UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Alzheimer's Disease Decreases Surface Glutamate Receptors By Regulating Actin Stabilization

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2012

The thesis of Vishnu Parthasarathy is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

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ABSTRACT OF THE THESIS

Alzheimer's Disease Decreases Surface Glutamate Receptors By Regulating Actin Stabilization

by

Vishnu Parthasarathy

Master of Science in Biology

University of California, San Diego, 2012

Professor Shelley Halpain, Chair

The early stage cognitive deficits in Alzheimer's disease (AD) have been attributed to soluble, oligomeric forms of Amyloid ß (sAß) and its effect on synaptic structure, function glutamate receptor content. Excitatory synapses in the brain mainly reside on dendritic spines. Their synaptic activity involves ionic currents that are mediated by glutamate receptors. Dendritic spines are rich in the cytoskeletal component actin, which mediates spine stability and morphology as well as anchors receptors to the spine surface. We observe that sAß reduces the expression of total and surface glutamate receptors through a mechanism that involves an increase in the activity of cofilin, an actin severing protein. In addition, we show that inhibition of cofilin's F-actin severing activity prevents the decrease in surface glutamate receptor expression witnessed in the presence of sAß. This provides a novel mechanism behind sAß-induced synaptic dysfunction in Alzheimer's Disease.

# I:

# Introduction

Alzheimer's Disease is the most common form of dementia with a prevalence rate of 60-80% in adults over the age of 65 (Plassman et al., 2007). It is characterized by a progressive decline in cognitive and memory functions, which have been attributed to soluble, oligomeric forms of Amyloid-ß (sAß) and its effect on synaptic function (Selkoe et al., 2002). Low-concentrations of cell-derived sAß have been shown to induce changes in dendritic spine stability and morphology (Calabrese et al., 2007; Shankar et al., 2008). Functionally, sAß attenuates synaptic plasticity by inhibiting long-term potentiation (LTP) and enhancing long-term depression (LTD) of glutamatergic transmission (Klyubin et al., 2005, Wei et al., 2007, Venkitaramani et al., 2007) along with inducing memory and learning dysfunction in animal models (Clearly et al., 2005). Although various studies show the effects of sAß on synaptic structure and function, the molecular mechanisms behind these changes remain unknown.

Glutamatergic receptors reside mainly on synapses and are important for synaptic plasticity mechanisms including those underlying LTP and LTD and provide a neuronal basis for learning and memory (Morris et at. 2003). These receptors transmit excitatory signals that produce a depolarizing current in the postsynaptic neuron in response to presynaptic activation. The two main ionotropic glutamate receptors are AMPA and NMDA. AMPARs are the most commonly found receptors in the nervous system. They are heterotetrameric consisting of an obligatory GluA2 subunit with a combination of GluA1, 3 or 4 subunits (Greger I.H. et al., 2007). NMDA receptors are also heterotetrameric complexes and contain two obligatory NR1 subunits with a combination of NR2A-D or NR3 subunits (Cull-Candy S. et al., 2001). These subunits are regulated spatially and developmentally with NR2B receptor subunits being preferentially located

in extrasynaptic regions of the neuron (Stocca et al., 1998, Rumbaugh et al., 1999, Tovar et al., 1999). While the consequences for synaptic plasticity (LTD, LTP, and depotentiation) of the potential differential localization of NR2A- and NR2B-containing NMDARs are not known, these receptors have been shown to traffic across the synapse in response to synaptic activity and also mediate the excitability of excitatory synapses (Shepard J.D. et al., 2007, Newpher T.M. et al., 2008).

Previous research has shown that sAß alters both the distribution and function of these receptors. Using biochemical and in-situ hybridization techniques, Mishizen-Eberez et al. (2004), showed that protein levels and mRNA expression for NR1/2B subunits were significantly reduced, while the NR2A subunit mRNA expression and protein levels were unchanged in the brain. In addition, whole cell patch clamp recordings by Snyder et al. (2005) showed that stimulation of NR1/NR2B receptors with NMDA/glycine revealed a decrease in NMDA-evoked currents in cells pre-exposed to synthetic Aß. This was also confirmed by Cissé et al. (2011) who showed that synthetic Aß decreased surface NR1 concentrations through a mechanism that involved interaction with the EphB2 receptor. Looking at Aß and AMPA receptors, Hsieh et al. (2006) showed that AMPA receptor removal underlies Aß-induced synaptic depression and spine loss.

Glutamate receptor expression at the synapse plays an important role in neuronal plasticity mechanisms like LTP and LTD (Shepard J.D. et al., 2007, Keifer J. et al. 2010) Consistent with the changes in expression levels of glutamate receptor subunits, it is plausible that the synaptic dysfunction seen under sAß could be occurring due to an alteration in glutamate receptor trafficking to and from the synapse. Changes in AMPA receptor trafficking at the synapse of dendritic spines has previously been observed in

response to LTP induction and activation of NMDA receptors. In response to LTP, AMPA receptors traffic to the surface of the spine. (Shi S., et al., 1999). Receptors on the surface of the dendrite are considered functional as they respond to synaptic stimulation. Internalized receptors on the other hand are non-functional and are often located in intracellular pools. Experiments by Snyder et al. (2005) showed that application of synthetic sAß decreases surface levels of NMDA receptors by promoting receptor endocytosis. Glutamate receptor endocytosis under sAß could be an underlying factor behind the inhibition of synaptic plasticity mechanisms like LTP.

It is important, however, to note that many of the studies mentioned above were conducted using synthetic oligomers of sAß. These require at lease a 2-fold magnitude of higher concentrations of Aß as well as longer incubation times. In our experiments, we used pathological concentrations of cell-derived sAß-42 as this more closely represents the endogenous form of Aß found in the Alzheimer's Disease brain (Selkoe et al., 2002). We look at the rapid effects of sAß with 2 hour incubation period to study the early stage effects of sAß on synaptic dysfunction.

While we are aware of some of the effects of sAß on glutamate receptor trafficking across synapses, the molecular mechanisms behind its modulation of the addition or removal of these receptors still remains unclear. We therefore first systematically characterized the surface and total glutamate receptor subunit expression of hippocampal neurons exposed to physiological concentrations of sAß. This is the first study to do a extensive analysis of the effects of sAß on individual glutamate receptor subunits. Our results show that sAß decreases the surface expression of all glutamate receptor subunits while decreasing the total values of the NMDA receptor subunits.

Studies have pointed to changes in actin dynamics as a possible mechanism behind the changes in glutamate receptor density at the synapse. In a review, Derkachet al. (2007) mentions that synaptic strength is correlated with glutamate receptor density, which in-turn is dependent on filamentous actin (F-actin) assembly. Dendritic spines are rich in F-actin and it is plausible that changes in actin dynamics could play a direct role in regulating the addition and internalization of these receptors. We hypothesize that sAß decreases glutamate receptor expression in neurons by decreasing actin stability. Through our experiments, we find that sAß reduces the F-actin concentration of dendritic spines and that these decreases in actin concentration and glutamate receptor expression under sAß occur due to changes in the activation level of cofilin, an F-actin severing molecule. By decreasing cofilin's F-actin severing activity, we not only prevented the decrease in F-actin concentration in dendritic spines of cells incubated with sAß, but also prevented the sAß induced loss of surface glutamate receptors. These findings may provide to a possible drug target for future treatments for Alzheimer's Disease.

# II:

# Results

In our experiments we use hippocampal cultures from rat brains that are 21 days in vitro (DIV), a point at which neurons are mature both in morphology as well as synaptic transmission. Secondly, the sAß-conditioned medium that we incubate our neurons in contains mainly oligomeric forms of Aß that have been shown to correlate directly with synaptic dysfunction. We added pathological concentrations (150 – 200 pM) of sAß to our cultures for only 2 hours prior to fixation since previous experiments by Calabrese et al., (2007) showed that you see extensive dendritic spine loss and changes in spine morphology within this time frame. The natural secreted Aß that we use can be administered at much lower concentrations than synthetic forms of Aß, thereby allowing us to mimic the levels of Aß that Alzheimer's Disease brains might be reasonably exposed to. All experiments reflect the effect of low concentrations on the early effects of sAß.

**sAß reduces surface expression of all AMPA and NMDA glutamate receptor subunit clusters**

To elucidate the effects of sAß on glutamate receptor expression, we first comprehensively characterized the surface expression levels of glutamate receptor subunits. Surface receptors were specifically detected by not permeablizing the membrane before antibody application and by using antibodies that specifically recognized only surface epitopes of glutamate receptor subunits. Dissociated rat hippocampal cultures (21-days in vitro) were transfected with eGFP and incubated with 150-200 pM of sAß 48 hours later for 2 hours. We found that selective staining for surface NR1, NR2A, and NR2B receptor subunits following exposure to sAß showed

statistically significant decreased in the area, integrated intensity and density of surface clusters (Fig 1B-D).

For AMPA receptors, the size, number and fluorescence intensity of surface GluA1 subunit clusters decreased (Fig 1B-D). GluA2 antibody staining revealed a decrease in size and density of the GluA2 clusters in the presence of sAß. However, fluorescence intensity remained unchanged. Our results therefore show that sAß decreases surface values of glutamate receptor subunits.

**sAß reduces total values of NR1, NR2A, and NR2B receptor subunits in dendrites**

Based on this data, we next looked at changes in the total level of glutamate receptor subunits. This data could be compared with surface values and prove useful in determining whether glutamate receptors are endocytosed or degraded with sAß application. Total receptor values were obtained by permeablizing the membrane prior to antibody application. Staining for total receptor subunit values for each individual glutamate receptor subunit we observed that sAß decreased the total values for the amount and concentration of NR1/2A and increased the amount and concentration of NR2B clusters. Soluble Aß also decreased the total density of NR1 and NR2A/B clusters (Fig 2A-C). These data shows that for NR1, NR2A, and NR2B receptor subunits, both the total and surface level cluster numbers decrease in the presence of sAß.

