Morphological Plasticity of Motor Axons in *Drosophila* Mutants with Altered Excitability

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An anatomical and electrophysiological study of *Drosophila* mutants has been made to determine the effect of altered electrical activity on the development and maintenance of larval neuromuscular junctions. We examined motor axon terminals of (1) hyperexcitable mutants *Shaker* (*Sh*), *ether a go-go* (*eag*), *Hyperkinetic* (*Hk*), and *Duplication of para⁺* (*Dp para⁺*); and (2) mutants with reduced excitability, *no action potential* (*nap^ts*) and *paralytic* (*para^{ts1}*).

Nerve terminals innervating larval body-wall muscles were visualized by using anti-HRP immunocytochemistry, which specifically stains neurons in insect species. In wild-type larvae, motor axon terminals were distributed in a sterotypic fashion. However, in combinations of *eag* and *Sh* alleles, the basic pattern of innervation was altered. There was an increase in both the number of higher-order axonal branches over the muscles and the number of varicosities on the neurites. A similar phenomenon was found in the double mutant *Hk eag* and, to a lesser extent, in *Dp para⁺* and *Dp para⁺ Sh* mutants. It is known that at permissive temperature the *nap^ts*, but not *para^{ts1}*, mutation decreases excitability of larval motor axons and suppresses the behavioral phenotypes of *Sh*, *eag*, and *Hk*. In the mutant *nap^ts* (reared at permissive temperature), a slight decrease in the extent of branching was observed. Yet, when combined with *eag Sh*, *nap^ts* completely reversed the morphological abnormality in *eag Sh* mutants. No such reversion was observed in *para^{ts1} eag Sh* mutants.

The endogenous patterns of electrical activity at the neuromuscular junction were analyzed by extracellular recordings in a semi-intact larval preparation. Recordings from wild-type body-wall muscles revealed rhythmic bursts of spikes. In *eag Sh* mutants, this rhythmic activity was accompanied by or superimposed on periods of strong tonic activity. This abnormal pattern of activity could be partially suppressed by *nap^ts* in combination with *eag Sh*.

The invertebrate CNS has long been viewed as having a rigid stereotypic organization that precludes much plasticity (see Murphey, 1986). In recent years, however, studies of long-term physiological and anatomical changes in *Aplysia* during behav-ioral conditioning (Kandel et al., 1986; Bailey and Chen, 1988a,b), as well as studies of regeneration (Murphey and Lemere, 1984; Shepherd and Murphey, 1986; Loer et al., 1987; Loer and Kristan, 1989), have changed this view. Instead, it has become apparent that, even though the behavioral repertoire of invertebrates is less complex than that of vertebrates, developmental and physiological plasticity is a common feature (reviewed in Lnenicka and Murphey, 1989; see also Technau, 1984; Meinertzhagen, 1989).

The mechanisms underlying neuronal plasticity remain unknown in most cases. However, a factor that has been widely implicated in plastic processes is electrical activity (Hubel et al., 1977; Harris, 1981; Meyer, 1982; Sanes and Constantine-Paton, 1983; Lnenicka and Murphey, 1989; Schmidt and Tieman, 1989). In the optic tectum of vertebrates, for example, it has been shown that correlated activity in retinal ganglion cells determines the fine distribution of retinal terminals in the tectum. Any perturbation of this pattern of activity leads to defects in the final pattern of connections and therefore in the size and/ or shape of the receptive field of these neurons in the tectum (Harris, 1980; Meyer, 1982; Schmidt and Edwards, 1983). In the vertebrate neuromuscular junction, it has also been shown that disruptions in the level of activity of motor axons lead to changes in morphology of axon terminals (Duchen and Strich, 1968; Brown and Ironton, 1977; Holland and Brown, 1980).

Very little is known about how activity levels affect the development of neuronal connectivity in invertebrates (reviewed in Lnenicka and Murphey, 1989). Lnenicka et al. (1986) showed that chronic stimulation of relatively silent phasic motoneurons in the crayfish changed the ultrastructure of its synaptic terminals. This change was reflected in an increased number of varicosities in the nerve projecting to the muscle, larger terminals, and greater synaptic contact area. This altered phenotype resembled the properties of tonic motor axon terminals in arthropods (Atwood and Johnston, 1968; Atwood and Jahromi, 1978; Hill and Govind, 1981; Titmus, 1981). On the other hand, Cohan and Kater (1986) have shown that electrical activity suppresses neurite elongation and growth cone motility in some cultured *Helisoma* neurons.

A genetic approach to the study of neuronal plasticity can be made in *Drosophila* by using a number of neural mutations that either eliminate nerve activity or increase excitability (Burg and Wu, 1986, 1989). We have initiated an anatomical and physiological study of neuromuscular junctions in the hyperexcitable mutants—*Shaker* (*Sh*), *ether a go-go* (*eag*), and *Hyperkinetic* (*Hk*)—and in mutants with reduced excitability—*no action potential* (*nap^ts*) and *paralytic* (*para^{ts1}*).

Sh, eag, and Hk were isolated by their phenotype of anesthesia-induced leg shaking (Kaplan and Trout, 1969). The Sh gene has been well characterized at the molecular level and found to code for the I_A potassium-channel protein (Bauman et al., 1987; Kamb et al., 1987; Papazian et al., 1987; Tempel et al., 1987). Sh mutant alleles increase transmitter release at the larval neuromuscular junction (Jan et al., 1977; Ganetzky and Wu, 1982a) and decrease or eliminate the transient K$^+$-current I_A from pupal and larval muscles (Salkoff and Wyman, 1981; Wu and Haugland, 1985). Mutants at the eag locus display spontaneous action potentials in larval motor axons (Ganetzky and Wu, 1983). Voltage-clamp analysis of larval muscle currents reveals that the delayed rectifier I_K and, in some alleles, I_A, are affected (Wu et al., 1983; Y. Zhong and C.-F. Wu, unpublished observations). The double mutant eag Sh shows synergistic interactions: both the release of neurotransmitter at the larval neuromuscular junction and the degree of adult leg shaking are greatly enhanced (Ganetzky and Wu, 1983). Hk mutants show abnormal neuronal activity in adult ventral ganglia (Ikeda and Kaplan, 1970) and stimulus-induced repetitive activity at the larval neuromuscular junction (Stern and Ganetzky, 1989). However, as for eag, the molecular nature of this mutation is still unknown.

The mutants para (Suzuki et al., 1971; Siddiqi and Benzer, 1976) and nap (Wu et al., 1978) were isolated on the basis of their temperature-dependent paralytic phenotype. Recent molecular studies indicate that the para gene most probably corresponds to the structural sodium channel gene, and that nap appears to be a regulatory gene, controlling the expression of para (Kernan et al., 1989; Loughney et al., 1989). At permissive temperature, the mutant allele para^{ts1} appears physiologically normal, whereas nap^{ts} mutants, because of a reduction in Na$^+$ channel number (Kauvar, 1982; Jackson et al., 1984), already show alterations in axonal conduction even at room temperature (Wu and Ganetzky, 1980). At room temperature, nap can suppress the shaking phenotype of eag Sh, as well as some physiological defects of eag and Sh (reviewed in Ganetzky and Wu, 1985).

Many of the above physiological studies have been carried out in the body-wall muscles of Drosophila larvae. In these muscles, 4 classes of potassium currents and a calcium current have been well characterized (Wu and Haugland, 1985; Gho and Mallart, 1986; Singh and Wu, 1989). Some of these currents have also been described at the single-channel level (Zagotta et al., 1988; Komatsu et al., 1990; Gorczyca and Wu, 1991). Several mutations that disrupt muscle currents, or alter neuromuscular transmission, have been characterized in the larval preparation (for review, see Ganetzky and Wu, 1986). In addition, anatomical aspects of the development and distribution of axon terminals in these muscles have been described (Johansen et al., 1989a,b). In this study, we used the larval neuromuscular preparation to examine the possible effect of altered activity on the morphology of motor axon terminals. We show that increasing excitability by simultaneous mutations in the eag and Sh genes or in the eag and Hk genes, or by augmenting the number of para$^+$ genes, leads to an increase in the ramification of branches on the muscles. In addition, there is a greater number of putative synaptic sites. These effects seen in eag Sh double mutants can be reversed by the mutation nap^{ts}. Although the mechanisms underlying the morphological changes are not known, these results provide an independent line of evidence for the involvement of nerve activity in shaping neuronal arborizations in

invertebrates. In addition, they also provide background for possible reinterpretation of the physiological defects observed on the neuromuscular junctions of these mutants (Jan et al., 1977; Ganetzky and Wu, 1982a,b; Stern and Ganetzky, 1989).

Some of the results have appeared in abstract form (Budnik et al., 1989b).

Materials and Methods

Fly stocks. All fly stocks were reared at room temperature (22–25°C) in standard *Drosophila* food. As a wild type, the strain Canton-Special (CS) was used. Chromosome markers are described in Lindsley and Grell (1968). The mutations used are listed below.

eag^1: Recessive ethyl methane sulfonate (EMS)-induced mutation located at map position 1-48 (Kaplan and Trout, 1969). This mutation removes about 50% of I_K (Wu et al., 1983; Y. Zhong and C.-F. Wu, unpublished observations).

eag^{4PM}: This eag allele preferentially affects I_A rather than I_K in larval muscles (Ganetzky and Wu, 1983; Y. Zhong and C.-F. Wu, unpublished observations).

Hk^1: Recessive EMS-induced mutation located at map position 1-30 (Kaplan and Trout, 1969). This mutant shows reduced I_A (Wu and Ganetzky, 1988) and, at high-frequency stimulation, displays prolonged excitatory junctional potentials (EJPs) at the larval neuromuscular junction (Stern and Ganetzky, 1989).

nap^{ts}: Isolated by EMS mutagenesis (Wu et al., 1978). Flies bearing this mutation become paralyzed at 37°C due to action potentials blockade. Maps to position 2-56.

para^{ts1}: First described by Suzuki et al. (1971). These mutants become paralyzed at 29°C. At this temperature, nerve conduction is completely blocked. Physiologically normal at permissive temperature (Wu and Ganetzky, 1980).

