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dHb9 expressing larval motor neurons persist through metamorphosis to innervate adult-specific muscle targets and function in *Drosophila* eclosion

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Abstract

The *Drosophila* larval nervous system is radically restructured during metamorphosis to produce adult specific neural circuits and behaviors. Genesis of new neurons, death of larval neurons and remodeling of those neurons that persistent collectively act to shape the adult nervous system. Here we examine the fate of a subset of larval motor neurons during this restructuring process. We used a dHb9 reporter, in combination with the FLP/FRT system to individually identify abdominal motor neurons in the larval to adult transition using a combination of relative cell body (CB) location, axonal position and muscle targets. We found that segment specific cell death of some dHb9 expressing motor neurons occurs throughout the metamorphosis period and continues into the post-eclosion period. Many dHb9>GFP expressing neurons however persist in the two anterior hemisegments, A1 and A2, which have segment specific muscles required for eclosion while a smaller proportion also persist in A2–A5. Consistent with a functional requirement for these neurons, ablating them during the pupal period produces defects in adult eclosion. In adults, subsequent to the execution of eclosion behaviors, the NMJs of some of these neurons were found to be dismantled and their muscle targets degenerate. Our studies demonstrate a critical continuity of some larval motor neurons into adults and reveal that multiple aspects of motor neuron remodeling and plasticity that are essential adult motor behaviors.

Keywords

Motor neuron remodeling; *Drosophila*; metamorphosis; dHb9; eclosion

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INTRODUCTION

The nervous system of holometabolous insects, such as the fruit fly, *Drosophila*, and the moth, *Manduca*, is extensively reorganized during metamorphosis (Consoulas et al., 2000; Tissot and Stocker, 2000; Truman, 1990), so that new neural circuits are established to execute stage specific behaviors such as eclosion, flight, copulation etc. The re-organization is particularly dramatic in the thorax, where the ganglion expands primarily due to extensive neurogenesis and becomes the seat of locomotor control. Studies in *Manduca* were among the first to demonstrate that dendritic and terminal arbors of persistent larval motor neurons are re-organized during metamorphosis, a process termed respecification (Levine and Truman, 1982, 1985; Thorn and Truman, 1989). The relatively smaller size of *Drosophila* makes it challenging to label individual cells; nevertheless, one study has succeeded in identifying the larval counterparts of some adult flight motor neurons (Consoulas et al., 2002). LacZ reporter lines have been used to demonstrate persistence of pharyngeal motor neurons (Tissot et al., 1998); MARCM lineage analysis shows that a few embryonically generated motor neurons innervate adult leg muscles (Baek and Mann, 2009). In the abdomen, although it is known that larval innervation is reorganized during metamorphosis to generate an adult complement (Currie and Bate, 1991; Hebbar et al., 2006), the pre-synaptic motor neurons that innervate these muscles have not been identified.

Adult innervation patterns of thoracic and abdominal muscles are established during metamorphosis, after the larval terminals have been withdrawn (Currie and Bate, 1991; Hebbar et al., 2006). Muscle patterns at the larval and adult stages are radically different and serve distinct functions, which makes it interesting to determine to what extent the adult complement is made up of new motor neurons vs. respecified larval motor neurons. In the thorax a gain of locomotory function (Truman, 1990) is matched by an expansion of the thoracic ganglion. The adult abdomen, by contrast, does not directly participate in locomotion, and correspondingly the abdominal ganglion is reduced in size. In light of this shift in locomotor focus, what is the fate of the 30 well characterized larval motor neurons, and what functions might they subserve? In this study, a subset of 6–11 abdominal motor neurons that express the *Drosophila* neuronal identity gene, dHb9, a conserved homeodomain protein, were examined through metamorphosis (Arber et al., 1999; Broihier and Skeath, 2002; Odden et al., 2002). The availability of genetic tools to restrict the number of GFP- labelled motor neurons in each hemisegment, and the ability to mark cell bodies and follow axonal trajectories into the periphery allowed us to identify and track individual motor neurons during the 4 day period of metamorphosis. The segmental pattern of dHb9 expressing motor neurons is refined during two defined periods that span the 4 day pupal phase. The pattern in the anterior abdominal segments is additionally refined during a 2 day post-eclosion period; these include motor neurons which innervate persistent larval muscles engaged in the behavior of eclosion. Targeting Diphtheria toxin-1 (DT-1), a protein synthesis inhibitor (Bellen et al., 1992) to dHb9 expressing motor neurons, severely affected eclosion, suggesting a role for dHb9 expressing neurons in this adult specific behavior.

MATERIALS AND METHODS

Fly Strains for developmental analysis

(1) dHb9-Gal4 (Broihier and Skeath, 2002) was used as a driver to target the membrane targeted reporter, UASmCD8GFP (Bloomington stock center), which allowed detection of axonal projections from cell bodies into segmental nerves and as they made their way into the periphery to terminate on their muscle targets. (2) The FLP/FRT system (Struhl and Basler, 1993) was used to restrict the expression of dHb9-Gal4 driven mCD8GFP to 2–4 neurons per hemisegment. yw122 [HS-FLP]; Sp/CyO; UAS-FRT-CD2, y+-FRT-CD8-GFP/TM6B flies (Gift of L. Vosshall) were crossed with dHb9-Gal4/TM6B females. (3) OK371-Gal4 (Bloomington stock center), known to drive expression in glutamatergic neurons including motor neurons (Mahr and Aberle, 2006) was used as a reference label to detect cell bodies in the nerve cord.

Generating FLP/FRT induced GFP expression

Crosses were raised at 25°C and egg-lay plates with early first instar larva were treated with a heat shock at 37°C for 30–60 minutes. Following heat shock, the plates were returned to 25°C. Late second instar larvae (non-TM6B) were examined live under a Nikon E600 microscope to identify those that had GFP expressing NMJs in anterior abdominal segments (A1–A4) in the bodywall. The yield of animals with GFP positive cells was approximately 20% (more than 1000 larvae were screened for this study). Animals were collected in vials and aged to appropriate stages-third instar larva, pupal stages, or adults, for dissections. 165 individually labeled cells from 74 larvae were observed to identify cell bodies based on their characteristic NMJs at target muscles (Table 1). Overlays of labeled CNSs were used to create maps of relative cell body locations.

Longitudinal imaging of specific motor neurons

Larvae with GFP labels in MN12-Ib, MN13-Ib, and MN14-Ib were examined “live” through the cuticle to identify fluorescent neuromuscular endings. Since each muscle has characteristic morphologies of NMJs, the presence of fluorescent junctions can indicate with certainty that the cognate motor neuron has been labeled. Images of NMJs were taken using a Nikon D300s camera mounted on a Nikon E600 fluorescence microscope. Each larva with GFP labeled NMJs on MFs 13/12/14 was kept individually in an eppendorf tube with fly food, aged to the desired time points (third larval instar, 30h APF, 60h APF, and adult), dissected and immunostained. The number of labeled neurons examined during pupal and adult stages in A1 and A2 segments were as follows: MN13-Ib (26 pupal, 10 adult), MN12-Ib (24 pupal, 20 adult) and MN14-Ib (18 pupal, 16 adult) (Table 1).

Staging and Dissection

Third instar larvae, pupae, virgin and 2-day-old adult flies were dissected along the dorsal midline to expose the VNC. For pupal dissections, animals were collected as white pre-pupae (0 h APF, hours after puparium formation), placed on moist filter paper in a petri dish and aged in a 25°C incubator. The pupae were dissected at key stages during the 4-day period of metamorphosis. Virgin adults were collected from bottles and dissected within 4

hrs of emergence. Virgins aged to 2 days represented the 2-day old adult stage. Dissected preparations were fixed in 4% paraformaldehyde, washed in 0.3% Triton-X buffered PBS (PBS-TX), blocked with 10% donkey serum in 0.1% BSA and 0.3% PBS-TX, and processed for immunohistochemistry.

Immunohistochemistry

dHb9-Gal4 driven UASmCD8GFP reporter expression was visualized using rabbit anti-GFP (1:1000; Abcam). Neuronal cell bodies in the VNC were also labeled with mouse-anti Even-skipped (1:50; DSHB) and rabbit or guinea pig-anti Hb9 (1:500; Skeath lab). To visualize the overall innervation patterns, goat anti-HRP (1:200; Abcam) or mouse-anti 22C10 antibodies (1:100; DSHB) were used. Following incubation in primary antibodies, all preparations were blocked in 10% donkey serum in 0.1% BSA and 0.3% PBS-TX for one hour. Secondary antibodies were usually applied for 2 hours at room temperature, and preparations were subsequently washed in 0.3% PBS-TX for one hour. The following Alexa Fluor secondary antibodies were used: donkey anti-rabbit 488 (1:400), donkey anti guinea pig 488 (1:400), donkey anti-goat 555 (1:400), donkey anti-mouse 555 (1:400) and donkey anti-goat 660 (1:200). To observe body-wall musculature, Phalloidin 546 (1:200; Invitrogen) or phalloidin 647 (1:100; Invitrogen) were used.

Image Acquisition & Data Analysis

All immunostained preparations were imaged using an Olympus FV500 confocal microscope. Fluorescent dyes were excited using argon 488, helium-neon 546 and 647 lasers. All images were taken as 1–3 μm optical sections that were stacked using Fluoview software to obtain a two dimensional projection. Morphological attributes of dHb9>GFP-positive motor neurons such as number of cell bodies, axons and NMJs were quantified on 2D projections of confocal stacks. To identify positions of the cell bodies within the D/V axis of the CNS, 3D projections of confocal stacks were color-coded based on their distance from the dorsal surface of the CNS (Zen software). All statistical analysis such as mean, standard error of mean, two-tailed Student's t-test were performed in Microsoft Excel.

