Dual role for Hox genes and Hox co-factors in conferring leg motoneuron survival and identity in Drosophila

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SUMMARY

Adult Drosophila walk using six multi-jointed legs, each controlled by ~50 leg motoneurons (MNs). Although MNs have stereotyped morphologies, little is known about how they are specified. Here, we describe the function of Hox genes and homothorax (hth), which encodes a Hox co-factor, in Drosophila leg MN development. Removing either Hox or hth function from a single neuroblast (NB) lineage results in MN apoptosis. A single Hox gene, Antennapedia (Antp), is primarily responsible for MN survival in all three thoracic segments. When cell death is blocked, partially penetrant axon branching errors are observed in Hox mutant MNs. When single MNs are mutant, errors in both dendritic and axon arborizations are observed. Our data also suggest that Antp levels in post-mitotic MNs are important for specifying their identities. Thus, in addition to being essential for survival, Hox and hth are required to specify accurate MN morphologies in a level-dependent manner.

KEY WORDS: Drosophila melanogaster, Hox genes, Motoneurons, Cell death and survival, Neuroblasts

INTRODUCTION

Animal locomotion requires coordinated muscle contractions that are controlled by motoneurons. Motoneurons receive commands in the CNS and execute these commands by controlling muscle contractions in the periphery. Most animals capable of movement adopt one of two types of locomotion. Undulatory movements, exhibited by Drosophila larvae and Caenorhabditis elegans, require coordinated body wall muscle contractions along the dorsoventral (DV) and anteroposterior (AP) axes. By contrast, walking by animals such as mice and adult Drosophila requires well-coordinated muscle contractions in each leg. Accordingly, walking depends on the accurate specification of and connections between the motoneurons, sensory neurons and interneurons that make up these circuits (Dasen and Jessell, 2009; Arber, 2012).

How the exquisitely stereotyped morphologies exhibited by neurons are established during development is an important unsolved problem in biology. In insects such as Drosophila, neurons are derived from neural stem cells, called neuroblasts (NBs). Each NB is located in a stereotyped AP and mediolateral position in the ventral nerve cord (VNC) and gives rise to a unique set of progeny (Schmid et al., 1999; Truman et al., 2004). A series of transcription factors expressed at sequential times during the life of each NB provides temporal information that is integrated with spatial information to generate neuronal identities (Pearson and Doe, 2004). NBs divide in one of two waves of activity, the first during embryogenesis, nine NBs give rise to ~50 MNs, which target 14 muscles in each adult leg (Baek and Mann, 2009).

In both vertebrates and invertebrates, the Hox family of transcription factors has emerged as a group of key players in nervous system development. Previous work on non-leg MN Drosophila lineages demonstrated that, depending on the context, Hox genes can regulate both apoptosis and the timing of cell cycle exit of NBs, and apoptosis in post-mitotic neurons. For example, in the abdomen the Hox gene abdominal A (abd-A) induces NB apoptosis in a subset of lineages that continue to produce progeny in thoracic segments (Bello et al., 2003; Cenci and Gould, 2005; Maurange et al., 2008). Abdominal Hox genes also have the ability to induce NB cell cycle exit in some abdominal lineages (Karlisson et al., 2010). Finally, the Hox gene Abdominal-B (Abd-B) can block or promote apoptosis of neurons, depending on the context (Miguel-Aliaga and Thor, 2004; Suska et al., 2011). In each of these cases, Hox genes execute functions that diversify the nervous system along the AP axis, analogous to their more classically defined functions in other tissues.

In vertebrates, Hox genes have been shown to be important regulators of MN identity in the spinal cord (Dasen et al., 2003; Dasen et al., 2005; Jung et al., 2010). In the mouse, Hox6 and Hox10 paralogs are needed to define the lateral motor columns (LMCs) present at brachial and lumbar levels, respectively, whereas Hox9 paralogs suppress the LMC fate at thoracic levels. Notably, the Hox accessory factor encoded by Foxp1 gates Hox functions in a level dependent manner, depending on the column (Dasen et al., 2008). Within the brachial LMC, cross-regulatory interactions between 11 of the 39 mouse Hox genes establish the identities of MN pools, providing them with their muscle-specific targeting properties in the limb (Dasen et al., 2005). The specification of pool identities by Hox genes is apparently distinct from their role in AP patterning, because these cross-regulatory interactions are restricted to limb-forming regions at brachial and lumbar levels of the rostral-caudal axis.

