Developmental Changes in Dendritic Shape and Synapse Location Tune Single-Neuron Computations to Changing Behavioral Functions

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Meseké M, Evers JF, Duch C. Developmental changes in dendritic shape and synapse location tune single-neuron computations to changing behavioral functions. J Neurophysiol 102: 41–58, 2009. First published April 22, 2009; doi:10.1152/jn.90899.2008. During nervous system development, different classes of neurons obtain different dendritic architectures, each of which receives a large number of input synapses. However, it is not clear whether synaptic inputs are targeted to specific regions within a dendritic tree and whether dendritic tree geometry and subdendritic synapse distributions might be optimized to support proper neuronal input-output computations. This study uses an insect model where structure and function of an individually identifiable neuron, motoneuron 5 (MN5), are changed while it develops from a slow larval crawling into a fast adult flight motoneuron during metamorphosis. This allows for relating postembryonic dendritic remodeling of an individual motoneuron to developmental changes in behavioral function. Dendritic architecture of MN5 is analyzed by three-dimensional geometric reconstructions and quantitative co-localization analysis to address the distribution of synaptic terminals. Postembryonic development of MN5 comprises distinct changes in dendritic shape and in the subdendritic distribution of GABAergic input synapses onto MN5. Subdendritic synapse targeting is not a consequence of neuropil structure but must rely on specific subdendritic recognition mechanisms. Passive multicompartiment simulations indicate that postembryonic changes in dendritic architecture and in subdendritic input synapse distributions may tune the passive computational properties of MN5 toward stage-specific behavioral requirements.

INTRODUCTION

During brain development, a large number of neurons must be connected appropriately to form the complex neural circuitry essential for adequate brain function. At the single-neuron level, hundreds or thousands of input synapses are scattered throughout the dendrites. Both dendritic geometry and the subdendritic distribution of synapses may affect input-output computations (Koch and Segev 2000). However, few examples indicate that dendritic geometry might be tuned toward behaviorally relevant computations (London and Haeusser 2005; Single and Borst 1998). Furthermore, it remains unclear whether synapses are targeted to identifiable subdendritic domains, and whether this might play a role for behaviorally relevant computations. In addition, evolution might have acted to maximize synapse density (Chklovskii et al. 2000) at a given unit length of dendritic cable.

Subcellular targeting of synaptic inputs takes place in neocortex where different types of GABAergic inputs are directed to perisomatic and distal dendritic regions of pyramidal neurons (Di Cristo et al. 2004; Somogyi et al. 1998). Input targeting to the perisomatic region is crucial for temporal input integration (Pouille and Scanziani 2001), but the behavioral consequences remain unknown. Similarly, AMPA-type glutamate receptor density is increased at distal dendrites of pyramidal cells (Andrasfalvy and Magee 2001; Magee and Cook 2000; Stricker et al. 1996). Such findings suggest rules for synapse distribution along the proximal to distal dendritic dimension. Moreover, individual dendritic branches may have distinct molecular compositions, rendering precise subdendritic targeting possible (Horton and Ehlers 2003). Here we investigate whether complex dendritic trees exhibit distinct addresses for subdendritic input synapse targeting, and whether dendritic architecture and synapse distributions might have an influence on adequate input-output computations.

To address these questions, we combined high-resolution confocal laser scanning microscopy (CLSM) with precise three-dimensional dendritic surface reconstruction (Schmitt et al. 2004) and automated co-localization analysis to map the distribution of potential input synapses through dendritic trees (Evers et al. 2005). We define “potential synapses” as contact between labeled axon terminals and dendrites as observed on the light microscopy level (Stepanyants and Chklovskii 2005; Stepanyants et al. 2002). We fully employ CLSM resolution to map the contacts of GABAergic terminals onto dendritic surface reconstructions of the motoneuron 5 (MN5) in the ventral nerve cord of the moth, Manduca sexta. The use of an insect preparation allows for relating dendritic architecture and intricate subdendritic synapse distribution rules to behavioral function. MN5 can be individually identified from animal to animal, even between different developmental stages. During metamorphosis, synaptic input and dendritic structure are remodeled while MN5 becomes transformed from a slow larval crawling into a fast adult flight motoneuron (Duch and Levine 2000). Flight motor output requires high spike timing precision, but motoneuron bursting during larval crawling does not (Duch and Levine 2000). Therefore synaptic input summation efficacy should be highly dependent on temporal input coherence in the adult flight but not in the larval crawling MN5. Our results show that stage-specific rules exist for the targeting of GABAergic input synapses onto the dendritic tree of MN5. Theoretical multicompartiment simulations indicate that dendritic shape and subdendritic synapse targeting may tune the computational properties of MN5 toward stage-specific functions and that this liaison of structure and function can be adjusted to meet changing behavioral requirements during postembryonic life.

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METHODS

Animals

*Manduca sexta* were obtained from a laboratory culture reared on artificial diet (Bell and Joachim 1976) under a long-day photoperiod regime (17:7 h light/dark cycle) at 26°C. Both chronologically and morphological criteria were used for staging of animals (Bell and Joachim 1976; Reinecke et al. 1980; Tolbert et al. 1983). In summary, L5 represents an animal from the fifth larval instar and P0 the day of pupal ecdisis. Pupal life was divided into 18 stages, each of which lasted approximately one day. In this study, the stages L5, P0, P4, and adult were used.

Intracellular labeling, immunocytochemistry, image acquisition, and quantitative morphometry

Intracellular staining and immunohistochemistry were conducted as described in Duch and Levine (2000) and Evers et al. (2006). Briefly, for intracellular labeling of MN5 a thin-walled glass micropipette (25–35 MΩ tip resistance) was filled with a mixture of 7% neurobiotin (Linaris GmbH, Wertheim-Bettingen, Germany) and rhodamin-dextran (Invitrogen, Carlsbad, CA) in 2 M potassium acetate. An air bubble was left between the dye-filled tip and the shaft filled with 2 M potassium acetate to avoid dye dilution. Following intracellular penetration of MN5 (identified by antidromic spike initiation via extracellular nerve 1 stimulation), the dyes were injected iontophoretically by a constant depolarizing current of 3 nA for 40–60 min. Then the electrode was removed, and the ganglia were fixed in 4% paraformaldehyde in phosphate-buffer solution (PBS, 0.1 M) for 2 h at room temperature. Ganglia were washed in PBS (0.1 M) six times for 15 min each. Then tissue was treated in a conventional microwave at 750 W for three times for 3 min each at 4°C to enhance antibody penetration and binding. Tissue was placed into a PBS (0.1 M)-filled W for three times for 3 min each. Then tissue was treated in a conventional microwave at 750 W for three times for 3 min each at 4°C to enhance antibody penetration and binding. Tissue was placed into a PBS (0.1 M)-filled eppendorf tube, which was completely submerged in a 500-ml beaker filled with ice to keep the temperature of the buffer at ~4°C. Ice was renewed following each microwave run. This was followed by additional two washes in 0.1 M PBS for 15 min each and dehydration in an ethanol-series (50, 70, 90, and twice 100%, 15 min each). Preparations were treated in a 1:1 mixture of pure ethanol and methyl salicylate for 5 min and cleared in methylsalicylate. This was followed by 5-min treatment in a 1:1 mixture of pure ethanol and methyl salicylate, rehydration in a descending ethanol series, and four washes in PBS-triton x (0.5% triton in 0.1 M PBS). Primary antibody incubation and anti-Synapsin I (SyrOrF1, kindly provided by Prof. Buchner, University Würzburg; 1:100) and anti-GABA (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany; 1:400) were conducted for 36 h in PBS-triton x (0.3%). This was followed by six washes in PBS (15 min each) and incubation with secondary antibodies. Neurobiotin was visualized with Cy2-streptavidin (Invitrogen, Karlsruhe, Germany; 1:750); GABAergic processes were visualized by incubation with Cy3-coupled mouse anti-Rabbit (Jackson Immunochemicals, Suffolk, UK; 1:200), and synaptic terminals were visualized by incubation with Cy5-coupled rabbit anti-Mouse antibody (Jackson Immunochemicals; 1:200). This was followed by six washes in PBS (0.1 M), dehydration in an ethanol series (see preceding text), 5-min treatment in a 1:1 mixture of pure ethanol and methyl salicylate and clearing and mounting in methyl salicylate.

