Chapter II

LITERATURE REVIEW

Cadherins

The cadherins are a large superfamily of calcium-dependent cell adhesion proteins. The cadherin superfamily includes classic cadherins, desmosomal cadherins, protocadherins, and other cadherin-related proteins, all of which have multiple repeats of an approximately 110 amino acid cadherin-specific motif (the extracellular cadherin repeat, or EC), in their extracellular domain (figure 1) (reviewed in Angst and Marcozzi, 2001; Takeichi and Abe 2005; Suzuki 1996). The classic cadherins, of which there are ~20, were the first discovered and are the best studied. Examples of classic cadherins include neural (N)-, retinal (R)-, vascular endothelial (VE)-, epithelial (E)-, and placental (P)-cadherin, named by the tissues in which they were originally discovered, but now known to have much broader tissue distributions. Classic cadherins are characterized by a highly conserved C-terminal cytoplasmic domain, a single transmembrane domain, and an N-terminal ectodomain consisting of five EC repeats. Calcium ions, which are required for adhesive binding, intercalate between the EC domains to produce a rigidified, rodlike ectodomain (Nagar, Overduin et al. 1996).

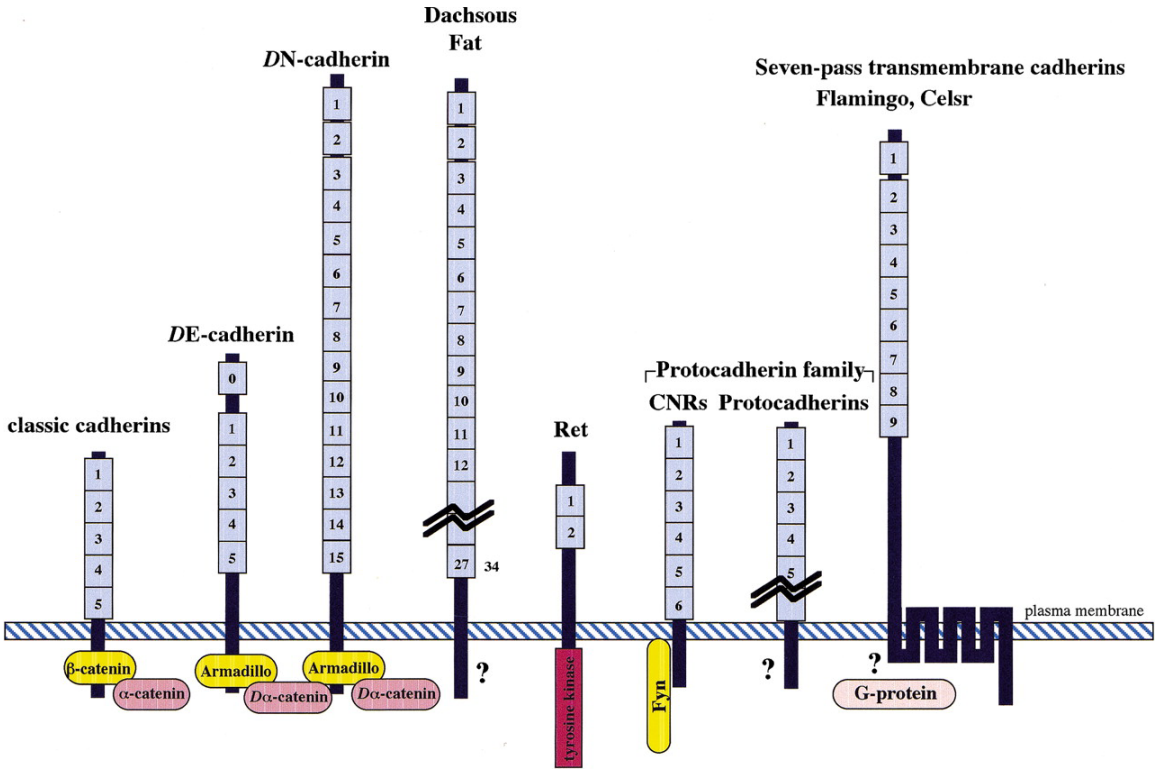

Figure 1. Schematic diagram of the molecular structure of the cadherin superfamily (blue) and their cytoplasmically associated proteins (yellow, pink) (from Yagi and Takeichi 2000).

