Video Article

Analysis of Dendritic Spine Morphology in Cultured CNS Neurons

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Abstract

Dendritic spines are the sites of the majority of excitatory connections within the brain, and form the post-synaptic compartment of synapses. These structures are rich in actin and have been shown to be highly dynamic. In response to classical Hebbian plasticity as well as neuromodulatory signals, dendritic spines can change shape and number, which is thought to be critical for the refinement of neural circuits and the processing and storage of information within the brain. Within dendritic spines, a complex network of proteins link extracellular signals with the actin cytoskeleton allowing for control of dendritic spine morphology and number. Neuropathological studies have demonstrated that a number of disease states, ranging from schizophrenia to autism spectrum disorders, display abnormal dendritic spine morphology or numbers. Moreover, recent genetic studies have identified mutations in numerous genes that encode synaptic proteins, leading to suggestions that these proteins may contribute to aberrant spine plasticity that, in part, underlie the pathophysiology of these disorders. In order to study the potential role of these proteins in controlling dendritic spine morphologies/number, the use of cultured cortical neurons offers several advantages. Firstly, this system allows for high-resolution imaging of dendritic spines in fixed cells as well as time-lapse imaging of live cells. Secondly, this in vitro system allows for easy manipulation of protein function by expression of mutant proteins, knockdown by shRNA constructs, or pharmacological treatments. These techniques allow researchers to begin to dissect the role of disease-associated proteins and to predict how mutations of these proteins may function *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/2794/

Protocol

The protocol described here can be used to examine dendritic spine morphology and dynamics in any primary cultured system.

1. Preparation of primary cortical neuron cultures

1. Prepare high-density cortical neuron cultures from Sprague-Dawley rat E18 embryos and culture in glia-conditioned serum-free medium[1-2].
2. Euthanize one pregnant rat (E18) according to ACUC procedures; quickly remove uterus (with fetuses in it) and place in a 100 mm Petri dish on ice.
3. Cut open uterus and amniotic membrane, hold fetus by the neck (umbilical cord intact) with one forceps, use another forceps to peel scalp from back to front, and slit the skull open with a sharp forceps tip along the midline from back to front.
4. Remove the whole brain with a curved forceps (in a scoop motion) and place in a 100 mm petri dish containing 10 ml ice-cold $Ca2^{+}$- and Mg^{2}+-free HBSS.
5. Hold brain stem with one forceps, separate hemispheres with another forceps, remove hippocampus and striatum, separate cortex and carefully peel the cortical tissue off from meninges.
6. Pool dissected cortical tissues, mince briefly and transfer to a 15 ml tube containing 4 ml pre-warmed trypsin/EDTA (0.25% trypsin, 0.53 mM EDTA; HyQ Trypsin from HyClone SH30042.01); incubate in 37 °C water bath for ~15 min; remove trypsin/EDTA as much as possible with a disposable plastic pipette, add 1.5 ml neuronal medium.
7. Dissociate the digested tissues mechanically by gentle pipetting with a 5 ml pipette (~10 strokes, avoid creating air bubbles), add 2 ml medium, let settle for 30 sec, then transfer 2 ml supernatant into a 15 ml tube.
8. Repeat step 6 twice (should take about ~15 min total).
9. Spin cell suspension at 200g for 2 min.
10. Remove supernatant (with a 5 ml pipette – do not vacuum aspirate); loosen the pellet by flipping fingers against the bottom; resuspend cell pellet in 5 ml medium and filter through a 40 µm cell strainer into a 50 ml tube.
11. Mix 10 µl cell suspension and 10 µl trypan blue; Count viable cells (trypan blue exclusion) with a hematocytometer.
12. Typical yield: 5 x 10^{6} cells/brain; dilute to desired density (e.g. 1.5 x 10^{6} cells/ml) for plating.

