Chapter 6: Discussion and Conclusions

CaMKII and PSD-95 colocalization studies

The spatial and temporal localization of signaling molecules are crucial for the form and endurance of synaptic plasticity [1-3]. Though the postsynaptic density acts as an integrated macromolecular machine, the spatial and temporal dynamics of individual PSD proteins are integral to the nature and strength of a postsynaptic response to impinging signals. Ca^{2+}/ calmodulin-dependent protein kinase II (CaMKII) is a major component of the PSD and is a crucial regulator of neuronal and behavioral plasticity [4-8]. Ca^{2+} signaling at the postsynaptic membrane occurs upon stimulation of the NMDA receptor and the subsequent activation of CaMKII. Consequently, the location of CaMKII relative to activated NMDA receptors is crucial to its subsequent signal transduction.

Numerous proteins of the PSD are known to bind and interact with CaMKII [9]. However, strategic interactions that specifically position CaMKII for optimal activation by Ca^{2+} influx and the subsequent phosphorylation of proteins important for LTP are particularly crucial. The binding of CaMKII with NR2A and NR2B subunits of the NMDA receptor is one such strategic interaction [1, 10-13]. We have hypothesized that Densin is another [14, 15].

Both the NR1 and NR2 subunits are required to form functional NMDA receptors at the synapse [16-18]. Deletion of synaptic NMDA receptors can thus be achieved by deletion of the c-terminal tails of the NR2 subunits [19, 20] or by deletion of the NR1 subunit [21]. I have shown that in NR2A x NR2B double tailless mutant animals,

CaMKII colocalization with PSD-95 is significantly reduced. Similarly, in NR1 knockout mice, CaMKII colocalization with PSD-95 decreases by the same amount. The loss of CaMKII colocalization with PSD-95 in both the NR1 and NR2A/ NR2B double tailless mice supports the notion that these genetic mutations are functionally equivalent with respect to their effect on localization of the NMDA receptors to synapses. Moreover, these studies support the role of the NMDA receptor as a PSD docking site for CaMKII.

I next investigated the localization of CaMKII at the tips of spines in Densin knockout mice. In the absence of Densin, CaMKII colocalization with PSD-95 is significantly decreased, supporting our hypothesis that Densin can act as a docking site for CaMKII at the PSD. Interestingly, the loss of CaMKII colocalization with PSD-95 in Densin knockout animals was equivalent to the loss of CaMKII colocalization with PSD-95 in both the NR2 double tailless and NR1 knockout mice. These results suggest that Densin and the NMDA receptor dock an equivalent sized pool of CaMKII in the PSD.

Finally, we investigated the effects of removing both the NMDA receptor and Densin on the localization of CaMKII. This was achieved by crossing mice heterozygous for both Densin and NR1. Our results showed a 60% decrease in the colocalization of CaMKII with PSD-95. Single knockouts of either Densin or NR1 resulted in only 23.9% and 18.7% decreases in CaMKII colocalization with PSD-95. The loss of ~60% CaMKII colocalization with PSD-95 suggests that the loss of both Densin and the NMDA receptor results in a synergistic decrease of CaMKII colocalization with PSD-95. A caveat to this interpretation is the fact that the levels of CaMKII is globally decreased in our primary hippocampal cultures. Consequently, the loss of CaMKII colocalization with PSD-95

may simply reflect the decrease in global CaMKII levels. Further investigation will be required to more fully interpret these observations.

A "compensatory" hypothesis would predict that in the Densin knockout mouse, more CaMKII would be bound to the tails of the NMDA receptor. I would propose testing this hypothesis by stimulating synaptic NMDA receptors in hippocampal slices from Densin knockout mice with bicuculline. If my hypothesis is correct, I should be able to detect an increase in CaMKII autophosphorylation and in phosphorylation of the NMDA receptor compared to wildtype. Such a result would provide support for a "compensatory" hypothesis.

