Chapter V

DISCUSSION AND FUTURE DIRECTIONS

Members of the cadherin family of cell surface glycoproteins have long been known to be involved in many aspects of cell-cell adhesion and recognition. In recent years, numerous studies have suggested a role for cadherins in the regulation of synaptic plasticity, both morphological (structural) and functional. The work described here contributed to expanding knowledge of cadherin function, and eventually the role thereof in synaptic modulation through the development of reporters of cadherin adhesive state. These reporters should prove valuable tools in the study of cadherins and their roles, both in the nervous system and elsewhere.

Construction of cadherin FRET reporters

In chapter IV of my thesis work, I have described the development of fluorescent cadherin molecules for use in FRET experiments. As described, there were many difficulties in generating cadherins that were both properly membrane expressed and fluorescent. A particular impediment to this was the apparent sensitivity of cadherin to intramolecular insertions, especially near its N-terminus. This sensitivity may be due, in part, to the proteolytic cleavage known to be required for production of mature cadherin from its preformed, inactive precursor (Ozawa and Kemler 1990; Wheeler, Parker et al. 1991). As is known to be the case for other proteins matured in the constitutive secretory pathway as cadherin is, precursors are synthesized in the endoplasmic reticulum, and are then trafficked through the *cis* and *trans* Golgi (Pasdar, Krzeminski et al. 1991). Pro-protein cleavage then needs to occur before cadherins can be trafficked to the cell membrane. It is currently not known which factors control and trigger the initiation of cadherin processing. However, Posthaus et al. have provided some evidence concerning

the enzyme(s) mediating E-cadherin and the desmosomal cadherins maturation (Posthaus, Dubois et al. 1998, 2003). Using a baculovirus co-expression system it was demonstrated that furin, a member of the subtilisin-like convertase family, is a proE-cadherin processing enzyme (although they were not able to determine with certainty that furin was the sole enzyme responsible).

Initial insertions in the first two EC repeats (EC1 and EC2), whether small epitope tag, or XFP fusions, yielded cadherins that were misprocessed and sequestered in ER and Golgi. The insertion points chosen were not near the regions known to be important for the pro-processing of cadherin, but may still have interfered with the action of the molecules necessary for cleavage or trafficking

The one site arrived at by making "intelligent guesses" that I was able to successfully membrane express an XFP-cadherin fusion at was on a loop in EC3, between β-strands C and D. These fusion proteins were properly expressed in the membrane, yet not fluorescent, as discussed in chapter IV. Interestingly, the site where GFP was inserted by random transposon insertion in clone TS25 was 4 amino acids away from the site I had chosen based on the published EC structures. This distance of 4 residues apparently made all the difference, as TS25 is properly expressed in the membrane and fluorescent, while my initial EC3-XFP fusions did not fluoresce. I think this demonstrates the power of a "shotgunned" and selectable approach, such as the transposon insertion technique.

Geometry of cadherins in cell junctions

As I have demonstrated, FRET occurs between the Venus- and Cerulean-TS25-based-Ncad constructs when they are expressed either both in the same cell, or separately in adjacent cells, indicating that the same FRET pair can be used to detect both *cis* and *trans* interactions. This is not something that would necessarily be expected and may offer hints as to the geometry of cadherin molecules in an intact cell junction. It is not likely that FRET acceptor and donor fusions with EC2 would yield similar levels of FRET in both *cis* and *trans* experiments if the cadherin ectodomains were fully interdigitated, as in models proposed by Chappuis-Flament, Wong et al. (2001) and Sivasankar, Gumbiner et al. (2001). The distance between donor and acceptor displayed on EC2 would be expected to be shorter when donor- and acceptor-fusion cadherins are expressed on the same cell and considerably longer when the cadherin pair are expressed on adjacent cells, which would likely result in different levels of FRET (figure 12). As very similar levels of FRET were observed in my *cis* and *trans* interaction experiments, this model is not likely to be accurate.

