

# A QUANTITATIVE STUDY OF SYNAPSES ON MOTOR NEURON DENDRITIC GROWTH CONES IN DEVELOPING MOUSE SPINAL CORD

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## ABSTRACT

The proportion of synaptic contacts occurring on dendrites as well as on dendritic growth cones and filopodia was determined from electron micrographs of developing mouse (C57BL/6J) spinal cord. Comparable areas of the marginal zone adjacent to the lateral motor nucleus were sampled from specimens on the 13th–16th days of embryonic development (E13–E16). At the beginning of this period, synapses upon growth cones and filopodia comprise about 80% of the observed synaptic junctions, but this proportion decreases with developmental time so that in E16 specimens growth cone synapses account for slightly less than 30% of the synaptic population. Conversely, at E13, synapses upon dendrites comprise less than 20% of the total number of synapses, but increase with developmental time so that they account for about 65% of the synaptic population of E16 specimens.

From these data, we suggest the following temporal sequence for the formation of synaptic junctions on motor neuron dendrites growing into the marginal zone. New synapses are initially made upon the filopodia of dendritic growth cones. A synaptically contacted filopodium expands to become a growth cone while the original growth cone begins to differentiate into a dendrite. This process is repeated as the dendrite grows farther into the marginal zone so that synapses originally made with filopodia come to be located upon dendrites. This speculation is briefly discussed in relation to the work and ideas of others concerning synaptogenesis and dendritic development.

## INTRODUCTION

Several investigators studying developing central nervous system (CNS) with the electron microscope have recently described synapses upon dendritic growth cones and filopodia (1–4). However, few inferences have been drawn by these investigators concerning the possible significance of growth cone synapses for mechanisms of synaptogenesis and dendritic maturation. One reason for this may be that the proportion of the synaptic population comprised by growth cone

synapses at sequential developmental ages is not known. While Mores's (5, 6) light microscope investigations of Golgi-stained developing CNS have indicated that close associations between dendritic growth cones and afferent axon terminals are common, it is unclear from ultrastructural studies whether or not synaptic junctions with growth cones represent a common phenomenon.

In our view, adequate criteria for classifying growth cones in electron micrographs have now

been established by studies of both *in vitro* (e.g., see references 7, 8) and *in vivo* (e.g., see references 1, 2, 9, 10) systems to allow for a reliable classification of isolated profiles as either dendritic growth cones or dendrites. Consequently, we have carried out a quantitative study of synapses in the motor neuropil of developing mouse spinal cord in an attempt to define the percentage of synaptic junctions occurring on growth cones and filopodia as compared with the percentage occurring on dendrites.

#### MATERIALS AND METHODS

The animals used in this study were fetuses of the inbred mouse strain C57BL/6J. Adult male and female mice were placed together in breeding cages for 4 h during the night period of a reversed day-night cycle. The females were then separated from males and examined for the presence of vaginal plugs. 24 h after a vaginal plug was observed was designated as embryonic day 1 (E1).

Fetuses were removed from their anesthetized (0.5–0.8 ml of 3.5% chloral hydrate per 25–30 g body weight, intraperitoneally) mothers by Caesarean section on embryonic days 13–16. The spinal cords of these fetuses were fixed for electron microscope examination by perfusion with a fixative consisting of 2% glutaraldehyde, 0.5% acrolein, 2% methanol-free formaldehyde, and 0.5% dimethyl sulfoxide in a 0.15 M cacodylate buffer containing 0.003 M  $\text{CaCl}_2$ . The pH of the final fixative was 7.2–7.4 and the solution was used at room temperature. Specimen perfusion was carried out in the following manner: The umbilical cord was cut and the fetus was placed in a small well underneath a dissecting microscope. The thoracic cavity was opened and a 30- or 32-gauge needle, attached to the perfusion apparatus, was driven into the heart using a micromanipulator. The right atrium was cut for return flow. Perfusion flow was produced by regulated air pressure and monitored by a microdrip chamber. Flow rates varied from  $\sim 0.1$  ml/min for E13 fetuses to  $\sim 1$  ml/min for E16 fetuses and the duration of vascular perfusions was generally 15–30 min. After vascular perfusions, the needle was withdrawn from the heart and inserted into the lateral cerebral ventricle. Approximately the caudal one-third of the fetus was then cut off and the fixative was perfused through the ventricular system at flow rates of  $\sim 0.1$  ml/min for about 30 min.

