Dendritic spine heterogeneity and calcium dynamics in basolateral amygdala principal neurons

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Power JM, Sah P. Dendritic spine heterogeneity and calcium dynamics in basolateral amygdala principal neurons. J Neurophysiol 112: 1616–1627, 2014. First published June 18, 2014; doi:10.1152/jn.00770.2013.—Glutamatergic synapses on pyramidal neurons are formed on dendritic spines where glutamate activates ionotrophic receptors, and calcium influx via N-methyl-D-aspartate receptors leads to a localized rise in spine calcium that is critical for the induction of synaptic plasticity. In the basolateral amygdala, activation of metabotropic receptors is also required for synaptic plasticity and amygdala-dependent learning. Here, using acute brain slices from rats, we show that, in basolateral amygdala principal neurons, high-frequency synaptic stimulation activates metabotropic glutamate receptors and raises spine calcium by releasing calcium from inositol trisphosphate-sensitive calcium stores. This spine calcium release is unevenly distributed, being present in proximal spines, but largely absent in more distal spines. Activation of metabotropic receptors also generated calcium waves that differentially invaded spines as they propagated toward the soma. Dendritic wave invasion was dependent on diffusional coupling between the spine and parent dendrite which was determined by spine neck length, with waves preferentially invading spines with short necks. Spine calcium is a critical trigger for the induction of synaptic plasticity, and our findings suggest that calcium release from inositol trisphosphate-sensitive calcium stores may modulate homosynaptic plasticity through store-release in the spine head, and heterosynaptic plasticity of unstimulated inputs via dendritic calcium wave invasion of the spine head.

SYNAPTIC CONTACTS BETWEEN glutamatergic neurons in the brain are largely made on dendritic spines. These specialized compartments are enriched in glutamate receptors and a host of other molecules involved in synaptic transmission and plasticity (Okabe 2007; Sheng and Kim 2011). In hippocampal and cortical pyramidal neurons, synaptically released glutamate evokes a rise in spine calcium, primarily through activation of ionotropic N-methyl-D-aspartate (NMDA) receptors (Kovalchuk et al. 2000; Yuste and Denk 1995), although voltage-dependent calcium channels (Bloodgood and Sabatini 2007a) and calcium permeable \textalpha-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) or kainate receptors (Yuste et al. 1999) have also been reported to contribute in some instances. This rise in spine calcium triggers a series of downstream effectors that can initiate different forms of synaptic plasticity (Rochefort and Konnerth 2012). The thin spine neck connecting the spine head to its parent dendrite limits diffusion between the two compartments, allowing calcium and other signaling molecules to be concentrated at active synaptic inputs and thus limiting synaptic plasticity to only active synapses (Noguchi et al. 2005). Such synapse specificity is a central tenet of most cellular models of associative learning (Bliss and Collingridge 1993; Morris 2003).

In addition to ionotropic glutamate receptors, activation of metabotropic glutamate receptors (mGluRs) has long been known to contribute to learning and memory formation, and the role of these receptors in synaptic plasticity is well recognized (Anwyl 2009; Fendt and Schmid 2002; Rodrigues et al. 2002). In the hippocampus, cortex and basolateral amygdala (BLA), activation of mGluRs in pyramidal neurons evokes focal rises in dendritic calcium that propagate as waves along the dendritic tree (Hagenston et al. 2008; Jaffie and Brown 1994; Nakamura et al. 1999b; Power and Sah 2007; Pozzo-Miller et al. 1996). mGluRs are G protein-coupled receptors that generate inositol trisphosphate (IP\textsubscript{3}), and calcium waves result from activation of IP\textsubscript{3} receptors (IP\textsubscript{3}Rs), and release of calcium from intracellular stores by regenerative calcium induced calcium release (Power and Sah 2007; Ross 2012). As these calcium waves can enter the nucleus, it has been suggested that they may convey synaptic signals to the nucleus, initiating changes in gene transcription (Bengtson and Bading 2012; Berридge 1998; Power and Sah 2002).

Dendritic spines, the site of most glutamatergic synapses, express mGluRs (Lujan et al. 1997; Rodrigues et al. 2002) and also contain smooth endoplasmic reticulum (ER) (Ostroff et al. 2010; Spacek and Harris 1997) that is a major site of intracellular calcium stores. In cerebellar Purkinje neurons, high-frequency stimulation (HFS) of parallel fibers activates mGluRs, and the resultant calcium release within the spine head (Finch and Augustine 1998; Takechi et al. 1998) is critical for the induction of long-term depression (LTD) at these synapses (Miyata et al. 2000). However, for most pyramidal-like neurons, calcium release from spine ER calcium stores has been primarily attributed to calcium-induced calcium release via activation of ryanodine receptors (RyRs) (Bloodgood and Sabatini 2007b; Emplage et al. 1999; Korkotian and Segal 1999; Raymond and Redman 2006). Here we show that calcium release from IP\textsubscript{3}-sensitive stores contributes to the spine calcium signal by two different mechanisms. Synaptic activation of mGluRs releases calcium from calcium stores within the spine head and generates a propagating calcium wave in the parent dendrite. This propagating dendritic calcium wave also invades some spines, thereby delivering a heterosynaptic signal to nearby spines.
MATERIALS AND METHODS

