Changes in Calcium Signaling During Postembryonic Dendritic Growth in *Manduca sexta*

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Duch, C. and R. B. Levine. Changes in calcium signaling during postembryonic dendritic growth in *Manduca sexta*. J Neurophysiol 87: 1415–1425, 2002; 10.1152/jn.00524.2001. Activity-dependent Ca\(^{2+}\) influx plays crucial roles in adult and developing nervous systems through its influence on signal processing, synaptic plasticity, and neuronal differentiation. The responses to internal Ca\(^{2+}\) elevations vary depending on the spatial distribution of Ca\(^{2+}\) accumulation in different cell compartments. In this study, the mechanisms and the distribution of Ca\(^{2+}\) accumulation are addressed by in situ Ca\(^{2+}\) imaging of an identified insect motoneuron, MN5, at critical stages of postembryonic life. During metamorphosis of *Manduca sexta*, MN5 undergoes extensive dendritic regression followed by regrowth. The time course, amplitude, and distribution of Ca\(^{2+}\) accumulation within MN5 change during development. During the initial stage of rapid dendritic growth and branching, dendritic growth cones are present, and voltage-dependent Ca\(^{2+}\) currents are small. At this stage, activity-induced elevations of internal Ca\(^{2+}\) are largest in the distal dendrites, suggesting that the density of voltage-gated Ca\(^{2+}\) channels is highest in these regions. Later phases of dendritic growth are accompanied by the transient occurrence of prominent Ca\(^{2+}\) spikes. Single Ca\(^{2+}\) spikes cause robust Ca\(^{2+}\) influx of similar amplitudes and time courses in all central compartments of MN5. The resting Ca\(^{2+}\) levels also increase during development. Ca\(^{2+}\)-induced Ca\(^{2+}\) release from intracellular stores did not contribute to the elevations measured at either stage, although Ca\(^{2+}\) stores are present in the dendrites. These developmental changes of the internal Ca\(^{2+}\) signaling are consistent with a regulatory role for activity-dependent Ca\(^{2+}\) influx in postembryonic dendritic growth.

INTRODUCTION

Intracellular free Ca\(^{2+}\) plays crucial roles in adult and developing nervous systems. Dendritic Ca\(^{2+}\) signals are important for information processing (Borst and Egelhaaf 1992; Hirsch et al. 1995; Sobel and Tank 1994) and synaptic plasticity (Bliss and Collingridge 1993; Yuste and Tank 1996). The somatic Ca\(^{2+}\) concentration can influence gene transcription (Hardingham et al. 1997). Internal Ca\(^{2+}\) affects aspects of neuronal differentiation such as axon extension and growth-cone motility (Gomez and Spitzer 1999; Gomez et al. 1995; Gu and Spitzer 1995; Kater and Mills 1991; Kater et al. 1988; Lnenicka et al. 1998). Elevations of free intracellular Ca\(^{2+}\) may occur by a variety of mechanisms, including release from intracellular stores (Berridge 1998; Libscobome et al. 1988; Wang and Augustine 1995), activity of the Na\(^+\)-Ca\(^{2+}\) exchanger operating in the reverse mode (Blaustein 1988), and through ligand-gated and voltage-dependent channels (MacDermott et al. 1986; Malinow et al. 1994; Regehr and Tank 1992). Dendritic Ca\(^{2+}\) spikes as occurring in cerebellar Purkinje cells (Llinsas and Sugimori 1980) cause Ca\(^{2+}\) influx into dendrites (Tank et al. 1988). Furthermore, backpropagating Na\(^+\) action potentials can cause dendritic Ca\(^{2+}\) influx through voltage-dependent calcium channels (Christie et al. 1995; Spruston et al. 1995), thereby mediating activity-dependent influences on dendritic plasticity (Magee and Johnston 1997). The responses to Ca\(^{2+}\) elevations differ among neurons and depend on the spatial distribution of Ca\(^{2+}\) accumulation. This study addresses the mechanisms and the distribution of Ca\(^{2+}\) accumulation in different cell compartments of an identified motoneuron by in situ Ca\(^{2+}\)-imaging experiments at critical stages of postembryonic development.

During the metamorphosis of the moth, *Manduca sexta*, many motoneurons undergo dramatic structural and functional modifications for the acquisition of new adult behavior (Conoulas et al. 2000). For example, changes in the dendritic morphology and membrane currents allow an identified flight motoneuron, MN5, to change its behavioral role (Duch and Levine 2000). The larval Ca\(^{2+}\) currents become negligible during early pupal stages but increase dramatically during mid-pupal life and then remain unchanged until adulthood (Duch and Levine 2000). The loss of most larval dendrites is followed by the formation of dendritic growth cones, rapid dendritic growth, and new branch formation until about 50% of adult development (Duch and Levine 2000). During the second half of adult development, growth cones are no longer present and branching is limited to the perimeter of the dendritic field, which continues to increase in size (F. Libersat and C. Duch, unpublished observations). This “switch” in the mode of dendritic growth coincides temporally with the occurrence of prominent Ca\(^{2+}\) spikes, which are allowed by a delay of several days between the development of the adult Ca\(^{2+}\) currents and the subsequent increase in K\(^{+}\) currents (Duch and Levine 2000). Because activity-dependent Ca\(^{2+}\) influx can affect neuronal differentiation (Cohan et al. 1987; Fields et al. 1990; Gomez and Spitzer 1999; Kater and Mills 1991; Kater et

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al. 1988; Mattson et al. 1988), the Ca\(^{2+}\) spikes may influence dendritic growth.

