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, 85287 AZ, USA

Running title: Intrinsic excitability and dendritic growth

With 28 text pages and 7 figures
Number of words: Abstract: 148
                 Introduction: 500
                 Discussion: 1360

Key words: activity, dendrite, CNS, insect, potassium currents, dendritic growth

Corresponding author: C. Duch at the above address
Email: Carsten.duch@asu.edu
Fax: 1 480 727 9440
Tel: 1 480 727 9442
Abstract

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 non-inactivating potassium channel decrease intrinsic excitability \textit{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 which can be addressed selectively \textit{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 (Montague and Friedlander 1989; 1991; Spatkowski and Schilling 2003; Gao and Bogert 2003; Mizrahi et al. 2000; Scott et al. 2002; 2003), external molecular cues (Kim and Chiba 2004; Landgraf and Thor 2006), humoral cues (Toran-Allerand et al. 1999; Weeks and Levine 1995; Cooke and Wooley 2005) and by neural activity (Duch and Mentel 2004; Libersat and Duch 2004; Wong and Gosh 2002). However, the degree to which neural activity affects developing dendrites varies considerably between different species, different types of neurons and different times during development (Libersat 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 at 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 in the adult fly (Ikeda and Koenig 1988; Fernandes and Keshishian 1998). We use a well described GAL4 driver that restricts expression of transgenes to a sub-set 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 3-D reconstructions (Schmitt et al. 2004; Evers et al. 2005) 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 sub-sets 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 d after eclosion. All electrophysiological experiments and all morphometric analysis were conducted with female flies. All behavioral experiments were conducted with male 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 Gal80), which has been shown to suppress GAL4 activity in all cholinergic neurons (Aberle et al. 2002). We have received a recombinant C380-GAL4, UAS-CD8-GFP; Cha-GAL80 line from Dr. S Sanyal (Emory University, Atlanta, Georgia, 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 recombinant 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 EKO (electrical knock-out) transgene, a modified non-inactivating 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 wildtype (Berlin wild) males.

Electrophysiology

Female flies were dissected dorsal side up in a 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 superfused with standard solution composed of the following (in mM): 128 NaCl, 2 KCl, 1.8 CaCl2, 4 MgCl2, 5 HEPES,
35.5 sucrose, pH was adjusted with 1M NaOH to 7.2, osmolality 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, osmolality 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 standard solution. The enzyme was filled into a patch pipette with about 1 MΩ tip resistance. Protease was applied focally over the cell body of MN5 and the sheath was removed mechanically by applying gentle suction. After proteasing the preparation was washed for 2 minutes in standard extracellular recording solution. For our recordings we used the Axopatch 200B patch clamp amplifier (Molecular Devices). After obtaining a gigaseal 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 10μs. 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 (2ml/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 offline-subtracted. For further analysis we used Microsoft Excel. Signals were lowpass 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 microelectrode (75-95 MΩ tip resistance) were filled with a mixture of 7% Neurobiotin (Linaris GmbH, Wertheim-Bettingen, Germany) and Rhodamin-Dextran (Invitrogen, Carlsbad, CA, USA) 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
iontophoretically by a constant depolarizing current of 0.5 nA for 10 to 12 minutes. 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 minutes each. This was followed by dehydration in an ethanol-series (50, 70, 90, and 2 times 100 %, 15 minutes each). Preparations were treated in a 1:1 mixture of pure ethanol and methyl salicylate for 5 minutes and cleared in methyl salicylate. This was followed by 5 minutes treatment in a 1:1 mixture of pure ethanol and methyl salicylate, rehydration in a descending ethanol series, 4 washes in PBS-triton x (0.5 % triton in 0.1 M PBS). This was followed by 6 washes in PBS (15 minutes 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 above), 5 minutes 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 40x oil immersion objective (numerical aperture 1.25). Cy2 was scanned by using excitation wavelengths of 488 nm (argon laser), and emission was detected between 495 and 530 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 above 300 nm diameter.

