C h a p t e r I

Introduction and

Literature Review

INTRODUCTION

The question of how an organism learns and remembers information essential for its survival has intrigued philosophers and scientists for hundreds of years. An old debate surrounding this issue as it relates to human beings is that of mind-body dualism, a concept first introduced in written form by the French philosopher and mathematician René Descartes. In his initial description of mental function, Descartes envisioned the mind and body as separate entities, with the soul — by way of the pineal gland — acting as the emissary between the two. Although the foundations of this argument were ultimately erroneous, Descartes made an important step toward describing something we now take for granted: the fact that physical events occurring inside the brain are responsible for all psychological phenomena.

While we now understand that the brain is the source of consciousness, it remains to be determined precisely how the brain is capable of storing and retrieving the information we describe as our thoughts, feelings and memories. Without a precise mechanistic description of the sequence of physiological events involved in storing or retrieving a memory, it may seem impossible to embark on studies of something so complicated as animal behavior. This has not, however, deterred the many scientists, philosophers and psychologists who have put forth their various theories on the subject over the course of time.

To a cellular and molecular biologist, perhaps the most influential of these theories was described in 1949 by Donald Hebb. The central premise of Hebb's postulate is as follows: When two neurons are connected to one another such that one neuron consistently and repeatedly causes the other neuron to fire, some growth process or metabolic change takes place that ultimately results in strengthening the connection between the two cells (Hebb, 1949). The essence of Hebbian plasticity has made its way into the thoughts of the general public, and is eloquently captured in the following quote by the popular novelist Tom Robbins:

> *The hand is the most wonderful instrument ever created, but it cannot act of its own accord; it is the servant of the brain. (Author's note: Well, that's the brain's story anyhow.) It reflects the kind of brain behind it by the manner and intelligence with which it performs its duties. The hand is the external reservoir of our most acute sensations. Sensations, when repeated frequently, have the capacity to mold and mark.*
>
> *Even Cowgirls Get the Blues*

Long-Term Potentiation

While it now seems intuitively obvious to a neuroscientist that repetitive, correlated activation of one neuron by another will enhance the communication between the two cells, direct experimental evidence of synaptic plasticity — the dynamic modification of synaptic strength between neurons of the central nervous system — was not described in a mammalian system until 1973, nearly 25 years after Hebb's insightful hypothesis. This seminal finding was the initial characterization of long-term potentiation (LTP). Working *in vivo* in the hippocampus, a region of the brain previously implicated in memory

formation, Bliss and Lømo induced LTP by delivering a series of high-frequency electrical pulses to the perforant path axons that innervate the dentate gyrus (Bliss and Gardner-Medwin, 1973). When the field excitatory postsynaptic potential (fEPSP) was measured following the high-frequency stimulus, Bliss and Lømo observed a sustained increase in the strength of activated synapses (shown schematically in Figure 1.1D).