This study tested whether activity-dependent Ca\(^{2+}\) influx occurred in the dendrites of MN5, whether Ca\(^{2+}\) influx occurred in the growth cones during early pupal life, how the Ca\(^{2+}\) spikes during later stages affected the internal Ca\(^{2+}\) concentrations, and whether the Ca\(^{2+}\) signaling changed during metamorphosis. The results are consistent with a regulatory role of Ca\(^{2+}\) in postembryonic dendritic growth.

METHODS

Animals

*Manduca sexta* obtained from the laboratory culture of the Division of Neurobiology at the University of Arizona were reared on artificial diet (Bell and Joachim 1976) under a long-day photoperiod regimen (17:7 h light/dark cycle) at 26°C and ~60% humidity. Both chronological and morphological criteria were used for staging of animals (Bell and Joachim 1976; Consoulas et al. 1996; Nijhout and Williams 1974; Tolbert et al. 1983). In summary, L5 represents the fifth larval instar, W0–W4 signify the 5 days of wandering, P0 indicates the day of the pupal molt, and P1–P18 are the following 18 days of pupal development. In this study, pupal stages P4 and P8 were used for a developmental comparison of cytosolic Ca\(^{2+}\) signaling during dendritic growth. P4 is the stage where prominent growth cones are formed, and rapid dendritic growth and new branch formation takes place (Fig. 1A). P8 is a stage where growth cones are no longer observed and further branching is limited to the perimeter of the dendritic field (Fig. 1B) (Duch and Levine 2000).

**FIG. 1.** A: stack of optical sections projected into 1 focal plane taken of the dendritic field of MN5 at pupal stage P4 after intracellular injection of rhodamine dextran, fixation, clearing, and mounting of the preparation. At stage P4, the tips of all dendrites show growth-cone-like structures, some of which are indicated with white arrowheads. One growth cone is shown as a selective enlargement in the bottom right corner. B: stack of optical sections taken from the dendritic region of MN5 at pupal stage P8. Same staining procedure as in A. C: single focal plane of the dendritic region of MN5 in situ at pupal stage P4 taken at 380-nm excitation wavelength with a cooled CCD camera after intracellular injection with fura-2. Growth cones are marked with white arrowheads. One growth cone is shown at higher magnification in the bottom right corner. D: single focal plane of the dendritic region of MN5 in situ at pupal stage P8 taken at 380-nm excitation wavelength with a cooled CCD camera after intracellular injection with fura-2. The black ovals in C and D indicate region 1 that was used for intensity measurements in subsequent figures. Scale is 50 μM.

Dissection for intracellular recordings

The animals were anesthetized by chilling on ice for 15 min. Animals were dissected along the dorsal midline and superfused with saline. The thoracic and the first two abdominal ganglia were removed, transferred into a silicone-elastomer (Sylgard)-coated petri dish, and pinned down at the cut ends of their lateral nerves in saline. The ganglionic sheath was removed mechanically with a fine pair of forceps.

Nerve 1 of the mesothoracic ganglion was left intact toward its specific peripheral branch, which contains only the axons of the five dorsal longitudinal flight muscle motoneurons. MN5 is the only motoneuron in the mesothoracic ganglion, which carries an axon in this particular nerve branch (Duch et al. 2000). Antidromic stimulation from this nerve branch was used to identify MN5 during intracellular recordings.

**Injections of fura-2**

Fura-2 pentapotassium salt (Molecular Probes, Eugene, OR) was used for calcium-imaging experiments. The tips of thin-walled borosilicate electrodes (resistances, 35–40 MΩ) were filled with 12 mM fura-2 in 100 mM potassium acetate. The electrode shafts were filled with 1 M potassium acetate, and an air bubble was left between the tip and the shaft to prevent dye dilution. Following intracellular penetration and antidromic identification of MN5, dye was injected iontophoretically by applying hyperpolarizing current of 1 nA amplitude for 10 min in preparations of pupal stage P4 and for 20 min in preparations of pupal stage P8. Pupal stage P8 was injected for a longer duration because the volume of MN5 increases considerably between the pupal stages P4 and P8 (Duch and Levine 2000). Following dye injection, ganglia were left in saline for an additional 15 min to allow dye diffusion.

