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Axon Guidance Autonomy and Tensional Requirement for Synaptic Function: Is Learning a Forced Response?

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AXON GUIDANCE AUTONOMY AND TENSIONAL REQUIREMENT FOR SYNAPTIC FUNCTION: IS LEARNING A FORCED RESPONSE?

BY

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Abstract

Individual neurons extend multiple processes whose growth cones exhibit different responses to their environment. In culture, detached growth cones display guidance autonomy, contain mRNA for cytoskeletal and other axonal components, and are capable of synthesizing protein locally. However, the extent to which growth cone's autonomy contributes to its pathfinding function within the complex *in vivo* environment is unknown. Here, we show that detached axonal growth cones from identified *Drosophila* motoneurons maintain balanced filopodial activities as they extend, navigate and target postsynaptic partner cells normally. After detachment, the growth cones continue to synthesize the synaptic vesicle protein Synaptotagmin but, upon contacting targets, fail to concentrate it at the presynaptic site normally. However, if held by a micropipette that resupplies mechanical tension, the growth cones' ability to localize synaptic vesicles is restored. Our results demonstrate functional autonomy of axonal growth cones as they navigate and initiate synaptogenesis, while implicating intercellular tension as a novel "retrograde" mechanism to adjust synaptic function.

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Chapter 1

Growth Cone Autonomy

Summary

The neuronal growth cone is guided to its appropriate target by molecular cues in the surrounding environment. Receptors on the neuronal cell surface signal, through second messengers, to cause cytoskeletal rearrangements giving rise to pathfinding behaviors such as extension and turning. Studies in which neurites were transected by a glass pipette showed that isolated growth cones are capable of turning toward attractive cues or away from repulsive ones when they were bath applied in culture or *in situ* preparations. But would autonomy be retained *in vivo* where a growth cone must discern the proper respond to multiple, even conflicting, guidance cues by expressing the correct balance of cue receptors and downstream signaling cascades which give rise to cytoskeletal rearrangements leading to extension and navigation? To answer this question, we used a laser microbeam to detach identified motoneuron axonal growth cones from their cell bodies in one hemisegment of otherwise intact, developing Drosophila embryos to determine the extent to which they are autonomous from their cell bodies. Detached motoneuron axons extend into the periphery and navigate to their target muscles just as well as their intact counterparts. Furthermore, the speed with which filopodia extend and retract, while reduced, remains equal and the ratio of extension to retraction events is retained in detached growth cones. Thus, axonal growth cones are substantially autonomous from the cell body *in vivo* with respect to pathfinding. This level of autonomy suggests that sub-cellular regions may act quite independently of the nucleus; a

situation required most dramatically in neurons whose single nucleus must coordinate elaborate and precise morphological and physiological activities in quite disparate projections, dendrites and axons.

Introduction

In complex neural networks, a single cell can generate and maintain thousands of synapses with extreme precision, acting as both an upstream and downstream partner to many other cells. Since the genetic material from a single nucleus must orchestrate the growth cone guidance of multiple neurites (axons and dendrites) we may expect the neurites themselves to be largely autonomous once they extend away from their cell body. This study is largely concerned with determining the extent of this autonomy in navigating to and maintaining synapses with downstream partner cells.

In order to generate appropriate connectivity, neurons are uniquely capable of extending long, stable processes to find and form synapses with partner cells. In 1890 Ramon y Cajal first described the swollen tip of growing nerves of fixed chick embryos and correctly predicted the function of this tip in exploring its surroundings as the nerve sought its partners. Since then we have learned much about this remarkable structure, the neuronal growth cone, though how it carries out its functions remains the focus of active and productive study.

Cytoskeletal Anatomy of a Growth Cone

Guidance cues are received by receptors on the growth cone and transduced into extension and navigation (Dickson, 2002; Goodman, 1996). Ultimately, however, the function of the growth cone is based on rearrangements of the cytoskeletal structures they contain (Dent and Gertler, 2003; Kalil and Dent, 2005; Suter and Forscher, 2000). The growth cone can be subdivided into three domains, the Peripheral (P), Transitional (T) and Central (C) domains, based on their cytoskeletal and morphological characteristics. The outermost (P) region of the growth cone is largely actin based. Unbranched bundles of actin form filopodia that actively extend and retract, apparently exploring their surroundings for guidance cues. A highly branched actin meshwork also forms in the P region, generating a thin lamellipodia, like webbing between the filopodial "fingers". Microtubules actively explore the (P) domain, especially along filopodia, and are themselves regulated by guidance cues (Buck and Zheng, 2002; Gordon-Weeks, 2004; Suter et al., 2004). The cell body-proximal (C) domain represents the termination of the axon shaft and is microtubule based. It also contains organelles such as mitochondria and vesicles, which have been transported along the axon by motor proteins. Between the (P) and (C) domains is the (T) domain where microtubules meet the actin network.

