γ-Secretase and metalloproteinase activity regulate the distribution of endoplasmic reticulum to hippocampal neuron dendritic spines

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ABSTRACT The neuronal endoplasmic reticulum (ER) contributes to many physiological and pathological processes in the brain. A subset of dendritic spines on hippocampal neurons contains ER that may contribute to synapse-specific intracellular signaling. Distribution of ER to spines is dynamic, but knowledge of the regulatory mechanisms is lacking. In live cell imaging experiments we now show that cultured hippocampal neurons rapidly lost ER from spines after phorbol ester treatment. ER loss was reduced by inhibiting γ-secretase (DAPT at 2 μM) and metalloproteinase (TAPI-0 and GM6001 at 4 μM) activity. Inhibition of protein kinase C also diminished loss of ER by preventing exit of ER from spines. Furthermore, γ-secretase and metalloproteinase inhibition, in the absence of phorbol ester, triggered a dramatic increase in spine ER content. Metalloproteinases and γ-secretase cleave several transmembrane proteins. Many of these substrates are known to localize to adherens junctions, a structural specialization with which spine ER interacts. One interesting possibility is thus that ER content within spines may be regulated by proteolytic activity affecting adherens junctions. Our data demonstrate a hitherto unknown role for these two proteolytic activities in regulating dynamic aspects of cellular ultrastructure, which is potentially important for cellular calcium homeostasis and several intracellular signaling pathways.—Ng, A. N., Toresson, H. γ-Secretase and metalloproteinase activity regulate the distribution of endoplasmic reticulum to hippocampal neuron dendritic spines. FASEB J. 22, 000–000 (2008)

Key Words: synapse • primary culture • live cell imaging

Dendritic spines on several classes of neurons contain tubules and cisterns of endoplasmic reticulum (ER) (1, 2). ER within spines is connected to the remainder of the ER that extends throughout the dendrites and soma of the neuron (3, 4). ER within dendritic spines contains many of the molecules involved in ER protein processing as well as calcium signaling and may thus contribute with these processes in a synapse-specific way (5–11). Indeed, the ER has been demonstrated to play an important role in synap-
genes. The PS proteins are essential components of the γ-secretase complex, and several FAD mutations have been reported to affect ER calcium load as well as store operated calcium entry (SOCE) (31, 32).

MATERIALS AND METHODS

Cell culture and transfection

Primary hippocampal neuronal cultures were established from embryonic day 17 mouse fetuses (NMRI; Scanbur, Sollentuna, Sweden) as described previously (3). Briefly, dissected hippocampi were disaggregated and plated on poly-d-lysine/laminin-coated (Sigma-Aldrich, St. Louis, MO, USA) chambered cover glass (LabTek, Campbell, CA, USA) at a density of $2 \times 10^4$ cells/well in Neurobasal medium with 2% B27, 0.5 mM t-glutamine, 25 μM glutamate, and antibiotics (all from Gibco-Invitrogen, Carlsbad, CA, USA). Transfection (on day 4 or 5 in vitro) was done with lipofectamine2000 (Invitrogen). The vectors pEGFP-N1 and pDsRed2-ER (Clontech, Palo Alto, CA, USA) were cotransfected at 0.6 μg each/well. Cells were imaged after 17 to 25 days in vitro.

Microscopy and image processing

Cultures were imaged at 37°C (a lens heater was used) on an inverted Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) as described previously (3). Typically, 4 to 6 neurons in one well were imaged and the x, y coordinates were stored so that the cells could be imaged repetitively. Images were projected and exported as tagged image files (TIFFs) for quantification using Object-Image 2.12 (freeware: http://simon.bio.uva.nl/object-image.html). Channels were split in Photoshop (Adobe, San Jose, CA, USA) and mounted as a stack in Object-Image in the following order: time point (TP) 1, green; TP1, red; TP2, red; TP2, green. This allowed the red (ER) signal to be compared between the two time points and correlated with the green.