Intracellularly, classic cadherins associate with a number of different kinds of molecules that mediate downstream signaling, as well as the actin cytoskeleton. The catenins (α, β, and γ) link classic cadherins to the actin cytoskeleton; this linkage is essential for adhesive function (Nagafuchi and Takeichi 1988). Interactions with other intracellular proteins such as p120, δ-catenin, and presenilin are thought to regulate dimerization of cadherins expressed in the membrane of the same cell (as known as *cis* interactions, discussed in the next section below). This has been shown to modulate the strength of interactions with cadherins on adjacent cells (known as *trans* interactions)

presumably by laterally clustering cadherins on the cell surface (Yap, Niessen et al. 1998; Baki, Marambaud et al. 2001).

Cadherin structure and adhesive function

Initial structural studies of cadherins (Shapiro, Fannon et al. 1995; Overduin, Harvey et al. 1995) utilized X-ray crystallography and NMR spectroscopy to determine the structure of the most N-terminal (most distal from the cell membrane) EC repeat, as this repeat was known from earlier work to be necessary for homophilic binding specificity (Nose, Tsuji et al. 1990). This work showed the EC structure to consist of 7 β strands and 2 α helices in a β-barrel topology similar to the immunoglobulin fold. Earlier studies had demonstrated that a conserved amino acid sequence, the HAV region (residues 79–81 of E-cadherin), which is common to the first EC of all cadherins, was required for adhesive function (Blaschuk, Sullivan et al. 1990). The surface displaying this HAV sequence, as well as the residues responsible for Ca^{2+} ion binding, were also determined.

Based on crystal lattice contacts observed in the structure of the first EC repeat, a two-step cadherin association mechanism was proposed (Shapiro, Fannon et al. 1995). First, a *cis* interaction pair (that was dubbed the "strand dimer") is formed between two parallel molecules by mutual exchange of a β-strand due to binding of a tryptophan (Trp2) from one molecule into a hydrophobic pocket of the partner molecule. Second, a *cis* dimerized pair undergoes a *trans* interaction (dubbed the "adhesion dimer") with a complementary antiparallel unit. Alternating *cis* and *trans* interactions then form a zipperlike superstructure.

These initial structural studies implicate only the first 2 ECs in these interactions. Several later studies, including a crystallization of the entire extracellular domain of C-cadherin (Boggon, 2002) and an electron tomographic study of epithelial desmosomes (He, Cowin et al. 2003) in which this C-cadherin ectodomain structure was fit to electron densities, representing cadherins in their "in situ habitat", confirmed the involvement of only the first 2 ECs. In constrast to the crystallographic studies, the tomography showed a much less ordered packing of cadherins, with the cadherin N-terminal interactions not forming a regular lattice. Cadherins were seen to assemble into groups of 10 to 20 molecules, with their N-terminal domains forming a series of "knots" at the midline of the cell-cell junction. Similar to the latticed packing seen in crystals, this grouping facilitates extensive interactions between EC domains.

In contrast to this N-terminal model for cadherin interactions, a number of biophysical studies have, through the use of surface force probes (Perret, Leung et al. 2004; Sivasankar, Gumbiner et al. 2001; Zhu, Chappuis-Flament et al. 2003) and cadherin-coated beads in a flow chamber (Perret, Benoliel et al. 2002), suggested that all ECs contribute to the interactions and interestingly, the strongest interaction is detected when all ECs are bound to each other (Perret, Leung et al. 2004), with the cadherin ectodomains fully interdigitated.

Functional inactivation of E-cadherin in CHO cells by mutating several conserved cysteine residues in EC5, the EC closest to the plasma membrane, further supports the idea that all ECs contribute to the interaction (Chappuis-Flament, Wong et al. 2001). Taking all of this data together, it appears that the most distal ECs (1 and 2)

are likely the most important for homophilic interactions, but the other ECs (3-5) also participate.