The Growth Cone Is The Leading Edge of Neurite Extension

The actin filaments of the P zone of the growth cone are constantly moved toward the (C) region, probably driven by myosin II motors (Medeiros et al., 2006). This retrograde actin flow is offset by polymerization at the leading edge. Lin and Forscher, (1995)

coated beads with Cell Adhesion Molecule (CAM) ligands and attached the beads to growth cones. By withholding the beads, they were able to restrain the retrograde flow and showed that this reduction the retrograde flow was inversely proportional to the extension velocity of the growth cone. This lead to a "clutch" model of growth cone extension in which the constitutive retrograde flow force is turned into forward movement by linking it with the extracellular substrate. A surprising finding about the Arp2/3 complex provides another possible contribution to forward extension of the neuronal growth cone. Arp2/3 is a complex of molecules that nucleates actin branches (Welch and Mullins, 2002). As Arp2/3 nucleates actin polymerization, one might expect it to cause more protrusion of the growth cone. However, Strasser et al., (2004) found that inhibition of Arp2/3 in neuronal growth cones caused them to extend more rapidly, though this was not the case in other migrating cell types. They hypothesize that microtubules in the (C) domain of neurons provide some of the protrusive force for axon extension. The actin meshworks generated by Arp2/3-mediated branching might disperse this protrusive force and lead to an inhibition of axon extension. So, while migrating cells and neuronal growth cones share many signaling molecules and actin-based structures at the leading edge, there are at least some significant differences in their details (Ridley et al., 2003).

Growth Cones Transduce Guidance Cues Into Turning Behaviors

The neuronal growth cone, the tip of the extending neurite, senses and responds to molecular cues in the surrounding environment by turning toward or collapsing away

from them (Goodman, 1996). Families of highly conserved cue/receptor pairs have been identified and, in Drosophila, include the slit/robo, netrins, Dunc5, Semaphorins/Plexins and Ephrins/Ephrin receptor families (Dickson, 2002; Guan and Rao, 2003). The signaling mechanisms that link cue receptors to the cytoskeletal rearrangements involved in turning remain under investigation, though progress is being made in elucidating the specific roles of many of the molecules involved. Rho family GTPases play a major role in many cellular functions, including growth cone turning. By binding GTP and subsequently hydrolyzing it to GDP, they are reversibly activatable. For example, Rac and CDC42 are clearly involved in the cytoskeletal dynamics of growth cone guidance, though how each molecule contributes to the behavior of different neurons at different stages remains unresolved (Kim et al., 2002; Ng and Luo, 2004; Ng et al., 2002). Cyclic nucleotides are another important component of the signaling cascade leading to growth cone turning. Indeed, simply changing the Cyclic AMP/GMP ratio was elegantly shown to reverse the response of cultured neurons to a netrin signal (Nishiyama et al., 2003). As with extension, the cytoskeletal dynamics controlled by these molecular cascades ultimately provide the physical and structural basis of growth cone guidance (Kalil and Dent, 2005). A growth cone responds to a negative cue by asymmetric actin depolymerization, causing it to collapse away from the cue. A positive guidance cue causes microtubule intrusion and stabilization in the (P) domain on one side of a growth cone causing it to grow toward the cue (Kalil and Dent, 2005). Thus, a growth cone is steered toward its target. However, *in vivo* a growth cone encounters many guidance cues, even simultaneously opposing ones, and must modulate its response very precisely

in order to find its way through the complex environment (Grunwald et al., 2001).

Growth Cone Autonomy Has Been Studied *in vitro*

Studies in which neurites were transected by a glass pipette showed that isolated growth cones are capable of turning toward attractive cues or away from repulsive ones when they were bath applied in culture or *in situ* preparations (Campbell and Holt, 2001; Harris et al., 1987; Shaw and Bray, 1977). But the complex *in vivo* environment, with a balanced interplay of multiple guidance cues and the their receptors and the proper expression and localization of molecules in signaling cascades that cause the cytoskeletal rearrangements required for growth cones to accurately navigate to and synapse with their correct partner cells, places greater demands on the growth cone if it is to be autonomous than a single guidance cue applied in a dish.

The *Drosophila in vivo* System

The *Drosophila* embryo contains eight abdominal segments (A1-8), of which A3-6 are highly similar. (Fig. 1A) The cell bodies of motoneurons reside in the CNS surrounding the ventral midline. They extend axons laterally around the periphery to innervate their target muscles. (Fig. 1B) Apposed (contralateral) half-segments (hemi-segments) are nearly identical and provide an ideal internal control for the experimental manipulations in this study. All motoneurons of the developing embryo, and the target muscles they innervate, have been uniquely identified (Fig. 1). The trajectories established by "pioneer" axons are followed by axons of other cells in several bundled (fasciculated)

nerves. The Inter-Segmental Nerve (ISN) is pioneered by the aCC motoneuron axon which innervates the most dorsal muscle, M1. aCC is followed closely by the RP2 axon which defasciculates to innervate M2 (Bate and Broadie, 1995; Keshishian et al., 1996).

Question

Directed motility is an inherent property of many cell types. Detached axons of cultured neurons and even lamellar fragments of fish keratocytes remain motile, although the mechanisms that drive them in the absence of guidance cues remains unclear.(Pollard and Borisy, 2003) But would extension be preserved in a detached *in vivo* growth cone?

