NIH Public Access
Author Manuscript

Calcineurin inhibition with systemic FK506 treatment increases dendritic branching and dendritic spine density in healthy adult mouse brain

Tara L Spires-Jones[1], **Kevin Kay**[1], **Roland Matsouka**[2], **Anete Rozkalne**[1], **Rebecca A Betensky**[2], and **Bradley T Hyman**[1]

[1]Massachusetts General Hospital/Harvard Medical School, MassGeneral Institute for Neurodegenerative Diseases, Charlestown, MA USA 02129

[2]Harvard School of Public Health, Boston, Massachusetts 02115

Abstract

Calcineurin has been implicated as part of a critical signaling pathway for learning and memory, and recent data suggest that calcineurin activation mediates some of the neurotoxicity of the Alzheimer related neurotoxin Aβ. Immunosuppression via calcineurin inhibition with the compound FK506 is an important treatment for organ transplant patients. Here we use Golgi impregnation techniques, along with a new survival analysis-based statistical approach for analysis of dendritic complexity, to show that in healthy adult mice one week of treatment with FK506 affects both the branching patterns and dendritic spine density of cortical neurons. These results indicate that calcineurin inhibition leads to readily detectable changes in brain morphology, further implicating calcineurin related pathways in both the function and structure of the adult brain.

Keywords

tacrolimus; FK506; dendritic spine; dendrite branching; Sholl plot; survival analysis

Introduction

Calcineurin has been long established as a critical mediator of cellular pathways underlying both electrophysiologic and behavioural measures of learning and memory, likely through mechanisms involving dephosphorylation of spine associated proteins as well as activation of specific transcriptional pathways [3,5–7,11,12,17,21,23]. Upregulation of calcineurin activity has also been implicated in Alzheimer disease models, where it is believed to be important for amyloid-beta induced loss of glutamate receptors and dendritic spine loss [2,16,27,31]. Inhibition of calcineurin has accordingly been shown to enhance memory performance in some paradigms in normal rodents, as well as in APP overexpressing Alzheimer models [8,20–22,32]. To better understand the underlying neurobiology of these

Corresponding Author: Bradley T Hyman, MD, PhD, 114 16th Street, Charlestown, MA 02129, Phone: 617-726-2299 Fax: 61-724-1480, bhyman@partners.org.

effects, we asked whether short term inhibition of calcineurin would impact brain structure in normal adult animals.

The calcineurin inhibitor FK506 (tacrolimus), is an inhibitor that acts via the complex of FK506 and FK506-binding protein binding to calcineurin to prevent calcineurin-mediated dephosphorylation [25]. FK506 is a commonly used immunosuppressant to combat graft versus host disease after transplant surgery [1]; however, serious neurological side effects occur in over 10% of FK506 treated patients including tremor, aphasia, cortical blindness, hallucinations, and memory impairment [19,30], underlying the need to clarify the effects of FK506 on healthy brain. FK506 (tacrolimus) is administered long-term after transplantation, but neurological side effects can manifest within days [10], thus we examined acute effects of systemic administration of FK506 on the brain.

Materials and Methods

Non-transgenic mice from Jackson laboratories (B6C3F1/J mice 6–8 months of age) were group housed in standard rodent cages with ad libitum access to water and mouse chow. A bolus of 0.1 mL/ 10g of mouse of 10mg/mL FK506 (Sigma Aldrich, St. Louis, MO - dissolved in 10% ethanol with 1% tween 80 in PBS [4], or vehicle (10% ethanol with 1% tween 80 in PBS without drug) was administered i.p. daily for 7 days (n=4 animals FK506, 4 animals vehicle treated).

After FK506 or vehicle treatment, mice were euthanized, perfused with PBS followed by 10% formalin, and post-fixed for 48 hours. All animals were sacrificed in one session (2 hours) to prevent variability in spine density due to the time of day. Brains were shipped to Neurostructural Research Labs (Tampa, FL) for rapid Golgi impregnation and sectioning to 120 µm. Layer II-III cortical neurons (located throughout the cortex) were sampled and analyzed for spine density and dendritic branching using Nikon Photobot and camera lucida. To analyze the dendritic branching, a Scholl analysis using concentric circles spaced 10 microns apart was used. To analyze spine density, dendrite segments from apical branches (n=83 dendrites from 4 FK506 treated animals, 85 from 4 vehicle treated animals) and basal dendrites (n=105 dendrites from 4 FK506 treated animals, 100 dendrites from 4 vehicle treated animals) were traced in fine detail including spines; the number of spines per neurite segment counted, and the image was scanned and the length of the dendrite measured using NIH software Image J to calculate linear spine density. Photomicrographs of dendritic spines were obtained on an Olympus BX51 microscope mounted with a DP70 camera and contrast enhanced in Adobe Photoshop.