All experiments were performed on 3- to 6-wk-old Wistar rats, of either sex in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Animal Ethics Committee for the University of Queensland. Coronial brain slices (350 μm) were prepared using standard techniques (Power and Sah 2007). Slices were incubated at 33°C for 30 min and then maintained at room temperature in artificial cerebral spinal fluid (ACSF) solution containing (in mM) 119 NaCl, 2.5 KCl, 1.3 MgCl₂, 2.5 CaCl₂, 1.0 Na₂HPO₄, 26.2 NaHCO₃, 11 glucose, equilibrated with 95% CO₂, 5% O₂. Slices were perfused with ACSF heated to 33°C, and whole cell recordings were obtained from principal neurons in the basal nucleus of the BLA. Patch pipettes (2–5 MΩ) were filled with an internal solution containing (in mM) 135 KMeSO₄, 8 NaCl, 10 HEPES, 2 Mg₂-ATP, 0.3 Na₃-GTP, 0.1 spermine (pH 7.3 with KOH, osmolality 280–290 mosM) and fluorescent indicators Fluo 5F and Alexa 594, as indicated. Electrophysiological signals were amplified with a Multiclamp 700A amplifier (Molecular Devices, Union City, CA), filtered at 2–5 kHz and digitized at 5–10 kHz with an ITC-18 board (Instrutech, Port Washington, NY), and controlled using Axograph (Axograph Scientific, Sydney, Australia). Only cells that had resting potentials more negative than −55 mV, action potential (AP) amplitudes (>100 mV, and input resistances >60 MΩ), were included in the data set. Projection neurons were distinguished from local circuit interneurons based on their AP half-width (>0.7 ms), their relatively small fast afterhyperpolarization (<15 mV), and their frequency-dependent spike broadening.

Two-photon fluorescence images were obtained with a Zeiss Axioskop 2FS with a 510 laser scanning head equipped with a Chameleon laser (Coherent, Santa Clara, CA) for two-photon excitation, as described previously (Power and Sah 2008). Neurons were loaded with the green fluorescent calcium indicator Fluo 5F (50–300 μM; Kd 2 μM; Invitrogen, Carlsbad, CA) and the red fluorescent calcium-insensitive Alexa 594 (30 μM; Invitrogen). Fluorescence images were acquired in line scan-mode (260 Hz) at a resolution of 10–20 pixels/μm. Calcium signals were calculated as the change in green fluorescence (Fluo 5F) normalized to the red fluorescence (Alexa 594), ΔG/R(r), for each region of interest. Data were restricted to spines perpendicular to the dendrite whose spine head extended at least 0.5 μm from the dendrite. For spines with short necks (<0.5 μm), the region of interest was limited to the distal portion of the spine head to avoid dendritic fluorescence contamination. Calcium signals were analyzed offline using custom software written using LabVIEW (National Instruments, Austin, TX).

Synaptic stimulation was performed using a bipolar stimulating electrode fabricated from double barreled theta glass (Harvard Apparatus TGC150–7.5, Kent, UK) pulled using a patch clamp puller to an −1-μm tip, filled with ACSF and placed 30–50 μm from the dendrite of interest located less than 100 μm from the soma. The stimulus used to stimulate calcium waves typically evoked a 5–10 mV excitatory postsynaptic potential (EPSP) prior to application of ionotropic antagonists. APs were evoked by trains (4 at 100 Hz) of brief (5 ms) depolarizing current injections (400–500 pA). Responses to AP trains and brief synaptic trains represent an average of three to six presentations. As the onset and time course of the calcium wave varied between stimulations, calcium wave measurements were performed on individual episodes, and the measurements were averaged. To improve the signal-to-noise ratio, calcium wave responses were digitally filtered with a 10-Hz low-pass zero phase-shift Bessel filter. Fluorescence signals were converted to calcium using methods described by Yasuda (Yasuda et al. 2004). Calcium wave measurements included the onset (10% peak), integrated area, peak (11.5-ms window) and half-width. On rare occasions, the peak fluorescence signal was well beyond the linear range of the indicator and quite unreliable. To minimize the effects of these error-prone values, estimates of peak calcium were capped at 10 μM. Depletion of intracellular calcium stores was prevented by maintaining the neuron at a slightly depolarized membrane potential (~50 to ~55 mV) (Power and Sah 2005).

Ruthenium red and low molecular weight heparin were added via the patch pipette. All other drugs were bath applied. Cyclopiazonic acid (CPA), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f][1]oxazol-7-sulfonamide (NBQX), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were prepared as stock solutions in DMSO. (RS)-α-methyl-4-carboxyphenylglycine (MCPG) was prepared as 200× stock solution in 100 mM NaOH. MCPG, NBQX and 2-amino-5-phosphonovaleric acid (APV) were purchased from Tocris (Bristol, UK). All other drugs were obtained from Sigma-Aldrich (St. Louis, MO). Unless otherwise specified, drugs were prepared as 1,000× stock solutions and stored frozen (~20°C) until required.