Cell death assay

Dissected pupae and adult preps were fixed for 30 minutes at room temperature in 4% paraformaldehyde, rinsed briefly in PBS-TX (0.5% Triton X-100 in phosphate-buffered saline), incubated in 100 mM SSC with 0.1% Triton X-100 at 65°C for 30 minutes, and rinsed twice in PBS-TX. Preparations were washed in 1 $\times$ terminal deoxynucleotidyl transferase (TdT) buffer (Roche) for 10 minutes and followed by an incubation in TdT reaction mix (Roche) at 37°C for 4 hour. The preps were then washed in 0.5% Triton-X for 2 hours at room temperature and incubated overnight with rabbit anti-GFP antibody (1:1000; Abcam) at 4°C to label dHb9-positive cell bodies in the VNC. After washes in 0.3% Triton-X (TBS) for one hour, the secondary antibody (Alexa Fluor donkey anti-rabbit 488, 1:400) was applied for 2 hour at room temperature to detect GFP. Cell death was detected in the red channel (helium-neon 546).

Induction of DT-1 expression: Temporal control of targeted expression of transgenes

dHb9-Gal4; Gal80^{ts} flies were crossed to UAS-DT-1 lines with UAS-mCD8GFP in the background. Since the dHb9-Gal4 promoter is turned on at the embryonic stage (Broihier and Skeath, 2002; Odden et al., 2002), it was essential to “bypass” expression of the DT-1 transgene (Diphtheria toxin, (Thum et al., 2006) prior to the pupal phase. The temporal control of targeted transgene expression was achieved by using Gal4/Gal80^{ts} system (McGuire et al., 2003). We chose to use DT-1 for cell ablations (Thum et al., 2006) as reaper (cell death) induction was not as effective in generating eclosion defects (17% compared to 94% with DT-1). The less severe effect of *reaper* manipulation is consistent with a previous study which reported that *rpr*-dependent ablation is not very effective during the pupal stage (Thum et al., 2006). Flies of a dHb9-Gal4; Gal80^{ts} line were crossed to UAS-DT-1 lines with UAS-mCD8GFP in the background. Crosses were maintained at permissive temperatures (18°C), and pupae were moved to 29°C as needed. Neurons expressing dHb9-Gal4 were targeted for manipulation using three heat pulse treatments.

Temperature shift regimens—Fly stocks for the transgene expression were raised and maintained at 18°C with 12hour light/dark cycles before beginning of each treatment. Treatment 1 (T1): 0hr control and experimental pupae were transferred to 29°C for the duration of metamorphosis. Treatment 2 (T2): 0hr control and experimental pupae were transferred to 29°C until the 48h APF (mid pupal stage) and transferred back to 18°C for the remainder of metamorphosis. Treatment 3 (T3): 0hr control and experimental pupae were maintained at 18°C until the mid-pupal stage and transferred to 29°C for the remainder of metamorphosis. Following the completion of each treatment, eclosion rates were measured (Table 5) and animals were dissected as virgin adults to examine cell bodies and NMJs.

Analysis of eclosion rate and cellular analysis

T1–T3 flies were divided into two groups- (1) Normally eclosed and (2) Un-eclosed. Based on a sampling of 30 flies, those that eclosed by 115h APF were considered ‘normally eclosed’. A very small proportion of flies can protrude their head, but do not fully eclose; these ‘half eclosed’ flies were considered un-eclosed. Flies from each category were dissected and imaged to examine the pattern of identified dHb9>GFP expressing motor neurons. Two different controls were used for this study. Control 1 (UASmCD8GFP/Gal80^{ts}; dHb9-Gal4/Tb) was used to compare eclosion rates. Control 2 (UASmCD8GFP/+; dHb9-Gal4/+) was to compare innervation patterns and dHb9>GFP expressing cell bodies.

Video Analysis for monitoring eclosion defects

Emergence of flies was recorded using a Sony HDR-CX210 video camcorder mounted on a stand in a 29°C incubator. Eclosion behavior was recorded during the 12 hr ‘light’ cycle in order to prevent any disruption of the natural light-dependent biorhythms. Control and experimental pupae were collected at 0h APF and placed in a moist chamber at 29°C. Video recording was usually performed on the fourth day after collection, for a period of 12 hours, and included the anticipated eclosion time. Multiple recordings were performed with a total of 107 control and 117 experimental flies. Video clips of 36 control flies and 36 experimental flies (includes 4 that eclosed, and 2 that half-eclosed) were used for behavioral

analysis. These analyses consisted of examining (1) head inflation (2) Operculum rupture (3) head emergence (4) abdominal contractions after head emergence.

Relative GFP intensity measurement of dHb9-positive cell bodies in the VNC

Relative GFP fluorescence intensity of cell bodies in the ventral nerve cord of control ($n=5$) and treatment1 experimental flies ($n=5$) was quantified. Both control and experimental preparations were treated in similar with identical exposure times (similar PMT value). IMAGEJv1.44 software was used to measure the signal intensity of the pixels (RGB) on the experimental as well as on control tissues and the ratio of the fluorescence intensity was measured. Differences in pixel intensity between control and experimental flies were evaluated using Student's *t*-test.

RESULTS

The larval body wall musculature is stereotyped, consisting of a repeated array of 30 muscle fibers each of which can be distinguished by their unique positions in the body wall, and the characteristic shape of NMJs (Johansen et al., 1989). There is usually a one-to-one correspondence between muscle fibers and their pre-synaptic partners, and the identities and positions of specific motor neurons have been well established at the embryonic stage (Landgraf et al., 1997). In the embryo, the neuronal identity gene, dHb9, is expressed in motor neurons that project to a subset of ventral and dorsal muscle targets (Broihier and Skeath, 2002; Odden et al., 2002). This was inferred based on the presence of a GFP label in four of five well-characterized nerve branches that project to body wall muscles. dHb9 expression has not been examined in the larva, and this was a necessary starting point for the current study which aims to establish the larval origins of adult abdominal motor neurons.

dHb9>GFP positive motor neurons innervate a subset of ventral and dorsal muscle targets in the larva

The dHb9-Gal4 driver (Broihier and Skeath, 2002) in conjunction with a membrane bound GFP reporter, mCD8GFP (dHb9-Gal4>mCD8GFP), allowed the observation of cell bodies in the CNS, axonal projections into the periphery, and the expanse of neuromuscular junctions. Labeled NMJs were present in every abdominal segment and were found on several prominent ventral muscles and a smaller subset of dorsal muscles (Fig 1A). The average number of NMJs seen in A1 and A2 is 10.83 0.16, and 10.5 0.12 respectively; fewer are seen in A3 and A4 (Table 2). NMJs at some muscle fibers, for example, MFs 12 and 13, express GFP strongly in all segments. In A1 and A2, these fibers are known to persist through metamorphosis and are eliminated after eclosion (Kimura and Truman, 1990). At most other muscle fibers, GFP expression is less intense in posterior hemisegments; for example, the cleft of MFs 15/16. GFP-positive sensory neurons (LBD) (Gorczyca et al., 1994) are found in the transverse nerve (TN), which runs along the segmental border; they too are more prominent in anterior abdominal segments.

The nerve branches populated in the third instar larva (Fig 1B, Table 3) are the same as those reported in the embryonic stage (Broihier and Skeath, 2002; Odden et al., 2002). These include, SNb, SNc, and SNd, all of which innervate ventral targets, and the ISN, which

carries axons that innervate dorsal muscles (nomenclature according to Johanssen et al., 1989). SNa, which carries axons to lateral muscles is the only nerve branch that does not contain axons of dHb9>GFP expressing motor neurons. Double labeling conducted with anti-HRP (all junctions) and anti-GFP (dHb9 junctions) revealed that most of the dHb9>GFP expressing axon terminals are the largest size, Type Ib, (Budnik et al., 1996; Gorczyca et al., 1993; Jia et al., 1993; Johansen et al., 1989), except for a single motor neuron that innervates the characteristic cleft NMJ of muscles 15/16, which elaborates intermediate sized Type I terminals (Fig 2; Table 3). This motor neuron is the only axon revealed in the SNd branch. The motor neuron innervating muscle 26 was not identified by Hoang and Chiba (2001), and our studies designate it as MN26-Ib. It is the only axon revealed in the SNc nerve branch (Fig 1B). ISN and SNb branches carry two or more axons of dHb9>GFP expressing neurons.

A typical arrangement of labeled cell bodies is shown in Fig 1C. There are many more cell bodies in A1 and A2 as compared with A3–A5 (Fig 1G, Table 2). This trend resembles what is seen for NMJs. A prominent set of dHb9>GFP expressing cells is present in the terminal segments (A7–A8) of the CNS, and the corresponding nerve bundles are always heavily labeled. We did not detect more cell bodies than NMJs that are strongly labeled with GFP, and for the most part, there is a close correspondence between the number of cell bodies and NMJs for each abdominal hemisegment (Fig 1G, Table 2), suggesting that the robust dHb9>GFP positive cells at the third larval instar are mostly motor neurons. We do not rule out that dHb9>GFP may be expressed at lower levels in interneurons or additional motor neurons. Many more muscles in A1 and A2 exhibit strong GFP labeling than in posterior segments (A3 onwards).

