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Studying the Forest, Not The Trees: Dendritic Morphology In Populations Of Neurons

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Introduction

In his foreword to the English translation of Ramon y Cajal's Histology of the Nervous System, W. Maxwell Cowan ascribes Cajal's genius in understanding neuronal structure and its functional consequence to his ability to "recognize representative trees within a forest of neurons and connections" [18, p.vi]. Establishing the morphological and functional characteristics of individual neurons has indeed been extremely valuable in our elucidation of the structure and function of the nervous system, but something may be lost in the study of these individual "trees". For several years, we have been studying the development and adult plasticity of dendritic morphology in small populations of spinal motoneurons in rats, (Fig.l) visualized after retro-

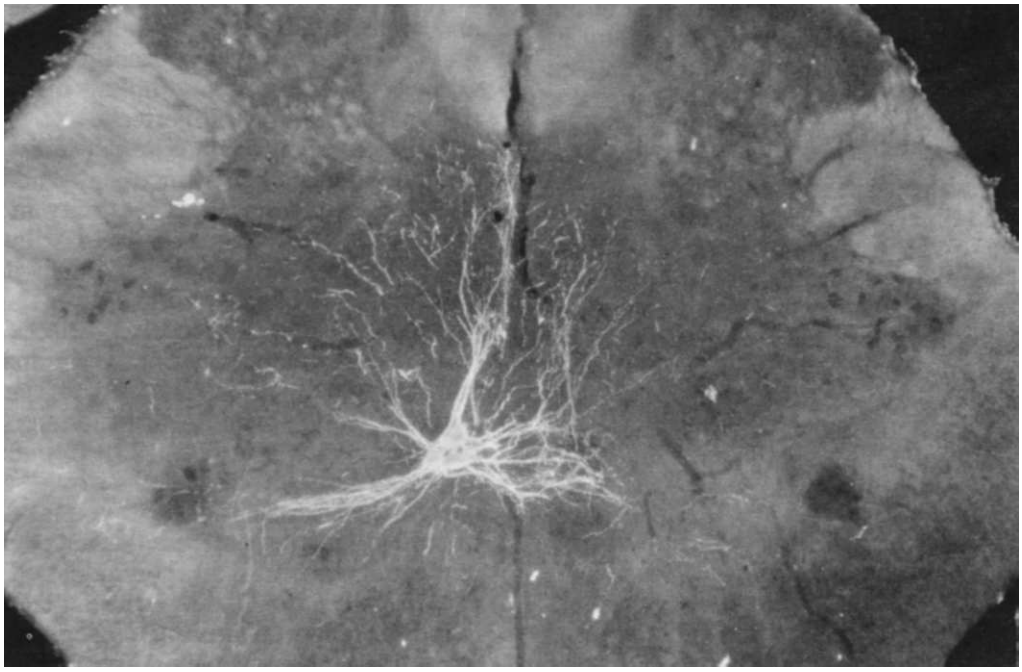
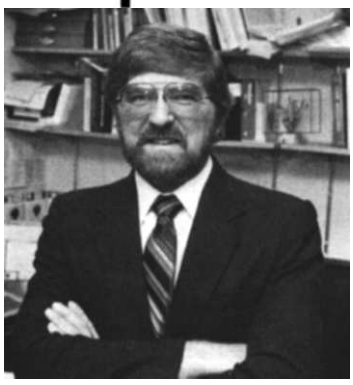


Figure 1. Darkfield photomicrograph of a transverse section through the lumbar spinal cord of a male rat showing retrogradely motoneurons after BHRP injection into the bulbocavernosus muscle.



Editor's Column

Is it really almost the last of summer? As I write this, it is the day after Labor Day and the swimming pools have closed for the season here in Kansas City. Baseball is still being played, but the Kansas City Royals are not doing well. The Kansas City Chiefs football team is a question mark for the impending season. Yes, it is almost the end of summer. There are other signs of summer demise; the leaves are showing some age, the sun does not seem to come up as early, and the baby racoons in the racoon family that frequents our back deck, have almost grown up.