I have shown that in the absence of the NMDA receptor, CaMKII can still be docked in the PSD. Previous work by Shen and co-workers [2, 22] has shown that CaMKII dynamically translocates upon synaptic stimulation with NMDA. Given that both the NMDA receptor and Densin can bind inactive CaMKII and that autophosphorylation of CaMKII increases its interaction with both proteins [14, 15], understanding the biological significance of this differential localization should be an exciting future line of investigation.

Quantitative Immunoblot Studies

Results from my quantitative immunoblot studies show that PSD-95, the NR1 and NR2A subunits of the NMDA receptor, Erbin and αCaMKII are all significantly decreased in the forebrain of knockout mice. I hypothesized that these decreases were the result of: 1) a decrease in gene transcription, 2) a decrease in the translation of these particular proteins, and/ or 3) an increase in their degradation. I investigated the first

hypothesis in collaboration with Barbara Wold's lab by utilizing RNA seq- a new, high-throughput sequencing method that allowed me to analyze transcriptional changes in all genes expressed in the forebrain and hippocampus in knockout mice [23].

Transcript levels for PSD-95, NR1, NR2A, Erbin, and αCaMKII did not decrease in a manner concomitant with the observed changes in their protein levels. Consequently, the decrease in protein levels may be due to changes in protein translation or protein degradation. It is possible that Densin selectively interacts with PSD-95 complexes that contain a specific composition of proteins, including NR1, NR2A, Erbin, and CaMKII. If this is the case, then the loss of Densin may result in the destabilization of these particular complexes, and potentially an increase in protein degradation.

Changes in Spine Density and Morphology

Changes in spine density are most often associated with homeostatic mechanisms aimed at regulating global synaptic input [24-26]. Furthermore, spine shape is correlated with the relative strength and maturity of a synapse [27, 28]. For example mushroom spines have larger, more complex PSDs [28]. Thin spines are associated with structural flexibility and smaller PSDs [29, 30]. Finally, stubby spines have been hypothesized to be a type of intermediate step between filapodial outgrowths and mature spines [31]. However, the clearest correlation between a spine, its functional strength and its degree of maturity is the size of its head [27, 28, 32-34].

In our analysis of dendritic spines from the CA1 area of the hippocampus of Densin knockout mice, we found a statistically significant increase in spine density (+13%) and a statistically significant decrease in spine volume (-11%); a trend towards

an increase in spine length (+4%) was observed, but not statistically significant. The increase in spine density along with a concomitant decrease in the size of the spine heads suggest that the loss of Densin likely affects synaptic input. However, the phenotypic changes observed may also suggest that the loss of Densin results in a stunted ability to form fully mature spines. Finally, as discussed later, the Densin knockout mice exhibited seizures when they were anesthetized with Nembutal and exhibited seizures. Given that seizures are known to affect spine dynamics and morphology, our results may be confounded by the seizures that these animals had prior to their perfusion . Additional work will be required to better understand the source of the observed spine phenotypes.

Dendritic Arborization

In culture, Densin knockout neurons exhibit broad and flattened dendritic trunks, and an overall decrease in the number of dendritic branches. These results are consistent with work demonstrating that Densin overexpression leads to an increase in dendritic branching [35]. Furthermore, results from my quantitative immunoblot studies shows that there is a significant decrease in the protein level of Erbin. Recent work by Reichardt and co-workers demonstrated that miRNA mediated knockdown of Erbin severely impairs dendritic morphogenesis [36]. They also showed that the loss of Erbin resulted in a mislocalization of δ-catenin.

δ-Catenin is part of a synaptic cell adhesion complex that includes N-cadherin [37]. Cadherin-catenin adhesion complexes have been shown to play important roles in synaptic plasticity, synaptogenesis and dendritic arborization [36, 38, 39]. Densin is known to directly bind δ-Catenin in the brain [40], a finding also observed in our lab

(Mary Kennedy, personal communication). δ-catenin is hypothesized to affect dendritic morphology through its regulation of the F-actin cytoskeleton via the Rho family of GTPases and cortactin activities [41]. Consequently, the mislocalization of δ-catenin results in aberrant dendritic structure through its regulation of the F-actin structure. The arborization changes seen in the Densin knockout mouse may thus be the direct result of a loss of δ-catenin localization or of the loss of Erbin.