Cadherin-mediated adhesion generates stable, tight junctions. In adults, interactions between cadherins on adjacent cells help to maintain the structural integrity of solid tissues and regulate the turnover and reorganization of tissue structures. For example, in adherens junctions between epithelial cells, E-cadherin forms the transport barrier between the intestine and bloodstream, while vascular endothelial cadherin (VE-cadherin) maintains the barrier properties of blood vessels. In addition to being stable enough to maintain tissue integrity, these junctions must be sufficiently plastic to permit rapid remodeling of tissue interfaces, allow for cell motility, and enable cellular rearrangements during different stages in development.

Understanding how cadherins form cell-cell junctions is central to understanding their role in biology. A fundamental challenge is to determine the mechanism of intercellular adhesive bond formation.

Neuronal cadherins

Cadherins are expressed in many tissues, including the central nervous system where they are most well known for their role in target recognition and stabilization during synaptogenesis. Crucial to synaptogenesis is the ability of axons to grow to their targets and form synapses with the correct postsynaptic cell type. Axons often travel long distances before reaching their final target, and although they come in contact with many potential partners along the way, they do not establish synapses on inappropriate cells.

Given the large number of targets, the system (or systems) mediating this recognition would need to be complex, and this requirement could only be met by adhesion proteins that offer sufficient combinatorial possibilities. This "lock and key" mechanism was first hypothesized by Sperry in 1963, and implies the existence of specific adhesion molecules that pair axons with their targets. There are now known to be at least 80 cadherins likely to be expressed in nervous tissue, including the classical cadherins and the protocadherins; this positions cadherins as likely candidates for a role as "synaptic specifiers."

There are many lines of evidence to support the idea that cadherins are responsible for proper synapse formation: Blockade of N-cadherin function in cultured hippocampal neurons by overexpression of a dominant negative cadherin (which lacks its ectodomain) results in a significant block of synapse assembly, detected as a loss of spines and an increase in the number of filopodia (Togashi, Abe et al. 2002). These effects are more pronounced in younger neurons, demonstrating that cadherins are active at earlier stages of synapse development. In addition, synapses that form on neurons that overexpress the dominant negative cadherin are smaller than controls and have decreased synaptic vesicle recycling and a decreased frequency of spontaneous EPSCs. They also fail to acquire resistance to F-actin depolymerization, which is a hallmark of mature, stable synapses. (Bozdagi, Valcin et al. 2004).

In the retinotectal system, treatment with antibodies that block N-cadherin adhesion causes retinal ganglion cells to overshoot their targets (Inoue and Sanes 1997). In the cerebellum, cadherins have been shown to appear in distinct cortical "stripes,"

expressed in cells that project to underlying nuclei expressing the same cadherin, forming zones of topographically organized connections (Arndt, Nakagawa et al. 1998). N-cadherin has been proposed to mediate the initial synaptic adhesion of the thalamocortical projections from the ventrobasal nucleus (that represent the facial vibrissae, or "whiskers") to the barrel field of somatosensory cortex. Levels of N-cadherin in thalamic axons decrease at postnatal day 9, however, αN- and β-catenin persist at the adult synapse (Huntley and Benson 1999). In hippocampal cultures N-cadherin first appears at all synapses but rapidly disappears in all but excitatory glutamatergic synapses (Benson and Tanaka 1998). In these last two studies, the persistence of β-catenin at synapses at which N-cadherin is eventually not present suggests that other cadherins may become important at these synapses during the course of maturation. This indicates that N-cadherin adhesion may stabilize early synapses that can then be remodeled and express a different cadherin.

These studies demonstrate the diverse roles of cadherins in synaptogenesis, and taken together, show cadherins to be deeply involved in regulation of axonal target specificity.

