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Yuncen Atticus He April 16, 2012

Effects of Glutamate Receptor Activation on Gld2-Dependent Synthesis of CaMKIIα and NMDA Receptor Subunits

by

Yuncen Atticus He

Dr. Gary Bassell
Adviser

Department of Biology

Dr. Kathleen Campbell

Committee Member

Dr. Kristen Frenzel

Committee Member

Dr. Dieter Jaeger

Committee Member

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Abstract

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The alteration of protein compositions at synapses following receptor stimulation is a crucial event that is thought to underlie processes such as memory storage and learning. However, the mechanisms behind this regulation and the functional units involved are not well known. Studies pointing to a group of proteins encoded by mRNAs possessing *cis*-acting elements that interact with mRNA binding proteins like the cytoplasmic element binding protein (CPEB) provide us with motivation to investigate the regulation of postsynaptic protein subunits such as the α-subunit of Ca2+/calmodulin-dependent protein kinase (CaMKIIα) and the 2A subunit of N-methyl-D-aspartate receptors (NR2A) after activation of glutamate receptors. Based upon preliminary data from the Bassell Lab, we hypothesize that the poly(A) polymerase Gld2 positively regulates activity induced synthesis of CaMKIIα and NR2A in dendrites and that chemical long-term potentiation (LTP) stimulation will induce protein synthesis-dependent insertion of NR2A in the plasma membrane. To assess our propositions, we stimulated hippocampal neurons with either glutamate or a chemical-LTP paradigm and observed the effects of the stimulations on CaMKIIα and NMDAR subunits through immunocytochemistry. Anisomycin, a protein synthesis inhibitor, and Gld2 short hairpin RNA (shRNA) lentiviruses were used to investigate the Gld2-dependent protein synthesis of these subunits. From this study, we determined that there is strong evidence suggesting glutamate stimulation increases the Gld-2 dependent expression of CaMKIIα and NR2A protein in distal dendritic regions. Although we did not observe significant changes in the surface NMDAR subunit expressions, we provide some suggestions for future experiments that could better support the second part of our hypothesis.

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Introduction

Hippocampal synaptic plasticity is critical for learning and memory (Kandel 2001). These experience-dependent changes in synaptic strength are mediated by modifying existing synaptic proteins or altering the synaptic protein composition in a synapse-specific manner. Two non-mutually exclusive mechanisms have been proposed for the delivery of proteins to activated synapses with such specificity: (1) synthesis of proteins locally or (2) targeted trafficking of proteins from the soma. The detection of mRNAs, polyribosomes, and translation factors beneath dendritic spines indicates that local protein synthesis could enable activity-dependent modification of specific synapses (Costa-Mattioli et al., 2009; Eberwine et al., 2002; Poon et al., 2006; Steward and Levy, 1982). Furthermore, there is support for local protein synthesis as two forms of synaptic plasticity, late-phase long term potentiation (L-LTP) and mGluR-mediated long term depression (mGluR-LTD), require dendritic protein synthesis (Huber et al., 2000; Kang and Schuman, 1996). Moreover, many dendritic mRNAs have been identified in hippocampal neurons including mRNAs that encode proteins important for synaptic plasticity and long-term memory such as the α-subunit of Ca2+/calmodulin-dependent protein kinase (CaMKIIα), microtubule-associated protein 2, postsynaptic density protein-95 (Bramham and Wells, 2007) as well as NR2A, the 2A subunit of N-methyl-D-aspartate (NMDA) receptors (unpublished data). Therefore, spatial control of mRNA translation is important for many different cellular functions involving plasticity.

Neuronal activity can regulate global dendritic translation through modulation of the translational machinery. However, given the many forms of protein synthesis-dependent

plasticity, it is likely that distinct, but perhaps overlapping sets of newly synthesized proteins determine and mediate different types of plasticity. Consequently, it is imperative to understand how local translation of specific mRNAs is regulated in dendrites. One hypothesis is that neural activity modulates dendritically localized mRNA binding proteins (RBP) and that these proteins regulate transport and/or translation of a subset of dendritic mRNAs through specific interactions with *cis*-acting elements (Richter and Klann, 2009). One such RNA binding protein is the cytoplasmic polyadenylation element binding protein (CPEB). CPEB is important in the regulation of synaptic plasticity, learning, and memory as CPEB knockout mice exhibit hippocampal LTP defects and reduced memory extinction (Alarcon et al., 2004; Berger-Sweeney et al., 2006; Zearfoss et al., 2008). In *Drosophila*, a homolog of CPEB, Orb2, has an important role in courtship plasticity (Keleman et al., 2007). Moreover, synaptic activity induces CPEB phosphorylation at synapses, and transgenic mice expressing non-phosphorylatable CPEB in Purkinje neurons have defects in cerebellar plasticity and motor behaviors (Huang et al., 2002; McEvoy et al., 2007). These findings emphasize the importance of elucidating the mechanisms by which CPEB controls mRNA translation in neurons.

The mechanisms regulating CPEB-mediated translation have been well-studied in *Xenopus* oocytes wherein CPEB is a critical regulator of translation during oocyte maturation (Richter 2007). In oocytes, CPEB is part of a cytoplasmic complex that regulates polyadenylation of cytoplasmic polyadenylation element (CPE)-containing mRNAs. The complex consists of CPEB, the poly(A) polymerase Gld2, the poly(A) ribonuclease PARN, and symplekin, a scaffold protein upon which the RNP complex is assembled (Figure 1). Under basal conditions, both PARN and Gld2 are active, but PARN is a more efficient enzyme; therefore, the

regulated mRNA's poly(A) tail remains short, and the mRNA is dormant. However, when progesterone stimulates CPEB phosphorylation, it induces the release of PARN from the complex and allows Gld2 to elongate the poly(A) tail. This event ultimately facilitates translational initiation (Richter 2007).

In the brain, CPEB is hypothesized to carry out its functions of regulating synaptic plasticity and some hippocampal-dependent memories by interacting with a subset of synaptic mRNAs that contain the *cis*-acting CPE sequence and regulating their translation (Du and Richter, 2005). Two dendritic mRNAs that have a CPE sequence in the 3' UTR are *CaMKIIα* (Wu et al., 1998) and *NR2A* (unpublished data) mRNAs. In cultured hippocampal neurons, the CPE sequences along with CPEB-1 regulate reporter RNA translation (Huang et al., 2003; Wells et al., 2001). The Bassell laboratory has shown that CPEB, Gld2, PARN, and symplekin co-localize in dendritic mRNA granules and that this complex is activated by neuronal activity to ultimately regulate protein synthesis (unpublished data). Furthermore, it has been shown that knockdown of the poly(A) polymerase Gld2 reduces dendritic poly(A) mRNA levels and occludes theta-burst long-term potentiation in the dentate gyrus region of the hippocampus (unpublished data). In addition, Gld2 activity is necessary for courtship memory formation in *Drosophila* (Kwak et al., 2008). Taken together, these findings and previous studies suggest that the CPEB mRNP complex, and specifically the poly(A) polymerase Gld2, regulates synapse function through polyadenylation of key mRNAs at hippocampal synapses. Stimulus-induced polyadenylation is hypothesized to provide the switch to active synaptic protein synthesis of critical molecules, such as CaMKIIα and NR2A (Figure 1). Therefore, this thesis investigates the role of Gld2 in activity-induced regulation of dendritic CaMKIIα and NR2A protein synthesis.

CaMKII is a critical protein in the postsynaptic density since its functions are important in changing the property and the population of proteins that are imbedded in the membrane of the dendritic spine. CaMKII is activated by increased intracellular calcium ions, which binds to calmodulin and activates the kinase. The enzyme is involved in many signaling pathways downstream of Ca^{2+} influx events, and the self-perpetuating CaMKII holoenzyme activity is proposed as a mechanism that maintains the altered function of potentiated synapses (Lisman et al., 2002). Currently, it is known that CaMKII is involved in the phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which increases the single channel membrane conductance in the event of glutamate binding (Hayashi et al., 2000). Moreover, CaMKII is also potentially involved in the trafficking and insertion of GluR1-containing AMPA receptors to the postsynaptic density (Hayashi et al., 2000), thus increasing the overall membrane conductance at the synapse when activated.

The dendritic synthesis of CaMKIIα has been shown to be critical for hippocampal long-term potentiation and spatial memory formation (Miller et al., 2002). CPEB interacts with CPEs in the 3'UTR of *CaMKIIα* mRNA, which undergoes activity-dependent polyadenylation in the brain and at synapses (Huang et al., 2002; Wu et al., 1998), but the poly(A) polymerase involved in this process remains unknown. Because of the crucial functions that CaMKII serves in the modification of synaptic properties and the established regulatory properties that CPEB-1 possesses in the translational regulation of CaMKIIα, we sought to investigate the role of the CPEB-associated poly(A) polymerase, Gld2, in regulating activity-induced expression of dendritic CaMKIIα.

NR2A is also of particular interest because NMDARs have a critical role in synapse function and are regulated by synaptic activity. NMDARs are composed of two NR1 subunits and two NR2 subunits. There are four types of NR2 subunits (A-D), but NR2A and NR2B are predominant in the hippocampus. NR2A and NR2B are different in their effects on NMDAR channel properties, protein interactions, and subcellular localization. This indicates that the regulation of the subunit expression is a significant determinant of synaptic function (Camilla and Nicoll 2007; Lau and Zukin 2007). However, the underlying molecular mechanisms behind the post-transcriptional regulation of key proteins involved in synaptic plasticity such as NR2A remain elusive. LTP induction in adult rat hippocampus leads to membrane insertion of NR2A-containing receptors (Grosshans et al. 2002), but it is unknown whether it involves local protein synthesis. The Bassell laboratory and collaborators have shown that CPEB interacts with NR2A mRNA through the CPE sequence in the 3'UTR and that Gld2 regulates NR2A mRNA polyadenylation and dendritic expression of NR2A protein (unpublished data). Consequently, the NMDA receptor subunits provide us with another molecule to examine for the potential role of Gld2-mediated dendritic protein synthesis.