Specimens were allowed to fix *in situ* for 4–5 h at room temperature with gentle agitation before being placed in fresh fixative overnight at 4°C. The next day the fifth and sixth cervical segments were removed from the fetal spinal cords and were placed in fresh fixative for an additional 24 h at

4°C. After primary fixation, the specimens were briefly rinsed in the same buffer as that used in the fixative and were then postfixed in 2%  $\text{OsO}_4$  (same buffer as above) for 2 h at room temperature. Subsequent to  $\text{OsO}_4$  postfixation, the specimens were briefly rinsed in buffer, dehydrated, and embedded in Epon-Araldite.

Ultrathin, transverse sections of the entire spinal cord face were cut with a diamond knife on an ultramicrotome. Serial sections were made from smaller block faces of an E14 specimen in order to confirm the identification of dendritic growth cones and filopodia. All sections were picked up on slot ( $1 \times 2$  mm) grids which had been previously coated with a carbon-stabilized Formvar film. The sections were stained with ethanolic uranyl acetate and lead citrate (11).

For the quantitative aspects of this investigation, the dorsolateral margin of the lateral motor nucleus was located (see reference 12) and 10–12 nonoverlapping electron micrographs of each specimen were taken at a primary magnification ( $\times 8,000$ ) insufficient to discern synaptic junctions clearly on the viewing screen. In the case of E13 specimens, the sample area extended about 50  $\mu\text{m}$  from the lateral-most motor neuron somata to the glia limitans. In older specimens, the lateral limit of the sample space was also approximately 50  $\mu\text{m}$  from the edge of the lateral motor nucleus despite the fact that the glia limitans had not been reached. The negatives of these samples were enlarged photographically 2.5 times so that synaptic junctions were readily identifiable. The number of synapses occurring on profiles designated as (a) dendritic growth cones or filopodia, (b) dendrites, and (c) unclassifiable profiles were counted, and the proportion of synaptic junctions formed with each category of postsynaptic element was calculated. Spinal cords from two littermates at each embryonic age were analysed for quantitative purposes.

#### OBSERVATIONS

##### *Identification of Motor Neuron Dendritic Growth Cones and Filopodia*

During the period of spinal cord development studied, it is common to encounter longitudinally sectioned profiles of dendrites emerging from the lateral aspect of the lateral motor nucleus. It is also possible to find such profiles in continuity with motor neuron somata (Fig. 1). For this reason, as well as ones we have presented elsewhere (12), we think it is reasonable to conclude that these profiles represent dendrites of motor neurons. In the region sampled in this study, bundles of these dendrites commonly run dorsally for some distance