Spine morphology. To estimate spine head, volume and neck length z-stacks of the calcium-insensitive Alexa 594 fluorescence were deconvolved using the expected maximum algorithm (Conchello 1998) in XCLONS (http://www.esrsl.wustl.edu/~preza/xclons/). The dendrite diameter was defined as its full-width at half-maximum intensity (FWHM). The spine-neck length was calculated as the smallest distance between the FWHM of the spine head and the FWHM of the parent dendrite. The spine-head volume was estimated by integrating the total fluorescence of the spine head (Noguchi et al. 2005). The conversion factor for estimating volume from fluorescence was calculated by measuring the cylindrical volume of a segment of dendrite within the same focal plane and dividing by its integrated fluorescence. To track spine morphological changes over time, Alexa 594 fluorescence z-stacks (<0.5-μm steps) were acquired at regular intervals before and after calcium waves were evoked. For each time point, the three to five focal plane images that included the spine of interest were selected and compressed into a single xy images by integrating the fluorescence intensity of each pixel across the focal planes. To control for general changes in fluorescence intensity, the spine fluorescence signals were normalized to that of a dendritic region of interest within the focal plane. Spine screening and selection were performed prior to HFS.

Fluorescence recovery after photobleach. Diffusional coupling between the spine head and its parent dendrite was assayed using fluorescence recovery after photobleach (FRAP). Small rectangular frames (2 × 256 pixels) encompassing the calcium-insensitive Alexa 594 fluorescent signals of the spine and parent dendrite were acquired at 100–500 Hz. After a baseline period, the fluorescence signal in the spine head was bleached by briefly scanning the spine head at higher laser intensity. The recovery of the spine-head fluorescence as new fluorescent indicators diffuse back into the photobleached area was plotted over time, and the time course of the recovery was fitted to a single exponential. The rate of fluorescence recovery is correlated with the diffusional coupling of the spine and dendrite (Svoboda et al. 1996).

Statistical analysis. Statistical analysis was performed using Statview (SAS Institute). Comparisons between spines and parent dendrites were performed using paired t-tests. The significance of correlations was determined using Fisher’s r to z test. Data are given as means ± SE, unless indicated otherwise.

RESULTS

Synaptic stimulation evokes store release in dendritic spines. Whole-cell patch-clamp recordings and high-speed two-photon fluorescence images were acquired from principal neurons within the basal division of the BLA (Fig. 1A). EPSPs (2–10 mV) were evoked using a bipolar stimulating electrode placed within the basal nucleus. Brief trains of synaptic stimulation (1–5 stimuli; 50 Hz) evoked a time-locked rise in cytosolic calcium that was largely restricted to the spine head [Fig. 1, B and C1; n = 23/15/14 (spines/neurons/animals)], with little or
no calcium rise in the parent dendritic shaft. The change in spine calcium had fast kinetics, rising within 5 ms of the first or second stimulus and decaying immediately after the final stimulus. As in pyramidal neurons of the hippocampus and cortex (Muller and Connor 1991; Sabatini et al. 2001), application of AMPA and NMDA ionotropic glutamate receptor antagonists (n = 20/12/11) or the NMDA receptor antagonist d-APV by itself (Fig. 1B; n = 3/3/3) abolished the rapid calcium rise, showing that it results from influx of calcium via synaptic NMDA receptors. When ionotropic glutamate receptors were blocked (Fig. 1C2), tetanic stimulation (50–100 Hz; 0.5–1 s) often evoked a delayed [onset: 386 ± 429 ms (mean ± SD) from the start of stimulation; n = 13/10/8] rise in spine calcium that was accompanied by a calcium rise in the parent dendritic shaft (Fig. 1C2). The temporal relationship between the calcium rise in the spine and shaft was variable (Fig. 1F) with some instances (n = 7) in which the spine calcium rise preceded, and was larger than, that in the parent dendrite (Fig. 1C2), while in others the spine response was smaller and delayed relative to that in the parent dendrite (n = 6, Fig. 1, D, F, G). Furthermore, in some spines (5/13), a biphasic response was evident with an early calcium rise that was restricted to the spine and was followed by a secondary slower calcium rise that was synchronous with a rise in shaft calcium (Fig. 1E).

Trains of high-frequency synaptic stimulation of input to pyramidal neurons have previously been shown to activate
metabotropic receptors that evoke propagating calcium waves in the dendritic tree (Power and Sah 2002, 2007; Ross 2012). This calcium wave results from generation of IP_3 and release of calcium from intracellular stores in the dendritic shaft. Thus it is possible that the NMDA-receptor-independent rise in spine calcium (Fig. 1C2) results from diffusion of calcium from the dendritic shaft. However, spine calcium rises that are larger than and precede that seen in the parent dendrite (Fig. 1, F and G) cannot be explained by passive diffusion of calcium from the dendritic shaft into the spine head and indicate a calcium source intrinsic to the spine head.