In addition to walking, each pair of legs in Drosophila executes segment-specific behaviors. For example, the second thoracic (T2) legs are important for flight takeoff, whereas the T1 and T3 legs are...
used for grooming the head and abdomen, respectively (Szemenyi, 1969; Kaplan and Trout, 1974; Dawkins and Dawkins, 1976; Trimmerchi and Schneiderman, 1993). These observations raise the question of whether Hox genes play a role in diversifying MN identities or circuitries between thoracic segments. In the work described here, we provide evidence that during the development of leg MNs Hox genes execute both AP patterning and segment-independent functions. Newly born leg MNs initially express the same Hox gene, Antennapedia (Antp), in all three thoracic segments. Genetic analysis demonstrates that Hox genes and the gene encoding the Hox co-factor homothorax (hth) are required for postmitotic MN survival in all three segments. By blocking cell death in these mutants and by examining individual mutant MNs, we show that these genes are not required to specify a leg MN identity or for their specific lineage identity. However, these experiments reveal a requirement for Hox genes to specify both axon and dendrite morphologies of these MNs. Interestingly, we also find that within a single NB lineage Antp is present at different levels that correlate with MN birthdate, and that these levels are important for instructing axon targeting. Thus, Hox genes are needed for both MN survival and for specifying their morphologies in a level-dependent manner in all three thoracic segments. Later in development, as leg MNs differentiate during metamorphosis, their Hox expression code changes in a segment-specific manner. The unique Hox code exhibited by pupal stage leg MNs suggests an additional late role for Hox genes in diversifying their properties in the different thoracic segments, perhaps to allow them to execute segment-specific functions.

MATERIALS AND METHODS

Fly stocks

Unless otherwise noted, fly stocks were obtained from the Bloomington Drosophila Stock Center.

- yw hs-flp; FRT82B
- yw hs-flp; FRT82B tubG80
- w hs-flp FRT19A
- w hs-flp tubG80 FRT19A
- SceFL Antp^NS-R3\Ubx^MG^II (Struhl, 1982)
- yw hs-flp; FRT 82B Antp^NS-R3/\Ubx/MKR2
- yw hs-flp tub-Gal4 UAS-GFP
- FRT82B Ubx^TM6B
- FRT82B hth^P2/MKR2
- w hs-flp FRT19A tubGal80
- vGlut-Gal4 (also called OK371-Gal4) (Mahr and Aberle, 2006)
- UAS-CD8GFP (on II)
- UAS-p35 (on II) (from Laura Johnston, Columbia University Medical Center, NY, USA)
- vGlut-lecA (C. S. Mendes and R.S.M., unpublished)
- LexO-rah3YFP (C. S. Mendes and R.S.M., unpublished)
- UAS-6myc-Antp (M. A. Crickmore and R.S.M., unpublished)
- UAS-AntpRNAi (Bloomington Drosophila Stock Center, stock number 27675)

Immunohistochemistry

Primary antibodies were: mouse anti-Abd-B [1:5; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Ubx (1:20; DSHB), mouse anti-Antp (8C11; DSHB), mouse anti-prospero (DSHB), mouse anti-d-CSP2 (1:200; DSHB), rat anti-Elav (1:50; DSHB), mouse anti-Repo (DSHB), rabbit anti-Exd (McGinnis and Abu-Shaar, 1996), guinea pig anti-Hth, (GP52) (Ryoo and Mann, 1999), rabbit anti-Antp-A (from B. Gebelein, Cincinnati Children’s Hospital Medical Center, OH, USA), rabbit anti-Sce-C (CLU395, 1:500) (Joshi et al., 2010), rabbit anti-Pb (e9, 1:100) (Cribbs et al., 1992), guinea pig anti-Dil (1:200; from W. Mccinnis, University California, San Diego, CA, USA), guinea pig anti-Lab (1:50,000; from B. Olstein, Columbia University Medical Center, NY, USA), guinea pig anti-Deadpan (1:1000, from J. Skeath, Washington University in St Louis, MO, USA), guinea pig anti-Dil (Estella et al., 2008) and rabbit anti-myc (Molecular Probes). Secondary antibodies were: AlexaFluor488, AlexaFluor555 and AlexaFluor647 conjugates (Molecular Probes).

CNS dissections and immunostaining were carried out as described (Baek and Mann, 2009). Samples were incubated with primary antibodies for two days and secondary antibodies for one day at 4°C. PBST (PBS with 0.1% Triton X-100, 0.5% BSA and 5% goat serum) was used for the incubation and washing steps. Adult legs attached to bodies were fixed overnight at 4°C and washed three times in PBST for 15 minutes at room temperature. CNS and legs were dissected and mounted onto glass slides using Vectashield mounting medium (Vector Labs).

