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Activity-dependent Regulation of Synapse and Dendritic Spine Morphology in Developing Barrel Cortex Requires Phospholipase C-β1 Signalling

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The phospholipase C-β1 (PLC-β1) signalling pathway, activated via metabotropic glutamate receptors (mGluRs), is implicated in activity-dependent development of the cerebral cortex, as both PLC-β1 and mGluR5 knockout mice exhibit disrupted barrel formation in somatosensory cortex. To characterize the effects of this signalling system on development of synaptic circuitry in barrel cortex, we have examined neuronal ultrastructure, synapse formation and dendritic spine morphology in PLC-β1 knockout mice. Qualitative ultrastructure of neurons and synapse density in layers 2–4 of barrel cortex were unchanged in PLC-β1 knockout mice during development [postnatal day (P) 5] and in mature cortex (P19–21). We found a decrease in the proportion of synapses with symmetric morphology at P5 that was gone by P19–21, indicating a transient imbalance in excitatory and inhibitory circuitry. We also investigated dendritic spines by back-labelling layer 5 pyramidal neurons with carbocyanine. We observed normal dendritic spine densities on apical dendrites as they passed through layer 4 of barrel cortex, but spine morphology was altered in PLC-β1 knockout mice at P9. These observations indicate that the PLC-β1 signalling pathway plays a role in the development of normal cortical circuitry. Interrupting this regulation leads to changes in synapse and dendritic spine morphology, possibly altering post-synaptic integration of signal.

Keywords: cortical development, mGluR5, PLC-β1, stereology, synapse morphology, synaptogenesis

Introduction

Orchestrating the development of intricately complex and highly ordered synaptic connections in the cerebral cortex requires tightly regulated biological mechanisms. In rodent cortex, neonatal neurons have relatively few synapses. During the first few postnatal weeks, they undergo a burst of synaptogenesis when excitatory and inhibitory synapses with distinct morphological characteristics form (Micheva and Beaulieu, 1996; White *et al.*, 1997). During this time, dendritic protrusions develop from long, highly motile filopodia and stubby protrusions into more mature thin and mushroom-shaped spines, each with an excitatory synapse on the tip (Miller and Peters, 1981; Fiala *et al.*, 1998; Harris, 1999b). Morphology of synapses and dendritic spines affects postsynaptic integration of signals, for example, allowing neurons to compensate for the attenuation of signals from synapses distant from the soma (reviewed by Hausser, 2001).

Maturation of cortical synaptic circuitry relies in part on neural activity. Sensory areas in particular undergo 'sensitive periods' when peripheral stimulation is essential to ensure correct cortical patterning. Mouse primary somatosensory cortex provides a useful model to study the mechanisms by which activity regulates cortical maturation because it develops barrels, distinct cytoarchitectonic structures in layer 4, composed of a cell-dense barrel wall and a cell-sparse barrel-hollow (Woolsey and Van der Loos, 1970). Rearrangement of layer 4 cells to form cortical barrels requires segregation of thalamocortical (TC) afferents into the barrel pattern (Van der Loos and Woolsey, 1973; O'Leary *et al.*, 1994; Cases *et al.*, 1996; Welker *et al.*, 1996; Abdel-Majid *et al.*, 1998), which, in turn, is regulated by presynaptic serotonergic receptor activity and signalling through adenylate cyclase (reviewed by Erzurumlu and Kind, 2001).

Recently, the role of postsynaptic activity in barrel formation has been investigated. Cortex-specific *N*-methyl-D-aspartate receptor (NMDAR1) knockout mice exhibit relatively normal segregation of TC afferents but layer 4 neurons fail to rearrange into cortical barrels (Iwasato *et al.*, 2000). Phospholipase C-β1 (PLC-β1) signalling, activated by metabotropic glutamate receptor (mGluR) stimulation, is also implicated in cortical barrel formation since both PLC-β1 and mGluR5 knockout mice also fail to form cortical barrels in response to TC axon segregation (Hannan *et al.*, 2001; Hannan *et al.*, 1998).

Although NMDAR1, PLC-β1 and mGluR5 all play a role in barrel formation, the precise cellular events that they regulate is not clear. Datwani *et al.* (2002) have shown that deletion of NR1 increases dendritic complexity and spine density, suggesting a role for NMDA receptors in synaptogenesis. To determine whether the PLC-β1 pathway also regulates the development of ultrastructural characteristics of barrel cortex, including synaptic connectivity, we examined the morphology of intracellular structures of cortical neurons and used unbiased stereological counting methods to assess synaptogenesis in layers 2–4 of barrel cortex in PLC-β1 knockout mice and wild-type (WT) mice. Spine morphology on apical dendrites of layer 5 pyramidal neurons as they pass through layer 4 in the barrel cortex was also examined.

We find that the development of synapse and dendritic spine morphology are disrupted in PLC-β1 knockout mice, indicating that this signalling system is necessary for normal development of cortical connectivity.

Materials and Methods

Animals

PLC-β1 knockout mice (originally donated by H.-S. Shin; Kim *et al.*, 1997) were bred as previously described (Hannan *et al.*, 2001). For all experiments, the observer was blind to the genotype of the mice.

Genotype was established by polymerase chain reaction amplification of purified genomic DNA. PLC-β1 knockout mice (KO) are homozygous for a null mutation of the gene coding for PLC-β1 (*PLC-β1*$^{-/-}$). Both heterozygous mice (*PLC-β1*$^{+/-}$) and those with two functional copies of the gene (*PLC-β1*$^{+/+}$), are considered WT mice since in every test we have performed, we have found no difference between these mice (Hannan *et al.*, 2001 and unpublished observations). All animal work conformed to UK Home Office regulations.