Results

Detached Axons Extend Normally

To address the question of whether detached growth cones would retain the ability to extend *in vivo*, we measured the distance extended by detached aCC motoneurons, the pioneer neuron of the Inter-Segmental Nerve (ISN), over a two-hour period of development in which they are rapidly extending toward their target muscle (Fig. 2). We compared the distance traveled by detached growth cones with that of their intact counterparts in adjacent or contralateral half-segments of the same embryo. Using gut morphology and CNS contraction to select embryos at 12 hours of development, we detached the growth cones of ISN motoneurons of abdominal hemisegment A4 or A5 using a laser micro-beam (see Materials & Methods). After allowing the embryos to continue development for two hours we fillet dissected, fixed and stained them

immunohistochemically with antibodies (1D4) raised against Fasciclin II (FasII), an antigen expressed in all Drosophila motoneurons. Images of detached and intact hemisegments were then acquired and analyzed using IPLab software on a Zeiss Axioskop microscope (Fig 3). Between hours 12 and 14 of embryonic development, intact aCC growth cones extend 27.5±2.5μm (n=36 growth cones). Detached growth cones extend 26.4±2.5μm (n=28 growth cones), the same distance as their intact counterparts ($p = 0.76$ in T-Test). Therefore, we find that detached growth cones extend through the *in vivo* environment as well as their intact counterparts.

Detached Axons Navigate the *in vivo* Environment Normally

Neuronal growth cones turn in response to guidance cues encountered in their environment. At hour 12 of development both the aCC and RP2 neuron axons have extended past the LBD sensory nerve cell body, which we used as a landmark for targeting the nerve for transection. During hour 13, the aCC and RP2 growth cones separate, or defasciculate, from each other and aCC continues laterally to innervate muscle 1 while RP2 turns posteriorly to innervate muscle 2. In order to determine if these axons were capable of carrying out these pathfinding behaviors without the benefit of their cell bodies, we again detached them using a laser microbeam at hour 12 (Fig. 4). By hour 14 the tip of intact RP2 has extended 10.8±1.0 μm away from the ISN. Even after laser-detachment, RP2 growth cones display the same maneuver, defasciculating and extending 10.3±1.3μm posteriorly, the same distance as their intact counterparts (n=14 growth cones $p=0.76$ in t-Test). Therefore, the computational ability required to respond

correctly to a complex set of environmental cues is largely self-contained within growth cones.

Filopodial Activity is Altered In Detached Growth Cones

Filopodia are actin-based extensions observed at the leading edge of neuronal growth cones, thought to act as guidance cue sensors as they extend into the surrounding territory and are required for proper growth cone guidance (Goodman, 1996; Wood and Martin, 2002). We used time-lapse video microscopy to analyze filopodial activity in detached and untreated growth cones in fillet-dissected embryos. In experimental and control dissections, growth cones were detached and then images were taken every 20 sec for the next forty-five minutes. Individual filopodia were measured in each frame of the "movie" (n=650 detached and 524 untreated, Fig. 5). The rate of filopodial movement was slowed in detached growth cones (average change 3.39±0.12μm/min) as compared to untreated (4.33±0.2μm/min, t-test $p<.001$). However, extension and retraction events of filopodia decrease in parallel, 21.4% and 23.9%, respectively, sustaining equilibrium in overall motility. Previous studies show that growth cones expressing mutant Rho GTPases, while displaying few guidance errors, shift their extension and retraction rates in parallel (Kim et al., 2002). Despite their well-conserved ability to migrate and target, growth cones show reduced filopodial activities when detached from the cell bodies. However, the current observation further supports growth cones' intrinsic ability to maintain equilibrium for filopodial motility.

Discussion

Our study finds that axonal growth cones are autonomous with respect to extension and navigation and that filopodia, while slowed, continue to extend and retract. While earlier work had shown that transected neurites in culture are capable of survival and significant behaviors associated with normal growth cone functions, it remained possible that these were simply stochastic behaviors or residual functions brought about by overwhelming and simplistic cues. By detaching axons in otherwise whole, developing embryos, we were able to determine that growth cones are capable of responding to complex environments in appropriate ways, which require intricate and balanced mechanisms. Thus, subcellular compartments, once differentiated, are endowed with the machinery required to carry out significant functions without constant monitoring and intervention by the genetic material in the nucleus.

Extension

By comparing length of transected axons with those of intact but otherwise identical axons of the same animal, we were able to show that they retain their *in vivo* ability to extend into the periphery. This implies that the requisite actin structures and the motors responsible for retrograde flow remain intact. The structural support and possible force contribution provided by microtubules remain. The detached axons remain functionally viable, at least over the two-hour period we observed.

Guidance/Turning

Detached axons of RP2 motoneurons were able to defasciculate normally from the ISN, turn posteriorly and contact their appropriate targets. These behaviors reveal an underlying network of molecules that our study suggests remain intact and functional, even without benefit of the cell body. Guidance cue receptors must decode the myriad cues in the environment into pathfinding behaviors that lead them to their proper tartet. Networks of signaling cascades, activators, kinases, phosphatases and their downstream effectors must all remain. Cytoskeletal elements must continue to respond properly to their regulators. Our results show that these molecular networds remain in place after axon transection, something that has not been previously seen.

Filopodial Activity

Filopodial activity provides the only example of the morphogenic function of the growth cone that displays a difference due to axon transection. We find it interesting that extension and retraction rates both decreased by the same amount and yet continue well after transection. Similarly, the numbers of extension and retraction events are decreased in synchrony, with the ration of extension to retraction remaining equivelant. The reason and significance of this parity is not immediately clear, but it suggests an underlying mechanistic cause rather than simply stochastic behavior.