In BLA projection neurons, group I mGluRs (Rodrigues et al. 2002) are expressed on spines, and many spines also contain smooth ER (Ostroff et al. 2010). Bath application of the mGluR antagonist MCPG (500 μM) reduced the NMDA-independent calcium response in the spine and its parent dendrite (Fig. 2A). The integrated area of the spine calcium response was reduced by 81 ± 9% (n = 3/2/2; P = 0.005). Consistent with the involvement of mGluRs, brief focal application of the mGluR agonist (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD) onto the proximal dendrites evoked a calcium rise in the dendritic shaft and attached spine (Fig. 2B; n = 21/12/12) that was similar to the synaptically evoked NMDA-independent response. Disrupting ER calcium stores with CPA blocked both the early and delayed components of the synaptically evoked spine calcium rises (Fig. 2C). CPA reduced the integrated area of the calcium response by 99 ± 0% (n = 3/3/3; P = 0.007). Both the early and delayed components of the NMDA-receptor independent spine calcium rise were still observed when the RyR blocker ruthenium red was included in the recording pipette (Fig. 2D). However, the integrated area of the NMDA-independent calcium rise tended to be smaller in the presence of ruthenium red in both the spine head (ruthenium red: median 0.075-s ΔG/R; n = 4/4/4; control: median 0.45-s ΔG/R; n = 18/14/9; Mann Whitney U, P = 0.057) and its parent dendrite (ruthenium red: median 0.22-s ΔG/R; n = 4/4/4; control: median 0.51-s ΔG/R; n = 14/14/9; Mann Whitney U, P = 0.18).

*Store release is restricted to proximal spines.* In BLA principal neurons, we have previously shown that intracellular store release within the dendritic shaft is prominent in proximal dendrites but less detectable in distal dendrites (Power and Sah 2007). We thus tested if calcium store release in dendritic spines is also differentially distributed throughout the dendritic tree. To avoid the confounding effects of calcium rises evoked by ionotropic glutamate receptors, mGluRs were selectively activated by focally applying ACPD onto different segments of the dendritic tree. Calcium is a co-agonist at IP_3Rs (Bezprozvanny et al. 1991; Finch et al. 1991; Nakamura et al. 1999b; Stutzmann et al. 2003); thus IP_3-mediated store release in spines will depend on both the concentration IP_3 and cytosolic calcium in the spine head. Thus, to maximize our ability to detect intracellular store release, AP trains were evoked during application of ACPD. AP-induced calcium influx through voltage-dependent calcium channels in the presence of IP_3 has been shown to initiate calcium release through IP_3Rs, resulting in an amplification of the AP-evoked calcium signal (Bezprozvanny et al. 1991; Finch et al. 1991; Nakamura et al. 1999b; Stutzmann et al. 2003). As in the dendritic shaft (Power and Sah 2007), in proximal spines, ACPD application efficiently amplified the AP-evoked calcium response (Fig. 3A), and this amplification was blocked by CPA (Fig. 3, B and D). The integrated area of the ACPD paired AP-evoked spine calcium response was 214 ± 25% (n = 5) of the unpaired AP-evoked response before application of CPA and 99 ± 15% of the unpaired AP-evoked response in the presence of CPA (P = 0.04; n = 53/2). Amplification of the calcium response was also blocked when the IP_3 antagonist heparin (500 μg/ml) was included in the patch pipette (Fig. 3, C and E). For spines located on primary dendrites, the AP-evoked calcium rise in the presence of ACPD in heparin loaded neurons was 99 ± 8% (median 107%; n = 5/5/2) of the control AP-evoked response, significantly smaller (Mann-Whitney U; P = 0.003) than the 207 ± 32% (median 156%; n = 16/9/9) amplification in control spines located on similar dendrites. These results show that amplification of AP-evoked calcium response by ACPD resulted from release of ER calcium stores.

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*Fig. 2. Metabotropic glutamate receptor (mGluR)-mediated store release raises spine calcium. A: calcium response (top traces) in the shaft and spine in response to tetanic stimulation (bottom trace) before (black traces) and after application of (RS)-α-methyl-4-carboxyphenylglycine (MCPG) (500 μM). B: iontophoretic application of the mGluR agonist (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD) evokes a delayed rise in spine calcium, but no change in whole cell current. The fluorescent calcium rise for the spine (black) and dendritic shaft (gray) are plotted. The whole cell current (V_m = 60 mV) is shown in the bottom trace. Solid bar indicates the timing of the 2-s ACPD application. C: calcium response (top traces) in the shaft and spine in response to tetanic stimulation (bottom trace) before (black traces) and after application of cyclopiazonic acid (CPA) (30 μM); D: cell loaded with ruthenium red (RR). Top traces show the calcium response in the shaft and spine for a cell loaded with RR. The bottom trace shows the voltage clamp response to synaptic stimulation. Ionotropic receptors have been blocked with APV and NBQX. CTL, control.*
As with store release in the dendritic shaft, calcium release in dendritic spines was also differentially distributed. In proximal dendrites, amplification of the AP-evoked calcium rise was evident in both dendritic shaft and spine (Fig. 3F). However, in distal dendrites, amplification was often not present in the shaft or the spine head (Fig. 3G). For spines located on primary dendritic branches \((n = 16/9/9)\), application of ACPD increased the peak and integrated area of the AP-evoked calcium rise in spines and shaft compared to control conditions, with the integrated area of the calcium rise evoked by APs with ACPD, normalized to the AP-evoked calcium rise, plotted for spines located on primary dendrites under CTL conditions and in the presence of heparin (Fig. 3H).