Electrophysiology

Subsequently, ganglia were transferred to the imaging setup and MN5 was re-impaled with a thin-walled borosilicate electrode (resistances, 100–120 MΩ) filled with 2 M potassium acetate. The re-impalment was conducted for two reasons. First, it prevented further uncontrolled dye filling of the cells during the imaging experiments. Second, simultaneous intracellular and optical recordings required very shallow electrode angles and the low-resistance electrodes that were used for dye injections injured the cells in such recording conditions. An Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) was used for all intracellular recordings. For antidromic stimulation of MN5, an extracellular suction electrode was placed at nerve 1. Antidromic spike initiation was confirmed by simultaneous intracellular recordings. The stimulation amplitude was set just above firing threshold. The manipulators for the electrodes were mounted to the microscope stage so that the preparation could be moved to image different fields of view from the same neuron. The intra- and extracellular recordings were synchronized with the imaging sequences by reading them simultaneously with a trigger trace provided by the imaging system into Clampex 8 (Axon Instruments) software. The trigger trace defined the time point of the start of each acquired frame.

Imaging

Fura-2 was excited in single (380 nm) or dual (340 nm/380 nm) wavelength illumination mode, and fluorescence images on the basis of emission light passing a 530-nm filter were captured with a cooled CCD camera (Hamamatsu 4742-95), mounted on a fluorescence microscope (Olympus BX50WI). The camera and a filter wheel were controlled with “Simple PCI” software (Compix). The same software was used for data acquisition and fluorescence intensity measurements. The intensity measurement data were further analyzed with
Cyclopiazonic acid (CPA, Sigma) was used at 200 M concentration in normal saline. Solutions for neuronal experiments using single wavelength mode were conducted at 8 x 8 binning, meaning that pictures of 128 x 128 pixels were transferred to the computer. With these settings, the exposure times per frame were 10 ms for the dendritic region and 2 ms for the cell body. All single-wavelength data shown were acquired under these conditions. In all experiments, Ca2+ responses of MN5 were measured as responses to either antidromic stimulation via nerve 1 or spikes elicited orthodromically with an electrode placed in the cell body. Regions of interest were placed at defined locations of MN5, as indicated in the figures, and the fluorescence intensities in each region were measured off-line with Simple PCI. Background was routinely subtracted. For all dendritic measurements, background was determined 50 mm anterior of the site where the major dendrite branches off the axon of MN5 because no arbors of MN5 are located in this region. For measurements from the soma or the link segment, background was measured 50 mm anterior of the soma, or 50 mm anterior of the respective site along the link segment. Photobleaching was not corrected. In all experiments using dual wavelength mode, changes in the fluorescein/fluorescein 380 ratio were taken to indicate changes in intracellular Ca2+ concentration ([Ca2+]i) (Gryniewicz et al. 1985). However, the absolute Ca2+ concentrations were not determined, given uncertainties concerning accurate calibration in situ. In all experiments, the resting ratio was determined after the re-impalement and prior to single-wavelength imaging. Preparations with a resting ratio >1.5 were likely to have been injured during the two intracellular impalements and were discarded. Similarly, the resting ratio was determined routinely between subsequent single-wavelength acquisitions to ensure the continued health of the cells. Experiments were terminated as soon as the resting ratio increased by >0.3 as compared with the value observed at the beginning of the experiment. In all experiments using single-wavelength mode, the index -ΔF/Fmean(380) was defined as is the usual case for insect motoneurons (Gwilliam and Burrows 1980). Single Na+ spikes evoked orthodromically by current injection into the soma did not evoke a detectable Ca2+ response in region 1 (Fig. 2C), or in any other region of the neuron (data not shown). Similarly, bursts of four to five orthodromic Na+ spikes at stage P4 did not evoke any detectable Ca2+ response in MN5 (Fig. 2E). In contrast, individual Ca2+ spikes, which occur spontaneously, or can be elicited orthodromically at pupal stage P8 (Duch and Levine 2000) led to a pronounced Ca2+ response in region 1 of MN5 (Fig. 2D). During multiple Ca2+ spikes, the amplitudes of the intracellular Ca2+ increases summed in an approximately linear fashion (Fig. 2F). At pupal stage P4, prolonged positive current injections of 3 nA led to high-frequency bursts of Na+ spikes, which caused slow Ca2+ responses of ~5% amplitude in region 1 (Fig. 2G). In contrast, at pupal stage P8 current injection of 3 nA for only 250 ms induced a brief burst of four Ca2+ spikes that led to rapid Ca2+ responses of >15% amplitude (Fig. 2H). In summary, Na+ spikes at pupal stage P4 evoked only minor increases in intracellular Ca2+ in MN5 that were only detectable during strong bursts, whereas individual Ca2+ spikes at pupal stage P8 evoked large elevations of the intracellular Ca2+ concentrations in region 1. Although the Na+ spikes that occur at pupal stage P4 cause only small depolarizations in the soma, the level of depolarization in region 1 must be quite high because action potentials are propagated actively along the axon. Therefore the difference in the Ca2+ responses at this site between the pupal stages P4 and P8 was probably not due simply to the level of depolarization, but to the density of Ca2+ channels in region 1. This is further supported by subthreshold current injections into MN5 at pupal stage P8 (Fig. 3). Single Ca2+ spikes elicited by current injections just over threshold led to Ca2+ responses of >5% amplitude. In contrast, the same cell showed weak Ca2+ responses of ~2% amplitude on subthreshold current injections (Fig. 3). In some preparations, it was possible to evoke a Na+ spike while staying subthreshold for the induction of a Ca2+ spike (Fig. 3). Like subthreshold current injections, single Na+ spikes evoked weak Ca2+ responses of ~2% amplitude at pupal stage P8 (Fig. 3). In contrast, at pupal stage P4, when Ca2+ spikes cannot be elicited (Duch and Levine 2000), single Na+ spikes and current injections just below the firing threshold did not cause a detectable Ca2+ response (Fig. 2), although the firing threshold is roughly ~40 mV at both stages (Duch and Levine 2000).