Sh^{KS133}: Described by Jan et al. (1977). Shows prolonged EJPs at the larval neuromuscular junction and completely removes I_A in muscle. (For further physiological characterization, see Salkoff and Wyman, 1981; Wu and Haugland, 1985; Haughland and Wu, 1990.)

Sh^{KO120}: Reduces but does not eliminate I_A in larval muscle. It also has abnormal neuromuscular transmission similar to Sh^{KS133} (Ganetzky and Wu, 1983; Wu and Haugland, 1985; Timpe and Jan, 1987). Maps to position 1-57.4.

Dp(1:4) r$^+$f$^+$. Translocation of the X-chromosome region 14A to 16A2 to the fourth chromosome. This duplication contains the entire para$^+$ gene and also covers l(1) l^{D23} and Df(1) r^{D17} (see below). In this paper, this duplication will be referred to as Dp para$^+$.

l(1) l^{D23}. This is a recessive lethal mutation that lies close to, but is distinct from, para (Ganetzky, 1984), and that is covered by Dp(1:4) r$^+$f$^+$.

Df(1) r^{D17}. This is a deletion from 14F6 to 15A6 of the X chromosome that also lies close to para. This deletion is also covered by Dp(1:4) r$^+$f$^+$ (Stern et al., 1990).

The strains containing Dp(1:4) r$^+$f$^+$, l(1) l^{D23}, and Df(1) r^{D17} were provided to us by Drs. M. Stern and B. Ganetzky.

Immunocytochemistry. Late third-instar larvae, staged by the morphology of the spiracles, were used for this study. Larval body-wall muscles were dissected in Ca$^+$-free saline (128 mM NaCl, 2 mM KCl, 4 mM MgCl$_2$, 5 mM HEPES, 35.5 mM sucrose, 5 mM EGTA). Samples were fixed either in nonalcoholic Bouin's solution [25 ml formalin (from a 37.7% stock solution, Fisher), 5 ml glacial acetic acid, 75 ml saturated picric acid solution] or in paraformaldehyde fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2) for 1–2 hr. We found that immunoreactivity was substantially higher and more reliable in Bouin's fixed tissue.

After fixation, samples were washed 2–5 times (15 min each) in 0.1 M phosphate buffer, (pH 7.2) containing 0.2 or 0.3% Triton X-100 (PBT). Then they were incubated overnight in 1:200 anti-HRP (Sigma) diluted in PBT. After 3 washes with PBT, samples were incubated for 4 hr in 1:20 goat anti-rabbit HRP-conjugated IgG (Cappel), washed 2 times in PBT, 1 time in 0.2 M Tris buffer (pH, 7.6), and reacted using diaminobenzidine (DAB, 5 mg/ml) and 0.03% H$_2$O$_2$.

Electron microscopy. Samples were dissected in Ca^{2+}-free saline, as above, and fixed in modified Trump's fixative [1% glutaraldehyde, 4% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.0 (CB)] for 2 hr. Samples were then washed 3 times with CB and postfixed in 2% OsO$_4$ for
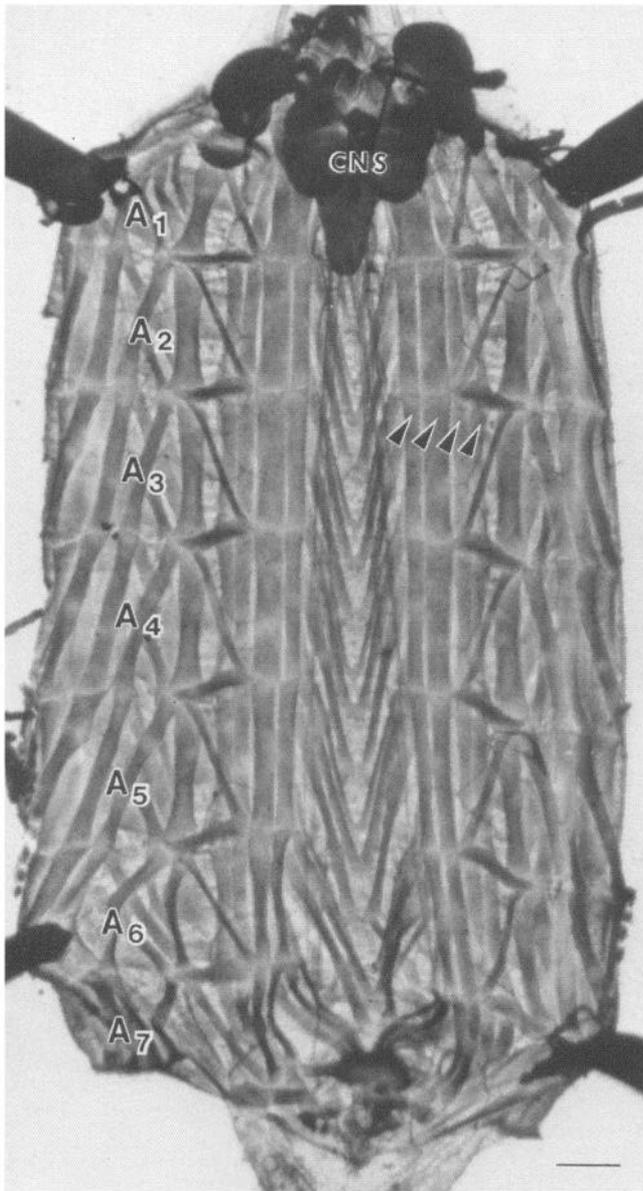
Figure 1. Toluidine-blue-stained preparation of body wall muscles of third-instar *Drosophila* larva. *Arrowheads* indicate (from *left to right*) longitudinal muscles 7, 6, 13, and 12 of the third abdominal segment. *A1–A7* indicate the abdominal segments 1–7. Note the *CNS* in the anterior *(top)* region. Scale bar = 200 μm.

30 min. After postfixation, samples were washed 3 times with CB and 3 times with distilled water and stained *en bloc* in 2% uranyl acetate for 1 hr. Samples were then dehydrated in an acetone series and embedded in Spurr's resin. Transverse thin sections were cut from muscles 6, 7, 12, and 13 (identified in thick sections; nomenclature for muscle identification is according to Crossley, 1978), using a diamond knife. Grids were poststained in 4% uranyl acetate (15–20 min) and 1% lead citrate (5–8 min) and visualized using a Zeiss EM 10 microscope.

Toluidine-blue staining. To visualize the distribution of muscle nuclei, toluidine blue (TB) was used. Briefly, whole larvae were heat fixed for about 3 sec in 60°C water. They were then dissected, stained for a few seconds in TB (6% borax, 1% boric acid, 1% toluidine blue), washed with water, and immediately photographed.

Electrophysiology. Extracellular recordings from body-wall muscles 6, 7, 12, and 13 were made by a differential amplifier (DAM-5, WPI) and a capillary glass suction electrode with a tip diameter of 10–20 μm. The positive and negative leads were chlorided Ag wires. Electrical activity was recorded upon touching the muscle fibers with the glass electrode (no suction applied). Signals were bandpass filtered at 100 Hz and 10 KHz and taped on a VCR recording system in conjunction with a pulse-code modulation unit (Neuro Data Instrument Corporation). All recordings were carried out in *Drosophila* normal saline (same as Ca^{2+}-free saline but with 1.8 mM $CaCl_2$ and no EGTA) at 19–20°C.

Results

Distribution of anti-HRP immunoreactive terminals in wild type

The body-wall muscles of larvae are composed of identifiable muscle fibers arranged in a segmentally repeated pattern in the thoracic and abdominal segments (Fig. 1). For a description of this pattern and its development, see Campos-Ortega and Hartenstein (1985) and Johansen et al. (1989b). Immunocytochemical and physiological studies have demonstrated that most of the innervation is glutamatergic (Jan and Jan, 1976; Johansen et al., 1989a). However, a subpopulation of muscle terminals also shows proctolin or octopamine immunoreactivity (Anderson et al., 1988; Halpern et al., 1988).

Most electrophysiological studies in *Drosophila* larvae have been performed using a particular subset of the body-wall muscles, the abdominal ventral longitudinal muscles 6, 7, 12, and 13 (nomenclature of Crossley, 1978). In this paper, we have centered on these muscles to study the morphology of motor terminals in mutants with altered excitability.

In order to visualize motor axon terminals, anti-HRP immunocytochemistry was used. In insects, the anti-HRP antibody binds to antigens exclusively located in neuronal membranes (Jan and Jan, 1982). The pattern of distribution of these terminals on each identified muscle fiber was characteristic. The description below and results of experiments in this paper were based on analyses of muscles 6, 7, 12, and 13 at the third abdominal segment (Fig. 1). This description is in general agreement with the description of glutamatelike immunoreactivity at these muscles made by Johansen et al. (1989a, b).

Muscles 6 and 7. Anti-HRP immunoreactivity in these muscles was located in sparsely ramified branches in the lateral aspect of these muscles (Figs. 2A, 3A). A branch that lies between muscles 6 and 7 gives rise to this innervation. Throughout this projection, as well as in the projections described below in muscles 12 and 13, enlargements of the neurites, or varicosities, are observed (Fig. 2). In muscles 6 and 7, most varicosities measure about 3–5 μm in diameter. However, some short ramifications show small varicosities in the range of ≤ 2 μm.

Muscles 12 and 13. At these more lateral muscles, a nerve branch contacts each fiber at its internal (visceral) face and gives off sparsely ramified branches that run anteriorly and posteriorly throughout the length of the muscle (Figs. 2B, 3A). Near the initial contact of the nerve with the muscle (proximal terminals), most neurites show large varicosities (3–5 μm). However, all distal neurites show varicosities of the small size (≤ 2 μm; Fig. 2B). Although most branches emerging from the initial branch point were restricted to the domain of the particular muscle fiber, in many occasions, the distal portions of the branches extended for a few tens of microns into the adjacent segment, or into a neighboring muscle in the same segment (see, for example, Fig. 4A). This occurred more frequently in muscles of more anterior segments (30% of muscle fibers in segment 2, 21% of muscle fibers in segment 3, 5% of muscle fibers in segment 6).