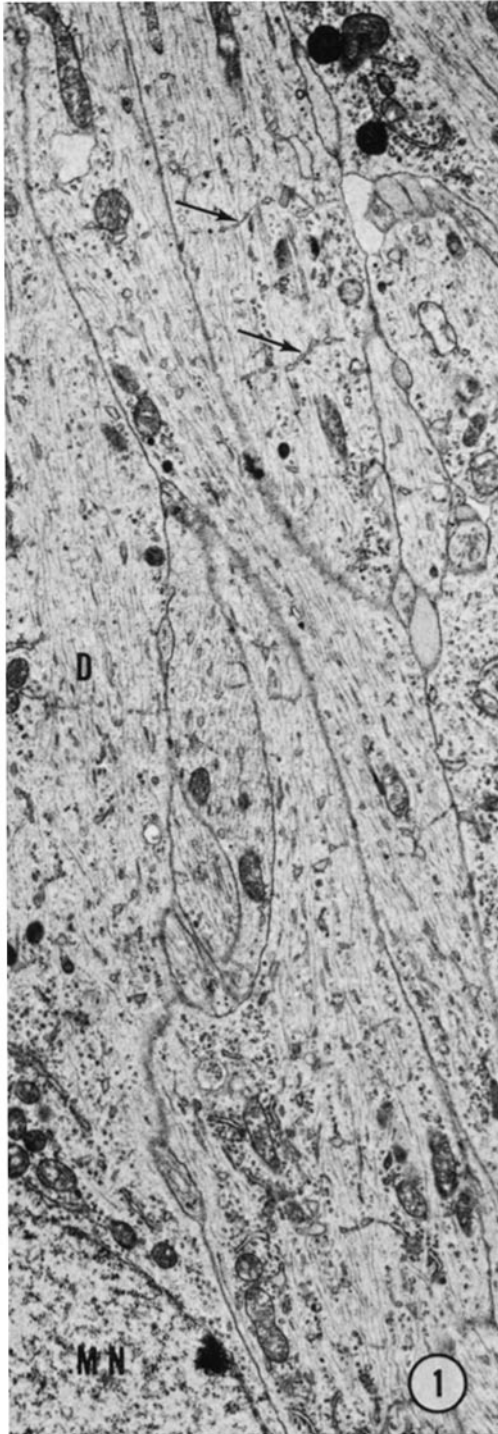


FIGURE 1 E14 spinal cord, lateral motor nucleus. A proximal dendrite (*D*) is shown in continuity with a motor neuron (*MN*) soma located near the marginal zone. This dendrite runs parallel with a number of

other dendrites from the lateral motor nucleus. The arrows denote transversely oriented, smooth-surfaced cisternae located within dendritic cytoplasm (also see Fig. 2).  $\times 9,300$ .

at the interface between the somata of the lateral motor nucleus and the adjacent marginal zone; they then bend laterally to course through the axonal profiles of the marginal zone. In their more proximal regions, the dendrites are characterized by ribosomes, mitochondria, and by longitudinally oriented microtubules and smooth-surfaced cisternae which intersect with transversely oriented, smooth-surfaced cisternae (Fig. 1). More distally, the dendrites gradually diminish in diameter and come to be characterized mainly by longitudinally oriented microtubules and by occasional ribosomes and smooth-surfaced cisternae (Figs. 2, 3). As the dendritic bundles penetrate into the marginal zone, an additional type of profile begins to occur. These new profiles are usually larger than those of the neighboring dendrites, have few microtubules, lack mitochondria, and contain a cytoplasmic material that appears to be arranged in a loose feltwork (e.g., see Figs. 4, 5). Frequently, large-bore, smooth-surfaced cisternae, vacuoles, and large vesicles are also located within these profiles (Figs. 2, 5, 6). We have observed such profiles to be in continuity with dendrites (Figs. 2, 3) and serial sections show that these profiles occur at the ends of dendrites as well as in more proximal dendritic regions. Generally, the cytological characteristics of these profiles are similar to those described for neurite growth cones in vitro (7, 8) and in vivo (9, 10) and, specifically, they are in accord with descriptions of dendritic growth cones in vivo (1, 2). Slender projections (Fig. 4) can be seen extending from such growth cone profiles and these projections are characterized by the presence of a filamentous material that appears to be oriented parallel with the long axis of the projections. The ultrastructure of these projections is identical to that of the dendritic growth cone filopodia in developing mouse olfactory bulb (1) and the "microspikes" projecting from dorsal root ganglion cell growth cones in vitro (7). Synaptic junctions occur upon dendrites (Fig. 3), dendritic growth cones (Figs. 3, 5, 6), and growth cone filopodia (Fig. 4). Occasionally we have observed presynaptic components of synaptic junctions whose cytological appearance (Fig. 5) suggests that they too may be growth