RNA-Seq Analysis and Identification of Candidate Transcripts Potentially Involved in the Seizure Phenotype

An immediate early gene (IEG) is defined as a gene whose transcript expression is increased rapidly and transiently in a protein synthesis-independent manner in response to extracellular stimuli [42]. Numerous experimental paradigms have been used to induce the expression of IEG including serum stimulation of fibroblasts, maximal electroconvulsive seizures (MECS), LTP paradigms, behavioral experiences in novel environments, drug and hormone challenge [43-48]. IEG have been catergorized into two functional classes: 1) regulatory transcription factors (RTF) which control the transcription of downstream genes; and 2) effector IEGs, which directly influence cellular functions [46, 47]. Examples of RTFs whose transcript increase is response to neural activity in the forebrain are c-fos, c-jun, zif268/ Egr1, and examples of effectors are homer1a, Arc, Bdnf, and Cox-2. By virtue of their transcriptional regulatory activities, RTFs are well positioned to affect global changes in response to neuronal activation, while effector IEGs are likely to immediately impact neuron specific events. Lanahan

and Worley suggest that the IEG response in neurons is likely limited to 30-40 genes, of which perhaps 10-15 are transcription factors [47].

Our analysis of the effect of Densin knockout on transcription in forebrain and hippocampal tissue uncovered a striking effect on a subset of immediate early genes- Arc, Egr1/ zif268, Egr2-4, c-fos, fos-b, and junb. Specifically, in the forebrain we discovered that these eight transcripts were strongly decreased. Interestingly, this same group of eight transcripts showed a striking increase in their transcript levels in the hippocampus. Furthermore, seven of the eight IEGs affected are all classified as regulatory transcription factors; Arc is classified as an effector. It is not yet clear why these eight IEGs would be affected by the loss of Densin or why they would have such striking differences in their level of transcript expression between the forebrain and the hippocampus. Furthermore, why IEGs with the classification as RTF would be over-represented is also unclear. Continued investigations into the primary literature may unearth a particular type of IEG induction paradigm that may more readily induce RTF as opposed to effector IEGs. If such a paradigm has been found, it may give us insight into the particular mechanisms affected by Densin.

Recent work by Greenberg and co-workers (Lin *et. al,* 2008) showed that Npas4 is an activity-dependent transcription factor, and that levels of Npas4 determine the number of functional GABAergic synapses that are formed via the induction of a program of gene transcription. Given that neurological disorders such as schizophrenia and epilepsy are known to be associated with an imbalance between excitatory and inhibitory synapses (Mohler, 2006; Wassef et al., 2003), our results suggest that the decreased expression of the Npas4 transcripts may contribute to the seizure phenotype.

Though the sedative properties of Nembutal are mediated by the $GABA_A\alpha1$ subunit (Rudolph et al., 1999), the $GABA_A\alpha2$ transcript (which increases by 81% in the forebrain of knockout mice) can specifically bind Nembutal and is the primary subunit that mediates the anxiolytic action to barbiturates (Low et al., 2000). The other $GABA_A$ receptors that bind and mediate the action of benzodiazepines are the $\alpha3$ and $\alpha5$ subunits. Little to no change in forebrain or hippocampal gene expression is seen for the $\alpha1$ (+1.4% fb; -14% hip), $\alpha3$ (-2.8% fb; +9% hip) or $\alpha5$ (+13.4% fb; -0.99% hip) subunits. Given that Nembutal is a GABA agonist, it should be sedating; this obviously is not the case for the Densin knockout animal. One possibility for the induction of seizures upon Nembutal administration may lay in the network properties effected by tonic inhibition. Tonic inhibition occurs when extrasynaptic $GABA_A$ receptors are activated by ambient GABA, thus prolonging inhibitory post-synaptic currents (Isaacson *et al*, 1993; Rossi and Hamann, 1998). By adjusting the excitability of neurons, tonic inhibition can regulate the oscillatory properties of neuronal networks (Towers et al., 2004). GABA is mainly associated with inhibition in adult animals. However, alterations in the oscillatory properties of networks can affect the balance between excitatory and inhibitory activity. The loss of Densin and the subsequent effect on Npas4 and $GABA_A\alpha2$, may result in an aberrant disinhibition of a network when the normal response would be inhibitory. It is not yet clear what roles Npas4 or $GABA_A\alpha2$ play in the seizures generated by Nembutal injections. However, if their protein levels are indeed affected, the balance between inhibitory-excitatory networks may result.