Mosaic analysis with a repressible cell marker (MARCM) analysis

To generate MARCM clones, embryos were collected for 12 hours and incubated for 24 hours at 25°C. First instar larva were transferred to vials and given heat shocks at appropriate time points. Entire lineage clones were generated from heat-shocks at 24-36 hours after egg laying (AEL). Strains used are listed below.

Standard MARCM clones

- yw hs-flp; vGlut-Gal4 UAS-CD8GFP; FRT82B tubGal80/FRT82B, FRT82B Scr^Antp^NS-R3\Ubx^MG^II, FRT82B Antp^NS-R3, FRT82B Ubx^P2 or FRT82B hth^P2.
- yw hs-flp/yw hs-flp tub-Gal4 UAS-GFP; vGlut-Gal4 UAS-CD8GFP/+; FRT82B tubGal80/FRT82B, FRT82B Scr^Antp^NS-R3\Ubx^MG^II, FRT82B Antp^NS-R3, FRT82B Ubx^P2 or FRT82B hth^P2.

Cell death inhibition

- vGlut-Gal4, UAS-rab3YFP/+; UAS-AntpRNAi/+. 

Microscopy and image analysis

Confocal images were taken as described (Baek and Mann, 2009) using the same confocal settings for each antibody. z-stack maximum merged images were generated and, if necessary, non-leg motoneuron clones were removed using ImageJ.

RESULTS

Hox gene expression patterns in leg MNs

In Drosophila, there are eight Hox genes: labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr), Antp, Ultrabithorax (Ubx), abd-A and Abd-B (McGinnis and Krumlauf, 1992). We examined the expression pattern of the proteins encoded by all eight Hox genes as well as hth and extradenticle (exd), which encode Hox co-factors. As in other tissues (Mann and Morata, 2000), nuclear Exd correlated with the presence of Hth, so only the pattern of Hth is reported here. Around 50 leg MNs are born from nine post-embryonic NB lineages in each thoracic hemi-segment (Baek and Mann, 2009). In the present study, we focused on LinA (also called Lin 15) (Truman et al., 2004) because it generates the largest number of leg MNs and can be readily identified by its position in the VNC (Fig. 1A-C). LinA gives rise to ~28 adult leg MNs (Baek and Mann, 2009; Truman et al., 2010). The timing and birth order for LinA MNs are stereotyped: LinA MNs are generated from ~50-90 hours after egg laying (AEL)
By late third instar (~120 hours AEL), LinA MN cell bodies reside medially in the VNC and send axons that exit the VNC towards the leg imaginal discs through the leg nerve (Fig. 1A-C) (Truman et al., 2004). At the pupal stage, LinA leg MN cell bodies move to their final positions, and dendritic and axonal arborizations begin to elaborate (Fig. 1A-C). In addition, we found that LinA also gives rise to ~22 glial cells that end up surrounding each thoracic neuropil in the adult VNC (Fig. 1D; data not shown).

We found that three Hox proteins, Pb, Antp and Ubx, and both Hox co-factors are expressed in the thoracic segments of the VNC, where leg MN cell bodies are located, at late larval and mid-pupal stages (supplementary material Fig. S1; Fig. S3A). Because there are no specific markers for the leg MNs, we used mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999), combined with the stereotyped position of LinA within the VNC to identify this lineage unambiguously. At the third larval stage, all LinA MNs in all three thoracic segments expressed Antp (Fig. 2B,C,E).

In the third larval VNC, most NBs, including the LinA NB, are positioned along the ventral side of the VNC and divide to produce progeny in the dorsal direction (Fig. 2A). Consequently, earlier-born neurons are located more dorsally and later-born neurons are located more ventrally, closer to the NB (Maurange et al., 2008). Interestingly, within single LinA MARCM clones, Antp was detected more strongly in ventrally located cells that end up surrounding each thoracic neuropil in the adult VNC (Fig. 1D; data not shown).