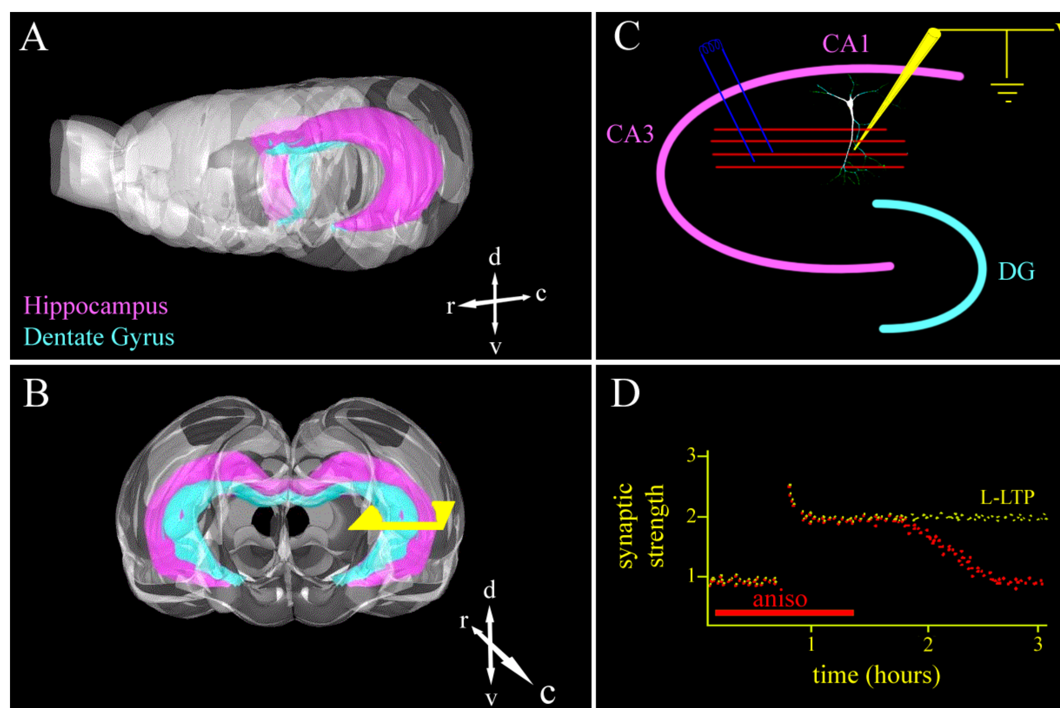


Figure 1.1 The Hippocampus and Long-Term Potentiation

Panels A and B show two 3-D models of the rat brain in different orientations as described by the anatomical coordinate axes (r: rostral, c: caudal, d: dorsal, and v: ventral). The locations of the Dentate Gyrus and Hippocampal Formation are shown as cyan and magenta surfaces, respectively. A representative slice setup of the hippocampus for an LTP experiment, taken from the yellow plane in panel B, is show as a schematic in C. The three subfields are labeled as DG (dentate gyrus), CA1 and CA3 (*cornu ammonis* fields 1 and 3). A stimulating electrode (blue) and recording electrode (yellow) are also indicated. The protein synthesis-dependence of L-LTP is schematized in panel D: long-lasting synaptic enhancement, measured as an increase in synaptic strength above the baseline, can endure for several hours (yellow dots), but when LTP is induced in the presence of the protein synthesis inhibitor anisomycin (aniso), the LTP decays back to baseline within a couple hours (red dots).

The question of whether or not LTP is the mechanism employed in the brain for memory formation has not been confirmed, although it is a widely held belief that some form of synaptic plasticity is an important physical mechanism underlying learning and memory (Stevens, 1998). Several key attributes of LTP in hippocampal area CA1 make it an attractive candidate mechanism for memory encoding: cooperativity, associativity, temporal persistence, and input-specificity. Of these properties, the most obvious in terms of understanding the relevance of LTP to memory formation is that of temporal persistence. In order for synaptic plasticity, such as that seen in LTP, to be considered as a plausible cellular mechanism for memory formation, it must be true that the synaptic enhancement lasts sufficiently long for the memory to be encoded. Indeed, certain forms of late-phase LTP (L-LTP) in hippocampal slices will endure as long as the slice remains alive (Reymann et al., 1985), and LTP recorded in living animals lasts from several days to weeks (Bliss and Gardner-Medwin, 1973; Leung and Shen, 1995; Staubli and Lynch, 1987). While memories can easily last longer than LTP has ever been recorded, this does not discount LTP as a potential memory encoding device: it is possible that a separate mechanism is responsible for the long-term storage of a memory once it has been encoded (Bliss and Collingridge, 1993).

The properties of cooperativity and associativity result from the fact that LTP in some regions of the brain, including the Schaffer collateral/CA1 synapses, requires coordinated activity of pre- and post-synaptic neurons in order to activate the N-methyl-D-aspartate (NMDA) type of glutamate receptors (Collingridge et al., 1983). At normal resting membrane potentials, NMDA receptor ion channels do not conduct any current

because they are blocked by a Mg^{2+} ion (Mayer et al., 1984). When a sufficiently large group of axons impinging on a given cell fire action potentials together (cooperativity) and/or when the postsynaptic neuron is sufficiently depolarized while receiving glutamatergic input (associativity), the Mg^{2+} blockade of NMDA receptors is relieved, allowing depolarizing ionic current — and calcium ions in particular — to pass through the open channels.

Silent Synapses

The molecular cascades responsible for LTP induction and maintenance have been the focus of extensive research for the past 25 years. From this work has emerged the description of an interesting phenomenon that may provide a molecular mechanism for the increased synaptic communication observed in potentiated neurons. This idea, known as the silent synapse hypothesis, initially emerged in the LTP literature from experiments investigating action potential-evoked transmitter release. Because evoked release of a neurotransmitter quantum (presumed to be a single vesicle) is a probabilistic event, succeeding only 10–40% of the time (Rosenmund et al., 1993), it is possible to quantify the success and failure rates. Interestingly, a number of researchers have described a decrease in evoked failure rates after LTP induction, which initially lead researchers to conclude that LTP induces an increase in presynaptic release probability (reviewed in Kullmann and Siegelbaum, 1995).

Another possible explanation for silent synapses involves the dynamics of postsynaptic surface expression of another type of glutamate-gated ion channel, the α-

amino-3-hydroxy-5-methylsoxazole-4-propionate (AMPA) receptor. AMPA receptors mediate a majority of the fast, excitatory synaptic transmission in the brain, and unlike NMDA receptors, AMPA receptors are not blocked by Mg^{2+} at resting membrane potentials. Because of this difference between the two receptor types, a synapse comprising exclusively NMDA receptors is functionally silent at a resting membrane potential whereas a synapse that contains both types of receptors is likely to generate an excitatory postsynaptic current (EPSC) in response to glutamate release.