Henceforth, the purpose of this research is to investigate how the activation of glutamate receptors affects the expression of proteins encoded by CPEB-target-mRNAs. To begin, we wanted to explore two targets that have been identified to interact with CPEB. While *CaMKIIα* is an established CPEB target (Wu et al., 1998), *NR2A* has recently been identified as a putative target by Sharon Swanger from the Bassell Lab and her collaborators (unpublished data). Therefore, we can divide the aims of our research into two separate parts. Our first aim focuses

on investigating the role of Gld2 in the activity-induced synthesis and dendritic expression of CaMKIIα and NR2A, while the 2nd aim looks to determine how chemically induced LTP affects the pattern of surface NMDA receptor expression levels in dendrites of cultured hippocampal neurons and whether differences are protein synthesis dependent. For aim 1, we hypothesized that Gld2 positively regulates activity-induced synthesis and dendritic expression of CaMKIIα and NR2A. Meanwhile for aim 2, we predicted that a particular form of chemical long-term potentiation stimulation will induce protein synthesis-dependent insertion of NR2A-containing NMDA receptors into the plasma membrane.

The results from this thesis research provide evidence that the dendritic expression of CaMKIIα and NR2A following glutamate stimulation is likely protein synthesis dependent and may be regulated by Gld2 in the CPEB complex. With an understanding of how protein synthesis and Gld2 affect the total protein levels of targets studied from aim 1, we turned our attention to the surface level expression of NMDA receptor subunits. We successfully implemented the chemical LTP paradigm in our culture system as we could show that it resulted in a significant increase in AMPA receptor surface expression in dendrites. However, this paradigm did not produce statistically significant effects on NMDA receptor surface expression. Thus, we propose some ways to improve upon our experimental design for future experiments in an attempt to reduce the amount of recorded variability.

Methods

Hippocampal neuron culture

Hippocampal neurons were isolated from embryonic day 18 (E18) rats and cultured as described in Goslin and Banker, 1998 with the following modifications. Neurons were plated on glass coverslips and grown in Neurobasal media (*Invitrogen*) with Glutamax (*Invitrogen*) and NS21. The dissection and neuron culture were performed by Sharon Swanger, a Ph.D. candidate in the Bassell laboratory.

Lentiviral short hairpin RNA (shRNA) knockdown

The shRNA lentiviral constructs were prepared on the pLentiLox3.7-Syn backbone. The Gld2 targeting sequence was: 5' atgcacaattcaactttca 3'. Lentiviruses were produced using the lentiviral vector mentioned above with packaging vectors pSPAX2 and pMD2.G in HEK293T cells (kind gift from J.D. Richter). For Gld2 knockdown experiments in cultured neurons, lentiviruses were added to the cultures at 14 day *in vitro* (*DIV*) and experiments were conducted 3 days later. To measure the level of knockdown, RT-PCR, western blotting, and immunostaining were performed (data not shown).

Neuron stimulation treatments

For glutamate treatments in aim 1, cultured primary hippocampal neurons from rat brains (E18, 17 *DIV*) were bathed in 10 μM glutamate for 10 minutes and then washed afterwards to remove neurotransmitters. For chem-LTP treatments in aim 2, cultured hippocampal neurons (E18, 17 *DIV*) were equilibrated in a chem-LTP buffer consisting of 140 mM NaCl, 1.3 mM

CaCl2, 1.3 mM KCl, 25 mM HEPES, 33 mM glucose, 0.5 μM TTx, 1 μM strychnine, and 20 μM bicuculline methiodide for 20 minutes with either 40 μM anisomycin (protein synthesis inhibitor) or dimethyl sulfoxide (negative control for protein synthesis inhibition). After equilibration, the chem-LTP buffer was then exchanged with either the vehicle (chem-LTP buffer) or the chem-LTP cocktail (chem-LTP buffer + 200 μM glycine) for 3 minutes. These cells were then incubated in the original chem-LTP buffer for 30 minutes.

Immunocytochemistry

Following stimulation and equilibration, the neurons on each coverslip were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 5 mM $MgCl_2$ for 20 minutes and then washed in PBS with 5 mM $MgCl_2$ for 5 minutes. The coverslips were then equilibrated in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) for 10 minutes and then in immunofluorescence buffer (IF buffer; 1% BSA heatshock, 1% fetal bovine serum in TBS) for 10 minutes before being incubated for an hour in a blocking buffer (2% BSA heat shock, 2% fetal bovine serum in TBS). In experiments quantifying the total dendritic protein levels, the cells were permeabilized in TBS containing 0.3% Triton X-100 and 0.1% Triton X-100 was also added to the IF buffer and blocking solution. After blocking, the coverslips were washed in IF buffer for 10 minutes 3 times and then immunostained for CaMKIIα (1:50 dilution, *BD Biosciences*) and NR2A (1:100 dilution, *Millipore*) in aim 1. Some cells were also stained with rabbit anti-synapsin (1:1000 dilution, *Sigma-Aldrich*), phalloidin (1:500 dilution, *Invitrogen*) for actin, and DAPI (*Sigma-Aldrich*) for nuclear DNA to help visualize the neurons. In aim 2, the cells were immunostained for individual NR2 subunits using either rabbit anti-NR2A (1:200 dilution, *Alomone Labs*) or rabbit anti-NR2B (1:100 dilution, *Alomone Labs*). Another group was simultaneously stained

with the mouse anti-NR1 subunit (1:200 dilution, *BD Biosciences*) and rabbit anti-GluR1 (1:20 dilution, *Millipore*), a positive control for successful long-term potentiation in our experiments (Lu et al., 2001). To visualize the signals, Cy2-coupled anti-rabbit and Cy3-coupled anti-mouse secondary antibodies (1:1000 dilution, *Jackson Immunoresearch*) were applied as needed to the cells. The coverslips were mounted onto microscope slides with polyvinyl alcohol mounting media containing propyl gallate to reduce fluorescence bleaching.

Image acquisition and processing

Images were acquired using a wide-field fluorescence microscope (Nikon Ti microscope) as Z-stacks (11 images, 0.15 μm steps), and then deconvolved to a 16-bit unsigned image using a 3D blind algorithm software (AutoQuant X, *CyberMetrics*). After deconvolution, the images from aim 1 were analyzed for mean intensity in ImageJ by summing the 5 most focused planes and then drawing a region of interest (ROI) in each neuron's distal dendrite (> 50 μm from the cell body). This region was measured for mean intensity and then the intensity of a nearby region with no cellular material was quantified and subtracted from the ROI's intensity value to account for background fluorescence.

For aim 2, we measured variables including integrated density, granule count, total granule area, and average granule size for granules filtered through a threshold intensity value using Fiji (ImageJ2). For each image acquired, the 5 stacks that were the most focused were summed into a single image and then a region containing a distal dendrite was selected using the 'Straighten' selection tool before converting the image to an 8-bit image and duplicating each file. After going through the same process for each image from a single imaging session, a

common lower-bound threshold was determined through the visualization of potential protein granules across every image, and then this threshold was applied to each duplicated region of interest. This process essentially filtered out all background noise below our applied threshold value by setting all pixels above the threshold to an intensity of 255 while setting those below the threshold to 0. With the threshold applied to the duplicated image, we used the 'Create Selection' function to create a masking region of interest over the original region. Finally, the integrated density and the total area above threshold were measured using the original image, and the granule count, total granule area and average granule size were collected using the 'Analyze particle' function on the duplicated region. Here, integrated density represents the total amount of protein (factoring in both intensity and area occupied) at the surface; granule count indicates the number of distinctly resolvable clusters of the receptor subunit; total area is the amount of pixels occupied by the proteins; average size represents the mean size of the protein clusters.

Data analysis

The raw data sets for each variable were normalized to the variable's average from the control group. In each experiment for aim 2 where GluR1 was quantified, if we did not observe an average increase > 20% in GluR1 integrated density for the whole experiment, then data from that experiment were excluded. To further process the data set, if for a certain protein or for a certain type of experiment where neurons exhibited uncharacteristically large increases in integrated density or total granule area, inter-quartile range (IQR) analysis for outliers was performed to remove cells whose integrated density or total area values exceeded the group median + 1.5*IQR.

Statistical analysis

To perform statistical analysis on the data from the different experiments, we first assessed whether the conditions for the parametric one-way ANOVA test were met. To do this, the normality of residual values based upon the difference between values expected from a general linear model and the actual values as well as the variance equality of the raw data were tested using the Shapiro-Wilk test and the Levene's homogeneity of variance test respectively in RExcel (*Statconn*). If the raw data failed to pass these conditions, power transformations by factors of 1 to 0.1 in decreasing steps were performed on the raw data until the transformed data set passed the conditions required for parametric statistical tests. Data sets that qualify for parametric tests were analyzed in SPSS (*IBM*) using the one-way ANOVA test with the Tukey post hoc test for comparison of group means. If the data failed to pass the conditions with the given power factor ranges, nonparametric ranking tests (Kruskal-Wallis and Mann–Whitney–Wilcoxon) were used in SPSS instead. Statistical significance is provided by p values less than 0.05.

Results

Protein-synthesis dependence of CaMKIIα expression in dendrites following glutamate treatment and the role of Gld2

To investigate whether postsynaptic stimulation regulates the dendritic expression of proteins encoded by CPEB target mRNAs, 17 day *in vitro* rat hippocampal neurons were treated with 10μM glutamate or vehicle for 10 minutes before being fixed and immunostained for CaMKIIα. We also tested whether protein synthesis mediated any glutamate-induced effects on CaMKIIα protein expression by treating neurons with 40 μM anisomycin or DMSO (vehicle) for 30 minutes prior to glutamate stimulation. Following acquisition of images, the neurons were analyzed as described above to quantify the mean intensity values along distal dendritic regions within individual neurons (Figure 2). Quantification of CaMKIIα in the dendrite showed a marginal increase (29.6%) in the protein levels following a 10 minute glutamate stimulation compared to the CaMKIIα levels from the control treatment (one-way ANOVA w/ Tukey HSD post hoc, $p = 0.055$; n = 27/28 cells, 2 experiments). In the presence of anisomycin, there was a non-significant reduction (14.2%) in CaMKIIα protein levels between cells treated with anisomycin and those treated with anisomycin and glutamate (one-way ANOVA w/ Tukey HSD post hoc, $p = 0.641$; n = 16/27 cells, 2 experiments), which points to the elimination of the increasing trend previously observed without anisomycin. However, when comparing the neurons treated with both glutamate and anisomycin versus only glutamate treated neurons, a significant increase (86%) in the dendritic CaMKIIα levels was observed in the latter group (MWW, $p<0.001$; n=27/28 cells, 2 experiments). These results suggest that CaMKIIα protein expression in dendrites may be regulated by protein synthesis following synaptic stimulation, but

because the data do not show statistical significance for all the appropriate pairwise comparisons, more experiments are necessary to confirm and validate this result.