13. Fill culture plates with plating media (Neurobasal media supplemented with 2% B27, 0.5 mM glutamine and 1% penicillin:stretomycin) (total volume *minus* plating volume) containing 18 mm round or 22x22 mm square coverslips, coated with poly-D-lysine (0.2 mg/ml, Sigma) dissolved in 0.1 M Borate buffer (3.1g/L Boric acid, 4.8g/L borax, pH 8.5, filter sterilized); total volume for 12-well plate = 0.8 ml/well.
14. Plate neurons at the density provided in the next step; disperse cells evenly by gentle rocking/tapping.
15. For 12-well plates (3.5cm^2/well): (hi-density 3 x 10^5/well=857/mm^2) (mid-density 1.5 x 10^5/well=430/mm^2); 6-well plates (9.6cm^2/well): (hi-density 9 x 10^5/well=624/mm^2) (mid-density 4.5 x 10^5/well=312/mm^2).
16. One hour after plating, replace all medium with fresh medium (Neurobasal media supplemented with 2% B27, 0.5 mM glutamine and 1% penicillin:stretomycin). Because cell debris tend to settle in the middle of the well, swirl the plates first to facilitate removal of debris; be careful not to let cells dry at any time.
17. Change ½ medium twice every week thereafter (remove ~300-350 µl from each well, and add 400 µl warmed fresh feeding medium to each well).
    Optional: On DIV 4 add 200 µM D,L-amino-phosphonovalerate acid (D,L-APV, Ascent Scientific) to feeding medium; culture neurons in Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine and 1% penicillin:stretomycin + 200 µM APV.
18. Grow primary cultures of cortical neurons for 24-28 days *in vitro* (DIV), as at this time-point dendritic spines display a mature morphology (i.e. they form connections with pre-synaptic partners, and have a clear head-like structure) and correspond to early adolescence in rodents[3-4]. Neurons grown on 18 mm round coverslips can be tranfected and treated pharmacologically, before being fixed, immunostained, and imaged using a confocal microscope and used for detailed morphometeric analysis of dendritic spines. Cells grown on 22x22 mm square coverslips can then be used in time-lapse imaging experiments to investigate dendritic spine dynamics.

2. Transfection of primary cultured cortical neurons

1. Cortical neurons are transfected 2-3 days before experiments using Lipofectamine 2000 (Invitrogen)[5].
2. Prepare “h-DMEM” by balancing the pH of DMEM (Dulbecco's Modified Eagle Medium; no glutamine, Invitrogen 11965-092) with 10 mM sterile HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;* MediaTech Cellgro 25-060-C I, 1M, pH 7). Warm to 37°C.
3. Transfer coverslips to 600/1000 µl (18/22x22 mm coverslips) pre-warmed (37°C) new antibiotic-free medium (Neurobasal medium, B27, 0.5 mM glutamine), and incubate cells in a humidified 37°C incubator, supplemented with 5% CO_2 for 30 minutes.
4. For each coverslip, add designated amount of DNA (one or multiple DNA plasmids, e.g. pEGFP to outline cell morphology and tagged-mutant synaptic protein; 1-2 µg depending on construct) to 50 µl of h-DMEM; allow to stand for 5 minutes.
5. For each coverslip, add 4/6 µl (18/22x22 mm coverslips) Lipofectamine 2000 to 50 µl h-DMEM; allow to stand for 5 minutes.
6. Thoroughly mix tubes from steps 1.3&1.4, keep for at least 20 minutes in a humidified 37°C incubator, supplemented with 5% CO_2. Complex is stable for up to 6 hours according to the manufacturer.
7. Add Lipofectamine 2000/DNA mixture from step 1.5 dropwise to cells. Incubate cells in a humidified 37°C incubator, supplemented with 5% CO2, for 4 hours.
8. Using feeding medium from step 1.1 (300/600 µl), supplement with pre-warmed (37°C) 400/800 µl fresh feeding medium (18/22x22 mm coverslips). Following step 1.6, transfer coverslips into medium containing old and fresh feeding medium.
9. Allow expression of plasmids to continue for 2-3 days.

3. Treatment of primary cultured cortical neurons grown on 18-mm coverslips

Neurons grown on 18 mm coverslips, previously transfected, can also be easily subjected to pharmacological treatments.

1. Prepare ACSF (artificial cerebrospinal fluid; in mM: 125 NaCl, 2.5 KCl, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 5 HEPES, 2.5 $CaCl_2$ and 1.25 $MgCl_2$ with 200 µM D,L-APV). Warm to 37°C. Pre-incubate coverslips in 900 µl warm ACSF for 30-60 minutes. Pre-treatment with inhibitors can be performed during this time.
2. Prepare pharmacological agents/vehicle from stock solutions to a 10X working concentration; perform dilutions of solutions in ACSF. Carefully add agent/vehicle to cells (final volume of 1000 µl, to a final concentration of 1X agent/vehicle), and allow treatment to carry on for desired time[5].
3. Following treatment(s), fix cells and process for immunocytohistochemistry.