Cadherin neuronal expression however, persists into adulthood, arguing for a cadherin role beyond synapse formation. In 1995, it was demonstrated by a combination of immunoprecipitation and Western blot analyses that N-cadherin is a major constituent of isolated rat forebrain postsynaptic densities (Beesley, Mummery et al. 1995). A subsequent study used confocal microscopy to optically section through synaptic junctions in adult mouse cerebellum, and, by rotating the stacked images, showed that in many cases, N-cadherin immunolabeling formed a ring that surrounded a central "pore"

of the presynaptic protein synaptophysin (Fannon and Colman 1996). αN and β-catenin have been shown to be localized to apposed pre- and postsynaptic membranes in circumscribed zones that flank the active zone presynaptically, the site of vesicle release, and the postsynaptic density, in which the neurotransmitter receptors are localized (Uchida, Honjo et al. 1996). Reconstruction from serial electron microscopy was used to determine the location and size of puncta adherens (cell junctions with enriched levels of adhesion molecules, including cadherins) in the stratum radiatum of hippocampal area CA1: puncta adherens were found at the edges of synapses on 33% of dendritic spines (Spacek and Harris 1998).

A number of more recent studies have used confocal microscopy to examine the distribution of various cadherins in relationship to immunolabeling for synaptic vesicle proteins such as synaptophysin (Arndt, Nakagawa et al. 1998; Benson and Tanaka 1998; Tang, Hung et al. 1998).

β-Catenin

As discussed above, β-catenin binds to the intracellular domain of cadherin and links it to the actin cytoskeleton through β-catenin's interaction with α-catenin, a vinculin-like actin-binding protein. This linkage is required for cadherin adhesive function – when cadherin with a deleted β-catenin binding site is expressed in L cells (a cell line lacking endogenous cadherins but expressing all catenins), the extracellular domain of the mutant E-cadherins was properly exposed on the cell surface, and had normal Ca^{2+}-sensitivity and molecular size. However, L cells expressing these mutant cadherins did not show any Ca^{2+}-dependent aggregation, indicating that the mutant molecules cannot mediate cell-cell binding (Nagafuchi and Takeichi 1988).

β-catenins interaction with cadherins is known to be negatively regulated by tyrosine phosphorylation (Roura, Miravet et al. 1999; Muller, Choidas et al. 1999; Pathre, Arregui et al. 2001; Sommers, Gelmann et al. 1994). Cyclin-dependent kinase 5 (Cdk5)/p35 kinase has been implicated as the responsible kinase, through its effect on the phosphorylation level of tyrosine-654 of β-catenin (Schuman and Murase 2003).

Our laboratory has shown that tyrosine kinase inhibitor promoted redistribution of β-catenin into spines, suggesting that under normal conditions, constitutive phosphorylation of β-catenin reserves substantial levels of β-catenin in the dendritic shaft (Murase et al., 2002). Consistent with this, postsynaptic expression of a mutant β-catenin in which phosphorylation was prevented resulted in its accumulation at spines. In contrast, expression of a phosphorylation-mimic mutant accumulated in the dendrite shafts. Accumulation of the dephosphorylation-mimic β-catenin mutant in spines was accompanied by an increase in the size of synapsin and PSD-95 clusters. Furthermore, presynaptic activity was elevated, as judged by an increase in FM4-64 dye uptake and the rate of spontaneous neurotransmitter release. We also showed that β-catenin moves from dendritic shafts into spines upon depolarization, increasing its association with cadherins ((Murase, Mosser et al. 2002) - my contributions to this work are discussed in chapter III). As a corollary to this, Togashi at al. observed that synapsin and PSD-95 puncta as well as FM4-64 dye uptake were decreased when spine localization of β-catenin was impaired by expression of a dominant-negative N-cadherin lacking a portion of the extracellular domain (Togashi, Abe et al. 2002).

In 2003, Bamji et al., used EM analysis to show a reduction in the number of synaptic vesicles in the neurons of β-catenin conditional knockout mice. Recently, the same group showed that brain derived neurotrophic factor (BDNF) mobilizes synaptic vesicles at existing synapses, causing small clusters of synaptic vesicles to "split" away from synaptic sites. This ability of BDNF to mobilize synaptic vesicle clusters depends on the dissociation of cadherin-β-catenin adhesion complexes that occurs after tyrosine phosphorylation of β-catenin. Overexpression of the phosphorylation-blocked mutant β-catenin increases cadherin-β-catenin interactions and abolishes the BDNF-mediated enhancement of synaptic vesicle mobility, as well as the longer-term BDNF-mediated increase in synapse number (Bamji, Rico et al. 2006).