The patterns of Hox expression changed by the mid-pupal stage (3-4 days post-pupariation), at a time when both axon targeting and dendritic arbors are elaborating. At this time, Antp was expressed strongly in all LinA MNs in the T2 segment but was undetectable in LinA MNs in T1 and T3 (Fig. 2G,J). By contrast, Ubx was observed in all LinA MNs of the T3 segment but not in T1 or T2 (Fig. 2H-J). As in the larval stage, no other Hox proteins were detected in LinA MNs at the mid-pupal stage. Hth and Exd were present in all LinA MNs at both larval and pupal stages.
In the adult, these segment-specific Hox expression patterns were maintained (supplementary material Fig. S3C-F). In summary, Hox expression in LinA is dynamic: in late third instar VNCs, all LinA MNs express Antp regardless of the thoracic segment (Fig. 2E). At this stage, the levels of Antp correlate with the MN birthdate within LinA: older MNs have lower levels of Antp than do younger MNs. Approximately 72 hours later, during metamorphosis, LinA MNs in T1 express no Hox genes, those in T2 express Antp and those in T3 express Ubx (Fig. 2J).

Hox genes and hth are required for MN survival
To determine the function of Hox genes and hth in LinA, we used the MARCM method to genetically remove their activities from this lineage. To ensure that we were examining the loss of all thoracic Hox function, most experiments used the triple-mutant chromosome Scr Antp Ubx, although, as described below, our key findings were confirmed with single Hox mutant chromosomes. Depending on the experiment, MARCM clones were labeled with mCD8GFP driven by either vGlut-Gal4, which is a post-mitotic MN driver, or by combining tub-Gal4, a ubiquitous driver, with vGlut-Gal4. The ubiquitous Gal4 driver was added to ensure that we were monitoring all LinA progeny, not just those that expressed vGlut-Gal4.

In either Scr Antp Ubx or hth mutant clones, the number of LinA-derived neurons (Elav+) was dramatically reduced when examined at the late third instar larval stage (~11 compared with 35 neurons in wild type) or in the adult [about four (hth) or nine (Scr Antp Ubx) compared with 25 MNs in wild type] (Fig. 3A,C,E,G; supplementary material Fig. S3A).
material Fig. S4A). These phenotypes were observed in all three thoracic segments. Because these experiments included the ubiquitous driver tub-Gal4, they argue against the possibility that MNs are being transformed to non-MN cell types in Hox or hth mutants.

Antp single mutant LinA clones also resulted in a reduced number of MNs in T1 and T2 but, curiously, not in T3 (in the adult, about nine LinA MNs were observed in T1 and T2 segments and ~25 neurons in T3) (supplementary material Fig. S4A). Ubx mutant LinA clones also did not show a reduction in MN number in T3: in the adult, ~25 MNs were observed in Ubx mutant LinA clones in all three segments (supplementary material Fig. S4A). We hypothesized that the difference in the phenotypes observed in T3 for Scr Antp Ubx clones (about nine MNs) and the singly mutant Antp or Ubx clones (~25 MNs) might be due to de-repression of Antp in Ubx mutant clones in this segment and a functional redundancy of these two genes for promoting survival. Consistent with this notion, when examined at the pupal stage, we observed Antp protein in Ubx mutant T3 LinA clones (supplementary material Fig. S4B).

Neuronal apoptosis in Hox and hth mutant LinA clones
The reduced number of MNs observed in Hox or hth mutant LinA clones could be caused by several mechanisms, which are not mutually exclusive: for example, early NB cell cycle exit, premature NB cell death, or post-mitotic MN cell death. To distinguish between these mechanisms, we first checked whether the LinA NB undergoes premature cell death. At the early third instar larva stage,
NBs, labeled by Deadpan (Dpn) (Lee et al., 2006), were observed in both wild-type and Hox mutant LinA clones (supplementary material Fig. S4C-F). At this time point, the LinA NB was associated with a similar number (~18) of neuronal progeny (as assessed by Elav+ staining) in both wild-type and mutant LinA clones (supplementary material Fig. S4C-F). These data argue against the idea that either premature LinA NB cell death or early cell cycle exit account for the lower number of LinA MNs. To determine whether apoptosis is responsible for the reduced number of MNs, we blocked cell death by expressing the caspase inhibitor p35 (Hay et al., 1995). When examined at the late third instar stage, the expression of p35 in Hox or hth mutant LinA clones (simultaneously using tub-Gal4 and vGlut-Gal4) rescued the reduced number of MNs (Fig. 3D,F,G). In addition, wild-type or mutant LinA clones expressing p35 generated a greater number of neurons than did wild-type LinA clones not expressing p35 (from ~35 to 45 cells; Fig. 3B,G). These findings are consistent with previous observations showing that a subset of LinA progeny normally undergoes apoptosis (Truman et al., 2010). An alternative explanation is that the LinA NB, which normally disappears at the mid-third instar stage (Truman et al., 2004), is kept alive by p35. When expression of p35 was limited to post-mitotic MNs (by using only vGlut-Gal4) we observed only a partial rescue (data not shown), suggesting that both mechanisms might be operational. However, we cannot rule out the possibility that p35 levels are too slow to accumulate in time to efficiently rescue MN survival, in part owing to perdurance of Gal80 in these MARCM clones.