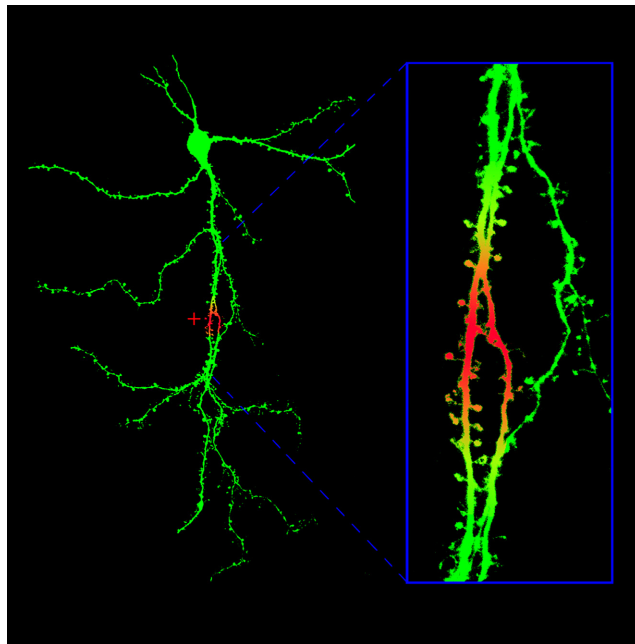
Two independent reports (Isaac et al., 1995; Liao et al., 1995) identified postsynaptically silent synapses using whole-cell voltage clamp recordings. Under low-intensity stimulus conditions, some synapses failed to show a postsynaptic response when the neuron was held at -60 to -65 mV (potentials at which NMDARs are blocked), but responses were detected at +50 to +60 mV. The responses at positive holding potentials were blocked by APV, indicating an NMDAR origin. Furthermore, the proportion of synapses exhibiting EPSCs at -60 mV was increased after LTP induction. Since these initial electrophysiological characterizations of postsynaptically silent synapses, a number of groups have also identified ‘morphological’ silent synapses, or synapses that contain NMDARs but no AMPARs as determined by immunocytochemical labeling (Liao et al., 1999; Petralia et al., 1999). While these results clearly implicate postsynaptic silent synapses in LTP, a detailed account of the molecular mechanisms governing conversion into active, AMPAR-containing synapses, remains to be described.

Input-Specificity

An individual neuron in the mammalian CNS may contain up to ten thousand synaptic connections. Furthermore, small groups of synapses on each neuron can be independently regulated: synaptic enhancement induced at one location on the dendritic arbor does not spread throughout the entire neuron (Andersen et al., 1977; Bonhoeffer et al., 1989; Engert and Bonhoeffer, 1997; Nishiyama et al., 2000; Schuman and Madison, 1994; Tao et al., 2001). The precision with which a neuron is able to fine-tune its synaptic inputs is directly related to the encoding capacity of that individual cell: the greater the input-specificity, the more information the cell will be able to encode. In the absence of input-specificity, all inputs onto a given neuron would likely carry the same information. While the number of neurons in the brain may be sufficiently large that a single cell *could* be responsible for encoding just a single bit of information, input-specific synaptic enhancement has been observed in a number of systems, and is likely involved in the capacity of information a given network of neurons is capable of storing.

Figure 1.2 Input-Specific Synaptic Enhancement

Shown is a simulated representation of a single hippocampal neuron during induction of synaptic plasticity. The region stimulated to induce plasticity is shown by the red plus sign in the full-scale image. As seen in the high-magnification inset image, the region of synaptic enhancement, as illustrated by the red color, is confined to only a small group of synapses in close proximity to the site of the plasticity-inducing stimulus.



Modulatory Transmitters and LTP

It has been clearly established that NMDA receptor-mediated calcium influx is required for LTP induction, although a variety of modulatory signals have also been implicated in the induction and persistence of hippocampal synaptic plasticity. These include the peptide growth factors brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), as well as more classical neuromodulators such as acetylcholine, norepinephrine, and dopamine. While all of these transmitter systems likely function in a concerted, complex fashion to regulate plasticity in-vivo, my work has focused on the effects of dopaminergic signaling for the following reasons: dopamine receptor signaling can regulate levels of cyclic adenosine monophosphate (cAMP); dopaminergic signaling is critically involved in the late phase of LTP; certain forms of learning behavior require dopamine receptor activity.

The five cloned types of dopamine receptors are characterized by their G-protein-coupled effector molecules, with activation of the D1/D5 class eliciting increased cAMP production through stimulation of adenylyl cyclase (Figure 1.3). Cyclic AMP is a vital second messenger, activating the cAMP-dependent protein kinase (PKA), and the cAMP responsive element binding protein (CREB), a transcription factor that regulates the expression of genes involved in various forms of plasticity and learning and memory (Bito et al., 1996; Dash et al., 1995; Deisseroth et al., 1996; Pittenger et al., 2002).

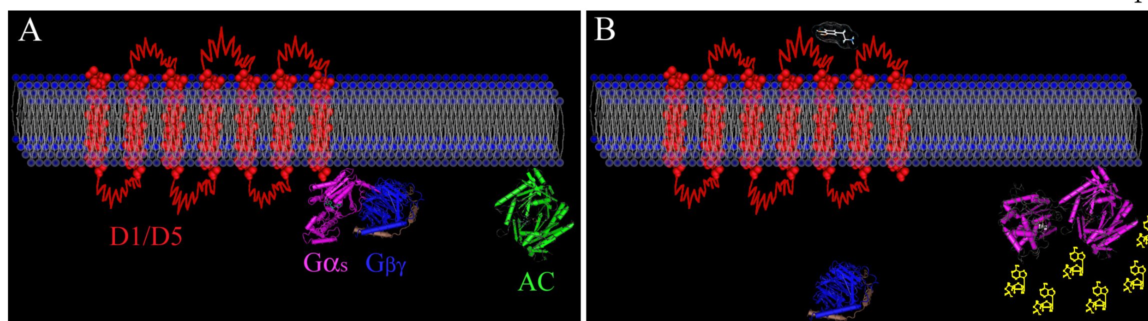


Figure 1.3 D1/D5 Receptor Signaling

The G-protein-coupled D1/D5 receptor (shown in red as a 7-helix transmembrane protein) signals to adenylyl cyclase (AC) through the heterotrimeric G-protein complex. In the absence of Dopamine (panel A), the Gαs and Gβγ subunits are together in a complex, associated with the C-terminal end of the receptor. Upon dopamine binding (panel B), the Gαs subunit dissociates from the βγ subunit, and then stimulates the production of cAMP (yellow) via an interaction with adenylyl cyclase.