Next, to discover the role of the CPEB complex in the regulation of CaMKIIα levels in dendrites, Gld2 expression was knocked down in cultured neurons via lentiviral shRNA transfections. Neurons with Gld2 knockdown were compared to those that received a control transfection following vehicle or glutamate treatment (Figure 3). To ensure that successfully transfected neurons were analyzed, only cells with identifiable GFP fluorescence were imaged as GFP was co-expressed with the shRNA in the lentivirus (figure not shown). Quantification for CaMKIIα levels along distal dendrites of non-Gld2 knockdown neurons showed a significant increase (17.8%) in protein levels following glutamate stimulation (MWW, $p = 0.004$; $n = 37/38$ cells, 2 experiments). Moreover, there was also a significant difference between the glutamate treated cells and the glutamate treated Gld2 knockdown cells (one-way ANOVA w/ Tukey HSD post hoc, $p < 0.001$; $n = 41/36$ cells, 2 experiments). Between the two Gld2 knockdown groups however, there was only a 5.1% change in CaMKIIα levels following glutamate stimulation as compared to the vehicle treated cells (MWW, $p=0.966$; $n=43/38$ cells, 2 experiments). From these results, it is evident that the glutamate-induced increases in dendritic CaMKIIα are in fact dependent upon the presence of Gld2 as any glutamate-induced changes seen in the control-transfected groups were eliminated when Gld2 levels were reduced by the lentivirus.

Protein-synthesis dependence of NR2A expression following glutamate treatment and the role of Gld2

To further investigate the regulation of synaptic proteins by protein synthesis and Gld2, we turned our attention to another target of CPEB, the NMDA receptor subunit NR2A. To first determine if there was a protein synthesis-dependent change in dendritic NR2A protein levels following stimulation by glutamate, we treated neurons in a similar manner as described above and immunostained for NR2A. Using the same imaging analysis techniques, NR2A mean intensities in dendrites were quantified and compared over the different treatment groups (Figure 4). Here, we did observe a significant increase (39.4%) in NR2A levels with the application of glutamate for 10 minutes when compared to the control treated cells (one-way ANOVA w/ Tukey HSD post hoc, $p<0.001$; n=29/29 cells, 2 experiments). Furthermore, we also observed a significant difference between the glutamate treated cells and the cells treated with glutamate plus anisomycin (MWW, $p < 0.001$; n = 29/29 cells, 2 experiments), which corroborates the previous result. However, when the cells were treated with anisomycin, this glutamate-induced increase in NR2A was eliminated as only a 4.4% increase was observed (one-way ANOVA w/ Tukey HSD post hoc, $p=0.961$; n=29/29 cells, 2 experiments). From this set of experiments, there is significant evidence supporting a protein synthesis-dependent increase in NR2A levels in dendrites following exogenous glutamate application.

Now to determine whether Gld2 is crucial in regulating this glutamate-induced NR2A synthesis, we compared NR2A levels in Gld2 knockdown neurons to control-transfected cells following glutamate or vehicle treatment (Figure 5). Between cells transfected with the control lentivirus, we observed a slight, but statistically non-significant increase (16.6%) in NR2A mean

intensity (MWW, $p = 0.083$; n = 26-28 cells, 2 experiments) in neurons that were stimulated with glutamate over the control group. These data indicate that this stimulation may not lead to significant increases in NR2A levels. However, when we compared the control-transfected cells treated with glutamate versus the Gld2 knockdown neurons treated with glutamate, there was 64% increase in dendritic NR2A for the control-transfected neurons in comparison to the Gld2 knockdowns (MWW, $p < 0.001$; n = 28/27 cells, 2 experiments). All of this taken together suggests that the knockdown of Gld2 leads to a significant decrease in dendritic NR2A protein levels under both basal conditions and following treatment with glutamate when compared to the control-transfected neurons. After glutamate was applied to the Gld2 knockdown neurons, no changes in NR2A levels were observed when compared to the control treated knockdown neurons (MWW, $p=0.725$; n=30-27 cells, 2 experiments), suggesting the block of glutamate-induced expression for NR2A. Although the control-transfected groups do not demonstrate significant differences in their mean intensities for this experiment, the marginal increase after glutamate stimulation is not seen in the Gld2 knockdown neurons, suggesting that the possible differential expression of NR2A in the distal dendrites may be Gld2 dependent.

Protein-synthesis dependence of NMDAR subunit surface expression following chemical LTP treatment

After observing an increase in the levels of NR2A along dendrites of hippocampal neurons treated with 10 μM glutamate, we decided to focus on surface level expression of NMDAR subunits and how they are affected by a more specific form of stimulation with 3 minutes of glycine treatment. This paradigm is known to induce plasticity in neurons equilibrated with the buffer mentioned in the Methods section above through the specific co-activation of

NMDARs by glycine and spontaneous glutamate releases (Lu et al., 2001). Additionally, rather than simply measuring the mean intensity of the proteins of interest, we used a threshold method to quantify different parameters of the immunofluorescence signals detected above an intensity threshold such as integrated density, granule count, total granule area, and average granule size in an attempt to reduce background noise and collect additional characteristic measurements.

To assess the validity of our experimental treatments, we first looked for significant increases in variables such as integrated density, count, and total area of our positive control GluR1 in the cell membrane following chemical LTP stimulation (Figure 6). GluR1 serves as the positive control for this study since it is known to be inserted into the postsynaptic density during LTP and it was previously shown that GluR1 surface expression increases with this paradigm (Lu et al., 2001). Experiments in which we did not observe sizeable increases (20% or more) in the previously mentioned measurements were excluded from analysis. When comparing neurons treated with the chemical LTP paradigm to the control group, significant increases in integrated density by 150% (one-way ANOVA w/ Tukey HSD post hoc, $p = 0.011$; $n = 32/31$ cells, 3 experiments), granule count by 83.6% (one-way ANOVA w/ Tukey HSD post hoc, $p = 0.008$), and total granule area by 156% (one-way ANOVA w/ Tukey HSD post hoc, $p = 0.009$) were observed but not in the average granule size, although the value changed by 32.8% (one-way ANOVA w/ Tukey HSD post hoc, $p = 0.149$). Furthermore, we assessed the protein synthesis dependence of these changes in GluR1 surface expression following the chemical LTP stimulation. In this part of the experiment, we recorded no significant changes in any of the variables when comparing the chemical LTP treatment to the control treatment for cells bathed in anisomycin (one-way ANOVA w/ Tukey HSD post hoc, intden: $p = 0.951$; count: $p = 0.609$;

area: p = 0.952; n = 23/23 cells, 2 experiments). These data verify the previous observations and suggest that our stimulation paradigm and measurement techniques are usable methods to test our hypothesis.

With expected results in the positive control experiments, we next turned our attention to NR2A. While analyzing NR2A, it was important to see how the effects of chemical LTP on the surface levels (Figure 7) compared to the total expression following glutamate stimulation. When neurons were stimulated with our chemical LTP buffer, a non-significant 19% increase in the group means for integrated density was observed between the chemical LTP treated cells and the control cells (one-way ANOVA w/ Tukey HSD post hoc, p = 0.816; n = 31/29 cells, 3 experiments). The data here were highly variable with a large standard error when compared to the normalized values (8-18%), so additional experiments must be completed in order to discern whether protein synthesis has a role in chemical LTP-induced surface expression of NR2A. Similar results were seen with granule count (one-way ANOVA w/ Tukey HSD post hoc, p=0.419), total area (one-way ANOVA w/ Tukey HSD post hoc, p=0.739), and average granule size (Kruskal-Wallis test, p=0.816). While no significant increases were observed in these parameters between chemical LTP treated and control groups, NR2A levels were significantly reduced in neurons treated with chemical LTP and anisomycin when compared to neurons treated with chemical LTP alone (one-way ANOVA w/ Tukey HSD post hoc, intden: p=0.011; count: p=0.001; area: p=0.016; n=27/27 cells, 2 experiments). These data indicate that the presence of anisomycin might block the accumulation of NR2A at post synaptic sites following synaptic stimulation. However, it is unclear why anisomycin in combination with glycine treatment would result in a reduction of surface expression when compared to anisomycin

treatment alone. Further work needs to be completed to understand how protein synthesis, and perhaps degradation, regulates NR2A surface expression.

Since NR2A and NR2B are the two predominant NMDAR subunits in these neurons, we also wanted to determine whether NR2B in the membrane is affected by the chemical LTP stimulation. Cells immunostained for NR2B were also quantified in the same manner (Figure 8), but no significant differences in any of the variables were observed among any of the treatment groups (one-way ANOVA, intden: $p = 0.323$; count: $p = 0.091$; area: $p = 0.295$; Kruskal-Wallis test, size: $p = 0.277$; n = 19-31 cells, 2-3 experiments). From these results, we can infer that surface NR2B is not affected by this chemical LTP stimulation protocol and is not regulated by protein synthesis.

Finally to investigate whether NR1 levels were affected by the chem-LTP stimulation, cells that were co-stained for GluR1 and NR1 were quantified for NR1 (Figure 9). After recording the variables for this set of test groups, we also found that no observable differences in any of the measurements were apparent across all treatments (one-way ANOVA, intden: $p=0.129$; count: $p=0.057$; area: $p=0.132$; size: $p=0.407$; n=20-31 cells, 2-3 experiments). Here again, we observe a significant amount of variability for all measurements with the standard errors ranging from 6% to 35%. Consequently, the results are difficult to interpret as the percent changes are sometimes smaller than the standard errors of the means. Because the groups are similar in all of the characteristics measured, we cannot conclude that surface NR1 levels are affected by our chemically induced LTP paradigm.

Discussion

The goal of this study was to understand how the activation of glutamate receptors, through the use of different stimulation paradigms, affects the expression of proteins encoded by CPEB-targeted mRNAs. In the first part of this thesis, we determined that non-specific glutamate stimulation increases the expression of CaMKIIα and NR2A protein in distal dendritic regions. Importantly, we found that protein synthesis is necessary for the glutamate-induced increase in NR2A protein expression, and there was a strong trend suggesting that protein synthesis regulates CaMKIIα dendritic expression. Moreover, the glutamate-induced effect on CaMKIIα protein expression in dendrites was eliminated when expression of the poly(A) polymerase Gld2 was reduced, and a strong trend suggests that Gld2 might similarly regulate NR2A protein expression in dendrites.