In addition to its role in linking cadherin to the actin cytoskeleton, β-catenin has long been known for the part it plays through the Wnt signaling pathway, in tumorigenesis. Cell-cell interactions are known to be a factor in cancer, and it has been suggested that β-catenin may be a key player in linking cell adhesion to transcriptional signaling through the Wnt pathway. Control of these dual functions is crucial for maintaining normal cellular function. Deregulation, which promotes the transcriptional function of β-catenin over its function in adhesion, is a major factor in the development and progression of many human malignancies (reviewed in Brembeck, Rosario et al. 2006).

It is clear that cadherins and their associated proteins, such as the catenins, play an important role in coordinating surface adhesion and cell recognition with cytoskeletal activity and cell signaling.

Synaptic plasticity

Although initial views of cadherin function in the nervous system were dominated by cadherin's roles in establishing brain structure and synaptic connectivity during development, their function at the synapse involves more than a merely structural role and includes a capability for modulation of the synaptic signal itself.

The phrase "synaptic plasticity" is used to describe changes in the strength of a synaptic signal as recorded electrophysiologically, as well as changes in synapse morphology or number. These two different forms of plasticity (functional vs. structural) are thought to be related as changes in synapse structure or number are thought to contribute to functional changes in synaptic strength. There is evidence for a cadherin role in both of these forms of plasticity. Cadherins have been studied, in this regard, mostly in the context of hippocampal long-term potentiation (LTP). LTP is characterized by an increase in synaptic strength lasting from several hours to days or longer, and is the primary cellular model for how neurons enable learning and memory formation (first described in Anderson and Lomo 1966; Bliss and Lomo 1973). In hippocampal area CA1, LTP shows two distinct phases: an early phase (E-LTP) that does not require protein synthesis and lasts ~1 to 2 h, and is thought to reflect posttranslational modifications and translocation of pre- and postsynaptic proteins (reviewed in Nicoll, 1999); and a more slowly developing, but long-lasting late phase (L-LTP) that requires gene transcription and protein synthesis and lasts many hours to days or longer (reviewed by Bailey, Bartsch et al. 1996). This phase may also reflect synaptic structural changes. Induction of late-phase LTP in hippocampal area CA1 appears to cause the growth of new dendritic spines or filopodia (suggested to serve as spine precursors (reviewed in

Yuste and Bonhoeffer 2004)) on the dendrites postsynaptic to the stimulated synapses (Engert and Bonhoeffer 1999; Maletic-Savatic, Malinow et al. 1999). LTP induction has also been found to result in ultrastructural modifications to existing synapses, with apparent increases in the number of perforated synapses, the size of the apposition zones between pre-and postsynaptic structures, and the length of the PSD (Buchs and Muller 1996). As discussed, this sort of structural remodeling brings with it a requirement for a stable, yet plastic means of regulating cellular adhesion. As mentioned above, cadherins have been shown to have a role in both functional and structural plasticity.

Induction of late-phase LTP has been shown to cause a significant increase in the number of synaptic puncta identified by visualizing synaptophysin and N-cadherin. This increase was PKA- and protein synthesis dependent. This induction also caused an increase in N-cadherin dimerization indicating a specific role for N-cadherin in the signal transduction cascade that leads to lasting synaptic potentiation (Bozdagi, Shan et al. 2000).

When cultured hippocampal cells are stimulated using high K^+ to depolarize them, N-cadherin dimerizes and acquires a long-lasting protease resistance (Tanaka, Shan et al. 2000). This has been shown to be protein synthesis independent and is not accompanied by N-cadherin internalization. In addition, N-cadherin, normally localized in discrete puncta in close proximity to the PSD, is rapidly and actively dispersed laterally along the plasma membrane upon depolarization with high K+. Furthermore, the presence of the NMDA receptor antagonist APV attenuated this acquisition of protease resistance and dimerization while direct stimulation with NMDA induced a high level of protease resistance and dimerization but does not induce cadherin dispersion.

