Chapter 4: The Role of Densin in the Postsynaptic Density and Docking of CaMKII

Introduction

Because proteins of the PSD apparatus work as an integrated whole, their differential spatial and temporal interactions are integral to the nature and strength of a postsynaptic response to impinging signals. Furthermore, the subcellular targeting and dynamic alterations in the localization of signaling and regulatory proteins may produce diversity in signaling complexes and increased specificity for target substrates [1]. The behavior of CaMKII enriched in the PSD reflects this dynamic spatial and temporal activity.

CaMKII is a Ser/ Thr kinase that is central to the coordination and execution of signal transduction of Ca^{2+} signals [2]. CaMKII is a dodecameric holoenzyme that is assembled in stochastic combinations from two homologous catalytic subunits, alpha and beta [3, 4]. These isoforms appear to differentially affect synaptic and dendritic morphology. In particular, αCaMKII is important for regulating synaptic strength and stabilization of dendritic arbors [5]. βCaMKII seems to have a greater effect on the degree of dendritic arborization, as well as the formation and number of synapses [6]. These differences may reflect the different developmental time courses of their expression. In addition to the markedly different roles in neuronal plasticity, the α and β subunits also impart differential subcellular distribution and translocation dynamics in response to NMDA receptor stimulation[7-9].

CaMKII is highly concentrated in the PSD [10, 11] suggesting that one or more docking sites within the PSD likely mediate its postsynaptic accumulation. Recent work indicates that the cytocolic tails of the NR2A and NR2B subunits of the NMDA receptor bind to CaMKII, thus serving as docking sites within the PSD [12-14]. Hell and co-workers found that both phosphorylated and unphosphorylated CaMKII can bind to these NMDA receptor subunits (Leonard, et al., 1999). However, activation of CaMKII, via NMDA receptor stimulation, greatly increases its affinity for the NMDA receptor.

In addition to their role in binding CaMKII, the c-terminal tails of the NR2A and NR2B subunits are also required for proper localization of functional NMDA receptor to the synapse [15]. The carboxyl terminal tails of the NR2 subunits contain protein binding sequences required for their interaction with MAGUK family proteins (PSD-95, SAP102, PSD-93 and SAP97) [15-17]. This interaction facilitates their localization to the synapse. Deletion of the NR2A or NR2B c-terminal tails results in the near total loss of synaptic NMDA localization [15, 18-21]. Furthermore, the loss of the NR2B c-terminus results in neonatal death, phenocopying the full length knockout of the NR2B subunit [18, 21, 22] and deletion of the NR1 subunit [23, 24].

Given the potential importance of the NMDA receptor's NR2 subunits for nucleating CaMKII at the PSD, our lab acquired two knockin mutant mouse lines created in the Seeburg lab; NR2A$^{\Delta c/\Delta c}$ and NR2B$^{\Delta c/\Delta c}$. Both of these mouse lines contain deletions of their carboxyl terminal cytoplamsic tails [18]. These two mouse strains were crossed to produce animals heterozygous for both NR2A$^{\Delta c/\Delta c}$ and NR2B$^{\Delta c/\Delta c}$. Progeny heterozygous for both mutations were interbred to generate NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ primary neuronal cultures. These "double-tailless" neuronal cultures were subsequently

used to study the effects of the loss of cytoplasmic tails on CaMKII localization to the PSD.

In addition to the NMDA receptor, we hypothesized that Densin may act as a second docking site for CaMKII in the PSD. Biochemical studies performed in the Kennedy lab found that Densin can bind CaMKII in its intracellular domain [25]. Similar to the interaction of CaMKII with the NR2 tails, binding of the kinase to Densin does not require autophosphorylation. However, activation by autophosphorylation increases the affinity of CaMKII for Densin ~100 fold [25].

In the following series of experiments, we set out to test the hypothesis that Densin and the NMDA receptor are the primary docking sites for CaMKII in the PSD. we proposed to do this by analyzing changes in co-localization of CaMKII with PSD-95 in dissociated hippocampal cultures via immunofluorescent image analysis. We hypothesize that the loss of the NR2 tails will result in a decrease of CaMKII localization at the PSD. Consequently, we first analyze colocalization of CaMKII with PSD-95 in NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ primary hippocampal cultures. Loss of the NR1 subunit causes the NR2 subunits to be retained in the endoplasmic reticulum, resulting in the loss of NMDA receptor at the synapse [26]. Consequently, we hypothesize that primary neuronal cultures made from NR1 knockout embryos should phenocopy the NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ primary neuronal cultures. we then analyzed the colocalization of CaMKII with PSD-95 in the Densin knockout mouse. Finally, we cross the Densin and NR1 knockout lines to generate Densin$^{-/-}$ x NR1$^{-/-}$ primary hippocampal cultures to investigate CaMKII colocalization with PSD-95 when both Densin and the NMDA receptor are removed from the PSD.