Geometric reconstructions and quantitative morphometry

Confocal image stacks were further processed with Amira-4.1 software (TGS). For 3-dimensional reconstruction of dendritic segments software plugins as published previously (Schmitt et al., 2004; Evers et al., 2005) 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 (StatSof, 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 figure 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 originating from the primary neurite as 1st 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 3-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, three days old male flies were immobilized by cold anesthesia for 20 seconds and glued [clear glass adhesive (Duro; Pacer Technology, Rancho Cucamonga, CA)] with head and thorax to a triangle-shaped copper hook (0.02 mm diameter). Adhesion was achieved by exposure to UV light for 10 s. The animals are 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 above, 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 completed and the total flight
time was recorded (extended flight). Every 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 (Sanyal et al. 2003; Budnik et al. 1996) was combined with a choline-acetyl transferase (cha) GAL-80 construct (Kitamato 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 about 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 figure 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 (Figs 1B, 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 figure 1C). Since 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, 3 dimensional geometric reconstructions of the central structure of MN5 were made from confocal image stacks (Fig 1 Ci, for methods see Evers et al. 2005). The length of the link segment is indicated by two white arrows in figure 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 0 order branch. The dendritic tree branching diagram with the link segment defined as a 0 order branch, all
dendrites branching off the link segment as 1st order branches, and all dendrites branching off a 1st order branch as a 2nd order branches, and so on is shown in figure 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 upon current injections into its soma (Fig 1D). The spiking threshold is $-19.7 \pm 10.9$ mV. Consequently, about 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 (Figs 1Di, Dii). 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 upon current injection (Fig 1E). A representative control recording is depicted in figure 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 named ‘EKI’ (for electrical knock-in) 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 non-inactivating Sh potassium channel (White et al.
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, 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 non-firer (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 (> 1nA). To account for variability in the excitability of MN5, the firing responses to current injection were divided into 5 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 1nA amplitude, and cells that responded with tonic firing to 1 nA current injection. For each genotype, each of these 5 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 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 membrane 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 to 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 sub-set of motoneurons under the control of the C380-GAL80, Cha-GAL80 driver (see figure 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 and the flight duration. 51 % of all control animals responded with flight behavior to a wind stimulus (Fig 3B). In double knock-downs for eag and Sh potassium channels 92% of all flies showed flight behavior, whereas EKO expression reduced the percentage of flyers to 23% (Fig 3B). Moreover, both the initial and the total flight time were significantly increased in eag and Sh double knock-downs, but significantly decreased by expression of EKO (Figs 3C, D). Consequently, flight motor performance was influences in a manner consistent with alterations of intrinsic motoneuron excitability and/or the resulting changes in dendritic morphology.

Overviews of representative examples of the dendritic structure of MN5 as occurring in all genotypes tested in this study are depicted in figure 4. All images are projections of all optical sections into one focal plane using the maximum intensity projection method to enable the viewer to get a comprehensive overview of the overall branching structure. Qualitatively, all genotypes tested exhibit the characteristic overall branching structure of the dendritic field of MN5 within its wildtype-like neuropil borders. Therefore, hyper- or hypoexcitability do not cause growth into completely different neuropil areas as compared to controls. In addition, characteristic major dendritic branches can be identified in all genotypes tested. However, overview images also show that both, hypo- and hyperexcitability cause dendritic overgrowth. In fact, eag/Sh double knock downs cause an increase in total dendritic length of more than 25 percent or 1500 µm (Fig 5A). In complex dendritic trees as in MN5 even 1 to 2 mm of dendritic overgrowth can hardly be seen on overview images, which shows the importance of thorough quantification. In order to analyze
quantitatively which aspects of dendritic architecture are affected by increased versus decreased intrinsic excitability, 5 representative intracellular stainings for each genotype were reconstructed in 3 dimensions from confocal images stacks (see overlays in figures 4Ai to Di). All geometric reconstructions were conducted with recently developed methods which have proven highly accurate (Schmitt et al., 2004; Evers et al., 2005). These reconstructions allow for an accurate quantitative comparison of dendritic structure from different genotypes. Dendrograms depicting the branching structures are shown in figures 4 Aii to Dii. This analysis clearly demonstrated that both increased (eag and Sh double knock down, see Fig 2C) 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 to 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 hypoexcitable 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 to controls (Fig 5D). This demonstrates that both increases and decreases in subsequent branch formation occur mostly at parts of the dendritic tree which 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 to controls. Both hyperexcitability 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 below).

