Dendritic spines do not split during hippocampal LTP or maturation

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Synapse splitting is considered a viable mechanism for increasing synaptic coupling between neurons[1–5]. We investigated this issue by reconstructing dendrites, axons and synapses from hippocampus during long-term potentiation (LTP) and maturation. Long, mature axons passed between neighboring dendritic spine synapses, precluding their formation by splitting.

Each dendritic spine in the hippocampus typically receives one excitatory synapse on its head, which is occasionally segmented into multiple active zones. These 'segmented synapses' have evoked much speculation regarding their possible role in synaptic plasticity[6]. One suggestion is that they represent an intermediate stage during the formation of a new synapse by splitting[1]. The spine would also split to regain the normal configuration of one synapse per spine head. Recent reports show that segmented synapses increase transiently after LTP induction in the hippocampus, then return to control levels within an hour[7,8]. This decline is accompanied by an increase in spine pairs from a single dendrite that contact the same presynaptic bouton. These 'same-dendrite, multiple-synapse boutons' (sdMSBs) are now widely interpreted as evidence for spine splitting as a basis for storing information and permanently increasing synaptic strength[2–5].

There are mechanistic and functional implications of this hypothesis that may be tested empirically. Mechanistically, if sdMSBs are the result of spine splitting, stable structures should not exist between the split spines, as they would have prevented the spines from moving laterally on the dendrite. Functionally, if sdMSBs are the signature of synapses strengthened by experience, they should accumulate as synaptic networks mature. To address these issues, we investigated the structure and frequency of sdMSBs during LTP and during maturation in hippocampal area CA1.

LTP was induced by tetanic stimulation to one of two sites in stratum radiatum in each of four hippocampal slices from male Long-Evans rats at postnatal day 15 (PN15)[9] (**Fig. 1a**). The other site received control low-frequency stimulation. LTP was expressed only in response to stimulation from the tetanization electrode, demonstrating that different axons were stimulated from the two sites (**Fig. 1b**). Slices were fixed 2 h after tetanic stimulation. Synapses were analyzed along dendrite reconstructions from serial section electron microscopy located within 100 μm of each stimulating electrode (trapezoids in **Fig. 1a**). A total of 69 dendritic segments were reconstructed, 36 averaging 5.04 ± 0.49 μm from the control and 33 averaging 5.65 ± 0.10 μm from the tetanized sites. The density of spines did not differ significantly between the control (1.55 ± 0.16 per μm) and tetanized (1.62 ± 0.24 per μm, $p = 0.93$) sites. The presynaptic boutons were distinguished as having one or multiple synapses (MSBs). The difference between the relative frequency of MSBs at control versus tetanized sites did not reach statistical significance (control, 0.28 ± 11; tetanized, 0.48 ± 0.19 per μm; $p = 0.06$). Seven sdMSBs (**Fig. 1c**) were found at the tetanized sites on six different dendrites in three of the experiments, while no spines contacted sdMSBs from any of the control dendrites. The mean frequency of sdMSB synapses at tetanized sites was 0.07 ± 0.027 per μm of dendrite (LTP, **Fig. 1d**), and is consistent with the low frequency of sdMSBs detected after theta-burst LTP in organotypic hippocampal slice cultures[7].

To determine if the sdMSB synapses could have arisen from splitting, the intervening neuropil was reconstructed in each case (**Fig. 2**). The gap that separated the two spines (**Fig. 1c**) had on average 3.1 ± 0.86 axons running through it. One gap contained seven axons (**Fig. 2a,b**). Interestingly, one of the synapses that shared this sdMSB was on a branched spine, the other head of which synapsed with a different axon. This observation shows that different heads of branched spines synapse with different axons[10], and that two spines on an sdMSB are not simply 'daughter' spines formed from splitting. The axons extending through the gap were traced through serial sections; they had a mature appearance and formed synapses with other dendrites (**Fig. 2c,d**). The maximum gap width varied from 0.3 to 0.95 μm, which is too small to accommodate the passage of axonal growth cones. Moreover, the convergence of many axonal growth cones through these small gaps during the 2 h after tetanization is extremely improbable. Therefore, tetanus-induced LTP did not produce sdMSBs by splitting a pre-existing spine synapse.