For AMPA receptors, changes in total values for GluA1 receptors did not mimic that of surface values; where the size, fluorescence intensity, and number of clusters remain unchanged in the presence of sAß (Fig 2A-C). Staining for the GluA2 receptor subunit showed a decrease in amount and concentration, but failed to show any decrease

in the number of clusters with sAß application. However, sAß did decrease the size and fluorescence intensity of these clusters. We see that while sAß decreased the surface levels of the GluA1 and GluA2 subunit, it did not affect total cluster population. Due to the extremely short time course of sAß application and low concentration of sAß used, it is plausible that the failure to see a decrease in the total value of the AMPA receptor subunits may be a temporal effect with total values decreasing over a larger time interval. Further experiments with different time courses are required to determine the temporal effect of sAß on total AMPA receptor values.

Lastly, staining for PSD-95, a post synaptic scaffolding protein that binds to NMDA receptors, revealed that PSD-95 area, concentration, and density levels did not change with sAß incubation (Fig 2A-C). This result is consistent with previous findings that loss of PSD-95-eGFP enrichment is not a prerequisite for spine retraction and that PSD-95-eGFP continues to be enriched in the shaft of the dendrite following spine elimination (Woods et al., 2011). Similarly, in this case, sAß induced spine elimination did not alter PSD-95 levels.

**sAß causes a decreases in the F-actin concentration of dendritic spines**

Having characterized the effect of sAß on both the total and surface levels of glutamate subunits in hippocampal cultures, we next conducted experiments to elucidate a mechanism behind the observed decrease in the surface glutamate receptor population with sAß application. We first focused on actin, a cytoskeletal component of dendritic spines that has been previously shown to play a role in stabilizing glutamate receptors on the surface of dendritic spines (Zhao et al., 2001). Neurons were transfected with an

eGFP cell filler and stained with phalloidin, a fungal toxin that binds F-actin. A quantification of the staining in individual dendritic spines of neurons incubated with sAß revealed that sAß decreased the average F-actin concentration of dendritic spines by 20 percent. (Fig 3A).

**LIM Kinase overexpression rescues sAß induced decrease in actin concentration of dendritic spines**

The decrease in F-actin concentration allowed us to determine if actin-regulating factors were behind the synaptic effects induced by sAß. Of the several actin-regulating factors present in dendritic spines, we focused our attention on cofilin, an F-actin severing molecule. Cofilin plays important roles in both actin polymerization and depolymerization. In depolymerization, cofilin severs actin creating more free barbed ends (FBEs) and G-actin/cofilin complexes. When cofilin is phosphorylated, this G-actin/cofilin complex dissociates releasing cofilin and G-actin monomer (Fig 4B). In polymerization, with help of additional proteins, the free barbed ends that cofilin creates by severing actin provide new sites for actin polymerization (Fig 4A). Rust M.B. et al (2010) showed that cofilin is required for proper AMPA receptor (AMPAR) diffusion from extrasynaptic to synaptic regions. In addition, Gu J., et. al., (2010) have shown that cofilin-mediated actin dynamics regulate AMPAR trafficking during synaptic plasticity and increases in cofilin activity upregulates insertion of AMPARs to the spine surface during LTP. With regards to Alzheimer's Disease, Zhao et al., (2006) showed that there are increased levels of cofilin in the AD brain. Shanker et., al (2007) also showed that inhibiting cofilin activation through expression of a constitutively inactive form of cofilin

prevented sAß induced spine loss. Lastly, previous research in our lab has shown that sAß increases the active form of cofilin and alters actin populations by increasing the ratio of dynamic versus stable F-actin in a manner that favors the dynamic pool relative to the stable pool. (data not shown).

In order to modulate actin stability, we decided to decrease cofilin's F-actin severing activity. We did this by overexpressing LIM kinase, an upstream kinase and regulator of cofilin. LIMK phosphorylates cofilin at Ser-3 and thereby inactivates its ability to bind and sever F-actin (Lin et al., 2010). Neurons were transfected with 6ug eGFP and 6ug LIMK prior to a 2 hour sAß application and fixation and were stained with phalloidin. To assess the effect of LIMK overexpression on actin concentration levels in dendritic spines, we quantified the levels of phalloidin staining in individual dendritic spines. LIMK overexpression prevented the sAß induced decrease in actin concentration of dendritic spines, pointing to an active role of LIMK in preventing loss of actin in the presence of sAß (Fig 3B). It should be noted however that LIMK overexpression significantly increased the baseline control concentration for actin in dendritic spines. This suggests the possible role of other pathways in regulating actin content in dendritic spines under sAß. Further experiments will be needed to assess these additional pathways.

**LIM Kinase overexpression prevents sAß induced decrease in surface NMDA receptor subunits**

Having observed the effect of LIMK in preventing the sAß-induced decrease in actin concentration of dendritic spine, we therefore tested whether overexpression of

LIMK prevented the sAß induced decrease in the surface levels of the NMDA receptor subunit clusters as well. We focused this study on NMDA receptors NR1 staining showed the most prominent changes in receptor expression with sAß. LIMK was overexpressed in dissociated hippocampal cultures prior to sAß exposure. In the absence of LIMK overexpression, we observed a significant decrease in the density of the surface levels of the NR1, NR2B, and NR2A subunits in the presence of sAß (Fig 5 A, B, C). However, when cofilin activity was suppressed by increased LIMK expression, sAß application failed to induce any changes to the density of all surface NMDA receptor subunits (Fig 5 A, B, C). These data indicated that cofilin activity induced by sAß may underlie the loss of surface NMDA receptors.

A.

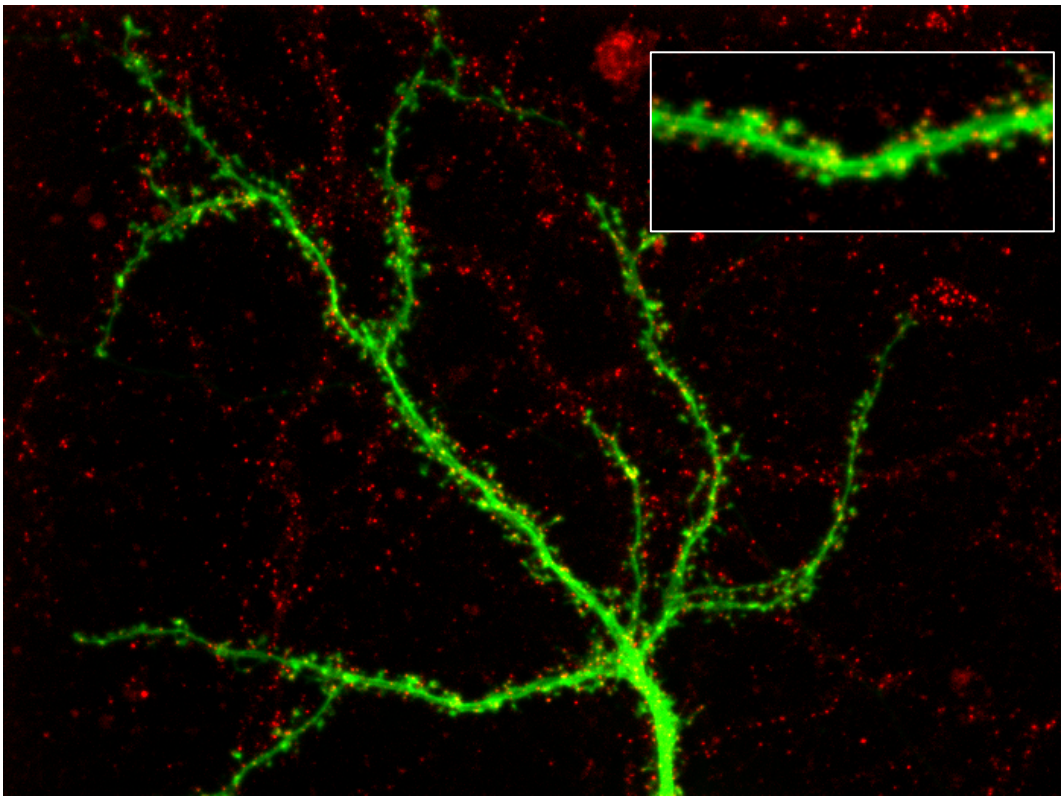
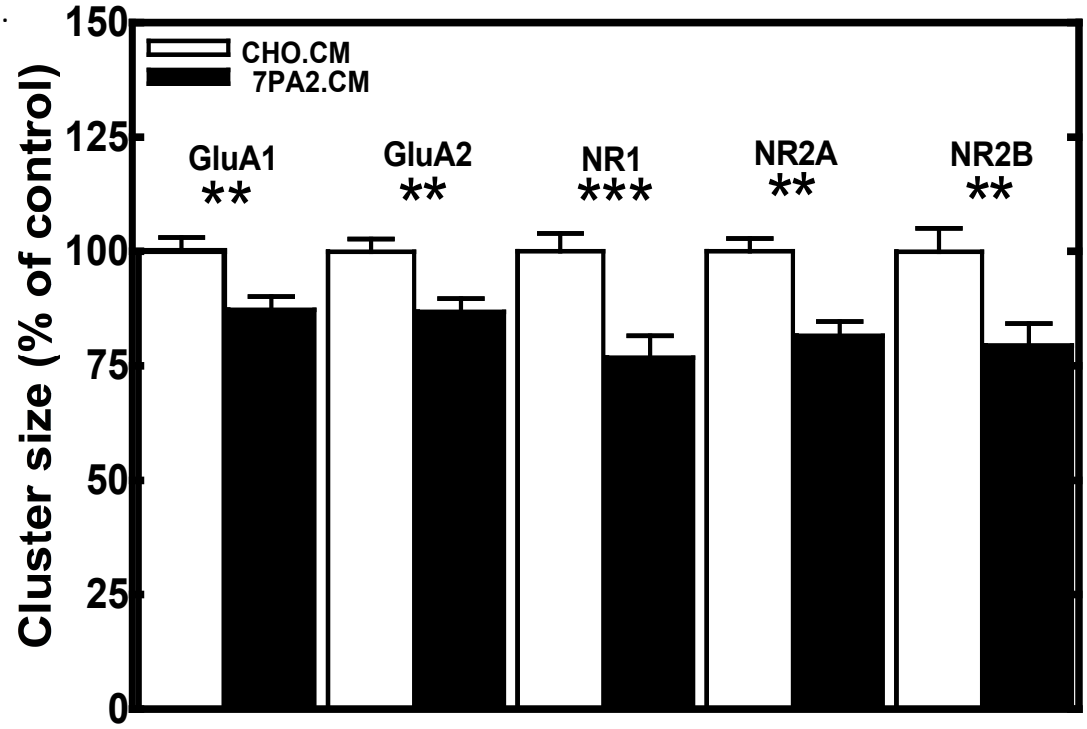