**Fig. 3.** Metabotropic glutamate agonists raise spine calcium via activation of inositol trisphosphate receptors (IP₃Rs). A: calcium response in the shaft and spine evoked by 2 action potential (AP) trains (bottom traces) are shown in the absence (left; APs) and presence (right; ACPD + APs) of ACPD. B: calcium response to APs, before (gray) and after ACPD (black) from the shaft and spine have been superimposed. Traces on the left are baseline, and on the right follow application of CPA (30 μM). C: example of calcium rises evoked by APs (gray traces) and APs + ACPD (black traces) are shown when the IP₃R antagonist heparin was included in the patch pipette. D: integrated area of the AP-evoked calcium rise in the presence of ACPD, normalized to the AP-evoked response in the absence of ACPD, is plotted before and after bath application of CPA (30 μM). Solid circles indicate means ± SE. *\(P < 0.05\). E: integrated area of the AP-evoked calcium rise with ACPD, normalized to the AP-evoked calcium rise is plotted for spines located on primary dendrites under CTL conditions and in the presence of heparin. **\(P < 0.01\). F: representative calcium rise evoked by APs (gray traces) and APs + ACPD (black traces) are plotted overlaid for a spine and its parent dendrite located on primary dendrites. G: representative calcium rise evoked by APs and APs + ACPD for a spine and its parent dendrite located on a higher order dendrite. H: cumulative histogram of integrated area of the calcium response evoked by APs paired with ACPD normalized to that evoked by APs in the absence of ACPD, for spines located on the primary (1, thin; \(n = 16\)), secondary (2, thick; \(n = 31\)), and higher order (3+, gray; \(n = 15\)) dendritic branches. I: an example of spine located on a higher order dendrite in which ACPD amplified the AP-evoked calcium rise. J: amplification of calcium response in the spines calculated as the integrated area of the calcium rise evoked by APs with ACPD, normalized to the AP-evoked calcium rise, is plotted against the amplification in the parent shaft dendrite. The line is the unity slope line indicating equal amplification. K: top: a fluorescent image of a dendritic and spine showing scan line of interest (gray box) and photobleach region (white box). Middle: fluorescence (F) of the scan line over time. The time of spine photobleach is indicated by the arrow. Bottom: time course of spine fluorescence recovery [fluorescence recovery after photobleach (FRAP)] has been fit with a single exponential (thin line) with the indicated time constant. L: FRAP recovery time constant is plotted against the extent of spine invasion calculated as the integrated area of the ACPD component of the spine calcium response normalized to the dendritic ACPD component.
calcium rise by 19 ± 7% (P < 0.05) and 107 ± 32% (P < 0.001), respectively. For spines located on secondary branches (n = 31/17/12), the peak and area increased by 23 ± 5% (P < 0.001) and 100 ± 45% (P < 0.01), respectively. In contrast, in distal dendrites, only 3/15 spines showed any amplification, and on average there was no significant amplification of either the peak (9 ± 5%; P = 0.16) or integrated area (12 ± 8%; P = 0.78) of the AP-induced calcium response (n = 15/10/10) (Fig. 3, H and I).

Amplification of the AP-evoked calcium response by mGluR activation was present in both spines and the parent dendrite, and the spine store calcium response was highly correlated with that of its parent dendrite (Fig. 3J; r = 0.785; P < 0.0001; n = 60). However, the amplified portion of the calcium response in the spine was generally smaller than in the shaft (Fig. 3J). This difference could be due to an intrinsic difference in the store release capacity of the two compartments, or simply result from calcium release in the dendritic shaft and its diffusion into the spine. Dendritic spines are diverse in both shape and intracellular constituents, even along the same dendritic segment (Sorra and Harris 2000), and these differences can control the diffusional coupling between the shaft and spine head. If the mGluR-evoked amplification of spine calcium rise primarily reflects diffusion of calcium from the shaft into the spine, then its amplitude should be dependent on the extent of diffusional coupling between the two compartments. To measure diffusional coupling between the spine and its parent dendrite, we photobleached Alexa 594 fluorescence in the spine head and determined the rate of FRAP (Svoboda et al. 1996). FRAP in spines of BLA neurons could be fit with a single exponential that showed wide variation in recovery time constants indicate greater diffusional isolation. There was no correlation (r = 0.25; P = 0.32; n = 17/86/6) between the FRAP time constant and the relative area of the ACPD-augmented portion of the calcium rise (Fig. 3L), indicating that the spine calcium response evoked by mGluR stimulation is derived, at least in part, from within the spine head. Moreover, AP trains evoked a rapid rise in calcium in every spine examined, confirming that the absence of amplification is due to lack of intracellular store release.