Activation of D1/D5 dopamine receptors in the hippocampus is both necessary and sufficient for the protein synthesis-dependent late phase of LTP (Frey et al., 1991; Huang and Kandel, 1995; Matthies et al., 1997). In the study by Huang and Kandel, bath application of SKF-38393, a D1/D5-selective agonist, was sufficient to induce a long-lasting form of LTP that was blocked by the protein synthesis inhibitor anisomycin (similar to the results shown in Figure 1.1D). In another study, the well-established decline in spatial memory performance observed in aged rats was attenuated by drugs that enhance the cAMP signaling pathway, including a dopamine D1/D5 agonist (Bach et al., 1999). In normal adult animals, dopamine appears to be involved in another interesting behavioral phenomenon (Li et al., 2003). In these experiments, exposure to a novel environment reduced the threshold for LTP induction in the rat hippocampus. Importantly, this decrease in the LTP threshold required dopaminergic signaling through the D1/D5 receptors, although the role of protein synthesis was not addressed. While other pathways are known

to activate cAMP signaling in neurons, such as the NMDA receptor-dependent activation of the alpha subunit of calcium/calmodulin-dependent kinase (CamKIIα; Chetkovich and Sweatt, 1993), the fact that dopamine receptor activity is both necessary and sufficient for L-LTP illustrates the importance of dopaminergic signaling in the protein synthesis-dependent phase of long-term synaptic enhancement.

Ultimately, changes in synaptic strength are achieved by modifying the complement, concentration and functional state of synaptic proteins. Such changes are affected by a variety of cellular processes, including trafficking and transport, buffering or sequestration, as well as protein synthesis and degradation. For example, in the presynaptic compartment, rapid axonal transport can provide many of the proteins and vesicles required to maintain and modulate synaptic function. In addition, the calcium-binding protein calmodulin can be buffered by the synaptic protein GAP43 (Baudier et al., 1991; Frey et al., 2000); the activity of some kinases is also effectively buffered by the binding of accessory subunits, such as the functional sequestration of PKA catalytic subunits by regulatory subunit binding. The most direct determinants of protein concentration, however, are protein synthesis and degradation. Translation and degradation are both critically involved in the regulation of protein expression; however the focus of the work described here is on the role of local protein synthesis in synaptic plasticity.

Local Protein Synthesis in Neurons

While there are forms of short-lasting plasticity that rely only on transient enzymatic cascades, long-lasting synaptic changes, as well as enduring memories, require

protein synthesis (Frey et al., 1988; Otani et al., 1989; Stanton and Sarvey, 1984). This requirement for protein synthesis, when considered in the context of the complexity of the dendritic tree and the input-specificity discussed above, poses an interesting question: How are newly synthesized proteins made available only at the synapses where they are needed?

At least three mechanisms may explain how neurons deliver the appropriate subset of proteins specifically to the modified synapses. In two of these, the proteins are synthesized in the cell body and then either shipped out to the correct synapses, or specifically sequestered at the enhanced synapses via an activity-dependent synaptic tag (Frey and Morris, 1997; Martin and Kosik, 2002; Schuman, 1999a). A more elegant solution to this problem, however, is provided by the local synthesis hypothesis. According to this idea, protein synthesis occurs specifically at or near synaptic sites where the new proteins are needed, thereby reducing the metabolic cost of activity-driven protein synthesis while simultaneously achieving region-specific protein delivery. Here I present the various studies that demonstrate the occurrence of local protein synthesis (LPS) and the possible roles for this cellular process in neural plasticity.

Translation machinery

One of the earliest hints at the existence of local protein synthesis in neuronal processes was the electron microscopic detection of polyribosomes in the dendrites of dentate granule cells in the hippocampus (Steward and Levy, 1982). In addition to observing these structures in the distal dendritic compartment, Steward and Levy noted the preferential localization of ribosomal clusters near dendritic spines. These synapse-

associated polyribosome clusters (SPRCs), situated in close proximity to the fundamental input units on CNS neurons, are positioned to respond rapidly to activity at nearby synapses.

