Dendrite Elongation and Dendritic Branching Are Affected Separately by Different Forms of Intrinsic Motoneuron Excitability

Carsten Duch, Fernando Vonhoff, and Stefanie Ryglewski
School of Life Sciences, Arizona State University, Tempe, Arizona

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Duch C, Vonhoff F, Ryglewski S. Dendrite elongation and dendritic branching are affected separately by different forms of intrinsic motoneuron excitability. J Neurophysiol 100: 2525–2536, 2008. First published August 20, 2008; doi:10.1152/jn.90758.2008. Dendrites are the fundamental determinant of neuronal wiring. Consequently dendritic defects are associated with numerous neurological diseases and mental retardation. Neuronal activity can have profound effects on dendritic structure, but the mechanisms controlling distinct aspects of dendritic architecture are not fully understood. We use the Drosophila genetic model system to test the effects of altered intrinsic excitability on postembryonic dendritic architecture development. Targeted dominant negative knock-downs of potassium channel subunits allow for selectively increasing the intrinsic excitability of a selected subset of motoneurons, whereas targeted expression of a genetically modified noninactivating potassium channel decrease intrinsic excitability in vivo. Both manipulations cause significant dendritic overgrowth, but by different mechanisms. Increased excitability causes increased dendritic branch formation, whereas decreased excitability causes increased dendritic branch elongation. Therefore dendritic branching and branch elongation are controlled by separate mechanisms that can be addressed selectively in vivo by different manipulations of neuronal intrinsic excitability.

INTRODUCTION

The dendritic shape of individual neurons is important for adequate neural network function because it ensures connections with the correct synaptic partners and affects synaptic input integration (Connors and Regehr 1996; Häusser et al. 2000). During development, dendritic shape is regulated by innate genetic factors (Gao and Bogert 2003; Mizrahi et al. 2000; Montague and Friedlander 1989; 1991; Scott et al. 2002, 2003; Sapatkowski and Schilling 2003), external molecular cues (Kim and Chiba 2004; Landgraf and Thor 2006), humoral cues (Cooke and Wooley 2005; Toran-Allerand et al. 1999; Weeks and Levine 1995), and neural activity (Duch and Mentel 2004; Libersat and Duch 2004; Wong and Ghosh 2002). However, the degree to which neuronal activity affects developing dendrites varies considerably between different species, different types of neurons, and different times during development (Liber sat and Duch 2004; Lohmann and Wong 2005). Within individual neurons different types of activity have to be distinguished. Intrinsic excitability regulates the number of spikes generated by a neuron and may affect global calcium concentrations that control dendritic properties by regulating transcription (West et al. 2002). By contrast, the detailed branching patterns of dendritic arbors and site-specific synaptogenesis may be regulated by calcium signals triggered by strictly local synaptic input (Lohmann et al. 2002; Niell et al. 2004). This study focuses on the role of endogenous intrinsic excitability for the development of dendritic architecture of identified motoneurons in the genetic model system Drosophila melanogaster.

Insect metamorphosis offers a useful model to study mechanisms underlying behaviorally relevant modifications of dendritic architecture during postembryonic development because individually identified neurons acquire their behavioral function, their geometry and their physiology during the transformation from the larval into the adult stage (Consoulas et al. 2000). We make use of the genetic power of Drosophila to manipulate the intrinsic excitability of a subset of motoneurons by targeted genetic manipulation of their potassium membrane conductances. Postembryonic dendritic growth has been described in detail for the identified flight motoneuron, MN5 (Consoulas et al. 2002), which innervates the dorsal longitudinal flight muscle (DLM) in the adult fly (Fernandes and Keshishian 1998; Ikeda and Koenig 1988). We use a well-described GAL4 driver that restricts expression of transgenes to a subset of motoneurons (Kraft et al. 2006) to express either dominant negative knock-downs for Shaker (Sh) (Mosca et al. 2005) and eag (Broughton et al. 2004) potassium channels, which are involved in A-type potassium currents, or to express a modified constitutively open Sh potassium channel (White et al. 2001). In situ patch-clamp recordings demonstrate that the first manipulation causes significant increases in intrinsic excitability of MN5, whereas the latter causes significantly decreased intrinsic excitability. Quantitative three-dimensional (3D) reconstructions (Evers et al. 2005; Schmitt et al. 2004) of MN5’s dendritic architecture demonstrate that both manipulations cause dendritic overgrowth, but by different mechanisms. Dendrite elongation and dendritic branching can be separated mechanistically and are affected differentially by increased and decreased intrinsic neuronal excitability. Finally, behavioral testing demonstrates that manipulations of excitability in identified subsets of motoneurons affect flight motor behavior.