It has been a busy summer but I am not sure I am really ready for the start of the school year (but it has started), conventions, talks and so forth. But they are here. On one of our trips this summer, we went to Montreal. What a beautiful city - so historical, so cosmopolitan. I really enjoyed lunch at a sidewalk cafe in the old town, then a wonderful dinner in a small, elegant restaurant. Delightful! We look forward to seeing many of you at the Society for Neuroscience meetings in Miami at the end of October. Please stop by the booth (see the back page for booth numbers) to see the new items and to make sure a new catalog is sent or pick one up there).

The article in this issue of the Carrier is a very interesting and timely one. Dr. Sengelaub and his student have given a great overview of their methods of studying dendritic morphology. Not only is the topic interesting and on the forefront of our field, but their insights are certain to be of real help to many in the area, and come from a vast amount of experience.

Please come by to see us at the Society for Neuroscience Meeting.

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grade labeling. In this article we will outline our method for studying three dimensional dendritic morphology in populations of neurons, describing the process, pluses, and pitfalls of this approach.

Why study populations?

Electrophysiological recording from single units provides information about the bioelectrical behavior of individual neurons, which may be more fully understood in consideration of how ensembles of neurons process information; indeed, the techniques and approaches for simultaneous recording from multiple neurons are on the rise [16]. Anatomical study of neuronal morphology at a population level can be equally enlightening, potentially providing information that could be obscured in the examination of individual neurons. Assessing the morphology of individual neurons, whether it be by classical Golgi methods or contemporary intracellular labeling techniques, has been greatly aided by the advent of computer-based morphology systems that integrate microscope images with video displays, and utilize sophisticated and powerful analysis-data is still a difficult and/or time consuming process. As a result, the number of neurons collected from an individual animal (often as few as one) or overall within a study and analyzed is understandably small, and the conclusions reached rely on the assumption that the chosen neurons are representative of the populations they are drawn from. However, variability in the population being assessed or sampling bias can potentially weaken (or create) effects.

Individual neurons are in fact individuals, and as individuals their functional and structural characteristics, as well as plasticity in those features, can differ dramatically. To illustrate this point, Figures 2 through 4 contain data drawn from our and other's work. Figure 2 shows the distribution of total dendritic lengths measured from Golgi impregnated hippocampal neurons from aged (22 month old) male rats, with or without supplemental testosterone treatment (n=30 neurons per group) [20]. In male rats, testosterone levels at 22 months of age are significantly lower than those of young adults, and treatment with testosterone clearly increases the average lengths of CA1 pyramidal cell dendrites. Additionally, there is both substantial variance in dendritic length as well as considerable overlap in both groups, indicating that individual neurons clearly show differential responses to normal or manipulated hormone levels. Figure 3 shows the distribution of basilar dendritic lengths measured from Golgi-impregnated frontal cortical neurons from lamina II-III in adult male rats, after either sham or

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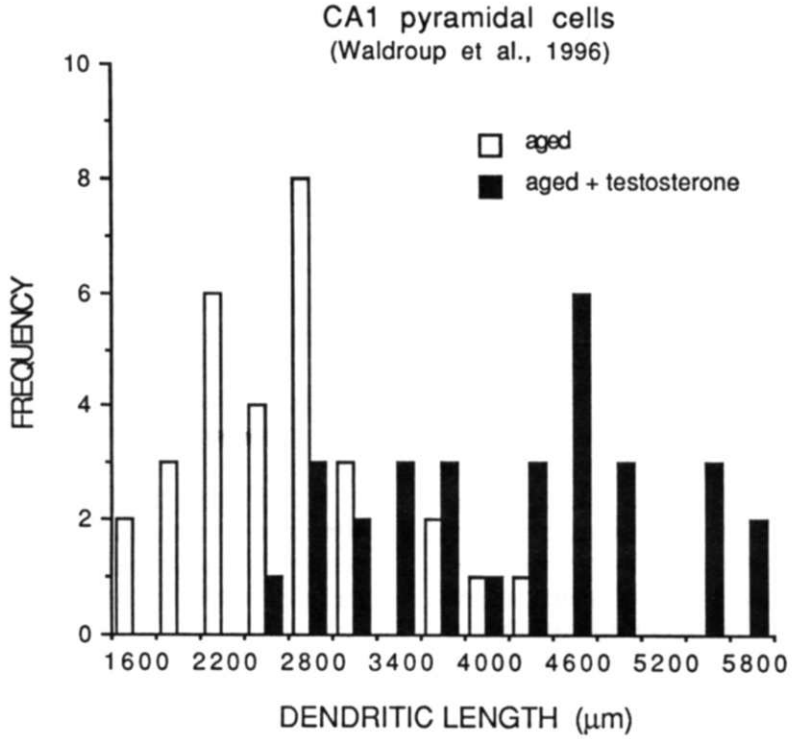