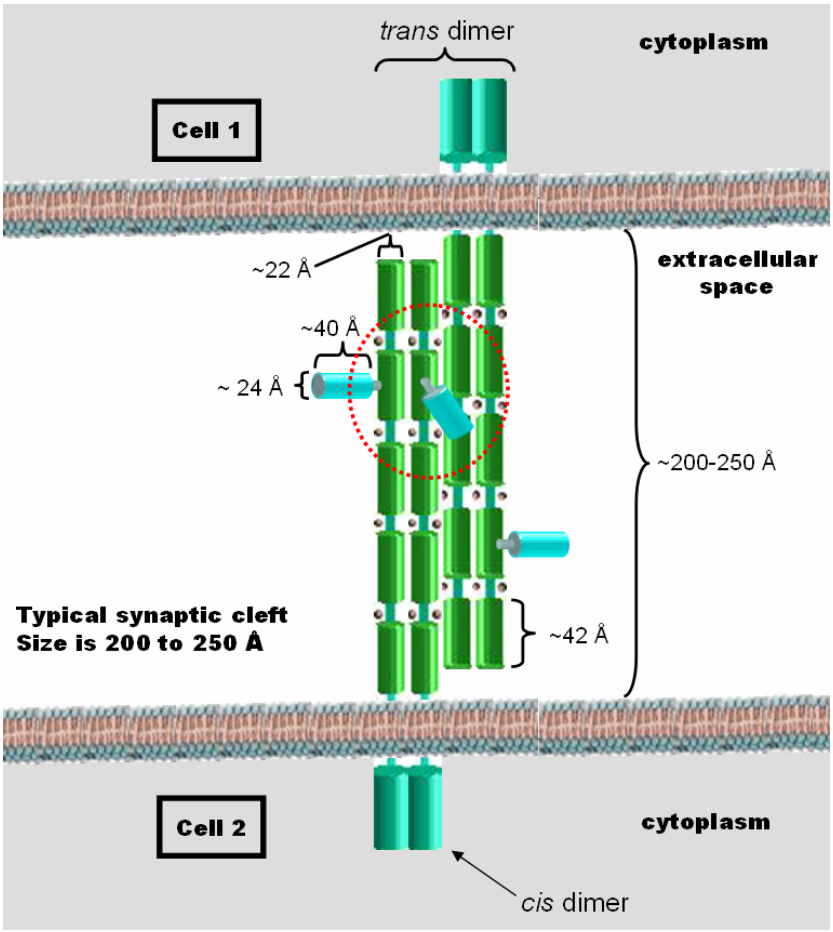

Figure 12. Quasi-to-scale schematic of fully interdigitated ectodomain model of cadherin interactions.
The symbols in the diagram represent the following: green cylinders, EC domains; brown circles, Ca^{2+} ions; blue cylinders, XFP FRET donor or acceptor fluorophores; grey cylinders, linker between XFP and cadherin created by transposon insertion. The dotted red circle represents the area within which FRET involving the circled XFP is expected to be strong.

However, if the models proposed by Boggon, (2002) and He, Cowin et al. (2003) were accurate, one might expect approximately similar levels of FRET in the *cis* and *trans* interaction experiments, as the distance between EC2s on both *cis* and *trans* interacting cadherins are approximately equivalent.

As an extension of this, it may be feasible to attempt to map the architecture of the cadherin interaction in intact cells through the combinatorial application of a small library of multiple FRET pairs with differing XFP insertion sites. As discussed in chapter IV, a single round of XFP insertion and selection yielded multiple viable insertion sites. It may be possible to repeat this process and obtain further cadherin-XFP fusions that would provide thorough coverage of the entire ectodomain (it also may be that, as no clones were obtained with insertions in the EC3-EC5 region, the Tn5-mediated insertion process is not entirely random and may be unable to create insertions in this region). If sufficient coverage of the ectodomain with was obtained, the FRET levels of different combinations of XFP-fusions could be compared in both *cis* and *trans* experiments and used to generate a FRET efficiency "map" of the cadherin interaction.