Since Steward and Levy's initial characterization of SPRCs twenty years ago, the anatomical evidence supporting local synthesis in neurons has steadily accrued. In fact, an entire complement of translation machinery has been detected in mature neurites, including messenger RNAs, endoplasmic reticulum, and markers for Golgi membranes (Gardiol et al., 1999; Kacharmina et al., 2000; Pierce et al., 2001; Steward and Reeves, 1988; Torre and Steward, 1996). Polyribosomes found at great distances from the nucleus are typically packaged into RNA granules: aggregates of mRNA, translation initiation and elongation factors, and a host of other molecules (Ainger et al., 1993; Knowles et al., 1996; Krichevsky and Kosik, 2001). These granules, which are actively transported from the nucleus to the dendritic and axonal compartments, contain many of the components necessary to carry out regulated protein synthesis in the processes.

Of critical importance in understanding the role of LPS in synaptic plasticity has been the identification of mRNAs present in the dendrites and axons. Using standard *in situ* hybridization techniques, a number of mRNAs have been shown to exhibit somatodendritic localization (reviewed in Steward and Schuman, 2001). These include structural proteins such as MAP2 and β-actin, as well as plasticity-related proteins like CamKIIα and the NR1 subunit of the NMDA receptor. Not all of these messages, however, show identical subcellular distributions within the dendritic compartment. For

example, the NR1 mRNA appears to be limited to the proximal domain of dendrites in the hippocampus (Gazzaley et al., 1997; Miyashiro et al., 1994), whereas the mRNA for CamKIIα is seen throughout the dendritic arbor (Burgin et al., 1990; Mayford et al., 1996).

mRNA trafficking

A number of studies have illustrated that the subcellular distribution of some mRNA transcripts is dynamically regulated in response to neuronal activity. Using high-resolution *in situ* hybridization and immunofluorescence microscopy, Bassell and colleagues investigated β-actin mRNA dynamics in developing cortical neurons in culture. In these experiments, the mRNA appeared in distinct granules that colocalized with components of the translation machinery. Furthermore, these granules were rapidly translocated into the dendritic and axonal growth cones in response to cAMP stimulation (Bassell et al., 1998). In more mature cultured neurons, the mRNAs for BDNF and the TrkB receptor exhibited similar behavior: high potassium-induced depolarization of cultured hippocampal neurons resulted in a redistribution of these mRNA species from a proximal to a more distal dendritic localization (Tongiorgi et al., 1997).

Perhaps the most thoroughly studied example of mRNA redistribution is the dynamic regulation of the mRNA for Arc – the activity-regulated cytoskeleton-associated protein (Lyford et al., 1995; Steward et al., 1998; Wallace et al., 1998). This series of studies took advantage of an ideal hippocampal anatomy: axonal fibers innervating the dentate gyrus of the rat hippocampus are topographically distributed such that axons from a specific region of entorhinal cortex terminate in a specific layer of the dentate granule cell

dendrites. This laminar organization of the hippocampal formation allowed the authors to specifically stimulate one region of the entorhinal cortex resulting in Arc mRNA and protein accumulation precisely in the corresponding synaptic layer of the granule cell dendrites. Because these experiments were performed in mature animals in vivo, they provide very strong evidence for activity-dependent mRNA targeting to the dendrites. A caveat to this study, however, is the fact that maximum electroconvulsive shock (MECS) was used to induce the observed Arc redistribution. While this provided the authors with sufficient Arc signal to be detectable, the stimulation patterns associated with MECS may result in a pathological brain state more than the subtle stimuli that result in synaptic plasticity.

Cis-acting RNA elements

A considerable amount of research effort has been devoted to characterizing the cis-regulatory regions involved in dendritic transport of specific mRNA molecules. For example, sequences in the 3' untranslated region (UTR) of the CamKIIα mRNA are necessary and sufficient for dendritic RNA trafficking (Mayford et al., 1996). Transgenic mice were constructed in which the lacZ coding sequence was placed upstream of either the 3'UTR of CamKIIα or, in a control construct, the polyadenylation sequence for bovine growth hormone (BGH). In mice expressing the lacZ-CamK3'UTR, the lacZ mRNA and protein exhibited punctate distribution in the distal dendrites, while the control construct showed little or no dendritic localization. More recent data from the Mayford lab indicate that the 3'UTR of CamKIIα is necessary for sustained long-term potentiation (LTP) and memory consolidation (Miller et al., 2002). Using a targeted transgene approach, the

authors replaced the 3'UTR of CamKIIα in mice with the BGH 3'UTR. The mutant mice showed dramatically altered CamKIIα mRNA distribution, with a majority of the transcripts being confined to the cell body layers in hippocampus and cortex. Consequently, the dendritic localization of the CamKIIα protein was reduced by over 75 percent. Hippocampal slices prepared from these mutant mice exhibited decreased late-phase LTP. In addition, in an important step toward implicating LPS in behavior, the mutants in this study were impaired in spatial as well as non-spatial memory tasks.

The trafficking of mRNAs in dendrites and axons is likely as dynamic as the trafficking of proteins. In an elegant set of experiments, Kosik and colleagues labeled the 3'UTR of the CamKIIα mRNA using a GFP/MS2 bacteriophage tagging system (Bertrand et al., 1998; Rook et al., 2000). In cultured hippocampal neurons, the authors described three distinct types of motion of the GFP-tagged 3'UTR: oscillatory motion, anterograde transport and retrograde transport, with motion being skewed toward anterograde transport upon depolarization. Of particular interest in this study was the fact that the GFP-tagged CamKIIα 3'UTR was shown to colocalize with synaptic markers as determined by immunofluorescence microscopy. Taken together, these results demonstrate that depolarizing stimuli are capable of driving CamKIIα mRNA into synaptic sites.