Figure 1. sAß decreases size, intensity, and number of surface glutamate receptor subunit clusters.

(A) Representative picture of a hippocampal neuron transfected with eGFP (green) as cell filler and immunostained for the NR1 subunit (red). Inset shows a close up of NR1 Clusters

(B,C,D) Quantification of area (B), fluorescence intensity (C), and cluster density (D) of surface GluR1, GluR2, NR1, NR2A, and NR2B receptor clusters in cells incubated with control (CHO.CM) or sAß (7PA2.CM-150 pM) medium for 2 hours. Immunofluorescence was determined on thresholded images using a routine in ImageJ.

B.

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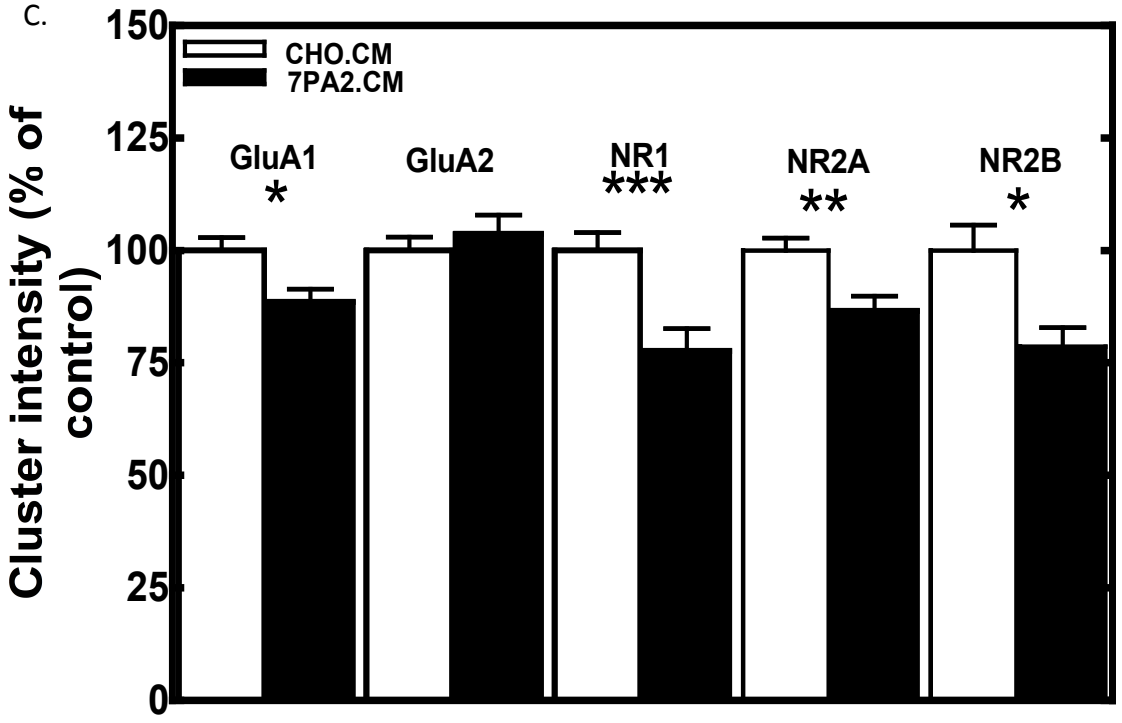



Figure 1 continued.

D.

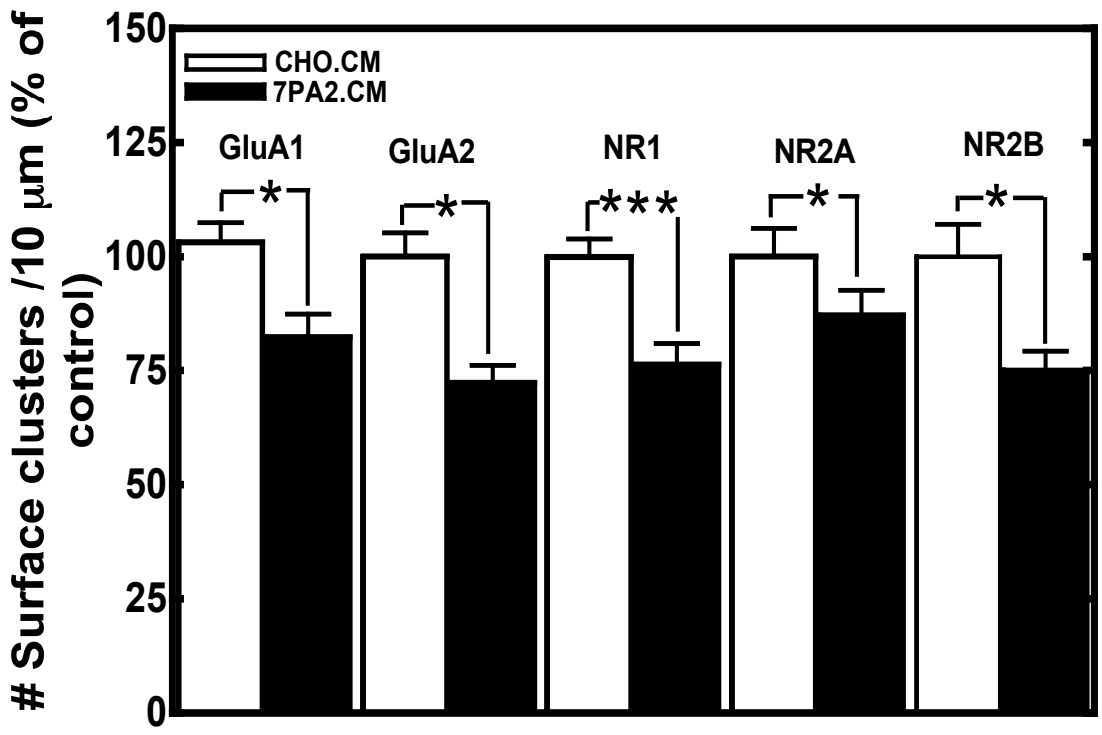



Figure 1 continued.