METHODS

Animals

Drosophila melanogaster flies were kept in standard 68-ml vials with cotton stoppers on a yeast–syrup–cornmeal–agar diet at 25°C and 50–60% humidity with a 12-h light/dark regimen. Flies were used for experiments 1 day after eclosion. All electrophysiological experiments and all morphometric analyses were conducted with female flies. All behavioral experiments were conducted with male flies. All experiments and all animal care procedures were in accordance with the National Institute of Health guidelines. Insects were raised for 2 weeks in the stock culture room (18°C, 80% humidity, 12-h light/dark regimen) and then transferred to the experimental room (25°C, 68% humidity, 12-h light/dark regimen) 1 day before experiments. Flies were kept in 68-ml vials in standard cages (50 flies/cage) and given ad libitum access to a yeast–syrup–cornmeal–agar diet and water. Male flies were used for behavioral experiments to minimize the influence of sex hormones on behavior. Flies were kept bivirginal (i.e., without mating) in the experimental room. For behavioral experiments, flies were used 1 day after eclosion. They were elicted until they showed no behavioral response. For morphometric experiments, flies were used 1 day to 1 week after eclosion. Flies for electrophysiological experiments were used 1 week after eclosion. Flies were sexed under a stereomicroscope to verify that males were used for behavioral experiments and that males and females were used for electrophysiological experiments. Flies were kept on a 12-h light/dark regimen and were kept at 25°C in the dark for at least 16 hours before each experiment. They were transferred to the experimental room 60 min before each experiment and were handled as little as possible during the experiment.

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flies because differences in ovarian load among females might have affected flight motor performance. Various strains were used for the experiments. All experiments were conducted with the C380-GAL4 line that has previously been described and expresses predominantly in motoneurons (Kraft et al. 2006). To visualize GAL4 expression in MN5 and other motoneurons, we used UAS-CD8-GFP as reporter. To avoid expression of GAL4 in interneurons, we used the Cha-GAL80 (choline-acetyl transferase promoter driven GAL4), which has been shown to suppress GAL4 activity in all cholinergic neurons (Aberle et al. 2002). We have received a recombiant C380-GAL4, UAS-CD8-GFP; Cha-GAL80 line from Dr. S. Sanyal (Emory University, Atlanta, GA, C380-GAL4, UAS-CD8-GFP;+;chaGAL80). Potassium membrane currents were genetically manipulated in two different ways. First, we expressed dominant negative transgenes for Sh and for eag potassium channel proteins [UAS-Sh(DN), UAS-eag(DN)], both of which have been reported to inhibit potassium currents. We used single knock-downs for either Sh or eag, or we used a recombiant chromosome named “EKI” (for electrical knock-in), which contains both transgenes (obtained from Dr. S. Sanyal). To decrease intrinsic excitability of MN5, we expressed two copies of the electrical knock-out (EKO) transgene, a modified noninactivating Sh potassium channel (White et al. 2001). All dominant negatives and EKO were expressed heterozygously by crossing the males to female C380-GAL4, UAS-CD8-GFP;+;chaGAL80 flies. As controls, C380-GAL4, UAS-CD8-GFP;+;chaGAL80 females were crossed to wild-type (Berlin wild) males.

Electrophysiology

Female flies were dissected dorsal side up in a silicone elastomer (Sylgard)-coated Petri dish. After removal of the gut and the esophagus, the ventral nerve cord was exposed. The Petri dish was then mounted onto a Zeiss fluorescence microscope, and the recording chamber was superposed with standard solution composed of the following (in mM): 128 NaCl, 2 KCl, 1.8 CaCl₂, 4 MgCl₂, 5 HEPES, and 35.5 sucrose, pH was adjusted with 1 M NaOH to 7.2, osmolarity was 295 mosmol/kg, adjusted with sucrose. Experiments were done at room temperature (∼21°C). The patch pipettes were pulled from filamented glass microelectrodes with a DMZ Universal Puller (Dagan) and fire polished to a resistance of 5 to 7 MΩ. Standard internal solution consisted of (in mM): 140 Kgluconate, 2 MgCl₂, 11 EGTA, 10 HEPES, 2 MgATP, pH was adjusted to 7.2 with 1M KOH, osmolarity was adjusted to 300 mosmol/kg with glucose if necessary. Before performing patch-clamp experiments, a thin sheath lying above the MN5 had to be removed enzymatically with 2% protease in 10 HEPES, 2 MgATP, pH was adjusted to 7.2 with 1M KOH, and the sheath was removed mechanically by applying gentle suction. After proteasing, the preparation was washed for 2 min in standard extracellular recording solution. For our recordings, we used the Axopatch 200B patch-clamp amplifier (Molecular Devices). After obtaining a gigascal, the membrane was clamped to −60 mV. Before going to whole cell configuration, we compensated for pipette capacitance. Series resistance was 8–25 MΩ, series resistance compensation was 42–45%. The prediction was set to 98%, and then we compensated for slow capacitances. The lag was 10 μs. During the recordings the cells were held at −60 mV. After establishing stable conditions, we switched to current-clamp mode. In most experiments, we worked with steady perfusion (2 ml/min) of the recording chamber to avoid the lack of oxygen.

