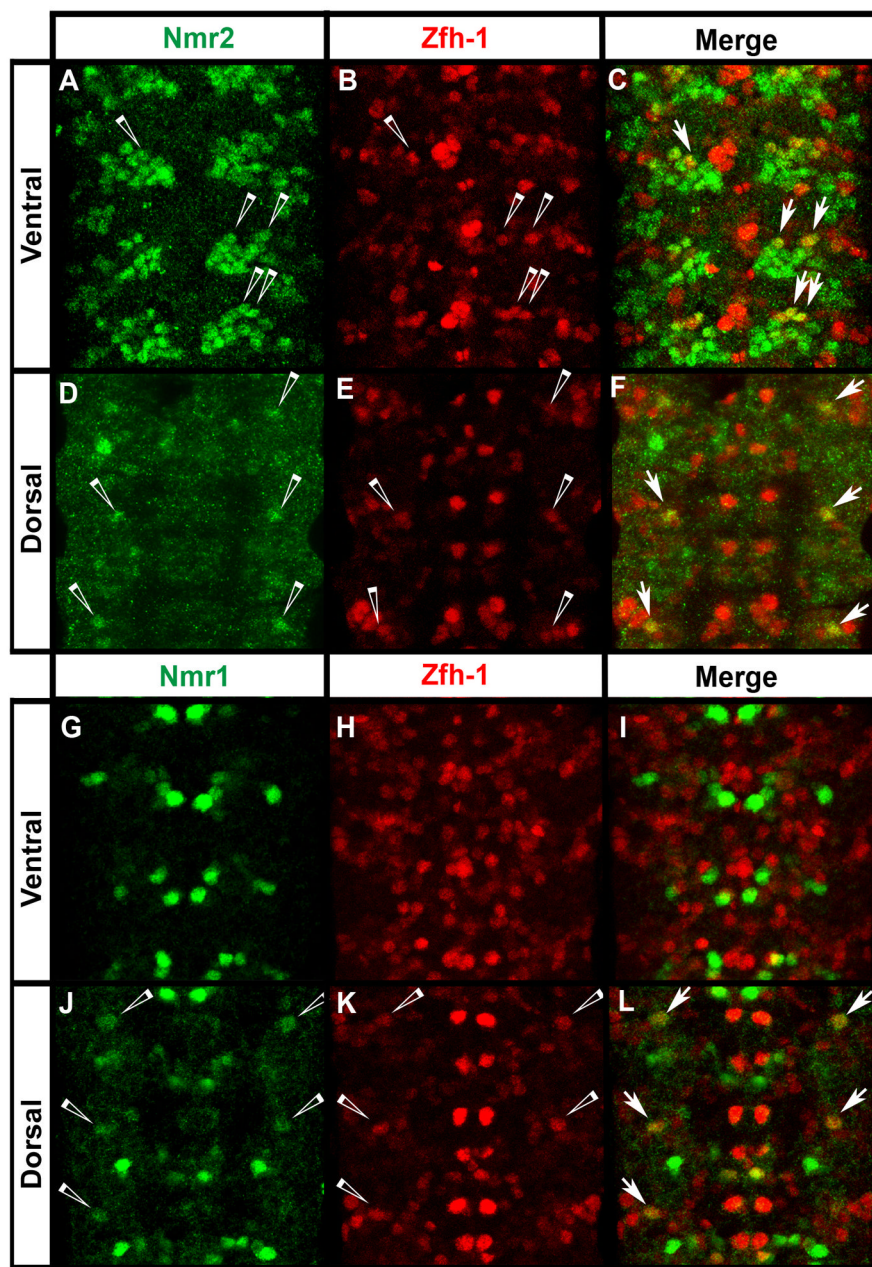
Figure 5.
Nmr1 and Nmr2 are detected in a few Zfh-1-expressing motor neurons.