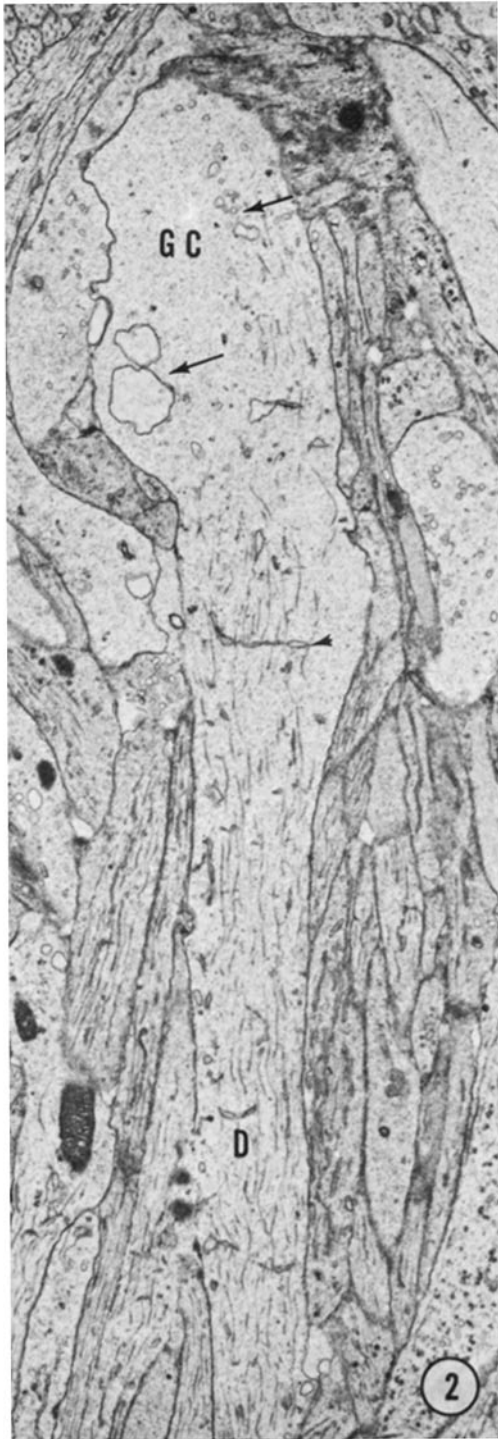


FIGURE 2 An electron micrograph of a more distal portion of a dendritic bundle from the same specimen as shown in Fig. 1. A dendritic profile (*D*) expands into a structure exhibiting the cytological features of a

cones or, at least, be located close to the growing portion of axons.

### *Quantitative Analysis of Growth Cone Synapses*

For quantitative purposes, dendritic growth cones and filopodia were both grouped as a single postsynaptic type that we have designated "growth cones" (Fig. 7). When the portion of a postsynaptic profile included within the section was too small to contain sufficient cytological details for accurate identification, it was designated as unclassifiable. As can be seen in Fig. 7, our data indicate that synapses upon the growth cones of E13–E16 mouse spinal cords constitute an appreciable proportion of the total synaptic population. The bulk of the synapses in E13 specimens are located upon growth cones (~80%), but the proportion of synapses in this category decreases during the next 2 embryonic days until it is slightly less than 30% of the total number of synaptic junctions observed in E16 specimens (Fig. 7). Conversely, synapses on dendritic profiles account for less than 20% of the total number of synaptic contacts at E13 and the proportion of synapses in this category increases during the next 2 embryonic days until dendritic synapses represent approximately 65% of the synaptic junctions counted in E16 specimens (Fig. 7). As was mentioned above, the sample area for specimens of all 4 embryonic days was held approximately constant.

### DISCUSSION

We interpret the temporal relationship observed between the proportions of growth cone and dendritic synapses in E13–E16 specimens (Fig. 7) to mean that presynaptic boutons initially make contacts with dendritic filopodia and growth cones, and that, as these synapses are formed, the dendritic growth regions move farther distally into the marginal zone leaving behind the synapses that have already been made. We envision this as taking place in the following sequence. The region of filopodial membrane where a synaptic contact is made becomes fixed with relation to its distance along the dendrite. Growth occurs by a

growth cone (*GC*) including smooth-surfaced vesicles (upper arrow) and vacuoles (lower arrow). The dendrite contains a transversely oriented, smooth-surfaced cistern (arrow head) near its transition into a growth cone.  $\times 14,000$ .



FIGURE 3 A small dendrite (*D*) expands into a growth cone (*GC*) in the marginal zone adjacent to the lateral motor nucleus of an E14 specimen. The dendrite receives a synaptic contact (*1*) and another synaptic junction (*2*) is located near the transition of the dendrite into its growth cone. Two more synapses (*3* and *4*) are found on a profile which is clearly an extension of the growth cone as seen in serial sections.  $\times 23,000$ .

