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Simultaneous analysis of dendritic spine density, morphology and glutamate receptors during neuron maturation *in vitro* by quantitative immunocytochemistry

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Abstract

Alterations in the density and morphology of dendritic spines are characteristic of multiple cognitive disorders. Elucidating the molecular mechanisms underlying spine alterations are facilitated by the use of experimental and analytical methods that permit concurrent evaluation of changes in spine density, morphology and composition. Here, an automated and quantitative immunocytochemical method for the simultaneous analysis of changes in the density and morphology of spines and excitatory glutamate receptors was established to analyze neuron maturation, *in vitro*. In neurons of long-term neuron-glia co-cultures, spine density as measured by drebrin cluster fluorescence, increased from DIV (days *in vitro*)10 to DIV18 (formation phase), remained stable from DIV18 to DIV21 (maintenance phase), and decreased from DIV21 to DIV26 (loss phase). The densities of spine-localized NMDAR and AMPAR clusters followed a similar trend. Spine head sizes as measured by the fluorescence intensities of drebrin clusters increased from DIV10 to DIV21 and decreased from DIV21 to DIV26. Changes in the densities of NR1-only, GluR2-only, and NR1+GluR2 spines were measured by the colocalizations of NR1 and GluR2 clusters with drebrin clusters. The densities of NR1-only spines remained stable from the maintenance to the loss phases, while GluR2-only and NR1+GluR2 spines decreased during the loss phase, thus suggesting GluR2 loss as a proximal molecular event that may underlie spine alterations during neuron maturation. This study demonstrates a sensitive and quantitative immunocytochemical method for the concurrent analysis of changes in spine density, morphology and composition, a valuable tool for determining molecular events involved in dendritic spine alterations.

1. Introduction

Dendritic spines are small extensions on dendrites that form the postsynaptic component of excitatory synapses in the brain. As spines form approximately one-to-one connections with pre-synaptic terminals, spine density reflects synapse density. The morphology of spines predicts the stability, plasticity, and strength of associated synapses. Spine morphological subtypes include thin (small head and long neck), mushroom-shaped (large head and short

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neck) and stubby spines (head and neck of same width). The bulbous heads and long necks of spines play important roles in signaling potentiation and propagation by sequestering and compartmentalizing key excitatory signaling effectors (Ashby et al., 2006; Korkotian et al., 2004; Majewska et al., 2000a; Majewska et al., 2000b). Consequently, loss or altered morphology of spines influence cognition and is a salient feature of multiple neurological disorders.

It is unclear whether observed decreases in spine density of mature neurons result from reduced formation of new spines or enhanced elimination of existing spines. Additionally, the order of morphological changes that spines undergo during degeneration is unclear. For example, both conversion of degenerating mushroom spines back to thin immature spine morphology and conversion into larger giant spines have been reported (For review see (Fiala et al., 2002)). These uncertainties and apparent contradictory results are likely due to the use of *in vitro* and *in vivo* experimental methods that are limited to analysis of either the density, morphology or composition of spines. An experimental model that permits concurrent evaluation of changes in spine density, morphology and molecular composition would be valuable for identifying the role of altered formation and elimination of spines, the order of morphological changes, and the proximal molecular mechanisms involved in spine alterations.

In vitro models are valuable tools for investigating molecular mechanisms in specific cell types in isolation. *In vitro* models also facilitate analysis and detection of sub-cellular or localized changes. In long-term primary neuron culture preparations, neurons undergo stereotypical developmental steps to transform into mature neurons that possess fully developed and functional spines (Papa et al., 1995; Zito et al., 2009). Thus, primary neuron cultures have been utilized to study neuron and spine developmental and regulatory changes. However, the analytical methods currently used for spine analysis are time-consuming, restrict sample size and are qualitative or semi-quantitative.

Here, using long-term primary hippocampal neuron-glia co-cultures, the temporal sequence of changes in the density and morphology of spines and excitatory glutamate receptors with increasing neuron age were examined using an automated quantitative immunocytochemical method for spine analysis. Analysis of spine density during neuron maturation in culture revealed phases of spine formation, maintenance and loss, providing a model with which to determine whether changes in spine density result from inhibited or delayed formation of new spines, or from enhanced or accelerated elimination of existing spines, *in vitro*. While NMDAR subunit (NR1), AMPAR subunit (GluR2), and spines increased during the formation phase, GluR2 loss preceded loss of NR1 and spines in later phases. This model reveals the order of molecular events involved in spine alterations during neuron development and aging as a continuum, which is essential for understanding mechanisms that may underlie age-related synapse dysfunction and loss.