Solutions

External saline for dissection and recording consisted of (in mM): 140 NaCl, 5 KCl, 4 CaCl2, 28 d-glucose, and 5 HEPES, pH was adjusted to 7.4 using 1 M NaOH. Zero-Ca2+ saline contained 140 NaCl, 5 KCl, 4 MgCl2, 28 d-glucose, 5 HEPES, and 0.5 EGTA. Cyclopiazonic acid (CPA, Sigma) was used at 20 μM concentration in normal saline.
Ca$^{2+}$ signals were induced at pupal stage P8 but not at pupal stage P4. At pupal stage P4, only much stronger depolarizations, such as those induced by trains of orthodromic (Fig. 2) or antidromic spikes (see following text) led to Ca$^{2+}$ responses in region 1.

To test whether Ca$^{2+}$-spike-induced Ca$^{2+}$ elevations were distributed evenly throughout different regions of the neuron at pupal stage P8, different compartments of MN5 were analyzed (Fig. 4). The same eight regions of interest were defined and imaged in eight different preparations of pupal stage P8. Region 1 was defined as described in the preceding text. Region 2 was set in a distance of 200 μm from region 1 along the link segment toward the soma (Fig. 4A). Region 3 was set in a distance of 100 μm from region 1 along the axon toward nerve 1 (Fig. 4A). Region 4 was set in distance of 150 μm from region 1 along the primary dendrite of MN5 (Fig. 4A). The somatic Ca$^{2+}$ responses of the same cells were measured in a different field of view (region 8, Fig. 4B). In an additional field of view, three regions along the link segment with distances of 100 μm relative to each other were analyzed (Fig. 4C, regions 5–7). Region 5 was set 50 μm to region 1 on the link segment. Individual Ca$^{2+}$ spikes evoked very similar Ca$^{2+}$ responses in all 8 regions analyzed (Fig. 4, D–F). The time constants of the decay of the signal were not significantly different among any of the regions shown in Fig. 4A ($P \geq 0.1$; Fig. 4, D and G). The time constants measured from the somata of the same preparations were considerably longer ($2.9 \pm 0.8$; $P = 0.042$). This difference could reflect a true difference in calcium dynamics but may have been due to the different volume to surface ratio of the soma at the two stages, or the excessive dye concentrations in the soma when the cells were filled to visualize dendritic and axonal regions. Therefore in three preparations of pupal stage P8, MN5 was filled for only 5 min. In these preparations, the time constant of decay in the soma was similar to that observed in all other regions ($1.4 \pm 0.7$; $P \geq 0.2$). This also explains the slightly longer time constant in region 2 in Fig. 4G because this region was closest to the soma. Therefore the time constant of decay of the intracellular Ca$^{2+}$ elevations in response to a single Ca$^{2+}$ spike was similar in all compartments of MN5. The same was true for the signal amplitude ($P \geq 0.1$; Fig. 4, H and I).

In summary, the Ca$^{2+}$ spikes that occur at pupal stage P8 led to strong elevations of the internal Ca$^{2+}$ concentration throughout all compartments of MN5. In contrast, single Na$^+$ spikes at
Differences were detected among the 4 regions. HSoma and the dendritic fi

A. C

E (bottom fi

spikes in the soma (average of 3 preparations in which the soma was

response to single Ca^2+ sequences, i.e., the somatic values to all other regions, and the 3 regions shown in

isons of values obtained from the same preparation but from different imaging

cord recordings that were conducted with high time resolution. As in

currents that were not detectable in somatic recordings due to

space-clamp problems. The following experiments were con-
ducted to explore this possibility and to further compare Ca^2+ signals at the two developmental stages.