Drugs

Phorbol 12-myristate 13-acetate (PMA) and GF109203X (G66850), LY294002, KN-93, DNQX, and MK-801 were from Tocris Bioscience (Bristol, UK). GM6001, U0126, Go6983, Go6976, DAPT, and TAPI-0 were from Calbiochem (San Jose, CA, USA). GM6001 was purchased as a 10 mM solution in dimethyl sulfoxide (DMSO) and stored at 4°C; all other compounds (except MK-801, dissolved in water) were diluted in DMSO and frozen in aliquots at 1000× or higher concentration.

Quantification and statistics

In a small subset of PMA-treated cells we noted odd structural features in that dendrites were studded with lamellalike structures and proper spines were very few in number. Such alterations have been described after higher PMA concentrations (33) or mutant MARCKS expression (34). These neurons were excluded. In the morphologically normal neurons, only stable spines were included in the analysis. Counting was done by a person masked to experimental conditions. ER-containing spines were classified into five different groups: stable spine with stable ER, stable spine that lost ER, stable spine that gained ER, deleted ER-containing spine, and new ER-containing spine. A spine was considered stable as long as its length was within 50% of its initial value; otherwise lost. ER was considered stable within a spine if the pixel area (intensity not considered) was within 25% of its initial value. The observer was trained to recognize the different categories, and the maintenance of the criteria was evaluated by randomly subjecting 10% of the neurons for careful analysis by a second observer. The distribution of the data was tested with Kolmogorov-Smirnov (with Lilliefors significance correction) and Shapiro-Wilks tests. Data in all groups except two (DAPT+PMA and TG+PMA) were normally distributed, and significance was assessed with Student’s t test. Because normality was rejected in two cases, significance was assessed with the Mann-Whitney U test. After these findings we retested all other results with the Mann-Whitney U test, and the highest P values are reported (in all of these cases the t test gave the least significance). Results are reported as average ± sd. Values of $P < 0.05$ were considered significant. Statistical calculations were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Phorbol ester causes a reduction in the number of ER-containing spines

Primary hippocampal cultures were established from embryonic day 17 mouse fetuses and used for experiments 17–23 days later. To visualize the structure of single neurons along with their ER content, cultures were cotransfected with vectors encoding enhanced green fluorescent protein (EGFP) and ER-targeted DsRed2 (RedER). EGFP diffuses freely in the cytosol and reveals the complete outline of the neuron, whereas RedER is targeted to and retained in the ER lumen. Phorbol esters are analogues of diacylglycerol (DAG) and thus activate numerous signaling pathways, most prominently those controlled by protein kinase C (PKC). We used PMA at 200 nM for 120 min, after which spine ER content was analyzed. We have noticed that ER structure is sensitive to fixation artifacts; hence, spine ER content was assessed by confocal microscopy in living multipolar and spiny neurons. PMA-treated cells displayed significantly fewer ER-containing spines (hereafter termed ER-spines) (Fig. 1A). In DMSO-treated cultures, the density of ER-spines was $17.6 ± 11.8/100 \mu m$, whereas in PMA-treated neurons, the density was $9.5 ± 7.5 \text{ ER-spines/100 \mu m}$ (Fig. 1B) ($n = 15$ neurons/group; 4 rounds of culture; $P < 0.05$, Student’s t test).

To analyze the effect of PMA in more detail, we decided to analyze the dynamics of changes in spine ER content. We therefore selected and imaged neurons once prior to the addition of PMA. After PMA, a second image of the same cell was captured, and dynamic changes were assessed (Fig. 2A). ER loss from spines after PMA occurred rapidly, such that practically all ER loss had occurred after 15 min (not shown). To gain time by allowing the collection of data from many neurons in each well, the second image was hereafter captured after 60–120 min (average 90 min). DMSO-treated cultures displayed a modest reduction in spine
ER content; over the time of observation we noted a 9.3 ± 10.8% reduction in the number of ER-spines compared with the number at $t=0$. PMA treatment triggered a more profound reduction: 44.0 ± 22.1% compared to $t=0$ (Fig. 2B) ($n=29$ neurons/group; 7 rounds of culture; $P<0.0001$, Student’s $t$ test).