In the MARCM experiments using tub-Gal4, which labels both MNs and glia, we found that the wild-type LinA NB also gives rise to ~22 glial cells (as assessed by anti-Repo staining) that end up surrounding the leg neuropil (Fig. 1D; data not shown). As with MNs, the number of glia was strongly reduced in Hox or hth mutant LinA clones (Fig. 3H). Surprisingly, however, although ectopic p35 expression in mutant LinA clones rescued the number of MNs, the number of glia was not rescued (Fig. 3H), arguing that apoptosis is not the cause of the reduced number of glia in Hox or hth mutant LinA clones. With the exception of very low levels of Antp, we also did not detect expression of Hox genes, hth or exd in LinA-derived glia (supplementary material Fig. S3B). Taken together, these data suggest that Hox and Hox co-factors might be required in the LinA NB to specify the glia cell fate.

**Defects in axon targeting of Hox and hth mutant leg MNs**

To determine whether, in addition to survival, Hox genes and hth are required for MN identities, we examined both axon targeting and dendritic arbors of mutant leg MN clones. Wild-type LinA MNs mainly target the femur and tibia and have a complex dendritic morphology in the neuropil (Fig. 4A) (Baek and Mann, 2009; Brierley et al., 2009; Brown and Truman, 2009). In either Hox (Scr, Antp, Ubx) or hth mutant LinA clones, the surviving MN axons targeted the same region of the leg as did wild-type LinA clones. Compared with wild-type clones, fewer branches were observed, consistent with the fewer number of cells (Fig. 4A shows examples in T2; see supplementary material Fig. S5A,B for examples in T1 and T3). The distal region of the tibia was most highly affected; other axon branching defects appeared to be randomly distributed within the LinA-targeted region of the leg (summarized in supplementary material Fig. S5D). Although axon branching errors were not observed in Ubx mutant LinA clones, Antp mutant LinA...
clones displayed targeting defects in all three segments, including T3, despite no affect on MN cell number in this segment (supplementary material Fig. S5C). These results argue that Hox genes, and \textit{Antp} in particular, play a crucial role in specifying MN morphologies in addition to maintaining their survival. Although the dendritic phenotype is more difficult to assess owing to the large number of MNs in a LinA clone, some defects could be observed. Most clearly, we found that a small number of midline-crossing dendrites were absent in \textit{hth} mutant LinA clones, in all three thoracic segments (Fig. 4A; supplementary material Fig. S5A,B). This phenotype was not observed in Hox mutant clones (Fig. 4A; supplementary material Fig. S5A,B).

To explore further the idea that Hox genes are required for conferring MN identities, we examined the phenotypes of Hox mutant LinA-derived MNs in which survival was rescued by the expression of p35. p35 expression in otherwise wild-type LinA clones generated MNs with axon-targeting properties very similar to those of wild-type LinA clones (Fig. 4B). Hox mutant axons that were rescued by p35 also targeted the same region along the proximodistal axis of the leg as did wild-type LinA MNs (Fig. 4B for examples in T2; supplementary material Fig. S5A,B for examples in T1 and T3). However, most samples exhibited some targeting defects (summarized in supplementary material Fig. S5D). These errors were not limited to one region of the LinA-targeted leg and typically consisted of an absence of terminal arborizations. In the neuropil, \textit{hth}; p35\textsuperscript+ clones exhibited a similar midline-crossing defect as seen in \textit{hth} clones not rescued by p35 (Fig. 4B; supplementary material Fig. S5A,B). Together, these data demonstrate that in the absence of Hox or \textit{hth} inputs LinA MNs are not appropriately specified, even when death is blocked. However, they also show that in this lineage Hox genes and \textit{hth} are not required for forming leg MNs and for specifying a LinA identity.