2. Materials and methods

2.1. Primary neuron culture

Animals were handled according to the Institutional Animal Care and Use Committee (IACUC) protocols at the University of Illinois at Chicago, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Pure hippocampal neuron cultures were prepared from E17 C57Bl6 (Charles River, Jackson Labs) mouse embryos. All culture materials were purchased from Gibco-invitrogen (Carlsbad, CA), except where otherwise noted. Neurons were cultured at low density, together with a feeder layer of astrocyte cells, as described in the Banker protocol (Banker and Goslin, 1991). Prior to culture preparation, sterile coverslips were dotted with paraffin wax and coated with 0.1mg/

ml of poly-d-lysine for at least 12 hrs. The coated coverslips were placed in a 60mm culture dish, washed extensively with ddH₂O, and incubated in MEM-alpha containing 10% fetal bovine serum and 1% pen-strep overnight at 37°C and 5% CO₂. On day of culture, media in coverslip-containing dishes were switched to neuron-plating media (1X MEM, 0.6% Glucose, 2mM Glutamine, 2.5% FBS). For hippocampal neuron culture preparation, the hippocampi were dissected out of mouse embryo brains in dissection medium (1:1 mixture of MEM and HBSS) and trypsinized with 2.5% Trypsin for 15 minutes at 37°C. Trypsin activity was diluted out with three 5-minute changes of dissection medium. Tissues were dissociated by gentle trituration using fire-polished pipettes. Cell suspension was filtered through a 40µm cell strainer, and cells in filtrate were plated at a density of 70cells/mm² on the poly-d-lysine-coated coverslips in 60mm dishes containing neuron-plating media. 2–4hrs later, the medium was switched to a neurobasal medium containing 1X B27 supplement from Gemini bio-products, West Sacramento, CA, and 1X Glutamax (NB-B27-GlutaMax). After 48hrs, 5µM Cytosine-β-d-arabinoside was added to cells for 72hrs to eliminate proliferating glial cells. Neurons on coverslips were then transferred into 6-well plates for co-culture with primary glial cells.

2.2. Glial culture

Glial cultures were prepared from postnatal day 2–3 apoE2-targeted replacement mice (Sullivan et al., 1998) as described in (Hu and Van Eldik, 1996; LaDu et al., 2000; Manelli et al., 2007). Tertiary glial cultures were used in this study to obtain glial compositions of ~95% astrocytes, and less than 5% microglia (Manelli et al., 2007). Cortices were dissected out and dissociated by trypsinization in 0.5% trypsin-EDTA for 10mins at 37°C. Cells were triturated and filtered once through a 100µm cell strainer and twice through 40µm cell strainers. Cells were plated in T150 flasks (1½ brain per T150 flask) in a MEM-alpha medium containing 10% FBS, 1% pen-strep. After 72–96 hours (day 3–4), the medium was refreshed completely to remove floating cellular debris. On day 10, confluent cultures were trypsinized and passaged into new T150 flasks. Cells in one T150 flask was split into two T150 flasks. Five days later (on day of primary neuron culture preparation), glial cells were passaged into 6-well plates, at a density of 7.5×10^4 cell per well in MEM-alpha medium containing 10% FBS and 1% pen-strep. 96hrs after plating, medium was changed to NB-B27-Glutamax. Glial cells were used for co-culture the following day.

2.3. Co-culture

Primary neurons on 12 or 15mm diameter coverslips were transferred into 6-well plates containing tertiary glial cells in NB-B27-Glutamax media. 2 or 3 coverslips were placed in each well with the neurons facing the glia. The paraffin wax spots on the coverslips prevented direct contact between neurons and astrocytes. One-tenth of the media was changed every 3–4 days. On days 10, 14, 18, 21, and 26, sets of neurons on coverslips were fixed for immunocytochemical analysis.

2.4. Transfection of primary neurons

DIV 12 neurons on coverslips in 12-well plates were transfected for 2 hours with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's recommendations, except that a mixture of 0.5µg of pmax-*GFP* construct (AMAXA) in 25µl neurobasal medium and 1.25µl of Lipofectamine 2000 in 25µl neurobasal medium was used.