The robust expression of GFP at the pre-synaptic terminals and in the cell bodies was crucial to enable an analysis of the fate of larval motor neurons during the pupal phase. The cell body positions of dHb9>GFP expressing neurons can be categorized into three groups based on D/V positions in the VNC (Fig 1C)- dorsal, intermediate and ventral. The dorsal cluster of neurons is very prominent in anterior segments (T2–A3), barely detectable in A4–A6, and seen prominently in the terminal segments. Paired clusters of five dorsally located motor neurons have been individually identified through intracellular dye-fills (Choi et al., 2004). Of these, three are dHb9-positive in A1 and A2, corresponding to the motor neurons innervating MFs 6/7, 30, and 14 (Fig 1D and 1E). The other 2 cells express the transcription factor, even-skipped (Fig 1F). dHb9 and even-skipped are known to be cross repressive and therefore expressed in non-overlapping subsets of neurons (Broihier and Skeath, 2002). The three dHb9 expressing neurons are arranged in a predominant configuration which is triangular (A1: 26 out of 36 hemisegments; A2: 23 out of 36 hemisegments) and a less predominant linear cluster. Based on a previously published characterization of this cluster (Choi et al., 2004), we propose that in A1 and A2, the dorsally located dHb9>GFP expressing neurons correspond to motor neurons that innervate MFs 6/7, 30, and 14.

dHb9-expressing NMJs in the adult abdomen are observed at ventral and dorsal muscles

The adult abdominal muscles develop during a four day period of metamorphosis (Currie and Bate, 1991). By 72h APF, a complete adult specific muscle pattern can be seen (Fig 3A). Similar to the larval pattern, the dHb9 innervation of adult muscles is restricted to ventral

and dorsal muscle fibers. In A1, this ventral muscle group is large and consists of 2–3 muscles arranged in a triangular conformation (Fig 3A–C). They have been previously identified as 80 and 81 (Miller, 1950). Ventral muscle groups in A2 onwards are much smaller in size and have fewer fibers. The dHb9 positive innervation of MFs 80 & 81 in A1, and the midline ventral muscles in A2 onwards, is maintained through eclosion and the 2-day old adult stage. The complement of ventral muscles in A1 and A2 of virgin adults includes persistent larval muscle fibers 13 and 12 (PLMF13, 12, Fig 3A, B). PLMFs12 and 13 are used for eclosion and subsequently degenerate (Kimura and Truman, 1990) (Fig 3C). This predicts a loss of dHb9>GFP-positive axons after eclosion. In virgin adults, five axons can be identified in the A2 nerve just after it exits the CNS (Fig 3D); of these, three project to ventral muscles (n=18; PMF12, PMF13 and MF 111), and two project dorsally (Fig 3D', D''). In the 2-day-old adult, 3 axons can be seen- one projecting to the ventral muscle at the midline, and two projecting to dorsal muscle fibers (n=12; Fig 3E). The reduction in ventrally projecting axons is significant ($p<0.0005$), as are the number of secondary axonal branches and boutons (data not shown). By contrast, there is no reduction of ventrally projecting inputs in A3 (Fig 3F); a single axon can be detected in newly eclosed flies as well as in 2-day old adults, which innervates the midline ventral muscle. No reduction of inputs to the dorsal muscles is observed in A1 and A2. Of the 18–22 dorsal muscle fibers normally found in A2–A5 (Currie and Bate, 1991), 9 fibers are innervated by dHb9 expressing neurons (Fig 3G, H; n=16 animals). None of the 20 lateral muscle fibers receive dHb9 innervation (Fig 3A–C). Non-dHb9>GFP NMJs are also observed in A1–A3 ventral muscles, suggesting that at least two distinct classes of motor neurons innervate these muscles in the adult (data not shown).

Establishing the relative positions of dHb9>GFP motor neurons in the larva

To follow the fate of dHb9 expressing motor neurons into the adult stage, it was important to individually identify each GFP labeled larval motor neuron, and to identify relative positions of neurons in the dorsal and intermediate clusters. This was made possible by the availability of genetic tools, and the ability to identify larval neurons by the characteristic shape of NMJs made on their target muscle fibers. Using a cell marking technique, the “Flip-out” strategy (Struhl and Basler, 1993) we restricted the GFP label to 1–3 neurons per hemisegment. Following the axonal trajectories of individual neurons to their muscle targets allowed individual identities to be assigned to motor neurons based on the Hoang and Chiba (2001) nomenclature. 165 labeled motor neurons (A1–A4) were individually analyzed to identify 11 dHb9 expressing neurons. The positions of seven (of 11) dHb9-expressing motor neurons in A1 and A2 were identified by FLIP-out induced GFP expression (Fig 4A–D). The identities of the remaining four, which have been previously described, were confirmed to be the single ventral MN12-Ib, and three that comprise the dorsal cluster, MN14-Ib, MN30-Ib and MN6/7-Ib. Axonal projections of individual motor neurons through the CNS provided an additional attribute for identification- motor neurons in the dorsal cluster and ventral clusters project contralaterally, whereas all neurons of the intermediate cluster project ipsilaterally (Fig 4, 5).

The number of dHb9 expressing motor neurons progressively decreases during metamorphosis

The three clusters continue to maintain their spatial separation along the D/V axis, making it easy to identify and follow them through metamorphosis and into the adult stages (Fig 6A–C, Fig 7A–C). A large number of neurons are known to undergo programmed cell death during the early phase (0h–20h APF) of metamorphosis (Choi et al., 2006; Winbush and Weeks, 2011), and to determine if dHb9>GFP expressing motor neurons were affected, the number of cell bodies in A1–A4 hemisegments of the VNC was quantified (Table 4). At 20h APF, compared to the larva, A1 and A2 show declines of 15% and 30% respectively, while A3 and A4 show about a 50–60% reduction. A reduction in number of GFP positive cells suggests that the cells may be eliminated or that they stop expressing GFP. Cell death assays, described later, support the elimination hypothesis. MN12-Ib which is the only motor neuron located most ventrally, remains intact in A1–A4 until 20h APF, and thus, most of the reduction occurs in the dorsal and intermediate clusters.

In A1, the total number of dHb9>GFP expressing neurons (approximately 8) does not change significantly between 20h APF and the newly eclosed adult (virgin) stage (Table 4); we propose that these are equivalent sets, and subsequently demonstrate that this is so, at least for the dorsal cluster (Fig 4, 5). By contrast, in A2, there is a reduction from 20h APF (7 ± 0.18 , $n=23$) to the virgin adult stage (5.33 ± 0.17 , $n=16$). During the post eclosion period, there is a significant reduction of the number of dHb9>GFP expressing neurons in A1 and A2 (Table 4; $p<0.005$). Quantitative analysis indicates that this elimination includes two neurons in each hemisegment, and these are likely to be motor neurons innervating PLMFs 12 and 13, which are implicated in eclosion (Kimura and Truman, 1990). The number of neurons in A3 and A4 is smaller at the virgin adult stage when compared with 20h APF, and there is no post-eclosion reduction. Consistent with a specialized muscle pattern that exists in A1, persistence of motor neurons is greatest in A1 (Table 4), where 70% of larval neurons are incorporated into the adult set. In A2, the persistence is 30%, and for A3 and A4 it is approximately 12%.

Development of the adult complement of dHb9 expressing motor neurons

Dorsal Cluster (0–5µm)—A progressive decrease in the number of cells is observed during the 0–12h APF period (Table 4). MN6/7-Ib, MN14-Ib and MN30-Ib can be easily identified at 8h APF (Fig 6A). These cells are present in 91% of A1 hemisegments (2.9 ± 0.29), and 54% of A2 hemisegments at 12h APF (2.54 ± 0.5 ; Fig 6B). At 10h APF, TUNEL signals are seen in one cell of the A1 dorsal cluster in 80% of the cases examined ($n=10$ hemisegments; Fig 6D, D'). A combination of TUNEL and FLP induced GFP labeling, muscle targets, and an examination of relative positions (based on the maps shown in figure 5) indicated that the dying neuron, located closer to the midline, and posteriorly in the cluster, is MN6/7-Ib (Fig 6I). Similarly, the identities of dying neurons in A2 were determined (Fig 6E–H). The progressive removal of cells is depicted in Fig 6I. Overall, cell death studies indicated that MN6/7-Ib is eliminated in both A1 and A2, and MN30-Ib is eliminated only in the A2 dorsal cluster by 10h APF (Fig 6E). In combination with FLP-induced GFP labels at the larval stage that were imaged into the adult stage [presented later], we conclude that dHb9>GFP expressing motor neurons that persist beyond 20h APF are

MN30-Ib and MN14-Ib in A1 and MN14-Ib in A2. Cells of the dorsal cluster do not persist in A3(68%), or A4 (100%).

The profile of dHb9>GFP expressing motor neurons seen at 20h APF, does not change drastically during the remainder of metamorphosis and until eclosion (Fig 7). The reduction seen during eclosion (Fig 7F–G) is likely associated with post-eclosion cell death. TUNEL labeling carried out at 2hr intervals following eclosion revealed TUNEL signals 4 hour after eclosion (Fig 7G, G') in one of two neurons in A1 (83%, n=12) and in the single neuron in A2 (83%, n=12). It is evident that equivalent neurons are eliminated in A1 and A2 and their identity was determined to be MN14-Ib (Fig 7D–F). Thus, in a post-eclosion animal, the only surviving neuron is in the A1 dorsal cluster- MN30-Ib (Table 4).

Intermediate cluster (5–25µm)—In the larva, an average of six dHb9>GFP expressing motor neurons is observed in the intermediate cluster of A1 and A2 (Table 4). A seventh neuron (MN26-Ib) is detected in 29% of A1 hemisegments and 21% of A2 hemisegments (n=21; Table 4). FLP- induced labeling has identified three that innervate dorsal muscles (MN20-Ib, MN19-Ib and MN11-Ib) and the remaining four innervate the ventral muscles (MN15/16-I, MN28-Ib, MN26-Ib, MN13-Ib; Fig 4). At 20h APF, this cluster remains intact in A1 (6.0 ± 0.17), but becomes reduced in A2 (4.75 ± 0.15). The A2 cluster further reduces by 48h APF (3.28 ± 0.18). From 48h APF onwards, there is no significant change in the number of dHb9>GFP expressing motor neurons of the intermediate cluster (Table 4).