Motor axon terminals distribute in an anteroposterior gradient. Even though the general pattern of distribution of motor
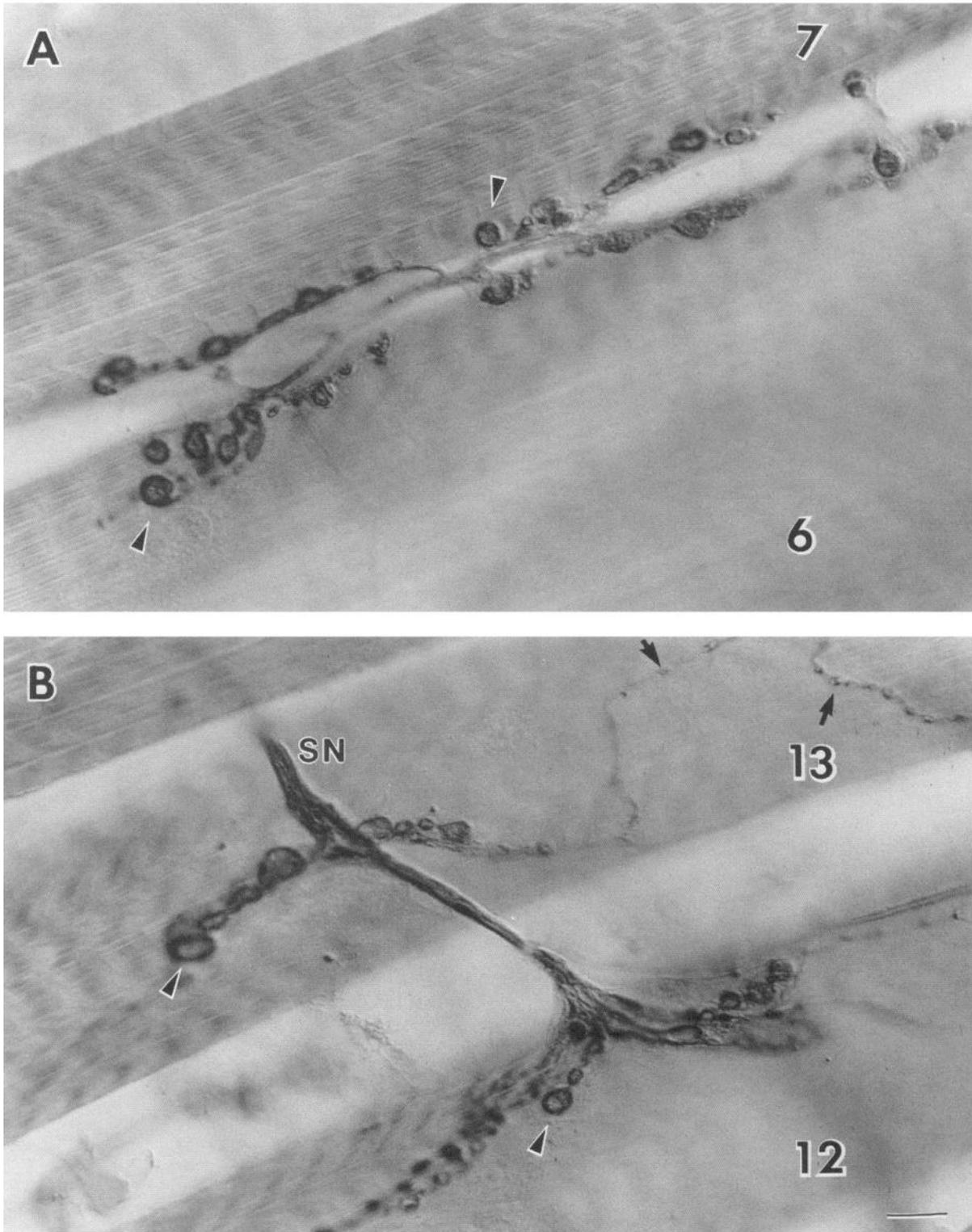
Figure 2. Innervation of muscles *6* and *7* (*A*) and *12* and *13* (*B*) as revealed by anti-HRP immunocytochemistry, visualized under Nomarski optics. Note the presence of prominent varicosities along neurites. *Arrowheads* point to the large-type varicosities, whereas *arrows* point to the small-type varicosities. *SN,* segmental nerve. Scale bar, 12 μm.

terminals is similar in all abdominal segments, it was noticed that anterior segments showed a higher degree of neurite ramification. The neurites of posterior segments displayed very little or no ramification, and the number of varicosities was also lower

(Fig. 4). This was particularly clear in muscles 12 and 13 from segment 6, where on many occasions one of the distal projections was missing. There was also a continuous change in the size and width of muscle fibers: posterior muscles become longer

A

B

C

wild type 7 6 13 12

eag Sh

eag Sh nap
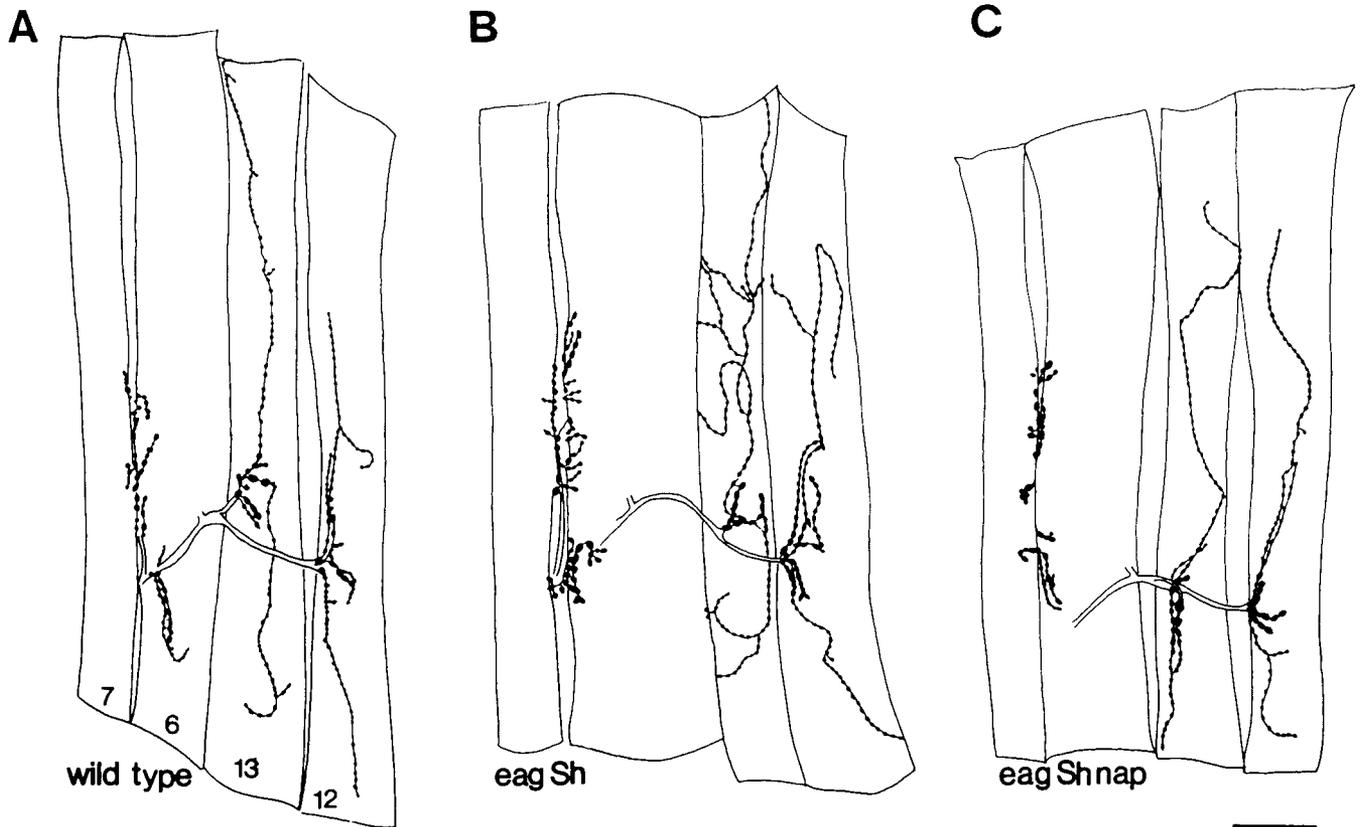
Figure 3. Camera lucida drawings of anti-HRP staining of axon terminals on muscles 6, 7, 12, and 13 of wild-type (*A*), eag^{4PM} Sh^{rKO120} double mutants (*B*), and eag^{4PM} Sh^{rKO120} nap^{ts} mutants (*C*). Notice that, in eag^{4PM} Sh^{rKO120}, the extent of ramification of neurites in muscles 12 and 13 is increased. This alteration is suppressed by nap^{ts}. Scale bar, 50 μm.

and thinner (see Figs. 1, 4*A*). Even after data were normalized using the surface area of the muscle fiber as drawn in camera lucida tracings (Fig. 4*A*), the anteroposterior difference persisted (Fig. 4*B,C*).