Material and Methods

4.1 Mouse strains

NR2A$^{\Delta c/\Delta c}$ animals were maintained as a homozygous mutant line while the NR2B$^{\Delta c/\Delta c}$ animals were maintained as heterozygous individuals because mice homozygous for the NR2B$^{-/-}$ mutation are embryo lethal; both NR2A$^{\Delta c/\Delta c}$ and NR2B$^{\Delta c/\Delta c}$ mutants were generous gifts from Peter Seeburg. NR2A$^{\Delta c/\Delta c}$ homozygous mice where crossed with NR2B$^{+/\Delta c}$ heterozygous mice to create NR2A$^{+/\Delta c}$ x NR2B$^{+/\Delta c}$ heterozygous animals. These animals where then crossed to generate NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ embryos for cell culture experiments.

Densin knockout animals were maintained as a heterozygotes. NR1 mutants, a generous gift from Peter Seeburg, were maintained as heterozygotes. NR1$^{+/-}$ mutants were crossed with F2 generation Densin$^{+/-}$ animals to produce NR1$^{+/-}$ x Densin$^{+/-}$ heterozygotes. NR1$^{+/-}$ x Densin$^{+/-}$ animals (F3) were subsequently crossed to produce NR1$^{-/-}$ x Densin$^{-/-}$ double knockout embryos for cell culture experiments.

Genomic DNA was isolated from mouse ear punch samples and used for PCR based genotyping.

4.2 Primary neuronal cultures and immunocytochemistry

Hippocampi from 16-day-old embryos (wildtype, NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$, NR1$^{-/-}$, Densin$^{-/-}$, and Densin$^{-/-}$ x NR1$^{-/-}$), were dissected and dissociated with trypsin and mechanical trituration. Cells were plated onto glass coverslips coated with Poly-D,L-

Lysine (Sigma, St. Louis, MO). Cultures were maintained in neurobasal media (Invitrogen, Carlsbad, CA) and supplemented with B27, glutamate, and Glutamax-I (Invitrogen). Genotypes of cell cultures were determined with genomic DNA isolated from embryonic tissue.

After 18-21 days *in vitro*, cover slips coated with cells were rinsed in ice-cold PBS and placed briefly in ice-cold methanol. The methanol was replaced with -20°C methanol and incubated at -20°C for 10-15 min. Cells were rinsed and incubated in h-PBS (450mM NaCl and 20mM phosphate buffer, pH 7.4) for 15 min and blocked with 5% normal goat serum and 0.05% Triton X-100 in h-PBS for 1 hour at 4°C. Fixed cultures were then placed in preblock buffer for one hour. Fixed cultures were then incubated overnight with primary antibodies; rabbit anti-PSD-95 (D27E11, 1:200; Cell Signaling, Beverly, MA) and mouse anti-αCaMKII (6G9, 1:1000; ABR, Golden, CO). Coverslips were washed three times (15 min per wash) in blocking buffer followed by incubation with goat anti-mouse conjugated to Alexa 568 and goat anti-rabbit conjugated to Alexa 488 secondary antibodies (Molecular Probes) at room temperature for 1 hour. Coverslips were then washed once in blocking buffer for 15min and twice in PBS for 15 min. Coverslips were then post-fixed in 2% paraformaldehyde in PBS for 10 min followed by two washes in PBS for 10 min each. Finally, coverslips were mounted on microscope slides with a drop of Prolong antifade reagent (Invitrogen) and allowed to dry overnight.

4.3   Fluorescent microscopy and image analysis

All immunofluorescent images were acquired on a Zeiss Axiovert 200M (Thornwood, NY) fluorescent microscope equipped with a 63x/1.4 oil objective and a high-resolution CCD camera (Axiocam MRm) controlled by Zeiss AxioVision 3.1 imaging software. Image exposure time was independently determined for each experiment by setting the exposure length to sub-maximal pixel brightness based on wildtype images. All images from the same experiment were acquired under identical settings.

Image analysis was conducted with the NIH ImageJ software program. PSD-95 was used as the marker for the PSD region. Threshold for PSD-95 immunostaining was set to allow all recognizable PSD-95 puncta to be included in the creation of the mask. The PSD-95 mask was then overlayed onto the CaMKII image. All CaMKII puncta that colocalized with the PSD-95 mask were measured for intensity. 15-20 neurons were analyzed per embryo. Each genotype was analyzed at least three different times from litters dissected from three different pregnant females. Mutant animals were always compared to wild type litter mates.

Values for brightness of CaMKII puncta normalized to wild type littermates were analyzed with the Prism statistical package (Graphpad, San Diego, CA) and normalized to wt. The one-sample t test (two-tailed) was used to determine whether the brightness of CaMKII puncta in mutant neurons was significantly different from that of wild type neurons. Data are presented as averages +/- standard error of the mean (SEM) with correlating p-value. All images are presented without alterations to brightness or contrast.