Chapter 2

Synaptogenesis

Summary

Synaptic development and maintenance require coordinated interaction between the pre- and post-synaptic cells. The function of ell adhesion molecules (CAMs), post-synaptically derived guidance cues, pre-synaptic vesicle proteins, neural transmitters and their receptors must all be orchestrated for a synapse to form and function properly. The ability of cells to locally synthesize and cluster these molecules pre- and post-synaptically has been an active area of research for the last few years. While the ability of neurites to carry out local translation has been well established, exactly which endogenous presynaptic proteins are locally synthesized *in vivo* has been difficult to determine. Furthermore, while many molecules are involved in clustering synaptic proteins, the retrograde signals inducing this function and the mechanisms by which these signals are transduced remain unclear. Here, we used quantitative immunofluorescence and pharmacological approaches to demonstrate that endogenous Synaptotagmin I (DSytI), an important synaptic vesicle protein, is locally synthesized in the Drosophila motor nerve ISN. Interestingly, DSytI, fails to cluster at the site of the nascent synapse of axons that have been detached from their cell bodies, though it does so in intact axons of adjacent hemisegments of the same animal. Our results show that DSytI is locally synthesized but that the mechanisms normally used to cluster it to synaptic sites are not functioning when the axons have been transected. Local DSytI protein synthesis begins while the growth

cone is still moving toward its target muscle. Therefore, this production of a molecule that functions in the release of synaptic vesicles must be happening in anticipation of the coming functions of synaptic communication rather than as a response to them.

Introduction

Synaptogenesis

The synapse is a highly regulated, intimate communication point between two cells. Both the presynaptic neuron and the postsynaptic cell, be it muscle or neuron, must provide CAMs at the right time and in the right amount for correct partnering (Prokop, 1999; Waites et al., 2005). The postsynaptic cell must present receptors for neurotransmitters released by its upstream partner. At the developing Drosophila NMJ, glutamate receptors are initially widely distributed throughout the muscle in a punctate pattern, but become tightly clustered at the site of the nascent synapse upon innervation (Marrus and DiAntonio, 2004; Saitoe et al., 1997). The Drosophila membrane-associated guanylate kinase (MAGUK) DLG (Discs Large) is thought to play a major, perhaps primary, role in recruiting at least some postsynaptic molecules (Chen and Featherstone, 2005; Thomas et al., 2000). Synaptic vesicles, containing transmitter, and the machinery to regulate and facilitate their docking, fusion and release are localized presynaptically (Littleton et al., 1993; Zhen and Jin, 2004).

Local Protein Synthesis in Axons

Local (akaryotic) protein synthesis, processing and degradation are important components of neuronal development and function that have enjoyed vigorous investigation in recent years (Martin, 2004). Historically, the idea of protein synthesis in neurites was dismissed because of an initial failure to find evidence of the requried rRNA components in squid giant axonal extracts (Edstrom et al., 1969). Theoretical considerations of the metabolic load on cell bodies and requisite half-lives of cytoskeletal proteins necessary for transport to the distal tips of axons, however, provided an impetus for continued investigation (reviewed in Alvarez et al., 2000). The initial failure to detect ribosomal subunits was explained by their clustering into discrete plaques trapped in the cytoskeleton at the axonal cortex and not extracted from the fixed preparations used at the time. We now know that neurites of many neuronal cell types contain rRNAs, tRNAs, mRNAs, membrane cisterns and elongation factors required for protein synthesis (Davis et al., 1992; Giuditta et al., 2002; Koenig, 1979; reviewed in van Minnen and Syed, 2001).

In growing vertebrate axons, protein synthesis has been shown to play a role in the response to guidance cues. Translation inhibitors (cycloheximide and rapamycin) caused errors in the response to netrin-1 and semaphorin-3A while eIF-4E-P (eukaryotic Initiation Factor-4E-P) and its binding partners, components of the TOR translation pathway, were shown by immunostaining to be activated in response to these cues(Campbell and Holt, 2001). Brittis et al., (2002) used a “fluorescent timer” to

indicate that newly synthesized proteins were found in growing axons beginning only after an intermediate target was reached. This suggests a role for local synthesis in the changing responsiveness of a growth cone to environmental cues. After synaptic initiation, local translation is required for synaptic plasticity. Postsynaptically, an increase in eIF-4E conglomerates is associated with increased Glutamate Receptor immunoreactivity and electrophysiological sensitivity increase (Sigrist et al., 2000). Ashraf et al., (2006) demonstrated that mRNA localization and translation of CaMKII occurred in areas of the olfactory bulb associated with an odorant when flies were learning to a task involving that odorant. However, a comprehensive list of which endogenous proteins are translated axonally remains to be generated. Furthermore, an integrated view of the timing and role of translation in synaptic development and function remains to be developed.

Synaptotagmin I Is Involved In Ca^{2+} Sensing and Vesicle Release

Molecular components of the synaptic machinery are brought to the site of the nascent synapse as it is developing.(Jin, 2002; Waites et al., 2005) *Drosophila* Synaptotagmin I (DSytI), a synaptic vesicle protein involved with the fast component of Ca^{2+}-mediated vesicle fusion and neurotransmitter release, is associated with vesicles which are localized to the synapse by stage 17 of Drosophila embryonic development (Kidokoro, 2003; Littleton et al., 1993; Tucker and Chapman, 2002). DSytI is required for normal synaptic function as mutant alleles cause neurons to release spontaneous vesicles more frequently than wild type while decreasing the amplitude of a nerve-evoked synaptic

response (DiAntonio and Schwarz, 1994). Furthermore, the number of synaptic vesicles localized to the active zones of NMJs are dramatically reduced in DSytI^{null} embryos (Loewen et al., 2006). Here, we use DSytI localization to the synapse as an assay for synapse development.