(A–L) Wild-type stage 15 embryos stained for Zfh-1 (*red*) and Nmr2 (*green*) or Nmr1
(*green*). (A–C) In the ventral region of the CNS, Nmr2-positive and Zfh-1-positive neurons
are co-expressed in approximately 2–3 neurons in the medial Nmr2-expressing cluster per
hemisegment (*merge*, *arrows*). (D–F) Dorsally in the CNS, a single lateral neuron co-expresses
Nmr2 and Zfh-1 per hemisegment (*merge*, *arrows*). (G–I) In the ventral region of the CNS, no
cells co-express Nmr1 (*green*) and Zfh-1 (*red*). (J–L) In the dorsal region of the CNS, one
lateral neuron weakly co-expresses Nmr1 and Zfh-1 per hemisegment (*merge*, *arrows*).
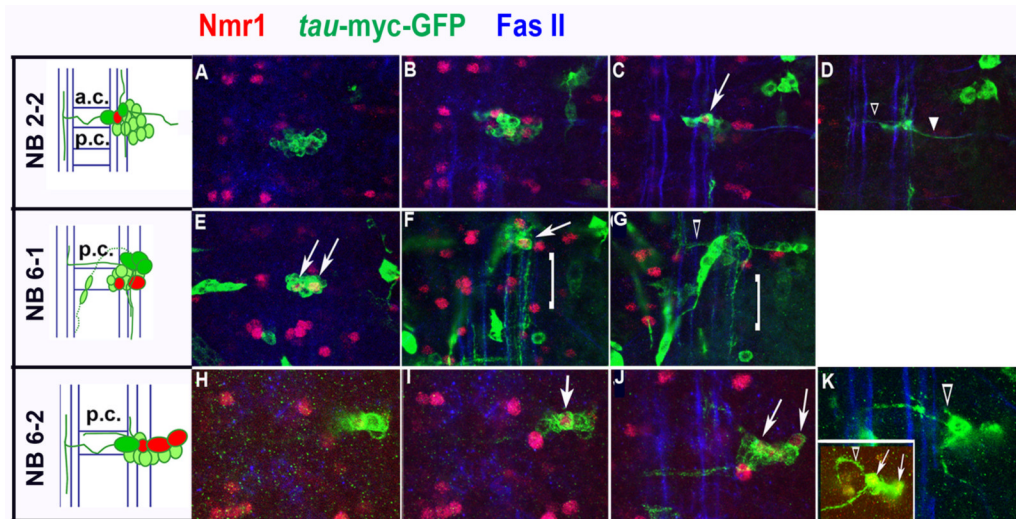
Figure 6.
A subset of Nmr1/−2 expressing interneurons derived from neuroblast lineages 2-2, 6-1, and 6-2.

Cartoons detail the positions and axon morphologies of Nmr1-positive interneurons (*red*) within identified NB clones in the CNS of wild-type, stage 15–17 embryos. The most ventral neurons are shaded *dark green*. Neurons are drawn in relation to the anterior and posterior commissures (AC/PC) and the longitudinal connectives (*blue*). From top (dorsal) to bottom (ventral) the compressed confocal images represent ~1 μM thick sections of Nmr1-positive clones. The nerve cord is triple-labeled for Nmr1 (*red*), axons (*green*, anti-GFP), and longitudinal connectives (*blue*, anti-FasII). (A–D) A lineage clone that contains one Nmr1-positive neuron (*arrow*) extends axons contralaterally (D, *open arrowhead*) and a motor neuron axon branch (D, *closed arrowhead*). (E–G) A lineage clone that contains two Nmr1-positive interneurons (*white arrows*) extends a collateral axon branch (G, *open arrowheads*) and a cascade of ipsilateral axons (F,G, *brackets*). (H–K) A lineage clone that contains three Nmr1-positive neurons (*arrows*) extends a small ipsilateral axon branch (J, *open arrowhead*) as well as anterior and posterior collateral axons. Occasionally, NB 6-2 clones exhibit an ipsilateral axon branch with a prominent arch and this axonal phenotype was detected in a similar clone (K, *inset*).