RNA-binding and transport proteins

Given that all mRNA synthesis occurs in the cell nucleus, an important question regarding mRNA transport concerns the identity of the molecular machinery involved in exporting RNA granules from the soma out to the dendrites. A tremendous amount of

information from developmental biology has been particularly instructive in this regard. For example, in the early stages of *Drosophila* development, a number of RNA-binding proteins are involved in the asymmetric distribution of mRNA and protein in the fertilized oocyte, which ultimately results in establishment of the anterior-posterior axis of the organism. A critical protein in this developmental process is the RNA-binding protein Staufen, which binds the 3'UTR of maternal mRNAs and transports them throughout the oocyte and developing embryo in a microtubule-dependent manner (Ferrandon et al., 1994; St. Johnston et al., 1991).

The essential role of Staufen in *Drosophila* development prompted the cloning of mammalian Staufen homologues by three independent groups (DesGroseillers et al., 2001; Kiebler et al., 1999; Tang et al., 2001; Wickham et al., 1999). As one of these studies illustrates, the mammalian Staufen proteins are also intimately involved with RNA transport in neurons; overexpression of full-length Staufen results in an increase in total RNA in dendrites (Tang et al., 2001). Conversely, overexpressing the Staufen RNA-binding domain in the absence of the putative microtubule-binding region actually reduces the overall amount of RNA detectable in neuronal processes (Tang et al., 2001). This important observation indicates that Staufen is not only sufficient for RNA transport into dendrites, but that interfering with the function of Staufen can affect overall RNA transport in the cell. While the actual necessity of Staufen for RNA transport during synaptic enhancement remains to be shown, it is clear that the RNA-binding and transport functions of this molecule serve an important function in the maintenance of RNA distributions in neurons.

Translation regulation

If dendritic protein synthesis plays a meaningful role in input-specific synaptic enhancement, there must be translation regulation exerted on mRNAs that are shipped out to the neuronal processes. Without such control, the mRNA may be translated anywhere within the cell and at any point in time after the mRNA has been transcribed. The post-transcriptional modification and regulation of mRNA destined for export from the soma is therefore a very meaningful component of the LPS hypothesis.

Significant progress has recently been made in understanding the regulation of mRNA translation. Rapamycin, a protein synthesis inhibitor with immunosuppressant activity, has been a useful pharmacological agent aiding investigations of regulated protein synthesis. Perhaps hinting at the existence of multiple translation regulatory systems, it has been shown that only a subset of mRNAs inside a cell at a given time are sensitive to translation inhibition by rapamycin (Jefferies et al., 1994; Terada et al., 1994). At the Aplysia sensory-motor neuron synapse, serotonin-induced facilitation is completely blocked by the general protein synthesis inhibitor emetine but only partially inhibited by rapamycin (Casadio et al., 1999). In adult hippocampal neurons, the mammalian target of rapamycin (mTOR) and other proteins involved in cap-dependent translation initiation are localized to synaptic sites (Tang et al., 2002). Furthermore, Tang and colleagues showed that rapamycin treatment of hippocampal slices selectively attenuates late-phase LTP, while completely blocking BDNF-induced synaptic enhancement. In this case, the inhibition of synaptic plasticity observed in the presence of rapamycin was remarkably similar to that observed in the presence of a general protein synthesis inhibitor (Kang and

Schuman, 1996; Kang et al., 1997). Taken together, these findings suggest that rapamycin-sensitive translation plays an important role in synaptic plasticity.

CPE and IRES sequences

Another potentially crucial element of translation control is the cytoplasmic polyadenylation element (CPE) and the cognate binding protein CPEB (Wells et al., 2000). Polyadenylation of mRNA transcripts is generally required for efficient translation, making temporal control over this process a candidate regulatory point in activity-driven protein synthesis. A detailed molecular analysis of CPEB signaling has revealed a putative role for this biochemical pathway in activity-driven polyadenylation and translation of the CPE-containing CamKIIα mRNA (Wu et al., 1998). In this model, an mRNA containing CPE sequences in its 3'UTR is rendered translationally dormant through an intricate series of protein-protein interactions involving CPEB, maskin, and the rate-limiting translation initiation factor eIF4E (Stebbins-Boaz et al., 1999). These messages become translationally competent as a result of aurora kinase-catalyzed polyadenylation (Huang et al., 2002), and subsequent dissolution of the maskin-eIF4E interaction via the polyadenylation binding protein (Cao and Richter, 2002). While there is much that remains to be learned in terms of understanding precisely how this process is involved in synaptic plasticity, the evidence that cytoplasmic polyadenylation is a potentially important check point has been clearly established. Further investigations of the interactions between mTOR and CPEB-mediated cascades may provide the basis for an understanding of the complete series of reactions required to initiate translation of mRNAs in an activity-dependent fashion.