Several studies have suggested that varicosities are regions of synaptic contacts (see, for example, Lnenicka et al., 1986). In order to address this possibility, we studied transverse sections of these muscles at the electron microscopical level. One-micron-thick sections were used to determine the presence of a varicosity. Then, the tissue was thin sectioned and stained with uranyl acetate and lead citrate. We analyzed 2 wild-type samples. We found that varicosities coincided with muscle sites showing synaptic terminals (Fig. 5; for descriptions of the ultrastructure of neuromuscular junctions in other insect species, see Osborne, 1975). These terminals were located in the peripheral sarcoplasm as invaginations into the muscle surface. Most terminals were characterized by the presence of 30-nm-diameter clear vesicles, as well as by a variable number of synaptic specializations in the presynaptic membrane (Fig. 5*A*). These vesicles were more concentrated in the region surrounding the synaptic specialization and seemed to be connected by a fibrous material. Terminals were separated from the postsynaptic membrane by a cleft of about 15–20 nm. At the postsynaptic region, and surrounding the whole terminal, a well-developed subsynaptic reticulum was observed (Fig. 5*A*).

Hyperexcitable double mutants eag Sh *show an increased number of branches and presumptive synaptic sites*

In order to investigate the effect of mutations that increased excitability, we studied double-mutant combinations of 2 different alleles of *eag* and *Sh*. We found that, in both eag^{4PM} innervation was altered (Fig. 3). The changes could be described as a substantial ramification of neurites throughout the muscle fiber. Analysis of these ramifications revealed that the number of higher order branches was particularly increased (Figs. 6, 7). In the wild type the number of primary branches was 7.2 ± 0.2 (41%), the number of secondary branches was 9.3 ± 1.1 (54%), and the number of tertiary branches was 0.9 ± 0.3 (5%). No quaternary branches were observed in all 9 samples analyzed. In eag^{1} Sh^{KS133} the number of primary and secondary branches was 8.1 ± 0.6 (31%) and 11.8 ± 1.1 (46%), respectively, and did not differ significantly from the wild type. In contrast, the number of tertiary (4.7 ± 0.5, 18%) and quaternary (1.3 ± 0.4, 5%) branches was significantly increased ($p < 0.05$). In addition, the number and, in some cases, the density, of varicosities in the neurites was also increased (Fig. 8).

The increase in the number of branches and varicosities was most noticeable in neurites containing predominantly the small-sized varicosities. Therefore, the effect was particularly clear in muscles 12 and 13 (Figs. 6, 8*A*). However, the same pattern of

alterations, though less dramatic, was observed in muscles 6 and 7 of *eag^1 Sh^{KS133}* mutants. In these muscles (6, 7), only the number of varicosities was analyzed, because the degree of ramification of neurites was difficult to quantify (Fig. 8*B*). No changes in the segmental or muscle specificity of the innervation were found in this and all other mutants analyzed in this study.

Only a slight but statistically significant decrease in the extent of branching was observed in temperature-sensitive *nap^{ts}* larvae, when reared at permissive temperature (Figs. 7*C*, 9). However, *nap^{ts}* in combination with *eag^{4PM} Sh^{rKO120}* completely reversed the *eag Sh* mutant phenotype in the body-wall motor axons (Figs. 3*C*, 7*B*, 8). In contrast, in *eag^{4PM} para^{ts1} Sh^{rKO120}* mutants reared at permissive temperature, the pattern of arborization of motor axons remained similar to that observed in *eag Sh* (Fig. 8). Only a slight but statistically insignificant increase in the number of branches and varicosities was observed in the single mutants *eag* and *Sh* (Fig. 9).

In vertebrate skeletal muscles, it has been noticed that, during synapse formation, myonuclei accumulate under the nerve terminal, in positions that coincided with clusters of ACh receptors (Nakai, 1968; Kelly and Zacks, 1969). Induction of ACh-receptor cluster formation elicited nuclei movements towards the newly formed cluster (Englander and Rubin, 1987). In *Drosophila* body-wall muscles, glutamate receptors seem to localize in regions of the muscle that are apposed to neurites (Johansen et al., 1989a.). Therefore, it was important to ascertain if the changes in distribution of varicosities in hyperexcitable mutants were correlated with changes of nuclear distribution. As in most muscles, the body-wall muscles of *Drosophila* larvae are polynucleated. These nuclei are distributed superficially in the internally facing (visceral) sarcolemma in a fashion characteristic for each muscle type. In muscles 7 and 13 of the third segment, for example, they distribute in a single row along the anteroposterior axis of the fiber (Fig. 10). In muscle 6, in contrast, nuclei are distributed in 2 alternating rows. In muscle 12, most nuclei were concentrated in the anterior half of the muscle fiber, often surrounding the region of contact of the nerve with the muscle fiber (Fig. 10). The distribution of nuclei in each segment was not identical. For example, in more posterior segments, the double row apparent in muscle 6 was transformed to a single row of nuclei. No change in the distribution or number of these nuclei was detected in any of the excitability mutants analyzed in this study.

Other mutants with increased excitability show alterations similar to eag Sh

Similar to the 2 *eag Sh* mutants, the appearance of nerve terminals in *Hk^1 eag^1* larvae was also altered. Again, the alteration was readily apparent in counts of the number of branches and varicosities, which showed an increase similar to that observed in *eag Sh* mutants (Fig. 11).

Because the molecular identity of the *eag* and *Hk* gene products is unknown, we wanted to address the question of whether increasing excitability by using other better-defined mutations would also produce the same changes in the morphology of motor axons. Stern et al. (1990) have demonstrated that increasing the number of normal *para* genes by introducing duplications of the gene (*Dp para^+*) has 2 prominent effects. It suppresses the temperature-sensitive paralysis of *nap^{ts}*, presumably by restoring the Na^+ channel density, and it enhances the leg-shaking phenotype of *Sh^{KS133}*, resulting in a behavioral phenotype similar to *eag^1 Sh^{KS133}*.
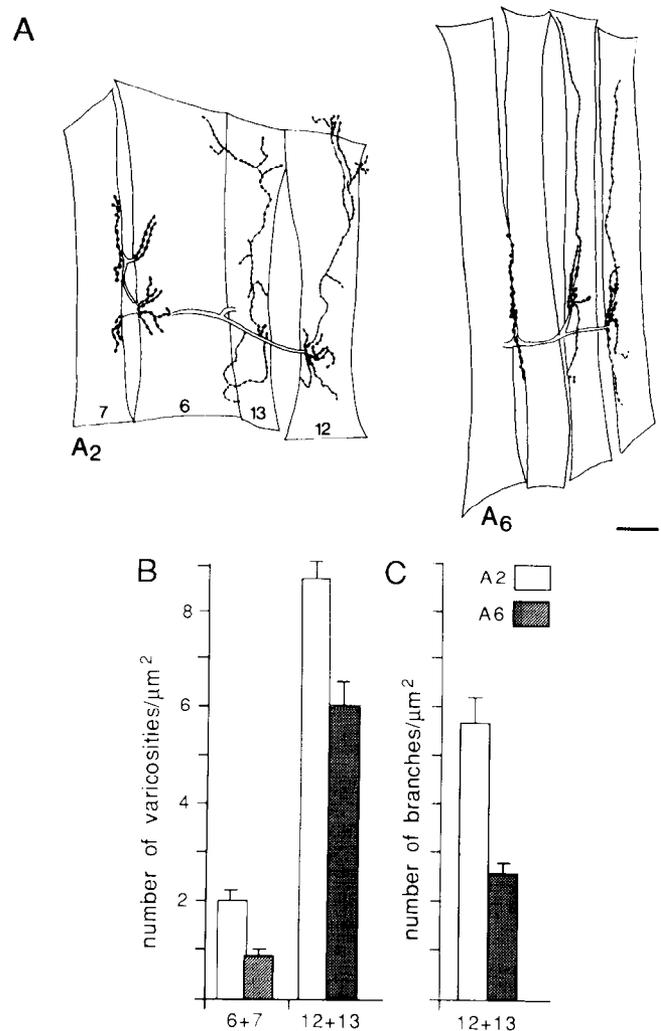
Figure 4. Anteroposterior gradient in extent of branching and number of varicosities of muscles *6, 7, 12,* and *13*. *A*, Camera lucida drawings of muscles 6, 7, 12, and 13 of an anterior segment (*A2*) and of a posterior segment (*A6*). Scale bar, 50 μm. *B* and *C*, Number of branches and varicosities in muscles 6, 7, 12, and 13 in A2 and A6. *B*, Number of varicosities per unit area × 10^3 in muscles 6 and 7 and 12 and 13. *C*, Number of branches per unit area × 10^4 in muscles 12 and 13. Data are expressed as mean ± SEM; $n = 10$. Note the decrease in both the number of varicosities and branches in the posterior segment.

We examined nerve terminals of male larvae that contained at least 1 extra copy of the normal *para^+* gene [*J^{P23}; Dp(1:4) r^+f^+*]. In addition, we analyzed a strain containing the mutation *Sh^{KS133}* in addition to the *Dp para^+* [*Df(1) r^{D17} Sh^{KS133}; Dp(1:4) r^+f^+*]. We found that, in both strains, the number of branches and varicosities was significantly increased, but to a lesser extent than that of *eag Sh* or *Hk eag* mutants (Fig. 11). Closer analysis of the data revealed a high variability not observed in any of the other strains analyzed. There appeared to be a group of samples with a phenotype similar to wild type, and a group of samples similar to *eag Sh* or *Hk eag* (Fig. 11). This could be the result of either variable penetrance or a different number of *para^+* gene copies (see Discussion).