To analyze the complexity of the dendritic tree from the Sholl plot data, we developed an algorithm using a survival analysis technique with follow-up "time" as the longest distance (from the cell body of a neuron) reached by each dendritic tree. The administrative censoring was set at the distance of 240µm from the soma. We considered two different events of interest: distance to zero intersections of dendrites with the concentric circles drawn around the soma and distance to one intersection. Due to the nature of the experiment, there were few "censored" (i.e., not observed all the way to 0 intersections) observations: 4 for distance to zero intersection and 3 for distance to one intersection. We tested the appropriate contrasts among the coefficients in the model to assess the effects of treatment.

For spine analyses, normality of data was confirmed using a Shapiro-Wilkes test. 1-way ANOVAs were used to asses the effects of treatment. These data are presented as means and standard deviations from the mean.

Results

To investigate the effects of calcineurin inhibition with FK506 on neuronal morphology, mice were treated for 1 week with daily i.p. injections of 0.1 mL/ 10g of mouse of 10mg/mL FK506, a dose known from previous rodent studies to achieve a brain level of FK506 of ~300 ng/gm tissue for at least 72 hours after the injection [4]. Mice were sacrificed after 7 days of i.p. injections and brains extracted for Golgi impregnation.

Golgi-impregnated layer II-III neurons from treated and untreated mice were traced using camera lucida. A total of 71 cells from 4 FK506 treated animals and 100 cells from 4 vehicle treated animals were traced (representative cells shown in figure 1A). Sholl plot analysis (Figure 1B, C) shows an increase in complexity of the basal dendritic tree of layer II–III neurons with FK506 treatment in wild-type mouse cortex. The treatment has a significant impact on the overall dendritic arbor (p-value=0.0104 and p-value = 0.0079 respectively for distance to zero and distance to one intersection). Those treated with FK506 have longer total dendritic arbor and more complex branching further away from the cell body compared to untreated animals.

Dendritic spines are known to be affected in many neurological disorders [9]. Basal and apical dendrites of layer II–II neurons differ in their inputs (basal and proximal apical dendrites receive excitatory inputs from layer IV neurons and local layer II–III neurons while more distal apical tufts receive inputs from thalamus and other cortical areas) and their distinct morphologies suggest that inputs to basal and apical dendrites might be integrated differently [28]. Thus we examined the dendritic spines along both apical and basal dendrites. We examined spines in FK506 and vehicle treated animals (basal dendrites n=105 dendrites from 4 FK506 treated animals, 100 dendrites from 4 vehicle treated animals; apical dendrites n=83 dendrites from 4 FK506 treated animals, 85 from 4 vehicle treated animals). We observed an increase in basal dendritic spine density (ANOVA $F[1,204]=11.8132$, p=0.0007) concomitant with the increased dendritic branching seen with Sholl plots.

Discussion

Dendrites integrate synaptic inputs to neurons, and their branching is thought to be related to their representational capacity [24]. Branching patterns of dendritic trees are related to the degree of compartmentalization of inputs to the cell and a stronger potential for compartmentalization (i.e. more complex branching) has been proposed to increase the representational power of the cell resulting in greater learning and memory capacity. [24] Dendritic structure appears to be regulated during development in part by calcineurin [26]. Dendritic spines, which comprise the post synaptic element of over 90% of cortical excitatory synapses, are thought to be particularly important for learning and memory [18]. Calcineurin activation has been implicated in the changes of dendritic spine morphology associated with long term depression [33]. Changes in dendritic spines and in dendritic branching are associated with many neurological disorders, underlying their importance in brain connectivity [9].

We investigated the effects of one week of systemic calcineurin inhibition using FK506 treatment on wild-type mouse neocortex to test the hypothesis that calcineurin affects neuroplasticity mechanisms, dendritic remodeling, and dendritic spines in the adult cortex using classical Golgi impregnation techniques, combined with a new statistical approach to analyze Sholl plot analyses of dendritic complexity based on survival analysis.