Question

In vivo, detached axons remain able to extend and navigate to their appropriate synaptic partners. But *in vivo* we can also assess their ability to form synapses with these partners correctly. We asked whether detached axons continue to produce and localize synapse-specific molecules *in vivo*?

Results

The total amount of DSytI immunostaining increases during development prior to synaptic clustering.

Using gut and CNS morphology, we chose 12- and 14-hour embryos to compare the total amount of DSytI in the distal part of the ISN. We fillet dissected and fluorescently labeled the embryos with an anti-HRP[FITC] antibody to stain nerves and with an ant-DSytI antibody detected with a [TRITC]-conjugated secondary antibody. We next took confocal images of ISN axons from young and old embryos using LSM software and a Zeiss Axiovert microscope. Using Adobe Photoshop to view only the green (FITC) channel we traced ISN axons and then summed the traced area of the Z-stack images from the red (TRITC) channel. We then quantitated DSytI immunoreactivity using

IPLab, which gives pixel intensity values, and Excel software to analyze those values. Total DSytI immunostaining intensity in 14hr embryos was 214% (n=19 pairs) of 12hr embryos, showing a doubling of DSyt in distal ISN axons between hour 12 and 14 (Fig. 6).

DSytI immunoreactivity is increased in ISN even without input from the cell body.

Next, we compared DSyt immunoreactivity in detached and intact axons of 14 hr embryos. We detached ISN axons in a single hemisegment of 12hr embryos and allowed them to continue development for 2 hours. We dissected, fixed, stained and analyzed them as above, comparing detached axons with intact ones in an adjacent or contralateral hemisegment of the same animal. Interestingly, total DSyt immunoreactivity in detached axons was 86% of that in intact ones (n = 28 pairs). When combined with the data comparing 12hr and 14hr DSyt levels, detached axons display an 84% increase in the amount of DSytI they had at the time of detachment 2 hours earlier. Since this increase occurs without input from the cell body or the nucleus, we considered the possibility that this increased protein detection was due to local translation.

Without translation, DSytI amount fails to increase.

To support the hypothesis that the subsequent increase in DSytI immunoreactivity of detached axons came from local translation, we employed the translation inhibitor Cycloheximide (CHX), a potent inhibitor of the translocation step of translation. We

bath applied 1.0 mg/ml CMI to whole embryos during the 2-hour period from 12 to 14hrAEL, then dissected, fixed and stained them in the same "mini-well" (see Methods and Materials) as others maintained in insect saline for the two hour period. DSytI detection in distal ISN axons of embryos kept in CMI was 31% of those maintained in insect saline (n = 5 experiments, far rignt bar, Fig 6). Thus, experiments employing CMI support the hypothesis that the increase of DSyt immuno-detection in detached axons was due to new protein synthesis during this period, preceding the onset of synaptogenesis.

DSyt increase is not through endocytosis

Because our DSytI antibody shows significant extraneuronal staining in discrete puncta, we thought it possible that the growth cone could acquire DSytI indirectly by endocytosis as it traveled through the periphery. In order to rule this out, we examined DSyt immunoreactivity in flies expressing a temperature-sensitive allele of *shibire*^{ts}. Shibire encodes the Drosophila Dynamin, a GTPase required for "pinching off" endocytic vesicles (De Camilli et al., 1995; Hinshaw and Schmid, 1995). At temperatures above 25°C, temperatures usually quite comfortable for embryos, endocytosis is rapidly eliminated in *shibire*^{ts} flies. We incubated 12hr embryos in insect saline either at restrictive (29°C) or permissive (18°C) temperatures for two hours then dissected, fixed, stained and analyzed DSyt immunoreactivity in distal ISN axons. DSyt I levels still increase within the axons, displaying 93% of control axons (n-9 pairs, p=0.62 in t-Test). Thus, DSytI is not endocytosed from the surrounding tissues as growth cones travel

toward their target muscles and local synthesis of synaptic proteins remains the most plausible explanation for the increase of immunoreactivity.

DSyt Clustering

Pathfinding growth cones display autonomy from their cell body with respect to extension and navigation. But does this autonomy extend beyond pathfinding into the role of a competent pre-synaptic partner? DSytI is clustered at the nascent motoneuron synapse late in embryogenesis and I used this clustering as an assay to test for synaptogenesis (Littleton et al., 1993). By summing Z-stacked confocal micrographs we was able to quantify the level of DSyt immuno-reactivity within axons along their length. Specifically, we wanted to examine the terminal 10%, the region anatomically correlated with the growth cone/nascent synapse, to see if DSyt reactivity was high in this region compared to the rest of the axon. Is DSyt clustered at the tip of the axon? More DSyt is concentrated at the synapse of intact axons than detached axons (Fig. 7). In intact axons, DSyt density (total pixel intensity value divided by the width of the axon) was detected at the tip to be 3 standard deviations above the average value of the entire axon. In contrast, the amount of DSyt was never more than 1 standard deviation above the average anywhere along the axon; it remains evenly distributed throughout the axon. So, while axons continue to produce DSyt after detachment, they fail to cluster it at the synapse.

Discussion

DSyt Levels Increase in Detached Axons.