Tissue Preparation

For synapse analysis, PLC-β1 knockout mice and littermate WT controls at postnatal (P) day 5 (n = 3 KO, 3 WT) and P19–21 (n = 4 KO, 3 WT) were terminally anaesthetized with intraperitoneal pentobarbitone (20%; J.M. Loveridge PLC, Southampton, UK). After disappearance of the tail-pinch reflex, animals were perfused transcardially with phosphate-buffered saline, pH 7.4 (Sigma-Aldrich, Ltd, Poole, UK), followed by 4% paraformaldehyde (TAAB, Aldermaston, UK) and 2% glutaraldehyde (BDH, Poole, UK) in phosphate buffer with a pH of 7.4 (PB, BDH). For dendritic spine analysis, PLC-β1 knockout mice and WT controls at P9 (n = 7 KO, 3 WT) were anaesthetized and perfused as described above except with 4% paraformaldehyde in PB, pH 7.4, as the fixative.

Body and Brain Weights

Wild-type and PLC-β1 knockout mice were weighed (N2B110, Ohous, Switzerland) at P5–7 (n = 16 WT, 6 KO), P9 (n = 12 WT, 4 KO), P14 (n = 53 WT, 30 KO) and P19–21 (n = 26 WT, 20 KO). After perfusion, brains were dissected and briefly touched to filter paper to remove excess liquid, then weighed (Precisa 125A, Precisa, Switzerland). Brains from the same four age groups were weighed (P5–7, n = 8 WT, 5 KO; P9 n = 5 WT, 2 KO; P14 n = 14 WT, 12 KO; and P19–21 n = 4 WT, 6 KO).

Processing for Electron Microscopy

Coronal sections 100 μm thick were cut through barrel cortex on a vibratome (General Scientific, Surrey, UK) at low speed and maximum vibration amplitude. Barrel cortex from the centre of the posteromedial barrel subfield (PMBSF) was dissected from these sections. In the case of PLC-β1 knockout cortex, which does not have cortical barrels, a section was dissected from the estimated centre of PMBSF. Sections were then processed for electron microscopy by treating with osmium tetroxide (1%, BDH) and uranyl acetate (0.5%, BDH), then embedding in resin (equal parts Araldite, and dodecenyl succinic anhydride (DDSA), with 0.5% BDMA catalyst; all three reagents from Agar Scientific, Stansted, UK). Embedded tissue sections were trimmed to contain only layers 2–4 of barrel cortex. These sections were wide enough to contain an entire barrel (wall and hollow). At this stage, blocks were coded and observations made blind to genotype. Blocks were resectioned on an ultracut microtome (Reichert-Jung) using a glass knife for semithin (1 μm) and a diamond knife (Diatome Ultra 45°, Leica Microsystems, Milton Keynes, UK) for ultrathin (~70 nm) serial sections. Semithin sections were collected on glass slides and stained with toluidine blue (1%, Emscope, Ashford, UK). Ultrathin sections were collected on Piloform-coated copper single-slot grids, then counterstained with lead citrate prior to observation.

Unbiased Synapse and Neuron Counting

Unbiased synapse counting using the physical disector method (Sterio, 1984) was carried out in a manner similar to that employed by Micheva and Beaulieu (1996). Tissue was prepared for electron microscopy as described above. Between two and five pairs of adjacent ultrathin sections per brain were photographed systematically through layers 2–4 on a Jeol EM (JEM-100CX, Tokyo, Japan). Photographs were taken diagonally across the section to ensure that all of layers 2–4 were sampled and to ensure equal sampling of barrel walls and hollows (where present). Individual pairs of sections were at least 3 μm apart to avoid counting the same synapse more than once. The thickness of each section was determined by taking half of the measure of the smallest fold on the section. A counting frame of 110 μm^2 (for P5 brains) or 60 μm^2 (for P19–21 brains) was placed over identical areas on the photo pairs using large fiduciary markers, such as mitochondria and nuclei, for orientation. Synapses that appeared in only one of the sections in the pair and did not touch the exclusion lines were counted as unbiased synapses. Synapses were defined as two opposing membranes separated by a cleft with at least three synaptic vesicles in the presynaptic terminal and a distinguishable postsynaptic density; and they were classified as asymmetric or symmetric based on morphology (Peters *et al.*, 1991). Synapse densities (D_s) were determined (in synapses per mm^3) according to the following equation:

$$D_s = N_s(1000\,\mu m/mm)^3/(N_p \times T \times A)$$

where N_s = number of unbiased synapses counted, N_p = number of photo pairs used, T = section thickness, and A = area of the counting frame. Neuron density was similarly determined using Toluidine Blue-stained semithin sections from the same tissue blocks. Neurons were identified by nuclear morphology and counted in counting frames of 15 625 μm^2.

Dendritic Spine Analysis with Confocal Microscopy

Callosally projecting layer 5 pyramidal cells were filled in P9 barrel cortex by implanting 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI; Molecular Probes, Leiden, Holland) crystals into the corpus callosum of each hemisphere immediately after perfusion. These neurons were chosen for this study because the specific subpopulation can be labelled, allowing analysis of a homogeneous population. Brains were incubated at 37°C for 18–21 days to allow dye transport. After dye transport was completed, hemispheres were embedded in 4% agar (BDH), sliced to 200 μm sections on a vibratome as described above, and counterstained with the nuclear stain bisbenzamide (2.5 μg/ml, Sigma-Aldrich) in 0.1 M PBS for 10 min. Sections were then rinsed in PBS, semi-permanently wet-mounted in PBS using paraseal (Sigma) to fix the coverslip, and observed using a Leica confocal microscope (Leica Microsystems UK Ltd, Milton Keynes, UK). To ensure only callosally projecting layer 5 cells were filled, we repeated the experiment with DiI implantation in the contralateral barrel field. Results did not differ from those with callosal implantation (data not shown).