Data acquisition and analysis

Data acquisition and analysis were performed with pClamp 10 (Molecular Devices). Liquid junction potential was calculated and off-line-subtracted. For further analysis, we used Microsoft Excel. Signals were low-pass filtered at 5 kHz, the sampling interval was 10 kHz. Experiments were performed without injecting current to stabilize the resting membrane potential.

Intracellular staining and histology

For intracellular labeling of MN5, thin-walled glass microelectrodes (75–95 MΩ tip resistance) were 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, the dyes were injected iontophotically by a constant depolarizing current of 0.5 nA for 10–12 min. Then the electrode was removed, the ganglia where fixed in 4% paraformaldehyde in phosphate-buffer solution (PBS, 0.1M) for 2 h at room temperature. Ganglia were washed in PBS (0.1M) six times for 15 min each. This was followed by dehydration in an ethanol-series (50, 70, 90, and 2 times 100%, 15 min each). Preparations were treated in a 1:1 mixture of pure ethanol and methyl salicylate for 5 min and cleared in methyl salicylate. This was followed by 5-min treatment in a 1:1 mixture of pure ethanol and methyl salicylate, dehydration in a descending ethanol series, four washes in PBS-triton × (0.5% triton in 0.1 M PBS). This was followed by six washes in PBS (15 min each) and incubation with Cy3-streptavidin (Invitrogen, Karlsruhe, Germany; 1:750). This was followed by 6 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.

Confocal microscopy

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). Cy3 was scanned by using excitation wavelengths of 568 nm (krypton laser), and emission was detected between 580 and 620 nm. By optimizing the sample preparation procedure as described previously (Evers et al. 2005), we can discriminate structures with a diameter of 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 >300 nm diameter.

Geometric reconstructions and quantitative morphometry

Confocal image stacks were further processed with Amira-4.1 software (TGS). For three-dimensional reconstruction of dendritic segments software, plug-ins as published previously (Evers et al. 2005; Schmitt et al. 2004) were used. These deliver precise quantification of midline and diameter as well as a triangulated surface definition fully exploiting optical resolution. 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 (StatSoft, Hamburg, Germany) and Microsoft Excel. ANOVA with Newman Keuls post hoc comparison were used to test for statistical differences among multiple experimental groups, and Student’s t-test was used for comparisons of morphometric parameters between two different genotypes.

The overall structure of MN5 is depicted in Fig. 1C. 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 origi-
nating 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 collapsed
the reconstruction onto one virtual origin. Values referred to as
relative to the collapsed origin, therefore regard the distance or
order on the respective sub-tree up to its insertion into the cell
body-axon link segments. Distances stated as along tree distance
are measured as path length from the 0 order branch of the sub-tree
along the midlines of the reconstruction. Air distance values refer
to the straight line length in three-dimensional space to the 0 order
branch point of the sub-tree.

**Behavioral testing**

Behavioral testing was conducted as previously described (Brembs
et al. 2007). Briefly, 3-day-old male flies were immobilized by cold
anesthesia for 20 s and glued [clear glass adhesive (Duro; Pacer
Technology, Rancho Cucamonga, CA)] with head and thorax to a
triangle-shaped copper hook (0.02 mm diam). Adhesion was achieved
by exposure to UV light for 10 s. The animals were then kept
individually in small chambers containing a few grains of sucrose
until testing (1–5 h). The fly, glued to the hook as described in the
preceding text, was attached to the experimental setup via a clamp to
accomplish stationary flight. For observation, the fly was illuminated
from behind and above (150 W, 15 V; Schott, Elmsford, NY) and fixed
in front of a polystyrene panel. Additionally, it was shielded by another
polystyrene panel from the experimenter. Tarsal contact with a bead of
polystyrene prevented flight initiation before the experiment started. A
digital high-speed camera (1000 pictures per second; Motion Scope;
Redlake Imaging, Morgan Hill, CA) was positioned behind the test
animal. To initiate flight, the fly was gently aspirated. The time until the
fly ceased flying was recorded (initial flight). The fly was aspirated as a
stimulation to fly each time it stopped flying. When no flight reaction was
shown after three consecutive stimulations, the experiment was com-
pleted and the total flight time was recorded (extended flight). Every