The stereotypical cap-dependent translation initiation through the CPEB pathway may only be part of the story, however. Another potentially interesting component of regulated protein synthesis is the cap-independent translation mediated by internal ribosome entry sites (IRESes) present in some mRNAs. Although traditionally associated with bicistronic messages found in viral genomes, IRES sequences may also serve as regulators of protein synthesis in eukaryotic mRNAs. Importantly, IRESes have been identified in a number of dendritically localized messages, including Arc, CamKIIα, and neurogranin (Pinkstaff et al., 2001). Of particular interest is the observation that the neurogranin IRES confers preferential cap-independent translation initiation in the dendrites, while protein synthesis regulation in the soma is mediated by a cap-dependent mechanism (Pinkstaff et al., 2001). This finding raises the possibility that distinct mechanisms may differentially regulate somatic versus dendritic translation of a single mRNA species.

Demonstrations of Local Protein Synthesis

With all of the components in place, and armed with the knowledge that mRNA granules containing translation machinery can be exported to the dendrites of living cells in an activity-dependent manner, it may seem a trivial leap to conclude that LPS is taking place in the dendritic compartment. However, the presence of mRNA and other requisite translation machinery does not necessarily indicate that all of these components are competent to produce functional proteins.

While most research in the field to date has focused on regulated translation in dendrites, the possible existence of axonal protein synthesis machinery has been described (Koenig and Giuditta, 1999). It has also been shown that protein synthesis in the presynaptic compartment of dorsal root ganglion neurons regulates axon regeneration in response to injury (Twiss et al., 2000; Zheng et al., 2001). More recent work has demonstrated a role for presynaptic LPS in growth cone responsiveness to local guidance cues during axon elongation (Brittis PA, 2002). Using a high-density retinal explant culture system, Brittis and colleagues determined that mechanically isolated axons continue to synthesize a GFP reporter. An important issue convincingly addressed in this study is the ability of distal processes to produce transmembrane proteins and deliver them to the cell surface. While it remains unclear whether axonal protein synthesis occurs in undamaged adult neurons, these data indicate that LPS plays a critical role in the presynaptic compartment during development and repair.

Radiolabeled amino acid uptake

One of the earliest studies providing compelling evidence that protein synthesis may take place in neuronal processes employed ventricular injection of ^{3}H-leucine into adult rats and subsequent autoradiographic and electron microscopic analysis (Kiss, 1977). From these early experiments, Kiss observed that a majority of protein synthesis in dendrites was restricted to the proximal domain, although a small amount of [^{3}H]leucine incorporation could be seen throughout the dendrites. It was not clear from this study,

however, that the signal detected in distal processes was significantly higher than background levels. Furthermore, given the nature of these experiments, and the considerable delay between injection of the radioisotope and fixation of the sample for analysis, a somatic source of the proteins detected in the dendrites could not be ruled out.

A similar approach with analogous results was described in acute hippocampal slices (Feig and Lipton, 1993). Pairing electrical stimulation of Schaffer collateral axons with the cholinergic agonist carbachol resulted in increased ^{3}H-leucine incorporation in the dendritic layers of hippocampal slices. While this further suggested the involvement of LPS in plasticity, it was unfortunate that the stimulation protocol used failed to elicit any type of synaptic modification. In addition, as with the study by Kiss, somatic protein synthesis as a source for the radiolabeled dendritic proteins could not be conclusively ruled out. Indeed, the fact that the cell body is such a large source of protein synthesis, with the neuronal processes being a relative sink, has present problems for researchers interested in the local protein synthesis hypothesis. In order to conclusively determine that the source of increased protein concentration in the dendrites cannot be attributed to proteins that were initially synthesized in the cell body, it is essential that the cell bodies and dendrites be somehow dissociated from one another.

Synaptosome preparations

Initial attempts at accomplishing this difficult task relied on subcellular fractionation techniques. Using a combination of biochemical tissue dissociation, filtration, and density gradient centrifugation of brain homogenates, it is possible to isolate small

membranous particles termed synaptosomes. These synaptosomes are pinched-off membranous structures which, when viewed through the electron microscope, appear to be presynaptic terminals and their associated postsynaptic structures. A number of groups have employed this method of synaptosome preparations as a means of addressing the local synthesis hypothesis without the confound of potential somatic contributions.

Perhaps unsurprisingly, the CamKIIα mRNA is enriched in synaptosomes. Upon NMDA receptor activation, CamKIIα protein is specifically upregulated in this biochemically-purified preparation, while overall translation is reduced (Scheetz et al., 2000). From this work, it is clear that synaptic stimulation is not simply activating translation of all proteins at random, but rather that a subset of proteins is preferentially increased. Other studies in synaptosomes have shown that activation of metabotropic glutamate receptors increases synthesis of the fragile-X mental retardation protein (FMRP) (Weiler et al., 1997). The RNA-binding properties of this protein are critically involved in neuronal development (Darnell et al., 2001), and deletion of the FMRP gene results in a dramatic loss of protein synthesis detected in synaptosomes (Greenough et al., 2001).

With sufficiently pure synaptosome fractions, it should be possible to evaluate the protein synthesis competence of these structures in the absence of cell bodies. There are, however, a number of complications involved in interpreting the synaptosome data (reviewed in Steward and Schuman, 2001). In particular, a number of mRNAs exist in synaptosomes that are entirely absent from the dendrites of neurons as determined by *in situ* hybridization (Steward and Schuman, 2001). These contaminants include the mRNA

encoding glial fibrillary acidic protein, a protein that is found exclusively in glial cells. The presence of such an impurity in synaptosomes warrants caution in the interpretation of data acquired with this technique.