Spine invasion by calcium waves. We have shown that synaptically released glutamate activates postsynaptic ionotropic glutamate receptors and results in a rise in cytosolic calcium that is restricted to the active spine head (Fig. 1). However, in proximal dendrites, repetitive stimulation of glutamatergic afferents leads to activation of mGluRs, generation of IP3, and release of intracellular calcium stores. This calcium release is not restricted to the spine head, but also raises calcium in the parent dendrite (Fig. 1) that can propagate through the dendritic tree (Power and Sah 2002; Ross 2012). We next tested if this propagating calcium wave could also invade neighboring unstimulated spines. Unstimulated spines were defined as those that showed no detectable calcium rise to brief synaptic trains (50 Hz, 100–200 ms) under control conditions, indicating they do not receive input from stimulated afferents. After blocking inotropic receptors (NBQX + d-APV) to avoid evoking APs due to synaptic summation, tetanic stimulation (50–100 Hz, 1 s) evoked a dendritic calcium wave (Fig. 4, A–C). As this calcium wave propagated in the dendrite, some spines were apparently shielded with little or no calcium rise in the spine (Fig. 4D). In contrast, other spines showed a robust calcium rise that closely matched that of the propagating wave in the parent dendrite (Fig. 4E). Spines that had a small or no calcium rise (Fig. 4D) were confirmed to be connected to the parent dendrite, and not photodamaged, as AP-induced rises in the shaft and spine were instantaneous and time locked (Fig. 4D, panel on right). Notably, this heterogeneity in spine calcium invasion was apparent even between spines located on the same dendritic segment (Fig. 4F), implying that the heterogeneity resulted from differences between spines.

In spines invaded by the propagating calcium wave, the calcium rise in the spine head was smaller (66 ± 9%; n = 21/13/12, P < 0.01) than in the parent dendrite (Fig. 4G), and its onset was delayed relative to that in the parent dendrite (234 ± 60 ms after the shaft, n = 20/13/12, P < 0.01) (Fig. 4H). The rise times for the calcium transient in the shaft and spine were similar, 496 ± 77 ms and 467 ± 98 ms, respectively, but the half-width of the calcium rise was significantly less in the spine (752 ± 100 ms) than in the parent dendrite (884 ± 107 ms; P = 0.01, n = 20/13/12). In contrast to synaptic stimulation, APs evoked a near instantaneous calcium rise in both spine and parent dendrite (Fig. 4D), and the calcium rise in the spine was generally larger in amplitude (160 ± 18% of that in the parent shaft; n = 18/11/10; P = 0.0016) and decayed faster (time constant 256 ± 39 ms, compared with 400 ± 30 ms; P = 0.0012, n = 18/11/10) in the spine compared with the shaft.

The delayed calcium response to synaptic stimulation was abolished in both the spine and dendrite when store release was disrupted by application of CPA, confirming that it required calcium release from ER calcium stores (Fig. 4I; n = 5/2/2). Blocking RyRs with ruthenium red had little or no effect in spine invasion (Fig. 4, J and K), with the relative area of the spine calcium response being 54 ± 14% in the presence of ruthenium red (n = 5/3/3) compared with 66 ± 9% in controls (n = 21/13/12; P = 0.53). Thus, in BLA principal neurons, RyRs are not required for the rise in spine calcium.

Role of spine geometry. We have shown that, as synthetically evoked calcium waves propagate along a dendritic segment, spines on that segment show a calcium rise that is highly variable in amplitude and is delayed with respect to that in the parent dendrite. As these spines were not innervated by the axons we were stimulating, it is unlikely that the spine calcium rise results from generation of IP3 within the spine. Thus the delayed rise in spine calcium could be due to diffusion of IP3, or calcium, into the spine from the parent dendrite. Consistent with a diffusible process, calcium wave invasion of spines was inversely correlated (Spearman’s rank correlation r = −0.8473; P < 0.0001; n = 19/11/10) with the FRAP time constant (Fig. 5, A and B), showing that spines diffusively isolated from the dendrite (long FRAP time constants) show little calcium wave invasion (Fig. 5, A and B).

The geometric features of the spine, such as its volume, neck width, and neck length, control its diffusional coupling to the parent dendrite (Holthoff et al. 2002; Nimchinsky et al. 2002; Noguchi et al. 2005). The spine head volume and neck length were estimated from deconvolved Z-stack images of the calcium-insensitive fluorophore Alexa 594. Invasion of spines by...
synaptically evoked calcium waves was inversely correlated with spine-neck length \( r = -0.76; n = 19/11/10; P < 0.01 \) (Fig. 5C), and positively correlated with the FRAP time constant \( r = 0.663; n = 19/11/10; P < 0.01 \) (Fig. 5D). However, there was no correlation between spine head volume and either spine invasion \( r = 0.16; P = 0.57 \) (Fig. 5C2), or diffusional coupling \( r = 0.032; P = 0.91 \). Nor was there any correlation between the dendrite diameter and spine invasion \( r = -0.42; P = 0.87 \) (Fig. 5C3) or diffusional coupling \( r = -0.095; P = 0.72 \). These correlations show that calcium entry into spines during wave propagation is dependent on their diffusional coupling to the dendritic shaft, which is a function of the length of the spine neck (Nimchinsky et al. 2002). Indicator buffering also reduces the probability of evoking store release and the spatial extent of wave propagation (Watanabe et al. 2006). To determine how spine invasion by propagating calcium waves is influenced by the exogenous buffer, we repeated the above experiments with different concentrations of calcium indicator. As the indicator concentration was lowered, tighter diffusional coupling was required for comparable levels of spine invasion (Fig. 5E). Extrapolating our results to indicator-free conditions, we estimate that invasion still occurs in the absence of calcium indicator, but is likely to be limited to those spines closely coupled to the dendrite (Fig. 5F).