Given the rather high rate of ER turnover within spines, the observed reduction in spine ER content after PMA could have been caused by increased ER exit, decreased ER entry, or a combination of the two. By analyzing the data further, we found that the reduced number of ER-spines following PMA treatment was indeed caused by a combination of increased exit of ER from stable spines and decreased entry of ER into stable spines. In control cultures, 45.2 ± 13.2% of the ER-spines had lost their ER after 90 min. This loss was significantly smaller than in PMA-treated cells, where 61.2 ± 11.4% of ER-spines lost ER (Fig. 2C) ($P<0.0001$, Student’s $t$ test). ER entry into spines that were devoid of ER at $t=0$ was much reduced following PMA treatment. Control neurons and PMA-treated neurons displayed 32.4 ± 13.5 and 17.4 ± 14.8% ER entry, respectively, (Fig. 2C) ($P<0.0001$, Student’s $t$ test).

**γ-Secretase and metalloproteinase activity mediate PMA induced loss of ER from spines**

Phorbol esters are known to trigger γ-secretase cleavage of a large number of plasma membrane proteins that are targets for metalloproteinase-mediated ectodomain shedding (20, 22). We wanted to test the involvement of these two proteolytic activities. Given the large variability in the response to PMA, we reasoned that inhibiting metalloproteinase as well as γ-secretase activity would increase our chances of detecting an effect. Therefore, in our first experiments addressing this question, we treated cultures with a combination of metalloproteinase (TAPI-0 and GM6001, both at 4 μM) and γ-secretase (DAPT at 2 μM) inhibitors. TAPI-0 and GM6001 are complementary and broad hydroxamate-based inhibitors of metalloproteinases of the matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase (ADAM) families. DAPT is a dipeptidic-specific inhibitor of γ-secretase cleavage of numerous type I transmembrane proteins (22). This TAPI-0/GM6001/DAPT (TGD) inhibitor cocktail indeed limited the PMA-induced loss of ER from spines (Fig. 3A).

**Figure 1.** PMA-treated cultures display fewer ER-containing spines. A) Representative images of dendrites on living neurons after treatment with either DMSO or PMA for 120 min. The displayed portion of the dendrite represents only a small part of the dendritic length quantified; the average length of dendrite counted is 480 ± 205 μm per neuron. B) ER-spine density (ER-spines/100 μm) in neurons treated with DMSO or PMA for 120 min.

**Figure 2.** Spine ER dynamics induced by PMA treatment. A) Representative images of the same dendrite before and after 90 min of PMA treatment. B) In live cell imaging experiments, PMA causes a significant reduction in the number of ER-spines after 90 min. C) Black bars show the fraction of ER-spines losing ER after $t=0$ (ER exit); white bars show the fraction of spines (devoid of ER at $t=0$) into which ER entered after $t=0$. PMA significantly increases ER exit and reduces ER entry ($n=29$ neurons/group; 430±174 μm of dendrite counted per neuron).

**Mechanisms of Spine ER Dynamics**

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Neurons in cultures pretreated with TGD 15 min prior to adding PMA displayed a 29.2 ± 29.5% reduction in the number of ER-spines, whereas 46.7 ± 24.1% of ER-spines lost their ER after PMA treatment (Fig. 3B) (n=21 and 20 for PMA and TGD, respectively; 423±167 μm of dendrite counted per neuron). The reduction in spine ER loss was equally contributed by reduced exit of ER from and increased entry of ER into spines (not shown).

We next asked whether the observed effect depended on inhibition of one of the proteolytic events or indeed required combined inhibition. We treated hippocampal cultures with 2 μM DAPT for 30 min prior to adding PMA. The net loss of ER from spines after DAPT + PMA was 21.6 ± 43.7%, whereas PMA alone caused a net loss of 43.9 ± 17.1% (Fig. 3C). When testing the distribution of the results, normality was rejected. Hence, a non-parametric test was used for comparison (Fig. 3D) (n=18; 4 rounds of culture; P=0.17, Mann-Whitney U test).