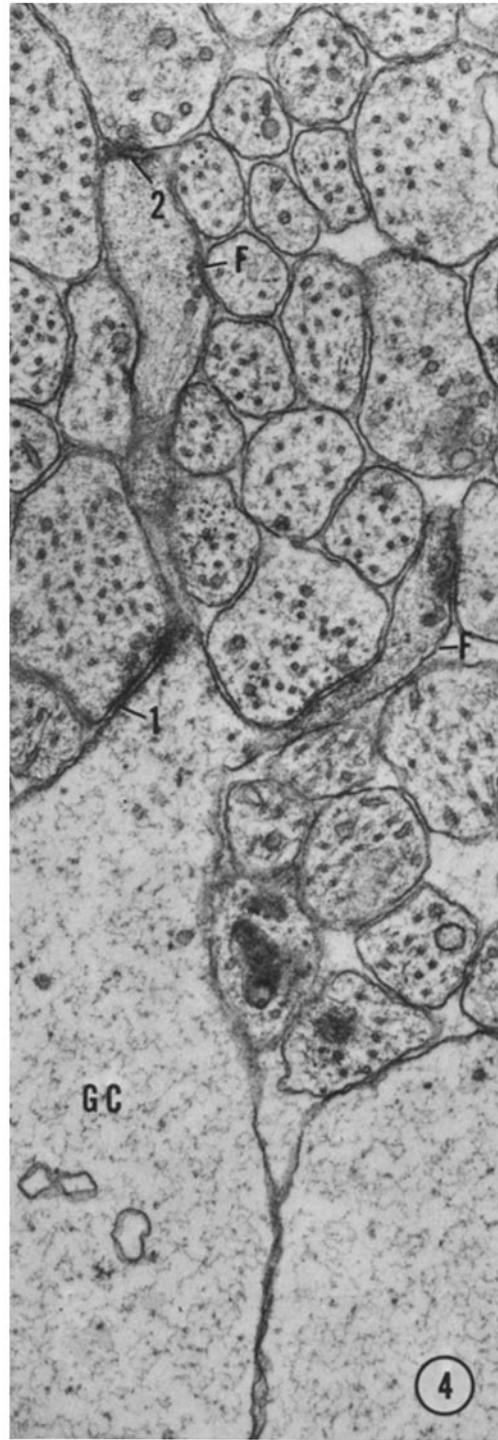
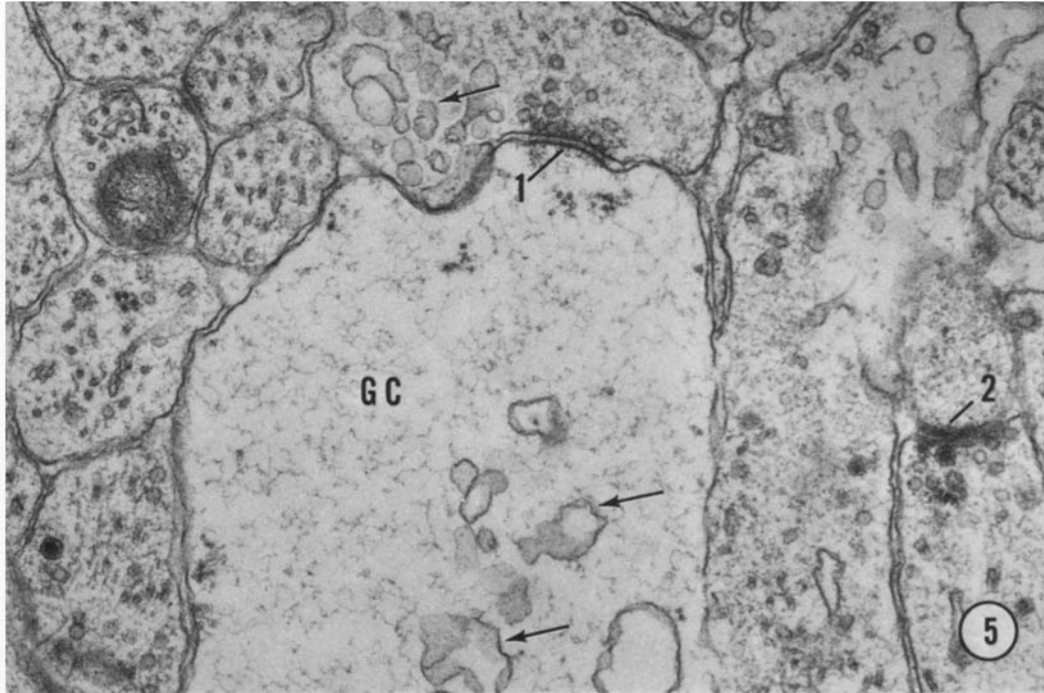


