Chapter V

Summary, Discussion and Future Directions

Summary of Results

Our work, described in detail in Chapter 2, provided the first direct visualization of dendritic protein synthesis dynamics in living hippocampal neurons (Aakalu et al., 2001). Taking advantage of the visible fluorescence signal provided by GFP, we flanked a membrane-tethered GFP coding sequence with the 3' and 5' UTRs of CamKIIα to both target the mRNA to the dendrites, and endow it with translation control elements. Neurons expressing this construct showed increased GFP production in response to brain-derived neurotrophic factor (BDNF). Increases in the distal dendrites were seen within 30 minutes of BDNF application, suggesting a local origin of the GFP signal. In an effort to rule out the cell body as a source of GFP signal detected in the distal dendrites, we successfully isolated dendrites using mechanical and optical techniques. In both cases, isolated dendrites continued to show increased GFP signal that was completely blocked in the presence of a protein synthesis inhibitor. Lending additional support to the putative synaptic localization of the increased GFP, we quantitatively demonstrated that GFP hotspots in the dendrites were spatially persistent over time, and were correlated with ribosomal and synaptic markers.

In more recent experiments, we investigated the role of dopaminergic signaling in LPS (Chapter 3). Given the similar temporal profiles of L-LTP inhibition by dopamine receptor agonists and protein synthesis inhibitors, we examined the ability of D1/D5 agonists to stimulate protein synthesis in the dendrites of cultured hippocampal neurons. In addition to direct observation of LPS stimulation by the agonists, our work provides a

possible mechanistic understanding of how dopamine may exert its effects on long-lasting synaptic enhancement: An important consequence of D1/D5-stimulated protein synthesis is the conversion of silent synapses into active synapses. This postsynaptic conversion was detected as increased surface GluR1 at synaptic sites, as well as an increase in the frequency, but not amplitude, of miniature excitatory postsynaptic currents. These results mark the first established connection between dopaminergic signaling, local protein synthesis, and silent synapse activation.

We have also provided the first freely available software tools for quantitative analysis of 3-D colocalization and 2-D spatial correlation (Chapter 4). While qualitative properties of colocalized signals in fluorescent images are frequently reported, they are rarely quantified. The method we have presented permits fully automated analysis of colocalization in 3-D image sets, as well as descriptive statistics about the particles from each image, regardless of their colocalization status. For cases in which spatial correlation may be of interest, for example, when proteins do not share the same space but may cluster in the same region of the cell, we have provided an analysis routine that quantifies this correlation, and expresses the result in a normalized fashion that facilitates between-group comparisons. Both programs include the ability for objective threshold determination and batch analysis, which will greatly reduce the potential of erroneous results due to user bias. These programs, when used within the constraints of the limitations addressed in Chapter 4, have the potential to create a consensus method for describing properties of colocalization and spatial correlation in immunofluorescence image data.

Discussion and Future Directions

While our work, along with the work of other researchers in the field (reviewed in Martin et al., 2000; Steward and Schuman, 2001; Steward and Schuman, 2003), has begun to define the importance of local protein synthesis in hippocampal neurons, the precise physiological function of this phenomenon remains largely unknown. Here I present a few of the outstanding issues related to LPS in synaptic plasticity, as well as some experiments that will help to address these questions.

Input-Specificity and LPS

A problem that has received considerable attention is the issue of input-specific synaptic enhancement, as briefly introduced in Chapter 1. Although it is generally accepted that some degree of input-specificity is maintained in neurons, the lower spatial limits of this specificity, as well as the role of LPS in this effect, have not been convincingly addressed. If plasticity is induced at just a few synapses on a distal dendritic segment, how far does that plasticity spread to adjacent synapses? In one study, this lower limit was determined to be approximately 70 μm from the site of LTP induction (Engert and Bonhoeffer, 1997). In these experiments, synaptic transmission was globally inhibited by bathing the cells in a cadmium-containing solution, which blocks the voltage-gated Ca^{++} channels responsible for evoked neurotransmitter release. Synaptic enhancement was induced by pairing whole-cell depolarization with local perfusion of a solution containing high Ca^{++} without cadmium.

A limitation to this experimental approach is that Engert and Bonhoeffer determined the spatial resolution of their stimulation and measurement technique to be approximately 30 μm, as estimated by visual inspection of a blue food coloring dye used in the perfusion solution. The use of this dye, when compared to a soluble fluorescent dye, gives a relatively poor estimate of the extent to which a perfused solution is spatially restricted (W. B. Smith, unpublished observations), raising the possibility that the authors were stimulating a much larger area than estimated. Even if this were a perfect assessment of the limit of their perfusate, a 30 μm section of dendrite could be too large an area to accurately assess the question of input-specificity. This result is further confounded by the fact that whole-cell depolarization, using an electrode at the cell soma, was used to induce plasticity. This may have disrupted voltage-gated ion channels in non-stimulated regions of the dendrite that under normal conditions could serve some critical function in maintaining input specificity.

More recently, an interesting set of experiments has examined, at a very crude spatial resolution, the role of LPS in input specificity (Bradshaw et al., 2003). In these experiments, Bradshaw and colleagues measured high frequency-induced L-LTP in two separate subfields of the hippocampal slice — stratum radiatum and stratum oriens — which are separated from one another by hundreds of microns of dendritic length, and are on opposite sides of the cell soma. The authors found that locally perfusing emetine, a protein synthesis inhibitor, selectively blocked L-LTP in the subfield where the drug was applied (for example, in radiatum), without altering potentiation in the other subfield (oriens). While this result successfully implicates LPS in input specificity, the scale at

which input specificity was examined is considerably larger than what has previously

been reported.