4.4   Quantitative immunoblot

Preparations of forebrain homogenates for immunobloting were performed as previously described (Section 2.5). Blots of five wt/ko sibling pairs were probed, in triplicate, for the following proteins: aCaMKII (6G9, 1:2500), synGAP (PA1-046, 1:1000), PSD-93 (PA1-043, 1:300), and PSD-95 (7E3, 1:2000; ABR, Golden, CO); GluR1 (1:500) and GluR2/3 (06-307, 1:200) from Upstate Biotechnology, Lake Placid, NY; α-Actinin (EA-53, 1:3000) and Actin (1:5000) from Sigma, St. Louis, MO; NR1 (1516, 1:700; Chemicon, Temecula, CA); β-Catenin (1:4000), NR2A (Uma, 1:2500), (NR2B Xandria, 1:2500), δ-Catenin/ NPRAP (1:300,) and Citron (CT261, 1:1000) developed in house; and Erbin, 1:1000 (a generous gift from Lin Mei, Medical College of Georgia, Augusta, GA). Blots were visualized with the Odyssey Infrared Imaging System (Li-Cor Bioscience, Lincoln, NE). Quantification of integrated IR fluorescence intensity was performed with the Li-Cor Odyssey analysis software. Statistical analysis was performed using the Prism software package. A one-sample t test (two-tailed) was used to determine whether fluorescence intensity of protein bands from knockout animals was significantly different from that of wild type litter mates. Data are presented as average +/- (SEM) with correlating p-value.

Results

4.5   Docking of CaMKII in the PSD

To investigate whether the loss of the NMDA receptor and Densin would affect the localization and accumulation of CaMKII in the PSD we measured the intensity of

CaMKII puncta colocalizing with PSD-95 in dissociated hippocampal neurons cultured from individual E16 mouse embryos.

In mature NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ mutant cultures (18 DIC), the CaMKII puncta colocalizing with PSD-95 are significantly less intense than wild type (-18.7% +/-1.2, p<0.0001; Fig 4.1). Similarly, cell cultures from NR1$^{-/-}$ embryos show a statistically significant decrease in the intensity of CaMKII puncta colocalizing with PSD-95 (-17.7% +/- 1.9, p<0.0001; Fig 4.1). When comparing the intensity of CaMKII puncta between the NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ and NR1$^{-/-}$ mutant cultures, no significant change is observed, suggesting that the NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ and NR1$^{-/-}$ mutants are indeed phenocopies.

In the Densin knockout we also see a significant decrease in the intensity of CaMKII puncta colocalizing with PSD-95 (-23.9% +/-3.7, p<0.0005; Fig 4.2). However, this result is confounded by our quantitative immunoblot studies that show a global decrease in αCaMKII protein expression (-18.12% +/-2.7, p<0.01; Fig 4.5). This result suggests that the loss of CaMKII colocalization with PSD-95 in the cell culture experiments may be due to the global decrease in CaMKII expression.

Densin$^{-/-}$ x NR1$^{-/-}$ cultures exhibited a still larger decrease in the intensity of CaMKII puncta colocalizing with PSD-95 (-57.1% +/-1.16, p<0.0001; Fig 4.3). Moreover, the Densin$^{-/-}$ x NR1$^{-/-}$ cultures exhibit a greater loss in intensity of CaMKII colocalizing with PSD-95 than the sum of the losses in single knockout cultures (-57.1% vs. -42.1%, respectively; Fig 4.4. These data may suggest that the NMDA receptor and Densin can compensate for each other in their effects on localization of CaMKII in the PSD. If this is true then their ability to maintain a pool of CaMKII in the PSD is synergistic. However, the decrease in CaMKII puncta intensity can also be the result of a

global decrease in CaMKII expression. This can result from a loss of activity dependent αCaMKII protein expression.

4.6   Decrease in the concentration of core PSD proteins in the Densin knockout

To determine if the loss of Densin affected the composition of the PSD we measured the level of several other core PSD proteins by quantitative immunoblot.

We found that the amounts of NR1 and NR2A subunits of the NMDA receptor significantly decreased (-22.0% +/- 4.3, $p<0.05$; -36.4% +/- 4.3 $P<0.01$, respectively) compared to wild type (Fig 4.5). Given that we found no significant decrease in the NR2B subunit, these data suggest that the composition of the NMDA receptor in the forebrain is shifted towards NR1-NR2B receptors in the Densin knockout mouse. Furthermore, we found a change in the ratio of expression of AMPA subunits. Specifically, the GluR2 subunit decreased by 24.24% +/- 1.9 ($p<0.01$) and GluR1 show no statistically significant change.

Erbin, a member of the LAP protein family of which Densin is also a member, is a PSD protein that has high sequence homology with Densin. We wondered whether its expression might increase as a compensatory response to the loss of Densin. In contrast, the level of Erbin decreased substantially by 45.1% +/- 3.7 ($p<0.0001$; Fig 4.5). Its level was the most significantly affected of any of the proteins investigated.

PSD-95, a major scaffolding molecule of the PSD, decreased by 33.31% +/-1.6 ($p<0.001$) in the forebrain homogenates (Fig 4.5). PSD-95 is known to interact with Densin through the dimerization of Maguin-1 (Ohtakara et al., 2002). Furthermore, PSD-

95 is known to bind directly to or mediate interactions with all of the PSD proteins whose expression levels are significantly decreased. Thus, our results suggest that the loss of Densin may destabilize a PSD complex that is organized by PSD-95.