Endogenous pattern of activity in the body-wall muscles

Many studies of *eag, Sh,* and *Hk,* as well as of their double-mutant combinations, have shown electrophysiological char-
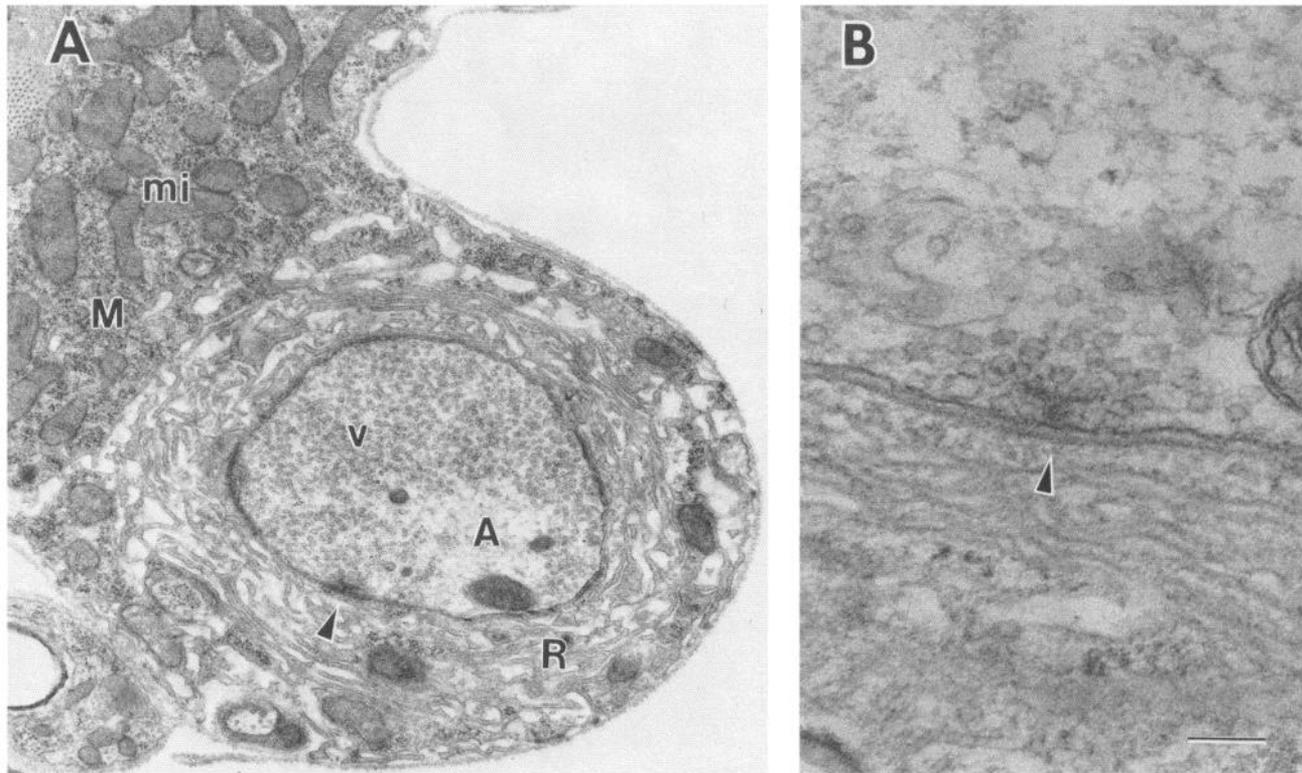
Figure 5. Electron micrographs of varicosities. *A,* A lower-magnification view showing an axon terminal (*A*) containing 30-nm clear vesicles (*v*) and a region of presumptive synaptic contact (*arrowhead*). *M,* muscle; *mi,* mitochondria; *R,* subsynaptic reticulum. *B,* Higher-magnification view of a synaptic region showing clear synaptic vesicles as well as an electron-dense body typical of insect neuromuscular synapses (*arrowhead*). Scale *bar*: 0.6 μm, *A*; 0.1 μm, *B*.

acteristics consistent with hyperexcitable behavioral phenotypes. However, most, if not all, of these studies have been conducted under conditions where the input from the CNS had been severed. In addition, many of the hyperexcitable phenotypes have been studied at low calcium concentrations (Ganetzky and Wu, 1983), at which reduced muscle contractions present less technical problems for intracellular EJP recordings. As a result of the striking changes in the morphology of axon terminals observed in these mutants, we were interested in verifying nerve hyperexcitability in these mutants at normal calcium concentration and with normal CNS inputs. We recorded from the muscles in a semi-intact preparation with the nerve connection between the CNS and the muscle left intact. Under these conditions, these supercontracting muscles contract considerably, making intracellular recordings technically very difficult. However, we found that the CNS-generated pattern as reflected by the muscle activity could be recorded extracellularly for 5–20 min.

Figure 12 shows the typical pattern of activity observed in the wild type, which was composed of a rhythmic pattern of bursting activity. Each burst consisted of a train of nerve-evoked spikes in the muscles. These spikes could correspond to action potentials at the muscle and/or to EJPs (Yamaoka and Ikeda 1988; M. Gho, personal communication). We believe that these spikes were nerve evoked and not spontaneous myogenic activity because they were completely blocked by TTX. Inward currents in these muscles are carried by Ca^{2+} (Gho and Mallart, 1986; Singh and Wu, 1989), but we observed no such spikes in wild-type and mutant larvae at normal Ca^{2+} concentrations in the absence of CNS input. Periods of bursting activity, usually

lasting on the order of minutes, were followed by periods of quiescence. Great variability was found from preparation to preparation regarding duration of bursts, frequency of spikes within a burst, duration of periods of bursting activity, and duration of periods of silence. Therefore, we did not attempt to quantify further these parameters of the pattern. Figure 13*A* shows the bursting pattern observed in 3 different wild-type preparations. A common feature of most wild-type patterns recorded was their predominantly bursting nature.

In *eag^{4PM} Sh^{rKO120}* mutants, bursting activity was also observed in the majority of the muscle fibers. However, in contrast to the wild type, periods of strong tonic activity, not organized into bursts, were also observed. This tonic activity was either superimposed on the bursting activity or observed alone. Figure 13*B* shows representative examples of patterns observed in 3 different *eag^{4PM} Sh^{rKO120}* preparations.

In order to characterize the distribution of patterns in these mutants, muscle activity observed in a period of 5–20 min was classified as follows.

Bursting (B): at least 3 bursts of spikes in approximately a 3-min period; all spikes grouped into bursts.

Bursting with some tonic component (B/T): spikes mostly grouped in bursts, but a low level of tonic activity (spikes not grouped into bursts) also observed.

Mixed tonic and bursting activity (T+B): Strong tonic non-bursting activity accompanied by periods of bursting activity, or the tonic activity seems to be superimposed on bursting activity.

Tonic (T): Activity is purely tonic with no clear signs of bursting organization.
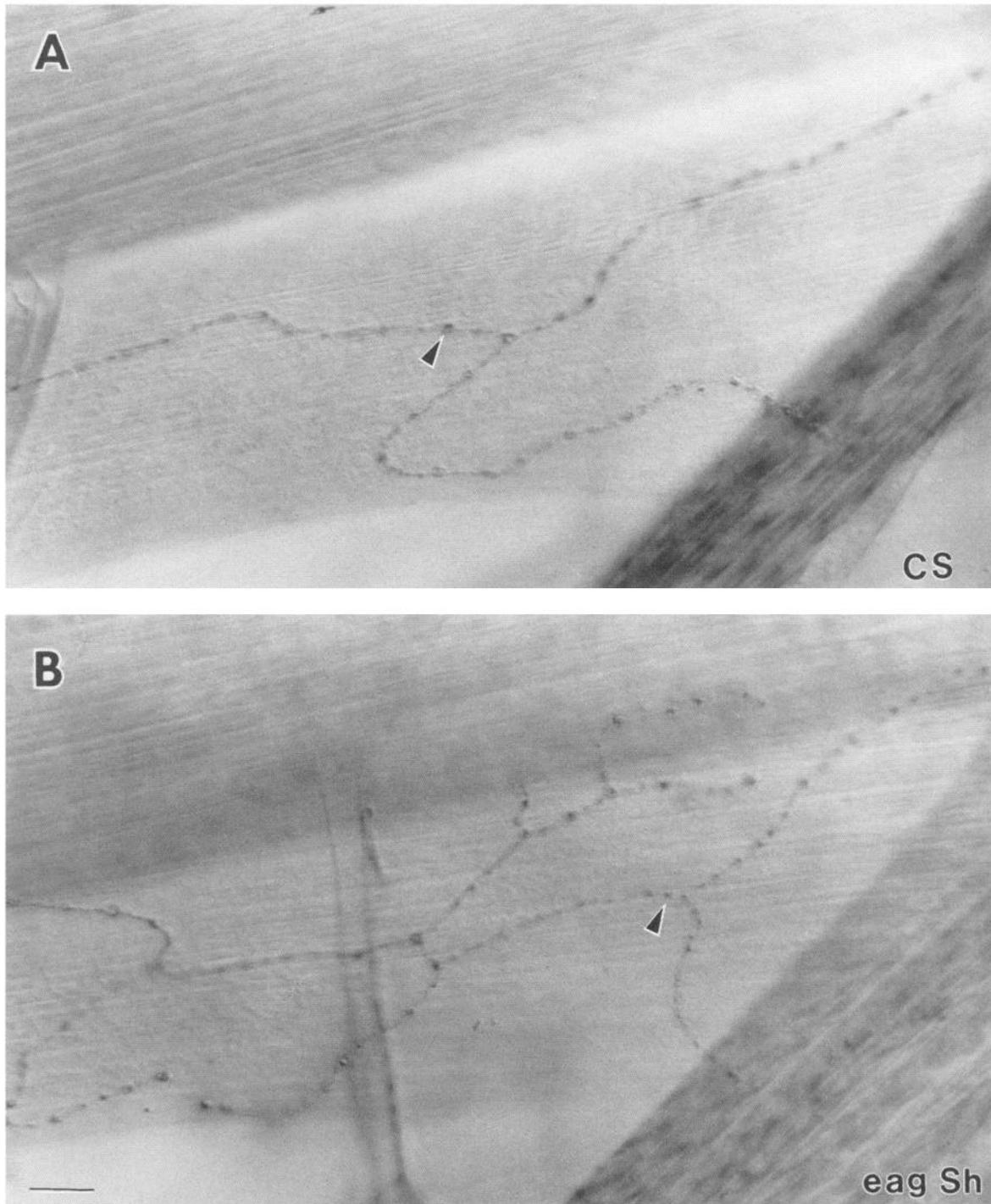
Figure 6. HRP immunoreactivity of neurites containing small-type varicosities (*arrowheads*) in *CS* (*A*) and *eag¹ Sh^KS133* (*B*) in distal axonal region of muscle 12. Notice the increased ramification of neurites in *eag¹ Sh^KS133*. Scale bar, 12 µm.