FIGURE 4 A higher magnification electron micrograph of a dendritic growth cone (*GC*) which gives rise to two filopodia (*F*). The uppermost filopodium is continuous with the growth cone as determined from adjacent serial sections. This particular section was chosen because it shows the synaptic contact (*2*) with the filopodium most clearly. Another synaptic junction (*1*) occurs on this growth cone at the base of a filopodium. E14 spinal cord.  $\times 36,500$ .



**FIGURE 5** A profile of a dendritic growth cone (*GC*) in the marginal zone adjacent to the lateral motor nucleus of an E14 spinal cord. Notice the feltwork of a fine cytoplasmic material and the profiles of smooth-surfaced cisternae or vacuoles (arrows) present within the growth cone's cytoplasm. A synaptic contact (1) is located on this growth cone and the presynaptic element also contains smooth-surfaced vesicles (arrow). What appears to be a second synaptic junction (2) is located in the lower right of this figure and the size and finely fibrillar appearance of the postsynaptic element suggest it may be a growth cone filopodium.  $\times 39,200$ .

protoplasmic movement from the growth cone into the synaptically contacted filopodium and as this filopodium expands into a new growth cone, the former growth cone develops the characteristics of a dendrite. The new growth cone then gives rise to new filopodia and the process repeats so that synapses initially made on filopodia come to reside sequentially upon growth cones and then upon dendrites. This idea is presented diagrammatically in Fig. 8, and it is consistent with Bray's (13) observations of the behavior of small particles attached to the elongating neurites of sympathetic neurons *in vitro*. Bray states that particles on growth cones "invariably" were observed to undergo a "backwards" or proximal motion. Thus, Bray's (13) work provides a precedent that structures becoming attached to growth cones are not necessarily propelled along with the growth cone in the direction of its advance. The idea that dendritic filopodia may be a preferential site for new synaptic contacts is in accord with the ob-

servations of Hayes and Roberts (4) that the earliest synapses in developing amphibian spinal cord are usually located upon "very small dendrites which might be filopodia from dendritic growth cones" and that with developmental time, more and more synapses are observed upon larger dendritic profiles. Hayes and Roberts (4) state this sequence suggests that postsynaptic components may increase in size after they have made a synaptic contact.

We believe the present study provides support for the idea (cf. references 5 and 6, for a thorough discussion) that the patterns of distal dendritic growth into their synaptic fields may be controlled to some extent by whether or not dendritic growth cones and, probably even more importantly, their filopodia come into synaptic relations. An obvious corollary of this speculation is that dendritic growth into zones not containing appropriate synaptic terminals would be retracted (or degenerate) when the growing dendrites fail to receive