Given the observed effects on total NR2A expression following a strong stimulus with 10 μM glutamate, we turned our attention to the surface expression of the NMDA receptor subunits as the protein synthesis-dependence of NMDA receptor insertion had not been previously addressed. For this part of the study, the experiments did not yield results with statistical significance when comparing surface expression of NMDA receptor subunits between the means of the control and chemical LTP treated groups. However, we did observe decreased NR2A levels in the distal dendrites of cells treated with both the chemical LTP paradigm and anisomycin. This unexpected result points to the possibility that the stimulation treatment may have increased the turnover rate of surface NR2A, and thus decreasing the membrane levels of the protein possibly through internalization. Furthermore, there was high variability in the measurements for NR2A and NR1 surface expression, so any potential differences between

control and chemical LTP treated groups were indiscernible. Later in this section, we speculate about causes for the variability and provide potential ways for addressing this issue. Additionally we also provide alternative experimental approaches for studying the role of protein synthesis in activity-induced NMDA receptor surface expression.

Modulation of CaMKII and NMDAR levels through protein synthesis in neurons following induction of plasticity is of critical concern in determining the mechanisms behind long term potentiation and the induction of late phase synaptic plasticity. Autophosphorylation among CaMKII holoenzymes is a potentially crucial mechanism that maintains the altered functions of potentiated synapses as the enzymes' activity are required for the phosphorylation of AMPA receptors that not only increases the individual single-channel conductance, but also increases the overall membrane conductance through these receptors via the insertion of additional GluR1-containing AMPA receptors to the post synaptic density (Lisman et al., 2002; Lisman and Zhabotinsky, 2001). With increased membrane conductance through AMPA receptors, depolarization from glutamate binding is facilitated in subsequent events. Therefore, the regulation of CaMKII subunits is of particular interest because their levels in the postsynaptic compartment can modulate activity in potentiated neurons during LTP.

While CaMKII is implicated in promoting plasticity through a pathway that regulates AMPA receptors in the post synaptic density, NMDAR and more specifically NR2A synthesis and surface insertion may also be important in the late phase of long term potentiation (Grosshans et al., 2002; Philpot et al., 2007). Because NMDA receptors work in conjunction with AMPA receptors to increase intracellular calcium concentrations, NR2A synthesis and

insertion into the post synaptic membrane would also amplify the efficacy of downstream pathways.

As a result of the critical roles that CaMKII and NMDAR expression modification take on during plasticity, it is therefore essential to clarify the mechanism behind how their expression and localization is regulated. Because these proteins take on similar roles in synaptic plasticity induced by long-term potentiation, it is not surprising that the expression of their subunits may also be regulated through similar mechanisms. Both the mRNAs for CaMKIIα and NR2A contain CPEs in the 3' UTR that may be targeted by CPEB-1 as a means of translational regulation. When synaptic activity induces the phosphorylation of CPEB, it leads to the exclusion of the ribonuclease PARN from the protein complex and ultimately causes the elongation of the poly(A) tail by Gld2, and thus facilitating translational initiation. Our results suggest that the polymerase activity of Gld2 might be responsible for glutamate-induced synthesis of CaMKIIα and NR2A. From literature that supports the localization of the CPEB-1 complex and other protein synthesis machinery such as ribosomes at the dendritic spine (Steward and Worley, 2002), we can only infer the local synthesis of these proteins might contribute to the increased dendritic expression during glutamate-stimulation.

Endogenous CaMKIIα mRNA has been shown to be localized to dendrites, but thus far most studies of the protein synthesis-dependent mechanisms regulating dendritic CaMKIIα protein expression have used recombinant proteins and biochemical fractions (Aakalu et al., 2001; Bagni et al., 2000; Gong et al., 2006; Scheetz et al., 2000). Importantly, in our study we found that glutamate-induced dendritic expression of endogenous CaMKIIα protein is regulated

not only by protein synthesis but also by the poly(A) polymerase Gld2. We postulate that Gld2 regulates *CaMKIIα* mRNA polyadenylation through CPEB as it is established that Gld2 interacts with CPEB (unpublished data) and that CPEB interacts with *CaMKIIα* mRNA (Wu et al., 1998). In future studies, it will be important to determine whether CPEB, and perhaps the CPE sequence, are also necessary for the glutamate-induced changes of CaMKIIα expression in dendrites.

For the experiments where strong trends were observed, we predict that given the effect size and the standard deviation in these experiments, it is likely that completion of a third independent experiment would provide a large enough sample size to show significant effects of the protein synthesis inhibitor and Gld2 knockdown. One interesting observation from our experiments is that the effect of glutamate on NR2A protein levels was reduced in the control lentiviral treated cells for the Gld2 knockdown studies as compared to the vehicle-treated cells in the anisomycin experiments. The same effect has been seen by additional members of the Bassell laboratory and collaborators when treating lentiviral transduced neurons (personal communication and unpublished results, S. A. Swanger). Given this trend, it will be important in future experiments using lentiviruses to take this reduction in stimulation-induced effect size into account when performing the *a priori* power analysis.

Although we did not observe results that supported our hypothesis for NMDA receptor surface expression, this study is novel in that it is the first to investigate exclusively the role of protein synthesis in activity-induced surface expression of NMDA receptor subunits in dendrites. In this study, we found that chemical LTP-induced GluR1 surface expression requires protein synthesis. This is a key finding as it is well-known that the surface expression of GluR1-

containing AMPA receptors is increased during LTP (Kessels and Malinow, 2009), but few studies have examined whether protein synthesis is required for this process. One previous study showed that increased surface expression of GluR1 in whole-neuron lysates is protein synthesis dependent during *in vivo* LTP (Williams et al., 2007). Importantly, we established that GluR1 insertion in distal dendrites is also protein synthesis-dependent using chemical LTP in cultured neurons.

In moving forward from the first aim of our study, changes were made in the method of how we obtained results as well as the measurements made in these experiments to investigate additional properties of these proteins that may have been masked by only quantifying and analyzing the mean intensity. Thus, instead of drawing an ROI, measuring the mean intensity, and then subtracting the mean intensity of a region beside the ROI to account for background intensity, we used a threshold technique that involves only quantifying densities above a certain intensity threshold perceived to be the lower limit of intensity emitted by a true protein granule. From this masking technique, we analyzed the integrated density of the pixels detected above threshold, the number of distinctly resolved particles, the total area occupied by the pixels above threshold, and the average size of the distinct particles to give us a more comprehensive idea of what is occurring in the various protein subunits analyzed.

Additionally, not only did we limit our area of study to the surface level expression, but we also wanted to use a more specific approach for stimulating postsynaptic neurons by the activation of NMDA receptors through mini-EPSPs caused by spontaneous presynaptic glutamate release. Therefore, instead of treating cells with 10 μM glutamate, we equilibrated the

cultures in a standard buffer with various inhibitors such as tetrodotoxin, strychnine, and bicuculline to prevent action potentials and the activation of inhibitory synapses during stimulation. Following equilibration, we treated the neurons by adding 200 μM glycine to specifically activate NMDA receptors. Therefore, rather than simply treating with excess glutamate, this stimulation paradigm relies on the release of glutamate from presynaptic cells to activate NMDA receptors when glycine is added to the buffer. While this approach is more specific in targeting the activation of NMDA receptors in postsynaptic neurons, the synaptic activation is directly affected by the level of synaptic connectivity in the cultured neuronal network, which introduces variability into the experiment. When visually scanning the coverslips for neurons through the microscope, it becomes apparent that certain coverslips contain smaller populations of neurons than others. With the switch from glutamate treatment to the chem-LTP paradigm, coverslips with fewer cells become problematic as the success of the stimulation largely depends upon the degree of innervation throughout the culture. Furthermore, coverslips that have inconsistent distribution of neurons in various parts of the coverslip also present an issue since cells from different regions of the coverslip might be subjected to incomparable degrees of innervation, thus leading to high variability within a treatment group from the same experiment. As a result of those coverslips that have only a small population of viable cells, the neurons from the groups generally appear to have very few glutamate receptor puncta.

Although the degree of innervation throughout the culture is one possible source of variability in the protein expression in dendrites, another cause is from the immunocytochemical analysis itself as a result of the difficulty in protecting the light-sensitive immunostainings from photo-bleaching. Therefore, prolonged exposure to light during imaging leads to the loss of

fluorescence, which can ultimately cause a large percentage of protein puncta to fluoresce at levels below the threshold. In general, there are many more AMPA receptors expressed on the surface of mature neurons as compared to NMDA receptors, and thus the immunofluorescence signals for GluR1 would be brighter and more stable than for an individual NMDA receptor subunit. Perhaps, this is one reason why we were able to detect the established effects on GluR1 surface expression, while our results regarding the NMDA receptors subunits were highly variable. Despite the sensitive nature of immunocytochemistry experiments, they allow us to look at endogenous proteins, which is critical for understanding protein expression. The use of recombinant protein analyses is useful in conjunction to immunocytochemistry, but alone, it is not indicative of how the native proteins are regulated.

Future Directions

To overcome the aforementioned caveats, we can improve upon our methods in some of the ways that are described below.

In our experiments, we were able to refine our analysis between aim 1 and aim 2 by using a different method of image processing and by collecting additional parameters other than mean intensity. In the current experiments with results presented here, each cell consists of only measurements from a single dendrite of approximate 20 μm in length. To improve the measurements for future experiments, we can lengthen the area of the dendrite that is analyzed to include the majority of that dendrite or to measure several regions or even multiple dendrites from the same cell and averaging the measured values to increase the amount of data collected. By averaging the measured variables across several regions or multiple dendrites from the same

cell, we can probably reduce the variability that we observe across different neurons for a certain treatment group.

While we can change the way that measurements are done for future experiments, we can also gather results and analyze immunocytochemical data from only within the dendritic spines as spines are known to be the postsynaptic compartment at glutamatergic synapses and will always contain glutamate receptors in the membrane. This way, we can further minimize the probability of quantifying background fluorescence and will isolate our analysis to the postsynaptic sites as opposed to the whole dendritic region, thus reducing the high degree of variability observed in the data.