Filled layer 5 pyramidal neurons in the barrel cortex were located using a Leica TCS confocal microscope (Leica) and images of their apical dendrites in layer 4 were acquired (an average of five cells per brain were analysed, n = 7 PLC-β1 knockout brains, 3 control). Images were taken every 1–3 μm through the thickness of the dendrite, then projected onto a two-dimensional plane using Leica TCS software. Dendritic spines were counted along a 50 μm length of apical dendrite through layer 4 to determine average spine density, and spines were classified into one of four morphological categories: thin, stubby, mushroom or branched (Peters and Kaiserman-Abramof, 1970; Harris *et al.*, 1992). A spine was classified as branched if it bifurcated into two spine heads. Mushroom, thin and stubby spines were assigned based on the ratio of spine head width (H) to neck width (n) according to the following ratios: mushroom spines, $H \geqslant 2n$; thin spines, $H < 2n$; stubby spines, $H < n$ (no neck visible).

Analysis

Statistical analyses were carried out using SPSS software (SPSS Inc., Chicago, IL). For each measure, mean and standard error of the mean (SEM) were calculated for every group tested. To compare means of weight and synapse density data, univariate analysis of variance (ANOVA) was used with genotype and age as independent variables. t-tests for independent samples (two-tailed; equal variance not assumed) were used to compare means of individual groups. When comparing percentages, Mann–Whitney non-parametric tests were used.

All values in the text and figures are reported as mean ± SEM.

Results

Qualitative Ultrastructure of PLC-β1 Knockout Barrel Cortex

The development of cytoarchitecture constituting barrel cortex is known to be disrupted in PLC-β1 knockout mice (Hannan *et al.*, 2001). However the underlying cellular basis of this anatomical defect is not known. Here, using electron microscopy, we investigated the fine structure of layers 2–4 of barrel cortex in PLC-β1 knockout and WT mice to determine whether

development of subcellular structures is also affected. At both P5 and P19–21, ultrastructure of PLC-β1 knockout barrel cortex appears qualitatively normal (Fig. 1). Neuronal nuclear membranes appear healthy and have no invaginations. Cytoplasmic organelles such as endoplasmic reticulum (ER), Golgi apparatus and mitochondria appear normal in the barrel cortex of knockout mice. There are no indications of cell stress (swollen mitochondria, large vacuoles, etc.) in the knockout neurons. Neuropil structures also appeared qualitatively similar in WT and PLC-β1 knockout cortex. Preservation of ultrastructure by aldehyde fixation was better at the later age as can be seen in Figure 1.

Synaptogenesis in PLC-β1 Knockout Mice

Involvement of the PLC-β1 signalling cascade in synapse formation in developing barrel cortex was investigated using the physical disector method (Sterio, 1984) to compare synapse densities in layers 2–4 of WT and PLC-β1 knockout barrel cortex. Unbiased counting indicates no difference in synapse density between the two groups at P5 — both groups have ~50 × 10^6 synapses/mm^3 (Fig. 2*a*). Synapse density was also examined at 3 weeks of age (P19–21) to determine whether the PLC-β1 signalling cascade affects the 'burst' of synaptogenesis that occurs during the second postnatal week of life (Micheva and Beaulieu, 1996; White *et al.*, 1997). At this age, there was no significant effect of the PLC genotype on synapse density; however, the knockout mice tend to have lower synapse density (209 × 10^6 ± 7.6 ×10^6 synapses/mm^3 in WT mice, 162 × 10^6 ± 17.6 × 10^6 synapses/mm^3 in knockout mice: Fig. 2*a*, *t*-test, $P = 0.071$). As expected, between P5 and P19–21, a large increase (3.7-fold) in synapse density occurs [two-way ANOVA, age $F(1,9) = 118.48$, $P < 0.0001$].

PLC-β1 knockout mice are lighter than their WT littermates when analysed across all ages tested [Fig. 2*b*, two-way ANOVA, $F(1,159) = 33.90$, $P < 0.0001$], and their brains tend to weigh less (Fig. 2*c*, two-way ANOVA, $F(1,48) = 3.76$, $P = 0.058$]. If the volumes of the brains are different, then the total number of synapses differs between groups since the synapse density is the same. To examine this possibility, neuron density was determined and a synapse per neuron ratio calculated since this ratio is volume-independent. Neuronal density is increased in knockout mice [Fig. 2*d*, two-way ANOVA, $F(1,9) = 8.702$, $P = 0.016$]; and the synapse:neuron ratio is decreased [Fig. 2*e*, two-way ANOVA, $F(1,9) = 8.544$, $P = 0.019$]. A Mann–Whitney test reveals that at P19–21, the decrease in synapse/neuron ratio in knockout mice is significant ($P = 0.034$). Taken together, these results demonstrate that although synapse density does not change in PLC-β1 knockout mice, there is an increase in neuron density and correspondingly fewer synapses per individual neuron on average, indicating that the PLC-β1 signalling pathway regulates synaptogenesis.

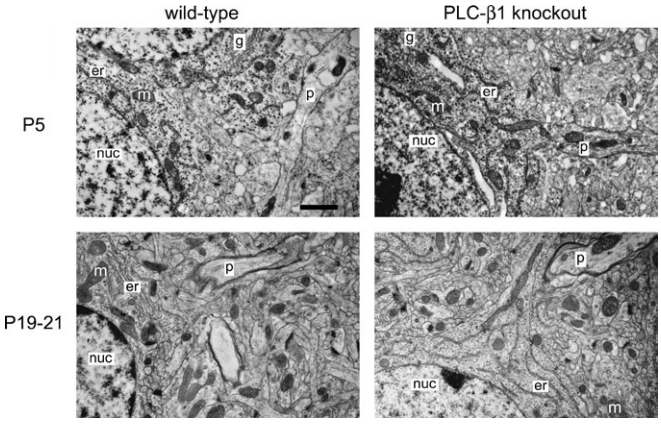

Figure 1. Ultrastructure of neurons in PLC-β1 knockout mouse barrel cortex appears normal. There are no qualitative differences between wild-type and knockout cortex at P5 or P19–21 in nuclear morphology (nuc), endoplasmic reticulum (er), Golgi apparatus (g), mitochondria (m), or processes in the neuropil (p). Scale bar = 1 μm.