A similar result was obtained when we treated cultures with the two metalloproteinase inhibitors TAPI-0 and GM6001 at 5 μM each (TG) prior to PMA. In this experiment, PMA reduced the number of ER-spines by 36.1 ± 28.4%, and in cultures pretreated with TG, the reduction was 15.2 ± 43.5%. The variability between cells in the TG group was very large, and results were not normally distributed (Fig. 3E) (n=22 and 20 in PMA and TG+PMA, respectively; 451±152 μm of dendrite counted per neuron).

Constitutive spine ER dynamics are sensitive to combined γ-secretase and metalloproteinase inhibition

Because spine ER is constantly turning over, we reasoned that another way of testing the involvement of γ-secretase and metalloproteinase activity in spine ER distribution was to assess the role of these proteolytic activities in the absence of PMA. Under this condition, the TGD cocktail led to a dramatic increase in the number of ER-spines after 90 min (Fig. 4A). The number of ER-spines in control neurons was reduced by 7.22 ± 15.3%, whereas TGD-treated cells showed a
14.1 ± 23.5% increase (Fig. 4B) \((n=26\) and 30 in control and TGD, respectively; 5 rounds of culture; \(P<0.0005\), Student’s \(t\) test). The increased number of ER-spines caused by TGD appeared to be mainly contributed by entry of ER into previously empty spines: ER entry in control cultures was 40.9 ± 12.1%, and in TGD-treated neurons it was 56.2 ± 21.2% \((P<0.001\), Student’s \(t\) test). We only noted a trend toward a contribution of decreased ER exit: 48.2 ± 13.1 and 42.1 ± 12.0% \((P=0.19\), Student’s \(t\) test) in control and TGD-treated neurons, respectively (Fig. 4C).

To determine the relative contribution of the two proteolytic activities we repeated the experiments with either DAPT (2 \(\mu\)M) or TG (5 \(\mu\)M each, TAPI-0 and GM6001). Somewhat surprisingly, we found that neither \(\gamma\)-secretase nor metalloproteinase inhibition had an effect on spine ER distribution (Fig. 4D). The change in the number of ER-spines was a loss of 6.76 ± 16.5% \((n=20)\) in control neurons. After DAPT or TG for 90 min, the net change was a loss of 4.0 ± 14.4% \((n=26)\) and 6.68 ± 20.0% \((n=21)\), respectively. Results were obtained from four rounds of culture for each condition.

**Involvement of other pathways downstream of PMA**

The classical and novel isozymes of PKC are well-characterized phorbol ester/DAG targets. We assessed the importance of PKC activation for PMA-induced loss of spine ER by pharmacological inhibition of these isozymes. Given that numerous PKC isozymes are expressed in the hippocampus and that no single inhibitor blocks the activity of all of these, we used the three inhibitors Gö6983, Gö6850, and Gö6976 in combination (GöX3) at 200 nM each. At this concentration, these bisindolylmaleimides have negligible effects on other kinases, and inhibition of PKC\(\mu\) and PKC\(\beta\) is ensured (35, 36). We found that adding GöX3 30 min prior to adding 200 nM PMA partially blocked the reduction in numbers of ER-containing spines (Fig. 5A). Control neurons \((n=13)\) displayed a 3.6 ± 13.9% reduction in the number of ER-spines, GöX3 + PMA-treated neurons \((n=15)\) displayed a 31.3 ± 20.3% reduction \((P<0.001\) vs. control, \(P=0.01\) vs. PMA, Student’s \(t\) test) and PMA-treated neurons \((n=15)\) had a 51.2 ± 18.3% reduction \((P<0.001\) vs. control, Student’s \(t\) test) (Fig. 5B). Results were obtained from four rounds of culture.