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 to 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 to 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 mean length of all dendrites (MDL) over their branching order clearly shows that hyperexcitability causes a slight but significant increase in MDL (ANOVA, $p \leq 0.05$) 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 figure 2) genetic manipulation of potassium channels under the control of the C380 Gal4 driver cause significant changes in intrinsic excitability, which can not 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 to control (data not shown). By contrast, expression of EKO, which caused hypoexcitability, significantly increased the mean radii of dendrites in all branch orders larger than 5 (Fig 7, ANOVA, p \leq 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 up-regulation 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 knock downs. Taken together, these data indicate that potassium channel manipulations in Drosophila motoneurons are not compensated adequately by up- or down-regulation 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 genetic manipulations of potassium channels, EKO expressing motoneurons show an increase in mean dendritic diameter by approximately 15%. According to neuronal cable theory this should reduce attenuation of postsynaptic potentials along the run of the dendrites, and, therefore, increase synaptically induced excitability. At present we have no further evidence that PSPs from a given dendritic site show larger amplitudes at the spike initiating zone of MN5, but our data suggest that in addition ion channel and synaptic strength regulation (Marder and Goaillard 2006), dendritic geometry is a potential additional mechanism of excitability homeostasis.
Intrinsic excitability affects dendritic growth

Genetically altered intrinsic excitability of MN5 significantly affects dendritic shape. However, the characteristic shape of MN5's dendritic field and its shape and overall boundaries remain unaltered. Major dendritic sub-trees can be identified in all potassium channel manipulations investigated in this study and no abnormal dendritic projections into different neuropil areas are observed. This agrees to the general idea that size and shape of a dendritic arbor are mainly determined by combined actions of intrinsic genetic signals, neurotrophins, guidance cues and dendo-dendritic interactions (Parrish et al. 2007). In cultured pyramidal neurons, the transcription factor neurogenin 2 is sufficient to establish the characteristic pyramidal cell morphology (Hand et al. 2005), and in Drosophila sensory neurons the relative levels of the factors cut, abrupt and spineless determine neuronal class-specific dendritic architecture (Grueber et al. 2003; Kim et al. 2006). In addition neurotrophins can guide developing dendrites (McAllister et al., 1997), and other guidance cues (Kim and Chiba 2004) have been demonstrated to establish dendritic architecture during the development of invertebrate and vertebrate neurons. Following genetic programs neural activity has been reported to play a major role in dendritic shape refining, remodeling and fine tuning (Parrish et al. 2007; Lohman and Wong 2005; Cline 2001; Wong and Gosh 2002; Libersat and Duch 2004).

Postembryonic dendritic remodeling and dendritic growth during insect metamorphosis has 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 growth in multiple systems (Cline, 2001). However, all three genetic manipulations of potassium membrane currents clearly caused changes in MN5 excitability, and all of them affected dendritic growth. It
seems unlikely that dominant negatives for Eag and Sh as well as EKO all cause changes in excitability, and that all of them also affect dendritic growth by some other mechanisms. Therefore, the most parsimonious explanation is that dendritic growth is affected by changes in MN5 excitability as induced by targeted manipulations of potassium membrane currents.

In general, two forms of activity may affect dendritic architecture. First, experience-dependent activity transmitted synaptically by afferent neurons or efference copies (Zhang et al. 2000), and second, endogenous activity in developing circuits that is independent of sensory input or motor output (Weliky and Katz 1999; Feller 1999). 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 Gosh, 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 \textit{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 above, intrinsic neuronal spiking patterns are most likely reflected by different global calcium signals (Redmond and Gosh 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 (Hu et al. 1999; Hardingham 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.