Synapses in layers 2–4 of barrel cortex were also classified based on morphology originally described by Gray as type 1 and type 2 synapses (Gray, 1959). Type 1 synapses, also called asymmetric synapses, are usually excitatory and are characterized by a wide synaptic cleft (~20 nm), pronounced postsynaptic density and small, round synaptic vesicles. Type 2 synapses, or symmetric synapses, are usually inhibitory and have a smaller synaptic cleft (~12 nm), less pronounced postsynaptic density and often exhibit pleomorphic (elongate) synaptic vesicles after aldehyde fixation (Fig. 3*a*, Peters *et al.*, 1991). During the sensitive period of development of barrel cortex (P5), PLC-β1 knockout mice have a higher proportion of symmetric (presumed inhibitory) synapses than WT mice (Fig. 3*b*, Mann–Whitney test $Z = -1.964$, $P \leq 0.050$). After the burst of synaptogenesis, *post hoc* tests reveal that there is no longer an increase in the proportion of symmetric synapses in knockout barrel cortex (Fig. 3*c*). This indicates a transient effect of PLC-β1 on inhibitory synaptic circuitry, which is compensated for by P19–21.

Dendritic Spine Formation in PLC-β1 Knockout Barrel Cortex

Over 90% of excitatory synapses in the brain occur onto dendritic spines (Peters *et al.*, 1991), protrusions which have been implicated in functions as important as learning and memory (reviewed by Fiala *et al.*, 2002; Nimchinsky *et al.*, 2002). Here we examine the effects of PLC-β1 signalling on dendritic spine density and morphology on apical dendrites of callosally projecting layer 5 pyramidal neurons as they pass through layer 4 of barrel cortex in PLC-β1 knockout mice and WT controls at P9 (Fig. 4*a*,*b*).

Spine density on these dendrites varied widely, but was not significantly different between WT and knockout mice (both groups have ~0.13 spines/μm: Fig. 4*c*). Spines were classified by morphology as mushroom, thin, stubby or branched (Fig. 5*a*) based on the ratio of spine head width to spine neck width and the number of heads. The length of dendritic spines of each morphological category was not affected by the PLC-β1 knockout genotype (Fig. 5*b*). However, as seen with synapse morphology, dendritic spine morphology is abnormal in knockout mice (Fig. 5*c*). PLC-β1 knockout mice have less than half as many spines with mushroom morphology in barrel cortex as WT mice with 13.8 ± 4.0% mushroom spines in knockouts and 32.3 ± 1.5% in WT mice (Mann–Whitney test, $Z = -2.165$, $P = 0.033$). There appears to be a compensatory increase in the proportion of thin and stubby spines in knockout mice, but neither of these increases reached significance.

Mushroom and thin spines become predominant morphologies during development — gradually replacing filopodia as the brain matures (Fiala *et al.*, 1998). Thus the deficit of mushroom spines in PLC-β1 knockout mice may reflect a disruption in spine maturation.