Antidromic stimulation of the axon of MN5 allows the motoneuron to be driven at high frequencies. Furthermore it
does not evoke Ca^2+ spikes at pupal stage P8 (Duch and Levine 2000). Thus antidromic stimulation is a useful method
for comparing the intracellular Ca^{2+} responses at equivalent levels of depolarization at both stages. Antidromic stimulation
with 60 Hz for 10 s led to Ca^{2+} responses in region 1 in both developmental stages (Fig. 5A). The amplitudes of the Ca^{2+}
responses were significantly higher in preparations of pupal stage P8 (Fig. 5, A and B). Fitted curves for the decay of the Ca-
signals are shown in the inset in Fig. 5A (P4 gray lines, P8 black lines). The time constant was significantly longer for
pupal stage P4 (Fig. 5C), indicating that either the Ca^{2+} buffering was stronger or the Ca^{2+} extrusion was slower at this
developmental stage. Thus Ca^{2+} responses during high-frequency Na^{+} spikes that were evoked by antidromic stimulation
were of higher amplitude and had a faster decay at pupal stage P8 than at pupal stage P4. This quantitative comparison of the
Ca^{2+} dynamics prompted the concern that the concentrations of free Ca^{2+} indicator differed in MN5 between the two
developmental stages, attributing to the different volumes and possibly also to different protein compositions. To test whether the higher signal amplitude measured in MN5 at pupal stage P8 as compared with pupal stage P4 was simply due to buffering effects of the Ca^{2+} indicator (Fig. 5, A and B), some preparations
were measured in dual-wavelength mode. Although this compromised the temporal resolution, dual-wavelength record-
ings are far less dependent on the dye concentration and thus serve as a useful comparison with the single-wavelength re-
cordings that were conducted with high time resolution. As in single-wavelength mode, dual-wavelength mode revealed that
antidromic stimulation with 60 Hz for 10 s led to Ca^{2+} responses in region 1 in both developmental stages (Fig. 5D).
Furthermore the amplitudes of the Ca^{2+} responses were about twice as large in preparations of pupal stage P8 (Fig. 5, D and
E), thus confirming the data obtained in single-wavelength mode (Fig. 5B). We could not definitively test whether the time
constant might have been influenced artificially by differences in the free indicator concentration among the stages. However,
varying the dye injection times by 30% for each stage (n = 2 for each stage), and test experiments with calcium green 5 (n = 3
for each stage) always showed that the time constant at pupal stage P4 was about twice as long compared with pupal stage
P8 (data not shown). This strongly suggests that the differences in time course found between developmental stages were not
artificially produced by different buffering effects of the indicator dye but rather due to real differences in the calcium

dynamics. Although the absolute Ca^{2+} concentrations were not
determined, the ratio of resting fluorescence at 340- and 380-nm excitation was significantly higher at pupal stage P8 than at pupal stage P4 \((P \leq 0.01; \text{ Fig. 5, D and F})\), indicating a higher resting intracellular Ca\(^{2+}\) level.

To test whether the Ca\(^{2+}\) responses that were evoked antidromically were caused by Ca\(^{2+}\) influx through the cell membrane, Ca\(^{2+}\) was replaced by Mg\(^{2+}\) in the extracellular solution (Fig. 6). The control stage P4 Ca\(^{2+}\) response in normal saline (Fig. 6, gray curve) was abolished after 10 min in Ca\(^{2+}\) free saline (Fig. 6). Washing in normal saline led to 50% recovery of the initial response amplitude after 5 min and to a full recovery after 10 min. Therefore the Ca\(^{2+}\) responses were dependent on external Ca\(^{2+}\). At pupal stage P8, the Ca\(^{2+}\) response to antidromic stimulation could also be abolished in Ca\(^{2+}\)-free saline (data not shown). However, this does not exclude the possibility that additional Ca\(^{2+}\) release from intracellular stores contributes to the higher amplitude of the Ca\(^{2+}\) response at pupal stage P8.

Pharmacological experiments were conducted to examine whether the Ca\(^{2+}\) influx through the cell membrane caused Ca\(^{2+}\) release from intracellular stores. The Ca\(^{2+}\) ATPase inhibitor cyclopiazonic acid (CPA) depletes intracellular Ca\(^{2+}\) stores (Lohr and Deitmer 1999). CPA (20 \(\mu\)M) clearly acted on the intracellular Ca\(^{2+}\) stores of MN5 in situ at both stages, P4 and P8, as shown by ratiometric imaging of the resting Ca\(^{2+}\) concentration. For example, in a stage P8
MN5, the resting ratio at region 1 increased significantly without depolarization after 90 s of CPA bath application (Fig. 7B). After an additional 90 s, the resting ratio again declined (Fig. 7B) probably due to the activity of Ca^{2+} pumps in the cytoplasmatic membrane. Imaging the Ca^{2+} responses to antidromic stimulation before and after this effect of CPA revealed no significant differences in the Ca^{2+} responses (Fig. 7C). In summary, MN5 showed CPA induced Ca^{2+} release from intracellular stores at both developmental stages, but this had no effect on the depolarization induced intracellular Ca^{2+} rise at stage P8.