**FIG. 1.** Structure and excitability of MN5. A: expression patterns of C380-GAL4: UAS-
mCD8-GFP, Cha-GAL80 in the ventral nerve cord of the adult fly. A shows the thoracic
neuromerexes with expression in the flight motoneurons MN1-5, a ventral unpaired me-
edian (VUM) neuron, and ~10 unidentified neurons in each thoracic hemisegment. The
locations of the somata of MN1-5 are marked by white arrows. Expression of
mCD8-GFP under the control of C380-
GAL4 in the abdominal neuromerexes is de-
picted in Ai. B: the location and overall
dendritic structure of the flight motoneurons
MN1-5 as revealed by selective retrograde
staining from the DLM flight muscle. The
fine structure of MN5 is depicted in C as a
projection view of all confocal optical sec-
tions into 1 image plane. The intracellular
label of MN5 is superimposed by a 3-dimen-
sional dendritic reconstruction in Ci. The run
of the link segment is indicated by 2 white
arrows (see text). The branching structure as
determined by geometric reconstruction is
shown as dendrogram in Cii. Representative
traces from in situ patch clamp recordings
from the soma of MN5 in current-clamp
mode are shown in D. Injecting current into the
soma of MN5 results in a phasic firing
response. The current injection protocol is
shown as inset in D. From the resting mem-
brane potential, 14 current injections of
200-ms duration and with increasing ampi-
tude in 100-pA increments were given. The
larger the amplitude of the injected current,
the shorter is the delay to action potential
initiation. This is shown as selective enlarge-
ment of the time scale in Di, Dii: every 3rd
sweep of the current injection is shown at a
lower time resolution for easier comparison.
To account for some variability as occurring
in the recordings in E, a nonrepresentative
example of a similar current-clamp
experiment is shown. The firing response to
somatic current injection is also phasic but
triplets of spikes occur. F: a representative
element of the existence of a sag potential
on negative somatic current injection.
stimulus after the first one, to which the fly showed a response, was recorded. Each fly was filmed during the first few seconds of flight, and the recordings were saved on a personal computer for later analysis. The person scoring the flight time was unaware of the treatment group of the animal. All animals were included in the study, including those that did not show any flight behavior.

RESULTS

To selectively express GFP, or other transgenes to manipulate potassium currents in a small set of neurons in the ventral nerve cord, including MN5, the C380-GAL4 driver (Budnik et al. 1996; Sanyal et al. 2003) was combined with a choline-acetyl transferase (cha) GAL-80 construct (Kitamoto 2002) to suppress GAL4 activity in all cholinergic neurons. It has previously been described that this construct restricts expression of GAL4 to a subset of motoneurons in the larval CNS (Sanyal et al. 2003). In the adult ventral nerve cord, this restricted GAL4 expression to the flight motoneurons MN1-5, a ventral unpaired median (VUM) neuron, and ~20 unidentified neurons in each thoracic neuromere (Fig. 1A). The flight motoneurons MN1-5 can be uniquely identified by retrograde dye labeling from the DLM flight muscle (Fig. 1B). The locations of MN1-5 within the pro- and the mesothoracic neuromere and the gross structure of these neurons have previously been described (Consoulas et al. 2002). In particular, the large dorsal cell body of MN5 makes it readily identifiable. Taken together, these results demonstrate that C380-GAL4; UAS-mCD8-GFP, Cha-GAL80 can be used to uniquely identify MN5 for patch-clamp recordings and for driving other transgenes in a subset of ventral nerve cord neurons. An overview of the structure of MN5 as revealed by intracellular dye filling and confocal microscopy is depicted in Fig. 1C. The axon of MN5 projects through the dorsal mesothoracic nerve (PDMN) (Power 1948) onto the DLM flight muscle (Ikeda and Koenig 1988; Trimarchi and Schneiderman 1994). The soma is located on the contralateral side of the dorsal mesothoracic neuromere (Fig. 1, B and C) (Consoulas et al. 2002). Many dendrites branch off the large primary neurite which connects the soma and the axon of MN5 (see arrow in Fig. 1C). Because MN5 is a monopolar cell, it is not clear which parts of the primary neurite are axonal or dendritic (Fig. 1C). For the purpose of morphometric analysis, we define the entire primary neurite between the soma and the site where the last dendrite branches off the primary neurite as link segment. To analyze dendritic architecture, 3D geometric reconstructions of the central structure of MN5 were made from confocal image stacks (Fig. 1Ci) (for methods, see Evers et al. 2005). The length of the link segment is indicated by two white arrows in Fig. 1Ci. Due to the fact that all dendrites of MN5 originate from the link segment, the integrative zone for synaptic input might spread out from the cell body to the origin of the axon. To account for this feature in our morphometric analysis, we defined all dendrites branching off the link segment as a first-order branch. To do this, we virtually eliminated the link segment and treated it as a zero-order branch. The dendritic tree branching diagram with the link segment defined as a zero-order branch, all dendrites branching off the link segment as first-order branches, and all dendrites branching off a first-order branch as a second-order branch, and so on is shown in Fig. 1Cii.