Visualizing changes in cadherin-cadherin interactions induced by synaptic activity

As discussed, the motivation for undertaking this work was the observation that cadherins were present in hippocampal synapses and involved in plasticity, with functional cadherin adhesion being necessary for LTP (Tang, Hung et al. 1998). Now that proof-of-principle experiments have proved positive in heterologous cells, the next step is the neuronal expression of the FRET reporters. Transient transfection of hippocampal neurons can be done with calcium phosphate as well as lipid-based transfection methods (such as lipofectamine), but neither method are likely to yield a high enough efficiency to conduct *trans* interaction FRET experiments. I have tested transfection efficiency by lipofectamine 2000 and was able to transfect cultured neurons with the Venus-cadherin fusion protein (figure 13), but efficiency was very low (on the

order of one neuron per 35 mm dish). A more efficient method of delivering DNA will have to be used for neuronal experiments.

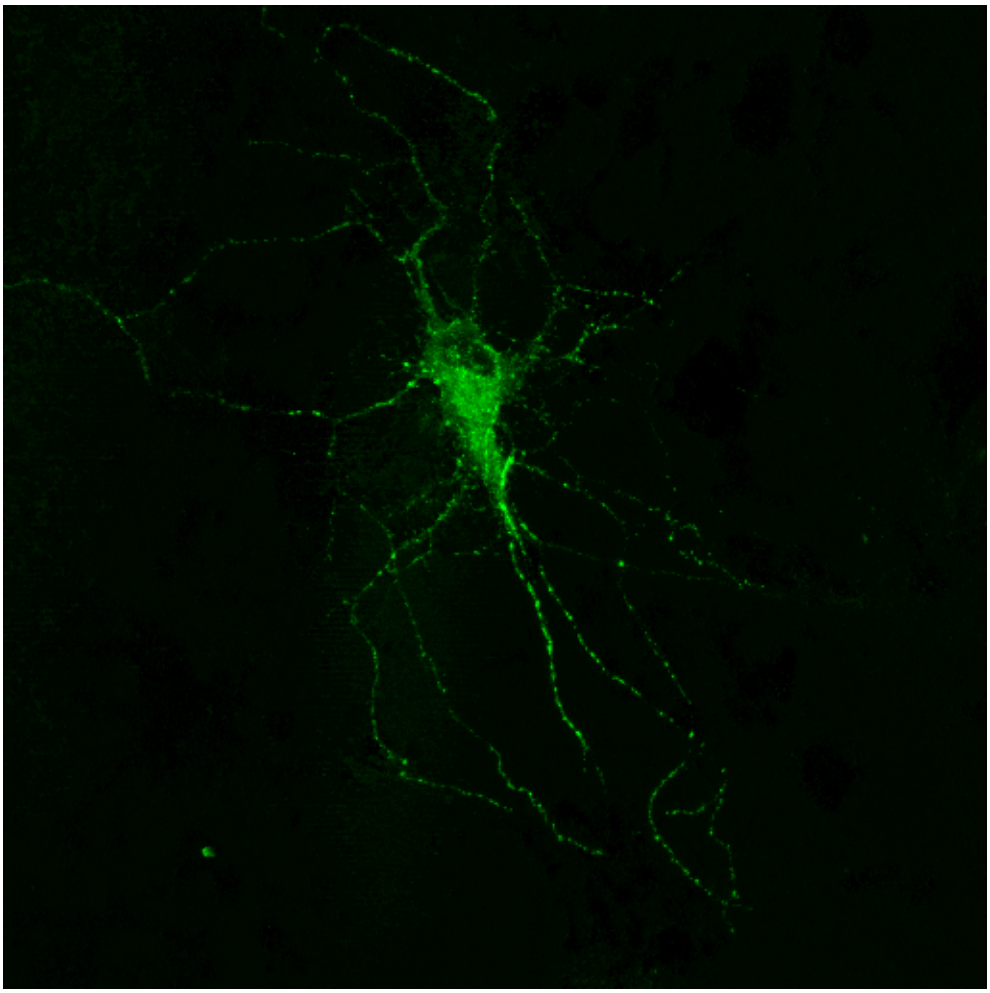

Figure 13. A hippocampal neuron expressing Venus-cadherin.
Cultured hippocampal neurons (12 DIV) were transfected with Venus-cadherin fusion construct. The image was taken 30 hours after transfection.