2.5. Immunocytochemistry

Neurons on coverslips were rinsed in artificial cerebrospinal fluid (ACSF: 5M NaCl, 3M KCl, 1M CaCl₂, 100mM HEPES, 1M MgCl₂, 8mM dextrose (D-glucose), then fixed with ice-cold methanol for 15mins. Neurons were further permeabilized with 1X phosphate-

buffered saline containing 0.025% triton-X detergent (1X PBS-TX) for 10mins and blocked with 3% BSA in 1X PBS for 30mins. For the double-staining of GFP-transfected neurons, a mixture of mouse anti-drebrin (Abcam, 1:200) and chicken anti-MAP2 (Abcam, 1:1000) antibodies was used. For quadruple-staining, neurons were incubated with a primary antibody mixture containing rabbit anti-GluR2 (Millipore, 1:50 dilution), mouse anti-NR1 (Pharmingen, 1:50 dilution), guinea pig anti-drebrin (Fitzgerald, 1:500 dilution), and chicken anti-MAP2 (Abcam, 1:500 dilution) for 1hr. Neurons were washed 3X for 5mins with 1X PBS-TX and incubated with a mixture of corresponding Alexa fluorophore-labeled secondary antibodies (invitrogen, 1:500 dilution): donkey anti-rabbit 750, donkey anti-mouse 647, donkey anti-pig 488, and goat anti-chicken 594, for 30mins. Following three 5-minute washes with 1X PBS-TX, coverslips were mounted on slides with prolong gold antifade reagent with DAPI (Invitrogen). Mounting media were allowed to cure overnight.

2.6. Image Acquisition and Dendrite Sampling

Confocal images of GFP-transfected neurons double-labeled with drebrin and MAP2 were captured with Zeiss LSM510 Meta microscope, using a 100X 1.4NA oil objective. Z-stack images were averaged 4 times and acquired every 0.5 μ m z-distance at 0.8X optical zoom and 1024 $\times$ 1024 pixel resolution. Wild-field images used for quantitative analysis were acquired using Zeiss Axio Imager M1 fluorescence microscope, equipped with Zeiss AxioCam HRm camera and controlled with Axiovision version 4.7 software. Images were captured with a 63X 1.4NA oil objective. 3 to 5 positions per coverslip were randomly selected and a 5 $\times$ 6 mosaic of 63X images per position was captured at 2776 $\times$ 2080 pixel resolution. Each mosaic contained an average of 15–20 neurons. For dendrite sampling, spiny neurons with discrete dendrites, as determined using MAP2/drebrin immunoreactivities, were marked. All channels were then turned off except for MAP2 and DAPI for dendrite sampling. Dendrite segments 20 μ m in length, from 2 to 3 dendrites per neuron, were sampled 50 μ m away from the cell body (Figure 1A). Distance and length measurements were performed using Axiovision software measurement tool. A jitbit macro recorder was used in conjunction with the Axiovision software to facilitate measurements and sampling. Images of sampled dendrites were exported as tiff images in 2 sets, one set with the MAP2 channel on (set 1) and a second set with the MAP2 channel turned off (set 2). Cluster quantification and intensity measurements were performed using set 2 images. Acquired 20 μ m dendrite images where 2 or more dendrites were in close proximity such that overlap of spines was suspected were discarded.

2.7. Image Processing/Quantitative Analysis

Quantitative analyses were performed with ImageJ NIH software using custom-written plugins. To evaluate use of drebrin cluster fluorescence as a marker for spine morphology, spine head size, as determined by area covered by GFP fluorescence, was compared with drebrin cluster fluorescence intensity. Only spines with distinct necks were used for this analysis. Tiff images of dendrite segments containing GFP and drebrin signals were obtained for analysis. The channels were split and the GFP image was selected and converted to a black and white image, with the background black and GFP signal white. GFP signals in the dendrite and neck of spines were erased, leaving signals in spine heads that outlined the spine area. The outlines of spine heads were converted to individual ROIs (region of interests) and the area of each ROI was measured. A mask of the ROIs was overlaid on the drebrin image. Drebrin fluorescence intensity within each ROI was measured. The areas of the individual ROIs obtained (from GFP signal) were plotted against corresponding integrated density values of drebrin cluster fluorescence intensities. Correlation analysis was assessed by Pearson correlation analysis (GraphPad Prism 4.0c).

For ROI selection of quadruply stained neurons, tiff images of 20 μ m dendrite segments containing MAP2 signal (set 1; MAP2 channel turned on) were used to draw ROI around spines and the parent dendrite to be analyzed. ROI selection for each image was saved. The second set of tiff images with no MAP2 signal (set 2; MAP2 channel turned off) were opened and ROI selections from set 1 images were overlaid on matching set 2 images. Immunoreactive signals outside the selected ROI were erased. Using the 'Find Maxima' command, background signal was set to luminance of diffuse fluorescence. For each 20 μ m dendrite segment image, drebrin, NR1 or GluR2 cluster intensities were automatically detected and point-selected, then automatically counted to obtain total counts per 20 μ m of dendrite length. For co-localization analysis, a method that permits detection of juxtaposed (within 1 pixel distance) and overlapping clusters as co-localized, was utilized. Illustrative images are shown in (Figure 1B). A gaussian circle of sigma 2.1 was automatically drawn around each point-selected cluster. A mask of the gaussian circles (1 per cluster) was created for each channel. Masks were overlaid one channel on another to generate colocalized points. Overlap regions (white) with area greater than 4 pixels were accepted as positive colocalizations. All measurements were performed on a per 20 μ m dendrite basis. Values were then averaged to obtain a mean and 95% confidence interval for all dendrites per time point. Statistical analyses were performed by unpaired students' t-tests with equal variance and statistical significance was set at $p < 0.05$.