In situ current-clamp recordings from the soma of MN5 with standard intra- and extracellular solutions (see METHODS) reveal that in control flies (C380-GAL4; UAS-mCD8-GFP, Cha-GAL80), MN5 has a resting membrane potential of $-56.1 \pm 5.1$ mV and an input resistance of $97 \pm 31$ MΩ and shows phasic spiking responses on current injections into its soma (Fig. 1D). The spiking threshold is $-19.7 \pm 10.9$ mV. Consequently, $-500$- to $600$-pA current injections into the soma are necessary to elicit an action potential (Fig. 1D), and the delay between current injection and spike initiation decreases with increased current injection amplitudes (Fig. 1D, i and ii). At large current injection amplitudes, additional action potentials can be induced, but MN5 does usually not fire tonically upon current injection. There is some variability in the excitability of MN5 in control flies, so that some recordings from MN5 showed short bursts of several spikes on current injection (Fig. 1E). A representative control recording is depicted in Fig. 1D. Injecting negative current into the soma of MN5 revealed a sag potential occurring at membrane potentials more hyperpolarized than $-100$ mV (Fig. 1F). Rebound spikes were usually not observed, but sag potentials decreased the threshold for spike initiation by positive current injections following hyperpolarization of the cell.

We used the C380-Gal4; ChaGal80 line to drive transgenic modifiers of neural activity in the GFP-labeled MN5. C380 expressed in MN5 only during the second half of pupal development. We intended to increase intrinsic activity of MN5 during this period of postembryonic development in vivo by expressing dominant negative transgenes for Sh and for eag potassium channel proteins [UAS-Sh(DN), UAS-eag(DN)] both of which have been reported to inhibit potassium currents. We used either single knock-downs for either Sh or eag, or a recombinant chromosome, EKI, which contains both transgenes. In Sh eag double mutants, motoneuron activity and axonal terminal branching over larval muscles are increased (Budnik et al. 1990). However, it was not clear whether adult motoneuron excitability could be altered in vivo by the expression of dominant negative transgenes for these potassium channels. In principle, knock-downs of Sh and eag potassium currents should increase a neuron’s excitability because the amplitude of voltage activated outward currents should be reduced. To decrease intrinsic excitability of MN5, we expressed two copies of the EKO (electrical knock-out) transgene, a modified noninactivating Sh potassium channel (White et al. 2001). The rationale is that depolarization-induced activation of the EKO channels should activate outward potassium currents that do not inactivate and therefore shunt depolarization.

In situ patch-clamp recordings in current-clamp mode from the adult MN5 demonstrate that its intrinsic excitability is significantly altered by expressing transgenes affecting potassium conductances. Under control conditions, MN5 shows a phasic firing response to current injections into the soma (Figs. 1D and 2A). Driving the expression of a dominant negative for eag potassium channel subunits slightly increases the excitability of MN5 (Fig. 2B). The firing response is still phasic, but more spikes occur in response to a current injection of defined amplitude although the amplitudes of the additional spikes are smaller (Fig. 2B). Expression of EKI transforms MN5 from a phasic into a tonic firer (Fig. 2C). In addition the action potential amplitude seems to be increased, but this was not
further quantified in this study. Expression of two copies of EKO transformed MN5 from a phasic firer into a nonfirer (Fig. 2D). The voltage response still showed a small peak during the first 5 ms of the current injection, but action potentials occurred in very few preparations and only as a response to large-amplitude current injections (1 nA). To account for variability in the excitability of MN5, the firing responses to current injection were divided into five classes, cells that showed no active response to current injection, cells that showed a graded peak that increased in amplitude with increased current injection amplitudes but showed no action potentials, cells that responded with one action potential to current injections of 1-nA amplitude, cells that responded with phasic spiking to current injections of 1-nA amplitude, and cells that responded with tonic firing to 1-nA current injection. For each genotype, each of these five responses was plotted as a percentage from the total number of recordings (Fig. 2E). This clearly demonstrated in a quantitative manner that EKO strongly decreased excitability, that eag single knock-downs slightly increased excitability, and that EKI double knock-down strongly increased excitability over controls (Fig. 2E). Therefore the genetic manipulations analyzed in this study can be used to test for possible effects of altered intrinsic excitability on motor behavior and also on dendritic growth. However, resting mem-