**Removing Hox functions from individual MNs results in axon- and dendritic-patterning errors**

The experiments described above examined entire LinA clones, each labeling >25 MNs. Consequently, these experiments do not have cellular resolution. To examine the role of Hox genes and \textit{hth}
in individual LinA MNs, we changed the MARCM protocol to generate individual MNs that are mutant for these genes (Baek and Mann, 2009). Altogether, we were able to recover about two-thirds of the MNs generated by LinA, suggesting that most LinA MNs can survive when the functions of these genes are removed at this last cell division. Moreover, more than half of these mutant MNs showed an aberrant morphology, although with variable penetrance (supplementary material Table S1). For many Hox or hth mutant MNs, dendritic or axonal arborization defects were observed, although the requirement for these factors often differed (Fig. 5; supplementary material Table S1). For example, a coxa-targeted MN had ectopic dendrites when mutant for hth, but not Hox (Fig. 5A, top row), whereas a tibia-targeted MN had ectopic dendrites when mutant for Hox, but not hth (Fig. 5A, bottom row). In both of these examples, axon branching appeared normal. By contrast, a femur-targeted MN had fewer axon branches when either Hox or hth functions were removed, but ectopic dendrites were only observed when the MN was mutant for hth (Fig. 5A, middle row). From these data it appears that individual MNs often have a unique requirement for hth and Hox functions.

In most cases, individual MNs mutant for hth or Hox exhibited defective, as opposed to transformed, dendritic and axonal morphologies and axon branches. However, in two examples (out of 125 total single MN clones), we observed MNs that appeared as though they were hybrids of two different MNs. In these cases, the mutant MNs had dendritic and axonal morphologies that are usually observed in different wild-type MNs, suggesting that the identities of these MNs were partially transformed (Fig. 5B,C). For example, Fig. 5B shows a Hox mutant MN that targets the distal femur but has a dendritic morphology normally found in a MN that targets the proximal tibia. Fig. 5C shows an hth mutant MN that targets the distal femur but has a dendritic morphology normally found in a MN that targets the proximal tibia. Although they are rare, these examples suggest that separable genetic programs control dendritic and axonal morphologies, and that Hox and hth often execute distinct functions at the single-cell level.

**Different levels of Antp are instructive for MN morphologies**

One of the intriguing observations described above is that Antp levels vary among LinA progeny such that younger MNs express higher levels than older MNs (Fig. 2B; supplementary material Fig. S2). In our previous analysis of this lineage (Baek and Mann, 2009), we found that older MNs from LinA had a tendency to target the proximal femur and proximal tibia, whereas younger MNs targeted the distal regions of these segments. To test whether Antp levels play a role in this targeting bias, we generated LinA MARCM clones in which Antp was ectopically expressed via the post-mitotic driver vGlut-Gal4. This manipulation did not affect MN survival or final cell number (data not shown). However, we found that there was a clear reduction of axon branches in the proximal femur and increased branching in the distal femur (Fig. 6; supplementary material Fig. S6). Weaker effects in the same direction were observed in the tibia. In a separate experiment, we knocked down Antp levels in all post-mitotic MNs by driving Antp-RNAi using vGlut-Gal4 and observed a distal-to-proximal shift in axon targeting (supplementary material Fig. S7). Together, these observations suggest that high levels of Antp, normally present in younger LinA progeny, promote the identity of MNs that target the distal regions of the femur and tibia. Conversely, MNs born earlier in the lineage target the proximal regions of these segments, in part owing to lower levels of Antp.

**No compensatory targeting from MNs from other lineages**

In all of the experiments described above, manipulating the activity of Hox or hth did not affect the MN or LinA identity of progeny cells. For example, cells derived from Hox mutant LinA and rescued by p35 expression remained MNs and targeted the same region of the leg as did wild-type LinA MNs. These observations suggest that the identities of progeny MNs are hardwired according to the NB lineage from which they are derived. As an additional test of this idea, we asked whether MNs derived from other lineages might be able to take the place of MNs that die in the absence of Hox or hth. To answer this question, we generated positively labeled Hox or hth mutant NB clones in a background in which all MNs were labeled by the expression of rab3-YFP (Zhang et al., 2007). In this background, we generated Hox and hth mutant LinA and LinB MARCM clones, labeled by mCD8GFP driven by vGlut-Gal4 (Fig. 7A). Axons and neuromuscular junctions (NMJs) in mutant MN clones were compared with those in wild-type MNs. For both LinA and LinB, legs innervated by either Hox or hth mutant clones
had regions that remained unlabeled by both rab3-YFP and GFP (Fig. 7B,C). We conclude that, despite the lower number of MNs produced by these mutant lineages, there was no compensatory targeting by wild-type MNs from wild-type non-LinA lineages. Thus, even when many muscle targets are available, leg MNs apparently maintain their lineage-derived identity.