4. Fixation and immunocytohistochemistry (ICC)

1. Fix neurons in either 4% formaldehyde/4% sucrose PBS (800 µl) for 20 minutes at room temperature, or in 4% formaldehyde/4% sucrose PBS (800 µl) for 10 minutes at room temperature, followed by 2X washes in PBS, followed by 10 minutes fix with pre-chilled (-20°C) methanol (800 µl) at 4°C. Methanol fixation works by denaturing and precipitating proteins. This procedure leads to an unmasking of the proteins in the post-synaptic density (PSD), as well as in lipid-rich areas.
2. Wash coverslips in PBS (800 µl), 2x, for 10 minutes each.
3. Permeabilize and block cells simultaneously in PBS containing 2% normal goat serum and 0.1% Triton-X-100 (800 µl) for 1 hr at room temperature.
4. Add primary antibodies raised against GFP, epitope tag (e.g. His, myc, V5) or endogenous proteins, to PBS containing 2% normal goat serum at the appropriate concentration. Take a 15 cm dish, divide it up into squares, number them, and cover with parafilm. Add about 80 µl of antibody and block mixture to parafilm (one drop per square), and place coverslip on to antibody/block mix with cells facing down. Incubated overnight at 4 oC.
5. Wash coverslips in PBS, 3x for 15 minutes each.
6. Dilute Alexa-conjugated secondary antibodies (Invitrogen) in PBS containing 2% normal goat serum at the appropriate concentration. Incubate the secondary antibodies in the same manner as in step 3.4, at room temperature for 1 hour, protected from light.
7. Wash coverslips a further 3x 15 minutes in PBS.
8. Mount coverslips onto standard microscope slides using ProLong Gold antifade reagent (Invitrogen).

5. Quantitative analysis of spine morphologies

We obtain confocal images of single- and double-stained neurons (cells expressing GFP and construct or endogenous protein of interest) using a Zeiss LSM5 Pascal confocal microscope. For all imaging experiments choose healthy pyramidal neurons to be imaged and used in subsequent analysis. Healthy neurons are cells that do not display any sign of blebbing or distress due to treatment or fixation and that contain a full, uninterrupted dendritic arbor.

1. Acquire images of neurons using a 63x oil-immersion objective (Zeiss) with a NA (numerical aperture) of 1.4, as a Z-series of 3-8 images, averaged 4 times, with 0.37 µm intervals, 1024x1024 pixel resolution at a scan speed of 2.5 seconds per section. Cultures that are to be directly compared need to be imaged with the same acquisition parameters. 10-20 neurons/condition, each from 3-5 separate experiments, and 2 dendrites of 100 µm per neuron should be analyzed. Experiments should be done blind and on sister cultures. Adjust detector gain and offset to include even dimly fluorescent thin spines without creating a halo around the dendrite, such that the transition between the fluorescent signal in the spine and the background is sharp, allowing precise quantification. Keep the acquisition parameters the same for all scans within the same experiment.
2. GFP images can be acquired using an argon laser line, exciting at 488 nm and collecting with a band-pass filter at 505-530 nm. Images of overexpressed proteins or endogenous proteins immunostained with an Alexa 568 secondary antibody (Invitrogen) can be collected using a HeNe laser exciting at 543 nm, and collecting using a long-pass filter at 560 nm. Keep laser powers to a minimum to avoid photobleaching of samples.
3. Using MetaMorph software (Molecular Devices, Inc.), two-dimensional, background-subtracted, maximum projection reconstructions of Z-series images are used for morphometric analysis and quantification. To examine the morphologies of dendritic spines, collapse Z-series images, calibrate the required distance, and then threshold to include all spines in a manner that the threshold corresponds exactly to the outline of the spines (Fig. 2E,F). Only spines on secondary and tertiary dendrites (total length of 100 µm per neuron) should be measured to reduce variability, and measurements of segments need to be made between branch points. Manually outline each spine so that it will become a closed perimeter (Fig. 2G).
4. Measure the following spine parameters automatically in MetaMorph: spine length, spine breadth, cross-sectional area, and dendritic spine linear density. Export the dendritic spine morphometric parameters into Excel for quantification. Use Student's unpaired t tests to determine the statistical significance of differences between two groups; one-way ANOVA can be used to compare three or more groups, followed by Tukey-b post hoc for multiple comparisons. Statistical analysis can be performed in Excel, GraphPad or SPSS. Analyze cumulative plots using Kolmogorov-Smirnov test (K-S test)[2,5-6].