**Effect of wave invasion on spine morphology.** Rises in spine calcium are known to trigger long-term changes in synaptic strength (Zucker 1999); therefore, we examined whether calcium from passing calcium waves alters the strength of synapses as they propagate along the dendrite. Changes in the
strength of the EPSPs during synaptic plasticity are associated with concomitant changes in the volume of the spine head receiving that input, with increases in synaptic strength being accompanied by an increase in spine volume (Matsuzaki et al. 2004), whereas a decrease in synaptic strength is accompanied by a reduction in spine volume (Zhou et al. 2004). In spines receiving direct synaptic input, as evidenced by a spine calcium response to brief stimulation, HFS (5 trains of 100 Hz, 1 s), which stimulates NMDA-receptor dependent LTP (Johansen et al. 2011), resulted in an increase in spine volume with an overall increase of 11% above baseline 10 min after HFS (Fig. 6, A, B, and G; P = 0.02; n = 7/5).}

We next examined spines that did not receive direct synaptic input, but tetanic stimulation generated a calcium wave that propagated in the parent dendrite. As calcium waves preferentially invade spines with short necks (Fig. 5), we separated the data into short-necked spines (< 0.7 μm) that are generally invaded by calcium waves, and long-necked spines (> 0.7 μm) that are less likely to be invaded. When waves propagated past short-necked spines, there was a reduction in spine head volume 10 min after HFS (89 ± 4% of baseline; n = 13; P = 0.035; Fig. 6, C–G). In contrast, spine volume was unchanged (102 ± 5% of baseline; n = 12; P = 0.68; short vs. long, P = 0.079) in long-necked spines, which are likely to be shielded from dendritic waves (Fig. 5C). In spines that did not receive synaptic inputs, and where no calcium waves were evoked in the parent dendrite, there was no change in spine volume, regardless of the neck length, being 99 ± 2% of baseline for short-necked spines (n = 34; P = 0.73) and 102 ± 3% of baseline for long-necked spines (n = 30; P = 0.46) 10 min after tetanic stimulation. Importantly, the reduction of spine volume was greater in short-necked spines located on dendritic segments, where the wave was evoked, than in short-necked spines on waveless dendrites (P = 0.04). In a cohort of dendrites, spine volume was followed for 20 min post-HFS, and short-necked spines on these dendrites spines with short necks also showed a reduction in volume at 20 min (88 ± 5% of baseline; n = 6; P = 0.06). Short-necked spines on waveless dendrites also showed a slight reduction in spine head volume at the 20-min time point (93 ± 3% of baseline; n = 24; P = 0.02), suggesting that short-necked spines may be more susceptible to changes in spine volume. In contrast, the volume of long-necked spines was unchanged in both wave (106 ± 5% of baseline; n = 5; P = 0.35) and waveless conditions (102 ± 5% of baseline; n = 14; P = 0.66).

**DISCUSSION**

As with other pyramidal neurons, glutamatergic afferents to principal neurons in the BLA preferentially make synapses...
onto dendritic spines. We have shown that, in BLA principal neurons, synaptically released glutamate can raise cytosolic calcium by multiple mechanisms. Single and brief synaptic trains activate postsynaptic ionotropic NMDA receptors and results in rapid influx of calcium that is restricted to the innervated spine head. Longer trains of stimuli activates metabotropic receptors that have two actions: the release of calcium from intracellular stores within the spine head, and the generation of calcium waves that spread through the parent dendrite, invading some nearby spines. All three mechanisms of calcium rise may initiate different forms of synaptic plasticity.

In the mammalian nervous system, glutamatergic synapses are largely made onto dendritic spines. These synapses are characterized by an electron-dense postsynaptic density (the PSD) within which are embedded ionotropic glutamate receptors, as well as a myriad of proteins involved in controlling synaptic stability and strength (Okabe 2012; Sheng and Kim 2011). Synaptically released glutamate activates ionotropic AMPA/kainate and NMDA receptors, and the resultant calcium influx leads to a rise in cytosolic calcium within the spine head (Guthrie et al. 1991; Yuste and Denk 1995). Dendritic spines are connected to the parent dendrite by a spine neck. The presence of calcium buffers, uptake and extrusion mechanisms within the spine head, as well as the narrow diameter of the neck, ensures that the calcium rise is restricted to the spine receiving synaptic input (Yuste et al. 2000). This rise of spine calcium can initiate several forms of synaptic plasticity, and its compartmentalization to spines receiving synaptic input underpins the input specificity of synaptic plasticity. As a result, many forms of synaptic plasticity are highly sensitive to blockade by agents that block calcium influx, such as NMDA-receptor antagonists (Johansen et al. 2011; Morris 2003; Pape and Pare 2010).