To determine whether the Ca^{2+} influx through voltage-dependent calcium channels (VDCCs) that was induced by antidromic stimulation was distributed evenly throughout different compartments of MN5, different regions were analyzed while applying the same antidromic stimulation protocol. The regions are indicated by the white ovals in Fig. 8, A (pupal stage P4) and B (pupal stage P8). The Ca^{2+} responses differed significantly among the different regions at both stages (Fig. 8, C and D). At pupal stage P4, the strongest Ca^{2+} response was observed in the dendrites (region 4). In some preparations, the resolution was high enough to image individual growth cones (Fig. 8E).

There the Ca^{2+} responses were as high as those observed in the dendrites. The Ca^{2+} responses observed in the axon ~100 μm away from region 1 were 50% of the amplitude observed in region 1 and only 15% of the amplitude observed in the growth cones and the dendrites (Fig. 8C). Antidromic stimulation evoked no Ca^{2+} signals in the link segment (Fig. 8C) or in the soma (Fig. 8F) at pupal stage P4.

In contrast, at pupal stage P8, a very different distribution of the Ca^{2+} responses within different parts of MN5 was found (Fig. 8D). The strongest response amplitudes were observed in the axon ~100 μm away from region 1 (Fig. 8D) in contrast to pupal stage P4 where the Ca^{2+} elevations were most pronounced in the dendrites. At pupal stage P8, the dendritic Ca^{2+} responses were ~60% of the amplitude that was observed in region 1. Furthermore at pupal stage P8, Ca^{2+} responses were observed in the link segment. The amplitude of these signals was similar to those observed in the dendritic region (Fig. 8D). In summary, at pupal stage P4, Ca^{2+} elevations in response to antidromically evoked Na^{+} spikes were mostly restricted to the dendritic regions, whereas at pupal stage P8 Ca^{2+} elevations were observed throughout all central compartments of the cell, with the highest amplitudes in the axon, where the antidromic spikes would be of greatest amplitude. Therefore at pupal stage P8, the largest Ca^{2+} influx was observed where the largest depolarization occurred. In contrast, at P4 the largest Ca^{2+} influx was observed in the dendrites despite the larger depolarization in the axonal region.

**DISCUSSION**

The key finding of this study is that activity-dependent Ca^{2+} influx via VDCCs occurs in the dendrites of MN5 but changes during development. The changes in Ca^{2+} signaling coincide with distinct phases of dendritic growth, suggesting that the developmental modifications in ionic currents are not only important for the new adult behavioral role of MN5, but also for specific aspects of the dendritic modifications that occur during the integration into the flight motor network.
Mechanisms of Ca\(^{2+}\) accumulation in MN5 at different developmental stages

At pupal stage P8, single Ca\(^{2+}\) spikes caused elevations of the internal Ca\(^{2+}\) concentration of >5% in all central compartments of MN5. This Ca\(^{2+}\) accumulation was likely to be caused entirely by influx through VDCCs because multiple Ca\(^{2+}\) spikes led to approximately linear increases in intracellular free Ca\(^{2+}\). Ca\(^{2+}\)-dependent Ca\(^{2+}\) release from internal stores did not appear necessary, and removal of extracellular Ca\(^{2+}\) abolished the responses. Although substantial Ca\(^{2+}\) responses occurred in response to propagating Ca\(^{2+}\) spikes, subthreshold depolarizations alone could induce low levels of Ca\(^{2+}\) influx, probably through low-voltage-activated Ca\(^{2+}\) channels, as in the dendrites of fly visual interneurons (Oertner et al. 2001). No significant differences in Ca\(^{2+}\) response amplitudes were observed among soma, axon, and dendrites of MN5 following the Ca\(^{2+}\) spike, indicating a rather even distribution of VDCCs throughout the neuron.

Even at stage P4, when whole cell Ca\(^{2+}\) currents are negligible (Duch and Levine 2000) and no Ca\(^{2+}\) spikes can be elicited, slow elevations in intracellular Ca\(^{2+}\) levels occurred in the dendrites in response to trains of action potentials. Surprisingly, the largest Ca\(^{2+}\) influx on antidromic stimulation did not occur in the axon where depolarizations caused by the Na\(^{+}\) spikes would be highest but rather in the dendrites and even in the distal growth cones. The activity-dependent increase of internal Ca\(^{2+}\) at P4 depended on external Ca\(^{2+}\) as shown by Ca\(^{2+}\) replacement experiments. Furthermore, Ca\(^{2+}\) release from intracellular stores was probably not involved, although intracellular stores were present in the dendrites at both developmental stages as shown by elevations in internal free Ca\(^{2+}\) after CPA application. Second-messenger-induced Ca\(^{2+}\) release from internal stores might be an additional regulatory mechanism in dendritic Ca\(^{2+}\) signaling (David and Pitman 1996; Wand and Augustine 1995).