In the posterior abdominal hemisegments, of the approximately four neurons present in the intermediate cluster at the larval stage in A3 and A4, only one neuron survives into the adult stage (Table 4). This neuron comprises the outer “row” of dHb9 expressing cells in the posterior abdomen (Fig 6A–C; 7A–C), which is easily detected through metamorphosis. In the 12 h APF stage, it was identified to be MN13-Ib using the ‘Flip-out’ technique, and by examining the cell body position (intermediate cluster and closer to the edge of the VNC) and terminals on muscle fiber 13 at 12h APF (A3, n=2; A4, n=3; A5, n=2). In light of the elimination of persistent larval muscle 13, which was the larval target of MN13-Ib, this raises a possible scenario of target switching, which is discussed later.

Ventral cluster (25–35µm)—MN12-Ib is the only dHb9>GFP expressing neuron that is located most ventrally in the nerve cord (Fig 4). This location, along with its characteristic contralateral projection enables us to follow it during metamorphosis (Fig 6A–C, Fig 7A–C). The identity of this neuron during metamorphosis was confirmed by examining FLP- induced single cell labeling at 12h APF (A3, n=2; A4, n=2; A5, n=3), where GFP positive NMJs are seen on PLMF 12 (data not shown). In 2-day-old adults, MN12-Ib is not seen in the nerve cord in A1 and A2 (Fig 7E–F). This is in agreement with the loss of its target muscle during the post-eclosion period as previously described (Kimura and Truman, 1990). We were not successful in consistently obtaining TUNEL signals for MN12-Ib, presumably due to its deep location in the nerve cord. In the posterior abdominal segments (A3–A5), MN12-Ib can be seen at 30h APF (Fig 7A; n=10) but it is not present at 48h APF stage (Fig 7B; n=14) (Table 4). This corresponds well with persistence of the muscle targets only in A1 and A2. At 34h APF, MN12-Ib was occasionally absent in the posterior abdominal hemisegments (A3–A5) of the VNC (30%, n=36); however, at 38h APF, MN12-Ib was not

found 81% of cases examined in A3-A5 (n=16). Although we have not examined cell death, this motor neuron is a candidate for elimination, since its target does not persist.

Remodeling of NMJs on persistent larval muscles 12 and 13 during metamorphosis

It was of interest to closely follow MFs 12 and 13 and their respective pre-synaptic partners, since the persistence of these muscles in A1 and A2 is implicated in eclosion (Kimura and Truman, 1990). Between 0–12h APF, muscle histolysis has already been initiated as evidenced by loss of the myofibrillar structure (Fernandes et al., 1991); however, the muscle patterns and characteristically shaped NMJs can be easily discerned (Fig 8 A–B, D–E). At 12h APF, dismantling of the (cleft) innervation is evident for muscles 6/7, while the terminals on MFs 13 and 12 appear more intact. This is aligned with loss of MN6/7-Ib in abdominal segments and the persistence of MN12-Ib and MN13-Ib, that we have observed (Fig 6G, H; Fig 8B). The persistence of MFs 12 and 13 in A1 and A2 is evident at 20h APF (Fig 8C), at which time they are highly contracted in nature. At 30h APF, when the formation of adult specific innervation pattern has just initiated (Currie and Bate, 1991), a terminal “knob” of GFP expression can be seen on the persistent muscle fibers (Fig 8F). By 72h APF, when the adult neuromuscular pattern is evident, persistent MFs 12 and 13 are elongated and display elaborated GFP positive terminals (Fig 8G), suggesting that the muscles have undergone remodeling as has their innervation.

MN13-Ib switches muscle targets during the larval-adult transition

In the larval stage, ‘flip-out’ labeling revealed that MF13 is innervated by a dHb9-positive motor neuron in the intermediate cluster, MN13-Ib, with an ipsilaterally projecting axon (Fig 4H, 4I). However, in the adult stage, we observed that this PLMF 13 is innervated by a motor neuron of the dorsal cluster, which projects contralaterally (Fig 10A–H; n=8). Two possible scenarios were considered to explain this discrepancy. In the first, MN13-Ib dies during metamorphosis, and subsequently, MN14-Ib innervates PLMF 13. Alternatively, larval MN13-Ib relocates its synapse during metamorphosis, while MN14-Ib innervates PLMF 13 in the adult. To discern these two scenarios, a longitudinal imaging analysis was conducted. Using the ‘flip-out’ strategy, larvae were identified in which GFP labeled NMJs were present on MFs 13 and 12 (Fig 9A). Aging these animals to the virgin adult stage, reveals that MN12-Ib innervation is maintained on MF12; however MN13-Ib innervates the adult specific A1 triangular muscle, MF80 (Fig 9B, C). FLP induced GFP expression was also obtained for NMJs of MFs 14 and 12 (Fig 9D). When this animal is aged to the virgin adult stage, MF13 was revealed to be the postsynaptic target of MN14-Ib (Fig 9E, F). Taken together, these data indicate that MN13-Ib relocates its synapse during metamorphosis and that a “non-native” pre-synaptic cell, MN14-Ib, innervates persistent MF 13.

In FLP- induced GFP labeled animals examined at 30h APF (Fig 9G–J), the contralateral axon projection of MN14-Ib makes terminals on persistent MF 13 (n=3). At this time, the ipsilaterally projecting MN13-Ib is also seen to terminate on MF 13 (n=3). This is in agreement with the Currie and Bate (1991) study that first examined the development of adult abdominal innervation, which indicated that persistent larval muscles serve as anchoring points for motor neurons that withdraw their larval innervation. At 60h APF (Fig 9K–O), when the adult specific innervation pattern is already established (Currie and Bate,

1991; Hebbar et al., 2006), examination of FLP induced GFP shows that MN14-Ib elaborates terminals on MF 13 in segments A1 and A2 (n=12, A1 and A2), whereas MN13-Ib elaborates terminals on a newly generated adult ventral muscle (n=9, A1 & A2). Collectively, we conclude that MN13-Ib relocates its synapse between 30h-60h APF stages, despite the persistence of its larval postsynaptic target.

Postsynaptic partners of dHb9-expressing motor neurons at the adult stage

FLIP-out induced single cell labeling was used to match pre-and postsynaptic targets in the adult, similar to the approach we used for the larva (Fig 4).

Dorsal cluster—At the virgin adult stage, two neurons are present in A1 (Fig 7D, E), of which one innervates PLMF 13 (Fig 10A, B, D; n=15); the other innervates MF 81 (Fig 10E, F, H; n=8). In A2, the single dorsally located neuron innervates PLMF 13 (Fig 10A, C, D; n=8). In light of the longitudinal imaging data, we are able to conclude that MN14-Ib, a neuron of the dorsal cluster, innervates PLMF 13 in A1 and A2. This allowed us to further conclude that the second neuron in A1 is MN30-Ib, whose target is the newly generated A1 specific ventral muscle 81. PLMF 13 is conspicuously absent from segment A3 onwards, and correspondingly, no dorsally located neurons expressing dHb9>GFP are seen in the nerve cord.

Intermediate cluster—At the newly eclosed adult (virgin) stage, six dHb9>GFP-expressing neurons are seen in 71% of A1 hemisegments (Fig 7D, E; Table 4). Of these, three innervate ventral muscles (Fig 10I–N), suggesting that the remaining three innervate dorsal muscles. This was supported by individually labeled neurons seen in the A2, each of which innervates multiple, and mutually exclusive dorsal muscle fibers (Fig 10O–P’’).

Ventral cluster—MN12-Ib is the only neuron that is located in this cluster, and is found in A1 and A2 segments of virgin adults (Fig 10Q–T).

Manipulating dHb9-positive neurons: Effects on eclosion rate—Since MFs 12 and 13 have been implicated in eclosion, and they are innervated by dHb9 expressing motor neurons, we hypothesized that these neurons are part of the eclosion circuit. To test for a role in this behavior, the dHb9-Gal4 driver was used to target diphtheria toxin (DT-1), which is used for cell ablations (Han et al., 2000). To determine the most effective period during metamorphosis to carry out the manipulation, three treatments were devised (Table 5, Fig 11A). As a baseline, we determined that control flies eclose ~96% of the time (n =107). The DT1 manipulation during the entire pupal period resulted in a more dramatic reduction in eclosion (~6%; n=117) (Fig 11B; Table 5). The sequence of events that occur during eclosion have been previously described (Kimura and Truman, 1990; McNabb et al., 1997). Operculum rupture is a visible starting point of the eclosion program. Prior to the rupture, a positional adjustment occurs at the anterior end of the pupal case, which moves the head up against the pupal casing wall, a process that is thought to allow subsequent head and thoracic muscle contractions to bring about the rupture, followed by head emergence. A combination of thoracic and abdominal contractions then leads to full emergence of the virgin adult. A majority of experimental animals (70%; n=117) failed to display the positional adjustment,

such that a visible gap is seen between the head of the fully formed pharate adult and the anterior end of the pupal casing (Fig 11E and E'). A small number of flies (~4%) do exhibit a partial (half eclosed) or complete eclosion program, and these are considered escapers (Fig 11D, D'). The half-eclosed phenotype (3 out of 117; Fig 11F) is a fully formed adult fly with its abdomen stuck in the puparium. Large variations were observed in the time taken to display characteristic eclosion milestones; nevertheless, the behaviors deviated significantly from control animals (Table 6). The underlying mechanism for the observed eclosion defects observed is likely to be primarily due to a role for dHb9-positive neurons in the head and thorax, with significant roles for abdominal counterparts in the overall execution of the eclosion program

Cellular analysis of eclosion defects

To determine the effects of dHb9-Gal4 driven DT-1 manipulation at the cellular level, dHb9>GFP expressing cell bodies in A1 and A2 segments of the adult ventral nerve cord were examined (Fig 12 A–D). Interestingly, MN12-Ib, the sole representative of the ventral cluster which innervates PLMF 12, was intact in most of the manipulated animals. In control animals, two motor neurons of the dorsal cluster are seen in A1 (MN14-Ib and MN30-Ib) and one in A2 (MN14-Ib) at the virgin adult stage. In A1 hemisegments MN14-Ib, which innervates PLMF 13, was absent in 50% (n=26) of eclosed animals, 85% (n=26) of un-eclosed animals and 67% (n=12) of half-eclosed animals. This neuron which is the only persistent motor neuron in A2, was mostly absent. Examination of NMJs on PLMF 13 revealed that in control flies, two motor neurons innervate PLMF 13; one is dHb9>GFP positive and the other neuron is non-dHb9 (n=8; Fig 12 E–F). In experimental flies, the NMJs on PLMF 13, did not express detectable levels of GFP (80%, n= 8; Fig 12G). Since cell bodies could be detected in the CNS (Fig 12G), the GFP intensity of cell bodies was assessed between control and experimental flies. We found an approximately 75% decrease in total GFP intensity in experimental flies as compared to controls (n=5), indicative of decreased GFP synthesis in DT-1 treated experimental flies; this explains the lack of/very low GFP expression at NMJs (Fig 12H).