Finally, we found that αCaMKII was significantly decreases by 18.12% +/-2.7 (p<0.01). This global decrease may result from a loss of activity dependent translation or degradation.

Core PSD proteins that did not exhibit significant changes in their level of expression were α-Actinin, NR2B, SynGAP, PSD-93, δ-catenin, β-catenin, Citron, and the GluR1 subunit (data not shown).

References

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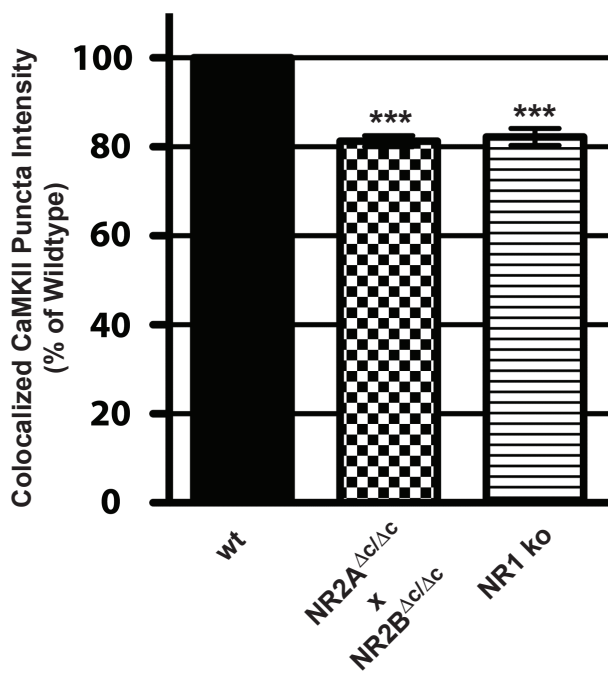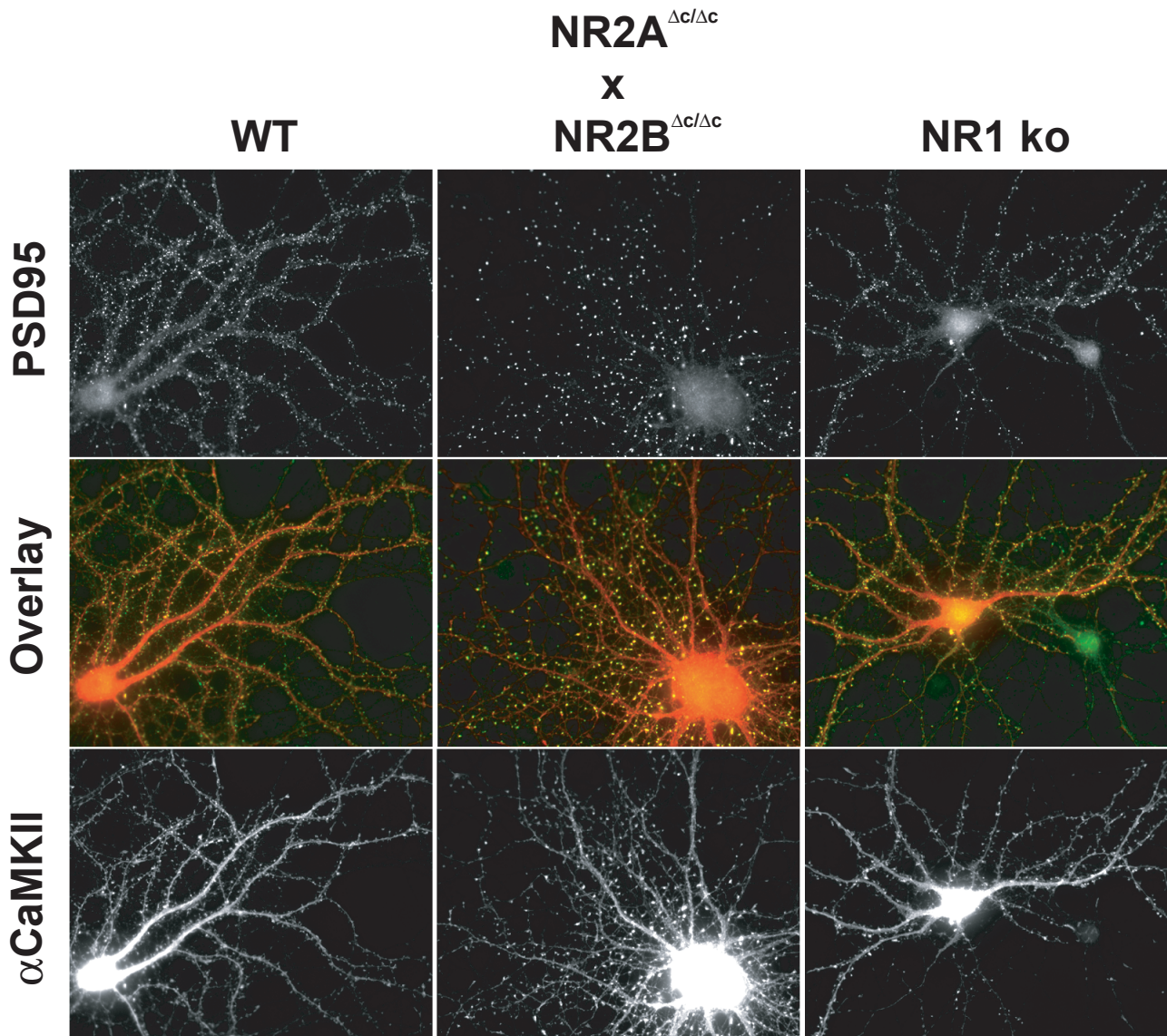