6. Quantitative immunofluorescence (IF)

Quantification of immunofluorescence using antibodies against specific synaptic proteins, visualized using an Alexa 568 secondary antibody (Invitrogen), can be used to examine changes in the clustering and synaptic localization of endogenous synaptic proteins.

1. Acquire images using a confocal microscope as described above. Use an argon and a HeNE laser (see above) to image double-stained neurons. Only image healthy pyramidal neurons that do not display any signs of distress.
2. For fluorescence intensity measurements, subtract the background corresponding to the dendritic shaft (Fig. 2I, yellow square) to generate a “background-subtracted” image (Fig. 2J) using MetaMorph. Equally threshold images to include clusters with intensity at least two fold above the adjacent dendrite (Fig. 2K). Outline regions along dendrites (100 µm per neuron) using the “Perimeters” utility (Fig. 2D) and automatically measure the linear density (number/100 µm dendrite length), and integrated intensity (total IF intensity) of each synaptic cluster using MetaMorph[7-9].
3. To measure relative spine content of a synaptic protein, first determine protein clustering by thresholding the GFP image to determine spine morphology (Fig. 2E,F) using MetaMorph. Transfer the regions of interest that only include spines to images of the protein of interest captured in the other channel (Fig. 2G,H). Count the number of protein clusters and measure the immunofluorescence integrated intensity inside spines to assess spine protein content.
4. Export the IF parameters of synaptic proteins into Excel for quantification. Use Student's unpaired t tests to determine the statistical significance of differences between two groups; one-way ANOVA can be used to compare three or more groups, followed by Tukey-b *post hoc* for multiple comparisons. Statistical analysis can be performed in Excel, GraphPad or SPSS.

7. Time-lapse imaging

To examine dendritic spine dynamics, such as changes in spine motility or in spine morphology of individual spines, in the presence or absence of disease-associated synaptic proteins, grow primary cortical neurons cultured on 22x22 mm square coverslips for 24-28 DIV. Neurons can be double-transfected with GFP/mCherry to define cell morphology, and fluorescently tagged mutant synaptic proteins as described above. For all imaging experiments, choose healthy pyramidal neurons expressing both constructs; these cells can be subjected to pharmacological treatment, imaged and used in subsequent analyses.

1. Pre-incubate neurons grown on 22x22 mm coverslips in 1.5 ml ACSF for 30-60 minutes in a humidified 37°C incubator, supplemented with 5% CO_2. Cells can also be pre-treated with drugs at this stage as well. Following pre-incubation/treatment, transfer cells to an enclosed imaging stage chamber (Warner, RC-30HV). Maintain a temperature of 37°C with a controller unit (Warner, TC-344B)[2,5-6,10].
2. To examine spine motility, pre-treat cells with drug or vehicle for up 30-60 minutes before transferring the cells to the imaging chamber. This allows the sufficient time for the drug/vehicle to initiate second messenger pathways within the neuron. Acquire images through a 63X objective (Ziess, N.A. 1.4) with 2X averaging at 10 minute intervals. Choose healthy neurons with overall pyramidal morphologies expressing GFP/mCherry or fluorescent-tagged protein. To minimize photodamage, reduce laser power to 0.5-1%. Comparisons of vehicle-treated versus drug-treated cells will demonstrate changes in spine motility. Alternatively, spine motility can be assessed before and after treatment, by perfusion of drug/vehicle (see below). Collect Z-stacks at each time point.