Figure 2. Individual variability in total dendritic lengths and responses to manipulation measured from Jjplgi impregnated hippocampal neurons from aged (22 month old) male rats, with or without supplemental testosterone treatment (n=30 neurons per group). Data from [20],

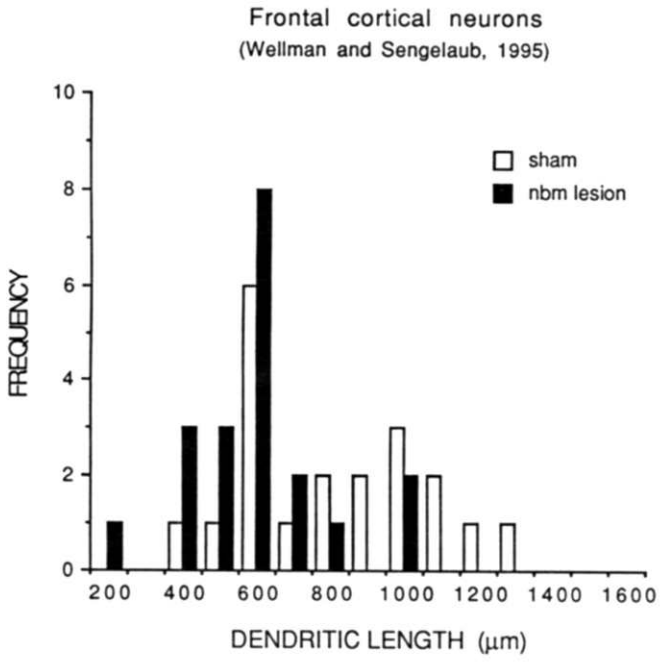

Figure 3. Individual variability in basilar dendritic lengths and responses to manipulation measured from Golgi impregnated frontal cortical neurons from lamina II-III in adult male rats, after either sham or excitotoxic lesions to their basal forebrain (nbm; n=20 neurons per group). Data from [21].

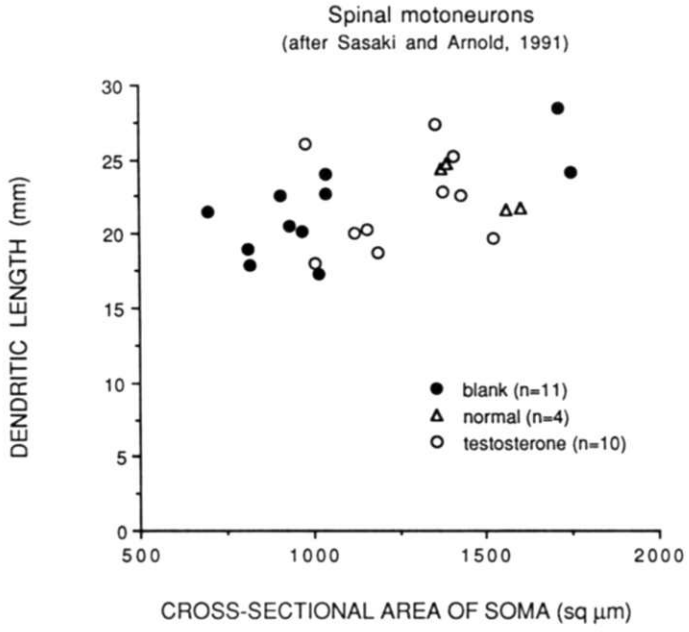

Figure 4. Individual variability in total dendritic lengths and responses to manipulation measured from intracellularly filled spinal motoneurons from intact adult male rats, or castrated adults with or without testosterone replacement (n=4-ll neurons per group). Data after [19].