Finally, both Densin and $GABA_A\alpha2$ can localize in the axon initial segment (AIS) [49-51]. It is reasonable to hypothesize that Densin may affect the localization of

$GABA_A\alpha2$ at the AIS. Consequently, in the absence of Densin, $GABA_A\alpha2$ may be mislocalized. Immunostaining experiments could shed light on this hypothesis.

Final Thoughts

One always hopes that the development of a knockout animal will not only provide support for proposed hypotheses, but will also yield information on how the system as a whole operates, thus leaving the researcher with many more questions than answers. Furthermore, one also hopes that the knockout animal will exhibit some exciting and unforeseen phenotype that will generate questions never even suggested based on current knowledge. On all these hopes, the Densin knockout animal has delivered.

Work conducted for my thesis is admittedly descriptive, but such is the nature of an initial characterization of a knockout mouse. Consequently, there is a wealth of experimental opportunities to be had. For example, in the course of making the full knockout animal, I also created a floxed/ conditional knockout. This conditional knockout strain has yet to be utilized for any experimental investigations. Many of the phenotypes I have identified (i.e., changes in spine morphology or dendritic arborization) or even the results from the RNA seq analysis certainly represent a system that has reached a new homeostatic set point. The conditional knockout can certainly be used to investigate the acute loss of Densin from neurons.

Another area ripe for further investigations is the affect that the deletion of Densin has on inhibitory networks. Does Densin play a role at the axon hillock? What protein(s) or complex(es) does Densin interact with at the AIS? Does Densin directly interact with

$GABA_A\alpha2$ containing receptors? Are transcriptional changes in Npas4 and $GABA_A\alpha2$ reflected at the protein level? If so, what effect does this have in relation to the sensitivity to Nembutal. Understanding the underlying mechanisms related to the loss of Densin and the susceptibility to seizures is of direct medical relevance. Furthermore, given that I have family members who suffer from epilepsy, I am well aware of the increased risk of complications from general anesthesia that epileptics face. Consequently, my interests are both scientific and personal.

Obviously, the continued investigations into the role of Densin within the PSD is central to the long-term interests of the Kennedy lab. Toward this end, we are currently engaged in a collaboration with Tom O'Dell's lab at UCLA to characterize any changes in the cellular physiology of the Densin knockout.

Currently, Holly Beale, another graduate student in the Kennedy lab, is moving forward on analyzing the data generated from the RNA seq collaboration with the Wold lab. This work has already identified candidate genes for some of the observed phenotypes and generated testable hypotheses. Furthermore, I would be remiss if I did not express excitement over being involved in the first ever use of this technology to investigate the transcriptional changes in a knockout mouse.

Given the host of cellular and molecular phenotypes exhibited by the Densin knockout mouse, it is highly likely that the Densin knockout mouse will also exhibit behavioral deficits. Tinh Luong, another graduate student in the Kennedy lab, has recently entered into a collaboration with Paul Patterson's lab to characterize behavioral deficits that impact learning and memory, motor coordination, or other aberrant behaviors.

One area or research outside the scope of the Kennedy lab, but interesting none-the-less, is the role of Densin in the kidney and pancreas. Densin is a component of the slit diaphragm complex of the kidney [52, 53] and autoimmune antibodies against Densin have been found in patients with type 1 diabetes [54] . Furthermore, the RNA seq analysis revealed that a host of kidney-, pancreas-, and diabetes-related transcripts were significantly changed in the Densin knockout. A future collaboration with a kidney biology laboratory could have immediate implications for understanding the molecular mechanisms and pathways in which Densin plays a role. Furthermore, the Densin knockout mouse can potentially help uncover disease mechanisms as they relate to diabetes.

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