Figure 4.1 Loss of the NMDA receptor results in a decrease of CaMKII co-localizing with PSD95 at the tips of spines. Representative images are from 18 DIC primary hippocampal cultures co-stained for αCaMKII and PSD-95. CaMKII puncta colocalizing with PSD-95 in NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ mutant cultures decrease by -18.7% +/- 1.2 (p<0.0001). CaMKII puncta colocalizing with PSD-95 in NR1$^{-/-}$ knockout cultures decrease by -17.7% +/- 1.9 (p<0.0001).
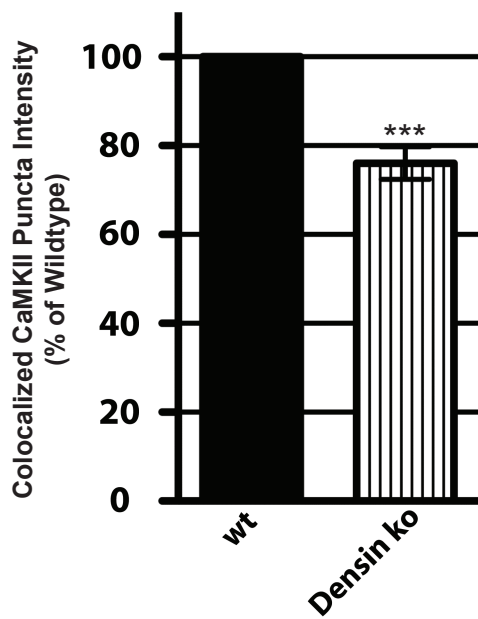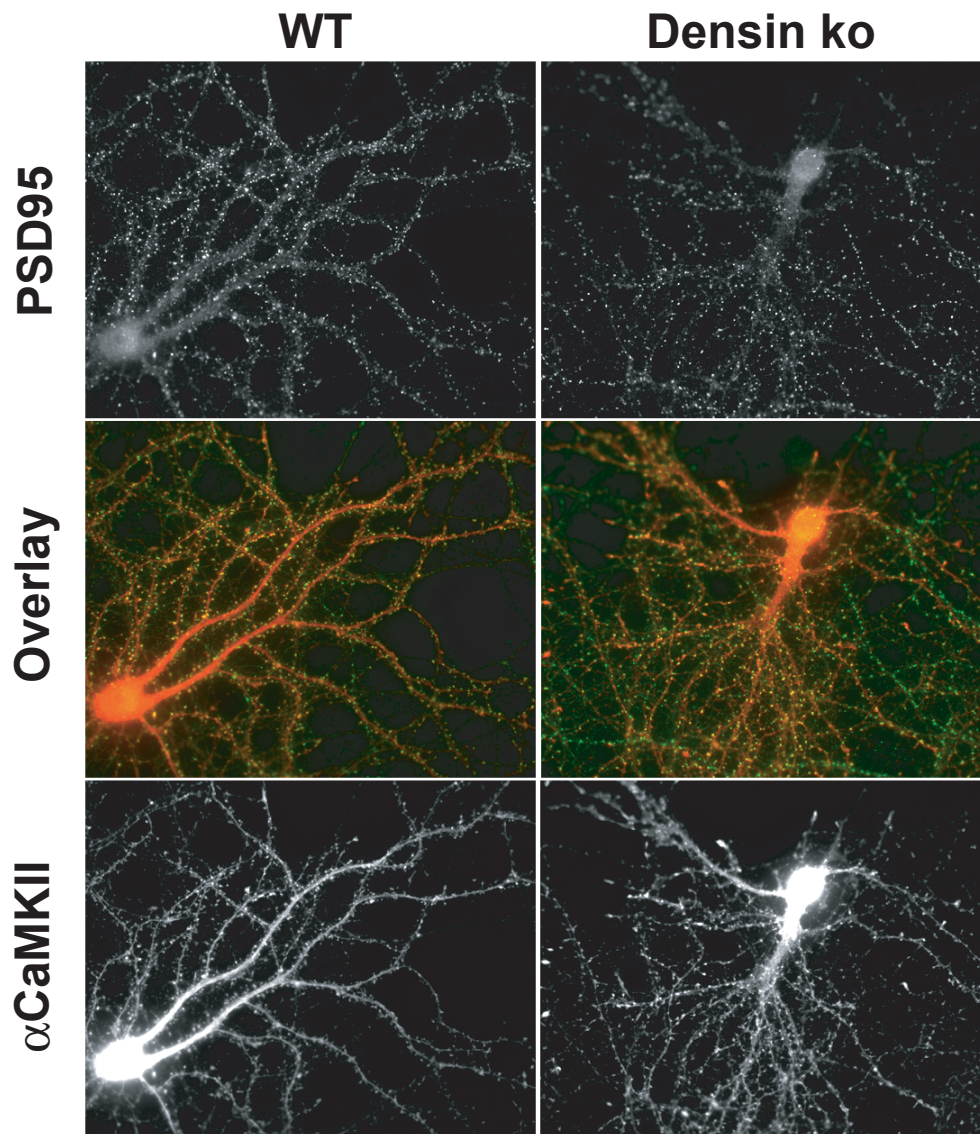
WT   **Densin ko**