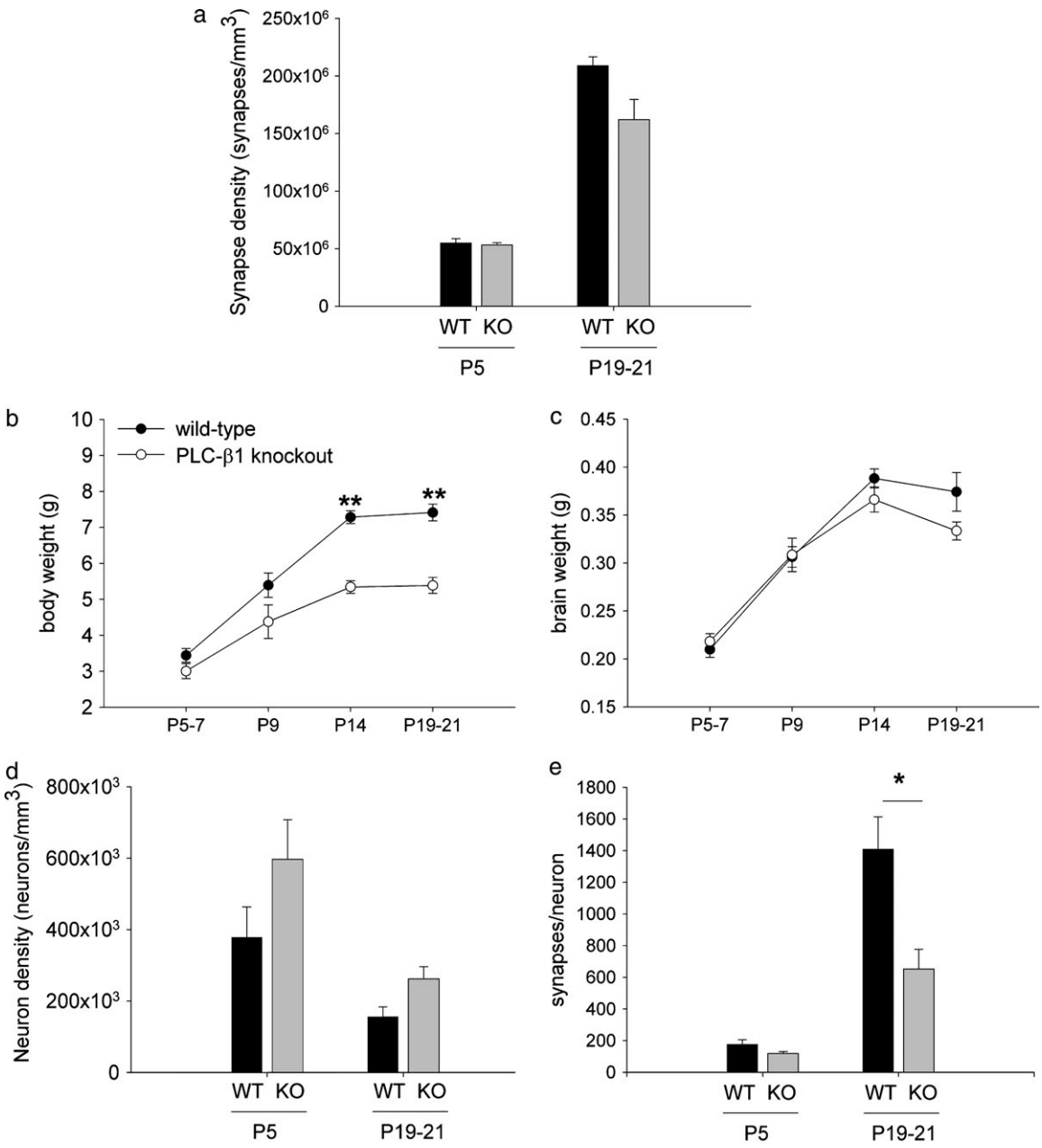

Figure 2. PLC-β1 signalling plays a role in synapse formation in developing barrel cortex. (*a*) There is a trend toward decreased synapse density in layers 2–4 of PLC-β1 knockout (KO) barrel cortex when compared to wild-type (WT) cortex [two-way ANOVA $F(1,9) = 4.051$, $P = 0.075$]. Between P5 ($n = 3$ knockout, 3 control brains) and P19–21 ($n = 4$ knockout, 3 control brains), a burst of synaptogenesis occurs, increasing synapse density 3.7-fold [two-way ANOVA $F(1,9) = 118.48$, $P < 0.0001$]. (*b*) Body weight of PLC-β1 knockout mice is significantly decreased [two-way ANOVA $F(1,159) = 33.90$, $P < 0.0001$] and (*c*) brain weight tends to be lower in PLC-β1 knockout mice [two-way ANOVA $F(1,48) = 3.76$, $P = 0.058$]. This implies that although synapse density is not significantly different in knockout mice, the number of synapses per neuron might be affected. (*d*) Knockout genotype increases neuron density in barrel cortex [two-way ANOVA $F(1,9) = 8.702$, $P = 0.016$]. (*e*) The calculated synapse per neuron ratio is also decreased in PLC-β1 knockout mice [two-way ANOVA, $F(1,8) = 8.544$, $P = 0.019$]. Asterisks represent *post hoc t*-tests (*$P < 0.05$, **$P < 0.0001$).

Dendritic spine analysis was carried out on two-dimensional projections of apical dendrites (as in Hayashi and Shirao, 1999; Marrs *et al.*, 2001), and no correction was made to account for spines lying below or above the dendrite, which were not counted. This may lead to an underestimate of spine density, but since experimental and control groups were treated identically, and spine lengths do not differ between groups, this underestimate should not affect the outcome of comparisons of density between groups. One potential confound arises, however, in dendritic morphology analysis. On a two-dimensional projection, the neck of a mushroom spine could potentially be occluded by the apical dendrite's shaft, making it appear to be a stubby spine. If the diameters of apical dendrites differ systematically between groups, an uneven bias in the proportion of mushroom spines observed could result. To find out whether such a bias occurred, we calculated average diameters of apical dendrites in the two groups and performed a correlation analysis between diameter and the percentage of mushroom spines observed. There is no difference between the average apical dendrite diameters of the two groups (WT: 3.1 ± 0.56 μm;