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excitotoxic lesions to their basal fore-brain (nucleus basalis magnocellularis; n=20 neurons per group) [21]. The basal forebrain is the primary source of cholinergic input to the frontal cortex, and lesions of the nbm result in a decline in average dendritic length in lamina II-III cortical neurons. Again, however, there is substantial variance in dendritic length as well as considerable overlap in the groups, indicating that individual neurons clearly show differential responses to cholinergic depletion. Finally, Figure 4 shows the distribution of total dendritic lengths measured from intracellularly filled spinal motoneurons from intact adult male rats, or castrated adults with or without testosterone replacement (n=4-ll neurons per group) [19]. As in the previous examples, there is both substantial variance in dendritic length as well as considerable overlap across the groups, indicating that individual neurons clearly show differential responses to normal or manipulated hormone levels. In all the above cases, the observed differential responses suggest that individual neurons are not necessarily representative of the populations they are drawn from, and small samples are especially vulnerable to the obvious consequences.

How neurons are selected for individual morphological analysis is another factor of major importance, and typically relies on a judgment by the investigator of whether candidate neurons appear "well impregnated" [17] or have dendrites filled "to their natural endings" [19]. Intracellular labeling techniques often depend not only on selecting neurons that are considered to be completely filled but on limiting which neurons get filled initially by physiological criteria such as stable membrane potentials or spike heights above particular values [15]. Because these selection criteria can be somewhat subjective or biased by size or other arbitrary criteria, they further compound the problem of inferring the general structure, function, and plasticity of a class of neurons from a sample of individual cells.

Methods

In studying the morphology of dendrites from a population of motoneurons within an animal, we have relied on retrograde labeling techniques to uniquely identify groups of motoneurons for subsequent analysis. This has proved particularly useful in examining classes of motoneurons that reside within nuclei containing mixed populations, each with their own peripheral target and unique structural/functional/plastic response natures. We are especially fond of horseradish per-oxidase conjugated to cholera toxin B subunit (BHRP, List Biological, Campbell, CA). Labeling populations of motoneurons with BHRP solves the two potentially confounding problems mentioned above; large numbers of motoneurons are labeled within each animal, limiting the degree to which individual neuronal variability can influence the results, and there can be no selection bias as to which motoneurons will be analyzed.

Labeling with BHRP permits sensitive detection and quantitative analysis of dendritic morphologies [10,3,9,2]. Injections of BHRP are usually made by simple pressure injection (0.05-0.5 pi, 0.2% solution) into the target muscle of choice, and because BHRP is not transported by primary muscle afferents, the only labeling that results in the spinal cord is retrograde labeling of the motoneurons and their dendritic arbors. After an appropriate survival time (typically 48 hours) to allow optimal labeling of the motoneurons, animals are deeply anesthetized and perfused intra-cardially with saline followed by cold 1% para-formaldehyde/1.25% glutaraldehyde. Spinal cords are removed, postfixed in the same solution for five hours, then transferred to sucrose phosphate buffer (10% w/v) overnight for cryop-rotection. Mixed groups of spinal cords are then embedded in gelatin and frozen-sectioned transversely at 40 |jm into four alternate series; every section is collected, with serial order maintained within each series. For visualization of BHRP, the tissue is immediately reacted using a modified tetramethyl benzidine protocol (TMB)[14], mounted on gelatin-coated slides, and counter-stained with thionin. Yoking groups together through sectioning and histochemistry protects against artifactual differences due to deviations in processing. Furthermore, all material is coded and each subsequent analysis performed by a single observer blind to the treatment groups.