Voltage-dependent dendritic Ca\(^{2+}\) influx has recently been reported to serve different functions among different types of neurons such as cricket giant interneurons (Ogawa et al. 2000), tangential cells of the fly visual system (Oertner et al. 2001), and hippocampal pyramidal cells (Christie et al. 1995; Isomura and Kato 1999). In pyramidal cells, dendritic Ca\(^{2+}\) influx through VDCCs plays critical roles in the induction of LTP (Magee and Johnston 1997), whereas in fly tangential cells, it might serve local adaptation to visual motion stimulation (Oertner et al. 2001). Its functional relevance for the adult MN5 remains to be investigated. In MN5, the net Ca\(^{2+}\) currents is unchanged from pupal stage P8 through adulthood (Duch and Levine 2000). Thus Ca\(^{2+}\) influx in the adult MN5 might be important for dendritic signal integration and for modulating the firing properties.

The activity-dependent Ca\(^{2+}\) signals at stage P4 in the absence of Ca\(^{2+}\) spikes might occur via voltage-dependent calcium channels in the dendritic regions. Action potentials cause large elevations of internal Ca\(^{2+}\) in hippocampal neurons by influx through VDCCs (Christie et al. 1995; Spruston et al. 1995). This mechanism would require that Na\(^{+}\) spikes depolarize distal dendritic regions of MN5. Action potentials ordinarily do not propagate actively into the dendritic region of insect motoneurons, but the distal dendrites are probably depolarized passively, especially in the reduced dendritic field at
stage P4. The distal dendrites and the growth cones show the largest Ca\(^{2+}\) response to trains of Na\(^+\) spikes either because the calcium channel density is highest there or because of restricted diffusion. The fact that we did not observe Ca\(^{2+}\) currents in voltage-clamp recordings from the soma at P4 (Duch and Levine 2000) is consistent with the former possibility. Alternatively, increases in internal Ca\(^{2+}\) at pupal stage P4 might reflect influx through the Na\(^+\)-Ca\(^{2+}\) exchanger stimulated by a rise in internal Na\(^+\) (Blautz 1988). In contrast, at pupal stage P8, the Na\(^+\)-spike-induced dendritic Ca\(^{2+}\) signals are smaller than the axonal ones. This is consistent with the hypothesis that VDCCs are evenly distributed throughout MN5 but that Na\(^+\) fails to depolarize distal dendrites in the more complex dendritic field at this stage.

**Putative role of Ca\(^{2+}\) spikes for dendritic growth**

The Ca\(^{2+}\) spikes that occur transiently during the developmental modifications of MN5 strongly affect the dendritic Ca\(^{2+}\) concentration. As shown previously, these spikes occur exclusively during pupal stages P7–P9 and thus correlate temporally with a switch in the mode of dendritic growth (Duch and Levine 2000). The extensive dendritic growth-cone branching that takes place in MN5 during earlier pupal stages ceases during the stages when these Ca\(^{2+}\) spikes occur. Growth cones are not observed during the second half of pupal development (Duch and Levine 2000; Libersat and Duch, unpublished morphometric analysis). Because the Ca\(^{2+}\) spikes occur spontaneously and can be evoked by sensory stimulation in the isolated ganglion preparation, it is likely that they also occur during normal development (Duch and Levine 2000). In cultured neurons, activity-dependent Ca\(^{2+}\) influx can operate as a growth-stopping signal (Baird et al. 1996), and high levels of Ca\(^{2+}\) influx suppress motile growth-cone structures (Mattson and Kater 1987). Membrane-potential-dependent Ca\(^{2+}\) influx into the growth cones and adjacent dendrites inhibits elongation and finally leads to a collapse of the growth cone (Kater and Mills 1991). The present study shows that strong Ca\(^{2+}\) influx into the dendrites of MN5 in situ correlates temporally with the cessation of growth-cone branching, suggesting that these spikes serve to stop growth-cone branching during metamorphosis. A restriction of dendritic branch number might have important consequences for the generation of a functional flight motor circuit.

Ca\(^{2+}\) homeostatic mechanisms can override depolarization induced growth-cone inhibition (Fields et al. 1990, 1993). Therefore it is important to note that the resting ratio of \(F_{340}/F_{380}\) in MN5 was significantly higher at pupal stage P8 than at pupal stage P4. Although the measurements were not calibrated to calculate the internal Ca\(^{2+}\) concentrations, they indicate a higher level at P8. This suggests that the higher Ca\(^{2+}\) influx at pupal stage P8 is not counteracted by developmental increases in the Ca\(^{2+}\) handling mechanisms. This is further supported by the relatively long-lasting increase in internal Ca\(^{2+}\) levels in response to a single Ca\(^{2+}\) spike.