3. To track changes in dendritic spine morphology over time, image coverslips for 1 hour to establish baseline changes in morphology. At this time, perfuse the drug/vehicle into the imaging chamber using a peristaltic pump (Gilson, Minipuls 3), and image neuron for a further hour. Acquire confocal Z-stacks using a 63X oil objective (Ziess, N.A. 1.4), with 2X averaging every 10 minutes. Reduce laser power to 0.5-1% to minimize photodamage.
4. At the end of each imaging session, obtain a 20X image of the entire neuron to ascertain level of photodamage. Any neurons exhibiting signs of distress should be omitted from quantification. Z-stacks of each time-point should be collapsed into 2D projections in Metamorph. Depending on image quality and level of transfection, a median-pass filter or background subtraction can be applied in Metamorph to produce clear images for analysis.
5. To evaluate spine morphing and motility, images taken at the beginning, middle and end of the 100 minute imaging session should be color-coded and overlaid in MetaMorph. At least 100 µm of dendrite per cell need to be analyzed. The total spine motility fraction is defined as the total number of motility events, i.e. extension, retraction, head morphing or protrusive motility normalized to spine number[6,11]. This method measures the frequency of events, without considering their magnitudes; it pools all types of events, and is a general estimate of overall motility. A protrusive event is defined as the appearance of a new, transient protrusion from a spine head or dendritic shaft. A retraction event is defined as the disappearance of an existing or transient protrusion located on a spine head or dendritic shaft. The sum of protrusion and extension events are divided by the total number of spines in the region of dendrite that is quantified.
6. To assess changes in individual spine morphology in response to drug treatment, measure the cross-sectional area of dendritic spines, or dendritic spine linear density at time point -1 hour (1 hour before perfusion), immediately preceding treatment and at each time point following treatment. Normalize each time point to the 1 hr prior-to-treatment time point. To determine that changes in dendritic spine morphology or linear density were not due to photodamage, analyze neurons perfused with vehicle. Differences in means can be determined by Student's unpaired t tests or one-sample t test.

8. Alternative approaches and keys to success

1. We have described culturing cortical neurons in the presence of D,L-APV, a NMDA receptor inhibitor, from DIV 4 until maturity (DIV24-28). It should be noted that while culturing cortical neurons in the presence of D,L-APV has the benefit of maintaining good health of cortical neuronal cultures long-term, the presence of D,L-APV may influence synapse development. As such an alternative is to culture neurons in the absence of D,L-APV[2,5]. When culturing neurons in the absence of D,L-APV extra attention should be paid to the overall health of cultures, as there is an increase chance of cell death due to excessive Ca2+ cytotoxicity via over-active NMDA receptor activation.
2. The neuronal cultures described in this protocol can be used to examine mechanisms that are relevant for regulating dendritic spine morphology and motility of dendritic spines displaying a mature morphology (i.e. they form connections with pre-synaptic partners, and have a clear head-like structure)2,4,5. Alternatively, use of cultures at an earlier time-point, e.g. DIV11-16, may be more suitable to address questions relevant for dendritic spine formation, or regulation of dendritic spines during early development.
3. An alternative to the use of confocal microscopy, is to use wide-field epi-fluorescence to acquire images of treated cultured cortical neurons. In combination with appropriate deconvolution algorithms, detailed morphometric analysis of spines can be made in fixed or live cells. Furthermore, it is possible to use alternative software, such as ImageJ (http://research.mssm.edu/cnic/tools-ns.html) or Neuronstudio (http://research.mssm.edu/cnic/tools-ns.html) which are free software, for the analysis of dendritic spine morphology.
4. When examining dendritic spine motility/morphing using time-lapse imaging, it should be noted that the use of shorter time-lapse intervals, e.g. 5-10 minutes, may provide a much more detailed analysis of dendritic spine turnover2,5. Use of shorter time intervals may also identify more rapid forms of spine morphing that would not be detected using longer time intervals.
5. The use of cultured cortical neurons has a number of disadvantages and concerns that need to be taken into consideration. Firstly, a concern of using cultured cortical neurons is that there may be variability between cultures that can impact parameters such as dendritic spine density. As such, it is essential that the following points are carefully adhered to ensure the least amount of variability. Firstly, culturing parameters and reagents should be kept as consistent as possible in addition to good culturing practices; this will greatly reduce the level of variability between cultures. Secondly, it is essential that all experiments be performed at the same time-point, and on sister cultures. In addition the time-point at which experiments are performed should be carefully chosen to allow the experimenter to ask the appropriate questions (see point 2 above). Finally, it is critical that appropriate controls are used in all experiments, so that treatment conditions are always examined relative to these internal controls. This will remove any concern of variability between cultures.
6. The use of cultured cortical neurons is a powerful tool in enabling researchers to critically examine the mechanisms that are essential for the regulation of dendritic spine morphology and motility. However, it is important to note that these cultures do not recapitulate an *in vivo* situation, and components such as cortical layer organization and the effect of other cell types, such as glial cells, on synapse formation cannot be addressed in the system described above. Nevertheless, such a system can be used to identify potentially crucial mechanisms underlying the regulation of dendritic spine morphology and motility.