Several possible methods are currently being explored: the use of biolistics, and viral infection. Once expression of the pair of FRET reporter constructs has been achieved in synaptically connected cells, one can begin to examine how activity regulates cadherin interactions.

Some consideration of the feasibility of detecting FRET signal changes at the synapse:

Spacek and Harris found puncta adherentia (PAs) at the edges of synapses on 33% of dendritic spines. The areas occupied by PAs were variable across different types of synapses, occupying 0.010 +/- 0.005 μm^2 at macular synapses and 0.034 +/- 0.031 μm^2 at perforated synapses (Spacek and Harris 1998). Based on electron tomographic data, C-cadherins are estimated to be present in desmosomes from neonatal mouse epidermis at a density of ~17,000/ μm^2 (He et al. 2003). Assuming N-cadherin is present at the same density in neuronal puncta adherentia as C-cadherin is in epithetial desmosomes, this yields an estimate of 85–255 cadherins at a single macular synapse, and 51–1105 cadherins at a single perforated synapse. This estimate also assumes that N-cadherin is only present at puncta adherentia, and is not expressed synaptically outside of PAs.

This, of course, is an estimate of the numbers of endogenous cadherins present at a single hippocampal synapse. It is possible that overexpression of an XFP-fusion cadherin may be able to push more cadherins into the synapse, although the cell may have a compensatory mechanism by which it might regulate the number of cadherins at a synapse regardless of overexpression.

Because the FRET signal is generated by exogenously transfected fluorescent fusion proteins, overexpressed over a background of endogenous cadherins, not every cadherin present in the synapse will be displaying a FRET-capable fluorophore. This will result in lower FRET signal than a cell junction expressing only fusion cadherins. However, my experiments in HEK-293 and COS7 cells were also done in the presence of

endogenous cadherins - HEK-293 and COS7 cells both express high levels of endogenous cadherins, so it is not likely that neuronal expression will exact a further penalty on the FRET signal due to cadherin background. It has also been shown that overexpression of dominant negative cadherin mutants causes the down-regulation of endogenous cadherins (Norvell and Green 1998; Nieman, Kim et al. 1999; Troxell, Chen et al. 1999). It has been indicated that the β-catenin binding domain on the mutant cadherin was required for this (Norvell and Green 1998; Troxell, Chen et al. 1999). It is possible that overexpressing the FRET fusion constructs (which, though they do not act as dominant negatives, do have intact β-catenin binding domains) could cause both heterologous cells lines and neurons to compensate by down-regulating endogenous cadherin expression.

Synaptic activity can be manipulated in many ways; when expression of a pair of FRET reporter constructs has been achieved the following protocols should be considered:

i) depolarization with KCl;

ii) application of the neurotransmitter agonists AMPA or NMDA;

iii) application of the $GABA_A$ receptor antagonist bicuculline (by blocking fast inhibitory transmission, bicuculline increases action potential firing and thus promotes release of endogenous transmitter at synaptic sites);

iv) application of antagonists that block excitatory synaptic transmission and action potential-dependent release (TTX, APV and CNQX cocktail);

v) electrical stimulation, which promotes the release of synaptic transmitter and also allows for flexibility in the rates and frequencies of stimulation which may produce different effects on cadherin interactions at synaptic junctions.

When combined, these techniques should yield a wealth of new information about how different activity patterns may alter the strength of cadherin associations.

Experiments made possible with cadherin FRET reporters

I'd like to describe some of the unanswered questions regarding the role of cadherins in the synapse we should be able to use these constructs to address. By expressing the constructs in hippocampal slices we can take advantage of the well-established paradigm of hippocampal LTP. We should be able to achieve expression in slices by using Sindbis or Lenti viral vectors to infect cells in areas CA1 and/or CA3. Our laboratory has in the past achieved specific expression of exogenous DNA in these subsets of hippocampal cells through the use of stereotaxic injections of viral vectors into the hippocampus of live rats, specifically targeting CA1 or CA3.