Data acquisition

Images were acquired with a Leica TCS SP2 confocal laser scanning microscope (Bensheim, Germany) using a Leica HCX PL APO CS ×40 oil-immersion objective (numerical aperture 1.25). To prevent cross-talk between overlapping fluorescent emission wavelength, sequential scanning mode was used. Cy2 and Cy5 were scanned simultaneously by using excitation wavelengths of 488 nm (argon laser) and 633 nm (helium neon laser). Cy2 emission was detected between 495 and 530 nm, and Cy5 emission was detected between 640 and 670 nm. No cross-talk between the channels was detected. Cy3 was excited with a green neon laser at 543 nm, and detection wavelength was between 550 and 590 nm. Switching between Cy3 scanning and Cy2/Cy5 scanning was conducted after every frame. By optimizing the sample preparation procedure as described previously (Evers et al. 2005), we can discriminate structures with a diameter below the emitting wavelength, approaching the theoretical limit of half the emitting wavelength (300 nm), at least in XY (Evers et al. 2005, 2006). The smallest dendritic diameters we find in MN5 are >400 nm. Electron microscopy of *Manduca* thoracic motor neuropil at the developmental stages relevant for this study demonstrates that synapsin I immunolabeled synaptic terminals appear as distinct puncta of 300–3,000 nm diameter (Hohensee et al. 2008). Therefore precise three-dimensional reconstructions of dendritic surface (see following text) can be used to map the distribution of immunolabeled synaptic terminals onto the dendrites of identified motoneurons.

Image analysis

Confocal image stacks were further processed with Amira-3.1.1 software (TGS). We used custom-developed Amira plug-ins for three-dimensional reconstruction of dendritic segments as published previously (Evers et al. 2005; Schmitt et al. 2004). These deliver precise quantification of midline and diameter as well as a triangulated surface definition fully exploiting optical resolution. To address the distribution of putative input synapses into the dendritic tree of MN5, the distribution of immunolabeled presynaptic profiles within the distance of optical resolution was evaluated. For quantification of the distribution of immunolabeled profiles along dendrites, the generated surface description was used to calculate the staining density within 300 nm from each surface element, a triangle. The position of the triangle was determined perpendicular to the midline of the corresponding reconstructed segment (for details, see Evers et al. 2005). A recent electron microscopy study demonstrated that this procedure accounted for all synaptic terminals located in the thoracic motor neuropil of *Manduca* but that it produced ~20% false positive synapses if only one immunolabel was used (Hohensee et al. 2008). In this study, this procedure was done for two immunolabels, anti-synapsin-I and anti-GABA. The correlation of both was used to indicate sites of putative GABAergic input synapses. Snapshots of scenes rendered in Amira-3.1.1 were arranged into figures with Adobe Illustrator CS and Photoshop CS (Adobe Systems Incorporated).

For statistical analyses, morphological parameters exported as ASCII-tables generated from Amira were imported into R (R Development Core Team 2004). Statistical analysis was conducted with the programs Statistica (StatSof, Hamburg, Germany) and Microsoft Excel. ANOVA with Newman Keuls post hoc comparison was used to test for statistical differences among multiple experimental groups or multiple classes of dendrites, e.g., between dendrites located in different Sholl spheres. Student’s t-test was used for comparisons of morphometric parameters between the larval and the adult MN5.

Morphometric analysis

The overall structure of MN5 is depicted in Fig. 1, A–D. MN5 is a unipolar cell. The axon projects through the mesothoracic nerve 1, and the cell body is located on the contralateral side of the ganglion. Axon and cell body are connected by a large primary neurite (link segment) from which all major dendritic branches arise. Therefore the integrative zone might be spread along the major primary neurites from the cell body up to the origin of the axon. To account for this feature in our morphometric analysis, we defined all dendritic branches originating from the primary neurite as first-order branches, virtually eliminating the link segments (which are treated as 0-order branches/branch points) between cell body and axon and therefore collapsing...
the reconstruction onto one virtual origin. Every branch branching off a first-order branch is defined as a second-order branch, and any branch branching off an \( n \)-order branch is defined as \( (n + 1) \)-order branch.

Values referred to as relative to the collapsed origin therefore regard the distance or order on the respective subtree up to its insertion into the cell body—axon link segments. Distances stated as along tree distance are measured as path length from the zero-order branch of the subtree along the midlines of the reconstruction. Air distance values refer to the straight line length in three-dimensional space to the zero-order branch point of the subtree. The Sholl analysis (Sholl 1953) measures the occurrence of a given metric parameter between two consecutive spherical shells with their center at the origin of the dendritic tree. This analysis gives information about the

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**FIG. 1. Morphometric comparison of the larval and the adult motoneuron 5 (MN5).** Representative confocal images of the larval (A) and the adult (B) MN5. Both images are projections views of all optical sections into 1 focal plane to visualize all central projections in one overview image. The cell body (cb) is located contralateral to the axon, and both are connected by a primary neurite, which also gives rise to all major dendritic branches. Images of 3-dimensional reconstructions are depicted in C for the larval MN5 and in D for the adult MN5. Both cells are shown in \( xy \), \( zy \), and \( zx \) view. Quantitative morphometric parameters as averaged from 3 representative larval (■, □) and 4 representative adult (●) reconstructions are shown in E–L. Total dendritic length (TDL) is significantly increased in the adult MN5 (E). Mean dendritic length (MDL; F), the number of dendrites (G) and dendritic density (I) are higher in the adult than in the larval MN5, but these differences are not statistically significant. Mean dendritic radius (H) is larger in the adult MN5 as compared with the larval MN5. Branch order analysis shows that the number of dendrites (J) of the adult MN5 is higher in all branch orders as compared with the larval MN5, but peaks in the branch orders 5–35. In the adult MN5, mean dendritic radii (K) are larger in all branch orders as compared with the larval MN5. Mean dendritic length (L) is increased in the adult in the branch orders 15–45, but is similar in the larval and the adult MN5 throughout all other branch orders. Statistical significance was tested with unpaired Student’s \( t \)-test, **, \( P < 0.01 \); *, \( P < 0.05 \). Scale is 100 \( \mu \)m.
distribution of metric parameters (e.g., number of dendrites, dendritic length, number of synapses etc.) throughout the three dimensions of the dendritic tree.

**Synaptic density counts**

Density counts for synaptic terminals were conducted in volumes of the following dimensions: $50 \times 50 \times 12.3 \, \mu m^3$. Within each cube, the number of GABA- and synapsin-I-immunopositive voxels was counted using ImageJ software (National Institutes of Health) with a plug-in that is based on intensity thresholds and a window value for the number of connected voxels that are accepted as one object (3D objects counter by Fabrice Cordelières, Institut Curie, Orsay, France; ImageJ). Most methods for the detection of immunopositive voxels in confocal image stacks are susceptible to errors because of uneven antibody penetration through the tissue, point spread during confocal image data acquisition, and optical resolution issues. Therefore we here used of two different approaches for counting immunopositive voxels to estimate the data integrity. In a first approach, a manual threshold was determined by a third person using a threshold mask in ImageJ without knowledge of the type of the immunostaining and the developmental stage (blind definitions of thresholds per 1 person for all image stacks). Additionally, a second standardized method was applied to define thresholds automatically. For each immunostaining, the staining intensity of all voxels within a cube was plotted as a frequency distribution intending to find the noise peak of each staining within the different images of GABA and Synapsin I. After removal of depth-dependent background alterations with a rolling ball algorithm (a background value is determined for every voxel by averaging over a sphere of given radius around each pixel. This average is then subtracted from all voxels to remove spatial variations in background intensities), the noise peak (offset/detector/technical noise) was found within the first 10 gray values for all image stacks (gray scale: 0–255). For the purpose of finding a conservative approach to eliminate slight fluctuations of the voxel intensity, intensities were divided into bins of 10 on an 8-bit gray scale from 0 to 255. Normalization was applied with regard to the first bin (which represents background). Thereby a normalized voxel frequency distribution was achieved, and threshold for accepting a voxel as immunopositive in a conservative manner was defined as highest 10% of all voxels. In all cubes, the frequency distribution was steep at this 10% value. This represented a good approximation for the transition from signal to background.

**Multicompartment modeling**

Theoretic modeling of the passive integration of electric discharges of dendritic trees was conducted in NEURON. The import of the reconstructed geometries of MN5 at different developmental stages into NEURON was done as described earlier (Evers et al. 2005). Due to the lack of accurate values for the passive electrical properties of Manduca motorneurons, we used values commonly applied for vertebrate and invertebrate neurons (specific membrane capacity $C_m = 1.15 \times 10^{-6} \, F/cm^2$, axoplasmic resistance $R_a = 45 \, \Omega \cdot cm$ passive leak conductance $g_{pas} = 0.0001667 \, \Omega^{-1} \, cm^{-2}$, and equilibrium $e_{pas} = -65 \, mV$). The resting membrane potential was measured with sharp electrodes from the soma of MN5 in situ ($n = 42$). Equipping MN5 geometry with the above-mentioned values for membrane capacity, membrane resistance and axoplasmic resistance matches also input resistance and time constant as measured with single sharp electrodes from the soma of MN5 in situ. This indicates that commonly used values for these passive parameters can be used for passive multicompartment models of MN5.