9. Representative Results

The assays described above are designed to investigate changes in dendritic spine morphology, number and motility in response to pharmacological treatments and/or genetic manipulations of protein function in vitro. In our lab, we have utilized these techniques to characterize the role of synaptic proteins that regulate the actin cytoskeleton in dendritic spines, thus altering their structure[2,9]. Furthermore, we have used this assay to determine how neuroactive compounds, such as neuromodulators may drive changes in dendritic spine morphology, number or shape, either alone[4,6,10], or in the presence of activity-dependent stimuli[5].

To estimate the accuracy of our measurements of 1D or 2D parameters, we measured the diameters and areas of fluorescent latex microspheres (Duke Scientific) of known dimensions (0.2, 0.52, 1.0 µm) mounted in the same conditions as the neurons throughout the study. Using the same imaging conditions as for spine measurements (63X NA=1.4 oil-immersion objective, LSM 5 Pascal) we imaged the microspheres, and then determined their diameters and areas in Metamorph, similarly with our spine measurements. We then compared the actual measurements with the known dimensions of the microspheres.

The graph in figure 1 (Fig.1) shows that we could accurately measure the areas of microspheres of 0.52 and 1.0 µm (areas = 0.21 μm^2 and 0.78 μm^2 respectively) (measured standard deviation ~15%; manufacturers determined standard deviation ~9%). Even for the 0.2 µm microspheres

our measurements were fairly close to the actual dimensions (however with large SD). This is consistent with the calculated lateral resolution of our confocal microscope using this particular objective, 0.3 µm (using the equation $\Delta xcf = (0.61/v2)\sqrt{/NA}$)[12]. This is significantly better than the axial resolution, which is known to be poor for confocal and 2-photon microscopes. In addition, we experimentally determined the lateral (x/y axis) point spread function (PSF) by measuring green fluorescent microspheres of 200 nm diameter (Duke Scientific) to be ~ 0.5 µm. This estimation was performed with the pinhole open, and thus it can be assumed that when the pinhole is closed, that the PSF would actually be smaller. Nevertheless, this again indicates that we are able to accurately measure areas greater than 0.196 μm^2 (diameter = 0.5 µm). An indication of the actual lateral PSF is given in (Svoboda *et al.*)13 as "*not much less than 0.4 µm, which would result in an area of 0.16 μm^2*". These comparisons and theoretical considerations indicate that we can accurately measure areas of objects similar in size to spines, since the dimensions of the spines we measured were larger than this resolution limit (> 0.25 μm^2). For such conditions, "*classic morphometric techniques are quantitative*"[13], indicating that we can reliably measure spine areas, and accurately compare spine morphologies in different treatment conditions.

In order to accurately measure dendritic spine morphology, we have transfected DIV 23 cortical neurons with an EGFP construct for 2 days. Following transfection, cells can be subjected to treatment, and are fixed and processed for ICC, where the GFP signal is enhanced to allow for even distribution across the dendrite (or neuron). Figure 2 shows a representative image of a cortical neuron transfected with GFP, imaged using a confocal microscope with a 63X objective (N.A. 1.4) (Fig. 2A). This allows for detailed high-resolution images of dendritic spines (Fig. 2B). Detailed morphometric analyses of dendritic spines are performed in our lab using the Metamorph program. This program allows us not only to measure dendritic spine morphology, but also to examine the localization of endogenous proteins. An overview of how we perform this analysis is provided in Figure 2 (Fig. 2C-G).