\textbf{Acknowledgements}

We are grateful to Dr. RB Levine (University of Arizona, Tucson, AZ, USA) for help in obtaining fly strains used in this study, and also for many fruitful discussions during the course of the experiments. We thank Drs R.B. Levine and C. Consoulas for many helpful comments on the manuscript. This work was supported by grants of the German Research Foundation (DFG, Du 331/5-1 to C. Duch and Graduate College 837 to S. Ryglewski and C. Duch), by funds from Arizona State University to C. Duch and S. Ryglewski. F. Vonhoff was supported by a fellowship of the Interdisciplinary Neuroscience Graduate Program at Arizona State University.
References


Figure legends

Figure 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 neuromeres with expression in the flight motoneurons MN1-5, a ventral unpaired median (VUM) neuron, and about 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 neuromeres is depicted in (Ai). (B) shows 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 sections into one image plane. The intracellular label of MN5 is superimposed by a 3-dimensional dendritic reconstruction in (Ci). The run of the link segment is indicated by two 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 membrane potential 14 current injections of 200 ms duration and with increasing amplitude 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 enlargement of the time scale in (Di). (Dii) Every third 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 non-representative extreme 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) shows a representative example of the existence of a sag potential upon negative somatic current injection.

Figure 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 to D). Selective enlargements of the onset of the voltage response to 1.4 nA current injection are shown
in (Ai to 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 two copies of 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 four genotypes (E). (F) shows 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 two copies of EKO. Error bars represent standard deviation. No statistical differences are found (ANOVA).

Figure 3. *Genetic manipulations affect flight motor performance.*

(A) Time lapse images of restrained flight behavior show subsequent images of one wing beat filmed at 1000 frames per second. (B) shows the percentage of flies responding with flight motor behavior after tarsal contact with the substrate is terminated and a wind stimulus is applied to the animals head. (C) depicts the median and upper and lower quartiles of the flight duration to the first stop (initial flight. (D) shows the median and upper and lower quartiles of the total flight duration until the flies stopped responding with flight behavior to three consecutive wind stimuli. Green bars are control (n = 37), dark blue bars represent eagSDN (n = 38), and light blue bars are EKO (n = 25). Asterisks indicate statistical significance. Mann and Whitney U-test, p < 0.05.

Figure 4. *Genetic manipulations of potassium membrane currents do not affect overall but detailed dendritic branching structure*

The left column (A to D) shows representative intracellular stainings of MN5 from different genotypes. For visualization of all dendrites in one image, all optical sections from confocal image stacks are projected into one image plane using the maximum intensity method (A = control; B = eagSDN double knock-down; C = eagDN knock-down; D = expression of EKO). In (Ai to Di) the projection vies are overlaid with images of geometric reconstructions from
confocal image stacks. In (Aii to Dii) the branching diagrams (dendrograms) resulting from geometric reconstructions are shown.

Figure 5. *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 three genotypes is shown in (F). Green bars are control, dark blue bars represent eagSDN, and light blue bars are EKO. Error bars represent standard deviation. 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.

Figure 6. *Branch order analysis of the dendritic structure of MN5 from control, hyperexcitable (eagSDN knock-down) and hypoexcitable (expression EKO) MN5*

(A) shows the mean number of dendritic branches (y-axis) per branch order. (B) shows the mean dendritic branch length per branch order. Green bars are control, dark blue bars represent eagSDN, and light blue bars are EKO. The number of animals for eagSDN and EKO is 5 each, and the number of control animals is 7.

Figure 7. *Dendritic diameters are altered in MN5 with EKO mediated decreased intrinsic excitability.*

Mean dendritic diameters (y-axis) are plotted as a function of branching order. Green bars are control and light blue bars represent EKO. Error bars represent standard deviations. Data are from 7 control and 5 EKO reconstructions.
figure 1
A. Control

B. eag knock down (eagDN)

C. eag and shaker double knock down (eagSDN)

D. 2 x EKO

E. Firing patterns upon current injection

F. V_m and R_input

Figure 2
figure 3
figure 5
**Figure 6**

**A**

Number of branches per order

- Control
- eagSDN
- 2 x EKO

**B**

Mean dendritic branch length per order

Branch order
mean radii per order

[µm]

branch order

control

2 x EKO

figure 7