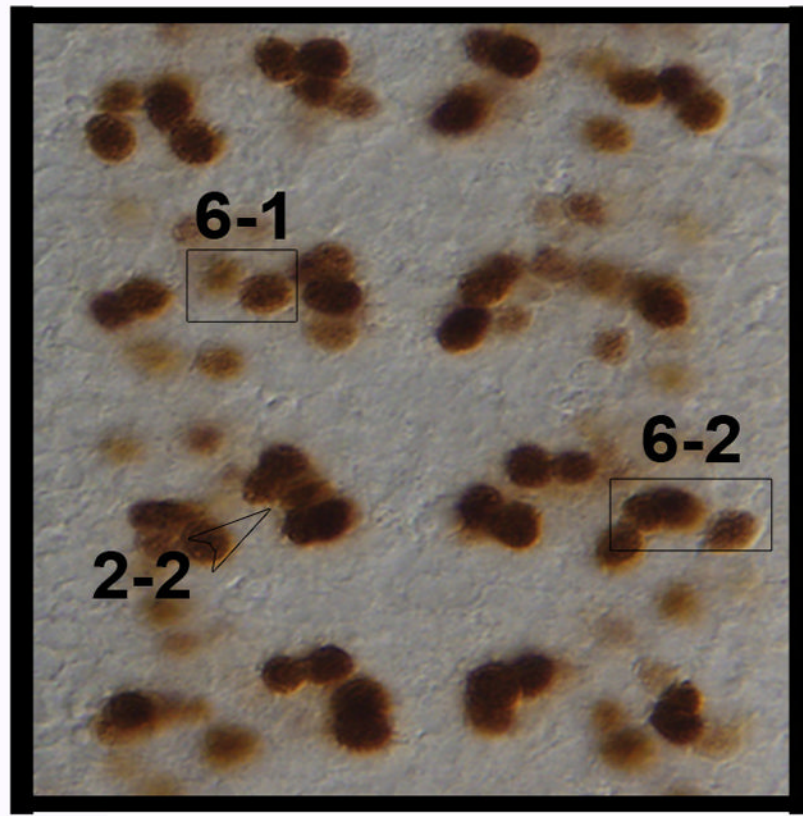
Figure 7.
Expression pattern of Nmr1-positive neurons derived from NBs 2-2, 6-1 and and 6-2.
The *boxed* sets of neurons highlight the Nmr1-positive neurons derived from NB lineages 6-1
(*left*) and 6-2 (*right*). An *open arrowhead* points to the Nmr1-positive neuron derived from the
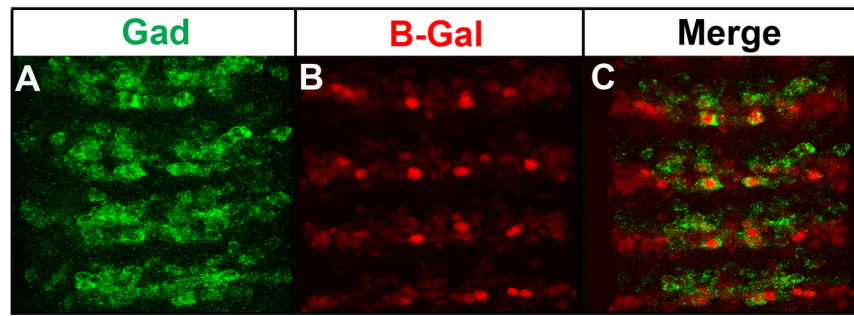NB 2-2 lineage.

Figure 8.
A subset of medial Nmr2 and Nmr1 interneurons co-express glutamic acid decarboxylase.