Additionally, we can also use an independent method to analyze the surface expression of NMDA receptors to provide possibly more conclusive validations of our immunocytochemistry results. One potential experiment would be to use a biochemical means to measure the surface expression, such as a surface protein biotinylation assay. In these experiments, the surface proteins would be precipitated from cell lysates and the levels would be analyzed by western blot. While this approach allows us to bypass any issues associated with imaging fluorescent molecules, it does not allow us to examine the surface expression specifically in distal dendritic regions as whole neuron lysates would be used. To analyze protein expression in distal dendrites without the use of antibodies, we could use fluorescence imaging of recombinant protein to study the role of protein synthesis and specifically the CPE sequence in activity-induced NR2A membrane insertion. We plan to transfect hippocampal neurons with a plasmid encoding NR2A fused to a modified green fluorescent protein (SEP; super elliptic pHluorin). Because SEP

fluorescence is pH-sensitive, it will be only be fluorescent when expressed on the cell surface and not internally, thus allowing specific detection of the surface population of recombinant NR2A protein. We can analyze fluorescence signal following chemical LTP stimulation in the presence of anisomycin to examine the role of protein synthesis in this process. To determine if the CPE sequence regulates NR2A levels or NMDAR surface expression, we can use a plasmid that contains the coding region of SEP-NR2A mRNA flanked by the 5' UTR and a portion of the 3' UTR of NR2A mRNA. Recombinant NR2A expression can be compared to plasmids having the wild type 3' UTR sequence or the 3' UTR with a mutated CPE sequence. Moreover, to investigate whether CPE-mediated regulation of NR2A translation regulates total NMDAR surface expression, hippocampal neurons can be treated as described earlier and immunostained for surface NR1. Using a combination of these independent techniques will provide a means for thoroughly evaluating NMDAR surface levels.

The key findings of this study are that glutamate-induced synthesis of CaMKIIα is regulated by Gld2, and that glutamate induces the synthesis of the NR2A of NMDA receptors. These studies have spurred many additional experiments performed by other members of the Bassell lab in parallel to those described in this thesis. For instance, the Bassell lab has shown that the chemical LTP paradigm, tested as part of this thesis, indeed activates the CPEB-associated complex in dendrites and induces a Gld2- and protein synthesis-dependent increase in the dendritic expression of NR2A protein (unpublished data). Although we cannot yet conclude whether surface NR2A is differentially expressed following our chemical LTP paradigm as a result of the high variability in our data, the finding that NR2A protein expression is increased by this stimulation is a promising result that stemmed from the initial work done in this thesis. By

completing additional immunofluorescence experiments and also using biochemistry and recombinant protein analyses, we can further evaluate the results observed in the current study and determine unequivocally whether protein synthesis regulates chemical LTP induced surface expression of NMDA receptors.

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Figures

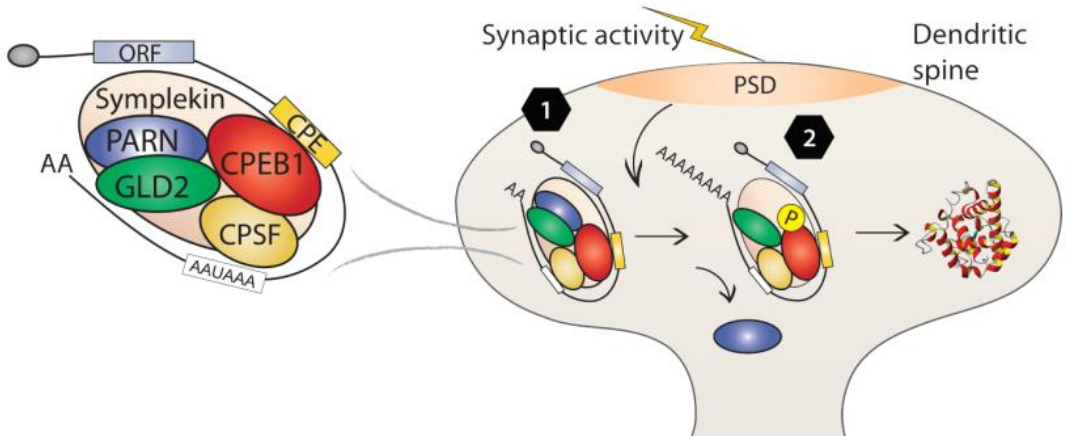

Figure 1. Model for the role of the CPEB1 complex in synaptic mRNA translation. (1) CPEB1 is part of a multi-protein synaptic complex that bidirectionally regulates polyadenylation of CPE-containing mRNAs. The complex contains CPEB1, the scaffolding protein Symplekin, the poly(A) polymerase Gld2, and poly(A) ribonuclease PARN. (2) Synaptic activity stimulates CPEB1 phosphorylation and extrusion of PARN from the complex, allowing poly(A) tail elongation by Gld2 and facilitating translation initiation at synapses.

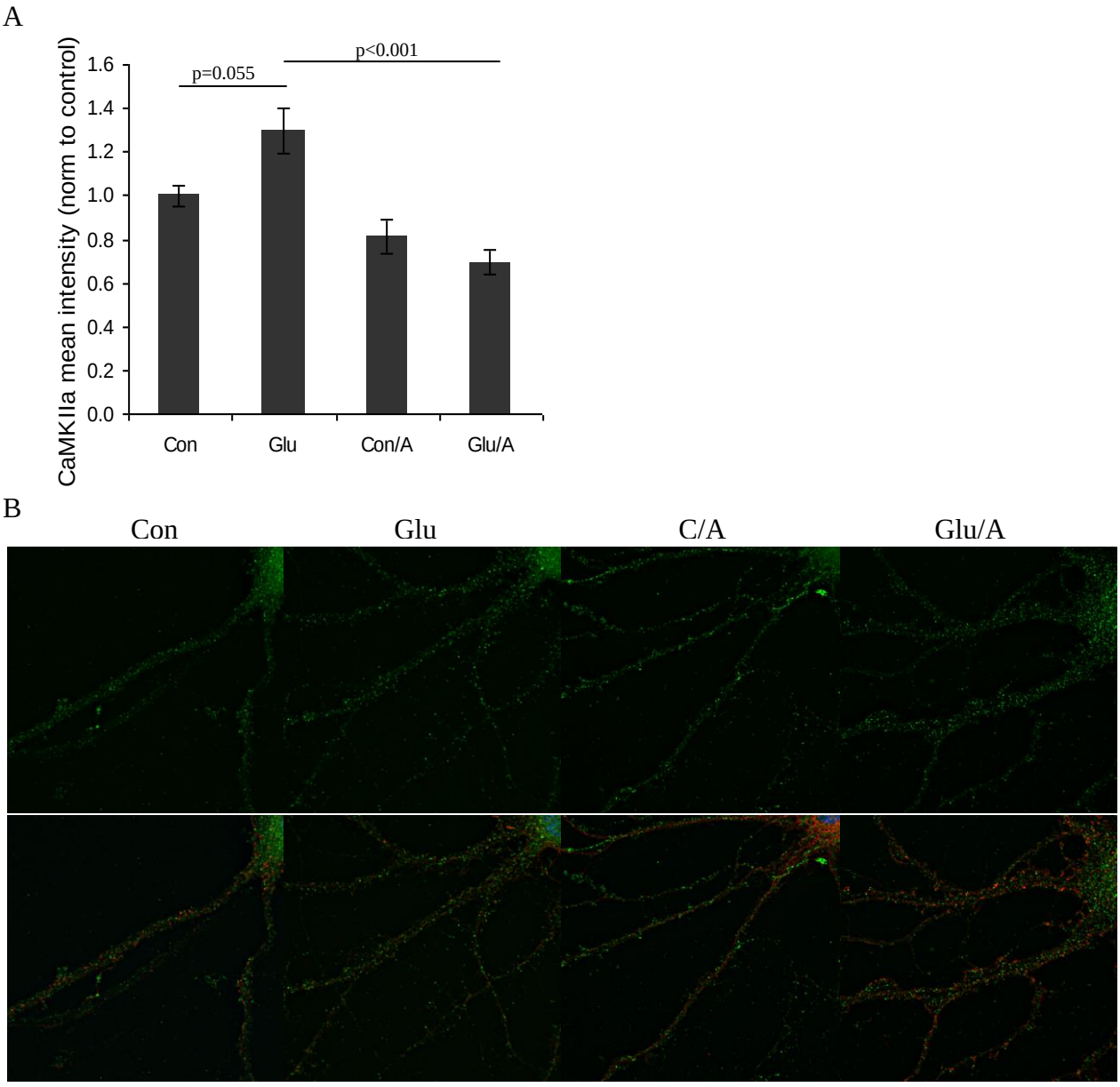

Figure 2. CaMKIIα mean intensity quantified in neurons that were treated with anisomycin (A) or DMSO as the protein synthesis control and 10 μM glutamate (Glu) or water as the stimulation control (Con). A. Glutamate treated cells exhibited a marginal increase in CaMKIIα mean intensity over the control (MWW, $p = 0.055$; $n = 28/27$ cells, 2 experiments), while there was a significant difference between the glutamate treated cells and the cells treated with glutamate plus anisomycin (MWW, $p < 0.001$; $n = 28/27$ cells, 2 experiments). B. Cells were immunolabeled for CaMKIIα (green), actin, (red), and DAPI stained for nuclear DNA (blue).