The number and labeling density of BHRP-filled motoneurons is assessed through the entire rostrocaudal extent of the nucleus of interest for all animals. Counts of labeled motoneurons in the nucleus of interest are made under bright-field illumination, where somata and nuclei can be visualized and cytoplasmic inclusion of BHRP reaction product confirmed. Adequacy of BHRP transport is further assessed by measuring the optical density of BHRP reaction product in motoneuron somata using a video-based morphometry system (JAVA, Jandel Scientific, San Rafael, CA), at a final magnification of 1350x.

For each animal, the morphology of labeled dendrites is assessed in alternate sections 320 pm apart in a single series through the entire rostrocaudal extent of the nucleus of interest. Dendritic tracing is performed under dark-field

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illumination, which allows for enhanced detection of small-caliber processes. BHRP-labeled fibers are traced in three dimensions using a computer based morphometry system (we currently use Neurolucida from MicroBrightField, Inc.) to yield both composite illustrations of the arbor (Figures 5 and 6) and measurements of individual fiber lengths. All BHRP-labeled fibers are drawn regardless of location, size, or contiguity with labeled cell bodies to ensure a complete assessment of dendritic morphology (final magnification, 250X). Additional measures of dendritic morphology such as the number of branches or process widths can also be easily obtained. Average dendritic arbor per labeled motoneuron is estimated by summing the measured dendritic lengths of the alternate sections, multiplying by two, then dividing the total by the number of labeled motoneuron somata in that series. This measure reflects only a fraction of the actual total dendritic label present and hence does not attempt to assess the actual total dendritic length of labeled motoneurons. However, it has been shown to be a sensitive and reliable indicator of changes in dendritic morphology in normal development [3,5], after hormonal, surgical, or neu-rochemical manipulation [2,10,12,6,7,8,9], and of morphological differences between motor nuclei [11,5].

The distribution, shape, and area of influence of the population arbor can also assessed by quantifying both the rostrocaudal and radial (me-diolateral and dorsoventral) distribution of the labeled dendrites. Morphological analyses using individual cells do not typically provide these kinds of data, as this would require systematic mapping of the population. The rostrocaudal extent of the dendritic arbor is determined after defining the rostrocaudal center of the motor nucle-