We have demonstrated an increase in DSytI protein in growing axons that have been detached from their cell bodies. This protein increase did not enter the detached axon through endocytosis and was sensitive to the translation inhibitor cycloheximide. During most of the developmental period observed, the axon was still growing toward its target muscle and synaptogenesis did not occur until close to the end. Therefore, the synthesis of this protein was in anticipation of its function. *Drosophila* embryogenesis occurs relatively rapidly, with fully motile larva hatching 24hrs after egg laying. In view of this need for a functional motoneuronetwork to develop quickly in order to coordinate crawling, perhaps this economy of early protein synthesis is necessary. We found evidence for the early, local production of an endogenous synaptic molecule.

Synaptogenic behaviors fail in detached axons

The distal tip of the axon is the location of two distinct but important functional domains across the development of the neuron. During pathfinding, the tip comprises the neuronal growth cone, which is charged with navigating its way toward the target cell. Upon reaching its postsynaptic partner, the axon tip changes function from pathfinding to presynaptic partner. One requirement for this functional change is the recruitment of new molecules. It is at this point that autonomy seemingly fails the axon tip. When detached from its cell body, the axon does not cluster DSyt, an important synaptic vesicle molecule, at the nascent synapse. However, while the distal axon has need of its

connection to the cell body at this juncture, the nature of this requirement is not molecular, but structural as we will see in the next chapter.

Chapter 3

Mechanical Force in Synaptic Plasticity

Summary

In recent years, it has become increasingly clear that the physical environment and forces applied to cells affect cellular development and function. It is possible that organisms are designed as tensigrity structures on several levels. If so, these structures that maintain their overall structure by balancing tension and compression forces, would provide an elegant means to transmit environmental information long distances in cells and tissues. Neurons, with axons that maintain tension and behave elastically when stretched, seem ideally situated to sense and respond to mechanical stimuli placed on them by their environment. However, there is a paucity of evidence for the function of intracellular axonal tension *in vivo*. DSytI, clustered presynaptically at synaptogenesis, diffuses away from the synapse if the axon is transected using a laser microbeam. By resupplying tension to the cut axon using a glass micro-pipette, we were able to maintain the clustering of DSytI at the neuromuscular synapse of fillet dissected embryos for two hours. Therefore, mechanical tension, in addition to molecular signals, is important for maintaining the function of the motoneuron. Animals develop and behave in the physical world and we feel that investigation of the mechanical forces imposed upon neurites at all stages of development will be necessary for a complete understanding of neuronal development and function.

Introduction

Mechanical Forces in Other Cells

Organisms, organs and tissues develop in the context of mechanical forces imposed on them from their physical environment. It is therefore not surprising that many cell types use the mechanical properties of their environment to inform their development and behavior (Discher et al., 2005). Vascular endothelial progenitor cells (EPCs), for example, respond to the sheer stress of media flow by up regulating transcription and translation of tissue-specific signaling and cell adhesion molecules (Tzima et al., 2005; Yamamoto et al., 2003). Fibroblasts also respond to mechanical stress, applied by a micropipette, by increasing the size and strength of their focal adhesions (Geiger et al., 2001; Riveline et al., 2001). The signaling molecules involved in the cellular response to these mechanical stimuli are specific to each case. However, in both EPCs and fibroblasts integrins play a central, though not exclusive, role as the cell-matrix adhesion molecule. As with growth cone guidance, cytoskeletal rearrangement is the final output of the morphological responses, but gene and protein profiles change as well.

Tensegrity

From Wikipedia:

"Tensegrity or tensional integrity refers to the integrity of structures as being based in a synergy between balanced tension and compression components.

The term "tensegrity" was first explored by artist Kenneth Snelson to produce sculptures such as his 18 metre high Needle Tower (1968). The term 'tensegrity' was coined for Snelson by Buckminster Fuller from tensional integrity."

Donald Ingber has made the case that organisms use tensegrity as a structural strategy on many scales (Ingber, 2003a; Ingber, 2003b). And, indeed, tensegrity seems attractive as a model for the responsive yet stable organization of cells, tissues and organisms. On one cellular scale, relatively stiff microtubules could provide the compressional elements with flexible actin filaments or meshworks providing the tensional ones. In other places, such as the leading edge of an axonal growth cone or migrating cell, the actin network could act as the compression-bearing unit with overlaying membrane providing the tensional resistance. By these means, tensegrity could underlie fast, flexible transmission of mechanical information over large cellular and tissue distances. This could be very useful to neurons whose microtubule-based axons can extend hundreds of cell body diameters. However, Heidemann and others maintain that tensegrity has a very specific meaning and that almost all of the data Ingber and colleagues use in support of his hypothesis can be explained in terms of more classical models of the mechanical properties of biological materials, though they themselves originally espoused the idea (Dennerll et al., 1988; Ingber, 2000). Whether we apply the word "Tensegrity" or not, it is becoming clear that the cytoskeletal elements act to transmit mechanical force over distances. Wang et. al. developed a FRET-based Src activation monitor and used laser tweezers to apply force to a bead crosslinked to the cytoskeleton of HeLa cells (Wang et

al., 2005). They observed the activation of Src both close to the bead and at a distance from it, but only when the actin cytoskeleton was intact and the bead was attached to it and not just to the membrane. The cytoskeletal structures are doing more than just providing structural support to cells.

Measured Forces in Neurons

In 1988 Dennerll et al. showed that chick neurons generated cytoskeleton dependant tension along their processes (Dennerll et al., 1988). By applying force to the axon with a glass needle, they demonstrated that cultured PC-12 neurites established a "rest tension" of 1-3 millidynes. After at least 15 to 20 rounds of repeated stretch the neurites returned to this rest tension in a cytoskeleton-dependant way. The tension generated by neurons has been suggested to contribute to brain morphology by physically moving more highly connected areas closer to each other (Van Essen, 1997). It has also been shown that applying ectopic tension to cultured neurons can cause de novo process extension and that these processes expressed axon-specific markers (Lamoureux et al., 2002). But might the tension generated by neurites contribute to neuron-specific synaptic functions?