PSD95

Overlay

αCaMKII

Figure 4.2 Loss of Densin results in a decrease of CaMKII (red channel) colocalizing with PSD95 (green channel) at the tips of spines in 18 DIC primary hippocampal cultures (-23.9% +/- 3.7; p<0.0005).
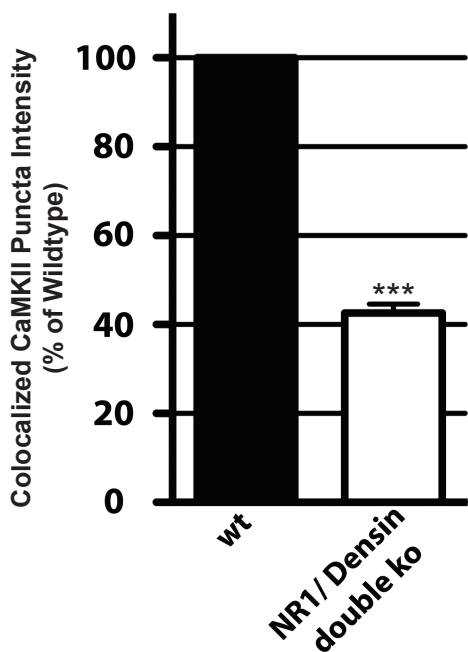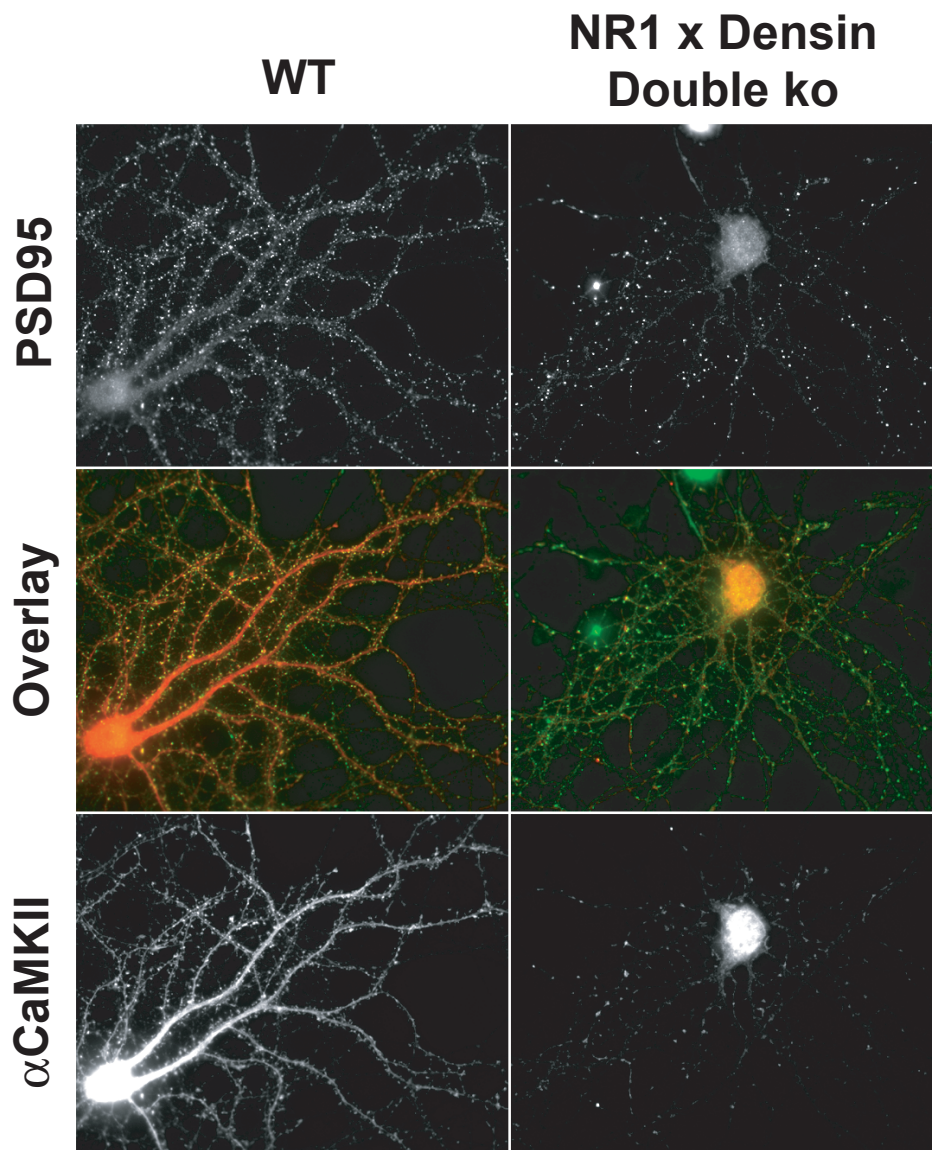
Figure 4.3 Loss of both Densin and NR1 results in a synergisitc decrease in CaMKII puncta intensity. The intensity of CaMKII punta colocalizing with PSD-95 shows a -57.1% +/- 1.16 (p<0.0001) decrease relative to wild type cultures.