One outstanding question is whether the Cadherin EC domain might be functioning as a synaptic cleft-Ca^{2+} sensor to detect synaptic transmission. To test this we can express the constructs in hippocampal slices and induce LTP. By monitoring FRET at the synapses formed by CA3 cells onto the CA1 cells, we can the follow changes in cadherin adhesion in response to the synaptic activity induced by a single test pulse as well as during the high frequency tetatanic stimulation used to induce LTP. Such a tetanus would be expected to cause a significant depletion of Ca^{2+} in the synaptic cleft, so we would expect to see a transient loss of cadherin adhesion during the tetanus, but would predict that no changes in cadherin adhesion would be observed during the single test pulses, as these were insufficient to allow for HAV peptide binding in Lixin's work (Tang, Hung et al. 1998).

Images can be taken at a temporal resolution on the order of milliseconds through line scans on a confocal microscope, or potentially using the higher speed made possible by a spinning (Nipkow) disk confocal microscope. This will allow us to capture cadherin interactions during these synaptic events.

We can also test the hypothesis that an increase in cadherin interactions contributes to an increase in synaptic strength. If this is true, we expect to measure an increase in cadherin adhesion after inducing potentiation. If we monitor FRET at CA3 -> CA1 synapses, this should be detected as an increase in FRET signal following LTP induction.

We can take advantage of the architecture of the hippocampus and the spatial resolution granted by imaging to confirm that observed changes in adhesion are the result of the strengthening of a specific synapse. If we express FRET reporter constructs in

CA1, CA3 and entorhinal cortex cells, we can selective potentiate either the CA3 or entorhinal cortex inputs to the CA1 cells and monitor the FRET signal at both sets of synapses. This gives us a within-cell control to verify if changes in adhesion are synapse-specific.

We can target donor and acceptor constructs to pre- and post-synaptic neurons to distinguish beween *cis* and *trans* interactions. To monitor *trans* interactions, we can, for example, express one of the pair in CA1 cells and the other in CA3 cells. To monitor *cis* interactions we can express both donor and acceptor cadherins in a single population of cells, for example the CA1 cells and not transfect the CA3 cells. By doing this, we should be able to monitor a loss of cadherin cis-interactions independently of trans-interactions.

It is unknown exactly the degree to which extracellular calcium concentrations are lowered by synaptic activity. As it is known that as calcium concentrations are lowered, the *trans* interactions are the first to be lost and *cis* interactions require a larger decrease in calcium concentrations, we can use this technique to determine if a loss of *cis* interactions ever occurs, and during which types of synaptic activity.

In addition to a potential role for N-cadherin in synaptic plasticity, N-cadherin best known for its role in axon targeting. It has been demonstrated by convergent evidence from vertebrate and invertebrate systems that axons of neurons expressing N-cadherin mutants cannot migrate normally to their targets. More recently, it has been demonstrated that N-cadherin also plays a role in dendritic branching. The generally accepted idea, although it has not been decisively demonstrated, is that cadherin - cadherin interactions play a key role in forming appropriate synaptic connections. If we

assume that this is the case, then an outstanding question is how N-cadherin function can be restricted to specific sets of axon-target connections? Since N-cadherin is expressed ubiquitously throughout the visual system, how do the neurons avoid forming a giant mass of cell-cell adhesions? One idea is that N-cadherin activity is controlled spatiotemporally and functions when necessary at different points in development. For example, in neural development, axons of a particular cell type often extend together in a bundle and when they reach their general target location, they branch off to make specific contacts. A general target location is often segregated into cell populations that develop at different time windows. One hypothesis is that perhaps N-cadherin is not expressed at the membrane until a time window at which a given set of neurons is ready to form connections. This is difficult to examine using typical imaging methods because it is impossible to distinguish when cadherins are interacting.

We could use the FRET constructs here to determine at what point in development a cell begins to express "functional" or adhesive cadherins.

As a final note, I'd like to add that, although I've spotlighted the role of N-cadherin in neural systems, it is also expressed in cardiac and skeletal muscle tissue, and it plays a prominent role in cell adhesion, differentiation, embyogenesis, invasion, and signaling. Undoubtedly, these FRET constructs will provide a resource to understand the role of cadherin-cadherin interactions in wide variety of cell processes.

We now have the tools in hand to monitor cadherin adhesion in active synapses, which should provide further insight into the role of cadherins in plasticity and a wide variety of cellular processes.