Because we found that PMA-induced reduction of ER-spines was caused by increased exit of ER from spines as well as reduced entry of ER into spines, we examined these aspects under PKC inhibition. Interestingly, PKC inhibition prevented PMA-induced exit of ER from spines but had no effect on ER entry into spines. In control neurons, 43.2 ± 14.1% of ER spines lost their ER, whereas PMA-treated spines displayed a 63.9 ± 16.3% reduction \((P<0.005\) vs. control, Student’s...
In neurons treated with GöX3 prior to PMA addition, the loss was 51.3 ± 16.8% (P<0.05 vs. PMA, P=0.19 vs. control, Student's t test) (Fig. 5C).

Numerous events downstream of phorbol esters and/or PKC have been reported in neurons. We have tested the involvement of several of these pathways. We found no significant effect on PMA-induced spine ER loss when treating the cultures with the MEK antagonist...
UO126 at 20 μM, a mix of antagonists of the AMPA and NMDA type of glutamate receptors; DNQX (at 20 μM) and MK-801 (at 25 μM), the PI3K inhibitor LY294002 at 100 μM; or the CamKII inhibitor KN-93 at 10 μM (not shown).

DISCUSSION

To facilitate comparing the different experimental conditions and evaluation of effects, our results are compiled in Table 1.

Mechanisms of PMA-regulated loss of ER from spines

An important question that arises from our work is how the proteolytic activity of metalloproteinas and γ-secretase can be mechanistically linked to dynamics in ER structure. In this respect, note that ultrastructural analysis has revealed that ER within spines often is associated with adherens junctions (18). At these sites, ER proteins are likely to interact with PM proteins. The latter category includes several type I transmembrane proteins that are targets for metalloprotease-mediated ectodomain shedding and ensuing γ-secretase cleavage (23–28). Furthermore, PSI has been shown to localize to adherens junctions at synapses (37). One possible interpretation of our data is thus that at least part of the reported PMA-induced loss of ER from spines reflects metalloprotease- and γ-secretase-mediated proteolytic remodeling of ER plasma membrane junctions (ERPMJs). The observed rapid loss of ER from spines indicates that shedding is an immediate event downstream of PMA. Such rapid shedding is most likely caused by metalloprotease activity at the cell surface (38), where γ-secretase has also been shown to be active (39–42), although other sites of action cannot be ruled out. Once the plasma membrane proteins interacting with ER resident proteins have been cleaved, the ER is likely to be expelled from the spine due to the forces driving ER motility (43). Based on the facts above and our data, we propose a hypothetical model of how the proteases exert their action on ER structure (Fig. 6).

In ectodomain shedding and ensuing γ-secretase processing, the rate-limiting step has been shown to be the metalloprotease cleavage (21). We were therefore somewhat puzzled to find that combined inhibition of the two proteolytic activities was required to obtain an effect. Although this may reflect a hitherto unknown mechanistic link between the shedding and γ-secretase activities, other explanations are also possible. For instance, it may be that several different transmembrane proteins are involved in forming ERPMJs (further discussed below). Within one spine, the ER might be tethered via several different combinations of proteins that may display different sensitivity to metalloprotease and γ-secretase processing. Nevertheless, even if combined inhibition was required to obtain a significant effect, treatment with either metalloprotease or γ-secretase inhibitors gave results that were not normally distributed (whereas in all other conditions they were). The possibility that our cultures contain different populations of neurons with respect to how their ERPMJs are constructed must therefore be considered. In this case, the potential effect of DAPT on its own could be related to the DAPT-induced stalling of endocytosis reported previously (44). Moreover, γ-secretase can cleave transmembrane proteins without prior metalloprotease cleavage (41, 45). Intriguingly, it was shown recently that different combinations of protocadherins have varying propensity to be cleaved by metalloprotease and γ-secretase in neurons (46).

Our results further show that the net spine loss observed after PMA is caused by increased ER exit from and decreased ER entry to spines. Importantly, our experiments using pharmacological inhibition of PKC suggest a mechanistic dissociation between the exit and entry of ER. Increased exit and decreased entry are both triggered by PMA, but only the former is mediated by PKC activation. Hence, it is possible that PKC is responsible for the induction of shedding and γ-secretase cleavage causing rapid loss of ER from spines. Another, as yet unknown, pathway is responsible for the decreased entry of ER into spines.