DISCUSSION

The present study has followed dHb9 expressing motor neurons through metamorphosis and into the adult stage, revealing multiple contexts for plasticity during the radical restructuring of the motor system. This includes generation of a new musculature with segment specific patterns, segment specific cell death and rewiring of larval motor neurons, switching of targets during the rewiring, and a role for persistent larval neurons in *Drosophila* eclosion. The correspondence between larval and adult pre-and postsynaptic partners is indicated in Figure 13 and 14.

Transition of the larval muscle pattern into the adult

Each hemisegment of the larval abdominal body wall has about 30 muscle fibers, the pattern of which is set up during embryonic myogenesis. Aside from minor variations in muscle insertion sites, the abdominal muscle pattern is mostly invariant from segment to segment, except for the terminal segments. In addition to muscle fibers 6/7, 12 and 13, which belong

to the internal layer of fibers (Hooper, 1986), dHb9>GFP expression identifies NMJs at other ventral muscle fibers, 26, and 27 which belong to the most external layer of muscle fibers, and 15/16, 14, 30 [ventral], 20, 19 and 11 [dorsal] which are part of an intermediate layer of fibers. All of these muscles degenerate at the beginning of metamorphosis, except MFs 12 and 13 in A1 and A2. These muscles function in eclosion and then undergo programmed cell death. In the adult, the pattern of muscles is simple, with a small set of 5–8 ventral muscles very close to the ventral midline, 20 closely apposed lateral fibers arranged dorso-ventrally, and 17–22 dorsal muscles that run longitudinally (Currie and Bate, 1991). Unlike the larval muscle fibers which are each innervated by a primary motor neuron, our data suggests that the persistent adult motor neuron can innervate multiple fibers.

The process of eclosion is attributed to many more muscle fibers in addition to PLMF 12 and 13. A set of 4 dorsal muscles (PLMF 1–4) and one lateral muscle (PLMF 8) are present in each hemisegment until the virgin adult stage and later degenerate (Kimura and Truman, 1990). PLMFs 12 and 13 do not remain larval like during the pupal period. They condense and lose birefringence as do most body wall muscles, but rather than degenerating, they elongate, widen, and a new set of motor terminals are generated at the NMJ (Fig 8). This remodeling is not surprising given the change in functional capability [crawling to eclosion], and bears some resemblance to the initial changes that are seen in the larval scaffolds for DLM thoracic muscles (Fernandes et al., 1991). It is not known if new myoblasts fuse with the persistent muscles as they do for the DLM.

Segment specific cell death refines the larval pattern of dHb9 expressing motor neurons to generate the adult pattern

The spatial separation of the dorsal, ventral, and intermediate cluster of motor neurons, and the characteristic axonal projections (contra lateral/ipsilateral) of the identified larval motor neurons allowed for easy identification. For example, the dorsal cluster of neurons in A1 and A2 with contralateral projections, can be tracked from the larva until the adult stage as a group that is close to the midline. The progressive reduction in the total number of neurons through metamorphosis, makes it unlikely that new dHb9 expressing motor neurons are born to generate the adult complement of dHb9 expressing abdominal motor neurons. This is aligned with studies conducted in the moth, *Manduca* as well as *Drosophila* (Tissot and Stocker, 2000; Truman, 1990), and allowed us to establish continuity from the larval to adult stages. Studies in *Manduca* have shown that many abdominal motor neurons are eliminated during the larval to adult transition and that this reflects a simplification of the abdominal musculature (Consoulas et al., 2000; Levine and Weeks, 1990; Tissot and Stocker, 2000). In *Drosophila*, two prominent periods of elimination have been documented for neurons in the CNS- one occurs during the first twenty four hours of metamorphosis, while the other occurs just after eclosion both are initiated by pulses of ecdysteroid (Choi et al., 2006; Kimura and Truman, 1990; Robinow et al., 1997; Robinow et al., 1993; Truman, 1990; Truman et al., 1992). The occurrence of cell death during the 0–24h APF period appears to clear the way for the onset of adult specific neurite outgrowth and the initiation of adult myogenesis in the abdomen, which is in progress by 28h APF (Currie and Bate, 1991). Post eclosion elimination is seen primarily in the motor neurons that innervate persistent larval muscle fibers [PLMFs] 13 and 12. MN14-Ib which innervates PLMF 13, and is while MN12-Ib

which innervates PLMF 12, are retained in A1 and A2, until the virgin adult stage, and eliminated 4 h after eclosion (Table 7, Fig 7). Segment specific cell death is most evident for the dorsal cluster, and the single neuron comprising the ventral cluster. These neurons derive from the same neuroblast, NB 3-1 and project contralateral axons (Landgraf et al., 1997; Schmid et al., 1999).

Neurons of the intermediate cluster project ipsilateral axons and are mostly derived from two neuroblasts (NB 3-2 and NB 4-2; Table 7). dHb9>GFP labeling in these neurons during the pupal period reveals that the cluster in A1 has the same number of motor neurons at the larval and at the adult stage. In the rest of the hemisegments, a reduction in number is seen during the 0–20h period (A2: 28%, A3: 71%; A4: 80%).

MN12-Ib, the sole dHb9 expressing motor neuron representing the ventral cluster, persists in A1–A6 beyond the 24h APF period (Fig 6, 7), although its muscle target, MF 12, is retained only in A1 and A2. It can be detected at 30h APF using dHb9>GFP, but is not present beyond 48h APF. Although, we have not tested for cell death during this period, it is likely that MN12-Ib is eliminated, as part of the simplification of the abdominal motor system. The persistence during the 24–30h period may have a bearing on the development of the adult neuromuscular system. The first myoblast fusions are detected around 28h APF, and nerve growth cones grow toward the nascent target muscles during the 26–36h period. Thus it is likely that cues from the periphery can bring about elimination of MN12-Ib in A3–A6.

Cell death is an important process that sculpts the nervous system, and has been reported during *Drosophila* embryogenesis. Two well-known pioneer motor neurons, dMP2 and MP1 undergo cell death after axon tracts have been laid down (Miguel-Aliaga and Thor, 2004). Segment specific death is also known to occur during embryogenesis in abdominal motor neurons which generates a set of thoracic motor neurons which innervate segment specific muscles (Rogulja-Ortmann et al., 2008). In *Manduca*, motor neurons are known to be eliminated in a segment specific fashion to generate sex-specific sets of motor neurons (Thorn and Truman, 1989).

Elimination of motor neurons has not been extensively studied in *Drosophila*. The only previous report of motor neuron cell death in the pupa identifies that RP2, a motor neuron innervating dorsal muscle targets, is removed during the 0–24h period, in A2–A6 (Winbush and Weeks, 2011). Interestingly, this muscle innervates dorsal muscle 2, which is reported to persist and to function in eclosion (Kimura and Truman, 1990). This raises the possibility that other motor neurons can innervate persistent muscles, a phenomenon that we have observed for PLMF 13.

Target switching during metamorphosis- possible clues from developmental history

Since larval MFs 12 and 13 are known to persist until eclosion, we expected that their cognate motor neurons would persist through metamorphosis and continue to innervate their larval targets. Through longitudinal (live) imaging we find that MN13-Ib switches targets to innervate newly generated adult muscles (MF80 in A1 and muscles along the ventral midline in A2 onwards), and that MN14-Ib innervates PLMF 13 in A1 and A2, subsequent to degeneration of its target muscle. In trying to understand the circumstances that lead to

MN14-Ib innervating PLMF 13, two attributes must be considered- the distinct axonal projections (of MN13-Ib & MN14-Ib), and the close proximity of MFs 14 & 13 in the embryo and larva. MN13-Ib (intermediate cluster) projects ipsilaterally in the larva, and ceases to innervate PLMF 13 during the pupal stage. MN14-Ib (dorsal cluster) projects contra-laterally in the larva, and after the death of its larval target, begins to elaborate terminals on PLMF 13. The “relationship” of these two neurons with PLMF 13 originates in the embryo. Embryonic studies show that two dorsally located neurons, RP1 and RP4, project contra-laterally, and innervate MF 13. Since these studies were done at stage 16 (Landgraf et al., 1997) when embryonic motor neurons have not yet elaborated terminals and may still be exploring muscle targets, the maps are not likely to represent the final targets. We propose that MN14-Ib is one of the embryonic pair of RP1 & RP4, that it explored the surface of MF13 located more internally in the bodywall, and later elaborated terminals on the more externally located MF14. The second candidate for the embryonic pair of RP1/RP4, is MN30-Ib, and just like MN14-Ib could have explored the surface of MF13, leading to the currently accepted embryonic map. The larval MN13-Ib is known to be ipsilateral (Hoang and Chiba, 2001; Kim et al., 2009), and is believed to be a new neuron that did not innervate targets in the embryo, in light of RP1 & RP4 being the contralaterally projecting embryonic motor neurons. We propose however, that the ipsilateral motor neuron depicted as innervating both MF 14, and MF 30 in the embryo, is the exploratory phase at stage 16 when the maps were made, and that it later “settles” for MF 13. We don’t yet understand why MN13-Ib would switch its target to an adult specific muscle while MN14-Ib; having lost its larval target begins innervating persistent muscle 13 in the pupa.