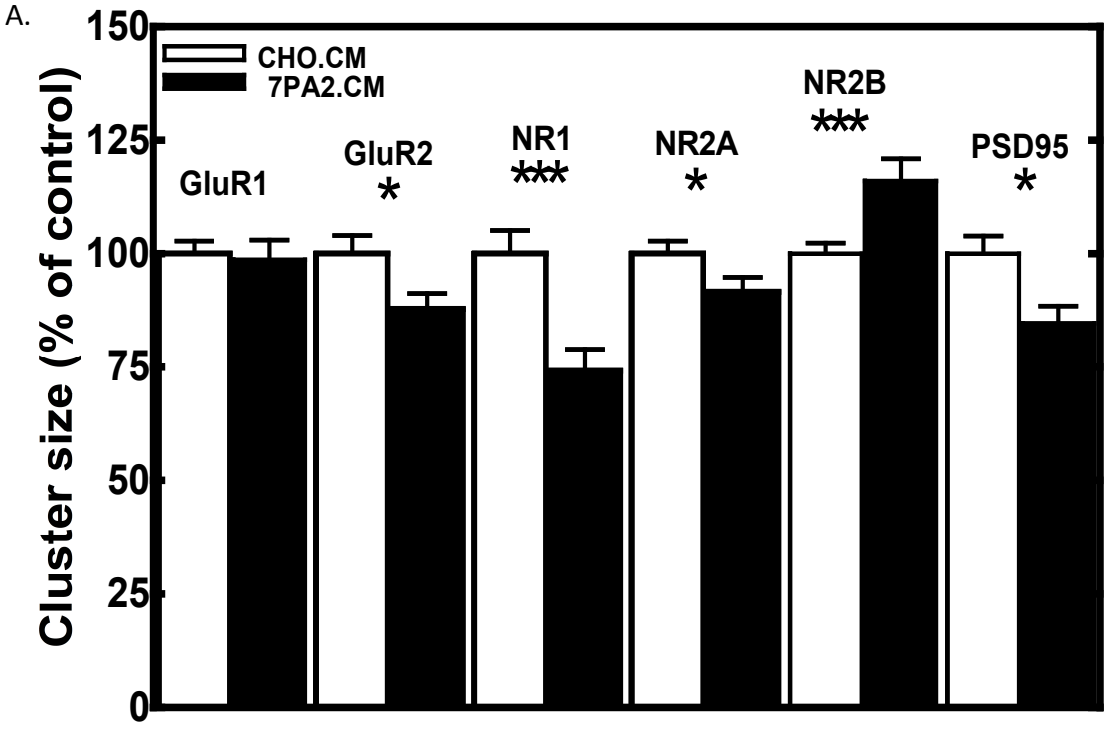
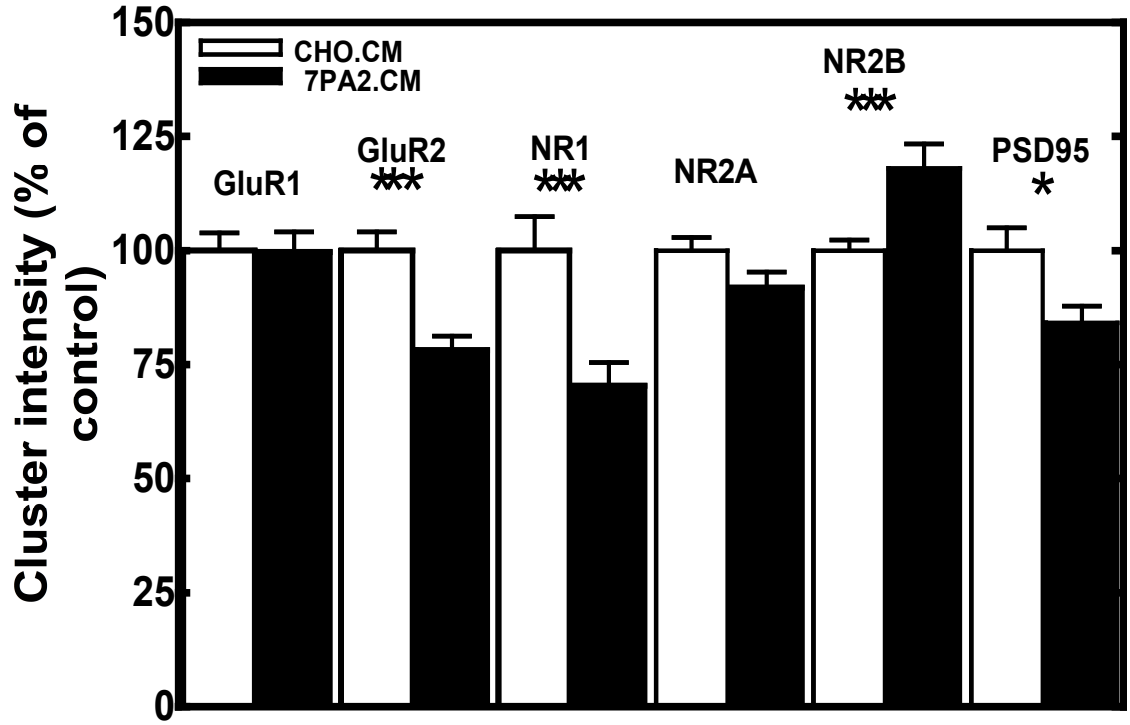



Figure 2. sAß decreases total NMDA subunit clusters

(A,B,C) Quantification of area (A), fluorescence intensity (B), and cluster density (C) of total GluR1, GluR2, NR1, NR2A, NR2B and PSD-95 clusters in cells incubated with control (CHO.CM) or sAß (7PA2.CM-150 pM) medium for 2 hours. Immunofluorescence was determined on thresholded images using a routine in ImageJ.

B.

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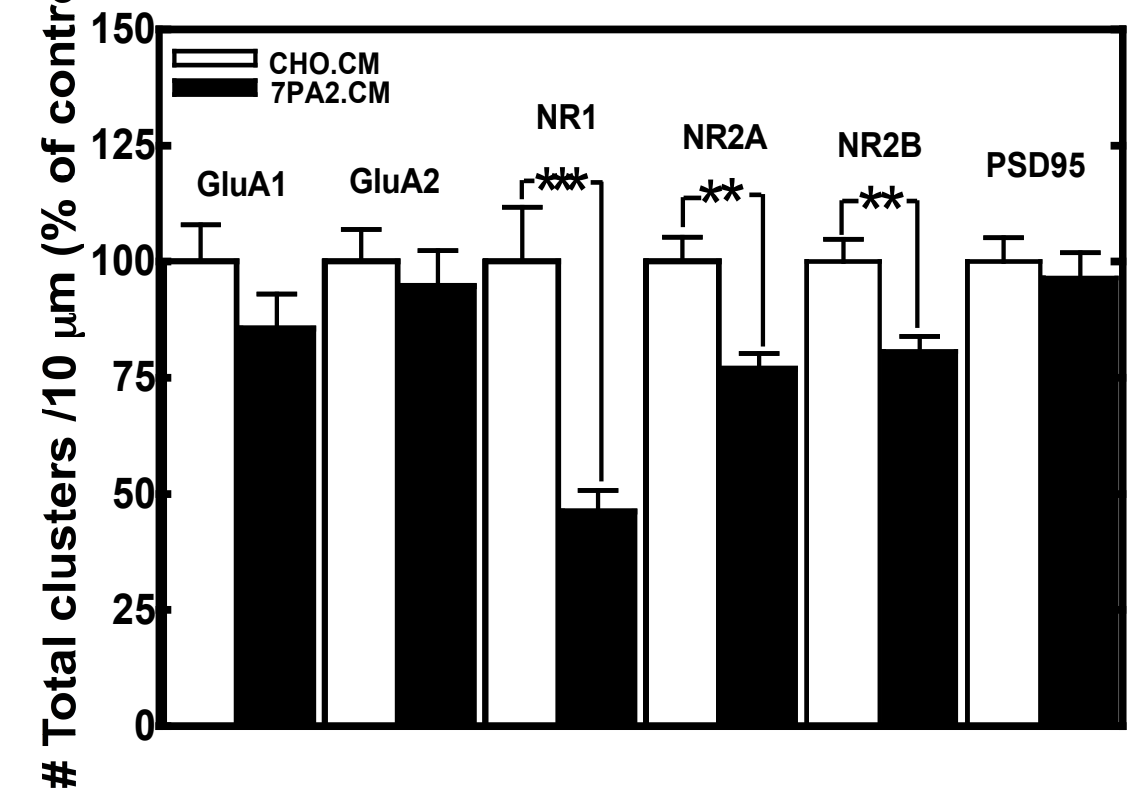



Figure 2 continued.

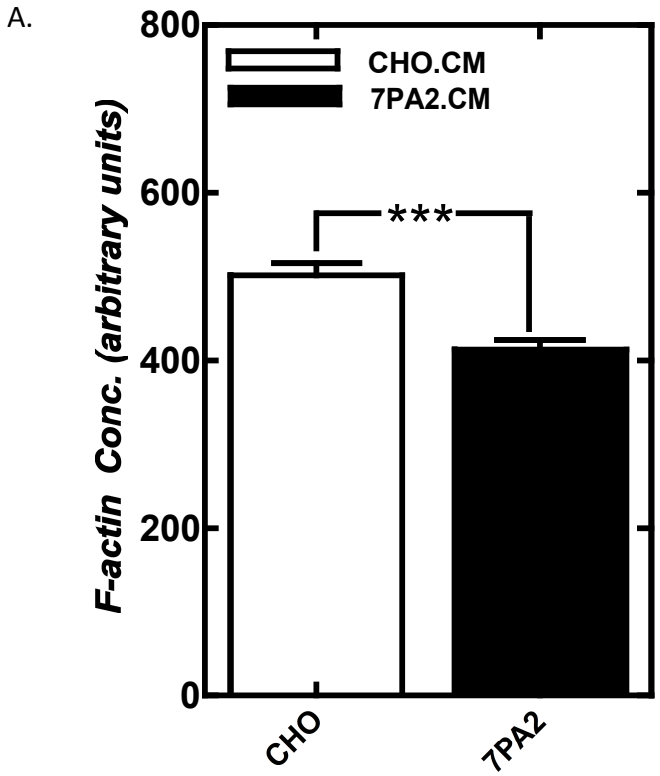
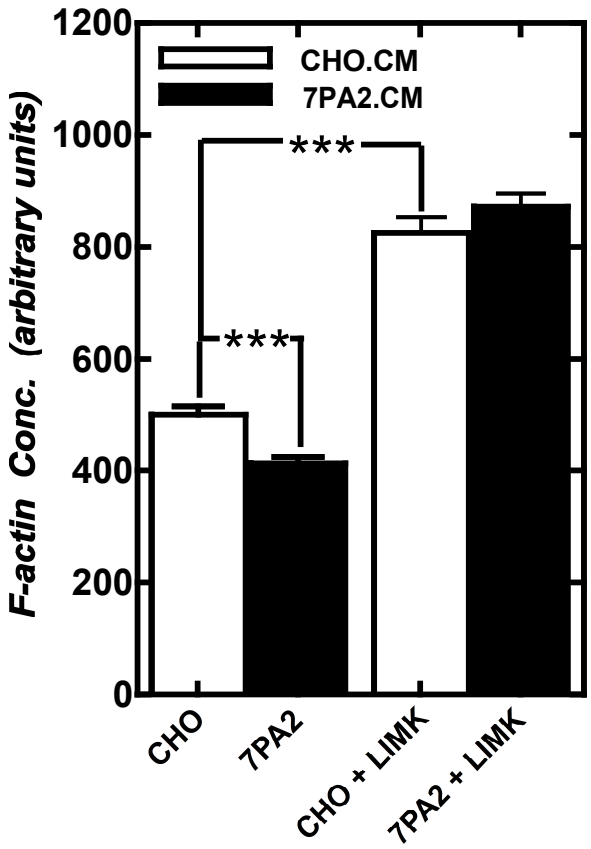



Figure 3. sAß decreases F-actin concentration which is prevented by LIMK overexpression

(A) Quantification of phalloidin fluorescence intensity over area of dendritic spines from neurons incubated with control (CHO.CM) or sAß (7PA2.CM-150 pM) medium for 2 hours. Immunofluorescence was determined on thresholded images using a routine in ImageJ. F-actin concentration decreases with sAß application

(B) Quantification of phallodin fluorescence intensity over area of dendritic spines from control neurons and neurons with LIMK overexpression with control (CHO.CM) or sAß (7PA2.CM-150 pM). LIMK overexpression prevented sAß-induced decrease in F-actin concentration

B.