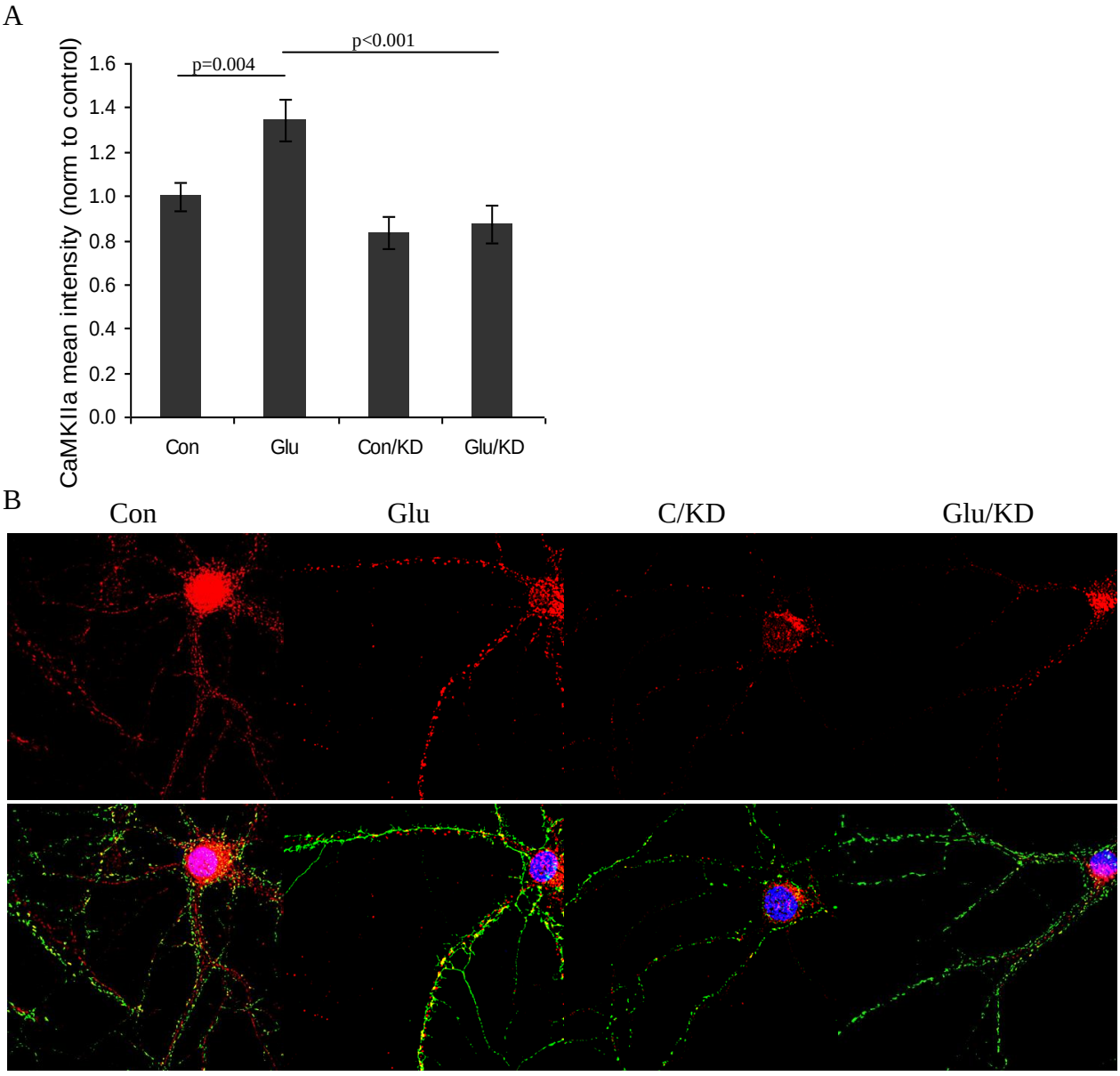

Figure 3. CaMKIIα mean intensity quantified in neurons transfected with Gld2 shRNA-expressing lentivirus (KD) or empty lentivirus as the transfection control and 10 μM glutamate (Glu) or water as the stimulation control (Con). A. Glutamate treated neurons transfected with the empty lentivirus exhibited an increase in CaMKIIα mean intensity over the controls (one-way ANOVA w/ Tukey HSD post hoc, $p = 0.004$; $n = 41/37$ cells, 2 experiments). There was also a significant difference between the glutamate treated cells and the Gld2 knockdown cells treated with glutamate (one-way ANOVA w/ Tukey HSD post hoc, $p < 0.001$; $n = 41/36$ cells, 2 experiments). B. Cells were immunolabeled for CaMKIIα (red), synapsin (green), and DAPI stained for nuclear DNA (blue).

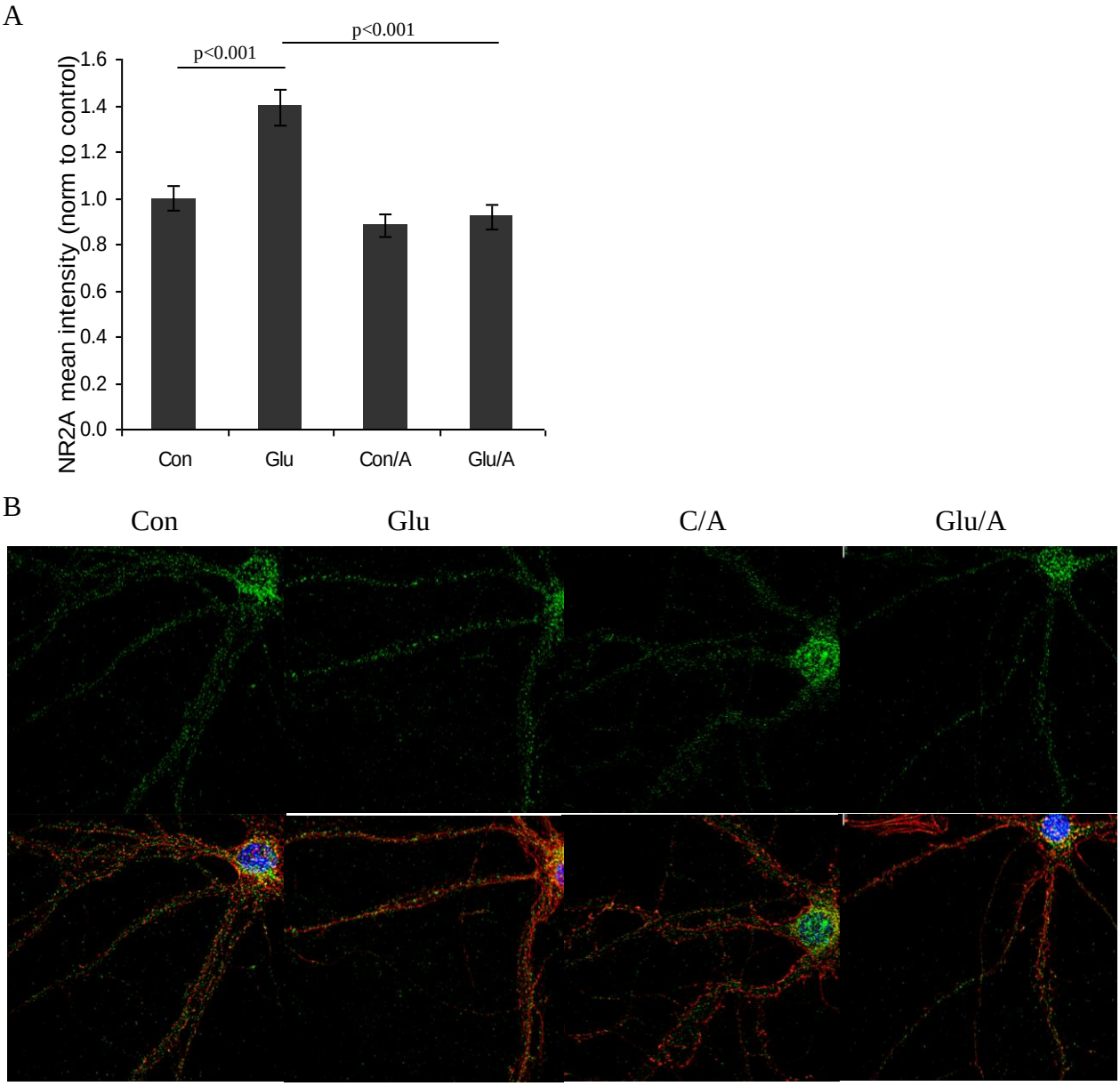

Figure 4. NR2A mean intensity quantified in neurons treated with anisomycin (A) or DMSO as the protein synthesis control and 10 μM glutamate (Glu) or water as the stimulation control (Con). A. Glutamate treated cells exhibited a significant increase in NR2A mean intensity over the control (MWW, $p < 0.001$; n = 29/29 cells, 2 experiments). Moreover, there was also a significant difference between the glutamate treated cells and the cells treated with glutamate plus anisomycin (MWW, $p < 0.001$; n = 29/29 cells, 2 experiments). B. Cells were immunolabeled for NR2A (green), actin, (red), and DAPI stained for nuclear DNA (blue).