**DISCUSSION**

In addition to their well-documented role in specifying differences along the AP axis during animal development (McGinnis and Krumlauf, 1992), Hox genes also set up differences along the AP axis within the nervous system. In *Drosophila*, some NB lineages produce more or less progeny depending on where they are along the AP axis and, therefore, which Hox gene they express (Bello et al., 2003; Maurange et al., 2008). In the vertebrate nervous system, sub-regions of the hindbrain, known as rhombomeres, obtain their identities as a result of which Hox genes are expressed (McGinnis and Krumlauf, 1992). And, more recently, *Hoxc9* has been shown to play a crucial role in specifying thoracic as opposed to limb-innervating motoneurons in the mouse spinal cord (Jung et al., 2010). In the work described here, we present evidence for a Hox function that is independent of AP patterning: the survival of post-mitotic MNs in the *Drosophila* thorax. Interestingly, in all three thoracic segments this function appears to be carried out primarily by *Antp*, although in T3 either *Antp* or *Ubx* can apparently execute this function. Moreover, for MN survival, similar (though not identical) phenotypes were observed for Hox and *hth* mutant LinA clones, suggesting that for this function, Hox and *hth* might function together to regulate the same target genes, as they do in other circumstances. Because our experiments depend on clonal analysis, our results also demonstrate that Hox genes are required autonomously within a single NB lineage for keeping motoneurons alive. Although this could represent a cell-autonomous function within individual MNs, we cannot exclude the possibility that the reduced number of LinA-derived glia in mutant LinA clones contributes to the poor survival of MNs.
In vertebrates, MN survival is typically dependent upon the reception of and retrograde signaling by neurotrophic factors derived from peripheral tissues (Zweifel et al., 2005; Kanning et al., 2010). By contrast, in Drosophila and other insects, MN survival is independent of the target muscle (Goodman and Bate, 1981; Whittington et al., 1982; Nässel et al., 1986; deLapeyrière and Henderson, 1997). Nevertheless, a family of neurotrophins that mediate neuronal survival has been identified in Drosophila that acts through Toll-like receptors (Zhu et al., 2008). Based on our results, we speculate that Hox and hth are required for the reception or production of these neurotrophins in LinA.

Although the number of MNs that die in these mutant backgrounds was consistent from sample to sample, the neurons that were affected were apparently randomly distributed within the lineage. One possible explanation for this observation is that without Hox or hth inputs the identities of surviving MNs become randomized, resulting in random targeting. However, our single-cell clone analysis argues against this idea, as MN identities remained largely intact despite exhibiting aberrant morphologies. Alternatively, we favor the idea that removing Hox or hth results in partial failure in the reception of survival factors, resulting in stochastic and partially penetrant cell death among all the MNs within the lineage.

**MN identities in the absence of Hox input**

In addition to promoting MN survival, our data also reveal a requirement for Hox gene functions in the specification of motoneuron morphologies. When entire LinA lineages were mutant for either Hox or hth, and MN survival rescued by p35, LinA MNs targeted the same positions along the proximodistal (PD) axis as did wild-type LinA MNs; axons targeting to ectopic positions along the leg were not observed. These observations suggest that Hox or hth inputs are not required for this level of MN specification; the cells remain MNs and their ‘LinA identity’ remains intact. However, within the correctly targeted region, mutant LinA MNs exhibited frequent axon-targeting defects. These defects were not limited to a specific subregion of the LinA-targeted domain, suggesting that no specific subset of LinA MNs has an absolute requirement for these functions.

The maintenance of a LinA identity by Hox or hth mutant lineages is reminiscent of the observation in vertebrates that the targeting of limb-level MNs appears superficially normal in the FoxP1 mutant (Dasen et al., 2008; Rousso et al., 2008). In this case, targeting defects were only revealed when individual MN pools were labeled by backfilling from specific muscles. Our single-cell mutant analysis argues against a similar scrambling of identity within LinA occurring in Hox or hth mutant MNs. Out of 87 individual Hox mutant LinA neurons that were analyzed (distributed across all three thoracic segments), 55 had a non-wild-type morphology. However, despite clear defects, these MNs did not appear to have a transformed identity. Instead, most mutant MNs continued to approach the same muscle target and either exhibited an aberrant dendritic (44/87) or axon branching (18/87) morphology. Moreover, for many LinA MNs that showed aberrant phenotypes when mutant, the same MNs had wild-type morphologies in other samples (supplementary material Table S1). One explanation to account for these partially penetrant phenotypes is that they result from the perdurance of the Hox or hth gene products following clone induction. It is also feasible that removing only a single component from potentially large transcription factor complexes has only a limited effect because of multiple, partially redundant inputs. Answering this question will ultimately require the identification and characterization of target genes that are directly regulated by Hox and hth in developing MNs.