A well characterized effect of activity-dependent stimuli is an increase in dendritic spine size[2]. Figure 3 shows representative time-lapse imaging of a dendritic spine of a DIV 24 cortical neuron expressing EGFP, imaged for 30 minutes before and after an activity-dependent stimulus. This time-lapse experiment confirms that activity-dependent stimuli increase dendritic spine size (Fig. 3A,B). To examine basal spine motility, images were acquired at time points 0, 50 and 100 minutes, and spine extensions, retractions, protrusive motility and head morphing were measured separately and combined as total motility. Figure 3C-D confirms that even under basal conditions, dendritic spines of cortical neurons display some level of motility.

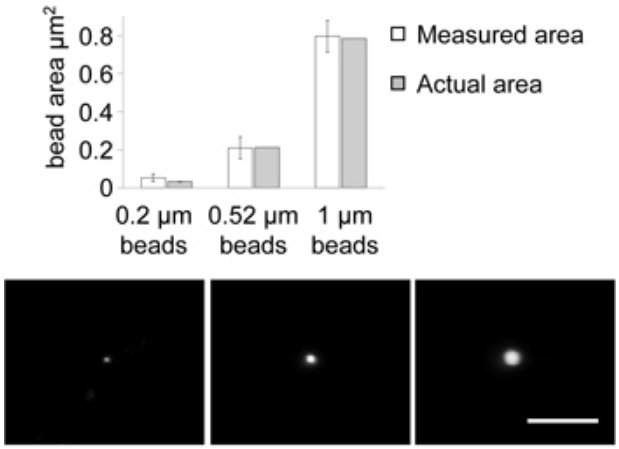

Fig. 1. Comparison of the measured and actual areas of fluorescent microspheres. Error bars represent standard deviation of measured areas. Images of microspheres of 0.2 µm, 0.52 µm and 1 µm. Scale bar = 5 µm.

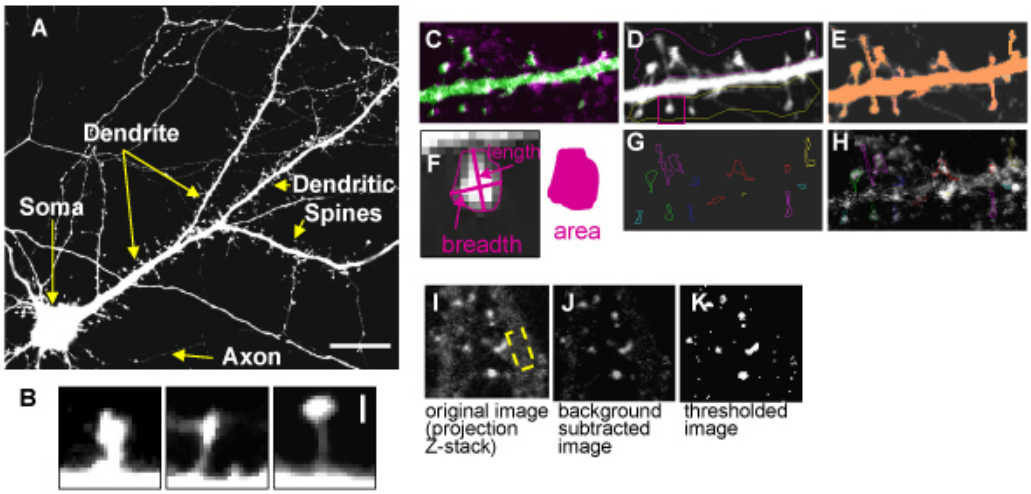

Fig. 2. Quantification of spine morphology and IF. A. Image of DIV 25 EGFP-expressing cultured cortical neuron. **B.** High magnifications of typical dendritic spine morphology found on cortical neurons. **C.** EGFP and endogenous protein (GluR1) overlay. **D.** Collapsed Z-series of an

EGFP-transfected pyramidal cell dendrite; the division between shaft and spines is traced manually. **E.** Distinct, superthreshold regions are then outlined by Metamorph and quantified. **F.** Hand-traced neurons used to determine spine length, breadth and cross-sectional area. **G.** Regions determined by Metamorph that correspond to spines. **H.** Spine outlines will be transferred to the red channel image (endogenous protein) to quantify spine-specific signal. **I-K.** To quantify receptor or synaptic protein cluster IF, projection images of Z-series are used. **I.** Dendritic shaft background IF (yellow square) is subtracted to generate a "background-subtracted" image. **J.** This image is then thresholded and the program automatically measures cluster area, linear density, and total grey value (IF intensity) **K.** Thresholded image from H. Scale bars, A = 15 µm, B = 1 µm.