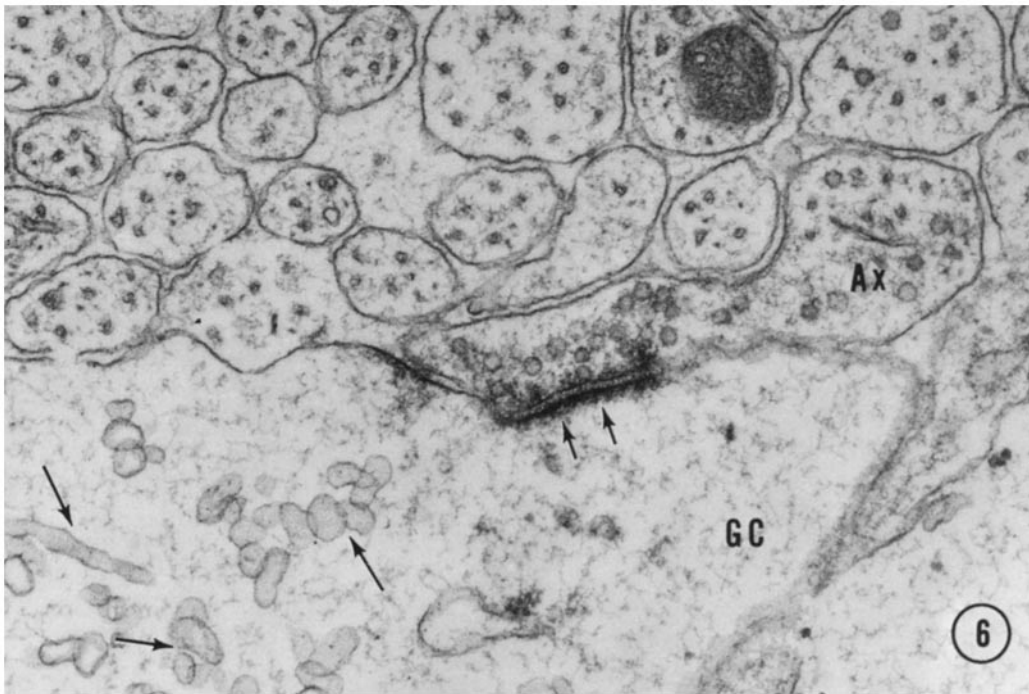


FIGURE 6 Same spinal cord region and specimen age as Fig. 5. A relatively well-differentiated synaptic junction (double arrows) is shown between a dendritic growth cone (GC) and what appears to be a collateral branch of a transversely sectioned axon (Ax). The single arrows point out smooth-surfaced cisternae or vacuoles within the growth cone.  $\times 44,000$ .

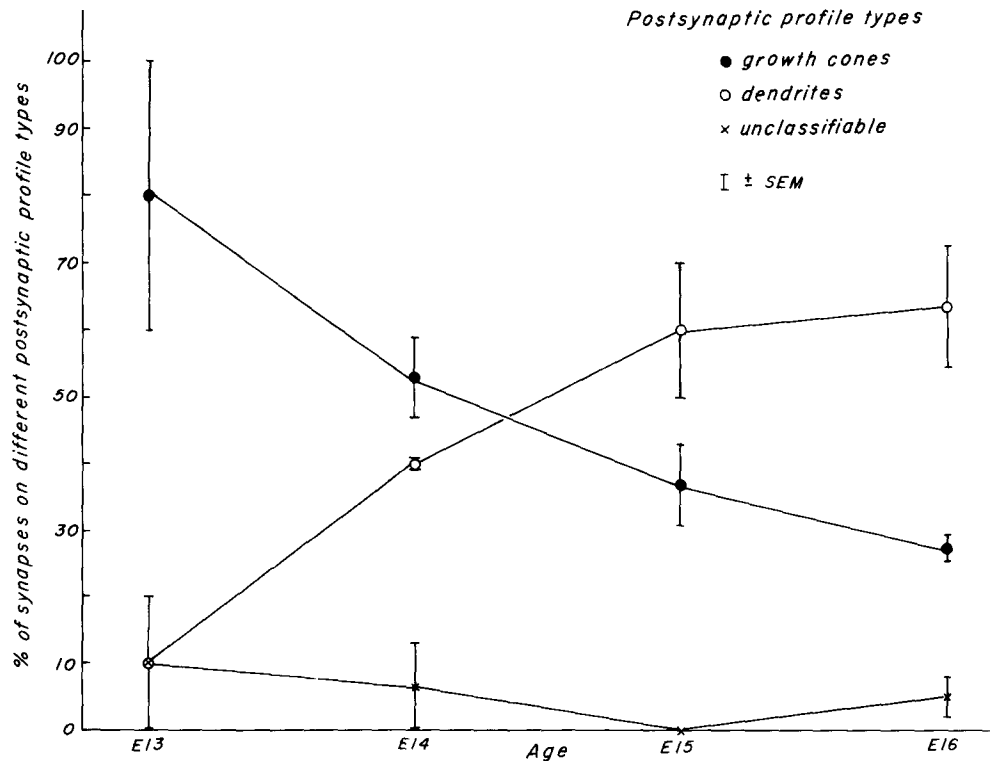


FIGURE 7 Graph showing the proportion of the total number of observed synapses comprised by different types of postsynaptic elements on 4 sequential days of development. Synapses upon both growth cones and growth cone filopodia have been grouped under the growth cone designation used in this figure. Each point represents the mean value of two littermates.