Question

Neurites not only achieve but also actively maintain at least some rest tension. This behavior cannot be accounted for by any passive viscoelastic properties (Heidemann et al., 1995). When the "growth cones" of chick sensory or cortical neurons were fixed to a

glass needle and slackened, they reattenuated to a tensional state. (Grinnell et al., 2003)also characterized an integrin dependant increase in the probability that vesicles will be released from frog motor neurons within milliseconds of an applied stretch of the muscle they innervate. Thus, it appears that neurites are positioning themselves so as to be tension sensors and using that tension to determine at least some aspects of synaptic function. Might Drosophila motoneurons be using tension to maintain synaptic function?

Results

To answer the question of whether ISN axons require tension to maintain the clustering of synaptic vesicles, we detached them in a single half-segment of pairs of 14:00 AEL embryos, which were then dissected on a single microscope slide. The cut end of one axon was secured using a micro-pipette and mechanical tension was re-supplied to the axon. Two hours later, the micro-pipette was removed and the embryos were fixed and immunostained for DSyt in the same solutions. Confocal micrographs of the detached axons were analyzed similarly to the earlier analysis performed on detached vs. intact axons. Thirteen pairs of axons were analyzed and on average there was a difference in the level of clustering of DSyt to the synapse. (Fig. 8) The axons that had tension restored clustered DSyt in the terminal 10% of the axon to 3 standard deviations above the average for the entire axons; conversely, detached axons that had been left untreated failed to cluster their DSyt to more that 1 standard deviation above the average anywhere along the axon. The mechanical tension under which axons are maintained is necessary to maintain synaptic clustering of DSyt1, an important synaptic protein.

Discussion

The activity-dependent long-term potentiation (LTP) of synapses presents several major challenges to the neuron functioning in a brain. Since a single neuron can synapse with many other cells, specificity is the first challenge. A still-hypothetical, presynaptic "tag" is being saught (Fonseca et al., 2004; Sajikumar et al., 2005). Furthermore, LTP requires transcription and the time required to send diffusible signals to the cell body and back seems to be prohibitive if they are also to allow for the specificity already mentioned (Huang et al., 2006). A role for mechanical tension would elegantly solve these difficulties by providing exquisite specificity and almost instantaneous signaling, through the cytoskeleton, across the entire cell.

Axons require mechanical tension to maintain synaptic molecular integrity. It is possible that mechanical force constitutes a "retrograde signal" to the axon that a successful anterograde signal has been sent. If this mechanical signal then induces the presynapse to potentiate (become more efficient) in the long term, then mechanical forces play a major role in synaptic plasticity. Coupled with the mechanical properties of cultured central neurons and the fact that dendritic spines are also shown to twitch in response to Ca^{2+} influx, it seems likely that mechanical force plays a significant role in memory formation and brain function.

Ongoing studies

Working with Shengyuan Yuang in the lab of Tahir Saif, we are continuing to determine the mechanical properties and mechanisms of *Drosophila* motoneurons. Using a probe to

manipulate *in situ* ISN axons, we are determining the tension maintained by them and the times required to generate the forces. We are also looking into how tightly the axon binds its target muscle. Further experiments will include applying forces on different time scales to evaluate the effects on synaptic vesicle clustering.

Materials and Methods

Immunocytochemistry

For the Extension and Navigation experiments, biotin conjugated 2° antibodies were detected with an ABC kit (Vector Labs) according to manufacturer's protocol. Sources of 1° antibodies used, at 1:1000 final dilution in TBS in all cases, were as follows: Mouse α-FasII (1D4) from Hybridoma Bank , Goat α-HRP[FITC] Jackson Labs, Rabbit α-DSytI Hugo Bellen. Fillet-dissected embryos were fixed in a 4% PFA solution for 15 minutes, blocked with BSA for two hours at RT and incubated in 1° antibodies overnight at 4° C.

The time-lapse movies for analyzing filopodial activity were generated by genetically supplying gap-GFP pan-neuronally (elav'-Gal4 X UAS-gap-GFP). Images were acquired and analyzed using IPLab imaging software from Scanalytics. Immunostainings of experimental and control preparations were all done in the same pools using the same solutions.

Laser detachment

A Laser Science, Inc (LSI) VSL 337-ND pulsed dye laser was used. Coumarin 440 dye, optimal for disruption of membrane, was used as per Keshishian, personal communication. The laser was aimed through a Newport attenuator and a plano-convex focusing lens and reflected by a 460dclp dichroic mirror into the back of a Zeiss Axioskop microscope and subsequently onto the preparation. The dichroic mirror allowed the passage of emission spectra of eGFP to allow for simultaneous visualization and laser-induced detachment of the axon. In all cases, axon detachment was determined by later immunostaining with an antigen other than GFP. Analyses were only done when axons of the hemisegment shot were detached but axons anterior and posterior remained intact to ensure that dissection alone had not damaged the axon.