Acute hippocampal slices

An important study providing very strong evidence of a direct link between LPS and synaptic enhancement used a brute-force method of removing principal cell bodies as a potential protein source: cell bodies in area CA1 of hippocampal slices were isolated from the synaptic neuropil by way of a microlesion with a dissecting knife (Kang and Schuman, 1996). Having previously demonstrated protein synthesis-dependent synaptic enhancement in hippocampal slices induced by neurotrophin treatment (Kang and Schuman, 1995a), Kang and Schuman used the microlesion technique to further demonstrate that this protein synthesis-dependent synaptic enhancement persisted even when the cell bodies were physically isolated from the dendrites. While this was the most convincing evidence to date, the argument could be made that the protein synthesis required for neurotrophin-induced enhancement was taking place in glial cells, interneurons or axons – all of which are present in the dendritic layer of the hippocampus.

In addition to long-term potentiation, neurons in hippocampal slices exhibit long-term depression, or LTD. This form of synaptic modification can be induced in slices by bath application of the group 1 metabotropic glutamate receptor agonist DHPG [(RS)-3,5-dihydroxyphenylglycine](Huber et al., 2000). Using a microlesion approach similar to that employed by Kang and Schuman, Huber and colleagues convincingly demonstrated that

DHPG-induced LTD in area CA1 is blocked by bath applied inhibitors of protein synthesis. Further strengthening the claim of LPS in the dendritic compartment, the authors also showed that inclusion of a protein synthesis inhibitor in the postsynaptic whole-cell recording pipette blocked mGluR-induced LTD. Because the net electrophysiological consequences of LTP and LTD are effectively opposite, it will be interesting to learn what proteins are differentially upregulated in response to these two forms of synaptic plasticity.

Dissociated culture systems

The dissociated cell culture system has provided the most definitive data illustrating the ability of neurites to synthesize proteins. In a beautiful series of experiments, Martin and colleagues used cultured Aplysia neurons to examine the possibility that LPS contributes to long-term facilitation (LTF) in these neurons (Martin et al., 1997). Given the large size and relatively robust nature of Aplysia neurons, the authors were able to culture a single bifurcating sensory neuron synapsing onto two spatially separated motor neurons. Taking great care to avoid the cell body, serotonin was locally perfused onto one of the connections, leaving the other sensory-motor connection unperturbed. The result of this precise serotonin application was input-specific LTF: only the synapses treated with serotonin exhibited synaptic enhancement. Importantly, the LTF was blocked by injection of a protein synthesis inhibitor into the presynaptic sensory neuron. Finally, to assess the ability of isolated neurites to synthesize proteins, 30-40 sensory neurons were cultured, their cell bodies removed, and then tested for their ability to incorporate ^{35}S-methionine.

Even in the absence of cell bodies, these isolated sensory neuron processes incorporated the radiolabeled amino acid in a protein synthesis-dependent manner.

These exciting results provided the first definitive proof that neurites, independent of the cell body, can produce proteins in response to synaptic enhancement-inducing stimuli. While intriguing, the results of Martin and colleagues may be limited due to the differences between vertebrate and invertebrate neurons. A presynaptic locus of protein synthesis-dependent synaptic enhancement is also inconsistent with the ultrastructural anatomical data in vertebrate neurons: SPRCs are present in dendrites but have not been detected in axons of mature vertebrate neurons in the CNS.

In order to conclusively demonstrate the occurrence of local, dendritic protein synthesis in mammalian neurons, we expressed a GFP-based protein synthesis reporter in cultured hippocampal neurons and monitored the GFP signal in real time using confocal microscopy (Aakalu et al., 2001). As detailed in Chapter 2, the GFP construct was translated in isolated dendrites in response to BDNF, and the highest intensity GFP signal was found near synaptic sites. Having developed a useful system for examining local translation, we next turned our attention to the effects of dopaminergic signaling on LPS. The results of these experiments, the focus of Chapter 3, show convincingly that D1/D5 dopamine receptor activity converts silent into active synapses by increasing surface GluR1 at synaptic sites in a protein synthesis-dependent manner.

In the course of these studies, it became apparent that more rigorous tools for analysis of colocalization and spatial correlation of immunolabeled proteins were required.

In Chapter 4, I present and provide a detailed methodology for the quantitative analysis of 3-D colocalization and 2-D correlation of protein clusters within the dendrites of cultured hippocampal neurons. The algorithms take into account important issues of image analysis including thresholding, signal quantity, and sensitivity of the results to user bias. Code for implementation of the algorithms in the MATLAB environment is provided in Appendices A and B.

Finally, while considerable progress has been made in the study of local protein synthesis, a number of intriguing questions remain. For example, relatively little is known about the degree to which input-specificity is expressed at the level of small groups of synapses. Furthermore, the role of local protein synthesis in establishing and maintaining input-specificity remains to be determined. Another issue concerns the relationship between NMDA receptors and dopaminergic signaling in the protein synthesis-dependent conversion of silent into active synapses. These questions, as well as a discussion of the potential future directions for this line of research, are discussed in Chapter 5.