The importance of Ca^{2+} ion

Cadherins and Ca^{2+}

Cadherin adhesion has a strict requirement for calcium, which rigidifies the interrepeat links and apparently activates *trans* interactions (Hyafil, Babinet et al. 1981). In the absence of calcium, cadherins undergo a reversible loss of their rodlike structure and collapse (Pokutta, Herrenknecht et al. 1994). Electron microscopic studies of oligomerized E-cadherin extracellular domains at different calcium concentrations have revealed that relatively low calcium concentrations (≥50 μM) are required to maintain the overall rodlike structure of the ectodomain, while higher concentrations (≥500 μM) are required for *cis* interactions (Pertz, Bozic et al. 1999). Ca^{2+} ions bind to pockets in the interrepeat regions of the cadherin extracellular domain with a stoichiometry of 3 Ca^{2+} per calcium-binding domain (Nagar, Overduin et al. 1996; Tamura, Shan et al. 1998). Interestingly, these sites bind Ca^{2+} with different affinities; when considered as an intact structure, the complete extracellular region binds Ca^{2+} with an average dissociation constant (K_d) of 30 μM (Koch, Pokutta et al. 1997), consistent with the minimal calcium concentrations required to maintain cadherin structure. The 3 Ca^{2+} binding sites between the two most N-terminal domains (EC1 and 2) were found to have K_ds at least an order of magnitude higher. One site of these 3, in particular, was shown to have a particularly high K_d (2 mM, as opposed to 330 μM for the other 2), implicating its role in the *trans* interactions only seen at higher calcium concentrations (>1 mM) (Pertz, Bozic et al. 1999).

Ca^{2+} in the extracellular space

It is interesting to consider the Ca^{2+} dependence of cadherin interactions in the context of the physiological concentrations of calcium found in the extracellular space in neurons. Average extracellular Ca^{2+} concentrations in mammalian brain range from 1.5 to 2.0 mM (Jones and Heinemann 1987), which is well within the range where cadherins engage in both *cis* and *trans* interactions. Experimental techniques have not yet provided direct, rapid measurements of Ca^{2+} concentration within individual synaptic clefts. However, computation approaches have yielded interesting results: simulations of active synaptic clefts (Egelman and Montague 1998, 1999; Wiest, Eagleman et al. 2000) predict that during synaptic activity, cleft Ca^{2+} concentrations will drop into the range where local synaptic and/or perisynaptic cadherins would lose *trans* interactions (~800 μM to 1 mM), and possibly (depending on synaptic parameters such as active zone size) to levels that would cause a loss of *cis* interactions as well (~200 μM). More recently, novel optical methods of measuring extracellular calcium have provided direct measurements of Ca^{2+} concentrations in active neuropil (Rusakov and Fine 2003). These experiments show that brief stimulation evokes significant depletion of extracellular Ca^{2+} in an NMDAR-dependent manner. Since the average synaptic cleft size (6-8 x 10^{-4} μm^3, assuming cleft width of ~20 nm and postsynaptic density area of 0.03 – 0.04 μm^2) (Rusakov and Kullmann 1998; Schikorski and Stevens 1997; Shepherd and Harris 1998) is below the optical resolution of laser scanning microscopy, direct measurements of Ca^{2+} concentration in the local microenvironment of individual synaptic clefts was not possible, but as the authors point out, the observed ~40-50 μM decrease in Ca^{2+} over the sampled neuropil volume of ~0.1 μm^3 could be interpreted as almost total depletion of

Ca^{2+} at all active synapse in the area. Thus, experimental data as well as simulations predict that Ca^{2+} is dynamically regulated in the synaptic cleft. This implies that alterations in cleft Ca^{2+} have important ramifications for cadherin-cadherin adhesion and signaling (Murase, Mosser et al. 2002; Tang, Hung et al. 1998).