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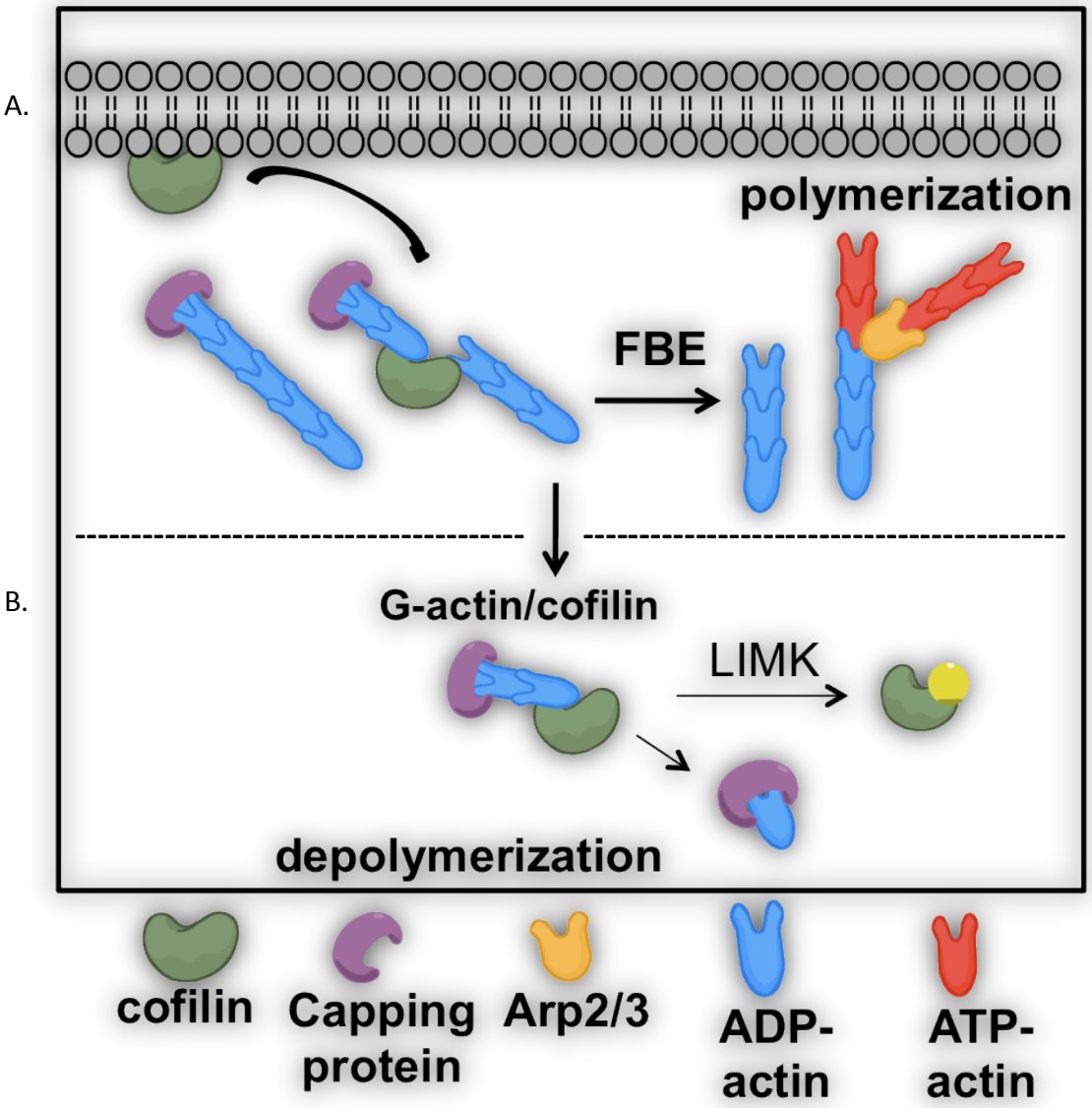



Figure 4: A model of cofilin activity modified from Oser et al., (2009)

(A) In polymerization, with help of additional proteins, the free barbed ends that cofilin creates by severing actin provide new sites for actin polymerization.

(B) In Depolymerization, cofilin severs actin creating more Free Barbed Ends (FBEs) and G-actin/cofilin complex. When cofilin is phosphorylated this complex dissociates releasing cofilin and g-actin monomers.

A.

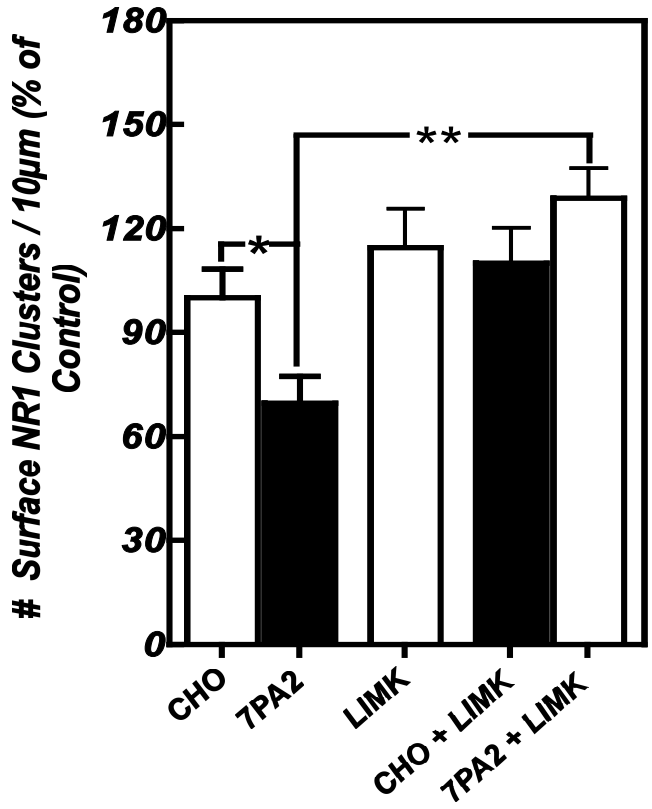
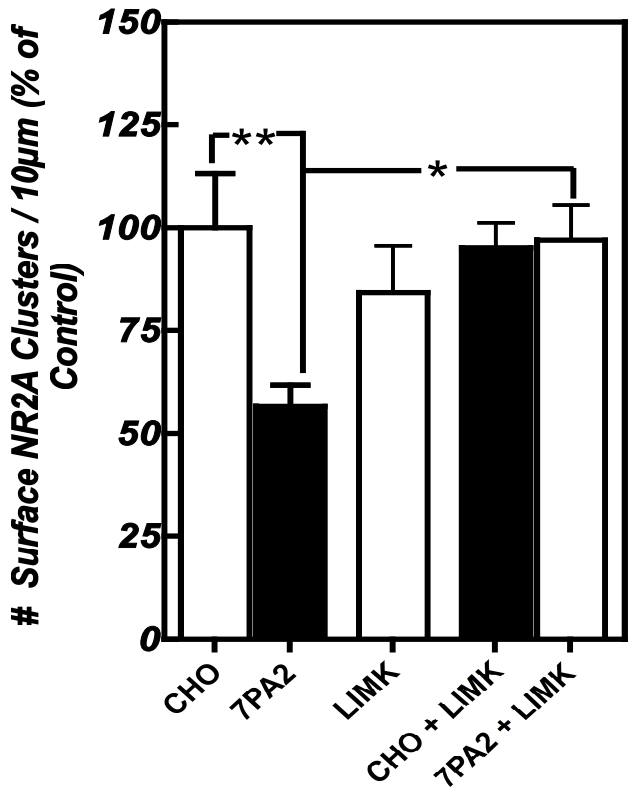



Figure 5. LIMK Overexpresession prevents sAß induced decrease in the density of surface NMDA receptor subunit clusters.

(A,B,C) Quantification of density on clusters of surface NR1 (A), NR2A (B) and NR2B (C) receptor subunits from neurons transfected with eGFP (cell filler) and LIM Kinase prior to incubation in control (CHO.CM) or sAb (7PA2.CM-150 pM) medium for 2 hours. Density of thresholded clusters containing NR1 fluorescence was calculated using routines in ImageJ and NeuronJ.

B.