We observe a marked increase (15%) in basal dendritic spine density and an increase in the complexity of basal dendritic trees of neurons in animals treated with systemic calcineurin

inhibition for only one week. Our new analysis method for branching data gives an indication of the complexity of the entire arbor instead of comparing mean numbers of intersections at individual distances from the soma with t-tests as is often seen in the literature [15].

These data are in accord with our recent observations that introduction of a constitutively active form of calcineurin into neurons in the adult cortex (via AAV mediated gene transfer) had the opposite effect: decrease in dendritic spines and diminution of dendritic arbor complexity [31]. Both of these effects – of spine loss after exposure to calcineurin activation and of spine increases after exposure to calcineurin inhibition – are of substantially greater magnitude than expected for week to week changes in adult cortex, in which only minor changes in spine density occur and dendritic arborizations (of pyramidal neurons) remain unchanged over several months of observation in vivo [13,14,29].

These results suggest that alterations in calcineurin dramatically impact the morphology of dendritic systems, thereby potentially altering neural system efficiency and responses, even in the adult brain. The data suggest a rather larger degree of plasticity in both dendritic spine number and in dendritic arborization patterns than perhaps would have been expected in non-pathological conditions in the adult brain, since dendritic trees and dendritic spines are stable over long periods of imaging in vivo as mentioned above. It is possible that these structural changes in dendrites underlie both the improved memory performance seen in some paradigms after calcineurin inhibition, as well as potentially some of the observed neurological side effects in patients treated with FK506.

Acknowledgments

References

1. A comparison of tacrolimus (FK 506) and cyclosporine for immunosuppression in liver transplantation. The U.S. Multicenter FK506 Liver Study Group. N Engl J Med. 1994; 331:1110–1115. [PubMed: 7523946]

2. Agostinho P, Lopes JP, Velez Z, Oliveira CR. Overactivation of calcineurin induced by amyloid-beta and prion proteins. Neurochem Int. 2008; 52:1226–1233. [PubMed: 18295934]

3. Baumgartel K, Genoux D, Welzl H, Tweedie-Cullen RY, Koshibu K, Livingstone-Zatchej M, Mamie C, Mansuy IM. Control of the establishment of aversive memory by calcineurin and Zif268. Nat Neurosci. 2008; 11:572–578. [PubMed: 18425121]

4. Butcher SP, Henshall DC, Teramura Y, Iwasaki K, Sharkey J. Neuroprotective actions of FK506 in experimental stroke: in vivo evidence against an antiexcitotoxic mechanism. J Neurosci. 1997; 17:6939–6946. [PubMed: 9278529]

5. Chan GC, Tonegawa S, Storm DR. Hippocampal neurons express a calcineurin-activated adenylyl cyclase. J Neurosci. 2005; 25:9913–9918. [PubMed: 16251439]

6. Christie-Fougere MM, Darby-King A, Harley CW, McLean JH. Calcineurin inhibition eliminates the normal inverted U curve, enhances acquisition and prolongs memory in a mammalian 3'-5'-cyclic AMP-dependent learning paradigm. Neuroscience. 2009; 158:1277–1283. [PubMed: 19041926]

7. Deng PY, Lei S. Long-term depression in identified stellate neurons of juvenile rat entorhinal cortex. J Neurophysiol. 2007; 97:727–737. [PubMed: 17135466]

8. Dineley KT, Hogan D, Zhang WR, Taglialatela G. Acute inhibition of calcineurin restores associative learning and memory in Tg2576 APP transgenic mice. Neurobiol Learn Mem. 2007; 88:217–224. [PubMed: 17521929]

9. Fiala JC, Spacek J, Harris KM. Dendritic spine pathology: cause or consequence of neurological disorders? Brain Res Brain Res Rev. 2002; 39:29–54. [PubMed: 12086707]

10. Forgacs B, Merhav HJ, Lappin J, Mieles L. Successful Conversion to Rapamycin for Calcineurin Inhibitor-Related Neurotoxicity Following Liver Transplantation. Transplantation Proceedings. 2005; 37:1912–1914. [PubMed: 15919502]

11. Havekes R, Nijholt IM, Luiten PG, Van der Zee EA. Differential involvement of hippocampal calcineurin during learning and reversal learning in a Y-maze task. Learn Mem. 2006; 13:753–759. [PubMed: 17101875]