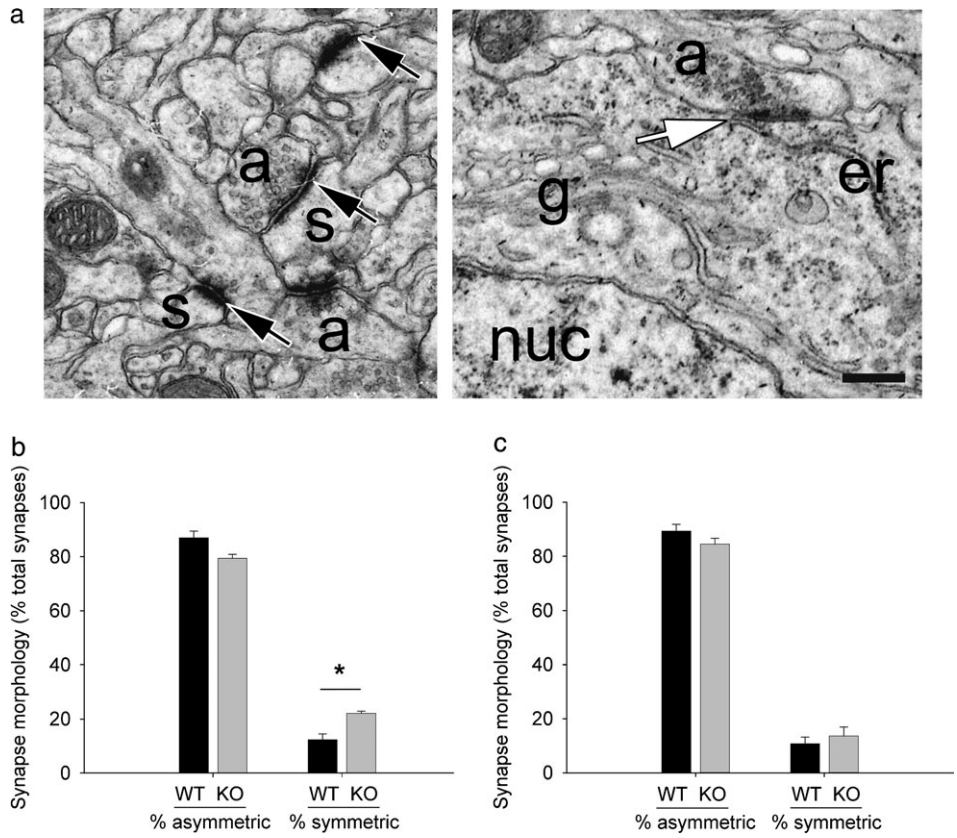

Figure 3. PLC-β1 signalling regulates synapse morphology. (*a*) Synapses were classified based on morphology as either asymmetric (black arrows) or symmetric (white arrow). Asymmetric synapses usually occur with an axon (a) as the presynaptic element and a dendritic spine (s) as the postsynaptic element. Symmetric synapses occur more often onto cell bodies, which can be distinguished by the presence of a nucleus (nuc) and cytoplasmic organelles such as endoplasmic reticulum (er) and Golgi apparatus (g). (*b*) PLC-β1 knockout (KO) barrel cortex has a higher proportion of symmetric synapses than that found in wild-type (WT) mice [two-way ANOVA, $F(1,13) = 10.625$, $P = 0.010$]. During the sensitive period of development (P5), *post hoc* tests confirm the increase in symmetric synapses is significant between wild-type and knockout mice (*Mann–Whitney test $Z = -1.964$, $P \leq 0.050$). (*c*) By 3 weeks of age (P19–21), this increase is no longer significant. Scale bar = 300 nm.

knockout: 2.90 ± 0.32 μm), and no correlation exists between apical dendrite diameter and the proportion of mushroom spines (Pearson's correlation $r = -0.202$, $P = 0.632$). Thus there should not be a systematic bias in calculating the proportion of mushroom spines between the groups.

Discussion

The results reported here demonstrate an important role for the phospholipase C-β1 signalling pathway in the development of cortical connectivity. Both synapse and dendritic spine development are disrupted in PLC-β1 knockout barrel cortex. These data suggest that these developmental events may be crucial for the formation of barrels, possibly as a result of their effects on signal integration in layer 4 neurons.

In normal mice, there is a burst of synaptogenesis in the barrel cortex during the second postnatal week (Micheva and Beaulieu, 1996; White *et al.*, 1997) that coincides with a critical period (in rat) during which activity mediates spine motility and concurrent refinement of cortical receptive fields (Vees *et al.*, 1998; Lendvai *et al.*, 2000; Stern *et al.*, 2001). Involvement of the PLC-β1 signalling pathway in activity-dependent regulation of spine morphology is suggested by the observation that in hippocampal spines, stimulation of group I mGluRs causes spine elongation (Vanderklish and Edelman, 2002). Calcium release from intracellular stores also affects spine morphology (Korkotian and Segal, 1999), and PLC-β1 regulates this release. When activated, PLC-β1 hydrolyses phosphatidyl inositol bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol triphosphate (IP3). DAG then activates protein kinase C and IP3 stimulates calcium release from intracellular stores.

We find using unbiased stereological counts of synapses and neurons that the absence of PLC-β1 signalling does indeed affect synapse and spine formation. While we observe no difference in synapse density between WT and knockout mice, there is an increase in neuron density and a decrease in synapse:neuron ratio in knockout barrel cortex. These changes are more pronounced at 3 weeks of age after the cortex has undergone a 'burst' of synaptogenesis. The 3.7-fold increase in synapse density between P5 and P19–21 reported here is within a factor of two of published increases of 7-fold in layer 4 of mouse barrel cortex (Micheva and Beaulieu, 1996) and 5.7-fold in all layers of rat barrel cortex (White *et al.*, 1997), a difference that may be accounted for by strain or species differences. The tendencies toward lower brain weight and increased neuron density at P19–21 in PLC-β1 knockout mice indicate a reduction in neuropil or changes in glial cells and perhaps a corresponding decrease in dendritic complexity. The reduced synapse per neuron ratio also indicates an overall decrease in synaptic connectivity in layers 2–4 of knockout barrel cortex.

The reduced synapse per neuron ratio is probably due to increased neuron packing density resulting from the smaller brain size of knockout animals. In support of this possibility,