Synapse localization probabilities determined as described in the preceding text were transformed into a discrete map of synaptic localizations as follows. To randomize synaptic positioning within the constraints of the specific synaptic distribution characteristics, we used a random number generator (equal distribution). We normalized to the total length of the dendritic arbor to position a given number of synapses on the reconstructed dendritic arbor. A value of 40% of the maximum synaptic likelihood (as determined in the individual data set) was used as the positive criterion to confirm synapse localization, and synapses at positions with lower synaptic likelihood were removed and redistributed. The synaptic activation onset was randomized for each individual synapse to be activated once during a simulation so that the synaptic activation sequence followed a Gaussian distribution with a given width (sigma) and its center to be at 10 ms after the start of the simulation. Synaptic activations were realized with NEURON’s AlphaSynapse model ($tau = 0.8 \, ms$, equilibrium potential $e = -10 \, mV$ and maximum conductivity $g = 10^{-7} \, \Omega^{-1}$). The AlphaSynapse model was used because no accurate data on the activation/inactivation time constants and maximum chloride conductances for central GABAergic synapses in Manduca are available. To scale the size of the larval dendritic tree to match the adult length, we changed each dendritic branch length by multiplication with the ratio of total adult to total larval tree length. All modeling results were exported as ASCII files and analyzed within R (http://www.r-project.org).

For statistical analyses, simulation results from NEURON were imported into Statistica. Statistical analysis was conducted with the programs Statistica (StatSoft, Hamburg, Germany) and Microsoft Excel. Data from different groups were not normally distributed, variances were not homogeneous between different groups, and differences in variances correlated with differences in means between different groups. Therefore nonparametric Kruskal Wallis ANOVA was used to test for statistical differences among multiple experimental groups. Mann and Whitney U test was used for comparisons of parameters between individual experimental groups. Significant differences were accepted at $P < 0.01$.

**RESULTS**

**Quantitative morphometric comparison of larval and adult dendritic structure**

For a quantitative comparison between the branching geometry of the larval and the adult MN5 dendritic tree, three-dimensional reconstructions (Fig. 1, C and D) were made from confocal image stacks (Fig. 1, A and B). Postembryonic changes in the structure of MN5 have previously been described qualitatively (Duch and Levine 2000), and quantification has been conducted by manual reconstruction (Liberstat and Duch 2002), but a thorough quantitative analysis based on improved novel semi-automatic reconstruction tools (Evers et al. 2005; Schmitt et al. 2004) has been lacking.

The postembryonic development of MN5 from a larval crawling into an adult flight motoneuron is accompanied by a significant increase in total dendritic length (adult: $39,716 \pm 3,838 \, \mu m$; larva: $26,227 \pm 267 \, \mu m$, means $\pm$ SD; Fig. 1E). This is correlated with a combined increase in the length of individual dendritic branches and an increase in the total number of branches. However, the difference in the mean length of the individual dendritic branches between the larval ($4.12 \pm 1.17 \, \mu m$) and the adult stage ($5.01 \pm 1.04 \, \mu m$, Fig. 1F) is not statistically significant. Similarly, if averaged over the whole dendritic tree, the difference in the total number of dendritic branches is not statistically significant (adult: $8,413 \pm 2,238$ dendritic branches; larva: $6,577 \pm 1,696$ dendritic branches; Fig. 1G). However, in the adult, significantly more branches are present in lower branch orders (Fig. 1J). The mean radius of the individual dendrites is increased signifi-
cantly from 0.28 ± 0.09 in the larval to 0.34 ± 0.07 μm in
the adult stage (Fig. 1H). The pervasion of the neuropil by the
dendritic tree of MN5 can be assessed by calculating the
relative space occupied by all dendrites within the volume
defined by the three-dimensional perimeter of the dendritic
field. We define this value as dendritic density. Dendritic
density is almost twice as large in the adult as compared with
the larval stage (Fig. 1A), but maximum dendritic path lengths
are similar between both stages. Branch order analysis was
conducted to test whether some morphometric parameters were
affected in specific branch orders only (e.g., MDL and the total
number of branches appeared larger in the adult, but no
statistical differences were observed by averaging over all
dendrites). A first-order dendrite is defined as any dendrite
branching off the primary neurite which connects the cell body
and the axon in MN5 (Fig. 1, A and B). A second-order branch
is defined as any dendrite branching off a first-order branch,
and any dendrite branching off an n-order dendrite is defined as
(n + 1)-order dendrite (see Libersat and Duch 2002). In the
branch orders 5–20, the adult MN5 contains significantly more
dendritic branches than the larval MN5 (ANOVA with Duncan
post hoc comparison, *P < 0.05; Fig. 1J). The mean radius of
all individual dendritic branches is larger in the adult than in
the larval MN5 (Fig. 1H). This is correlated with an increase of
the dendritic radius through all branch orders, although no
statistical significant differences were found in the branch
orders 34–52 (ANOVA with Duncan post hoc comparison *P
< 0.05; Fig. 1K). No statistical significant differences in
MDL (Fig. 1F) were found in any branch order, although adult
MDL showed higher mean values than larval MDL throughout
all branch order (Fig. 1L).

Localization of GABAergic input synapses to MN5 dendrites

Remodeling dendritic structure during postembryonic develop-
ment is directly related to changes in synaptic inputs im-
pinging onto the dendritic tree (Consoulas et al. 2000; Tissot
and Stocker 2000; Weeks 2003). For MN5 in Manduca, larval
synapses are eliminated during the disassembly of larval crawl-
ing circuits, and MN5 dendrites retract during late larval and
early pupal life (Duch and Mentel 2004). This is followed by
dendritic growth and synaptogenesis during the motoneuron’s
integration into the newly formed adult flight motor networks
(Duch and Levine 2000; Evers et al. 2006). Postembryonic
changes in dendritic architecture, membrane currents and mo-
tor function have been described for MN5 (Duch and Levine
2000). This renders MN5 a useful model for asking whether
postembryonic changes in the structure and function of indi-
vidual neurons are accompanied by stage-specific rules for
subcellular targeting of synaptic inputs through complex den-
dritic trees.

We examined the distribution of putative GABAergic input
sites on the dendritic tree of MN5. We conducted immuno-
labeing for the general presynaptic protein synapsin I and for the
neurotransmitter GABA. The co-localization of both labels
was used to highlight a putative GABAergic release site (see
METHODS, Evers et al. 2005). In the insect CNS, anti-synapsin I
immunolabeling can be used as reliable marker for synaptic
terminals and yields puncta of 300–3,000 nm diam that repre-
sent the synaptic terminal including active zone plus reserve
vesicle pool (Hohensee et al. 2008). Combining these immu-
nolabels with an intracellular staining of MN5 allowed us to
assess the distribution of GABAergic release sites in relation to
the dendritic arbor of MN5 (Evers et al. 2005; Schmitt et al.
2004) (see METHODS). Briefly, the cell surface of the neuronal
arborization is reconstructed from confocal image stacks, and
the co-localization probability of GABA and synapsin-I is
calculated for each surface element within a 300-nm distance.
The validity of this method for detecting 75% correct syn-
aptic inputs into postembryonic dendritic arbors of Manduca
motoneurons has recently been confirmed by a correlative
confocal and electron microscopy study (Hohensee et al.
2008). In addition we have previously demonstrated that rela-
tive synapse numbers detected with this method correlate with
electrophysiological analysis of the numbers and amplitudes of
postsynaptic potentials (Duch and Mentel 2004).

Using this method, we charted the distribution pattern of
GABAergic input synapses onto the dendritic tree of MN5
at different developmental stages (Fig. 2). As an example, in
Fig. 2A1 a single reconstructed dendritic branch is superim-
posed on an optical section from a whole-mount immuno-
stained neuropil against GABA (green). Every surface element
of the dendrite reconstruction is color coded for the mean
GABA-immunolabel staining intensity within 300-nm dis-
tance. In Fig. 2A2, the corresponding optical section of the
anti-synapsin I immunolabel (blue) is shown. Parts of the
dendritic surface reconstruction with an anti-synapsin I label
within a 300-nm distance to the dendritic surface are coded by
warmer colors, indicating a putative input synapse. To highlight
synaptic terminals that contain GABA, we calculated a correlation
coefficient of both, the anti-GABA and anti-synapsin I image
data. We extracted the average correlation value within 300-nm
distance from the neuronal surface to restrict the analysis to
GABAergic synapses that are in juxtaposition to the dendritic
surface of MN5 (Fig. 2A3). Applying this procedure to all optical
sections produced a complete map for the distribution of putative
GABAergic input synapses onto the entire dendritic tree (Fig. 2B).