To measure fluorescence intensity of each cluster, the gaussian circles around each were converted into individual ROIs and mean fluorescence intensity of each cluster was measured. Cluster intensity values for all dendrites per time point were represented as percent cumulative frequency. Data shown are from one representative experiment out of two independent experiments, each containing triplicate cultures.

3. Results

3.1. Methodology used to analyze spines, excitatory glutamate receptor distributions, and spine subtype

In developing neurons, dendrite maturation involves continuous elongation and branching, with subsequent formation of dendritic spines. We performed our analysis on dendrite segments at a fixed distance from the cell body, as the maturation state of dendritic spines may vary with distance from the cell body. Dendrite segments were sampled 50 μ m away from the cell body in order to accommodate the shorter length of DIV10 dendrites and sampling from secondary dendrites of apical branches.

Immunocytochemical analysis of endogenous markers was utilized for dendritic spine analysis in order to circumvent concerns associated with GFP transfection (use of high-density cultures and potential influences on neuron viability). MAP2 is a reliable marker for outlining dendrites in low-density neuron cultures. It is also a marker for changes in neuron viability. Thus, neurons with beaded or no MAP2 immunoreactivity were excluded from spine analysis to permit detection of spine alterations that occur prior to overall changes in neuron viability. Actin is enriched in spines to varying degrees, with higher actin content present in larger mushroom spines than in thin spines (Fischer et al., 1998; Shoji-Kasai et al., 2007; Takahashi et al., 2003). Drebrin A, an actin-binding protein, is expressed exclusively in dendritic spines (Hayashi et al., 1996) and enriched in spine heads (Figure 2). Drebrin has been used by several groups to label spines, *in vitro* (Garcia et al., 2010; Kobayashi et al., 2007; Takahashi et al., 2003). We compared drebrin-cluster distribution with GFP transfection to determine whether drebrin is a reliable marker for spines. Drebrin clusters were present on spine heads, while GFP was diffusely distributed throughout the dendrites and spines (Figure 2A). We further compared drebrin cluster intensity to area covered by GFP fluorescence in spine heads (Figure 2B). Drebrin cluster intensity correlated

directly with area covered by GFP, thus demonstrating drebrin clusters as reliable markers for spine morphology. This finding is in agreement with an earlier study that showed that drebrin content correlates with spine head size (Kobayashi et al., 2007).

Using drebrin cluster immunoreactivity as a spine marker, the relative distributions of NR1 and GluR2 clusters were analyzed (Figure 3). NR1 or GluR2 clusters present on spines (spine-localized) were defined by the co-localization of NR1 or GluR2 clusters with drebrin clusters. The calculated difference between the densities of total NR1 (or GluR2) and spine-localized NR1 (or GluR2) clusters was assumed to represent the density of NR1 (or GluR2) clusters on the dendrite shaft (shaft-localized). Thus, shaft-localized clusters include clusters on dendrite shafts and in dendrite protrusions that lack drebrin cluster immunoreactivity.

Spines were observed to contain only NR1 (NR1-only), GluR2 (GluR2-only) or both (NR1+GluR2) clusters. NR1+GluR2 immunoreactive spines represent mature spines and are identified by triple-colocalization of NR1, GluR2, and drebrin clusters. The difference between the density of total spine-localized NR1 (or GluR2) and density of mature NR1+GluR2 spines was used to define the density of immature NR1-only (or GluR2-only) spines.

3.2. Density and morphology of spines with increasing neuron age

To examine changes in spine density with increasing neuron age, the density of drebrin clusters on 20µm dendritic segments from DIV (days *in vitro*) 10, 14, 18, 21, and 26 hippocampal neurons were analyzed. Spine density significantly increased from 9.9 spines/10µm on DIV10 to 20.0 spine/10µm on DIV18, remained stable from DIV18 to DIV21, and significantly decreased to 17.1 spines/10µm on DIV26 (Figure 4B). This suggests that spines undergo phases of formation (DIV10-18), maintenance (DIV18-21) and loss (DIV21-26), *in vitro*.