Ca^{2+} and LTP

LTP and other forms of synaptic plasticity have long been studied as attractive cellular correlates of learning and memory. A widely accepted model for LTP induction involves activation of NMDA receptors, which allows Ca^{2+} to enter the dendritic spine (reviewed in Bliss and Collingridge 1993). The rise in intracellular Ca^{2+} that results is generally agreed upon to be the "trigger" for LTP. As discussed above, Ca^{2+} influx may also result in a Ca^{2+} decrease in the synaptic cleft. This local decrease in Ca^{2+} could cause a loss of cadherin rigidification and disruption of cadherin adhesion.

Treatments that interfere with cadherin function have been shown to significantly attenuate hippocampal LTP. When cadherin adhesion is blocked by either antibodies specific for the N- or E-cadherin ectodomain, or peptides that bind to the conserved HAV sequence discussed above, LTP is inhibited if the peptide or antibody is present during induction of LTP. However, if application of the peptide or antibody is delayed until 30 minutes after LTP induction, no inhibition of LTP occurs (Tang, Hung et al. 1998). The same treatment had no effect on normal synaptic transmission or short-term synaptic plasticity. This suggests that the synaptic activity that leads to induction of LTP may lower local cleft Ca^{2+} concentration and render the synaptic cadherins temporarily vulnerable to antibody or peptide binding. Consistent with this idea, the LTP blocking

effect could be overcome by raising the extracellular Ca^{2+} concentration, presumably compensating for the stimulation induced Ca^{2+} depletion (Tang, Hung et al. 1998). Additionally, during the maintenance phase after successful induction of LTP, extracellular Ca^{2+} was temporarily decreased and then returned to normal levels. When performed in the presence of a scrambled control peptide, LTP recovered to pre-Ca^{2+}-lowering levels. However, when performed in the presence of HAV peptides, this resulted in an inhibition of LTP, with potentiation levels never recovering (Tang and Schuman, unpublished observations).

Thus, HAV peptides inhibit LTP only when applied during induction, or in the presence of reduced extracellular Ca^{2+}. This implies that the inhibition of LTP occurred because the high frequency stimulation used to induce LTP caused a reduction in extracellular Ca^{2+}, disrupting cadherin interactions and allowing the HAV peptides access to their binding sites.

When considered as a whole, current data points strongly to an extracellular Ca^{2+}-mediated role for cadherin in synaptic plasticity.

FRET as a tool for studying protein dynamics

Fluorescence (or Förster) resonance energy transfer (FRET) has been shown to be a useful tool in determining spatiotemporal relationships between molecules (Sorkin, McClure et al. 2000; Broudy, Lin et al. 1998; Damjanovich, Bene et al. 1997; Kenworthy and Edidin 1998; Kenworthy, Petranova et al. 2000; Selvin 1995; Guo, Dower et al. 1995). FRET is a process in which energy is nonradiatively (by means of long-range dipole-dipole coupling) transferred from a donor fluorophore in an electronic excited

state to an acceptor (which may, but does not need to, be fluorescent). The efficiency of energy transfer varies inversely with the 6^{th} power of the donor-acceptor separation over the range of 1-10 nm. This range of distances is relevant for most biomolecules and their subdomains involved in complex formation and conformational changes. Energy transfer by FRET is also dependent on the extent of overlap of donor emission and acceptor adsorption spectra and the relative orientation of the donor and acceptor dipoles. Because of these factors it can be difficult to use FRET to determine absolute distances, but FRET can be quite effectively used to determine relative distances and changes in relative distance.

FRET is unique in its ability to provide signals that are sensitive to changes in intra- or intermolecular distances in the 1-10 nm range, well below the inherent diffraction limit of conventional fluorescence microscopy.

Thus, FRET is an excellent technique to monitor in living cells protein orientation and interactions, allowing us to observe changes in cadherin-cadherin interactions induced by changes in extracellular Ca^{2+} as well as any changes induced by synaptic activity in neurons.

In subsequent chapters, I will first describe my work in analyzing the dynamics of β-catenin in neurons. I will then describe my development of FRET-based cadherin homophilic interaction reporters and their use in studying changes in cadherin interactions in response to extracellular Ca^{2+} concentration.