Next we assessed whether sdMSBs might represent synapses strengthened by splitting during maturation. Spiny dendrites in the middle of stratum radiatum of hippocampal area CA1 were reconstructed from postnatal day 21 (PN21) rats, using 3 perfusion-fixed rats and 3 control slices fixed after 3 h *in vitro*. Sixty dendritic segments, each approximately 6 μm long, were reconstructed from the 6 volumes. The PN15 dendrites were less spiny (1.58 ± 0.08 per μm) than the PN21 dendrites (2.83 ± 0.10 per μm; $p < 0.0001$), which were of comparable spine density to adult dendrites (3.0 ± 0.8 per μm[11]). The PN21 dendrites made synapses with 828 boutons. Of these, 243 (29%) were MSBs, consistent with the average frequency observed in adults[12]. Ten sdMSBs were found on the PN21 dendrites, five in the perfusion-fixed material

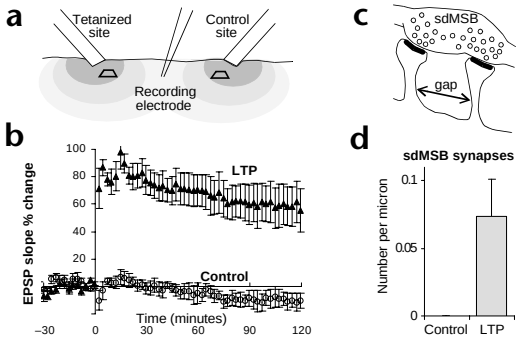

Fig. 1. sdMSB synapses are found only at tetanized sites in PN15 hippocampal slices. (**a**) Cross-section through stratum radiatum illustrates fall-off in density of synapses activated by each stimulating electrode. Serial EM samples were taken from the densely stimulated regions (trapezoids). (**b**) Pairs of tetanic stimuli (0.1-ms pulses at 100 Hz for 1 s, 20 s interval) were delivered to induce LTP (triangles)[9]. The same number of pulses were delivered pretetanus to the control site at low frequency (5 Hz). (**c**) sdMSBs form synapses with two spines, separated by a gap, and arising from the same dendrite. (**d**) sdMSBs were detected near the LTP electrode but not near the control electrode ($p < 0.02$). All procedures followed the National Institutes of Health guidelines and underwent yearly review by the Animal Care and Use Committees.

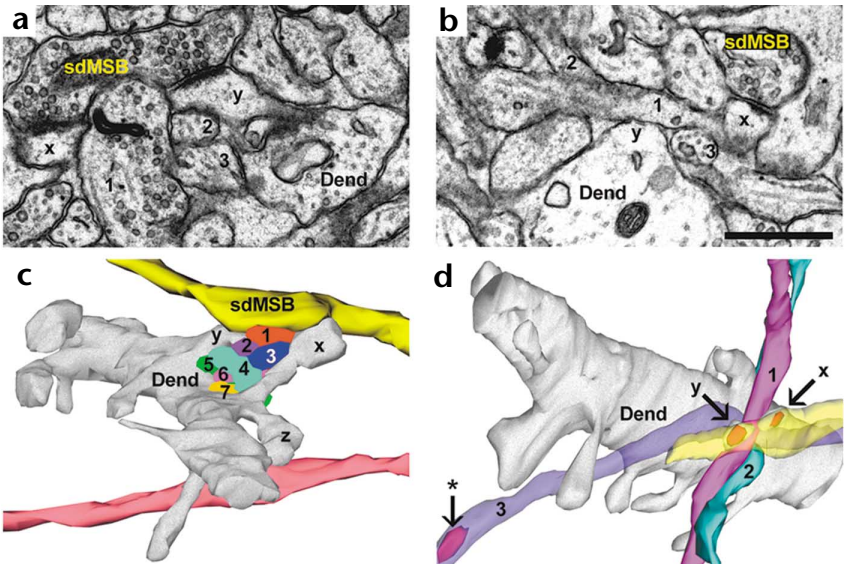

Fig. 2. Gaps between spines of sdMSBs contain mature axons at the LTP sites. (**a**,**b**) Seven axons (1–7) pass through the gap between two spines (x,y) contacting the same bouton (sdMSB). Three of these axons (1–3) are visible in a single section, with all seven partially reconstructed to show their position in the gap. The other branch (z) of spine x synapsed on a different axon (pink). (**c**,**d**) In another instance, three axons (1,2,3) are visible in the micrograph along with the head of one spine (x) and the origin of the other (y). In reconstruction, the axons extend between the spines (x,y) with synapses (red) on the yellow sdMSB. A synapse (*) is also illustrated on one of the other axons. Scale bar, 0.5 μm.