To examine spine morphological alterations, changes in spine head sizes as measured by drebrin cluster fluorescence intensities were analyzed. Analysis of the cumulative frequency distributions of drebrin cluster fluorescence intensities showed a shift towards higher fluorescence intensity from DIV10 to DIV21, indicative of a progressive increase in spine head sizes. This suggests a conversion of thin spines (small head size) to mushroom spines (large head size) (Figure 4C). On DIV26, relative to DIV21, a decrease in spine head sizes was observed as the cumulative frequency of drebrin clusters with mean fluorescence intensities higher than 40 shifted towards lower fluorescence. In addition, spines with smaller head sizes were lost as mean fluorescence intensities lower than 40 shifted towards higher fluorescence. Visual inspection of the dendrite images on DIV26 further supports this conclusion.

3.3. Densities and sizes of NMDAR and AMPAR subunit clusters with increasing neuron age

Both NMDA and AMPA receptors are heterotetrameric complexes. In the adult brain, NMDAR is composed mostly of its obligatory subunit NR1, along with either NR2A and/or NR2B. AMPAR complexes are mostly composed of GluR1-GluR2 or GluR2-GluR3 complexes. To analyze changes in NMDAR and AMPAR, changes in NR1 and GluR2 cluster immunoreactivities were examined. Total NR1 cluster density was substantially (~2-fold) higher than total GluR2 cluster density at each time point (Figure 5B). Total NR1 cluster density significantly increased during the formation phase (DIV10-18) from 16.0 to 30.6 clusters/10µm, remained stable during the maintenance phase (DIV18-21), and significantly decreased during the loss phase (DIV21-26) to 25.5 clusters/10µm. Total GluR2 cluster density increased significantly from 7.7 to 16.3 clusters/10µm during the

formation phase. However, in contrast to total NR1 (and drebrin), total GluR2 cluster density decreased significantly from 16.3 to 14.4 clusters/10 μ m during the maintenance phase (DIV18-DIV21). Total GluR2 cluster density decreased further to 7.6 clusters/10 μ m during the loss phase (DIV21-26).

Decreases in total NR1 and GluR2 cluster density may result from the combination of smaller-sized clusters to form larger-sized clusters, as can be expected during enrichment of receptors in spines. Thus, decreases in cluster densities may reflect loss only when accompanied by no change or by decreases in cluster sizes. NR1 and GluR2 cluster sizes were analyzed by measuring the mean fluorescence intensity of individual clusters. Analysis of the cumulative frequency distribution of NR1 cluster fluorescence intensities showed an increase from DIV10 to DIV18, a decrease from DIV18 to DIV21, and a subsequent increase from DIV21 to DIV26 (Figure 5C). Increase in the frequency of larger sized NR1 clusters on DIV26 may be due to a preferential loss of smaller-sized NR1 clusters.

Cumulative frequency distribution of GluR2 cluster fluorescence intensities revealed an increase from DIV10 to DIV18. However, GluR2 cluster fluorescence decreased progressively from DIV18 to DIV26 (Figure 5D). Thus, the decreased density of GluR2 clusters observed during the maintenance phase (DIV18-21) is accompanied by decreases in GluR2 cluster size, supporting actual loss of GluR2 clusters at this phase. These data suggest that loss of GluR2 clusters precede that of NR1 clusters with increasing neuron age.

3.4. Distribution of NMDAR and AMPAR subunit clusters with increasing neuron age

Spine development, maturation or maintenance involves targeting and enrichment of NMDAR and AMPAR in spines. Spine-associated NR1 and GluR2 cluster density increased significantly during the formation phase (5.5 to 14.1 clusters/10 μ m for NR1; 4.1 to 9.9 clusters/10 μ m for GluR2), remained stable during the maintenance phase (DIV18-21), and decreased significantly during the loss phase (11.2 clusters/10 μ m for NR1; 6.0 clusters/10 μ m for GluR2) (Figure 6B). Spine-localized NR1 cluster densities were higher than spine-localized GluR2 cluster densities at each time point.

The density of dendritic-shaft localized NR1 (shaft-NR1) clusters increased from 10.5 clusters/10 μ m on DIV10 to 16.2 clusters/10 μ m on DIV14 and remained stable up to DIV26. In contrast, shaft-GluR2 clusters increased from 3.6 clusters/10 μ m on DIV10 to 6.5 clusters/10 μ m on DIV14, remained stable up to DIV18, then decreased significantly and progressively to 1.6 clusters/10 μ m on DIV26 (Figure 6C). The absence of change in the densities of shaft-localized NR1 and GluR2 clusters from DIV14 to DIV18, when increased spine-associated receptor cluster densities and total cluster fluorescence intensities are observed, supports the targeting of NR1 and GluR2 clusters to spines during the formation phase. There was no change in the density of spine-localized GluR2 in the maintenance phase (DIV18-21) (Figure 6B). However, a significant decrease in the density of shaft-localized GluR2 clusters was observed (Figure 6C). This suggests that loss of shaft-localized GluR2 clusters occurs prior to loss of spine-localized clusters, and accounts for the observed decrease in total GluR2 cluster density during the maintenance phase.