Ultimately, the Ca\(^{2+}\) influx has to be translated into changes in the cytoskeleton to affect neuronal growth. Promising candidates via which Ca\(^{2+}\) signals might be read are the Ca\(^{2+}\)-sensitive enzymes CAM-K II and calcineurin. In the developing *Xenopus* optical tectum, CAM-K II is required to limit the elaboration of neuronal arbors (Zou and Cline 1999). In hippocampal neurons, CAM-K-II-dependent phosphorylation of MAPK is critical for dendritic spine morphology (Wu et al. 2001). During embryonic spinal cord development, Ca\(^{2+}\) transients inhibit the rate of axon outgrowth (Gomez and Spitzer 1999) by mediating an increase in the activity of calcineurin (Lautermilch and Spitzer 2000). The functional role of these pathways in insect motoneuron remains to be investigated.

The Ca\(^{2+}\) spikes probably propagate actively into the soma because somatic and dendritic Ca\(^{2+}\) responses to single Ca\(^{2+}\) spikes were similar in amplitude and time course. Although the functional importance of the somatic Ca\(^{2+}\) spikes in MN5 remains unclear, nuclear Ca\(^{2+}\) signals can affect gene transcription (Hardingham et al. 1997) and thus play a regulatory role during neuronal differentiation. Therefore it will be important to examine whether the somatic Ca\(^{2+}\) signals in MN5, which occur only during specific times of postembryonic development, are translated into nuclear Ca\(^{2+}\) elevations.

**Putative role for low levels of Ca\(^{2+}\) influx into distal dendrites and growth cones at P4**

The effects of intracellular Ca\(^{2+}\) on growth-cone behavior are concentration dependent (reviewed in Kater and Mills 1991). Briefly, at optimal levels of internal Ca\(^{2+}\), outgrowth is profuse, but lower or higher levels result in decreased outgrowth. Therefore the low levels of Ca\(^{2+}\) influx at stage P4 might allow MN5 to maintain optimal Ca\(^{2+}\) levels and promote growth-cone branching. During normal development, MN5 is probably never active at the high frequencies that were used for antidromic stimulation in this study, and thus fast elevations of the Ca\(^{2+}\) concentrations by >20% are unlikely to occur. Nevertheless, Na\(^+\)-spike-induced Ca\(^{2+}\) influx was also detectable at antidromic stimulation frequencies of 5 Hz, although the responses were more variable and less robust (data not shown). In cultured neurons, changes in the internal Ca\(^{2+}\) concentration as little as 30–50 nM reliably alter filopodia morphology (Rehder and Kater 1992). Therefore moderate activity at pupal stage P4 might allow low levels of Ca\(^{2+}\) influx within a permissive range for dendritic growth.

**Putative role for Ca\(^{2+}\) influx in the adult MN5**

The large net Ca\(^{2+}\) current in MN5 remains unchanged from pupal stage P8 through adulthood (Duch and Levine 2000). Voltage-dependent dendritic Ca\(^{2+}\) influx serves a variety of functions in different neurons (Christie et al. 1995; Isomura and Kato 1999; Oertner et al. 2001; Ogawa et al. 2000). In pyramidal cells, dendritic Ca\(^{2+}\) influx through VDCCs plays critical roles in the induction of LTP (Magee and Johnston 1997), whereas in fly tangential cells it might serve local adaptation to visual motion stimulation (Oertner et al. 2001). A testable possibility in the case of MN5 is that large Ca\(^{2+}\) currents, and perhaps elevations in internal Ca\(^{2+}\), are important for participation in flight. Ca\(^{2+}\) conductances play a major role in the generation of plateau potentials (Hancock and Pitman 1991, 1993; Hartline and Russel 1984; Kiehn 1991). Although the adult MN5 displayed no plateau potentials in the isolated ganglion preparation, prolonged Ca\(^{2+}\) action potentials could be evoked after applying TEA (Duch and Levine 2000). In many systems, plateau potentials can be induced by neuromodulators or any intervention that sufficiently reduces oppos-
ing outward currents (Hultborn and Kiehn 1992; Kiehn and Harris-Warrick 1992; Ramirez and Pearson 1991). Flight behavior, for example, is strongly influenced by the neuromodulator octopamine, which evokes plateau potentials in locust flight interneurons (Orchard et al. 1993). One possibility therefore is that the adult Ca\textsuperscript{2+} currents come into play because opposing outward currents are reduced by modulatory signals during flight. In particular, moths display a prolonged warm-up phase during which neuromodulators may readily prepare the process for development changes in the Ca\textsuperscript{2+} phase during which neuromodulators may readily prepare the opposing outward currents are reduced by modulatory signals during flight. In particular, moths display a prolonged warm-up phase during which neuromodulators may readily prepare the CNS for flight behavior (Claassen and Kammer 1986). To explore the function of developmental changes in the Ca\textsuperscript{2+} signaling in identified neurons, both developmental and behavioral consequences will have to be considered. A challenging task will be to understand how changes in the Ca\textsuperscript{2+} currents that are important for the adult firing properties of a neuron might also be used as signals for developmental modifications, such as dendritic remodeling.

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