**FIG. 2.** Intrinsic in vivo excitability of MN5 is altered by genetic manipulations of potassium channels. Representative examples of spiking responses to current injected into the soma of MN5 as determined by in situ patch-clamp recordings are depicted for 4 different genotypes in A–D. Selective enlargements of the onset of the voltage response to 1.4-nA current injection are shown in Ai–Di. The typical phasic firing responses of MN5 from heterozygous control animals is shown in A. Dominant negative eag potassium channel knock-down increases the number of spikes caused by current injections of a given amplitude, but the firing patterns are still phasic, and the amplitude of additional spikes becomes increasingly smaller (B). Dominant negative double knock-down of eag and Sh causes a tonic firing response (C). Expression of 2 copies of electrical knock-out (EKO) causes an absence of action potentials as response to somatic current injection. To account quantitatively for variability in the firing responses within each genotype, the firing responses were classified in 5 types (no response, graded peak, single spike, phasic firing, and tonic firing). The percentage of recordings falling into each of these categories is plotted for each of the 4 genotypes (E). F: the average resting membrane potential ($V_m$) and the average input resistance for MN5 from controls, double knock-downs for eag and Sh, and from MN5 expressing 2 copies of EKO. Error bars represent SD. No statistical differences are found (ANOVA).
brane potential or input resistance as measured by whole cell patch-clamp recordings from the soma of MN5 were not affected by expression of EKO or EKI in MN5 (Fig. 2F). We never observed spontaneous spiking in any of the genotypes investigated in this study (data not shown). In addition, only few spontaneously occurring postsynaptic potentials of small amplitude (1–3 mV) were observed in somatic whole cell current-clamp recordings, and we found no indications for altered synaptic input following targeted manipulations of potassium membrane currents. However, we did test whether synaptic drive to MN5 might be altered by stimulating neurons involved in shaping flight motor patterns, such as wing sensory cells.

To test whether genetic manipulation of excitability in a subset of motoneurons under the control of the C380-GAL80, Cha-GAL80 driver (see Fig. 1A for overview of expression patterns in ventral nerve cord) affected motor output, the flies were subjected to behavioral testing in a restrained flight assay. Flies were glued to a hook and attached to the experimental setup via a clamp to accomplish stationary flight (Fig. 3A). Tarsal contact with a bead of polystyrene prevented flight initiation before the experiment started. To initiate flight, the styrofoam ball was removed, and the fly was gently aspirated. The time until the fly first ceased flying was recorded (initial flight). Each time the fly stopped, it was aspirated as a stimulation to fly. When no flight reaction occurred after three consecutive stimulations, the experiment was completed and the total flight time was recorded (total flight). Targeted manipulation of excitability strongly affected the likelihood to fly. When no flight reaction occurred after three manipulations of the flight reaction, see Fig. 2D). The analyses clearly demonstrated that both increased (eag and Sh double knock-downs) and decreased intrinsic excitability (EKO expression, see Fig. 2D) cause significant increases in total dendritic length (Fig. 5A). However, overgrowth is significantly larger in eag and Sh double knock-downs as compared with MN5 expressing EKO (Fig. 5A). Although increased and decreased intrinsic excitability both cause increases in total dendritic length (TDL), each genetic manipulation affects different aspects of dendritic growth. The number of dendritic branches is significantly increased in the hyperexcitable MN5 (Fig. 5B), whereas it is not significantly altered in the hypoxi-
citabile MN5 (Fig. 5B). By contrast, in the hypoexcitable MN5 the mean length of the individual dendritic branches (mean dendritic length, MDL) is significantly longer than in control or in hyperexcitable neurons (Fig. 5C). This clearly demonstrates that dendritic segment growth and dendritic branching can be regulated separately and that both are affected differentially by different genetic manipulations of intrinsic motoneuron excitability. However, there is also a slight but statistically significant increase in MDL in hyperexcitable potassium channel double knock-downs (Fig. 5C), indicating that this manipulation induces not only the formation of more branches but also causes an increase in the average length of the individual branches. Neither the addition of many new dendritic branches in eag and Sh double knock-downs nor the slight reduction in dendritic branches in the hypoexcitable MN5 (Fig. 5B) cause significant changes in the maximum branch order as compared with controls (Fig. 5D). This demonstrates that both increases and decreases in subsequent branch formation occur mostly at parts of the dendritic tree that do not reach maximum branch order in controls.

Both types of dendritic overgrowth, hyperexcitability-mediated increases in branch formation and also hypoexcitability-mediated increases in MDL, lead to significant increases in the total space occupied by MN5 dendrites. The mean distance of all dendrites to the origin (Fig. 5E) and also the maximum path length are significantly longer (not shown) in both manipulations as compared with controls. Both hyper- and hypoexcitability-mediated dendritic overgrowth lead to increased dendritic surface (Fig. 5F), and, therefore also increase space for potential input synapses. Despite the finding that hyperexcitability mediated overgrowth causes significantly more total dendritic length than hypoexcitability-mediated increases in MDL, the resulting total dendritic surface does not differ between both genotypes (Fig. 5F). This is because dendritic radii are also affected by genetically induced changes in motoneuron excitability (see following text).