As shown in Figure 14, in both *eag^4PM Sh^rKO120* and *Hk¹ eag¹*, there is an increase in the percentage of fibers that show some level of activity not grouped into bursts (categories B/T and T+B). This is in contrast with wild-type muscle fibers where activity is mostly organized into bursts (category B). These results show that, in addition to the increased neurotransmitter release that is observed in these hyperexcitable mutants (Ganetzky and Wu, 1983), there are also alterations in the *pattern* of activity of the motoneurons. However, we found that in

Sh^KS133 Dp para+ mutants, though there was a dramatic increase in the amplitude of EJPs (Stern et al., 1990), the pattern of activity at the muscles was very similar to wild type (Fig. 14*D*).

In the morphological analysis of nerve terminals, we found that *nap^ts* could suppress the increased number of branches and varicosities of *eag^4PM Sh^rKO120*. In addition, *nap^ts* has been found to suppress some behavioral and physiological abnormalities of *eag* and *Sh* (Ganetzky and Wu, 1982a,b, 1983). Therefore, it was relevant to investigate whether the abnormalities in the

Figure 7. Number of primary, secondary, tertiary, and quaternary branches in muscles 12 and 13 in wild type ($n = 9$) and $eag^{4PM}\ Sh^{rKO120}$ ($n = 9$; *A*), $eag^{4PM}\ Sh^{rKO120}\ nap^{ts}$ ($n = 8$; *B*), and nap^{ts} ($n = 7$; *C*). Data are expressed as mean ± SEM. Note that the increase in branching observed in $eag^{4PM}\ Sh^{rKO120}$ is due to an increase of higher-order branches. Also notice that higher-order branches are reduced in nap^{ts} mutants.
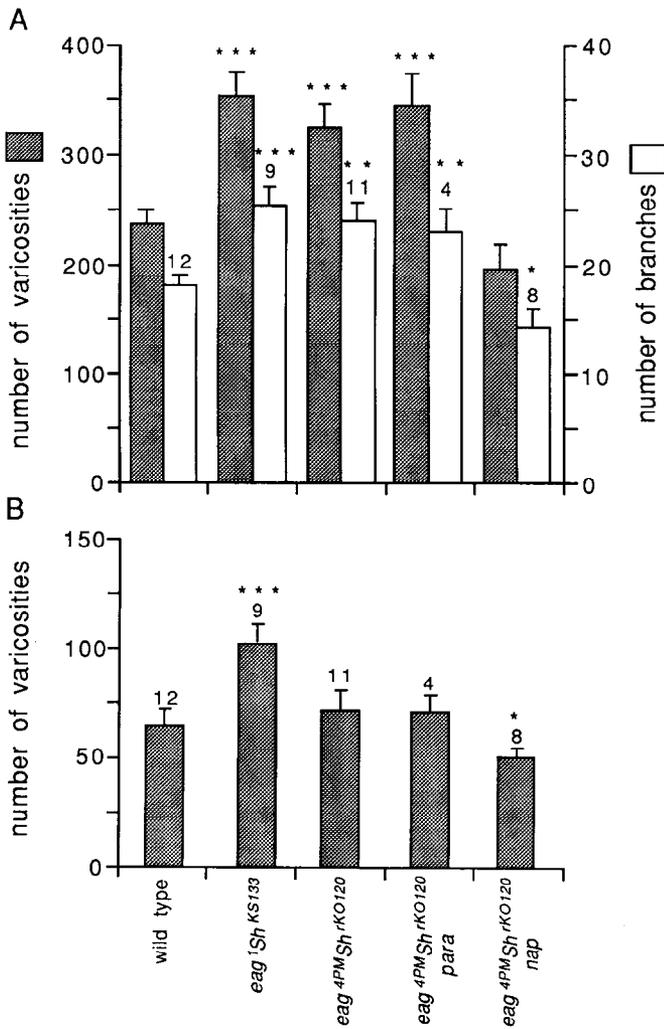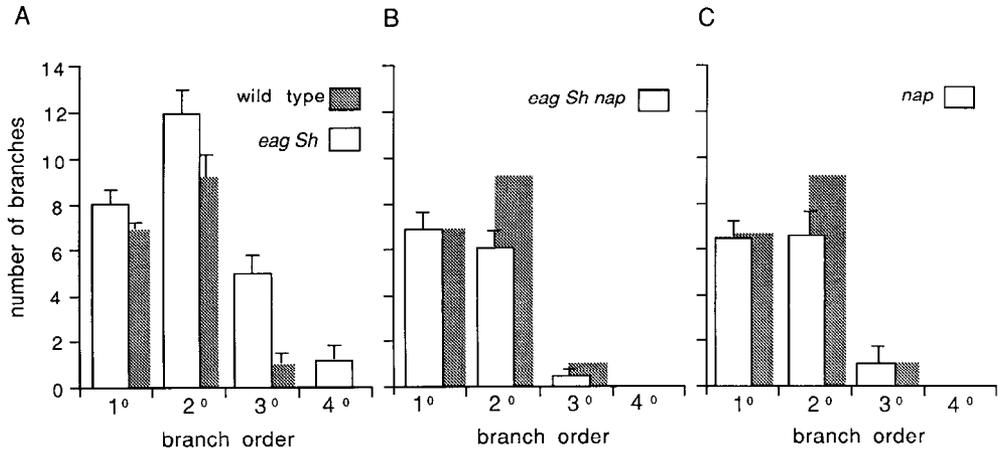
Figure 8. Graphical representation of number of varicosities and total number of branches in muscles 12 and 13 (*A*) and in muscles 6 and 7 (*B*). Note the increase in the number of varicosities in 2 different *eag Sh* double mutants. nap^{ts}, unlike $para^{ts1}$, in combination with *eag Sh*, completely reversed this altered phenotype. Data are expressed as mean ± SEM. The *number* of samples is indicated near the *error bars*. Means were compared to wild type using the Student's *t* test. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

pattern of activity of motor axons could also be rescued by nap^{ts}. As shown in Figures 13*C* and 14, the hyperexcitable pattern of activity observed in $eag^{4PM}\ Sh^{rKO120}$ was only partially suppressed by nap^{ts}. While there was an increase in the number of fibers with purely bursting activity, and a decrease in the number of fibers showing strong tonic activity (T+B), the distribution of activity types was not completely like the wild type. In most muscle fibers, bursting activity was readily apparent. However, in many fibers, a low level of abnormal tonic activity was also observed (Fig. 14).

In the single mutants nap^{ts} and eag^{4PM} ($n = 12$ and 11 fibers, respectively; data not shown), the distribution of activity types was almost as the wild type, though in eag^{4PM}, there was a slight increase in the number of muscle fibers that displayed a low level of tonic activity in addition to the bursting activity (B/T type).

Discussion

In this article, we report the effects of mutations that alter neuronal excitability on the morphology of motor axon innervation.
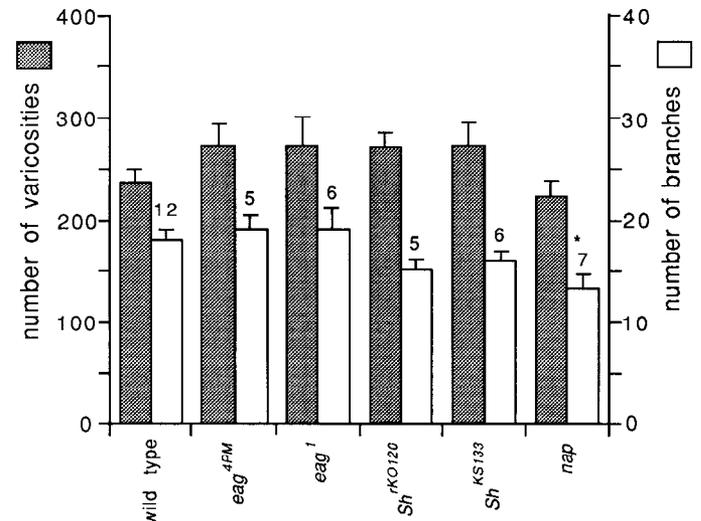
Figure 9. Number of branches and varicosities in single mutants. Data expressed as mean ± SEM. The *number* of samples is indicated near the *error bars*. *, $p < 0.05$.
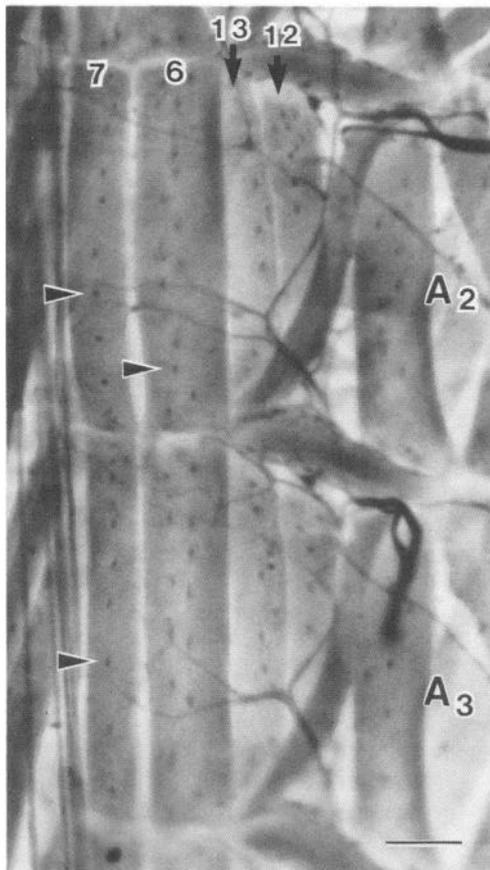
Figure 10. Distribution of nuclei (*arrowheads*) in muscles *6, 7, 12,* and *13* of the abdominal segments *A2* and *A3*, as revealed by toluidine-blue staining. Scale bar, 80 μm.