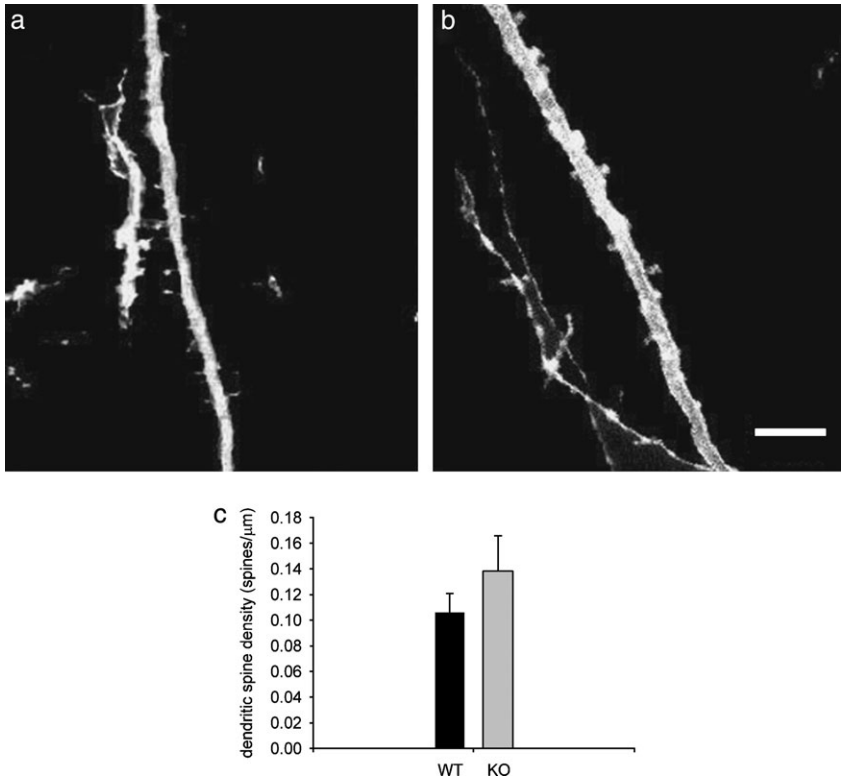

Figure 4. Dendritic spines were examined on apical dendrites of callosally projecting layer 5 pyramidal neurons filled with carbocyanine dye in wild-type barrel cortex (*a*) and PLC-β1 knockout barrel cortex (*b*). Dendritic spine density (*c*) did not differ between wild-type (WT) and PLC-β1 knockout (KO) mice. Scale bar = 10 μm.

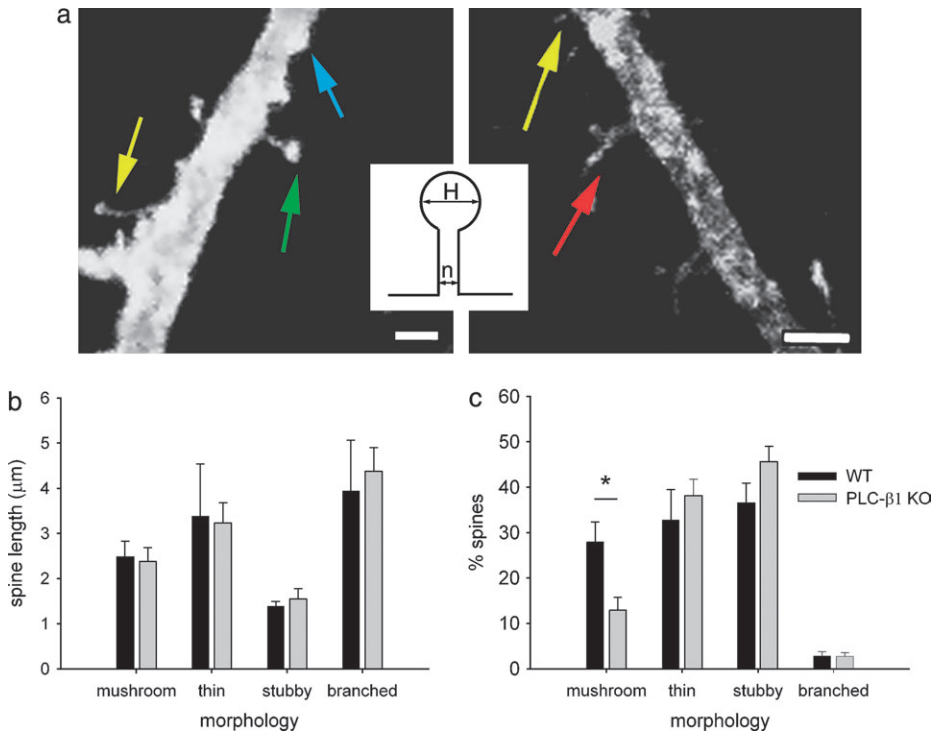

Figure 5. PLC-β1 signalling regulates dendritic spine morphology. (*a*) Dendritic spines were classified by morphology based on the number of spine heads (> 1 = branched spine) or the ratio of spine head width (H) to neck width (n, see Materials and Methods for explanation) as either mushroom (green arrow), thin (yellow arrows), stubby (blue arrow) or branched (red arrow). (*b*) Dendritic spine lengths did not differ between wild-type (WT) and PLC-β1 knockout (KO) mice. (*c*) The proportion of spines with mushroom morphology was decreased in PLC-β1 knockout barrel cortex indicating a possible disruption of spine maturation (*Mann–Whitney test, $Z = -2.165$, $P = 0.033$). Scale bars = 3 μm.

previous work in our laboratory showed a significant 17% decrease in the density of neurons between P14 and P21 in WT barrel cortex but no corresponding decrease in PLC-β1 knockout somatosensory cortex. Further, this study showed a 28% increase in neuronal packing density in all layers of PLC-β1 knockout somatosensory cortex when compared to WT cortex (V. Lane, C. Blakemore and A.J. Hannan, unpublished observations). We also have evidence that neuronal cross-sectional soma area is unchanged in PLC-β1 knockout barrel cortex at P21 in Golgi-impregnated sections (data not shown). Together, the data showing increased neuronal density and unchanged soma size in barrel cortex indicate the decreased synapse per neuron ratio calculated here is due to loss of neuropil.

The intriguing phenotype of reduced body weight in the PLC-β1 knockout mice raises the possibility that the plastic events mediated by PLC-β1 that we observe in the cortex may also occur in brain regions involved in regulating food intake. For example, plasticity affecting body weight has recently been observed in the hypothalamus in both developing (Bouret *et al.*, 2004) and adult mice (Pinto *et al.*, 2004). The PLC-β1 signalling pathway could be involved in hypothalamic plasticity since organelles resembling botrysomes, which contain PLC-β1 in the cortex (Kind *et al.*, 1997), have been observed in hypothalamic neurons in adult animals (Leranth *et al.*, 1985).