(A–C) Stage 15 nerve cords from the H15-LacZ reporter line, which labels all Nmr1-positive
and Nmr2-positive neurons. (A) Staining for glutamic acid decarboxylase (Gad) specifically
identifies GABAergic populations of interneurons (*green*). (B) Staining for β-Galactosidase
(B-Gal) marks all Nmr1 and Nmr2 positive neurons (*red*). (C) A prominent, medial pair of
previously identified Nmr1 and Nmr2 co-expressing neurons detected within each segment of
the nerve cord accumulates a high level of vesicular Gad at the plasma membrane (*arrows*,
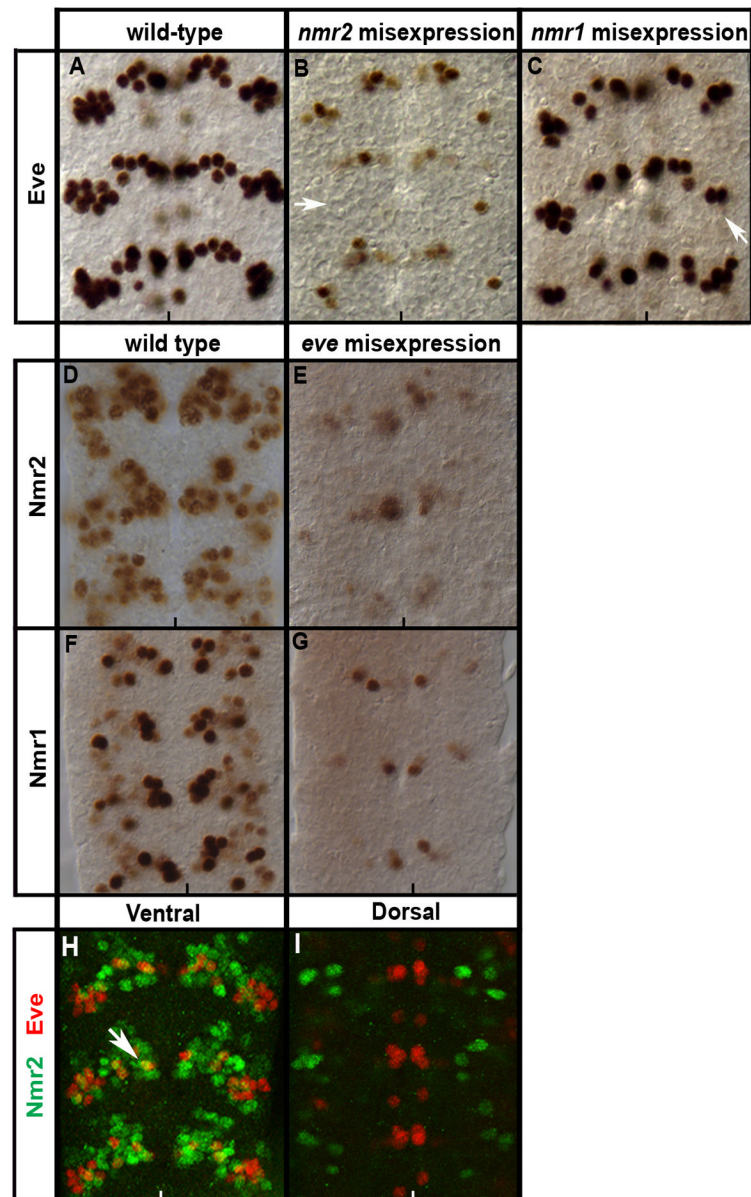*merge*).

Figure 9.
Nmr1 and Nmr2 repress Eve expression.
Wild-type stage 15 embryonic nerve cords stained for Eve (A–C, H,I), Nmr2 (D,E,H,I), and
Nmr1 (F,G). (A) Wild-type expression pattern of Eve within the ventral region of the CNS.
(B) Misexpression of Nmr2 in all post-mitotic neurons (*ElavGAL4::UAS-nmr2*) significantly
reduces Eve expression in all Eve-positive neurons (*arrow* points to location of ELs). (C)
Misexpression of Nmr1 throughout the CNS (*ElavGAL4::UAS-nmr1*) causes a mild decrease
of Eve expression (*arrow* points to ELs). (D,F) Wild-type expression pattern of Nmr2 (D) and
Nmr1 (F). (E,G) Expression of Eve throughout the CNS (*ElavGAL4::UAS-eve*) results in a
significant decrease of Nmr2 (E) and Nmr1 (G). (H,I) Eve (*red*) exhibits a predominantly
mutually exclusive expression pattern with Nmr2 (*green*) in the ventral (H) and dorsal (I)
regions of the nerve cord while a single Eve-positive U/CQ neuron (H) co-expresses Nmr2
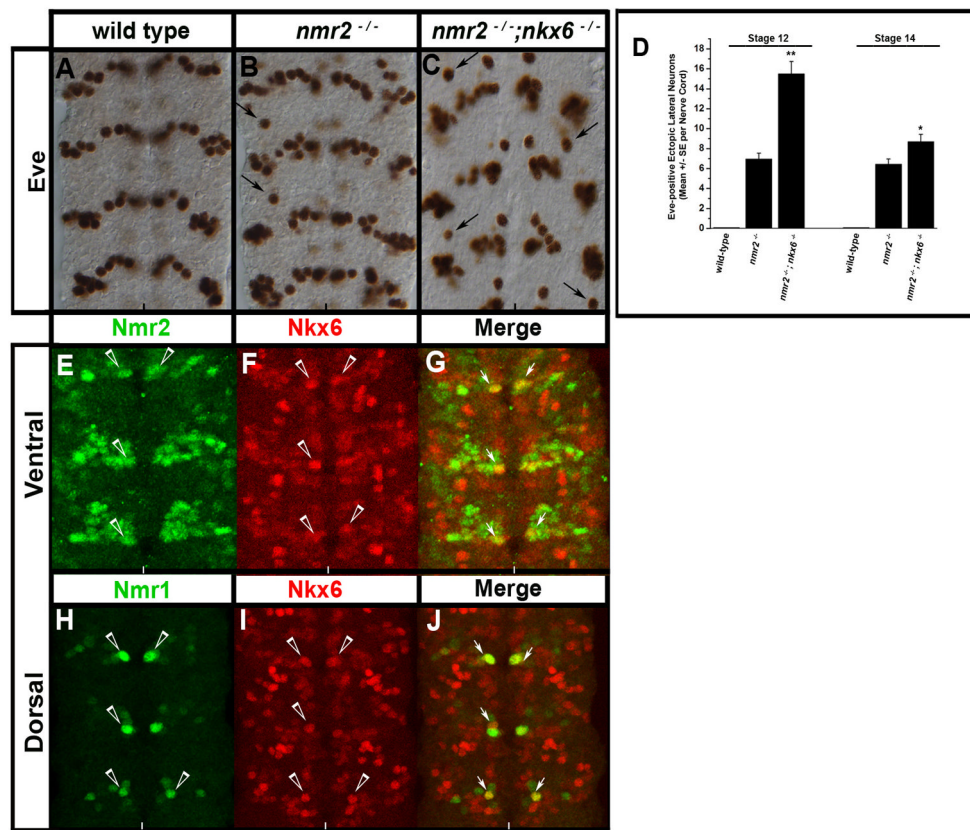(*arrows*).