Figure 11. Number of branches and varicosities of muscles 12 and 13 in CS, *Dp para*[+], *Sh*[KS133] *Dp para*[+], *Hk eag*[1] and *Hk*. Each *point* in this graph corresponds to counts in an individual sample. Notice the variability of *Dp para*[+] and *Dp para*[+] *Sh*[KS133] data. **, $p < 0.005$; ***, $p < 0.0005$.

We centered our study exclusively on the third abdominal segment.

An interesting observation is that each segment is mainly innervated by the corresponding segmental nerve, but that terminal neurites can cross the segmental boundaries and innervate the distal portions of muscles of adjacent segments. This implies that there is not strict segmental specificity for the body-wall muscle innervation.

Influence of activity on motoneuron terminal arbors

Comparisons of wild-type axon terminals with terminals of several hyperexcitable mutants revealed a significant increase in

We used the body-wall muscles of *Drosophila* larvae because their physiology, anatomy, and development have been well characterized (Ganetzky and Wu, 1986; Johansen et al., 1989b). Anti-HRP staining of axon terminals revealed that they displayed varicosities that could be classified into 2 populations according to their size (small type and large type; see also Johansen et al., 1989a). Ultramicroscopical analysis revealed that varicosities coincided with synaptic sites. However, because we did not serially section the tissue, we do not know whether varicosities are exclusive sites of synapses or whether there are synapses at other neuritic sites, as well. In crustacean neuromuscular junctions, it has been shown that, while varicosities are the region where most ultrastructural synaptic features are found, these are also seen at extravaricosity sites (Lnenicka et al., 1986). Studies are in progress to determine if the same is the case in *Drosophila*.

We found that, even though the distribution of axon terminals seemed to be characteristic for each identifiable muscle fiber in different segments, there were clear differences among body segments. The degree of branching as well as the number of varicosities decreased in more posterior segments. These intersegmental differences could be the result of a developmental gradient at a particular period of synaptogenesis. Alternatively, it could be the result of functional differences, for example, an anteroposterior gradient in the levels of activity of neuromuscular junctions, which in turn could result in variations in the size of the projection and presumably in the number of synaptic sites.

Figure 12. Extracellular recordings of CNS-evoked responses in larval body-wall muscle 6. Similar activity was observed in muscles 7, 12, and 13. In this preparation, connections between muscles and CNS were left intact (see Fig. 1). Recordings were carried out at 19–20°C in normal saline. *Vertical bar,* 150 μV; *horizontal bar,* 5 sec in *A,* 1 sec in *B,* 50 msec in *C*.

A

CS

B

eag Sh

C

eag Sh nap

20 s
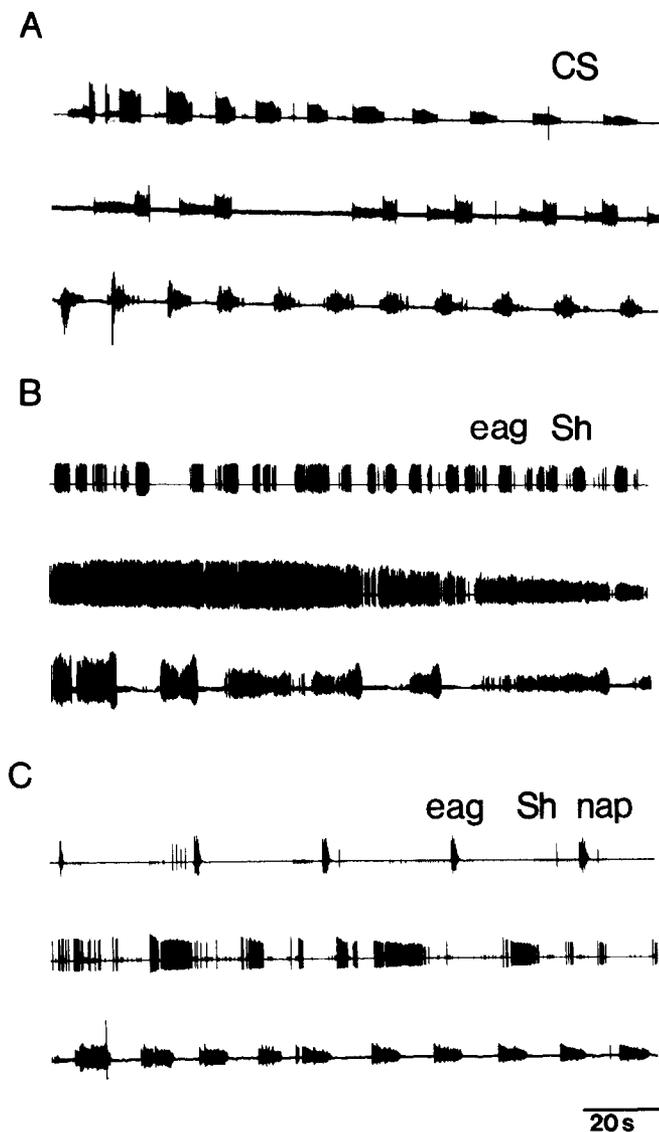
Figure 13. Patterns of activity in wild type (*A*), *eag⁴ᴾᴹ Shʳᴷᴼ¹²⁰* (*B*), and *eag⁴ᴾᴹ Shʳᴷᴼ¹²⁰ napᵗˢ* (*C*). Each trace in *A, B, and C* is a recording of a different larval preparation representing a typical pattern observed. Notice that in wild type, CNS-driven spikes are grouped in bursts. In contrast, in *eag⁴ᴾᴹ Shʳᴷᴼ¹²⁰*, tonic activity is dominant, though it is accompanied by periods of bursting activity. Most *eag⁴ᴾᴹ Shʳᴷᴼ¹²⁰ napᵗˢ* preparations show a bursting activity similar to wild type. However, a large proportion of muscle fibers in this genotype still show a low degree of abnormal tonic activity.

the extent of branching, in particular, higher-order branches, and an increase in the number of varicosities. This was most dramatic in the double mutants *eag Sh* and *Hk eag,* and to a lesser extent, in *Dp para⁺* and *Sh Dp para⁺*. None of the single mutants *eag, Sh,* or *Hk* showed statistically significant difference from the wild type. It is interesting to note that the mutants *eag Sh, Hk eag, Dp para⁺,* and *Sh Dp para⁺* have many common physiological and behavioral abnormalities related to hyperexcitability (Ganetzky and Wu, 1983; Stern and Ganetzky, 1989; Stern et al., 1990). However, the mechanism by which they increase excitability is different. For example, *eag Sh* mutants confer hyperexcitability by decreasing potassium currents (I_A and I_K), whereas *Dp para⁺* mutants produce an hyperexcitable

phenotype presumably by increasing the number of sodium channels (Stern et al., 1990).

Even though the number of varicosities and branches in both *Dp para⁺* and *Sh Dp para⁺* samples was significantly higher than the wild type, we observed great variability in the morphological data. There appeared to be some samples very similar to the wild type, and others with a phenotype similar to *eag Sh* or *Hk eag.* This variability is possibly related to the uncertainty in the number of duplicated *para⁺* genes present in the two strains used [*l(1) lᴰ²³; Dp(1,4) r⁺f⁺* or *Df(1) rᴰ²³; Dp(1,4) r⁺f⁺*]. Their genetic compositions assure that at least 1 extra copy of *para⁺* (in addition to the one normally present on the X chromosome) will be present in males (Stern et al., 1990). This is so because this duplication covers *l(1) lᴰ²³* and *Df(1) rᴰ¹⁷* that are otherwise lethal. However, there could be either 1 extra copy of the *para⁺* gene in heterozygotes or 2 extra copies in flies homozygous for *Dp(1,4) r⁺f⁺*. It is not possible to distinguish larvae of these 2 genotypes.

The fact that hyperexcitability derived from different molecular defects in *eag Sh, Hk eag, Dp para⁺,* and *Sh Dp para⁺* produces similar morphological alterations indicates a possible activity-dependent mechanism that could regulate the growth or maintenance of the motor terminals. Similar observations have been reported in the neuromuscular synapses of crustaceans (Lnenicka et al., 1986), where increasing the level of activity by chronic stimulation of a motor axon also produced an increase in the number of varicosities at the terminals.

Further evidence consistent with our hypothesis of activity-dependent morphological change is that the reduced excitability caused by *napᵗˢ* reversed the effect of *eag Sh* on motor axon terminals. This mutant has been shown to reverse hyperexcitable phenotypes of *eag, Sh,* and *Dp para⁺* physiologically and behaviorally (Ganetzky and Wu, 1985; Stern et al., 1990), presumably by decreasing the expression of the *para⁺* gene, thereby decreasing the number of sodium channels (Kauvar, 1982; Jackson et al., 1984). It is interesting to note that the *napᵗˢ* mutation alone shows a decrease in the degree of branching in comparison to the wild type. This could indicate that reduced excitability generates effects opposite to those caused by hyperexcitability in motor axon terminal morphology. In contrast to *napᵗˢ*, *paraᵗˢ¹* did not reverse the *eag Sh* phenotype. This is not surprising because no physiological defects have been found in *paraᵗˢ¹* when reared and tested at room temperature (Wu and Ganetzky, 1980; Kauvar, 1982; Jackson et al., 1984).

An intriguing observation is that the changes observed in the number of higher-order branches and varicosities were most dramatically expressed in neurites containing the smaller type of varicosities. At present, it is not known whether there is a functional difference between the larger- and smaller-type varicosities. Studies are in progress to determine if the 2 types can be differentiated ultrastructurally. A speculation that can be made is that neurites containing the smaller type of varicosities are plastic portions of motor terminals. These regions could be the sites at which the size of the projection and the number of synapses are regulated, for example, during the dramatic growth that the muscles undergo throughout the larval stages.