12. Havekes R, Nijholt IM, Visser AK, Eisel UL, Van der Zee EA. Transgenic inhibition of neuronal calcineurin activity in the forebrain facilitates fear conditioning, but inhibits the extinction of contextual fear memories. Neurobiol Learn Mem. 2008; 89:595–598. [PubMed: 17884610]

13. Holtmaat A, Svoboda K. Experience-dependent structural synaptic plasticity in the mammalian brain. Nat Rev Neurosci. 2009; 10:647–658. [PubMed: 19693029]

14. Holtmaat AJ, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW, Svoboda K. Transient and persistent dendritic spines in the neocortex in vivo. Neuron. 2005; 45:279–291. [PubMed: 15664179]

15. Hu F, Li G, Liang Z, Yang Y, Zhou Y. The morphological changes of pyramidal and spiny stellate cells in the primary visual cortex of chronic morphine treated cats. Brain Res Bull. 2008; 77:77–83. [PubMed: 18638530]

16. Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, Iwatsubo T, Sisodia S, Malinow R. APP processing and synaptic function. Neuron. 2003; 37:925–937. [PubMed: 12670422]

17. Kandel ER. The molecular biology of memory storage: a dialogue between genes and synapses. Science. 2001; 294:1030–1038. [PubMed: 11691980]

18. Knott G, Holtmaat A. Dendritic spine plasticity--current understanding from in vivo studies. Brain Res Rev. 2008; 58:282–289. [PubMed: 18353441]

19. Lee SH, Kim BC, Yang DH, Park MS, Choi SM, Kim MK, Cho KH. Calcineurin inhibitor-mediated bilateral hippocampal injury after bone marrow transplantation. J Neurol. 2008; 255:929–931. [PubMed: 18458866]

20. Lu YM, Mansuy IM, Kandel ER, Roder J. Calcineurin-mediated LTD of GABAergic inhibition underlies the increased excitability of CA1 neurons associated with LTP. Neuron. 2000; 26:197–205. [PubMed: 10798404]

21. Mansuy IM, Mayford M, Jacob B, Kandel ER, Bach ME. Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. Cell. 1998; 92:39–49. [PubMed: 9489698]

22. Miyakawa T, Leiter LM, Gerber DJ, Gainetdinov RR, Sotnikova TD, Zeng H, Caron MG, Tonegawa S. Conditional calcineurin knockout mice exhibit multiple abnormal behaviors related to schizophrenia. Proc Natl Acad Sci U S A. 2003; 100:8987–8992. [PubMed: 12851457]

23. Mulkey RM, Endo S, Shenolikar S, Malenka RC. Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature. 1994; 369:486–488. [PubMed: 7515479]

24. Poirazi P, Mel BW. Impact of active dendrites and structural plasticity on the memory capacity of neural tissue. Neuron. 2001; 29:779–796. [PubMed: 11301036]

25. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Immunol Today. 1992; 13:136–142. [PubMed: 1374612]

26. Schwartz N, Schohl A, Ruthazer ES. Neural activity regulates synaptic properties and dendritic structure in vivo through calcineurin/NFAT signaling. Neuron. 2009; 62:655–669. [PubMed: 19524525]

27. Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P. Regulation of NMDA receptor trafficking by amyloid-beta. Nat Neurosci. 2005; 8:1051–1058. [PubMed: 16025111]

28. Spruston N. Pyramidal neurons: dendritic structure and synaptic integration. Nat Rev Neurosci. 2008; 9:206–221. [PubMed: 18270515]

29. Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, Svoboda K. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature. 2002; 420:788–794. [PubMed: 12490942]

30. Wijdicks EF, Wiesner RH, Dahlke LJ, Krom RA. FK506-induced neurotoxicity in liver transplantation. Ann Neurol. 1994; 35:498–501. [PubMed: 7512320]

31. Wu HY, Hudry E, Hashimoto T, Kuchibhotla K, Rozkalne A, Fan Z, Spires-Jones T, Xie H, Arbel-Ornath M, Grosskreutz CL, Bacskai BJ, Hyman BT. Amyloid beta induces the morphological neurodegenerative triad of spine loss, dendritic simplification, and neuritic dystrophies through calcineurin activation. J Neurosci. 2010; 30:2636–2649. [PubMed: 20164348]