The differences between the role of Hox genes in Drosophila compared with their role in specifying MN identities in vertebrates might be a consequence of the additional requirement for establishing motoneuron pools in vertebrates, which, for forelimb MNs, depends on at least 11 different Hox gene products (Dasen et al., 2005). By contrast, the relatively lower number of motoneurons innervating the muscles of the Drosophila leg might not require such a complex combinatorial transcription factor code. We speculate that the expansion of Hox gene inputs governing MN development in vertebrates, itself a consequence of a large increase in Hox gene number, might have enabled the evolution of more complex limbs and musculature.

**Different requirements for Hox and Hox co-factor inputs**

As noted above, we found that both Hox and hth inputs are similarly required for MN survival, suggesting that these factors might function as co-regulators of the same target genes, as has been observed for other Hox functions (Riekho et al., 1997; Mann and Affolter, 1998). However, other phenotypes were different when Hox or hth functions were removed (Figs 4, 5). For example, for a femur-targeting MN we found that Hox inputs were required for accurate axonal targeting, whereas hth was required to block the aberrant growth of dendrites towards the midline in the CNS (Fig. 5). By contrast, for a tibia MN, we found that Hox functions were required to block aberrant dendrite growth, whereas hth appeared to play no role in either dendrite or axon morphology (Fig. 5). In a third example, hth was required for a wild-type dendritic pattern of a coxa-targeting MN, whereas Hox functions apparently played no role in this MN (Fig. 5). Taken together, these data support the idea that independent genetic programs control dendritic patterning and axonal patterning. In addition, for these aspects of MN morphology it appears that Hox proteins do not use Hth (and probably also not Exd) as a co-factor. Instead, these data suggest that Hox and hth are regulating different sets of target genes that independently affect dendritic or axonal targeting, depending on the MN. Previous work suggests that, for specifying dendrite morphologies, one pathway that may be targeted is the Robo/Slit pathway (Brierley et al., 2009).

**Antp levels as a timing mechanism**

The progeny of individual NBs in Drosophila differ in part owing to their birthdates within their respective lineages. For several NB lineages, this temporal information is controlled by a series of sequentially expressed transcription factors (Pearson and Doe, 2004). Here, we provide evidence that another mechanism to diversify cell fates within a lineage is by varying the levels of the Hox transcription factor Antp. The experiments supporting this conclusion were clearest when we ectopically expressed Antp in post-mitotic progeny using the vGlut-Gal4 driver. In these experiments, we observed a clear shift from MNs targeting the proximal femur, which are born early in the lineage, to distally targeting MNs, which are born later in the lineage. Conversely, knockdown of Antp levels in post-mitotic MNs by RNAi resulted in a distal-to-proximal shift in axon targeting. In contrast to these knockdown experiments, when we eliminate Hox gene expression using null alleles we found that both distally and proximally targeting MNs were affected. Based on these findings, we conclude that low levels of Antp results in a phenotype that is distinct from that observed in the absence of Antp. The data suggest that low Antp...
levels (normally present in early-born MNs) instruct MNs to target distal muscles, whereas MNs with no Antp show neither a proximal nor a distal bias in their axon targeting.

Although to our knowledge this mechanism has not been previously observed for a Hox protein, the levels of the BTB/zinc finger transcription factor Chinmo are instructive in NB lineages in the Drosophila mushroom body (Zhu et al., 2006). In this case, Chinmo levels are controlled translationally, by sequences in the 5’UTR of its mRNA. Although such a mechanism may account for varying Antp levels in LinA, it is also possible that Antp is continuously expressed in the LinA NB, but not in its progeny, resulting in its gradual decline in post-mitotic neurons. Level-dependent functions have also been observed for the Hox accessory protein FoxP1 in vertebrate MNs and for Hth in Drosophila embryonic lineages (Dassen et al., 2008; Karlsson et al., 2010). However, these two cases are distinct from the Chinmo and Antp examples, which influence cell fates within individual NB lineages. By contrast, in the case of FoxP1, its levels are important for establishing differences between MN columns: FoxP1 levels are high in limb-level LMCs, but low in intervening MNs that project to thoracic body wall muscles. In the case of Hth, an abrupt increase in levels during embryogenesis is important for accurate gene regulation in the progeny of NB 5-6 in the Drosophila thorax. Nevertheless, the findings presented here reinforce the idea that transcription factor levels play an important role in their function, and in conferring temporal information to NB lineages.

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