To better illustrate changes in the distribution of NR1 and GluR2, the density of NR1 and GluR2 clusters on dendrite shafts were expressed as a proportion of total cluster density (Figure 6D). With increasing neuron age, NR1 clusters on dendrite shaft significantly decreased up to DIV21 and increased on DIV26. On the other hand, GluR2 clusters on dendrite shafts decreased significantly and progressively up to DIV26.

3.5. Density of spine sub-types with increasing neuron age

Spine-localized NR1 cluster density includes clusters in NR1-only and NR1+GluR2 spines. Similarly, spine-localized GluR2 clusters include clusters in GluR2-only and NR1+GluR2 spines. To examine changes in the density of spine subpopulations, the density of immature (NR1-only or GluR2-only) and mature (NR1+GluR2) spines were analyzed. Significant increases in the densities of all three groups were observed during the formation phase (3.0 to 7.7 spines/10 μ m for NR1-only; 1.6 to 3.5 spines/10 μ m for GluR2-only, 2.5 to 6.4 spines/10 μ m for NR1+GluR2) (Figure 7). There was no change in the densities of any of the three groups during the maintenance phase (DIV18-21). During the loss phase (DIV21-26) however, the densities of NR1-only spines remained stable, while the densities of GluR2-only and NR1+GluR2 spines decreased significantly to 1.6 and 4.4 spines/10 μ m, respectively.

4. Discussion

The current study reveals changes in the density and morphology of spines, NMDAR and AMPAR distribution, and the temporal sequences with which they are affected during neuron maturation. This study further demonstrates an automated method of spine analysis that permits simultaneous measurement of spine density, morphology, and composition.

4.1. Advantages of the current model and method of analysis

Dendritic spine development is a dynamic process involving rounds of rapid extension and retraction of spines. Regulation of spine number and distribution, as observed by two-photon imaging *in vivo*, involves three phases that lead to 1) a net increase in the spine density, 2) no change in spine density, and 3) a net decrease in spine density (Holtmaat et al., 2005; Spires-Jones et al., 2007; Zuo et al., 2005). These phases are clearly identifiable in our current *in vitro* model as the phases of spine formation, maintenance, and loss, respectively. Identifying the phases of spine formation, maintenance and loss reveals the phase of neuron development wherein a change occurred. Additionally, when used in conjunction with an analytical method that reveals the maturation states of the spines, changes in spine density due to inhibited or delayed formation of new spines and/or enhanced elimination of existing mature spines can be delineated (see schematic in Figure 8).

Filopodia extensions lack actin-rich bulbous heads (Takahashi et al., 2003), thus are not identified by drebrin-cluster immunoreactivity. This provides the advantage of analyzing changes that are specific to spines. While, dendritic spines were originally thought to evolve from filopodia extensions whose high motility is proposed to play a crucial role in the successful encounter of pre- and post-synaptic units for the formation of synapses, accumulating data that show formation of shaft synapses prior to formation of spines (Fiala et al., 1998; Yuste and Bonhoeffer, 2004), formation of spines directly from dendrite shafts (Dailey and Smith, 1996), and that only ~0.2% of filopodia convert to spines (Majewska et al., 2006), suggest otherwise. This suggests that mechanisms independent of filopodia govern spine development and highlight the importance of differentiating filopodia from spines when analyzing changes in spine density and morphology.

Additional advantages of the current method include analysis of dendrite segments of same length sampled from a specified distance from the cell body. In this study, spine analysis was performed using 20 μ m dendritic segments, sampled 50 μ m away from the cell body. As the density and maturation state of spines vary along the length of dendrites limiting analysis to a specified distance from the cell body may prove advantageous for increasing sensitivity of analysis. A recent study using two-photon glutamate uncaging suggested that dendritic sub-regions serve as the functional units for long-term memory storage (Govindarajan et al., 2011). The observation that glutamate receptor clusters are redistributed between spine and

parent dendrites lends support for analysis of dendritic subregions, rather than individual spines and synapses, as functional units of learning and memory. It is interesting to note that the distribution of the glutamate receptor subunits examined was different at the different phases of spine development. This may explain variable outcomes/results associated with neuron culture studies. Thus reconciliation of experimental variability in neuron culture studies may require assessment of the developmental phases of viable neurons in culture.