Developmental changes in GABAergic input synapse density
density on MN5 dendrites

Before testing for site-specific subdendritic input synapse
targeting, the overall amount of putative GABAergic inputs per
dendritic surface was compared between different stages of
postembryonic development. Maps of all putative GABAergic
inputs through the dendritic tree of MN5 are shown for the
larval stage (Fig. 2B1), the day of pupal ecdysis (Fig. 2B2), the
fifth day of pupal life (Fig. 2B3), and for the adult stage (Fig.
2B4). These data are quantified and normalized by dividing the
dendritic surface area contacted by putative GABAergic ter-
minals by the total dendritic surface area for each of the four
developmental stages (Fig. 3A). More than 6% of the dendritic
surface of the adult flight MN5, but <4% of the dendritic
surface of the larval crawling MN5 receive contacts from
GABAergic terminals (Fig. 3A). This difference is statistically
significant (P < 0.01, Student’s t-test). The regression of larval
dendrites between the fifth larval instar (Fig. 2B1) and the day
of pupation (wanderer 4, Fig. 2B2) is accompanied by synapse
elimination (Fig. 3A) as has previously been demonstrated
(Duch and Mentel 2004). The regressed dendritic field at stage
W4 contains not only fewer GABAergic contacts than the
larval dendritic tree, but the density of putative GABAergic
inputs is 2.5-fold decreased as compared with the larval stage (Fig. 3). This indicates that during late larval life putative GABAergic synapses are also eliminated from dendrites that are not retracting. During the first 5 days of pupal life corresponding to the initial phase of rapid dendritic growth (Evers et al. 2006), the relative dendritic surface area contacted by putative GABAergic input synapses is further reduced by a factor of 2 (Fig. 3). This demonstrates that newly formed dendrites are not immediately contacted by GABAergic terminals. The values for stage W4 and P5 are not tested statistically because the data are based on two whole tree reconstructions for each of these stages. However, additional preparations with partial reconstructions are in accordance with the values for W4 and P5 shown in Fig. 3.

The second phase of dendritic growth and maturation between pupal stage 6 and adult (Duch and Levine 2000; Evers et al. 2006) is accompanied by the formation of many new putative GABAergic inputs (Fig. 2B4), so that the percentage of dendritic surface area in contact with putative GABAergic input synapses increases more than six-fold between pupal stage 5 and the adult stage (Fig. 3).

Input synapse density on MN5 dendrites is not a simple reflection of neuropil synapse density

The higher density of GABAergic terminals contacting the dendritic surface of the adult flight MN5 as compared with the larval crawling MN5 could be caused by a higher overall density of GABAergic terminals in the adult motor neuropil. Alternatively, more GABAergic terminals might specifically contact the adult dendritic tree as compared with the larval dendritic tree, at similar overall synapse densities at both stages. To distinguish between both possibilities, we counted the relative numbers of GABAergic terminals and total terminals in defined volumes in the larval and in the adult motor neuropil. Within the perimeter of the dendritic field of MN5, terminals were counted in three cubes of defined side length (see Methods). Within each cube the number of GABA (Fig. 3B1) and of synapsin I (B3) immunopositive voxels were counted and divided by the total number of voxels. Thresholds for immunopositive voxels were defined with two independent methods. First, manual threshold was determined blindly by a third person using a threshold mask in Image J (National Institutes of Health, see Methods). Second, we employed a standardized automated method to define thresholds at the brightest 10% of all voxels (Fig. 3C, see red lines, see Methods). Every voxel count was conducted with both methods (manual blind thresholding and standardized thresholding), and the results were processed separately. Because every GABAergic terminal contains GABA and synapsin I, the overlap of both was used to count voxels located in GABAergic terminals (Fig. 3B5, co-localized area). To determine the number of all voxels located in other than GABAergic synaptic terminals, the number of voxels
FIG. 3. The density of GABAergic terminals contacting on MN5 dendrites changes through different stages. 

**A**: the number of dendritic surface triangles in contact with a putative GABAergic terminal normalized to the total number of surface triangles for the stages larva 5 (black bar), wandering 4 (white bar), pupa 5 (dark gray bar), and adult (light gray bar). Error bars represent SD. The phases of dendritic regression and dendritic outgrowth as described in Duch and Levine (2000) are indicated by arrows and labeled boxes. **B**: the relative numbers of GABAergic terminals and other terminals within the perimeter of the dendritic field of MN5 (in 3 cubes of defined volume, see METHODS) were counted in defined volumes in the larval and in the adult motor neuropil. Within each cube the number of GABA (B1) and of synapsin I (B3)-immunopositive voxels was counted and divided by the total number of voxels. Thresholds for immunopositive voxels were defined with 2 independent methods. First, manual threshold was determined blindly by a 3rd person using a threshold mask in Image J (National Institutes of Health, see METHODS). Representative blindly defined threshold masks are shown for anti-GABA immunostaining (B2) and for synapsin immunostaining (B4). Co-localization was used to determine voxels belonging to putative GABAergic synapses (white mask in B5). Automated threshold determination is depicted in C. The staining intensity of all voxels within each cube is plotted as frequency distribution. Threshold for accepting a voxel as immunopositive was defined at the brightest 10% of all voxels as indicated by the red lines (C). To determine the number of all voxels located in other than GABAergic synaptic terminals, the number of voxels immunopositive for GABA and for synapsin I were subtracted from the total number of synapsin-I-positive voxels. To normalize these numbers, each voxel count was divided by the total number of voxels within each cube. **D**: the proportion of voxels that are GABA immunopositive for the larval (black bar) and the adult (gray bar) motor neuropil as determined with both methods (manual blind and automatic thresholding). Error bars represent SD. Each bar represents the mean of 12 cubes from 4 different animals. **E**: the respective bar diagram for voxels from putative GABAergic terminals. **F**: the respective diagram for the ratio of all voxels belonging to putative GABAergic terminals divided by all other synapsin-I-positive voxels within each cube. No significant differences for any of these voxel counts were found between larval and adult neuropil regions surrounding MN5 dendrites.
immunopositive for GABA and for synapsin I were subtracted from the total number of synapsin-I-positive voxels. For normalization, each voxel count was divided by the total number of voxels within each cube (percentage GABAergic or percentage Syn-I-positive voxels). The percentage of voxels that are GABA and synapsin I positive is similar in the larval and in the adult flight motor neuropil. Approximately 2% of the three-dimensional space is occupied with putative GABAergic terminals, no matter whether manual (Fig. 3D, black bars) or standardized (Fig. 3D, gray bars) thresholding is used. The same is true for the percentage of all other terminals containing synapsin-I-positive immunolabel (all Syn I immunopositive voxels minus all co-localized voxels). This most likely reflects all type I terminals, except GABAergic ones, in the respective neuropil regions. With both methods we find ~7–8% of the volume of both neuropil regions occupied with non-GABAergic type I terminals (Fig. 3E).

No statistical differences exist between both regions or between both methods (Student’s t-test, P > 0.65). As GABA is the predominant inhibitory transmitter in the insect CNS and most other type I terminals in the ventral nerve cord are excitatory cholinergic synapses (Burrows 1996), an inhibition/excitation ratio (I/E ratio) can be estimated by the ratio of both voxel counts (Fig. 3F). The I/E ratio is ~0.22, it is similar between the larval and the adult motor neuropil. Only manual thresholding in the larval motor neuropil yields higher I/E values (Fig. 3F, 0.31 for manual thresholding in the larva), but this difference is not statistically significant. In summary, these data demonstrate that overall densities of GABAergic and other type I terminals are similar in the larval and in the adult motor neuropil surrounding the dendrites of MN5. Consequently, the observed higher density of putative GABAergic terminals contacting adult MN5 dendrites as compared with larval ones is not a consequence of terminal availability.