32. Zeng H, Chattarji S, Barbarosie M, Rondi-Reig L, Philpot BD, Miyakawa T, Bear MF, Tonegawa S. Forebrain-specific calcineurin knockout selectively impairs bidirectional synaptic plasticity and working/episodic-like memory. Cell. 2001; 107:617–629. [PubMed: 11733061]

33. Zhou Q, Homma KJ, Poo MM. Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses. Neuron. 2004; 44:749–757. [PubMed: 15572107]
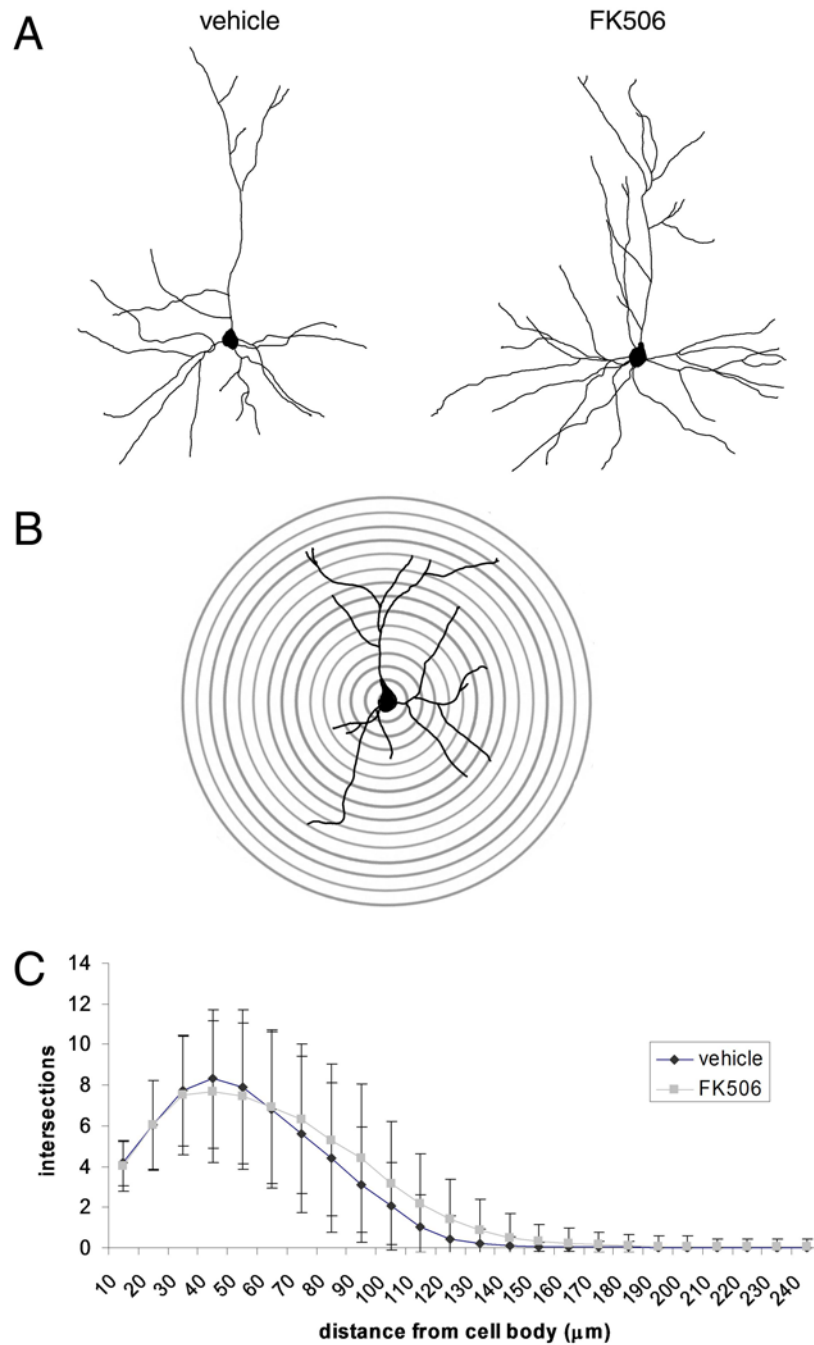
Figure 1.
Golgi-impregnated layer II–III cortical neurons were traced using camera lucida in FK506 and vehicle treated animals (A). A