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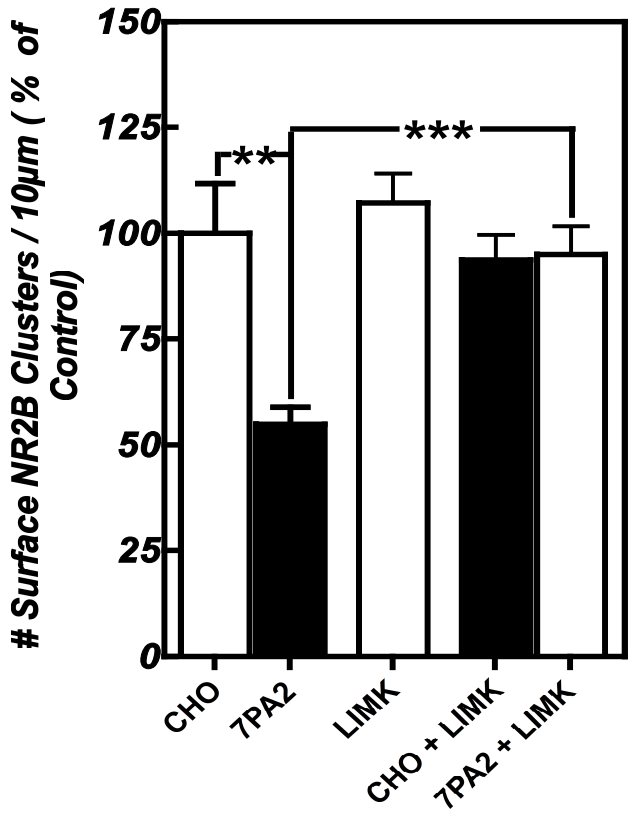



Figure 5 continued.

# III:

# Discussion

This study is aimed to comprehensively assess the short-term effect of physiological concentrations of cell derived sAß on expression levels of glutamate receptor subunits. I found that both surface AMPA and NMDA receptor subunits were reduced significantly in the presence of sAß. Additionally, this decrease in glutamate receptor expression was coincident with a decrease in F-actin concentration in dendritic spines. These effects were prevented by LIMK overexpression, suggesting that sAß decreases glutamate receptor expression on dendritic spines by altering actin stability.

**Cell derived sAß decreases surface glutamate receptors**

Numerous studies indicate that Aβ peptides are important for initiating the pathogenesis of Alzheimer's disease (Walsh et al., 2004). While the mechanisms by which this occurs are not known, experiments with the application of synthetic forms of Amyloid ß have indicated that decreased glutamate receptor expression levels could be responsible for the memory impairments seen in the early stages of Alzheimer's disease (Mishizen-Eberez et al. (2004), Snyder et al. (2005)). However, for synthetic peptides to have an effect, they require much higher concentrations of Aß along with longer incubation times. In our experiments, to more closely mimic *in vivo* conditions, we chose to use a soluble cell derived form of Amyloid ß and study its effects on surface and total glutamate receptors. The focus on surface receptors is based on the accepted assumption that only surface receptors participate in synaptic transmission. Staining dissociated hippocampal neurons for specific AMPA and NMDA surface receptor subunits after a short two hour incubation period with sAß, we observed a significant decrease in expression levels in all surface glutamate receptor subunits in comparison to those in

neurons incubated in a control medium. This data is consistent with previous experiments performed with synthetic forms of Aß.

However, it is important to note that only total levels of NMDA receptor subunits decreased in the presence of sAß, while leaving the total AMPA subunit expression levels unchanged. This different effect of sAß on total levels of AMPA versus NMDA receptors could reflect a difference in the way receptors are regulated when endocytosed from the synapse. Due to the short incubation period with sAß prior to fixation, we may be failing to see a change in the total levels of AMPA receptors due to a possibly larger time course required for the endocytosis of AMPA receptors compared to their NMDA receptor counterparts. On the other hand, AMPA receptors may remain intact when they are endocytosed into the cytosol, allowing antibodies that permeate the membrane to bind them, while NMDA receptors are immediately degraded following a sAß-induced endocytosis.

It is also important to keep in mind that because the experiments thus far are conducted on fixed samples, they do not directly propose a mechanism by which glutamate receptors disappear from the surface of dendritic spines. Other studies suggest NMDA receptors are in fact endocytosed (Snyder et al., 2005). Further experiments will have to be conducted to elucidate the mechanism behind the mechanism of glutamate receptor removal from the synapse.

**sAß decreases NMDA receptor expression by modulating F-actin stabilization**

Based on F-actin presence in dendritic spines, we hypothesized that sAß decreased glutamate receptor expression by modulating F-actin stability. We observed

that sAß not only affected glutamate receptor expression levels, but also significantly decreased F-actin concentration levels in dendritic spines. We found that inactivation of cofilin prevented both the decrease in glutamate receptor expression and actin concentration induced by sAß. Overexpression of LIMK, an upstream kinase that phosphorylates and inactivates cofilin, not only prevented the sAß induced decrease actin concentration of dendritic spines, but also prevented NMDA receptor subunit expression levels from decreasing. This data points to a possible role of actin stabilization in modulating sAß induced changes in glutamate receptor trafficking across the dendritic spine surface and could provide a possible drug target for Alzheimer's Disease treatments.

**Looking Forward**

In addition to observing the effects of sAß application on glutamate receptor expression and F-actin concentration, we also noticed that sAß induces heterogeneous changes in dendritic spine morphology, causing spines to either shrink, elongate or remain unchanged (Supplementary Fig 1). No study to date has looked at the molecular mechanisms behind these early and varied morphological changes nor tested if they are indicative of synaptic dysfunction, or how they relate to actin regulation. Answers to these questions may help us prevent these changes or even to determine which spines may be resistant to sAß insult. To study this, we first tested the effect of sAß on spine morphology. In preliminary experiments, we looked at how sAß affects spine subtype categorization in hippocampal neurons. By measuring their length, neck width, and head width, we categorized dendritic spines into four different subtypes: mushroom, stubby,

thin, and filiapodia (Supplementary Fig 2A-D). When incubated with sAß for two hours, neurons exhibited a significant decrease in mushroom type spines while the number of thin and filopodia type spines increased (Supplementary Fig 3). Further analysis of sAß's effect on morphology could help us understand whether certain types of spines are more resistant to sAß compared to others.

We would like to begin addressing whether a spine's subtype classification is a determinant of sAß effect on it. Does the initial morphology of the spine determine whether it will shrink, elongate or remain unchanged in the presence of sAß? Is spine resistance to sAß dependent on actin as well? Future experiments will strive to answer whether LIMK overexpression will prevent the changes in spine morphology and subtype observed with sAß. Lastly we hope to tie sAß's effect on glutamate receptor trafficking, actin concentration, and spine morphology into a model that gives us a clear understanding of the pathophysiology of Alzheimer's disease in the hopes of proposing possible treatments to prevent its early stage manifestations.

# IV:

# Materials and Methods

**Preparation of natural Aß**

Natural Aβ was prepared according to Walsh et al. (2002a). Regular CHO cells (control Chinese hamster ovary cells) or CHO cells expressing APP V717F mutation (referred as 7PA2 cells) were grown to confluency. Neurobasal medium (plus B27) was conditioned by incubation for 16 h with the cells, then cleared of cells and debris (200×g, 10 min, 4 °C), flash frozen in aliquots and kept at −80 °C prior to application to neuronal cultures. Total Aβ concentration was established by Enzyme- linked immunosorbent assay (ELISA) for Aβ using monoclonal antibody Ab9 as capture (Levites et al., 2006) and biotin-coupled 4G8 (Signet Labs, Dedham, MA) as reporter. The cells were grown and the media was collected in our lab. The ELISA was performed by the laboratory of Eddie Koo at UCSD.

**Hippocampal culture transfection and Aß application**

Hippocampal cultures were prepared according to Calabrese and Halpain (2005) at a density of 300 cells/$mm^2$ and maintained in neurobasal medium (GIBCO), supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine (Sigma). Neurons were transfected at 21 days in-vitro (DIV) using calcium phosphate precipitation, with 6µg eGFP-N1 or 6µg of eGFP and 6µg LIM Kinase per 1.9$cm^2$ well plate. Cells were incubated with the transfection mixture for 3 h in a 5% $CO_2$ incubator at 37 °C, washed twice with prewarmed HBS solution (in mM: 135 NaCl, 4 KCl, 1 $Na_2HPO_4$, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 20 HEPES, [pH, 7.35]) and replaced with the medium in which they had been growing before transfection. Cells were fixed within 2 days post-transfection after a 2 hour incubation in sAß. An equivalent volume of conditioned

medium from control CHO cells was applied in parallel to control hippocampal cultures.

**Immunocytochemistry**

Cultures were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) plus 120 mM sucrose for 20 min at 37 °C. They were then rinsed three times with PBS and blocked for 30 min with 2% bovine serum albumin (BSA) in PBS to prevent non-specific binding. For surface binding, coverslips were incubated for 1 hour at room temperature with mouse monoclonal anti-NMDAR1 antibody at 1:200 (clone 54.1, Millipore), rabbit polyclonal anti-NR2A at 1:500 (Invitrogen), mouse monoclonal anti-NR2B (Antibodies Incorporated), rabbit polyclonal anti-GluR1 (Calbiochem), or mouse monoclonal anti-GluR2 at 1:500 (Chemicon). This was followed by washing 3x with PBS and then the coverslips were incubated with AlexaFluor-568-conjugated secondary antibody (Molecular Probes) for 45 min at 37 °C. Following rinsing, the coverslips were mounted on to slides for image acquisition. Similar procedure was conducted for total receptors using the following antibodies: mouse polyclonal anti-NR1 at 5ug/ml (Pharmiren), rabbit polyclonal anti-NR2A at 4ug/ml (Millipore), rabbit polyclonal anti-NR2B at 2ug/ml (Millipore), rabbit polyclonal anti-GluA1 at 1:1000 (Millipore), rabbit polyclonal at 5ug/ml (Millipore), and mouse monoclonal anti-PSD95 at 1:100 (Sigma), along with permeablization with 0.02% Triton-X in PBS for 4 minutes prior to blocking.