Stage-specific rules exist for the distribution of GABAergic inputs on MN5 dendrites

Not only dendritic geometry and synapse density but also the distribution of synapses through the dendrites may affect synaptic input computations and neuronal function (Koch and Segev 2000). We tested whether the postembryonic change in MN5 motor function (Duch and Levine 2000) was accompanied by stage-specific rules for subdendritic placement of GABAergic inputs. To address this question, a modified Sholl (Sholl 1953) analysis was conducted. Most insect motoneurons, including MN5, are monopolar neurons that do not produce somatic action potentials. Therefore the integrative zone might be spread along the major primary neurites from the cell body up to the site of action potential initiation on the axon. To account for this feature in our morphometric analysis, we defined the entire link segment between cell body and axon as the origin of the dendritic tree and mathematically collapsed the link segment into one point in space (see METHODS). Consecutive three-dimensional concentric spheres of 40 μm radius were placed around this origin of the dendritic tree. To visualize all dendrites belonging to specific Sholl spheres, they are depicted in different colors in Fig. 4, A and B, for the dendritic tree of the larval MN5 (blue, Sholl sphere 40-80; red, 120-160; green, 240-280). Within each Sholl sphere, the surface area in contact with GABAergic terminals is divided by the total surface area. This normalizes for differences in dendritic surface area among different Sholl spheres. The GABA-positive fraction of dendritic surface is plotted for each Sholl sphere for both the larval and the adult MN5 (Fig. 4C). For the larval MN5, all Sholl spheres receive the same relative amount GABAergic contacts (Fig. 4C, ■, ANOVA, P = 0.29). By contrast for the adult MN5, a significant increase in the relative amount of GABAergic contacts per dendritic surface area occurs in the higher Sholl spheres (Fig. 4C, ◇, 240–360 μm spheres; ANOVA, P < 0.01). This means that putative GABAergic inputs occur predominantly more distally in the dendritic field. This shift of GABAergic contacts toward the perimeter of the dendritic field of the adult MN5 is also obvious when using actual distance along the run of the dendrites to the origin, instead of air distances, to define the radii of the Sholl spheres (Fig. 4D, tree distance, ANOVA, P < 0.01). Tree distance is a more functional measure, because postsynaptic potentials (PSPs) have to propagate along the run of the dendrites to the spike initiating zone. For both air distance and tree distance (ANOVA, P = 0.21) of the larval MN5, the relative dendritic surface contacted by putative GABAergic terminals is similar throughout all Sholl spheres (Fig. 4, C and D, black bars). These data demonstrate that the postembryonic change in function of MN5 is accompanied by a change in the distribution of GABAergic terminals contacting the dendritic surface of MN5 from a random distribution in the larval crawling motoneuron to a shift toward more distal dendrites in the adult.

Distribution of GABAergic contacts on MN5 dendrites is caused by specific subdendritic targeting mechanisms and not by neuropil structure

In principle, this postembryonic re-distribution of putative GABAergic inputs through the dendritic field of MN5 could be caused in two different ways. First, it might reflect a general re-organization of the neuropil. In this case, distal dendrites would be located in neuropil regions containing a higher density of GABAergic terminals than the neuropil regions around more proximal dendrites of MN5. If every GABAergic terminal had the same chance to make contact onto a neighboring dendrite, a higher density of terminals would result in a higher contact number per dendritic surface area. This case would not require specific subdendritic targeting of GABAergic terminals. Alternatively, the overall densities of excitatory and inhibitory terminals might be even throughout the neuropil. In this case, the observed shift of GABAergic input synapses toward higher Sholl spheres would require specific subdendritic targeting of contacts. To distinguish between these possibilities, we counted the relative numbers of GABAergic terminals and total terminals in different neuropil regions surrounding MN5 dendrites. We compared neuropil regions located in lower MN5 Sholl spheres (Fig. 5A, region 1, few GABAergic inputs) to neuropil regions located in higher MN5 Sholl spheres with large numbers of putative inputs impinging on MN5 (Fig. 5A, region 2). Within each region synaptic terminals were counted in three cubes of defined volume as described in the preceding text (Fig. 3, B and C, see also METHODS). The percentages of voxels that were GABA and synapsin I positive with respect to the total voxel numbers were similar in regions 1 and 2. In all cubes, ~2% of the three-dimensional space was occupied with putative GABAergic terminals, no matter whether manual or standardized thresholding was
used (Fig. 5B). Consequently, the accumulation of GABAergic contacts in higher Sholl spheres of the dendritic surface of MN5 was not caused by an overall higher density of GABAergic terminals in the respective neuropil regions. The same was true for the percentage of all other terminals containing synapsin-I-positive immunolabel (all Syn I immunopositive voxels minus all co-localized voxels). With both methods we found ~8% of the volume of both neuropil regions occupied with non-GABAergic type I terminals (Fig. 5C). No statistical differences existed between both regions or between both methods (Student’s t-test, \( P > 0.65 \)). The resulting I/E ratio was similar between regions 1 and 2, and no differences were found between both thresholding methods (0.27, data not shown). In summary, these data demonstrate that the dendritic tree of MN5 is embedded into a neuropil with homogenous type I synapse distributions (Fig. 5, B and C) and homogeneous I/E ratios.

GABAergic inputs are targeted to the roots of dendritic sub-trees in the adult MN5

Are there also specific strategic localizations of such contacts with regard to individual dendritic branches in addition to pushing putative GABAergic inputs toward the perimeter of the dendritic field? The procedure to test for this is depicted schematically in Fig. 6A. In a given dendritic tree, each individual dendritic branch was divided into 10 sections of equal length. We then tested whether all 10 sections contained the same density of GABAergic contacts and averaged these data over all dendrites of the tree. Even in a situation with GABAergic contacts located predominantly in higher Sholl spheres, these might be distributed evenly along the individual branches (Fig. 6A, middle). By contrast, one could also envision a scenario in which GABAergic contacts might be targeted so that they selectively shunt individual dendritic sub-trees. One way to achieve this would be that lower-order dendrites, which are thick and give rise to many higher-order dendrites, receive putative GABAergic inputs predominantly at their distal end at the root of the dendritic sub-tree where they house but not at their proximal end, which connects these dendrites to other sub-trees (Fig. 6A, right). By contrast, thin higher-order dendrites might receive an even distribution of inhibitory contacts. To test for this, all dendrites of any given dendritic tree were divided into two
classes; thick dendrites between 0.5 and 1 μm radius and thin dendrites with radii <0.5 μm. For both classes of dendrites, the total surface area and the total surface area contacted by putative GABAergic inputs were determined. The ratio yielded the percentage of dendritic surface contacted by GABAergic terminals. This ratio showed little variability for either the thick or the thin dendrites across different animals at a given developmental stage (thick dendrites adult, 4.6 ± 0.6%; thin dendrites adult, 9.3 ± 0.5%; larva thick dendrites 5.4 ± 0.13%; larva thin dendrites 5.7 ± 0.13%; 0.25%). These values were used to calculate an expected dendritic surface area contacted by GABAergic terminals within each of the 10 sections along all individual dendrites of a given class (Fig. 6 A, left). The null hypothesis was that on average, the surface of each of the 10 sections along each of the dendrites was contacted by an equal number of GABAergic inputs, i.e., no preferential targeting of inhibitory synapses was expected along individual branches. Then the average surface area contacted by GABAergic terminals was determined for each of the 10 sections along the dendrites by analyzing the putative GABAergic input distribution pattern found within the surface reconstruction. Finally, for each section, the surface area that was on average contacted by GABAergic terminals was divided by the expected surface area. Consequently, numbers >1 indicate that within a given section along the dendrites more GABAergic terminals than expected make contact. This procedure was conducted separately for all thick and all thin dendrites within each animal and subsequently averaged over four adult and three larval animals. For thick dendrites in the adult MN5, the number of GABAergic inputs was significantly increased in segment 10 and significantly decreased in segments 1–3 (Fig. 6B, □, ANOVA, Newman Keuls post hoc test P < 0.01). Therefore inhibitory inputs did not occur randomly along the run of individual thick dendrites (between 0.5 and 1 μm diam) but, by contrast, were located with a higher likelihood toward the end of thick dendrites, i.e., at the root of dendritic sub-trees arising from this dendrite but furthest away from other sub-trees.

In summary, the integration of MN5 into the newly formed flight motor networks during postembryonic development is accompanied by targeting putative GABAergic input synapses to specific subdendritic addresses. First, putative inhibitory inputs occur at a higher density at the perimeter of the dendritic field (Fig. 4), and second, low-
order branches are targeted with a higher likelihood at their distal end, which is the root of dendritic sub-trees (Fig. 6). These rules for subdendritic distribution of putative GABAergic inputs are not a reflection of varying overall terminal densities of this transmitter class in the neuropil (Fig. 5).