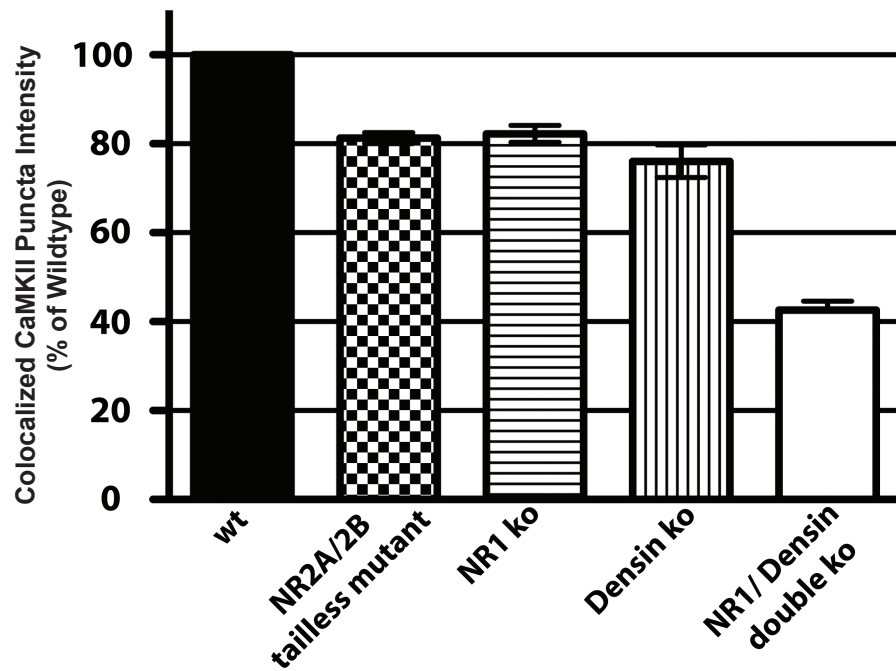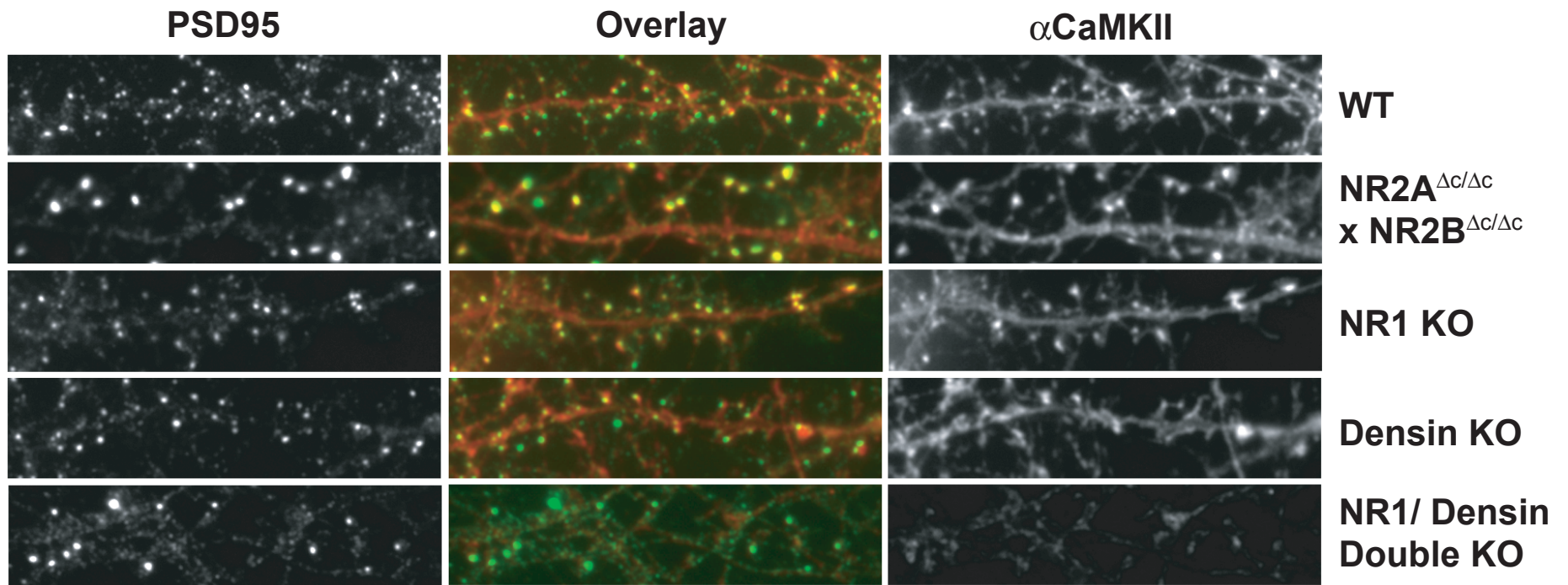
Figure 4.4 Representative images of dendritic branches from Figures 4.1, 4.2, and 4.3. The images shown here represent primary hippocampal cultures made from sibling embryos from a single pregnant female. NR2A$^{\Delta c/\Delta c}$ x NR2B$^{\Delta c/\Delta c}$ cultures were compared to their own wild type litter mates.
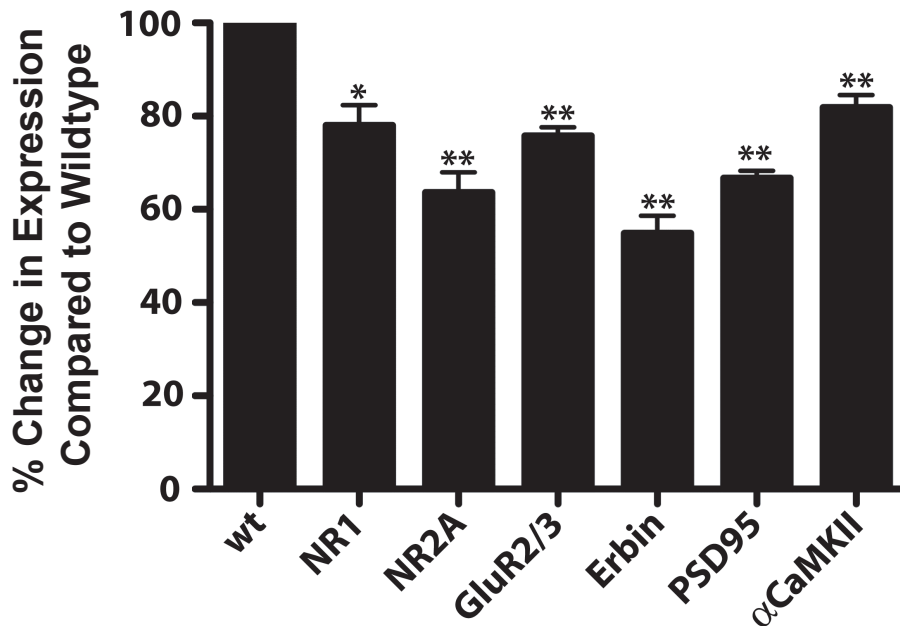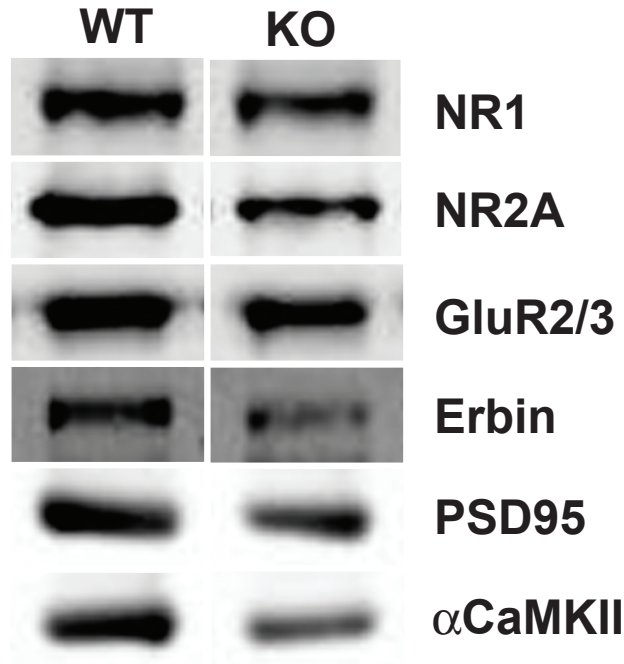
Figure 4.5 Loss of Densin results in an altered composition of the postsynaptic density complex. Quantitative immunoblotting of forebrain homogenates was performed to determine the change in the expression of core PSD proteins. Representative blots are shown for core PSD proteins with statistically significant changes. Not shown are immunoblots for proteins that showed no difference (α-Actinin, NR2B, SynGAP, PSD-93, δ-Catenin, Citron, β-Catenin, and GluR1). No protein assayed exhibited an increase in expression level. * $p<0.05$; ** $p<0.01$