Neurons also express a variety of mGluRs, and activation of group I mGluRs leads to hydrolysis of phosphoinositide and generation of IP₃ (Pin and Duvoisin 1995). Activation of IP₃Rs on smooth ER results in release of intracellular calcium stores, either by directly opening IP₃R channels, or due to the synergistic action of calcium at partially activated IP₃Rs (Nakamura...
et al. 1999a). In BLA principal neurons, as in other pyramidal cells, brief activation of glutamatergic synapses evokes a rapid, spine-specific rise in calcium that results from activation of ionotropic glutamate receptors and consequent calcium influx (see also Humeau et al. 2005). In this study, we have shown that tetanic stimulation of the same afferents activated mGluRs that evoked a delayed rise in calcium in both the spine and its parent dendritic shaft. Activation of mGluRs on pyramidal neurons is known to release calcium stores in the dendritic shaft (Power and Sah 2007; Ross 2012). Several lines of evidence indicate that the rise in spine calcium is not due to diffusion from the parent shaft, but results from calcium release from IP$_3$-sensitive ER calcium stores within the spine head. Firstly, if calcium was entering the spine from the shaft, the calcium rise in the shaft has to precede that seen in the spine head. However, in many cases, the delayed rise in spine calcium preceded that seen in the dendritic shaft. Second, in spines that received glutamatergic inputs, there was no correlation between the extent of diffusional coupling between the dendrite shaft and spine (as assessed by FRAP at the spine head), and the amplitude of the delayed spine calcium rise, contrary to the expectation from simple diffusion of calcium.

Third, a subset of spines is known to contain smooth ER (Ostroff et al. 2010; Spacek and Harris 1997), the source of released calcium, and disrupting this calcium store with CPA blocked the spine calcium response. Finally, mGluRs have been found to be located in the perisynaptic region of spines head, consistent with the requirement of repetitive stimulation (Lujan et al. 1997). While IP$_3$-mediated store release in spines is well established in Purkinje neurons (Finch and Augustine 1998; Takechi et al. 1998), in pyramidal neurons, calcium release from spine ER calcium stores has been attributed to activation of RyRs (Bloodgood and Sabatini 2007b; Empetage et al. 1999; Korkotian and Segal 1999; Raymond and Redman 2006). Our results are in agreement with the findings of Holbro and colleagues (2009), who also reported mGluR-dependent IP$_3$-mediated spine calcium rise in a subset of spines in hippocampal pyramidal neurons (Holbro et al. 2009).

The delayed rise in calcium evoked by mGluR stimulation was not restricted to the spine head, but was also apparent in the parent dendrite, where it propagated as a calcium wave (Power and Sah 2007). This propagating calcium wave invaded spines with short necks that show less diffusional isolation from their parent dendrite, but was excluded from long-necked spines. While diffusional coupling can also be partly influenced by spine head volume, we show that the spine neck is the key feature that determines spine invasion from the shaft. Thus, while spine geometry is known to be important for concentrating NMDA-evoked calcium within the spine head (Nimchinsky et al. 2002; Noguchi et al. 2005), our results show that it also acts to occlude dendritic calcium signals from the spine head.

During spine invasion by propagating calcium waves, either IP$_3$ diffuses from the dendrite into the spine head, activating IP$_3$Rs within the spine head, or calcium itself diffuses from the dendrite into the spine head. Our data are consistent with either diffusional process, and potentially both processes could occur. Diffusion of calcium is spatially and temporally limited by calcium pumps and exchangers, and calcium handling within the spine head is thought to be so rapid, due to its large surface-to-volume ratio, that calcium ions entering the spine head during synaptic stimulation are removed from the cytosol before they can traverse the spine neck (Nimchinsky et al. 2002). However, as calcium waves propagates in the dendrite, the sustained calcium rise in the larger proximal dendritic may overwhelm the calcium extrusion processes along short spine necks, explaining the relatively long-lasting calcium transients in these spines.

Synaptically released glutamate evoked large rises in spine calcium by activating NMDA receptors, and HFS at these synapses lead to an increase in spine volume. Tetanic stimulation of glutamatergic afferents to BLA principal neurons is well known to lead to potentiation of these synapses (Johansen et al. 2011; Morris 2003; Pape and Pare 2010), and the increase in spine volume we report is consistent with this (Matsuzaki et al. 2004). In contrast, short-necked spines, not innervated by activated afferents, but invaded by propagating calcium waves, showed a short-lived decrease in spine volume but long-necked spines, shielded from the dendritic calcium wave, were unaltered. While not directly demonstrated here, this finding suggests an (albeit brief) reduction in synaptic strength at these synapses (Zhou et al. 2004). It was notable that the rise in spine calcium as a result of wave invasion is considerably less than that evoked by calcium influx via NMDA receptors. Thus these results are in general agreement with the predictions of the Bienenstock-Cooper-Munro model for synaptic plasticity (Bienenstock et al. 1982; Yeung et al. 2004), in which large calcium rises are lead to synaptic enhancement, whereas smaller calcium rises result in a reduction in synaptic strength.

Calcium waves and IP$_3$Rs have been implicated in heterosynaptic depression of unstimulated inputs following the induction of LTP (Nagase et al. 2003; Nishiyama et al. 2000; Royer and Pare 2003), and our results provide one possible explanation for these results.

Finally, one interesting prediction of our results is that this heterosynaptic LTD will be restricted to short-necked spines in a dendrite showing a propagated calcium wave. In BLA principal neurons, store release is prominent in the proximal primary and secondary dendritic branches, but is largely absent in the distal dendrites (Power and Sah 2007). We find that, in these neurons, store release in dendritic spines is similarly distributed with little or no release in the distal dendrites. Thus such heterosynaptic plasticity is expected to be restricted to the proximal dendritic tree. The significance of these electrotonically privileged synapses is currently unclear, as the BLA is not a laminar structure, and inputs from different brain regions are interspersed along the dendrites (Humeau et al. 2003; Sah et al. 2003).

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).
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