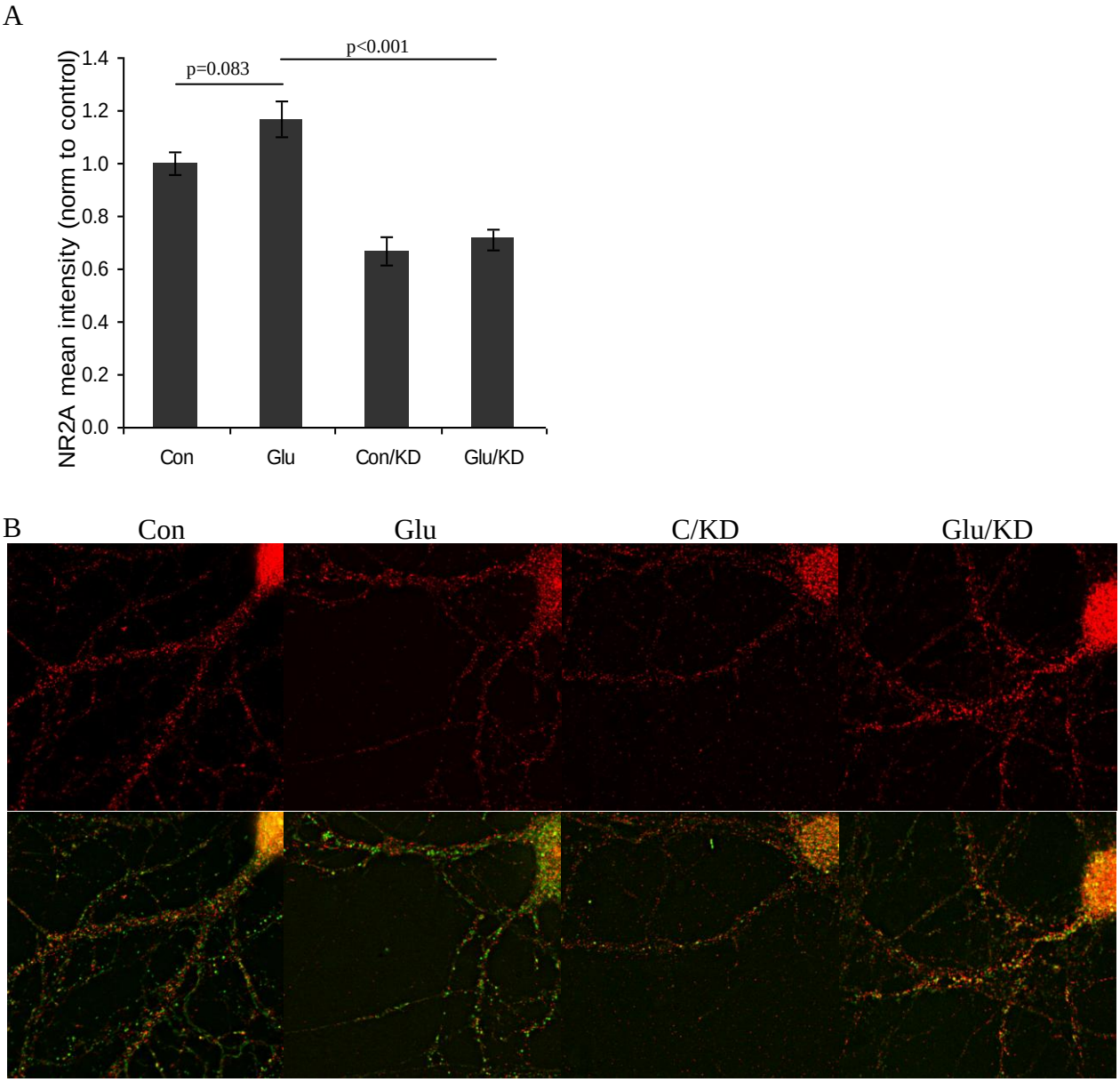

Figure 5. NR2A mean intensity quantified in neurons transfected with Gld2 shRNA-expressing lentivirus (KD) or empty lentivirus as the transfection control and 10 μM glutamate (Glu) or water as the stimulation control (Con). A. Glutamate treated neurons transfected with the empty lentivirus exhibited a marginal increase in NR2A mean intensity over the control (one-way ANOVA w/ Tukey HSD post hoc, $p = 0.083$; $n = 28/26$ cells, 2 experiments), while there was a significant difference between the glutamate treated cells and the Gld2 knockdown cells treated with glutamate (one-way ANOVA w/ Tukey HSD post hoc, $p < 0.001$; $n = 28/27$ cells, 2 experiments). B. Cells were immunolabeled for NR2A (red) and synapsin (green).

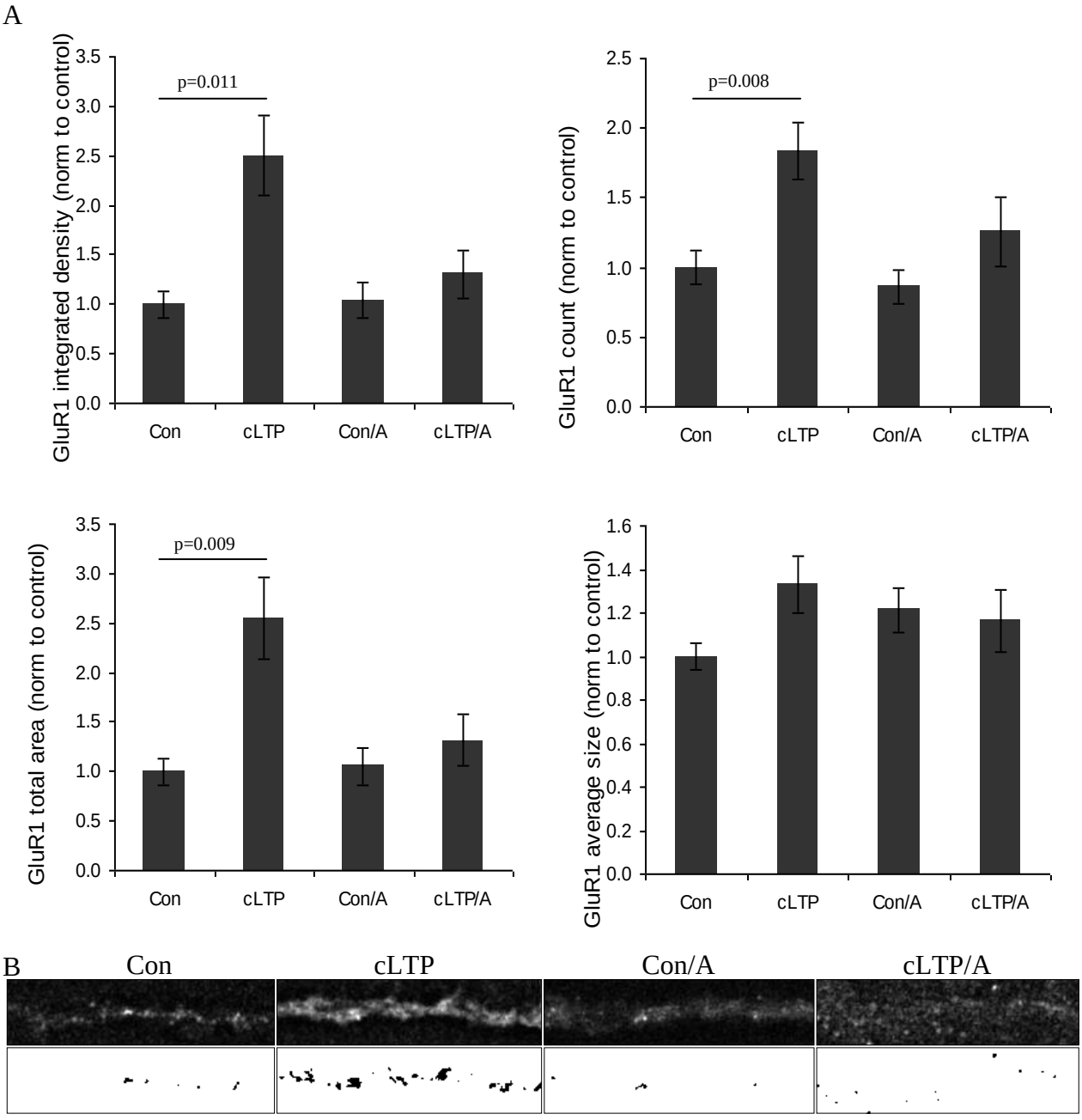

Figure 6. Surface GluR1 integrated density, count, total area, and average size quantified in neurons treated with anisomycin or DMSO as the protein synthesis control and cLTP or buffer exchange as the stimulation control. One experiment was excluded. A. cLTP treated cells exhibited a significant increase over the control cells in GluR1 integrated density, count, and total area (one-way ANOVA w/ Tukey HSD, intden: $p = 0.011$; count: $p = 0.008$; total area: $p = 0.009$; $n = 32/31$ cells, 3 experiments). This effect induced by cLTP was eliminated when anisomycin was added. No differences were observed in average size (one-way ANOVA, $p = 0.175$; n – 32/31 cells, 2- 3 experiments). B. Dendrites were immunolabeled for surface GluR1 with granules over threshold.

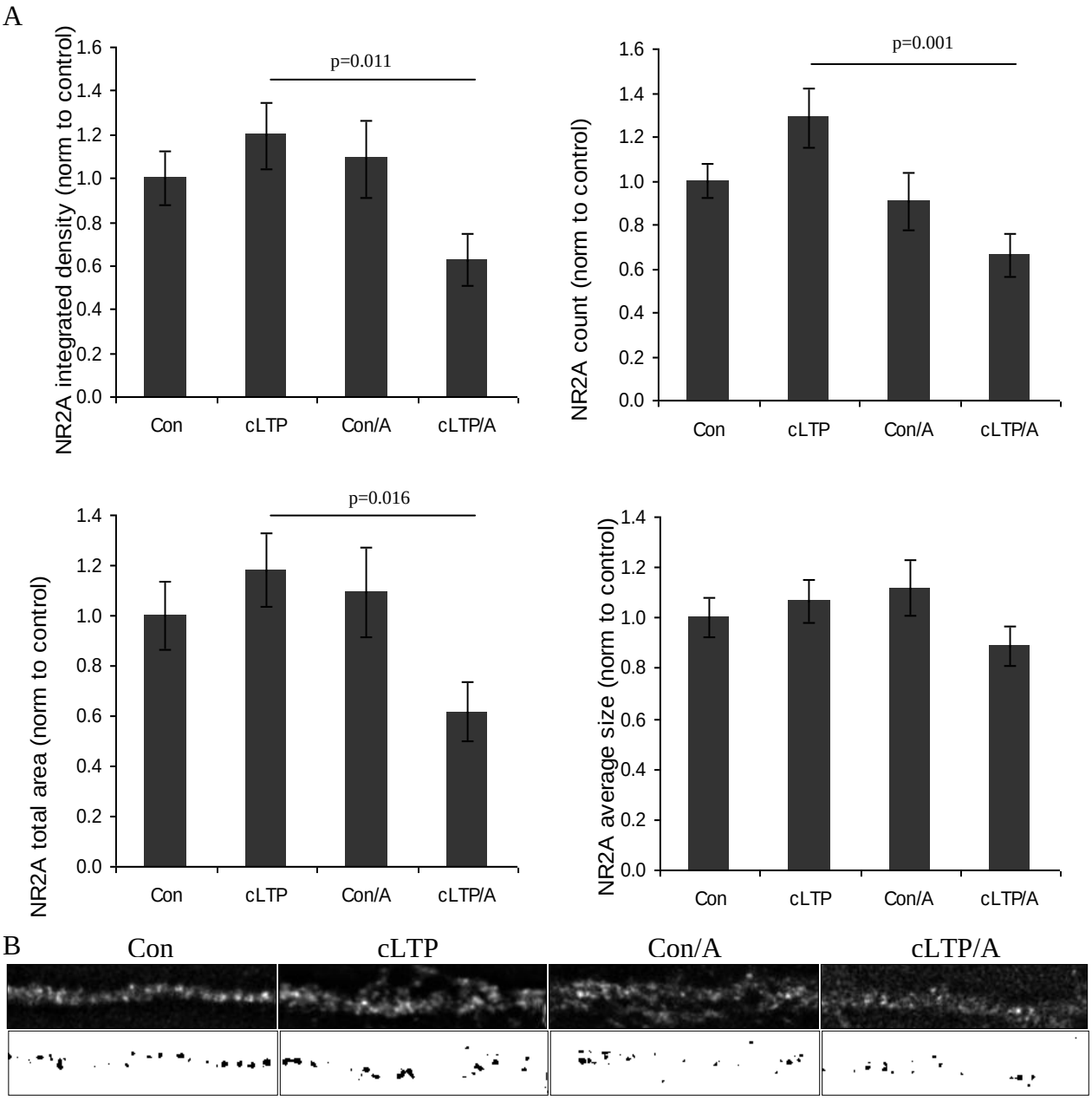

Figure 7. Surface NR2A integrated density, count, total area, and average size quantified in neurons treated with anisomycin or DMSO as the protein synthesis control and cLTP or buffer exchange as the stimulation control. A. cLTP treated cells did not exhibit significant increase over the control cells in NR2A for any of the measurements, but a significant increase was observed over the cLTP and anisomycin treated neurons for 3 of the four variables (one-way ANOVA w/ Tukey HSD, intden: $p = 0.011$; count: $p = 0.001$; total areal: $p = 0.016$; $n = 31/29$ cells, 3 experiments). No differences were observed in average size (Kruskal-Wallis test, $p = 0.599$; n – 31/29 cells, 3 experiments). B. Dendrites were immunolabeled for surface NR2A with granules over threshold intensity.