4.2. Phases of spine development, maintenance, and loss by drebrin cluster immunoreactivity

Our current analysis shows that spine density increased from day 10–18 (formation phase), remained stable from day 18–21 (maintenance phase) and decreased from day 21–26 (loss phase) with increasing neuron age (Figure 4). Analysis of morphological changes in spines shows increases in spine head sizes from day 10–21 (formation and maintenance phases) and a decrease on day 26 (loss phase). Increases in the frequency of drebrin clusters with higher fluorescence may result from a preferential loss of thin spines. However, since the density of drebrin clusters increased and remained stable up to DIV21, the observed increase in drebrin cluster fluorescence intensity suggests an overall increase in the head size of existing spines. In contrast, on DIV26, the decrease in total drebrin cluster density was accompanied by a decrease in drebrin cluster fluorescence intensity, suggesting a loss of spines and decreases in the head size of remaining spines. These findings are consistent with previous spine density and morphological analyses performed using DiI-filled fixed cultures (Papa et al., 1995).

4.3. Order of neuron aging-associated spine alterations

Our findings that the densities of NR1 and GluR2 increased and were enriched in spines during neuron and spine development is consistent with previously reported developmental analysis of NMDAR and AMPAR subunits in unstimulated neuron cultures (King et al., 2006; Lesuisse and Martin, 2002; Pickard et al., 2000). While studies show that spines and synapses acquire NMDARs prior to AMPARs, both NMDAR- and AMPAR-only synapses and spines have been observed, suggesting that the localization of these receptors are independently regulated (King et al., 2006; Lachamp et al., 2005; Rao et al., 1998). What remains to be determined are the changes in glutamate receptor distribution and density, and their temporal relationship to spine morphological changes during neuron aging.

Aging, the most common risk factor for multiple neurodegenerative disorders, leads to alterations in spine morphology and decreases in spine density (Bloss et al., 2011; Duan et al., 2003; Dumitriu et al., 2010). Interestingly, aging decreases the expression of NR1 (an NMDAR subunit) and GluR2 (an AMPAR subunit) in the brain (Hof et al., 2002). Synaptic expression of NMDAR and AMPAR is known to influence spine density, morphology and function. Whether loss of these receptors is causal to, or a consequence of, spine alterations during neuron aging is unclear. Our data show that while spine density remained stable and spine head sizes increased during the maintenance phase, an apparent declustering of NR1 and GluR2 clusters, and a significant decrease in the density of GluR2 clusters, were observed. Loss of shaft-localized GluR2 preceded loss of spine-localized GluR2. Analysis of changes in spine subpopulations revealed a decrease in the density of both NR1+GluR2 and GluR2-only spines in the loss phase, while NR1-only spine density remained stable. Decreases in NR1+GluR2 spines may result from the loss of either NR1 or GluR2 from mature spines to yield more GluR2-only or NR1-only spines, respectively. The observed decrease in the density of GluR2-only spines, but not NR1-only spines, during the loss phase suggests that mature spines lose their GluR2 clusters prior to losing their NR1 clusters as they age.

Regulation of synaptic GluR2 is mediated by the interaction of NSF and PICK1, where PICK1 is known to create and maintain extrasynaptic pools of GluR2, while NSF targets GluR2 to the synapse (Gardner et al., 2005). Interestingly, decreased levels of GluR2 and PICK1 have been reported in older rats, as compared to younger and adult rats (Yu et al., 2011).

5. Conclusion

Spine alterations are pathological features of multiple cognitive disorders. While new methodologies aid in the understanding of spine dynamics, determination of molecular mechanisms will require concurrent analysis of the density, morphology and molecular compositions of spines. This current study provides such a model *in vitro* as has been demonstrated by analysis of changes in spine density, morphology and glutamate receptors. The feasibility and sensitivity of the current method of analysis and model will facilitate determination of specific molecular events involved in spine density and morphological alteration, as well as the screening and determination of potential therapeutic targets and reagents.

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Research Highlights

- Established a method for the concurrent analysis of spine density, morphology and composition.
- Examined spine alterations during phases of formation, maintenance and loss.
- Loss of GluR2 clusters preceded loss of NR1 clusters and spines.

Figure 1. Dendrite sampling and flow chart of method used for quantitative analysis

(A) Discrete dendrites 20µm in length were sampled 50µm away from the cell body of neurons quadruple-stained for NR1, GluR2, Drebrin and MAP2. (B) Flow chart and images showing method used for quantitative analyses by ImageJ NIH software. Images are thresholded for the purpose of illustration. 20µm dendritic segments were sampled (1). Region of interest (ROI) was drawn around spines and the parent dendrite to be analyzed. Signals outside of drawn ROI were erased (2). With background set to luminance of diffuse fluorescence signal, clusters were automatically point-selected and counted (3). Point-selections were converted into individual circular ROIs and centered on each cluster. Mean fluorescence within individual circular ROI was used as a measure of individual cluster fluorescence intensity (4). For colocalization analyses, mask of circular ROIs for each channel was created. Masks of 2 channels were overlaid for double-colocalization analyses, and all three masks were overlaid for triple-colocalization analysis. Overlapped regions greater than 4 pixels were considered as positive colocalizations (shown in white) (5). Spines that contain NR1 and GluR2 on the analyzed dendrite are highlighted in white.