Any persistent larval motor neuron that does not innervate its larval target, engages in “switching” its muscle target, when it innervates a newly developing adult muscle fiber as is the case for most abdominal motor neurons. Studies conducted in *Manduca* have revealed that (Levine and Truman, 1985) during this process the dendritic arbors undergo morphological changes, which will aid the incorporation of these neurons into new neural circuits.

A role for dHb9>GFP expressing neurons in eclosion

The role of dHb9 in establishing neuronal identity is well known. Initially, during the process of specification, even skipped and Hb9 are expressed in non-overlapping subsets, serving to distinguish between dorsally projecting and ventrally projecting motor neurons (Broihier and Skeath, 2002; Odden et al., 2002). The persistence of dHb9 in neurons after the larval period may have some role to play in survival, prior to being re-specified to innervate muscle targets that will engage in the adult specific behavior of eclosion. Our manipulations of dHb9-expressing neurons by targeting DT-1 resulted in eclosion defects, suggesting the importance of these neurons in the eclosion circuit. However, since we have not examined dHb9 expression in thoracic and head regions, the involvement of neurons in addition to the abdomen cannot be ruled out. Also, the eclosion circuit must involve other non dHb9 expressing motor neurons that innervate dorsal and lateral muscles that persist from the larva (Kimura and Truman, 1990). Nevertheless, it is significant, that in DT-1 expressing flies, the majority of experimental flies (96%) failed to undergo eclosion, while the rest took 13- fold longer time to complete the eclosion process or managed to half-

eclose. Moreover, quantitative analysis of the rate of abdominal muscle contractions reveals that experimental flies exhibit slower contractions than control flies, thus, highlighting the importance of dHb9-expressing motor neurons in the proper execution of eclosion behavior. The defects in operculum rupture suggest that dHb9-positive neurons innervating thoracic/head muscles could be affected as a result of cell-specific targeting of DT-1. These are likely to be part of an eclosion circuit. Cellular analysis has revealed that MN14-Ib which innervates PLMF 13, plays a more important role than MN12-Ib, which innervates PLMF 12.

Quantitative analyses indicate that in DT-1 treated experimental flies, a majority of neurons survive when compared to controls. In reality, however, these neurons may be operating at physiologically insufficient levels to execute necessary muscular contractions due to partial inhibition of protein synthesis. Therefore, it is possible that many of the persistent dHb9>GFP expressing motor neurons do play a role in eclosion, with MN14-Ib possibly playing a larger role than others. Future studies can be aimed at dissecting the role of individual dHb9-positive motor neurons to further elucidate the functional significance of individual motor circuits in this complex behavior.

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Figure 1. Expression pattern of dHb9>GFP in the third instar larva

(A) A subset of NMJs in the body wall is labeled with GFP. A1n= A1 nerve; Arrowhead indicates cleft innervation of MF 15/16. (*)= Muscle fibers 13 and 12 in A2. (B) The number of dHb9 axons in each nerve tract is indicated. (C) Three clusters of dHb9>GFP expressing cell bodies are observed: dorsal (red), Intermediate (green and yellow) and ventral (blue). Dashed line indicates the dorsal midline of the CNS. (D–F) Of the five dorsal neurons (green) revealed in OK371>mCD8GFP, three are dHb9-positive neurons (E, red; D, green & red) and two are even-skipped positive (F, red). (G) Number of dHb9-positive neuronal cells closely matches with the number of NMJs. Bars represent 200 μ m in (A), 40 μ m in (B), 20 μ m in (D) and 35 μ m in (E and F).

Figure 2. Morphological attributes of NMJs made by dHb9>GFP expressing motor neurons
 (A) Schematic representation of the NMJs made by dHb9 expressing neurons which populate four of six known nerve branches: SNb, SNc, SNd and ISN. Muscle fibers innervated by the corresponding motor neurons are indicated in bold. Double labeling with HRP indicates that GFP reveals only a subset of boutons at each of the muscle fibers. Muscles closer to the cuticle are indicated in dashed lines. (B) A group of four ventral muscles that are innervated by dHb9>GFP expressing motor neurons. (C–F) Higher magnification images showing the NMJs at each of the 6 muscle fibers shown in (B). (G)

Schematic depicting a group of ventral muscles (dashed lines) that lie beneath the ones shown in B. These include (H) MF 26 (SNc), (I) MF 28 (SNb), (J) MFs 30, 14 (SNb). (K) Three dorsal muscles are innervated by dHb9>GFP expressing motor neurons, all of which receive axons through the ISN branch. These include MFs 20, 19 (L), and MF 11 (M). Scale bars represent 50 μm for (C–F), 65 μm for (H, I, J, L), 45 μm for (M) and 110 μm for (B and K),

Figure 3. Adult innervation revealed by dHb9>GFP in the third instar larva

(A–C) The mature pattern of dHb9>GFP innervation is present at 72h APF. White asterisks: Persistent larval muscle fibers 12 and 13; White dotted line: a large triangular muscle arrangement in A1 (MFs 80, 81; Crossley, 1980). In a 2-day old adult, MFs 12 and 13 are absent in A1 and A2. (D) Virgin Adult: Three NMJs can be observed in the A2 ventral muscle group (D') Close-up of the A2 segmental nerve, which carries a total of 5 axons, 3 to the ventral muscle group and 2 to the dorsal muscles (D''). (E) In a 2-day old adult, a single motor axon in A2 innervates the ventral muscles, and two motor axons project to the dorsal

muscles. (F) The number of motor axons to ventral muscles decreases from 3 to 1 in A2; in A3, there is no change. Dorsal muscle targets of dHb9 expressing neurons at virgin (G) and 2-day old adult (H) stages. (G' and H'): Schematic representation of G and H respectively. Filled gray muscles indicate targets innervated by dHb9>GFP neurons. Black dotted lines indicate the larger persistent larval muscle fibers, which are eliminated between virgin and 2-day old adult stages. Scale bar in A represents 80 μm for (A and B) and 95 μm for (C). Scale bars in (D and E) represent 50 μm , and 90 μm in (G and H).

Figure 4. Matching pre and post synaptic partners by FLP-induced GFP expressing single motor neurons

(A–D) 9 dHb9>GFP expressing motor neurons are represented in the four examples of larval CNS containing FLP induced GFP labeling in subsets of dHb9 neurons.

(E–G) Axons of MN6/7-Ib and MN14-Ib exit the CNS contralaterally to innervate MFs 6/7 and 14 respectively.

(H, I) Axon of MN12-Ib exits the CNS contralaterally to innervate MF 12, while the axon of MN13-Ib exits the CNS ipsilaterally to innervate MF 13.

(J, K) MN28-Ib exits the CNS ipsilaterally through the SNb nerve to innervate MF 28.

(L, M) Axon of MN15/16-I exits the CNS ipsilaterally through the SNd nerve to innervate MF 15/16

(N, O) Axon of MN26-Ib exits the CNS ipsilaterally through the SNc nerve to innervate MF 26.

(P, R) Cell body positions and motor axonal projections of MN20-Ib and MN11-Ib in segments A3 and A2 respectively. Axons of MN20-Ib and MN11-Ib exit the CNS ipsilaterally through the ISN nerve to innervate MF 20 (Q) and MF 11 (S) respectively. (T, U) dHb9>GFP flip out preps labeled with anti-Hb9 antibody (red) and anti-GFP antibody (green). The CNS midline is shown with a white dotted line.

(T) Relative positions of MN6/7-Ib, MN14-Ib, MN15/16-I, MN20-Ib, MN12-Ib and MN13-Ib in A2 and A3 segments of the VNC.

(U) Relative positions of MN11-Ib and MN13-Ib in A2 and A4 segments of the VNC.

Scale bars represent 25 μm in (E, H, J, L, P, R, T and U), 30 μm in (N), 55 μm in (F, G and I), 60 μm in (K, Q and S), 70 μm in (M), 100 μm in (G) 115 μm in (O).

Figure 5. Relative positions of dHb9-positive motor neurons in the third instar larva

(A) Schematic summarizing the relative positions of dHb9-positive motor neurons in the A1 and A2 segments of the larval CNS (based on an examination of 165 clones). A color spectrum, from red to blue has been used to depict their distance from the dorsal surface of the VNC. (B) Muscle targets and characteristic neuromuscular junctions of these motor neurons are shown. Neuromuscular junctions have been color coded to match with corresponding cell bodies.

Figure 6. Persistence of dHb9>GFP positive motor neurons during early metamorphosis (0–20h APF)

A–C: Pattern of cell bodies from 8h–20h APF. The lateral cluster in A1 and A2 (Arrows), and the dorsal cluster in A1 and A2 (ovals) are indicated in the ovals. Smaller dashed circular outlines (C) indicate cells that persist through early metamorphosis in A3–A6, and collectively form an outer row of cells. These were identified to be MN13-Ib. Red arrowheads (C) indicate MN12-Ib in A3–A6, which collectively form an inner row.