Mechanisms of constitutive spine ER dynamics

Increased or decreased spine ER content must involve an alteration in the spine’s affinity for ER. That such differences in affinity can exist is supported by the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Net effect on number of ER spines</th>
<th>Effect on ER entry</th>
<th>Effect on ER exit</th>
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<tr>
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<td>+/−</td>
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<td>TG, 90 min; PMA, 90 min</td>
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<td>DAPT, 90 min; PMA, 90 min</td>
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<td>TGD, 90 min</td>
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<td>GöX3, 30 min; PMA, 90 min</td>
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previously reported fact that hippocampal dendritic spines differ in their ability to maintain ER for longer periods of time (days) (3). ER that remains within spines for several days must be anchored there in some way, perhaps at ERPMJs as outlined above. The highly dynamic spine ER, however, may never be anchored at stationary ERPMJs but rather be an effect of the constantly ongoing dynamic movement of ER tubules and cisterns (43). According to this view, the spine cytoplasm is temporarily visited by ER that does not become integrated with the spine signaling machinery. One example supporting such a scenario is the phenotype of hippocampal dendritic spines in synaptopodin null mice that still can contain ER, although spine apparatuses are lacking. This structural abnormality correlates with synaptic plasticity and memory deficits (47), indicative of disturbed spine-specific intracellular signaling. Furthermore, it was previously established that release of calcium from stores within spines contributes in a major way to calcium transients (48), but other studies found the contrary (49, 50). Now, recent findings show that only a subset of ER-containing spines in hippocampal slices display an apparent store release of calcium on synaptic stimulation (51), indicating that, indeed, the presence of ER is not sufficient for it to participate in signaling.

We treated cultures with a combination of metalloproteinase and γ-secretase inhibitors (in the absence of PMA) and found that the number of ER-containing spines increased. Assuming that this effect is caused by reduced cleavage of PM proteins, three alternative mechanisms (possibly acting in combination) can be considered. First, it may hinder constitutive shedding of PM transmembrane proteins engaged in ER interactions. Second, it may inhibit the degradation of nonoccupied potential ER-binding transmembrane PM proteins in spines, hence increasing the number of available attachment-points for ER. The third alternative is similar to the second; only that the affected PM proteins may reside anywhere in the neuron and form motile ERPMJs that can move into spines. We deem the first alternative the most plausible. Clearly, ERPMJs have been observed in dendrites (18, 52–54), but whether they are motile or not is unknown.

Do PMA-regulated and constitutive ER dynamics have different mechanisms?

The second and third alternatives above suggest that constitutive shedding and γ-secretase cleavage limits the number of available points of interaction between ER and PM. Constitutive shedding has been described in many cell types and can be regulated by pathways other than those triggered by phorbol esters (55). PMA-induced shedding is typically mediated by PKC and MEK/ERK activation, although PKC-dependent and MEK/ERK-independent shedding was also reported previously (56). Many neuronal type I mem-

**Figure 6.** Model of dynamic physical interaction between ER and PM within a dendritic spine based on ultrastructural data (18). Junctions between ER and PM have been implicated in many aspects of calcium signaling and ER calcium homeostasis (14–16, 60, 61, 64). A1, A2) Based on our results, one class of spine ERPMJ may contain a PM type I transmembrane protein bound to an ER protein. The former is anchored to the extracellular matrix (ECM), as indicated in A2, or involved in cell–cell adhesion (not shown). We speculate that this currently unidentified transmembrane protein is a target for metalloproteinase and γ-secretase cleavage. The fact that we can implicate γ-secretase in the regulation of ER structure is interesting, given that ER calcium homeostasis (including SOCE) is affected by FAD mutations in the γ-secretase component PS1 (69–71) or in PS1−/− cells (69, 73) and that SOCE requires ER-PM contact (60, 61). B, C) Metalloproteinase and γ-secretase proteolytic processing of the PM-protein leads to the disassembly of the ERPMJ making the ER free to move within the cytosol (B). Due to the forces acting on the ER, it will eventually be lost from the spine.
brane proteins, including those that can be found in spines, are shed and processed at a basal level (24–28, 57). What “basal level” means in relation to our results is unclear; it may reflect a housekeeping function to clear old proteins but could also be the result of specific signaling events. Although our data are consistent with a model whereby PMA activates this constitutive system, until we have reliable markers for spine ERPMJs, we cannot exclude that different pathways are involved or even that PMA/PKC acts predominantly on one class of ERPMJs and metalloproteinase/γ-secretase on another.