To address the actual lower limit on the spatial restrictions of LPS-dependent input

specific plasticity, it may be necessary to examine the process in cultured hippocampal

neurons rather than using acute slices or slice cultures. There are certainly disadvantages to

this approach, given the potential differences in synapse density and connectivity between

the dissociated culture system and slice. However, the advantages of spatial precision and

temporal control over drug application make the dissociated culture system ideal for

investigations of this nature. Cultured neurons expressing GluR1 tagged with an

extracellular pH-sensitive GFP molecule could be used to investigate the spatial limits of

stimulus-induced AMPA receptor surface expression dynamics. With such a construct,

surface GluR1 expression could be followed in near real-time as follows: an image

acquired in the presence of a mildly acidic bath solution would be subtracted from a

previously acquired image at pH 7.4. With an appropriate GFP tag, such as the pHluorin

GFP molecule (Miesenbock et al., 1998), this subtraction will yield an image of the surface

population of GluR-containing AMPA receptors. This imaging protocol, used in

combination with local perfusion of a dopamine agonist, glutamate, or high K^+, would

facilitate the rapid visualization of activity-regulated GluR1 surface dynamics, a

phenomenon that has been correlated with synaptic plasticity (reviewed in Malenka, 2003).

Because an experiment monitoring receptor dynamics would be correlative in nature, direct demonstration of the input specificity limit would require combining this imaging approach with whole-cell or perforated patch recordings. By measuring evoked responses at a number of locations on the dendritic arbor, both before and after local perfusion at one location with a drug that induces plasticity, it should be possible to measure the spread of synaptic enhancement from one location to another. Ultimately, the merging of these two experimental approaches would provide the most accurate estimate of the degree to which synaptic enhancement is truly input-specific, and whether or not LPS is critically involved in this specificity.

Role of Dopaminergic Signaling

A number of interesting questions remain unanswered with regards to the dopamine studies. In particular, the necessity of this pathway for LPS induction has not been addressed. It has previously been shown that D1/D5 dopamine receptors can converge with various signaling systems via physical interactions with GABA and NMDA receptors (Lee et al., 2002; Liu et al., 2000; Pei et al., 2004). Given the critical involvement of NMDA receptors in various forms of synaptic plasticity, it will be interesting to examine the relationship between NMDA-R signaling and the effects of D1/D5 agonists we have described. It is possible that the D1/D5 receptors simply play a permissive role, somehow enhancing NMDA-R signaling to produce the observed increases in GluR1 surface expression and synaptic transmission. Alternatively, the dopaminergic signaling pathway may serve as a necessary and sufficient mechanism for the conversion of postsynaptic

silent synapses. A relatively simple set of experiments, in which the effects of D1/D5 agonists on GluR1 expression and synaptic transmission are examined in the presence of APV, should be sufficient to resolve this issue.

The source of the GluRs that are increased on the cell surface in response to dopamine receptor activation also remains to be determined. While we show that the increase is sensitive to protein synthesis inhibitors, as well as showing a small increase in total cellular levels of GluR1, we have not conclusively demonstrated that the newly synthesized receptors are the same receptors that appear on the surface. Distinguishing between the three possibilities (i.e., whether some, all, or none of the new receptors on the surface were synthesized in response to the agonist), will be an interesting topic to explore. There are a variety of ways to answer this question. One experiment involves ^{35}S-methionine/cysteine labeling followed by surface biotinylation and combined streptavidin/GluR1 immunoprecipitation. If D1/D5 agonists simulate synthesis and insertion of newly-synthesized GluRs, agonist treatment should result in higher levels of biotinylated ^{35}S-GluR1 as compared to control samples. Because there may be significant loss of the GluR1 product in the various steps of such a complex procedure, the ^{35}S pulse could instead be followed by chymotryptic digestion of surface proteins. Because the chymotrypsin is not membrane permeant, subsequent immunoprecipitation of GluR1 and autoradiography will show a reduced molecular weight GluR1 band if the ^{35}S-labeled receptors are inserted into the plasma membrane.

Concluding Remarks

The localized control of complex cellular processes, such as the regulation of protein synthesis at synaptic sites, is an important mechanism for achieving subcellular specificity in a variety of biological systems. In the single-celled yeast *Saccharomyces cerevisiae*, precise targeting of specific mRNA species from the mother to the daughter cell during budding is important for successful asexual reproduction (Beach and Bloom, 2001; Bertrand et al., 1998). The partitioning of mRNA and proteins is also a key component of developing embryos in *Drosophila*: when the normal spatial distributions of these factors are disrupted, proper development of the organism may fail (Berleth et al., 1988; Ding et al., 1993; Lieberfarb et al., 1996).

Here we have presented evidence that local protein synthesis takes place in the dendrites of mature hippocampal neurons, and that D1/D5-mediated dopaminergic signaling affects GluR1 receptor dynamics in a protein synthesis-dependent manner. Because dopamine is an important neuromodulator, playing a critical role in such diverse phenomena as drug addiction, Parkinson's disease, schizophrenia, and learning and memory, implicating the dopaminergic system in regulation of local protein synthesis opens up a large potential field for research. If it can be determined that the effects of dopamine in any of these behavioral or pathological conditions are mediated through protein synthesis-dependent pathways, new therapies may de developed to help treat addiction, and improve cognitive performance in both abnormal and normal brains.