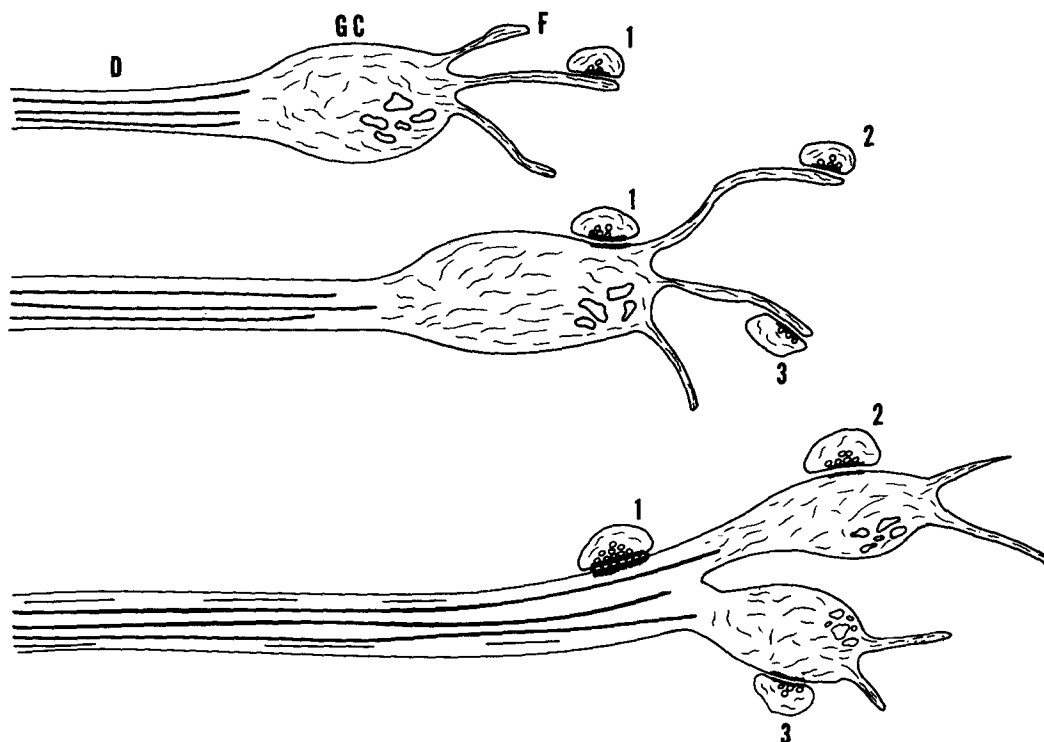


FIGURE 8 A diagrammatic representation of the temporal sequence suggested in the text for the formation of synaptic junctions on motor neuron dendrites. The abbreviations are the same as those used in previous figures. Common numbers are used to designate the same synaptic junction at different stages of dendritic growth.

synaptic validation that they are probing into appropriate synaptic fields.

Evidence has been presented by others (5, 6, 14-16) which indicates that the presence of the fibers and/or terminals of appropriate afferents may be responsible for "inducing," as well as for orienting, the dendritic development of target neurons and that synaptic or other physical contacts may be involved in such an induction (e.g., see reference 15). In the case of the spinal cord regions we are studying, axons are present within the early marginal zone bordering the motor nuclei before the time when dendritic profiles are obvious, and this is at least temporally consistent with a postulated induction of dendritic growth by their afferent fibers. However, in the spinal cord, it is not clear whether or not synaptic contacts occur on motor neuron somata early enough in development to qualify as possible inducing contacts. At the earliest stages which we have examined in detail (E13-E14), relatively long dendritic extents usually occur between motor

neuronal somata and the region where synaptic contacts become common. While this might argue against the necessity of physical contacts as part of a presumptive "induction" of motor neuron dendrites by afferent nerve fibers, it may be that the first and "inducing synapses" occur on or near somata before embryonic day 13. These synapses might then move along for some distance with the growth buds forming the proximal dendrites before they are left behind. While there is evidence that this may be the way in which the climbing fiber terminals of the cerebellum attain their final positions on the proximal dendrites of Purkinje cells (14, 15), a comprehensive analysis of dendritic and synaptic development before embryonic day 13 has to be carried out before data from fetal mouse spinal cord can be brought to bear upon this point.

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