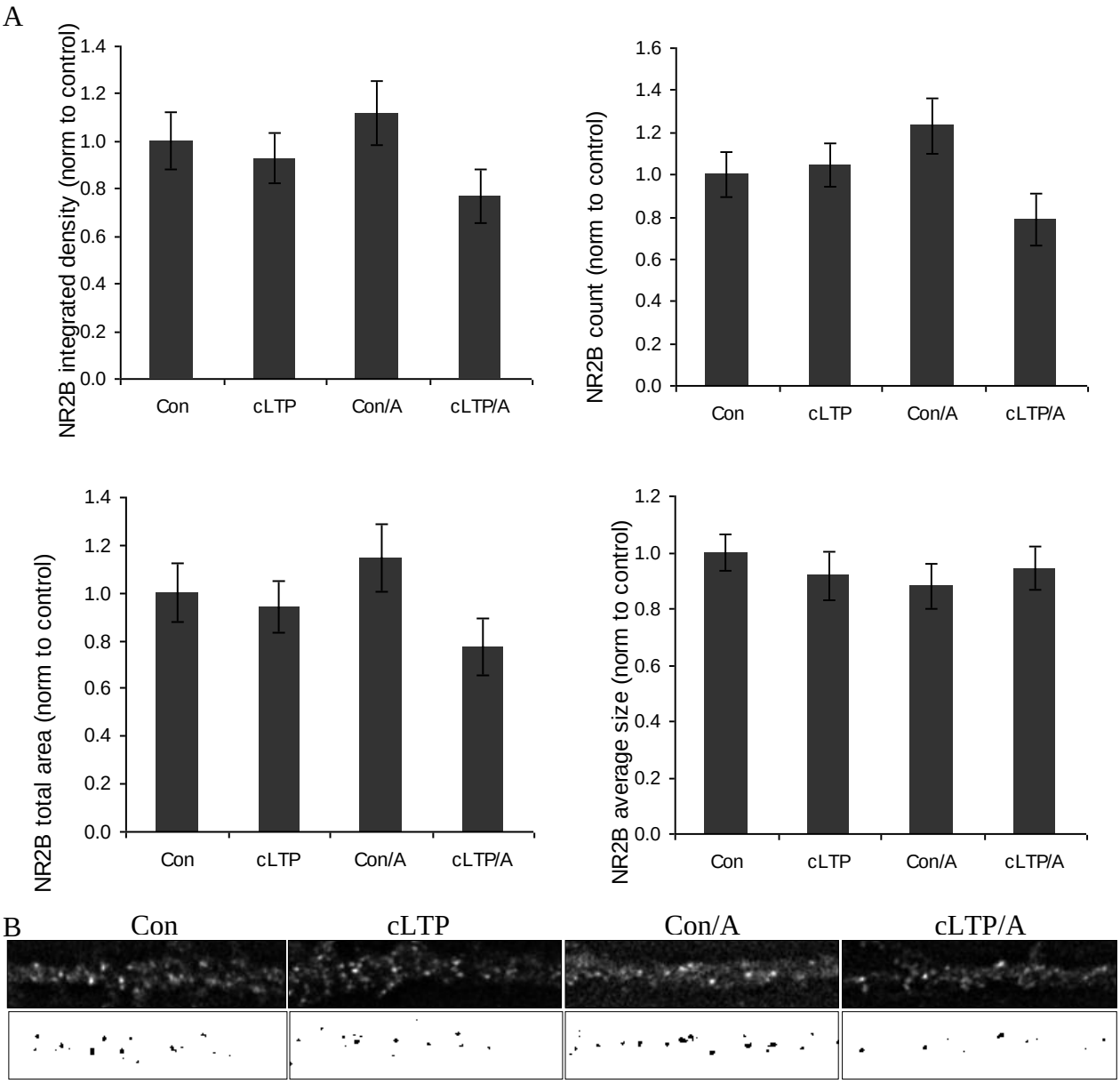

Figure 8. Surface NR2B integrated density, count, total area, and average size quantified in neurons treated with anisomycin or DMSO as the protein synthesis control and cLTP or buffer exchange as the stimulation control. A. No significant differences among the treatment groups were observed for any of the quantifications (one-way ANOVA, intden: $p = 0.323$; count: $p = 0.091$; total areal: $p = 0.295$; Kruskal-Wallis test, average size: $p=0.277$; 19-31 cells, 2-3 experiments). B. Dendrites were immunolabeled for surface NR2B with granules over threshold intensity.

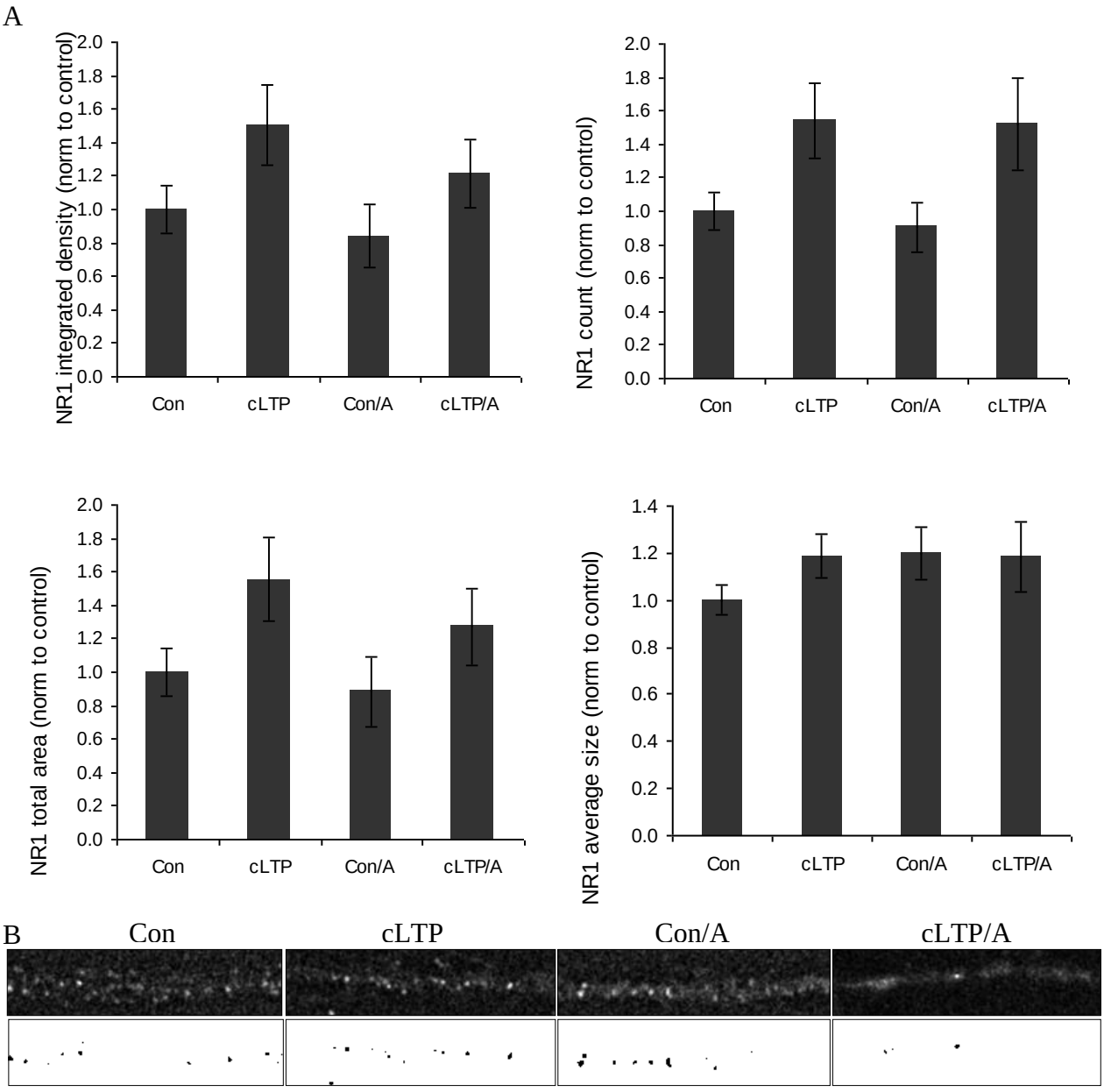

Figure 9. Surface NR1 integrated density, count, total area, and average size quantified in neurons treated with anisomycin or DMSO as the protein synthesis control and cLTP or buffer exchange as the stimulation control. A. No significant differences among the treatment groups were observed for any of the quantifications (one-way ANOVA, intden: $p = 0.129$; count: $p = 0.057$; total areal: $p = 0.132$; average size: $p=0.407$; 20-31 cells, 2-3 experiments). B. Dendrites were immunolabeled for surface NR1 with granules over threshold intensity.