Fluorescence Quantification

Confocal images were acquired using Zeiss LSM imaging software. In all experiments comparing immunofluorescence detection, confocal settings (e.g. image size, pinhole size, laser strength, detector gain etc.) were identical for experimental and control preparations. Z-stack images were then converted to PICT format using Adobe Photoshop and pixel intensity values were determined by IPLab imaging software. Values were transferred to Microsoft Excel for analysis. In experiments where it was not possible to compare axons of the same prep (e.g. Young vs. Old preps, CMI vs. Saline incubation, Pulled vs. Unpulled) pixel values were normalized to a non-expressing tissue background 25X25 pixel area taken from the axon hillock of the LBD Cell body.

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Zhen, M., and Y. Jin. 2004. Presynaptic terminal differentiation: transport and assembly. *Curr Opin Neurobiol*. 14:280-7.

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EDUCATION

Ph.D. University of Illinois, Urbana-Champaign
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Cell and Developmental Biology

B.A. Indiana University, Bloomington, Indiana
May 1987
Psychology, Minor in Business.

QUALIFICATIONS SUMMARY

- 9 Years professional laboratory experience
- Research focus on Neurobiology, microscopy (Nomarski, fluorescent, confocal) and image quantification
- 10 Years business experience, 5 years in management
- Extensive experience with scientific presentations and teaching

TECHNICAL SKILLS

Microscopy

- Designed laser integration with Zeiss Axioskop
- Fluorescence light-path optic design
- Confocal imaging and 3D analysis
- DiI single cell labeling
- *In vivo* single cell micropipette manipulation

Cell Biology

- Immunocytochemistry (histological and fluorescent)
- *In situ* mRNA hybridization
- Basic molecular biology techniques
- Protein purification: HPLC, Western blots
- *Arabidopsis* (plant) cell culture
- Pharmacological translation inhibitors

Computer Proficiency

- Imaging software: Adobe Photoshop, Canvas, NIH IPLab, OpenLab, Volocity, Zeiss LSM
- HTML and web page design

PROFESSIONAL EXPERIENCE

Pre-Doctoral Research University of Illinois, Urbana-Champaign
1999 – Present
Cell and Developmental Biology

- Developed immunofluorescence quantification of protein expression and intracellular localization
- Identified role of mechanical force/tension in synaptogenesis
- Characterized role of ECM molecule Tiggrin in axonal growth cone guidance
- Designed laser micro-beam technique for *in vivo* transection of fluorescently labeled neuronal axons

Undergraduate Research University of Illinois, Urbana-Champaign
1997-1999

Crop Sciences

- Investigated the biochemical source of plant resistance to glyphosate
- Used *in vivo* and spectraphotometric enzyme assays to study mutant enzymes
- Maintained and expanded transgenic lines of cultured plant cells for experimental use
- Ordered and tracked inventory and lab supplies
- Repaired and maintained laboratory equipment

Business Experience T.I.S. Bookstore, Champaign, Illinois
1993-1998

- Manager, Textbook Department
  1996-1998
  - Designed and implemented computerized inventory and accounts payable tracking system which improved bookkeeping efficiency and accuracy
  - Purchased and maintained $1 million inventory
  - Hired, trained and managed approximately 15 employees
- Assistant Manager, Textbook Department
  1993-1996
  - Developed and implemented database software to track special orders improving organization and customer service
  - Helped open new branch of growing corporation in highly competitive market
  - Maintained bookkeeping and inventory
  - Promoted to department manager in recognition of performance excellence

Teaching Assistant University of Illinois, Urbana-Champaign
2003-2006

School of Molecular and Cellular Biology

- Developed course web-site and lead discussion for 1st year Graduate students in Cell and Structural Biology Department
- Taught over 100 Undergraduate students in lab and lecture courses over 5 semesters
- Helped develop Biochemistry Merit course for pre-meds that emphasized group collaboration

Parkland Community College, Champaign
2002-2005

Department of Natural Sciences

- Taught on-line course on Evolution for non-scientists requiring excellent written communication skills

SELECTED PRESENTATIONS

"Role of Mechanical Force in Synaptic Formation and Maintenance"
Poster Presentation, 2005. Neurobiology of Drosophila Conference, Cold Spring Harbor Laboratory, NY

"Autonomous Growth Cones Require Cell Body at Synaptogenesis"

Poster presentation, 2005 46th Annual Drosophila Research Conference, San Diego, CA

"Local Government Gives Rise to Growth Cone Autonomy* in vivo*"

Poster Presentation, 2003 44th Annual Drosophila Research Conference, Chicago, IL

"Functional Autonomy of* Drosophila *neuronal Growth Cones"

Oral Presentation, 2002 15th Annual Cell and Molecular Biology/Molecular Biology Research Symposium, Urbana, IL

"Axonal Growth Cone Displays Functional Autonomy in vivo"

Poster presentation, 2001. Neurobiology of Drosophila Conference, Cold Spring Harbor Laboratory, NY

"Role of ECM Molecule Tiggrin in Drosophila Growth Cone Guidance"

Invited Speaker, 2000. Midwest Neurobiology Meeting, Aimes, IA

"ECM Molecule Triggrin Plays a Role in Nerve-Muscle Interactions"

Invited Speaker, 2000. 41st Annual Drosophila Research Conference, Pittsburgh, PA

HONORS

- 46th Annual Drosophila Research Conference Travel Grant, 2005
- Winner, Best Poster (out of more than 60). NIH Training Grant Symposium, 2003
- Fellow, NIH Cell and Molecular Biology Training Grant, 2000
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- Cold Spring Harbor Laboratory Travel Grant, 2005