FIG. 6. Strategic locations of GABAergic inputs into the adult MN5 at the roots dendritic sub-trees. A: the logic and the procedure of the analysis shown in B–E. For analysis, each dendrite of a tree is divided into 10 sections of identical length (A1). The hypothesis to test was whether, on average, putative GABAergic inputs are evenly distributed along all 10 sections of the dendrites. Random distribution of excitatory and inhibitory synapses along individual dendrites is schematized in A2 (random within branch distribution). A3: a schematic example of random distribution in higher-order branches but increased occurrences of GABAergic contacts to the roots of low-order branches. To test whether increased input incidences occurred on average in any of the 10 sections (A) along individual branches, a 5-step procedure was conducted. First, all dendrites of a tree were divided into thick low-order dendrites (0.5–1 μm radius) and thin higher-order dendrites (radius <0.5 μm). Second, for each reconstruction, the percentage dendritic surface that was contacted by putative GABAergic inputs was determined separately for thick and for thin dendrites. Third, total dendritic surface was determined for each of the 10 segments along individual dendrites. Fourth, with the null hypothesis of an even distribution, the overall percentage GABAergic input and the total surface area in each of the 10 dendritic segments (averaged over all dendrites) the expected surface area contacted by GABAergic terminals within each of the 10 segments was calculated, separately for thick and for thin dendrites. Fifth, for each of the 10 segments the measured surface areas contacted by putative GABAergic inputs were determined (averaged over all dendrites, but separately for thick and thin dendrites), and normalized with respect to expected surface area contacted by GABAergic terminals. Bars show normalized values averaged from 4 animals. A y value >1 means that within this section, averaged over all dendrites, more surface than expected is contacted by putative GABAergic inputs. This procedure was conducted separately for thick (B) and thin (C) dendrites in the adult MN5 and for thick (D) and thin dendrites in the larva (E). *.., expected value at even distribution for GABAergic contacts. Error bars represent SD. *, significance (ANOVA, Newman Keuls post hoc test P < 0.01).
As described in the preceding text, the development of MN5 from a slow larval crawling to a fast adult flight motoneuron is accompanied by postembryonic changes in its firing properties (Duch and Levine 2000). The larval MN5 is a typical slow insect motoneuron which has a low firing threshold and shows tonic firing upon current injection and during crawling behavior, whereas the adult MN5 has the properties of a typical fast insect motoneuron with a more depolarized firing threshold and phasic firing responses upon current injection. This is caused by postembryonic changes in voltage-activated membrane currents (Duch and Levine 2000). However, it remains unclear whether dendritic architecture remodeling as described in this study might also aid the different functions of MN5 at the two different stages. As insect flight is a well-investigated motor behavior (Burrows 1996), one prediction for the adult MN5 is that sharp volleys of synaptic drive that occur at defined times within each wing-beat cycle must get summated to cause one MN5 spike per wing down stroke. Therefore our working hypothesis is that the passive architecture of the adult MN5 should be tuned for effective summation of synaptic input that occurs within narrow time windows, but the efficiency of synaptic input summation should decrease markedly if input synapse firing synchronicity decreased. By contrast, the larval MN5 innervates a slowly contracting muscle. In a number of invertebrate preparations, the contraction slopes of slow muscles are mostly independent of brief changes in motoneuron spiking frequency and spike timing (Hooper et al. 2007; Morris and Hooper 1998). Accordingly, the larval MN5 fires bursts of multiple action potentials during crawling behavior without a clear demand on spike time precision within each burst (Mental and Duch 2003). In contrast to the millisecond range of cycle period during adult flight, cycle period during crawling measures multiple seconds. Therefore we hypothesize that the efficiency of synaptic input summation in the larval MN5 should be less dependent on input synapse firing synchronicity. It is apparent that active properties and altered circuit connectivity contribute significantly to the different firing behaviors of the larval and the adult MN5 (Duch and Levine 2000, 2002). However, here we tested whether the remodeling of the branching structure, and therefore the altered passive electrical properties of dendritic architecture might also contribute to differentiate the properties of a slow and a fast motoneuron. Solely passive multicompartment models based on the dendritic geometry and synaptic distribution characteristics as determined in the preceding text were used to test this hypothesis in simulation experiments. Simulations are not intended to realistically mimic the synaptic drive as occurring during behavior but rather to test whether larval and adult dendritic geometry revealed different properties with respect to their passive temporal and spatial summation properties. As a read-out of this, we determined the maximum voltage deflection at the root of the axon in response to activations of uniform synapses with different temporal coherences.

For these simulations we distributed 50, 100, or 200 synapses onto passive multicompartment models of the dendritic tree of a larval or the adult MN5. Synapse placement was derived from the experimentally determined GABAergic synapse distribution pattern as described in the preceding text (see Methods). Simulations did not take realistic conductances and reversal potentials of Manduca GABAergic synapses into account. The only purpose was to test whether the passive integration properties of stage-specific dendritic architectures could potentially cause differences in the effectiveness of synaptic input summation dependent on different input firing synchronicities. Because data on time constant and maximum conductance for Manduca central GABAergic synapses are not available, for simplicity, synaptic activations were realized with NEURON’s AlphaSynapse model (see Methods). Each synapse was activated once during one simulation iteration. The time point of firing onset was randomized for each individual synapse, so that the temporal distribution of the synaptic activation sequence followed a Gaussian distribution with a given width (sigma, see Methods). Simulations were run with eight different sigmas (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 4.8, and 6.4 ms) to compare different degrees of input synapse firing synchronicity. For example, at a sigma of 0.1 nearly all synapses (50, 100, or 200) were activated within a 0.1-ms window. By contrast, at a sigma of 6.4, the same numbers of synapses were activated within a 6.4-ms window. To account for both, different synapse firing sequences as occurring at any sigma larger than 0, and also for different sites of activated synapses within the dendritic tree, each simulation was run for 10 independently drawn synapse loci and for 20 different temporal sequences of activation. This resulted in 200 simulations per synapse number and sigma (degree of input synapse firing synchronicity). At eight different sigmas, this resulted in 1,600 simulations per stage and synapse number. For each of these simulations, the resulting maximum voltage at the root of the dendritic tree is plotted over time, and the dots in the resulting data distribution are color coded for the different sigmas (Fig. 7, A–C). Representative examples of voltage traces as resulting from simulations with 100 synapses and a sigma of 1.6 ms are depicted as insets for the larval, the scaled larval, and the adult dendritic tree (Fig. 7, A–C).

For the larval dendritic tree (Fig. 7A) and 50 synapses (A, left), different degrees of input synchronization (sigmas, color coded) do not result in different maximum depolarization amplitudes at the root of the dendritic tree. Consequently, the peak amplitudes of synaptically evoked depolarizations are mostly intermingled for different sigmas with regard to amplitude (y axis) and time of peak depolarization (x axis). Increasing the number of activated synapses to 100 (Fig. 7A, middle) or 200 (right) does not yield different maximum depolarization amplitudes for different input synchronizations (sigmas). These data are evaluated statistically in Fig. 7, D1 (50 synapses), E1 (100 synapses), and F1 (200 synapses). For each sigma, the median maximum depolarizations are plotted as resulting from 200 different simulations with different synapse activation sequences. For 50, 100, and 200 synapses, no statistical differences are found for any of the eight different input firing synchronicities (Fig. 7, D1 and E1, Kruskal Wallis ANOVA, \( P > 0.05 \)). In contrast, for the adult dendritic tree and 50 synapses (Fig. 7C, left), the maximum depolarization amplitudes resulting from 200 simulations for each input firing synchronicity segregate for different sigmas. This indicates that in the adult dendritic tree, the degree of input synapse firing synchronizion may affect membrane depolarization more than the activation sequence of the individual synapses or...
different loci within the synapse pool. Increasing the number of active synapses to 100 (Fig. 7C, middle) and 200 (Fig. 7C, right) further sharpens the segregation of maximum depolarization amplitudes in the adult dendritic tree. These data are evaluated statistically in Fig. 7D3 (50 synapses), E3 (100 synapses), and F3 (200 synapses). For each synapse number, the resulting median maximum depolarizations do not differ statistically between input firing synchronicities of 0.1 and 0.2 ms. By contrast to the larval MN5, lower input firing synchronicities (all sigmas >0.2) result in statistically significant lower effects on membrane potential (Kruskal Wallis ANOVA, P < 0.01). For the tested synapse numbers, each incremental increase of sigma (from 0.2 to 0.4 to 0.8 to 1.6 to 3.2 to 4.8 to 6.4 ms) yields significantly smaller changes in membrane potential (Mann and Whitney test, P < 0.01). Therefore the maximum depolarization amplitude is significantly stronger affected by input firing synchronization in passive models of the adult as compared with the larval dendritic tree.