and five in the slices with 0.06 ± 0.016 synapses occurring on sdMSBs per μm of dendrite. Overall, sdMSBs were 1–2% of synaptic boutons along both PN21 (10 of 828 boutons) and adult dendrites (4 of 202 boutons[12]). The frequency of sdMSBs seems more closely related to spine density than to the strengthening of synaptic networks with experience.

We investigated whether synapse splitting produced the 10 sdMSBs at PN21 by reconstructing all elements in their gaps (**Fig. 1c**). An average of 3.7 ± 0.68 axons were found per gap (**Fig. 3**), with each gap containing at least one axon. Two gaps contained a mature spiny dendrite ~0.5 μm in diameter, with 11–16 microtubules and a mitochondrion. The maximum gap widths ranged from 0.16 to 1.1 μm. Neither the axons nor the dendrites could easily have grown through after gap formation, suggesting that the sdMSBs did not form by synapse splitting.

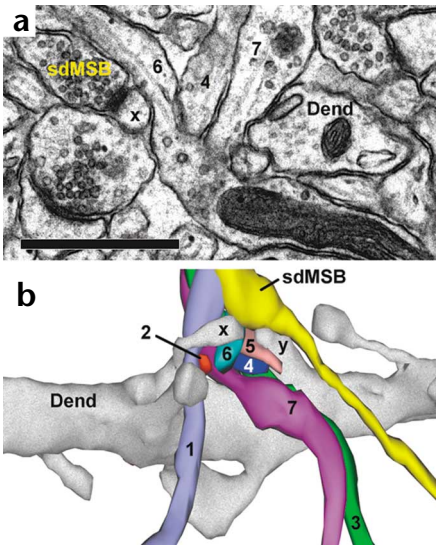

Fig. 3. Reconstruction of sdMSB and related structures in a PN21 slice. (**a**) The sdMSB makes a synapse with the head of one spine (x) on this section. Three of the axons (4,6,7) are visible between the spine head and the dendrite (Dend). (**b**) Three-dimensional reconstruction of the dendrite (gray), the sdMSB axon, and all seven axons (1–7) passing through the gap between the spines (x,y). Four of the axons (2,4,5,6) are cross-sectioned to avoid obscuring the other axons. Scale bar, 0.75 μm.

Despite continued evidence against spine splitting, attempts have been made to retain the hypothesis with the addition of spine retraction and subsequent regrowth[1]. But new spines can not grow out around existing axons while maintaining contact with the original presynaptic bouton. If a spine resulting from the split broke its synaptic connection and found a new path through the neuropil to the original bouton, the splitting of the original synapse would be irrelevant. The regrown spine would be a nonsynaptic protrusion that could have arisen from the dendrite *de novo*. Dynamic protrusion outgrowth has been observed on living dendrites during LTP in culture[13,14]. In this study, protrusions resembling dendritic filopodia[15] without synapses were also found. They extended from dendrites in all PN15 and PN21 tissue. In the PN15 slices, these non-synaptic protrusions occurred at both control (0.16 ± 0.04 per μm) and tetanized sites (0.23 ± 0.05 per μm; $p = 0.052$). Thus, sdMSBs may have formed from these non-stabilized protrusions rather than from synapse splitting.

We conclude that sdMSBs do not arise from synapse splitting because mature dendrites and axons pass through the gaps between spines synapsing on them. Furthermore, the crucial intermediate—partially split spines sharing the same bouton—has not been observed[7,10]. The rarity of sdMSBs in maturing hippocampus suggests that those transiently produced during LTP are not a long-term strengthening mechanism for hippocampal synaptic networks.

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Competing interests statement

The authors declare that they have no competing financial interests.

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