The observed reduction in average number of synapses per neuron probably results from the decrease of postsynaptic excitatory signalling via PLC-β1. Synaptogenesis in barrel cortex is known to rely in part on neuronal activity (Sadaka *et al.*, 2003). Another isoform of phospholipase C, PLC-β4, has also been shown to regulate synapse elimination in climbing fibres of the developing cerebellum (Kano *et al.*, 1998); thus it is plausible that the loss of PLC-β1 impacts directly upon synapse formation or elimination.

We also found that PLC-β1 signalling regulates the development of synapse morphology in barrel cortex. At P5, during the sensitive period of development of the barrel cortex, PLC-β1 knockout mice have an increased proportion of synapses with symmetric morphology (presumed inhibitory), which will result in an imbalance of inhibitory and excitatory inputs. The relative increase in inhibitory synapses is unexpected since PLC-β1 knockout mice often develop epileptic seizures from the fourth week of life, leading to the expectation of increased numbers of excitatory synapses and/or a decrease in inhibitory synapses. Indeed by P19–21, after the sensitive period, and before the onset of epileptic seizures, there is no longer a significant increase in symmetric synapses in knockout mice.

The transient increase in the proportion of symmetric synapses in PLC-β1 knockout mice may reflect an acceleration in the formation of inhibitory synapses, or delayed formation of excitatory synapses. This is very interesting since inhibitory synaptic activity regulates the critical period of changes in visual cortex in response to monocular deprivation (Fagiolini and Hensch, 2000). The change in proportion of symmetric synapses in PLC-β1 knockout mice may thus alter the fine balance of excitatory and inhibitory transmission and interfere with sensitive period plasticity in barrel cortex.

We also report that dendritic spine morphology during the sensitive period of development of the barrel cortex is regulated by PLC-β1 signalling. There is strong evidence that neural activity is necessary for spine formation, plasticity, and maintenance. Enucleation, lesioning of the lateral geniculate nucleus, dark-rearing and tetrodotoxin (TTX) injection in the retina result in a reduction in spine density on pyramidal cells of visual cortex (Valverde, 1967; Globus and Scheibel, 1966; Riccio and Matthews, 1985). In rat barrel cortex, whisker deprivation results in changes in spine morphology (Vees *et al.*, 1998). Spine density can also recover after deprivation (Valverde, 1971; Parnavelas *et al.*, 1974), further indicating the dependence of spine density on activity.

We find that apical dendrites of callosally projecting layer 5 pyramidal neurons as they pass through layer 4 bear significantly fewer spines with mushroom morphology in PLC-β1 knockout than in WT barrel cortex. These cells were chosen since a specific subpopulation (those projecting to the contralateral cortex via the corpus callosum) can be easily labelled, allowing detection of subtle changes in dendritic spines that might be obscured in a heterogeneous population. A difference in spine morphology implies altered postsynaptic integration of signals since spine morphology affects the calcium dynamics at the spine head and the amount of calcium that reaches the body of the dendrite (Majewska *et al.*, 2000). Similar studies of layer 4 neurons in these mice should be undertaken to further understand PLC-β1 regulation of dendritic spine development; however, these studies will be more difficult as the population of cells is more heterogeneous and harder to acces for staining.

Spines with mushroom morphology are presumably more mature than stubby spines since proportionally more synapses form on mushroom and thin spines than on stubby spines, filopodia and dendritic shafts as the cortex matures (Fiala *et al.*, 1998; Harris, 1999b). Thus the PLC-β1 signalling pathway could be involved in the maturation of spine morphology and of excitatory synaptic circuitry. Morphology and density of dendritic spines are affected in several forms of mental retardation (Kaufmann and Moser, 2000). Also, neuronal activity influences spine maintenance and plasticity (McKinney *et al.*, 1999; Lendvai *et al.*, 2000; Matus, 2000). An effect on spine morphology and plasticity appears to be functionally important in these animals: PLC-β1 KO mice show deficits in species-typical behaviours [KO mice displace ('burrow') fewer food pellets from a tube; nest construction is impaired; and they are unable to learn a whisker-discrimination learning task (A.L. Upton, unpublished observations].

We do not yet know how PLC-β1 signalling affects spine morphology. One possibility is that Ca^{2+} release from the ER due to PLC activation mediates morphological changes, since calcium from intracellular stores modifies spine shape in culture (Harris, 1999a). PLC-β1 could also regulate spine shape by controlling PIP2 concentrations, which affect peripheral actin structure formation in culture (Laux *et al.*, 2000). Transient expression of PLC-β1 in punctate-structures between ER and Golgi indicates a role for PLC in protein trafficking to dendrites (Kind *et al.*, 1997; Hannan *et al.*, 1998, 2001), which could also affect spine shape. Another possibility is that the PLC-β1 pathway could affect formation of mushroom spines via interactions with Shank and Homer, which are involved in regulating the morphology of dendritic spines (Naisbitt *et al.*, 1999; Sala *et al.*, 2001, 2003). Since Homer binds to type I mGluRs on the PSD and IP3 receptors on smooth ER, it can form a bridge between receptors that activate PLC-β1 (mGluR1 and 5) and the target receptor of IP3, a product of PLC-β1 activity.

In light of the results reported here, it would be interesting to perform assays of dendritic spine maturation and motility in PLC-β1 knockout mice using *in vitro* methods such as neurons expressing actin-tagged green fluorescent protein (Matus,

2000) or *in vivo* methods to test activity-dependent plasticity in spine motility and formation (Lendvai *et al.*, 2000; Knott *et al.*, 2002; Trachtenberg *et al.*, 2002).

Taken together, these results demonstrate that PLC-β1 signalling participates in the regulation of normal development of synapse and dendritic spine morphology. In PLC-β1 knockout mice, the abnormal cortical circuitry could contribute to the failure of layer 4 spiny stellate cells to rearrange into a barrel pattern in response to signals from thalamocortical axons.

Notes

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