The adult MN5 comprises significantly more total dendritic lengths than the larval MN5 (see Fig. 1). To test whether the higher sensitivity to input synchronization of the adult MN5 is simply caused by a different ratio of synapse number to total tree length, the same simulations were repeated with a larval dendritic geometry scaled to match the adult dendritic length (see Methods; Fig. 7B). The simulation results for the scaled larval dendritic tree (Fig. 7B) mostly match those of the original larval tree (Fig. 7A) in that no segregation of the maximum depolarizations occurred for different input synapse firing synchronicities. Statistical evaluation demonstrates that for 100 synapses no significant differences in the median maximum depolarization exist between any of the 8 tested sigmas (Fig. 7E2, Kruskal Wallis ANOVA, P > 0.01). For 200 synapses (Fig. 7D2), a sigma of 4.8 differs significantly from neighboring sigma values (Mann and Whitney test, P < 0.01), but no systematic differences exist for incremental increases in input firing synchronicity. Similarly, for 200 synapses, sigmas of 0.2 and 0.4 ms differ significantly from lower (sigma = 0.1 ms) and higher input firing synchronicities (sigma = 0.8), but no systematic differences exist between low- and high-input firing synchronicities (Fig. 7F2). Therefore independently from total tree length, in the adult dendritic geometry, the summation efficacy for synaptic input depends significantly stronger on the degree of synchronization of synaptic input firing than in the larval dendritic tree geometry. Summation efficacy in the larval tree depends more on the sequence of synaptic firing and specific synapse loci than on the degree of input synchronization. Vice versa, in the adult MN5 geometry, input synchronization between 0.4 and 6.4 ms affects the resulting membrane voltage more than the sequence of firing or specific synaptic loci.

In addition, for all synapse numbers tested (50, 100, 200), the median change in membrane potential pooled over all sigmas and synaptic firing sequences is significantly larger in the adult dendritic tree as compared with the larval dendritic tree (Fig. 7G, Mann and Whitney test, P < 0.01). This indicates that adult tree geometry may serve effective synaptic input summation better than the larval one. Scaling the larval dendritic tree to the total dendritic length of the adult MN5 dendritic tree further lowers the median change in membrane potential on synaptic firing significantly (Fig. 7G, Man and Whitney test, P < 0.01). Within the parameter space tested in this study, the resulting median depolarizations between the larval and the adult dendritic tree are only similar at low synapse numbers and high sigmas, e.g., for 50 synapses and a sigma of 6.4 ms the resulting median membrane potential is −27 mV for both tree geometries. However, as soon as input synapse firing synchronicity is increased, only the adult tree architecture, but not the larval one, shows significant changes in the maximum change in membrane potential (Fig. 7D, I and J). Scaling the larval tree to the adult size results in significantly lower changes in membrane potential over all sigmas and synapse numbers (Fig. 7, D2–F2 and D3–F3).

Evaluating the effects of synapse distributions on stage-specific dendritic computations

To test the specific contribution of dendritic tree topology and synapse distribution to the different sensitivities to input synapse firing synchronicity, we randomized the positioning of synapses with regard to the midline length of the dendritic tree (Fig. 8). Simulations were conducted for 100 synapses and eight different sigmas as described in the preceding text, and results are compared between randomized synapse distributions and those derived from the neuroanatomical data (Figs. 4 and 6). For the adult dendritic tree, randomizing synapse distributions does not affect the sensitivity of dendritic geometry to different sigmas (Fig. 8A). For both random and nonrandom synapse distributions, significant differences in the median maximum depolarization exist for all sigmas >0.2 ms (Fig. 8C, Kruskal Wallis ANOVA and Mann and Whitney test, P < 0.01). This indicates that the sensitivity of the adult dendritic tree to different degrees of input synapse firing synchronization might mainly be caused by tree geometry but not by the specific synapse distribution rules reported in this study. However, randomizing synapse distributions results in a small but statistically significant increase in the mean maximum voltage deflection for each sigma (Fig. 8C, Kruskal Wallis ANOVA and Mann and Whitney test, P < 0.01). In passive models of the larval dendritic tree, randomization of synapse distributions causes significantly larger changes in membrane potential for all sigmas (Fig. 8, B and D). In addition, small but statistically significant differences in the mean maximum depolarization occur at sigmas >3.2 ms (Fig. 8D). This indicates that random synapse distributions through the larval tree geometry cause more effective input summation and a higher sensitivity to input firing synchronization than experimentally derived synapse distributions. However, even with randomized synapse distributions membrane potentials resulting from synapse activations at sigmas <3.2 ms are not statistically different in the larval tree geometry (Fig. 8D), whereas in the adult tree geometry statistical significant differences are observed for all sigmas >0.4 ms (Fig. 8C).

Discussion

Our results demonstrate that postembryonic changes in the function of the identified motoneuron MN5 are accompanied by functional dendritic architecture remodeling and changes in the subdendritic distribution of putative GABAergic input synapses. Overall, the adult flight MN5 receives a significantly higher density of GABAergic inputs than the larval crawling MN5, indicating that flight spiking patterns might require more
inhibitory inputs per dendritic surface than larval crawling spiking patterns. The higher density of GABAergic contacts on the surface of the adult MN5 dendritic tree as compared with the larval MN5 is not a consequence of a stage-specific differences in neuropil synapse density. Therefore stage-spe-
cific differences must exist either with respect to targeting or to the maintenance of GABAergic inputs on the dendritic tree of MN5. The larval MN5 receives an even distribution of GABAergic inputs through different Sholl spheres and also along individual dendritic branches. By contrast, the adult
MN5 receives more putative GABAergic inputs at the periphery of the dendritic field. In addition, proximal putative GABAergic inputs close to the spike initiating zone (SIZ) are preferentially targeted to the roots of dendritic sub-trees. These synapse distribution patterns are not a reflection of neuropil structure, but recognition mechanisms for subdendritic targeting of GABAergic inputs must exist. Multicompartment simulations suggest that dendritic remodeling might tune the geometry of MN5 to stage specific motor functions.

**Subdendritic synapse targeting**

The overall densities of GABAergic terminals as revealed by counting puncta immunopositive for GABA and for Synapsin

**FIG. 7.** The efficacy of synaptic input summation depends on input firing synchronicity in the passive adult but not in the passive larval dendritic geometry. The peak depolarizations at the axo-dendritic junction (y axis) as a response to input synapse firing of different synchronicities are plotted over time to peak (x axis). Simulations are conducted in multicompartment models of a passive adult dendritic tree (A) and a passive larval dendritic tree (B). One hundred synapses were distributed either according to experimentally derived synaptic input patterns or randomly. Each synapse was activated once during each simulation. The temporal activation patterns was drawn from a Gaussian distribution with different widths [sigma equals 0.1 (black), 0.2 (red), 0.4 (green), 0.8 (dark blue), 1.6 (light blue), 3.2 (pink), 4.8 (yellow), and 6.4 (gray) ms]. The sequence of synapse activations was random. For each synapse number 10 independent sets of synapses were drawn (see METHODS). For each sigma and for each set of synapses, the simulation was repeated 20 times, resulting in 1,600 simulations per experimental group, each of which is depicted by 1 data point color coded for sigma. C: the medians, quartiles, and min/max values of maximum depolarization of the adult model for each sigma for the activation of 100 synapses distributed either according to confocal laser scanning microscope (CLSM) data (white boxes), or distributed randomly (gray boxes). D: the medians, quartiles and min/max values of maximum depolarization of the larval model for each sigma for the activation of 100 synapses distributed either according to confocal laser scanning microscope (CLSM) data (white boxes), or distributed randomly (gray boxes). In C and D, asterisks indicate statistical significance (Kruskal Wallis ANOVA with Mann and Whitney test, P < 0.01; n.s indicates P > 0.01).