Our results are in contrast with what was observed by Burg and Wu (1986, 1989) in sensory neurons of adult *Drosophila.* Genetic mosaics were generated in which a single sensory-bristle complex was genotypically *eag Sh* or *paraᵗˢ¹ napᵗˢ*. However, analysis of the morphology of the sensory neuron dendritic arborization revealed no obvious abnormality with either mutant.
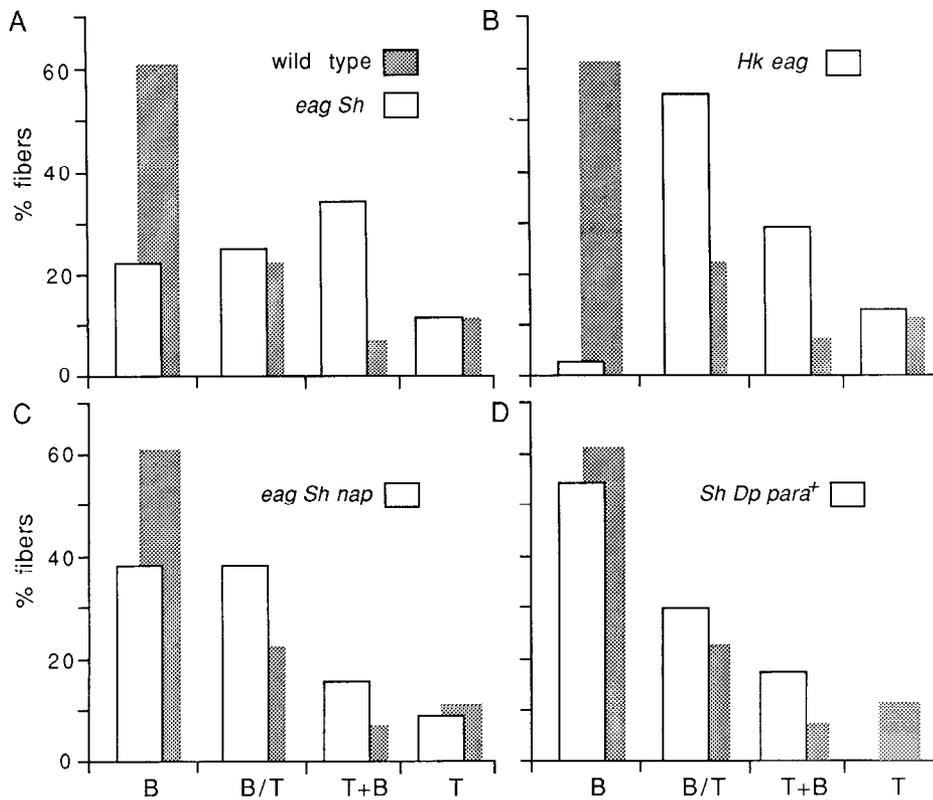
Figure 14. Distribution of muscle fibers with bursting (B), tonic (T), or a combination of both patterns of activity (B/T or T+B; see text) in wild type (n = 35 fibers) and eag^{4PM} Sh^{rKO120} (n = 34 fibers; A), Hk^1 eag^1 (n = 32 fibers; B), eag^{4PM} Sh^{rKO120} nap^{ts} (n = 32 fibers; C), and Sh^{KS133} Dp $para^+$ (n = 30 fibers; D).

Several alternative explanations for these results can be made. First, it is possible that activity-dependent changes of neural development are restricted to a selected population of neurons. In fact, sensory receptors are epithelial in origin and, thus, may differ from motoneurons derived from central neuroblasts. A second possibility is that, in order to produce changes in neuronal morphology and synaptogenesis, it is necessary for the mutation to elicit changes in both the presynaptic and postsynaptic sites. In their work, only the sensory neuron and not the postsynaptic target was most likely mutant as indicated by reflex behavior control of neighboring bristles (Burg and Wu, 1986, 1989).

A similar phenomenon, an increase in the extent of branching and the number of varicosities, was observed in a serotonin nerve projection to the digestive system of Drosophila larvae (Budnik et al., 1989a). This change was observed in larvae that were genetically deficient in serotonin and dopamine. It would be interesting to investigate if this change was also an activity-related phenomenon generated by the lack of neurotransmitter action. Unfortunately, nothing is known about the electrophysiology of that enteric serotonin projection.

The effect of activity on the innervation of neuromuscular junctions has been studied in vertebrates. It has been found that motoneurons are saved from cell death by pre- or postsynaptic activity block that results in polyinnervation of the muscles (Laing and Prestige, 1978; Pittman and Oppenheim, 1979; Oppenheim, 1981). Conversely, electrical stimulation of the nerve results in enhanced neuronal cell death (Oppenheim and Nuñez, 1982). In Drosophila, the fact that intracellular recordings reveal 2 EJP sizes with different stimulation thresholds (Jan and Jan, 1976) suggests the presence of at least 2 motoneurons. However, we found that the increase in branching observed in hyperex-

citable mutants was in higher-order branches and not in the number of primary branches or branches emerging from the nerve (Fig. 7). This suggests that the increased size of the projection is probably not due to changes in the number of motoneurons innervating the muscles.

In adult vertebrate skeletal muscle, it has been found that blocking pre- or postsynaptic activity produces sprouting of motor endings (Brown and Ironton, 1977; Holland and Brown, 1980). We found that increasing rather than blocking activity seems to produce an increase in the size of muscle projections in Drosophila. Furthermore, the mutation nap, which decreases excitability, slightly reduces the branching of the projection. Similar to our studies, in crustacean neuromuscular junctions, an increase in the number of varicosities as a result of chronic stimulation has been reported (Lnenicka et al., 1986). One possibility to explain the observation in vertebrates is that, in those studies, activity was drastically blocked. In this and crustacean studies, only the activity level was modified.

Possible mechanisms

A question that remains to be answered is what aspect of neuronal activity could produce the morphological changes observed. Many studies have shown that synergistic interactions between eag and Sh or between Hk and eag increase the amount of neurotransmitter released many fold. In addition, it has been shown that these mutants show repetitive spontaneous action potentials (Ganetzky and Wu, 1983, 1985; Zhong and Wu, unpublished observations). In this report, the endogenous (presumably neurally evoked) activity was recorded extracellularly, and we were not able to measure parameters such as the amount of neurotransmitter released or the size of the postsynaptic potential. However, we were able to obtain information about the

CNS-evoked *temporal pattern* of activity. We found that most wild-type muscle fibers displayed a rhythmic, bursting pattern of activity. In contrast, in most *eag Sh* and *Hk eag* muscle fibers, this bursting pattern was accompanied by a strong tonic (nonbursting) activity. Again, as with the morphological changes observed, *nap^ts* also reversed the alterations in the pattern of activity in *eag Sh*. However, in contrast to the morphological changes, this reversion was only partial. In *eag Sh nap* triple mutants, the predominant type of activity was similar to that of the wild type (bursting), but a low level of abnormal tonic activity was also present. These findings suggest that the level of activity (frequency of firing, size of the response, etc.) is not the only factor to be considered in activity-dependent changes, but that the temporal parameters need also to be taken into account.

Even though the implication is that hyperexcitability generates hyperinnervation, the mechanisms underlying this phenomenon are unknown. One possibility is that activity is necessary to stabilize newly generated synapses and neurites. In the presence of relatively low levels of activity, the probability that a given synapse would be stabilized is low, and therefore only a fraction of synapses are formed (Changeaux and Danchin, 1976). Another mechanism is that there are trophic interactions between nerve and muscle. For example, a trophic factor (which could be the neurotransmitter itself) whose release and/or transport is activity dependent could mediate both the increase in synaptic sites and the branching and growth of neurites. This suggestion seems to have some support in certain crayfish motoneurons. Lnenicka and Atwood (1989) applied an axonal conduction block to a small portion of a crayfish motor nerve and chronically stimulated the unblocked portion of the nerve, either proximal (near the neuronal cell body) or distal (near the neuromuscular synapse) to the block. They found that long-term physiological changes in the motoneuron nerve terminal activity (from phasic to tonic) could be produced only if the activity of the motoneuron cell body was altered. Changes of activity only in the axon and synaptic terminals did not produce any long-term change. In some vertebrate preparations, neuronal excitation is known to bring about gene expression in the nucleus via changes in the levels of second messengers (Morgan and Curran, 1989).

In vertebrates, it has been proposed that synapses undergo continuous turnover. In neuromuscular junctions, presynaptic terminals unapposed to postsynaptic specializations (newly formed terminals), and postsynaptic specializations unapposed by presynaptic terminals (regressing synapses) are normally seen (Wernig et al., 1980; Anzil et al., 1984; Wernig and Fisher, 1986), though the molecular mechanisms that might regulate this dynamic process are still unknown. In the present study, we only looked at neuromuscular junctions of third-instar larvae. Therefore, we do not know the time course of the changes observed, for example, whether in the wild type there could be an early period of hyperinnervation followed by pruning, and whether this pruning is altered in hyperexcitable mutants. However, Johansen et al. (1989b) have looked at the embryonic development of the innervation of the body-wall muscles. At least up to first instar, they did not see any profuse ramification of neurites like the ones observed in this report. In this regard, it is interesting to note that we found an anteroposterior gradient in the degree of branching of nerve terminals. A parallel gradient in the levels of activity at certain developmental stages could explain these segmental differences. This hypothesis could be

tested in our system by, for example, eliminating nerve activity in temperature-sensitive mutants for a relatively short period and examining the changes produced in the projections.

Based on our results, it can be suggested that the number of synaptic sites and the degree of branching of a given neuron could be the result of the degree of electrical activity of that neuron. Such a mechanism could provide the basis for both developmental and physiological plasticity. In *Drosophila,* a collection of mutations that affect different cellular growth and regulation mechanisms is available for further exploration of this activity-dependent phenomenon.

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