D–H: Programmed Cell death (PCD) detected in a subset of dHb9>mCD8GFP expressing neurons at 10h APF, using TUNEL assay. Circles indicate the dorsal clusters of dHb9-

positive neurons in A1 (D) and A2 (E). Arrows indicate apoptotic dHb9-positive neurons in A1 (D and D'), A2 (E and E') and A3 (D and D'') hemisegments. The boxed area in (F) shows a pair of MN6/7-Ib in A2 hemisegments which also label with TUNEL (red) at 10h APF. The boxed area is shown in (G, H) at low magnification, allowing the labeled NMJs in the periphery to be seen. The projections of MN6/7-Ib into the periphery reveals the typical cleft innervation at MFs 6/7.

(I) Schematic diagram summarizing the early metamorphic (0–12h APF) fate of dorsally located motor neurons (MN6/7-Ib, MN14-Ib and MN30-Ib).

Scale bars represent 40 μm in (D, E, F, G and H), 15 μm in (D', D'', E' and F'), 20 μm in (G').

Figure 7. Establishment of the adult pattern of dHb9>GFP expressing neurons (30h-Adult)
 (A) 30h APF: The pattern of cell bodies resembles what is seen at 20h APF. (B) 48h: Due to expansion of the thoracic neuromeres, the cell bodies of MN12-Ib move closer to the midline (white arrowheads). (C, C') MN12-Ib in A1 and A2 (arrowheads) send anteriorly projecting trajectories that then cross the midline. (D, E) Virgin Adults: Persistent cells include the dorsal clusters in A1 and A2, MN12-Ib in A1 and A2 segments (white arrowheads), lateral clusters in A1 and A2 (arrows), and MN13-Ib in posterior abdominal segments (dashed white circles). (F) 2-day adult: Fewer cell bodies are observed as

compared with virgin adults. 2 cells remain in the A1 dorsal cluster. MN12-Ib is absent in A1 and A2. Several cells in the lateral clusters in A1 and A2 persist. Fewer cells express dHb9 in A3 onwards.

(G, G') 4h after eclosion: A pair of dorsally located dHb9>GFP-positive neurons in A1 and A2 undergoes programmed cell death (PCD) as detected by TUNEL labeling.

Scale bars represent 70 μm in (A: larva), 85 μm in (A: 30h APF), 90 μm in (A: 20h APF and 48h APF), 95 μm in (A: adult), 50 μm in (B, C and F), 45 μm in (E), 10 μm in (G').

Figure 8. Remodeling of innervation to persistent larval muscles 12 and 13 during the larval-adult transition

(A–C) Retraction of larval NMJs and histolysis of larval muscles occurs during the 0–12 h APF period, and by 20h, most of the larval muscles have histolyzed, and persistent larval muscles 12 and 13 can be seen only in A1 and A2 (*).

(D–G) Remodeling of NMJs on MFs 12 and 13 during 0–72h APF.

Scale Bar in C= 90µm in A–C; 50µm in D–G

Figure 9. Longitudinal imaging of MN13-Ib and MN12-Ib from larva to the adult stage reveals that MN13-Ib switches its muscle target

(A) The NMJs made by MN13-Ib and MN12-Ib in the A1 hemi-segment of a second instar larva are imaged through the cuticle.

(B) The same animal imaged as a virgin adult, shows GFP labeled NMJs on the persistent MF 12, and on MF 80 but not on persistent MF 13.

(C) Schematic representation of an adult A1 hemisegment indicating the innervated muscles seen in (B): MF 13 (gray) and MF 80 (black).

(D) In a different animal, NMJs made by MN12-Ib and MN14-Ib are visualized through the cuticle. This animal imaged at the virgin adult stage (E) shows NMJs on persistent larval MFs 12 and 13.

(F) Schematic representation of an adult A1 hemisegment indicating the innervated muscles [MF 12, 13 (gray)], seen in E.

(G–H) The terminals of MN14-Ib visualized at 30h APF are seen on MF 13 (*).

(I–J) The terminals of MN13-Ib visualized at 30h APF are seen on MF 13 (*).

(K–M) The terminals of MN14-Ib visualized at 60h APF are seen on MF 13 (*).

(O–P) The terminals of MN13-Ib visualized at 60h APF are seen on MF 80.

Scale bar in A represents 50 μm in (B, E) and 70 μm in (A, D).

Scale bar in G represents 35 μm in (G), 40 μm in (O), 45 μm in (H, I, K, and O), 70 μm in (L), 100 μm in (J).

Figure 10. Adult muscle targets of dHb9-expressing neurons in A1 and A2 as revealed by FLP-induced labeling

For each pre-synaptic cell (s) labeled with GFP, the postsynaptic muscle target shown with the NMJs. Schematics identify the muscle targets in the context of the other surrounding muscle fibers in that hemisegment.

(A) MN14-Ib in A1 and A2 (dorsal cluster) innervate MF13 in corresponding bodywall segments (B, C).

(E) MN30-Ib and MN13-Ib which are cells of the dorsal cluster, persist to innervate MF 80 and 81 respectively (F, G).

(I–N) Subsets of neurons of the lateral cluster in A1 are labeled in (I) and (L); their muscle targets include MFs 80 and 81 (J, K, M, N).

(O–P'') Two distinct neurons in the lateral cluster of A2 are labeled. These innervate non-overlapping sets of dorsal muscles in the virgin adult.

(Q) MN12-Ib is labeled in A1 and A2, which contralaterally innervate the persistent larval muscle 12 in the bodywall (R, S).

Scale bars represent 25 μm in (B, F and J), 40 μm in (C), 35 μm in (G), 30 μm in (M and R), 45 μm in (S) and 40 μm in (O' and P').

Figure 11. Effects of DT-1 expression on dHb9 neurons

(A) Temperature regimens to manipulate dHb9-expressing neurons during metamorphosis. (B) Monitoring eclosion behavior defects with video recordings. White dotted lines indicate control flies. Video frames of control (C and C') and experimental flies (D and D'). (E and E') Successive video frames of an experimental fly showing a gap between the head and the anterior end of the pupal casing even after 115hr-equivalent age, indicating a lack of head expansion (F) A 'half-eclosed' experimental fly showing emergence of the head and thorax, but the abdomen is still within the case.

Figure 12. dHb9>GFP expressing cell bodies and NMJs in controls and DT-1 induced experimental flies

A–F: The ventral ganglion of control and experimental animals. The dorsal cluster of cells in A1 and A2 is indicated in the ovals.

(A) Control (dHb9-Gal4/UASmCD8GFP/W¹¹¹⁸): Four neurons are present in A1 and two neurons are present in A2.

(B) Un-eclosed: Three neurons are present in A1, and no dorsally located cell is seen in A2.

(C) Half-eclosed: Four neurons are present in A1, and none in A2.

(D) Eclosed: Four neurons are present in A1, and one A2.

Scale bar in A represents 50 μ m in (A), 55 μ m in (B–D).

E–H. Persistent larval muscle fibers 12 and 13 of the animals shown in A–F

(E and F) Control (dHb9/GFP/w¹¹¹⁸): A2 Ventral muscles (including PLMFs 13 and 12) receive inputs from both dHb9>GFP positive and non-dHb9 expressing neurons, indicating two distinct classes of motor neurons innervate this muscle. White dotted lines show the region of PLM 13. NMJs that do not express dHb9 are distinguished by visualizing boutons which are only HRP-positive (white arrows).

(G) Un-eclosed: White dotted lines show the area of PLM13. Non-dHb9-positive NMJs (HRP-positive; white arrows) are mainly seen on PLMF 13 in A2.

(H) Measurement of relative dHb9>GFP expression levels in cell bodies of control and experimental (treatment 1) flies. Error bars on the graph represent S.E.M. Asterisks represents significant differences ($p < 0.0005$).

Scale bar in E represents 30 μ m for (E and F), and 35 μ m for (G).

Figure 13. Schematic representation of the muscle targets of four prominent dHb9>GFP expressing motor neurons in A1–A3 during the larval and adult stages
 MN14-Ib and MN30-Ib are in the dorsal cluster and project contra-laterally to their muscle targets. In A1 and A2, MN14-Ib innervates persistent muscle fiber 13, and is absent in A3. MN30-Ib is only present in A1 and innervates a segment specific adult muscle, MF 81. MN13-Ib is in the intermediate cluster and projects ipsilaterally. In A1 it innervates a segment specific adult muscle (MF 80), whereas in A2 and A3 it innervates an adult muscle, MF 111. MN12-Ib represents the ventral cluster and innervates persistent muscle fiber 12 in A1 and A2.

Figure 14. Schematic representation of the location of dHb9>GFP expressing motor neurons and their muscle targets at adult stage

Segment specific patterns (A1–A3) of dHb9 expressing MNs are depicted in the newly eclosed adult. The colors red, green/yellow, blue, represent the dorsal, intermediate and ventral clusters respectively. Neuromuscular junctions have been color coded to match the corresponding cell bodies.

Table 1

Summary of analysis of abdominal motor neurons induced “Flip-out” HH9

Stages	Number of dissected animals	Number of labeled cells in A1–A4	Number of MN12-Ib in A1 and A2	Number of MN13-Ib in A1 and A2	Number of MN14-Ib in A1 and A2
Larva	74	165	14	12	4
6 APF	10	30	4	4	2
10h APF	14	35	6	7	0
12h APF	12	20	4	3	1 (only A2)
30h APF	15	22	4	3	3 (only A2)
60h APF	20	35	6	9	12
Adult	52	68	20	10	16

Table 2

NMJJs and cell bodies at the larval stage revealed by dHb9>GFP

NMJ on	A1 (n=6)	A2 (n=18)	A3 (n=25)	A4 (n=18)	A5 (n=21)
15/16	100%	100%	84%	44%	9%
26	83%	66%	72%	44%	42%
6/7	100%	100%	100%	100%	76%
28	100%	100%	100%	88%	90%
30	100%	100%	40%	16%	28%
14	100%	83%	80%	66%	33%
13	100%	100%	100%	100%	100%
12	100%	100%	100%	100%	100%
20	100%	100%	100%	100%	90%
19	100%	100%	100%	94%	80%
11	100%	100%	48%	11%	9%
Distribution of total # of NMJs	11= 83%; 10= 17%	11= 50%; 10= 50%	11= 16%, 10= 20%, 9= 44% 7-8= 20%	9= 22%, 8= 38% 7= 22%, 6= 16%	8= 4%, 7= 67% 6= 14%, 4-5= 15%
Av # of NMJs*	10.83± 0.16 (n=6)	10.5 ± 0.12 (n=18)	9.30 ± 0.22 (n=25)	7.66 ± 0.24 (n=18)	6.57 ± 0.20 (n=21)
Av # of Cell Bodies*	10.125 ± 0.1 (n=24)	10.04 ± 0.11 (n=24)	7.79 ± 0.77 (n=24)	7.5 ± 0.14 (n=14)	4.5 ± 0.27 (n=8)

* All values are mean ± S.E.M. Numbers in parentheses indicate sample size. The predominant numbers are indicated in bold.