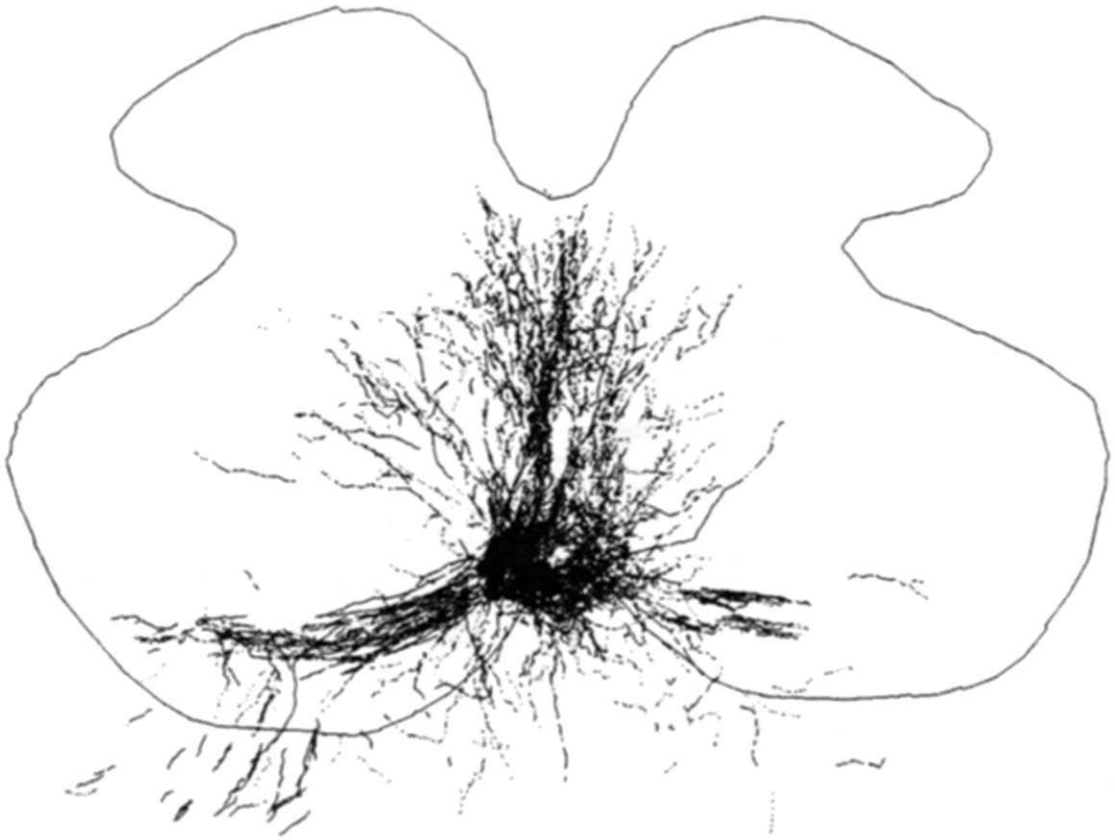

Figure 5. Computer-generated composite drawing of BHRP-labeled motoneuron somata and processes drawn at 320 urn intervals through the entire rostrocaudal extent of the spinal nucleus of the bulbocaver-nosus. Composite consists of reconstructions from 10 sections but illustrates only one eighth of the actual dendritic labeling achieved. The gray matter outline is taken from a single section in the middle of the rostrocaudal range of the label and serves only to orient the reconstruction.

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us and then recording the rostrocaudal distance spanned by labeled dendrites In the mediolateral/ dorsoventral planes, the distribution of dendritic material and the maximal radial extent of the dendritic arbor can be measured using a set of axes radially oriented around any structure of interest. We typically divide the spinal cord into 24 bins of 15 degrees each, and then measure all of the dendritic processes contained within those bins, or the distance between some reference point and the most distal BHRP-filled process. These analyses have been useful in revealing several interesting features of dendritic morphology, including preferential distribution to specific areas of the gray matter or selective changes in that distribution. For example, we often study a medially positioned population of motoneurons, the spinal nucleus of the bulbocavernosus (SNB; see Figs. 1,5,6). We found that the postnatal growth of dendrites of SNB motoneurons is bi-phasic, initially growing exuberantly and then retracting to adult lengths and distribution [3]; the retraction is most pronounced in areas where the dendritic fields from the two halves of the SNB overlap across the midline [4]. An earlier study [1] relying on Golgi labeling of individual motoneurons failed to detect either of these phenomena, most likely due to the small sample size (only 10 neurons per age) and the selection criteria used ("only neurons with the most extensive dendritic fields in each group were used", p. 78).

These measures are also useful in confirming completeness of labeling to the most distal processesreconstruction of intracellularly labeled motoneurons indicates that the highest-order branches occur at the same distances from the somata that we measure in our extent analyses. Because our technique relies on transport of BHRP throughout the targeted motoneurons, it is important to note that group differences in transport could potentially confound any result. This is an extremely important consideration, and one to which we have been especially sensitive. We have previously argued in detail that the degree