The possibility of different populations of ERPMJs in our cultures is highlighted by the fact that, although PMA has a dramatic effect on spine ER content, some ER-containing spines do not lose their ER after activation of PMA-induced pathways. Molecules that were previously reported to affect spine ER content include Homer1b and Shank1B. Their combined overexpression (or Shank1B alone) was shown to cause a dramatic increase of ER in hippocampal dendritic spines (10). The cytosolic adaptor protein Homer1 physically links IP3Rs in the ER with several plasma membrane proteins, such as metabotropic glutamate receptors (mGluRs) (58) and TRPC1 (59). A role for Homer1 in spine ER dynamics is so far supported only by overexpression experiments. Neither of its known binding partners, however, are type I or II membrane proteins (and hence are not targets for γ-secretase or metalloproteinase cleavage), and it is possible that a Homer-Shank complex is associated with ER that remains in spines after PMA.

Relevance of spine ER dynamics

As outlined above, one aspect of the observed spine ER dynamics may represent trafficking/diffusion of ER or even ERPMJs that transiently visit spines. Therefore, the mechanisms we have revealed by studying spine ER dynamics could also be relevant to the regulation of ERPMJ dynamics in the whole cell. Apart from the Homer-containing complexes discussed above, other protein interactions involving proteins localized to spines and dendrites have been implicated in forming ERPMJs. For instance, the recently discovered molecular basis for store-operated calcium entry (SOCE) is highly interesting in this respect. STIM1 and Orai1/CRACM1 have been shown to cluster in small domains in the ER and PM, respectively, in response to ER calcium store depletion (60, 61), thus providing the first example of a dynamic structural and functional link between the PM and the ER. STIM1 puncta also form in neurons and can be found in spines (unpublished results). The junctophilin (JP) family of proteins are also interesting in this respect, because they are known to accumulate at ERPMJs, and double-null mutants for JP3 and JP4 display memory and synaptic plasticity deficits (62). Furthermore, the sodium calcium exchanger (NCX) is active in synaptosomes (63), and NCX1 can be coimmunoprecipitated with ankyrin 2, the sarco- and endoplasmic reticulum calcium ATPase 2 (SERCA2), and the inositol 3-phosphate receptor 1 (IP3R1) (64). The ankyrin interaction with several ER proteins is particularly interesting, because many PM proteins also bind ankyrin, notably L1 (65). Another class of PM proteins worth mentioning is the lipoprotein receptors. Many of these proteins undergo ectodomain shedding and γ-secretase cleavage and interact with synaptic proteins such as the scaffolding protein PSD-95 (66). Finally, the Notch family of proteins should be considered given the reported learning and synaptic plasticity defects resulting from reduced levels of Notch1 (67, 68). Notch localization to spines, however, has not yet been demonstrated.

Our data linking γ-secretase activity to ER dynamics may be relevant for the reported disturbances in SOCE associated with FAD mutations in PS1 (69–71; but see ref. 72). Interestingly, in PS1+/− cells, SOCE is augmented (69) and in PS1+/− neurons SOCE is of such a magnitude that it can induce long-term potentiation (73). In addition, synaptosomes from FAD-PS1 transgenic mice exhibit increased release of ER calcium (74), a finding that can be explained by increased synaptosomal ER content. Experiments specifically addressing the relationship among FAD-PS1, SOCE, and ER structure are needed, as the effects of our short-term pharmacological inhibition of γ-secretase are likely to differ from long-term effects of FAD-PS1 expression.

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