series of concentric circles every 10 µm (B) was used for Sholl analysis. Quantification (C) shows an increase in branching of basal dendrites with FK506 treatment, which is significant using our survival analysis method. Error bars represent standard deviation from the mean.
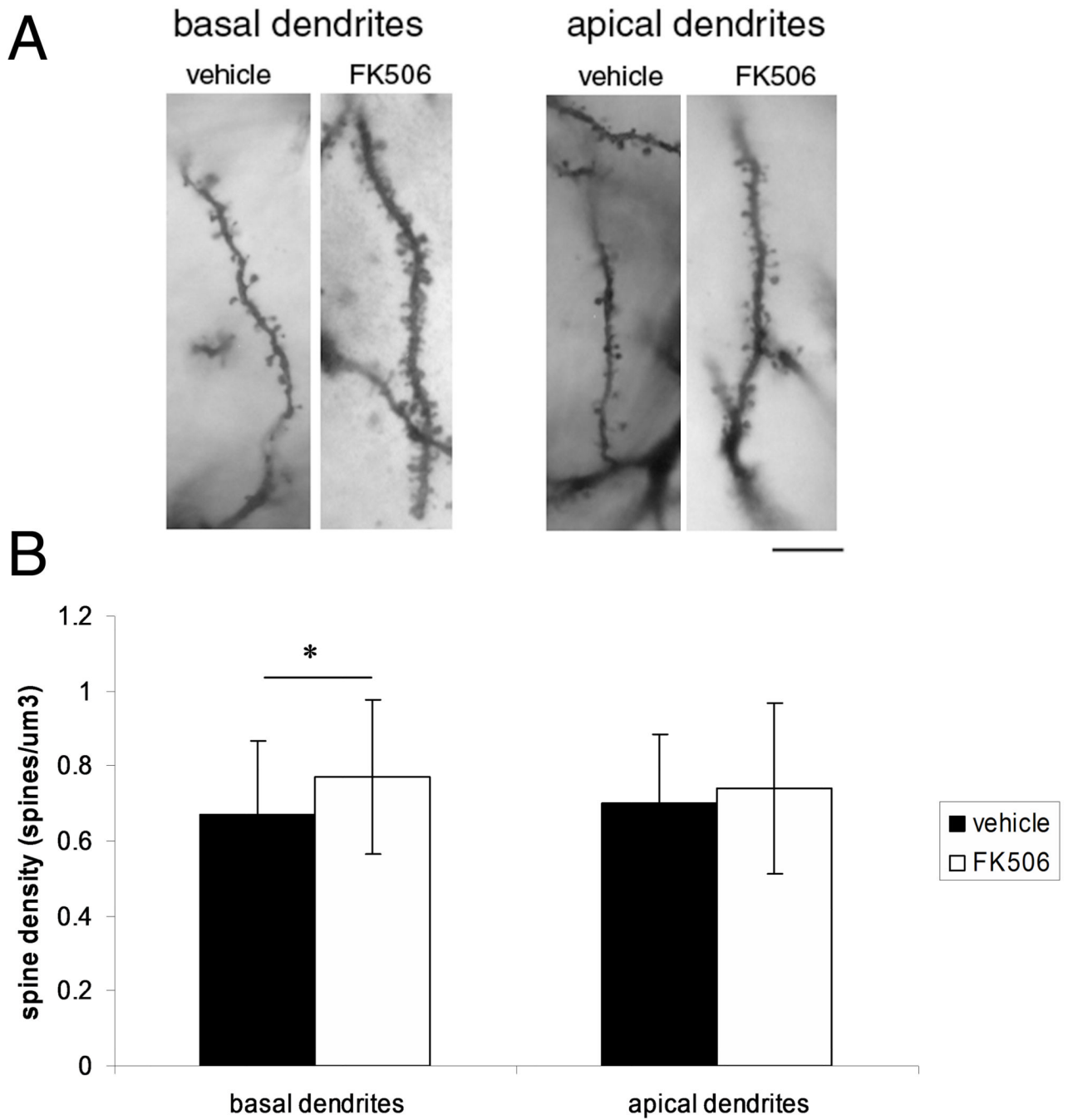
Figure 2.
Dendritic spines were examined on basal and apical dendrites of layer II–III pyramidal neurons (A). FK506 treatment increased spine density along basal dendrites (B)Scale bar represents 10 μm. Error bars represent standard deviation from the mean.