Branch order analysis was conducted to test whether the addition of dendritic branches in hyperexcitable motoneurons (Fig. 5B), the increase in the mean dendritic segment length in hypoexcitable motoneurons (Fig. 5C), or changes in the radii occurred in specific parts of the dendritic tree. For branch order analysis, every dendrite branching off the primary neurite (which was defined as origin, see METHODS) was defined as a first-order branch. Every dendrite branching off a first-order dendrite was defined as a second-order dendrite, and so on. Plotting the number of dendrites as a function of their branching order reveals that both excitability manipulations, potassium channel knock-down and expression of EKO, cause a significant reduction in the number of low order branches as compared with controls (Fig. 6A, branch orders 1–10, ANOVA, \( P \leq 0.05 \)). Hyperexcitability causes significant overgrowth of dendrites in all branch orders between 15 and 40 (ANOVA, \( P \leq 0.05 \)), whereas hypoexcitability causes a significant reduction (ANOVA, \( P \leq 0.05 \)) in the number of branches in these orders (Fig. 6A). Within the branch orders 15–44 hyperexcitability causes approximately the same magnitude of...
branch addition as the reduction in the number of branches caused by hypoexcitability (Fig. 6A). These data show that the initial formation of lower order branches is slightly impaired by both genetic manipulations, but for the formation of higher order branches opposing manipulations of intrinsic activity have opposite effects on new branch formation.

Plotting the MDL over their branching order clearly shows that hyperexcitability causes a slight but significant increase in MDL (ANOVA, *P* < 0.05), but hypoexcitability causes a larger increase of MDL throughout all branch orders (Fig. 6B). This demonstrates that EKO expression affects the elongation of individual dendritic branches during all phases of branch formation during pupal life.

As demonstrated by current-clamp recordings from the soma of MN5 (see Fig. 2) genetic manipulation of potassium channels under the control of the C380 Gal4 driver cause significant changes in intrinsic excitability, which cannot be compensated for in vivo by homeostatic mechanisms which have been described in various systems including cultured *Drosophila* neurons (see Discussion). However, we have not tested whether synaptic homeostatic mechanisms counteract the altered intrinsic excitability in our experiments, nor have we tested whether the expression of some other membrane currents was altered in MN5 with manipulated potassium currents. In any case, intrinsic excitability as determined by the firing responses to somatic current injections was clearly altered in MN5 with manipulated potassium conductances. This in turn caused significant alterations of distinct aspects of dendritic growth, depending on whether MN5 was manipulated to have increased or decreased intrinsic excitability. An additional possibility to compensate for altered excitability might be to change the diameter of dendrites. Thicker dendrites possess a lower inner resistance for passively conducted electrical signals, and thus the length constant, lambda, should be increased in dendrites with a larger diameter. Vice versa, lambda should be decreased in thinner diameter dendrites. However, potassium channel knock-down did not cause any significant differences in the radii of the dendrites as compared with control (data not shown). By contrast, expression of EKO,

![Figure 5](http://jn.physiology.org/) Quantitative comparison of the dendritic structure of MN5 from control, hyperexcitable (eagSDN knock-down) and hypoexcitable (expression EKO) MN5. The total dendritic length (TDL) from all 3 genotypes is shown in A. The number of branch points is depicted in B. Mean dendritic length of all dendritic segments is shown in C, and the mean maximum branch order for each genotype is depicted in D. The mean distance of all dendritic segments to the origin of the tree is depicted in E and mean surface of all 3 genotypes is shown in F. Green bars are control, dark blue bars represent eagSDN, and light blue bars are EKO. Error bars represent SD. The number of animals for eagSDN and EKO is 5 each, and the number of control animals is 7. Statistical significance is indicated by asterisks; * = *P* < 0.05, ** = *P* < 0.001.
which caused hypoexcitability, significantly increased the mean radii of dendrites in all branch orders larger than 5 (Fig. 7, ANOVA, $P < 0.05$). This might in principle cause an increased passive conductance of postsynaptic potentials (PSPs) along the dendritic field to the origin where the spike is generated. However, at present we have no further evidence as to whether PSPs from the same sites are larger in MN5 with EKO expression and whether this might be a compensatory mechanism for decreased intrinsic excitability.

**DISCUSSION**

**Genetic manipulations of potassium membrane channels alter motoneuron excitability in vivo**

Expression of dominant negatives for either eag, or Sh, or both potassium channels under the control of the C380 driver clearly changes the intrinsic excitability of MN5 in vivo as demonstrated by altered responses to current injection into the soma. Homeostatic compensation has been demonstrated for genetically altered excitability (Marder and Goaillard 2006). Even though such mechanisms may act during postembryonic CNS development in *Drosophila*, our data show that genetic manipulations of potassium currents are not fully compensated. Thus the excitability of selected central neurons can be manipulated by targeted expression of transgenes. By contrast, resting membrane potential and input resistance as measured from the soma of MN5 are not altered by these manipulations. In cultured division-arrested neuroblasts from *Drosophila* knock-out of the calcium channel $\alpha$ subunit, cacophony (cac), results in homeostatic upregulation of voltage activated potassium channels, such as Sh (Peng and Wu 2007). However, cac current expression is unaltered in various potassium channel mutants (Peng and Wu 2007). Similarly, at the *Drosophila* neuromuscular junction, endogenous neural activity is increased in eag/Sh double mutants (Budnik et al. 1990). In the adult fly, we find on the behavioral level that the likelihood to fly and flight durations are significantly increased in eag/Sh knockdowns. Taken together, these data indicate that potassium channel manipulations in *Drosophila* motoneurons are not compensated adequately by up- or downregulation of other ion channels.