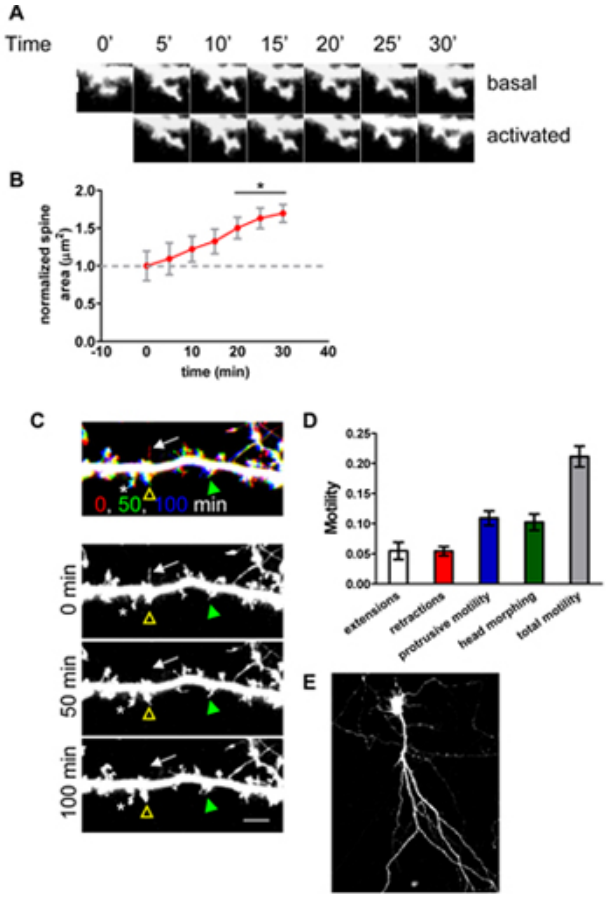

Fig. 3. Activity-dependent changes in spine morphology, and basal motility of dendritic spines. A. Time-lapse imaging of a representative dendritic spine before and after addition of an activity-dependent stimulus. Activity-dependent stimuli were induced by switching the experimental media from ASCF containing $Mg2^{+}$ and APV to ACSF without Mg2+ and APV, but containing 10 µm glycine **B.** Quantification of dendritic spine area (size), normalized to -30 minute time point (30 minutes prior to perfusion). **C.** Representative images of 0, 50 and 100 minute time points of dendritic spine motility. Top images shows overlay of 0 (red), 50 (green) and 100 (blue) minute time points, while images below are each time point separately. Asterisk denotes spine extension; white arrow denotes spine retraction; green arrow head denotes protrusive motility; open yellow arrow head denotes spine head morphing. **D.** Quantification of dendritic spine motility parameters and total motility. **E.** 20X image of cortical neurons expressing EGFP following imaging session to determine health of cell.

Discussion

The techniques described above for the detailed quantitative analysis of dendritic spine morphology, linear density and motility in either fixed or live primary cortical neurons are focused on understanding the effects of post-synaptic mechanisms that may contribute to neuropathologies. A similar approach can be used to quantify spine morphology or motility in any spiny neuron, including hippocampal pyramidal, Purkinje, or medium spiny neurons.

The protocol described here can be adapted to look at the fundamental properties of synaptic proteins and/or the effects of pharmacological treatments on dendritic spines. Furthermore, it can be used to assess changes in the localization of endogenous synaptic proteins ranging from scaffold proteins to glutamate receptors. In addition, it allows not only detailed morphometric analysis of fixed spines, but also measurement of the effects of synaptic proteins or pharmacological treatment on dendritic spine motility. These time-lapse imaging techniques can also be modified to examine trafficking of proteins, with or without simultaneously examining dendritic spine morphology.

Detailed analysis of dendritic spine morphology and motility is an essential tool for investigating the potential effects of mutant synaptic proteins, and how mutations in these disease-associated synaptic proteins may affect synapse structure and function, thereby contributing to the pathophysiology of a number of disorders of the brain.

Disclosures

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