Figure 10.

nmr2 and *nkx6* collaborate to inhibit *eve* expression.

(A–C) Stage 14 wild-type and mutant embryonic nerve cords stained for Eve, (D) a chart quantifies the number of Eve-positive lateral neurons in wild-type, $nmr2^{-/-}$, and $nmr2^{-/-};nkx6^{-/-}$ mutant backgrounds at stages 12 and 14, (E–J) immunofluorescent staining of Nmr2-, Nmr1-, and Nkx-6-positive neurons in the CNS of Stage 15 wild-type embryos. All images depict the ventral region of the CNS. (A) Stage 14 wild-type Eve expression pattern. (B) As shown previously, $nmr2^{-/-}$ mutant embryos exhibit Eve-positive ectopic lateral neurons (*arrows*, see Fig. 1B). (C) $nmr2^{-/-};nkx6^{-/-}$ double mutant embryos exhibit increased numbers of Eve-positive ectopic lateral neurons throughout the nerve cord as compared with $nmr2^{-/-}$ mutants (*arrows*). The nerve cords of $nmr2^{-/-};nkx6^{-/-}$ mutants also exhibit severe orientation defects that become pronounced at stages 15–16 (data not shown). (D) Each bar value represents the mean of 20 embryos scored for the number of Eve-positive ectopic lateral neurons observed throughout the nerve cord at stages 12 and 14. The error bars represent the S.E.M. and statistical significance was determined by One-way Analysis of Variance (ANOVA) where $p^{**} < 0.001$ and $p^* < 0.01$. (E–G) Nmr2 (*green*) and Nkx6 (*red*) exhibit a mutually exclusive expression pattern with the exception of one medial neuron per hemisegment that co-expresses Nmr2 and Nkx6 (*merge*, *arrows*). (H–J) Nmr1 (*green*) and Nkx6 (*red*) are also co-expressed in one medial neuron per hemisegment.