**Dendritic diameters might be a mean to compensate for altered intrinsic excitability**

Although on the level of intrinsic excitability we have not found evidence for compensatory mechanisms counter-acting...
Postembryonic dendritic remodeling and dendritic growth during insect metamorphosis have mainly been attributed to the action of ecdysteroids. Studies in Manduca and in Drosophila have shown that dendritic pruning is triggered by ecdysteroids (Schubiger et al. 1998; Streichert and Weeks 1995; Truman et al. 1994). In addition, ecdysteroids promote outgrowth of cultured motoneurons (Matheson and Levine 1998), and the dendritic shape of MN5 in Drosophila is affected by mutations in early ecdysteroid response genes (Consoulas et al. 2005). However, activity has been reported to act in concert with steroid-induced dendritic remodeling in Manduca (Duch and Mentel 2004). Our data clearly indicate that intrinsic excitability has significant effects on in vivo motoneuron postembryonic dendritic growth in Drosophila. An alternative possibility is that expression of dominant negatives for eag and Sh as well as EKO might cause protein-protein interactions, which in turn might affect dendritic growth. The eag channel is known to interact with CaMKII (Sun et al. 2004), and CaMKII can affect dendritic pruning (Schubiger et al. 1998; Streichert and Weeks 1995). Furthermore, within individual neurons, the detailed branching patterns of dendritic arbors can be regulated by calcium signals triggered by strictly local synaptic input (Lohmann et al. 2002; Niell et al. 2004), whereas intrinsic spiking activity may affect global calcium
concentrations that control dendritic properties by regulating transcription (West et al. 2002). The manipulations used in this study clearly affect the intrinsic excitability of MN5, but it remains unclear at this point whether this also causes different local responses to synaptic input. However, we do not find different amounts or different types of dendritic growth in different dendritic regions of MN5. Our geometric reconstructions of the entire dendritic tree allow characteristic parts of the dendritic tree, that are located in different neuropil regions to be compared. MDL, dendritic diameters, and branching frequencies were not affected differentially in specific parts of the dendritic field only (data not shown). Therefore, the most parsimonious explanation for our results is that altered excitability causes different amounts of intrinsic spiking activity during normal development in vivo, and this in turn affects dendritic growth as a global signal throughout the neuron. Global calcium signals are thought to affect dendritic growth via transcriptional regulation (Redmond and Ghosh 2005). Activity-dependent calcium influx preferentially activates calcium/calmodulin-dependent protein kinase (CaMK) and the Ras/mitogen-activated protein kinase (Ras/MAPK) pathways. Both pathways can regulate gene transcription via phosphorylation of cAMP response element binding protein (CREB). However, a dependence of activity-dependent dendritic growth during postembryonic Drosophila CNS development had not previously been demonstrated.

Dendrite elongation and branch formation are affected separately by different manipulations of intrinsic activity

The most striking finding of this study is that both increased and decreased intrinsic excitability of the same identified motoneuron cause dendritic overgrowth by different mechanisms in vivo. Increased excitability causes increased branch formation, whereas decreased intrinsic excitability causes increased dendritic branch elongation. This demonstrates that dendritic branch elongation and dendritic branch formation are mechanistically separable and that both are differentially affected by different kinds of intrinsic neuronal excitability. Therefore different neuronal activity patterns must be translated onto different intracellular signaling pathways. How can this be accomplished? As stated in the preceding text, intrinsic neuronal spiking patterns are most likely reflected by different global calcium signals (Redmond and Ghosh 2005). Different pathways of calcium entry can address distinct transcriptional events. Calcium entry via L-type calcium channels and calcium influx via ligand-gated ion channels contribute to different responses of CREB-mediated transcription (Hardingham et al. 1999; Hu et al. 1999). Furthermore, different degrees of calcium-dependent CaMK activation stimulate a CREB phosphatase (Bito et al. 1996), which in turn, may cause different CREB-induced transcriptional events, depending on the amount of activity-induced calcium entry. The mechanisms by which different types of intrinsic motoneuron excitability may be decoded by various intracellular signals to mediate different aspects of dendritic growth remain speculative. The genetic tools available in the Drosophila system bear the potential to unravel these mechanisms in the future.

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