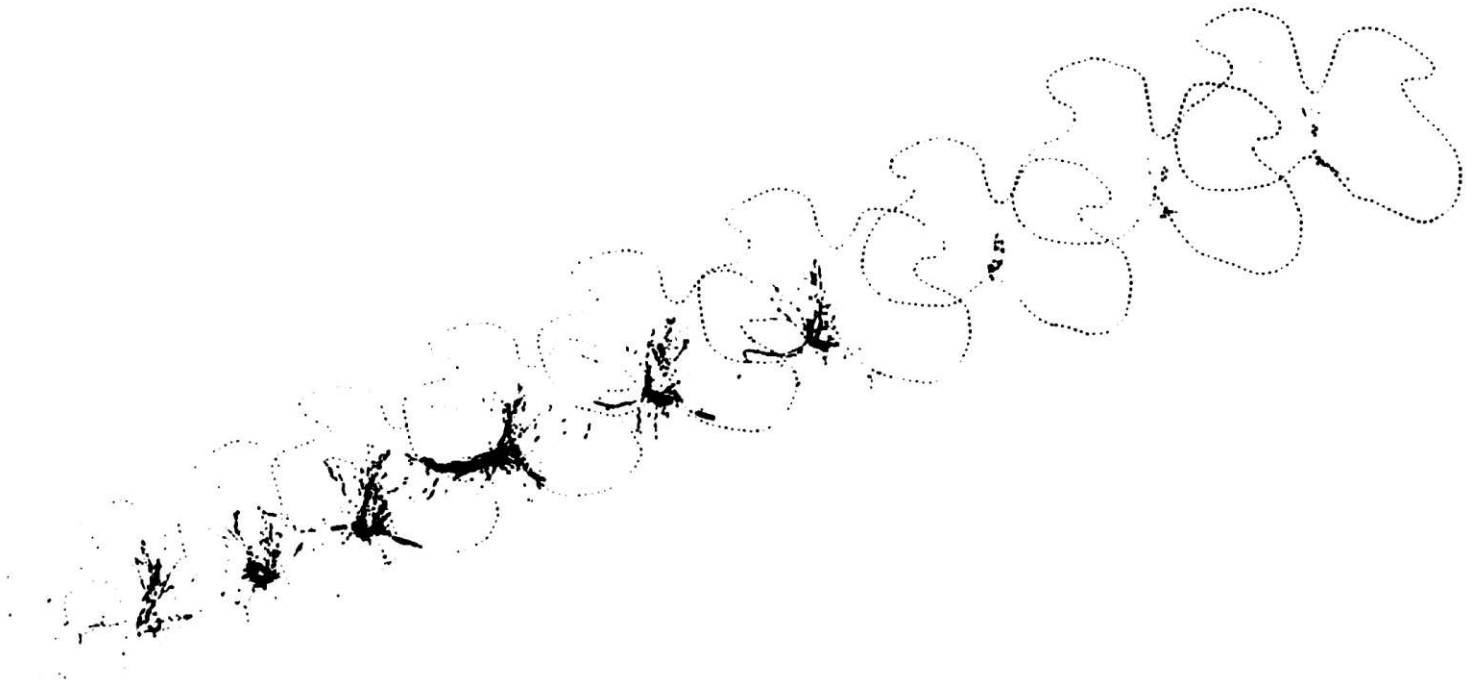

Figure 6. The same composite drawing of BHRP-labeling as in Figure 5, rotated and exploded to view differences in dendritic labeling across the population through the rostrocaudal axis.

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of dendritic labeling after a variety of hormonal manipulations is comparable, and that differences in dendritic morphology that arise between experimental groups are not likely due to transport artifacts [12,5,6]. For example, previous studies have demonstrated that neither axonal transport of BHRP [13], nor dendritic transport [12, 5] is affected by hormone levels. Retrograde labeling with BHRP across a variety of manipulations produces similar numbers of fibers filled, maximal fiber lengths, and frequency distributions of fiber length. Similarly, dendritic length and radial extent measures are unaffected by surgical manipulations. Moreover, we have never observed differences in the number or labeling density of motoneurons between groups. Overall, we are confident that the degree of dendritic labeling seen across groups is in fact comparable, and thus any differences observed reflect true differences in dendritic morphology rather than simple transport artifacts.

We believe that this multicellular level of analysis and the resulting "cumulative morphology" can be a powerful tool for elucidating the structure of neuronal dendrites that comprise a func-^»?^=rtienal population in the nervous system. Because neurons in populations must work together in the expression of their joint function, we further believe this method is an appropriate as well as sensitive tool.

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