Figure 2. Drebrin clusters reliably label spines and correlate with spine morphology

(A) DIV12 neurons were transfected with a plasmid expressing GFP. Four days after transfection (DIV16), neurons were fixed, permeabilized, and stained with Drebrin (red) and MAP2 (blue). Colocalization (yellow) of GFP and drebrin on spines in merged images demonstrates that drebrin clusters localize on spines (see circled region). Scale bar is 10 μm .

(B) Significant direct correlation between drebrin cluster fluorescence intensity and area covered by GFP in spine heads ($r^2 = 0.8899$, $p < 0.0001$).

Figure 3.
Flow chart of method used to analyze receptor subunit distributions and spine sub-types.

(A)

(B)

(C)

Figure 4. Phases of spine formation, maintenance, and loss as defined by drebrin cluster density and fluorescence intensity

(A) Representative images of quadruple-stained DIV10, 14, 18, 21, and 26 neurons (top) and dendrites (bottom) showing drebrin (green) and MAP2 (white) immunoreactivities. NR1 and GluR2 immunoreactivities are not shown. Scale bars are 10µm (top) and 5µm (bottom). (B) Density of drebrin clusters per 20µm dendrite segments from DIV10-26 neurons. Data are presented as ± 95% confidence interval; *p<0.05; n.s., no significance. (C) Percent cumulative frequency of drebrin cluster fluorescence intensities.

Figure 5. Total densities and sizes of NR1 and GluR2 clusters

(A) Representative images showing NR1 and GluR2 immunoreactivities in DIV10-26 dendrites that were quadruple-stained with drebrin (not shown), NR1 (blue), GluR2 (red) and MAP2 (white). Scale bar is 5 μ m. (B) Total densities of NR1 and GluR2 clusters per 20 μ m dendrite segments from DIV10-26 neurons. Data are presented as $\pm$ 95% confidence interval; $p < 0.05$: * for NR1, # for GluR2. (C) Percent cumulative frequencies of NR1 cluster fluorescence intensities from DIV10-26. (D) Percent cumulative frequency of GluR2 cluster fluorescence intensities from DIV10-26.

Figure 6. Distribution of NR1 and GluR2 clusters on spines and dendrite shafts

(A) Representative images showing NR1- and GluR2-cluster localizations in DIV10-26 dendrites that were quadruple-stained with drebrin (green), NR1 (blue), GluR2 (Red) and MAP2 (white). Scale bar is 5µm. **(B)** Total densities of spine-associated NR1 (NR1-only & NR1+GluR2) and GluR2 (GluR2-only & NR1+GluR2) clusters per 20µm dendrite segment from DIV10-26 neurons. **(C)** Densities of shaft-localized NR1 and GluR2 clusters per 20µm dendrite segments from DIV10-26 neurons. Values were obtained by subtracting spine-associated cluster density from total cluster density. **(D)** Proportion of NR1 and GluR2 cluster on shaft (not present on spines) from DIV10-26. Values were obtained by dividing density of shaft-localized clusters with total cluster density. Data are presented as $\pm$ 95% confidence interval; $p < 0.05$: * for NR1, # for GluR2, n.s., no significance.

Mature and Immature Spine Densities

Figure 7. Densities of mature and immature spines

Densities of NR1-only, GluR2-only, and NR1+GluR2 immunoreactive spines per 20μm dendrite segments from DIV10-26 neurons. Triple-colocalization of drebrin, NR1 and GluR2 was used to measure the density of NR1+GluR2 immunoreactive spines. The density of NR1-only (or GluR2-only) spines was obtained by subtracting NR1+GluR2 spine density from the total spine-localized NR1 (or GluR2) density. Data are presented as $\pm$ 95% confidence interval; $p < 0.05$: * for NR1, # for GluR2, & for NR1+GluR2, n.s., no significance.

Figure 8.

The schematic illustrates use of phases of spine formation, maintenance and loss as a model for identifying the role of delayed spine formation (red), accelerated loss of spines (blue), delayed formation and accelerated loss of spines (yellow), or inhibited formation of spines (green) in spine density alterations, when compared to neurons of the control group (black). The maintenance phase is identified as the spine stabilization phase, consistent with observed AMPAR enrichment and increases in the head sizes of existing spines.