Table 3

Identity of motor neurons revealed by dHb9>GFP labeled larval NMJs on target muscles in segment A1–A3.

Nerve Routes	No. of Axons	Target Muscles	Innervating motor neurons*	Cluster	Axonal projections in the VNC
SNd	1	15/16	MN15/16-I , MN15/16/17-Ib, MNSNb/d-Is, MNSNb/d-II	Intermediate	Ipsilateral
SNc	1	26	MN26-Ib %	Intermediate	Ipsilateral
SNb	6	6/7	MN6/7-Ib , MNSNb/d-Is	Dorsal	Contralateral
		28	MN28-Ib	Intermediate	Ipsilateral
		30	MN30-Ib , MNSNb/d-Is, MNSNb/d-II	Dorsal	Contralateral
		14	MN14-Ib , MNSNb/d-Is, MNSNb/d-II	Dorsal	Contralateral
		12	MN12-Ib , MNSNb/d-Is, MNSNb/d-II	Dorsal	Contralateral
		13	MN13-Ib , MNSNb/d-Is, MNSNb/d-II	Intermediate	Ipsilateral
ISN	3	20	MN20-Ib , MNISN-Is, MNISN-II	Intermediate	Ipsilateral
		19	MN19-Ib , MNISN-Is, MNISN-II	Intermediate	Ipsilateral
		11	MN11-Ib , MNISN-II	Intermediate	Ipsilateral

* Nomenclature based on Hoang and Chiba (2001). The corresponding motor neurons of dHb9>GFP labeled NMJs are indicated in bold.

% This motor neuron was not identified by Hoang and Chiba (2001).

Table 4

The number of dHb9>GFP expressing cell bodies at key stages during the larval to adult transition.

	A1	A2	A3	A4
Larva MNs *	10.125 ± 0.1 (n=24) Table 2	10.04 ± 0.11 (n=24) Table 2	7.79 ± 0.77 (n=24) Table 2	7.5 ± 0.14 (n=14) Table 2
20h MNs *	8.54 ± 0.15 (n=24) 15% reduction D: MN14-Ib, MN30-Ib I: MN13-Ib, +4 V: MN12-Ib	7 ± 0.18 (n=23) 30% reduction D: MN14-Ib I: MN13-Ib, +4 V: MN12-Ib	3.1 ± 0.28 (20) 50% reduction D: None I: MN13-Ib, +1 V: MN12-Ib	2 ± 0.0 (n=16) 74% reduction D: None I: MN13-Ib V: MN12-Ib
48h	8.36 ± 0.15 (11) D: MN14-Ib, MN30-Ib I: MN13-Ib, +4 V: MN12-Ib	5.77 ± 0.22 (9) D: MN14-Ib I: MN13-Ib, +2 V: MN12-Ib	1.71 ± 0.18 (11) D: none I: MN13-Ib, +1 V: none	1.14 ± 0.14 (17) D: none I: MN13-Ib, +1 V: none
Virgin MNs *	8.33 ± 0.21 (n=6) 15% reduction D: MN14-Ib, MN30-Ib I: MN13-Ib, +4 V: MN12-Ib	5.33 ± 0.28 (n=20) 61% reduction D: MN14-Ib I: MN13-Ib, +2 V: MN12-Ib	1.5 ± 0.13 (n=7) 81% reduction D: none I: MN13-Ib, +1 V: none	1 ± 0 (n=8) 87% reduction D: none I: MN13-Ib, +1 V: none
Adult MNs *	6.88 ± 0.11 (n=9) 33% reduction D: MN30-Ib I: MN13-Ib, +4 V: None	3.1 ± 0.31 (n=10) 70% reduction D: None I: MN13-Ib, +2 V: None	1.28 ± 0.16 (n=7) 84% reduction D: None I: MN13-Ib V: None	1 ± 0 (n=6) 87% reduction D: None I: MN13-Ib V: None

* Identities as determined by Figures 2, 4, 5, 6, 7, 9. Bold Red notation indicates a change from the previous stage

Table 5

Effects of targeted manipulations on eclosion rate

Genotypes	Eclosed (%)	Un-eclosed (%)	Sample Size
Control GFP/Gal80 ^{ts} ; dHb9/Tb	96.3%	3.7%	107
DT-1: Treatment-1 GFP/Gal80 ^{ts} ; dHb9-Gal4/UAS-DT1	5.6%	94.4%	117
DT-1: Treatment-2 GFP/Gal80 ^{ts} ; dHb9-Gal4/UAS-DT1	84%	16%	70
DT-1: Treatment-3 GFP/Gal80 ^{ts} ; dHb9-Gal4/UAS-DT1	82.5%	17.5%	70

DT-1 induced killing of dHb9-positive neurons reduced the rate of eclosion significantly as compared to controls ($p < 0.0005$).

Table 6

Analysis of the timing of eclosion behavior

	Control GFP/Gal80 ^{ts} ; dHb9 Gal4/Tb	Experimental GFP/Gal80 ^{ts} ; dHb9 Gal4/UAS-DT1
Un-successful Positional Adjustment of head	1/51 (~2%)	83/117 (70%)
Operculum rupture	49/51 (~98%)	7/117 (~6%)
Operculum rupture to head emergence	6.97 ± 0.4 n=36	159 ± 56.9 [*] n=6 (6%)
Head emergence to complete emergence	38.86 ± 6.26 n=36	511 ± 217.4 [*] n=4 (3.4%)
Operculum rupture to full emergence	45.83±6.6 n=36	594 ± 226.55 [*] n=4
# abdominal contractions after head emergence	15.13 ± 1.55 n=36	230.2 ± 109.3 n=4
# of abdominal contraction/10 sec	4.94 ± 0.2 n=36	Eclosed [*] 2.3±0.21 (n=6) Unclosed: 1.2±0.11 (n=19)

All times are in seconds; All values are ± S.E.M. Numbers in parentheses indicate sample size.

^{*}
p<0.0005

Table 7

The relationship of embryonically generated dHb9-positive motor neurons in A1 and A2 to their larval and adult counterparts.

Clusters	Embryo <i>I, 2</i>			Larva <i>3, 4, 5,*</i>		Adult [*]
	MN/NB/ Nerve route	Axonal projection	Target(s)	MN	Target (s)	
Dorsal	RP1 or RP4 NB 3-1/ISNa ^{1**}	Contralateral CB@midline	MF 13	MN14-Ib	14	A1, A2: PLMF 13
	RP1 or RP4 NB 3-1/ISNa ^{1**}	Contralateral CB@midline	MF 13	MN30-Ib	30	A1: MF81 A2: Cell death
	RP3 NB 3-1/ISNa ^{1**}	Contralateral CB@midline	MF 6/7	MN6/7-Ib	6/7	Dies at 6–10 APF
	NB 4-2	Ipsilateral	MF 14-1,2 (MF 14,30)	MN13-Ib	13	A1: MF 80 A2 onwards: ventral muscle
Intermediate	NB 4-2/SNb	Ipsilateral	MF 28	MN28-Ib [*]	28	Triangular muscles (A1) ^a A2 onwards: Absent
	NB4-2/SNc	Ipsilateral	MF 26	MN26-Ib [#]	26	Absent ^b
	NB7-1/SNd	Ipsilateral	MF 15/16	MN15/16-I	15/16	Triangular muscles (A1) ^a A2 onwards: Absent
	NB3-2/ISN	Ipsilateral	MF 20	MN20-Ib	20	A1, A2: Dorsal muscles [^]
Ventral	NB3-2/ISN	Ipsilateral	MF 19	MN19-Ib	19	A1, A2: Dorsal muscles [^]
	NB3-2/ISN	Ipsilateral	MF 11	MN11-Ib	11	A1, A2: Dorsal muscles [^]
	RP5 NB3-1/SNb	Contralateral CB@midline	MF 12	MN12-Ib	12	A1, A2: PLMF 12 A3 onwards: Absent

References:

- ¹-(Landgraf et al., 1997);
- ²-(Schmid et al., 1999);
- ³-(Hoang and Chiba, 2001);
- ⁴-(Choi et al., 2004);

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5-(Kim et al., 2009)

Abbreviations: MN-motor neuron; CB-Cell body; NB-Neuroblast; N.A.-Not applicable;

* Based on our studies;

Not identified by (Hoang and Chiba, 2001; Kim et al., 2009);

** Embryonic motor neurons known to express dHb9 (Broihier and Skeath, 2002; Odden et al., 2002).

(¹, *a*, *b*) Postsynaptic targets are inferred; two of the three motor neurons innervating dorsal muscles (¹) persist into the adult and innervate dorsal targets; of the remaining three motor neurons of the intermediate cluster, two innervate triangular muscles (*a*) while the third (*b*) is likely to not persist due to reduced GFP expression.