**Image acquisition**

Fluorescence images were collected using an Olympus Fluoview 500 confocal microscope by sequential illumination using the HeNe Green 543 laser and the HeNe Red

633 laser. Sequential acquisition eliminated bleed-through. A stack of images was acquired in the z dimension at optical slice thickness of 0.4 μm to cover entire neurons, using a 60×1.4 NA Plan APO oil immersion objective.

**Cluster Quantification**

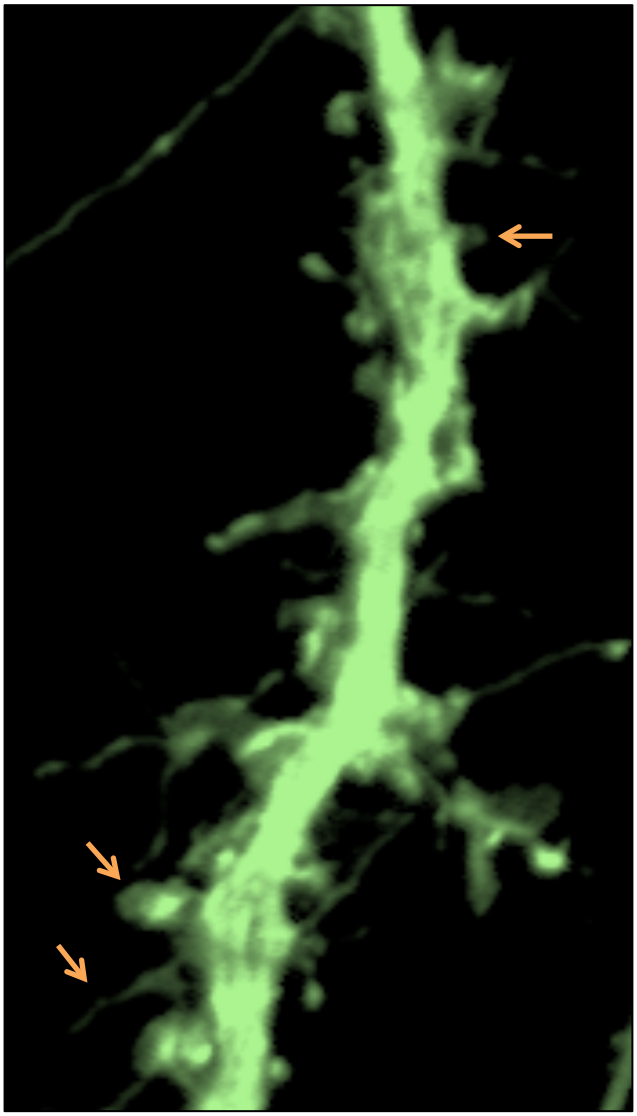
Glutamate receptors were quantified using the ImageJ software. The eGFP cell filler in the green channel was used to create a mask of the dendrite in each image (Sup. Fig 4A). This mask was used to define receptor clusters that lie only within the dendrite. Only clusters in the 568 channel that colocalized with areas that fell within the eGFP mask we quantified (Sup. Fig 4B). A threshold was then generated to determine a standard value for a cluster and this threshold was used to quantify the area (size), intensity (amount), and number of clusters that fell within the dendrite (Sup. Fig 4C). Lastly, the density of clusters on a given dendritic was calculated by dividing the total number of clusters within the dendritic by the compiled length of the dendrite which was obtained using the NeuronJ plugin of ImageJ.

**Statistics**

Statistical calculations (Student's t-test) were performed using GraphPad Prism. Significance was set at $p < 0.05$. For the receptor expression analysis, three sets of experiments were conducted with each experiment consisting of 12 - 16 cells/condition. For the spine subtype morphology quantification, 500 – 550 dendritic spines were analyzed for both the control and sAß conditions.

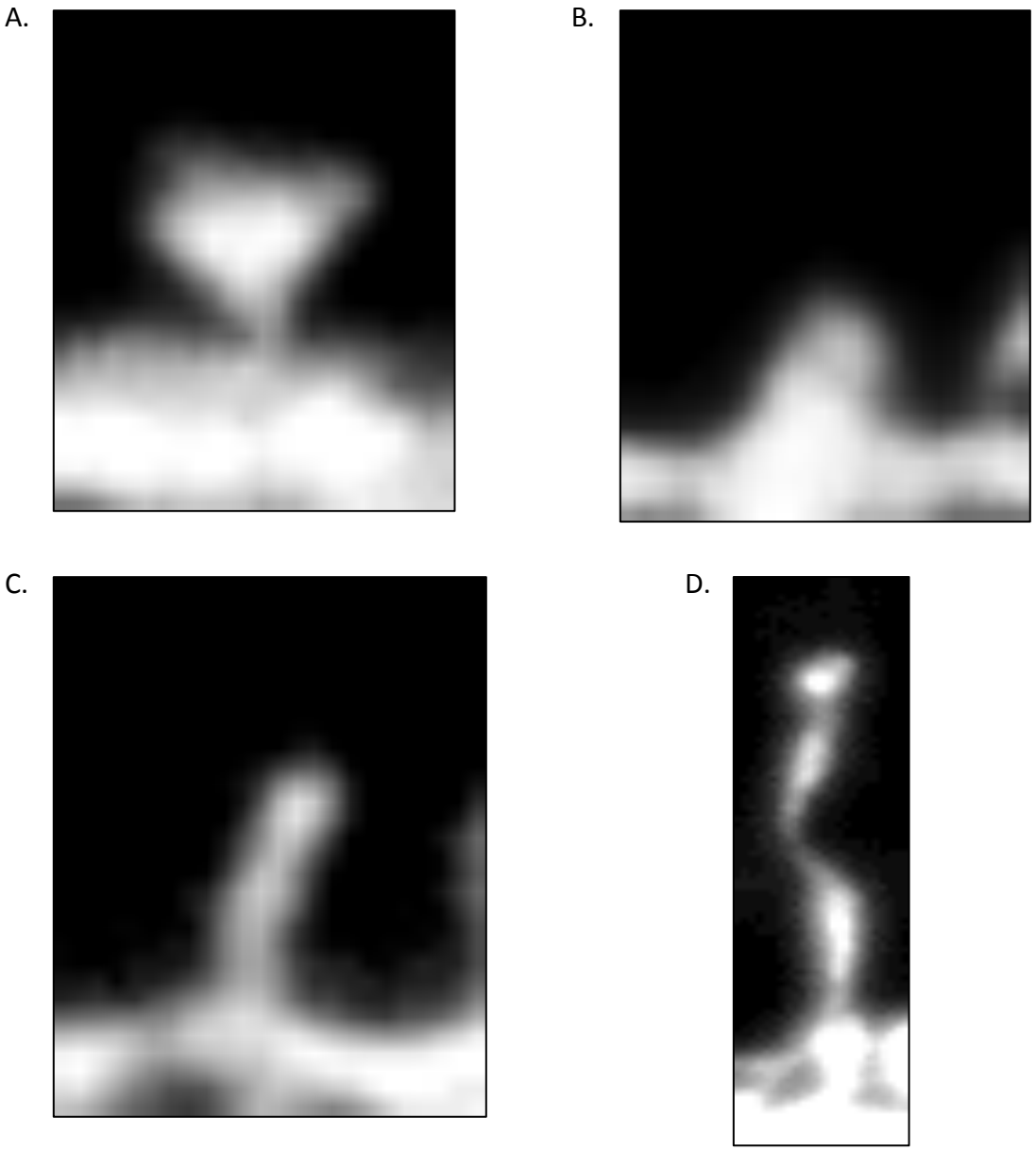
# V:

# Supplemental Figures




Supplementary Figure 1: sAß induces changes in dendritic spine morphology

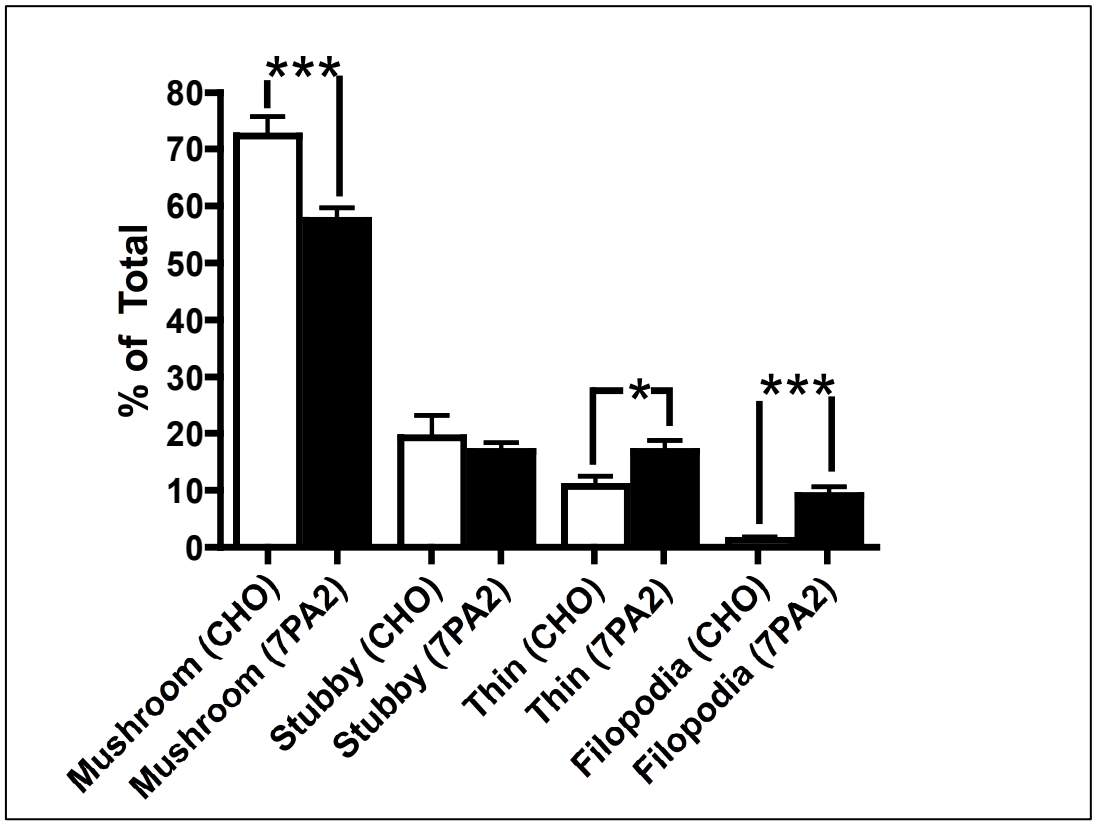
(A,B,C) Confocal image of a hippocampal neuron expressing eGFP cell filler and incubated with sAß for 2 hours prior to fixation. Arrows point to a stubby spine (A) with no neck or head, a mushroom spine (B) with a neck and head, and a filopodia (C) type spine with an elongated neck and no head.




Supplemental Figure 2: Dendritic spines can be classified as mushroom, stubby, thin and filopodia

(A,B,C) Confocal image of a dendritic spine that defined as mushroom (A), stubby (B), thin (C), and filopodia (D) type based on a quantification of spine length, neck width, and head width

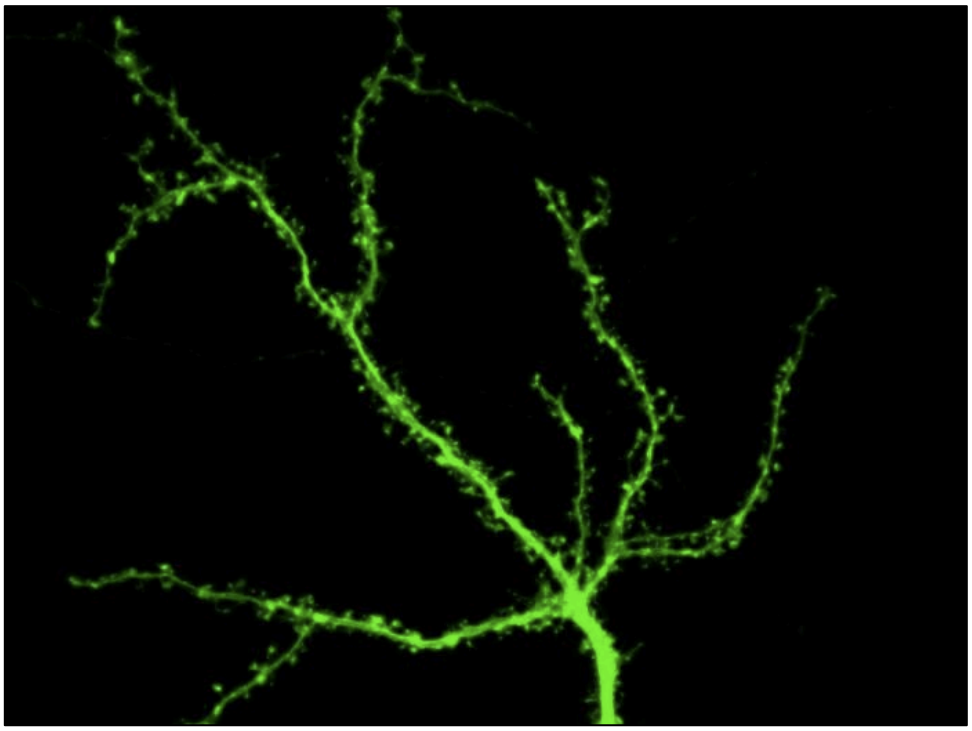
A.




Supplemental Figure 3: sAß decreases the number of mushroom type spines and increase the number of thin and filopodia type spines

(A) Quantification of the number of different spine subtypes in dendrites incubated with CHO (control) and 7PA2 (sAß). Sample size ranged from 450 to 500 dendritic spines

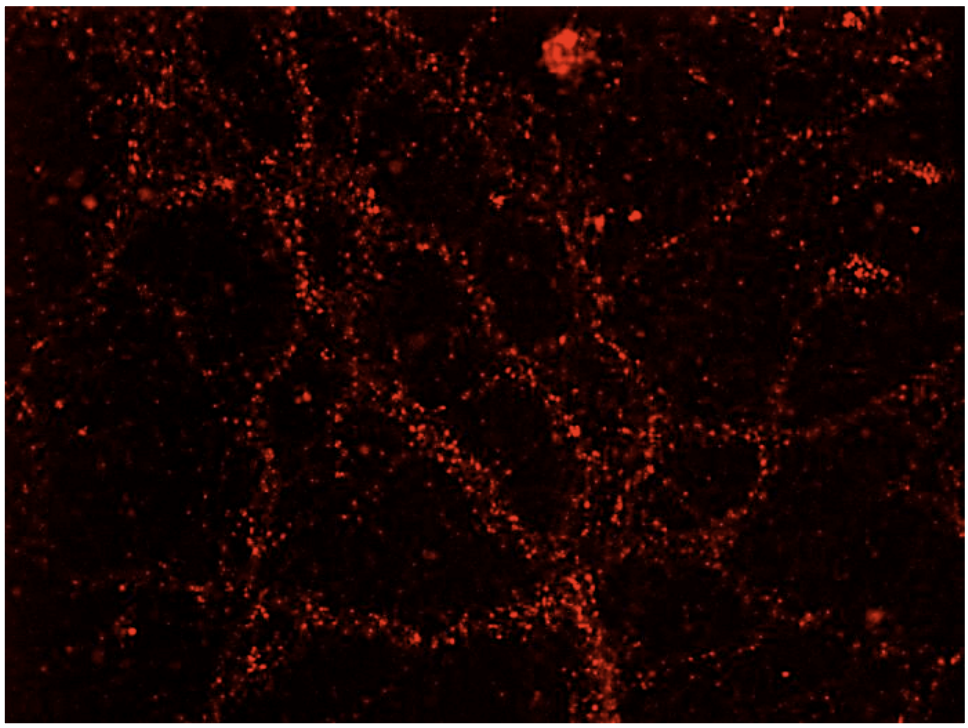
A.



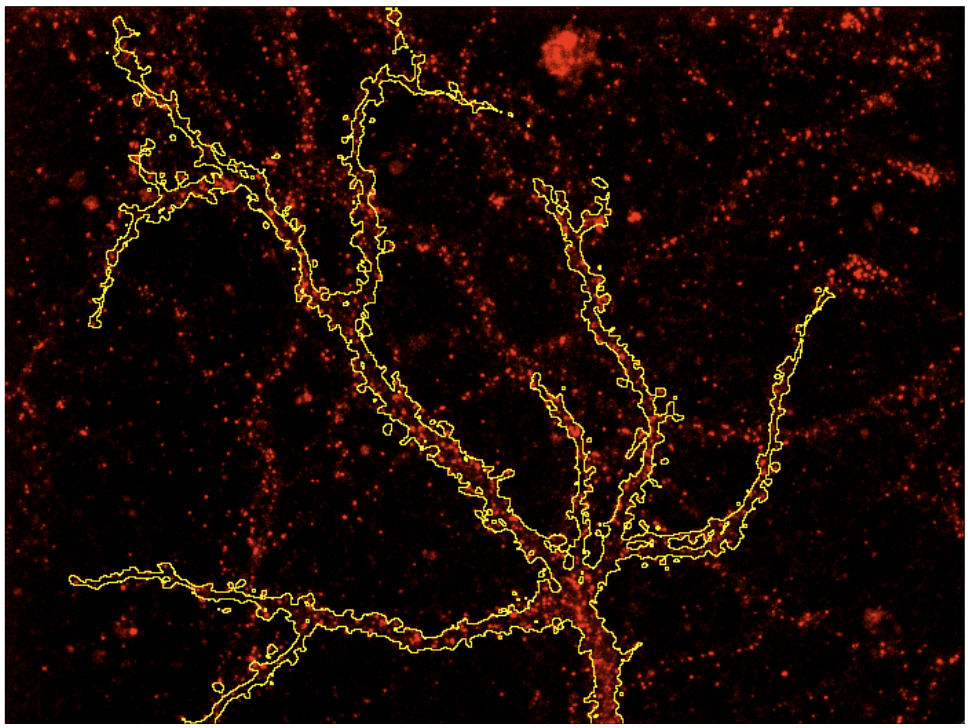
Supplementary Figure 4: Glutamate receptor cluster analysis

(A,B,C) A mask of the neuron is created using the eGFP cell filler in the green channel (A). This mask is superimposed on the corresponding immunostained image of glutamate receptor clusters in the red channel (B) to define clusters that lie within the given dendrite (C).

B.



C.



Supplementary Figure 4 continued.

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