**FIG. 8.** Sensitivity to input firing synchronization is not caused by specific synapse distributions but by dendritic tree geometry. The peak depolarizations at the axo-dendritic junction (y axis) as a response to input synapse firing of different synchronicities are plotted over time to peak (x axis). Simulations are conducted in multicompartment models of a passive adult dendritic tree (A) and a passive larval dendritic tree (B). One hundred synapses were distributed either according to experimentally derived synaptic input patterns or randomly. Each synapse was activated once during each simulation. The temporal activation patterns was drawn from a Gaussian distribution with different widths [sigma equals 0.1 (black), 0.2 (red), 0.4 (green), 0.8 (dark blue), 1.6 (light blue), 3.2 (pink), 4.8 (yellow), and 6.4 (gray) ms]. The sequence of synapse activations was random. For each synapse number 10 independent sets of synapses were drawn (see METHODS). For each sigma and for each set of synapses, the simulation was repeated 20 times, resulting in 1,600 simulations per experimental group, each of which is depicted by 1 data point color coded for sigma. A: the medians, quartiles, and min/max values of maximum depolarization of the adult model for each sigma for the activation of 100 synapses distributed either according to confocal laser scanning microscope (CLSM) data (white boxes), or distributed randomly (gray boxes). B: the medians, quartiles and min/max values of maximum depolarization of the larval model for each sigma for the activation of 100 synapses distributed either according to confocal laser scanning microscope (CLSM) data (white boxes), or distributed randomly (gray boxes). In A and B, asterisks indicate statistical significance (Kruskal Wallis ANOVA with Mann and Whitney test, P < 0.01; n.s indicates P > 0.01).
Three classes of GABAergic interneurons are known to make connections with motoneurons in the insect ventral nerve cord, local spiking interneurons (Watson and Burrows 1987), intersegmental spiking interneurons (Watson and Laurent 1990), and nonspiking local interneurons (Wildman et al. 2002). In accordance to our findings of homogenous neuropil distributions of GABAergic terminals, the projections of these classes of neurons are not restricted to specific parts of the motor neuropil. Therefore recognition mechanisms must exist for GABAergic interneurons to specifically distinguish between the larval and the adult dendrites of MN5 and between different subdomains of the adult dendritic tree.

In principle, two categories of mechanisms could account for subdendritic synapse targeting. First, “activity-dependent site-specific pruning” of dendrites, i.e., initial synaptic inputs form nonselectively through the dendritic field and subdendritic specificity is established by experience induced elimination of inappropriate inputs or by activity-dependent site-specific synapse maintenance. Alternatively, subdendritic targeting might be accomplished in an experience-independent manner by the presence of molecular signals on the appropriate dendritic compartments. Experience guided subcellular synapse elimination has been reported to confine inhibitory glycineric inputs to the somata of auditory interneurons in rodents (Kapfer et al. 2002). By contrast, specific subcellular targeting of GABAergic synapses on pyramidal neurons in visual cortex does not rely on experience-dependent thalamic input (Di Cristo et al. 2004). Subdendritic targeting of inhibitory inputs through different dendritic domains of MN5 is most likely also established in an experience-independent manner because adult synapse distribution patterns are already established at the day of adult emergence, and the pupa cannot fly. However, we cannot exclude the possibility that flight motor network activity in the pupal nervous system might occur at amplitudes subthreshold to cause muscle contractions but still instructive for restricting synapse locations to specific parts of the dendritic field. The central motor network for flight is established ≥2 day before adult emergence (Kinnamon et al. 1984; R. Vierk and C. Duch, unpublished results). However, adequately patterned sensory feedback cannot occur, and no flight muscle twitches were observed during pupal stages. In addition, systemic injections of the chloride channel blocker PTX do not affect the distribution rules for putative GABAergic inputs in the adult MN5 (Meseke et al. 2009). Therefore molecular cues that denote subdendritic domains remain the more parsimonious explanation for subdendritic synapse targeting.

The cellular polarization into axon and dendrites is manifested early during development by the presence of different sets of cell surface molecules (Craig and Banker 1994). However, dendrites themselves can be further polarized into apical or basolateral domains, for instance, and individual dendritic segments may have distinct molecular identities (Horton and Ehlers 2003). Furthermore, in cultured hippocampal neurons somato-dendritic targeting of Kv 2.1 potassium channels has been demonstrated (Lim et al. 2000), whereas AMPA receptor density is progressively increased toward the distal end of CA1 pyramidal neurons (Andrasfalvy and Magee 2001; Magee and Cook 2000), suggesting subdendritic specialization in the proximal to distal dimension. Our findings demonstrate a proximal to distal dendritic polarity as an evolutionary conserved principle present also in insect motoneurons. At present, it remains unclear which extrinsic or intrinsic signals, such as neurotrophins (Huang and Reichardt 2003) and cytoskeletal regulatory proteins, may establish and maintain proximal to distal dendritic polarity.

Possible function of dendritic architecture remodeling

In MN5, increasing demands on input synapse integration, as occurring during the development from a slow crawling into a fast flight motoneuron, are accompanied by the establishment of distinct subdendritic input synapse distributions. Functional significance for segregation of different types of synaptic input to different subdendritic domains has been suggested for GABAergic innervation of pyramidal cells (Pouille and Scanziani 2001), and evidence for behavioral significance has been found in an identified locust visual interneuron involved in collision avoidance during flight (Gabbiani et al. 2002). Many insect muscles with well-characterized functions are innervated by few individually identified motoneurons, allowing the relationship among dendritic architecture, input synapse distributions, and behavioral function to be tested. The larval crawling MN5 innervates a slowly contracting body wall muscle (Duch et al. 2000). In many invertebrates, the contraction slopes of such muscles are mostly independent of brief changes in motoneuron spiking frequency and motoneuron spike timing (Hooper et al. 2007; Morris and Hooper 1998). By contrast, the adult flight MN5 innervates a fast contracting flight muscle, which twitches once per MN5 spike, contracts 25 times per second during flight, and flight behavior relies on the millisecond precision (Tu and Daniel 2004). Therefore the computational demands for precise coding of synaptic input synchrony are much higher in the adult as compared with the larval dendritic tree of MN5. Correlatively, multicompartments simulation demonstrate that depolarization amplitude close to the SIZ (spike initiating zone) of the adult MN5 passive dendritic tree varies strongly with different degrees of input firing synchrony. By contrast, synaptic input firing input synchrony is not coded in depolarization amplitude close to spike initiation of the larval MN5 passive dendritic tree. However, because no data are available on the activation time constant and the conductance of GABAergic synapses located on Manduca motoneurons, our simulation experiments were conducted with the NEURON alpha synapse model. In addition, we did not simulate additional synaptic drive from other transmitter classes as must be occurring during flight motor behavior. Therefore the time values for statistical differences between different input synapse firing synchronicities as observed in this study might differ from the ones existing during synaptic drive in vivo. Nevertheless, input summation in passive fibers is likely to be qualitatively similar with respect to input firing synchronicity even if a different synapse model was used. Therefore the simulation results support the hypothesis that the dendritic geometry of the adult MN5 might be tuned toward effective summation of synchronous synaptic input, whereas input summation in the larval MN5 is much less sensitive to the temporal spread of synaptic firing. Randomizing synapse distribution revealed that the sensitivity of the passive tree of the adult MN5 to input firing synchronicity depends mainly on dendritic tree architecture but not on the
specific distributions of input synapses as found in this study. Similarly, in the larval dendritic tree, random synapse distributions cause significantly larger changes in membrane potential upon synapse activation for all synapse numbers and firing synchronicities tested, but it changes the low sensitivity of the larval tree to input firing synchronicity only mildly. This suggests that dendritic architecture remodeling during metamorphosis is well suited to serve the changing behavioral function of MN5.

These results, however, do not prove that dendritic geometry is really used as an essential component for stage-specific behavioral synaptic input computation because it remains unclear whether active properties, different types of input synapses, and modulators might be much more important than dendritic geometry. However, the simulation results demonstrate that passive dendritic geometry changes as occurring during normal development can in principle affect dendritic computation in a manner that matches predictions based on behavioral function. Functional roles of dendritic shape and synapse distribution for behaviorally relevant computations have been demonstrated in few systems (London and Haeusser 2005; Single and Borst 1998). To the best of our knowledge, this study provides the first evidence for postembryonic dendritic shape changes that might underlie behaviorally relevant dendritic computations. However, passive compartment models are a simplification because MN5 contains dendritic calcium conductances (Duch and Levine 2002) that may change the computational properties of dendrites significantly (London and Haeusser 2005). Furthermore, our simulations operate with the same conductance for all synapses, but in reality a variety of pre- and postsynaptic mechanisms can alter synaptic strength. Nevertheless, this reductionist approach identifies dendritic architecture as a potentially important variable for motor behavior specific computations of synaptic input timing.

During recent years, with increasing knowledge on dendritic properties, the role of individual neurons has evolved conceptually form that of a simple integrator of synaptic input to a much more sophisticated processor converting binary synaptic input into analogue variables, like postsynaptic membrane potential or intracellular calcium levels, which in turn, can be subject to computations (Koch 1997). Our results suggest that such computations might be aided by intricate rules for subdendritic spatial distributions of synapses. Unraveling the mechanisms for subdendritic synapse localization in identified insect motoneurons might allow for testing the behavioral function of synapse localization rules by manipulations on the single neuron level.

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