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Remodeling of dendrites and spines in the C1q knockout model of genetic epilepsy

Yunyong Ma^{*,1}, Anu Ramachandran^{*,2}, Naomi Ford³, Isabel Parada¹, and David A. Prince¹

¹Dept. of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA 94305 USA

²Dept. of Neuroscience, University of Southern California, Los Angeles, CA 90089 USA

³University of California Davis School of Veterinarian Medicine, Davis, CA 95616 USA

Summary

Purpose—To determine whether developmental synaptic pruning defects in epileptic C1q-knockout (KO) mice are accompanied by postsynaptic abnormalities in dendrites and/or spines.

Methods—Immunofluorescence staining was performed on biocytin-filled Layer Vb pyramidal neurons in sensorimotor cortex. Basal dendritic arbors and their spines were reconstructed with Neurolucida software and their morphological characteristics were quantitated in Neuroexplorer.

Key findings—Seven to nine completely-filled pyramidal neurons were analyzed from the wild type (WT) and C1q KO groups. Compared to WT controls, KO mice showed significant structural modifications in their basal dendrites including 1) higher density of dendritic spines ($0.60 \pm 0.03/\mu\text{m}$ vs. $0.49 \pm 0.03/\mu\text{m}$ dendritic length in WT, $p < 0.05$); 2) remarkably increased occurrence of thin spines ($0.26 \pm 0.02/\mu\text{m}$ vs. $0.14 \pm 0.02/\mu\text{m}$ dendritic length in control, $p < 0.01$); 3) longer dendritic length ($2680 \pm 159 \mu\text{m}$ vs. $2119 \pm 108 \mu\text{m}$ in control); and 4) increased branching (22.6 ± 1.9 vs. 16.2 ± 1.3 in WT at $80 \mu\text{m}$ from soma center, $p < 0.05$; 12.4 ± 1.4 vs. 8.2 ± 0.6 in WT at $120 \mu\text{m}$ from soma center respectively, $p < 0.05$). Dual immunolabeling demonstrated the expression of putative glutamate receptor 2 (GluR2) on some thin spines. These dendritic alterations are likely postsynaptic structural consequences of failure of synaptic pruning in the C1q KO mice.

Significance—Failure to prune excessive excitatory synapses in C1q KO mice is a likely mechanism underlying abnormalities in postsynaptic dendrites, including increased branching and alterations in spine type and density. It is also possible that seizure activity contributes to these abnormalities. These structural abnormalities, together with increased numbers of excitatory synapses, likely contribute to epileptogenesis in C1q KO mice.

Keywords

dendrite; spine; pruning; C1q; genetic epilepsy

Correspondence should be sent to: Dr. David Prince, Dept. of Neurology and Neurological Sciences, Stanford University, Room M016, Alway building, 300 Pasteur Drive, Stanford, CA 94305-5122 USA, daprice@stanford.edu; Tel: 650-723-5522; Fax: 650-723-1080.

*These authors contributed equally to the work.

Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Introduction

Early neural development is marked by excessive outgrowth of neurons, leading to the formation of extensive networks of synapses. As development progresses, the redundant synaptic connections are selectively removed, or “pruned” (O’Leary & Stanfield, 1986; Hua & Smith, 2004). Pruning is achieved by the coordinated involvement of several genes and proteins (Liu et al., 2005; Stevens et al., 2007; Pfeiffer et al., 2010; Paolicelli et al., 2011; Schafer et al., 2012). Recently, the C1q gene, the initiation protein of classical complement cascade associated with the immune inflammatory response, has been shown to be important in synaptic pruning (Stevens et al., 2007; Schafer et al., 2012), through actions involving microglia (Schafer et al., 2012; Stephan et al., 2012). Mice in which the C1q gene was deleted developed larger numbers of retinogeniculate synapses and increased innervation of lateral geniculate relay cells (Stevens et al., 2007). These findings prompted experiments in which we examined structural and functional consequences of the failure to prune synaptic connectivity in neocortex of C1q-knockout (KO) mice. Results showed an increased excitatory connectivity in the cortical network manifest by increased frequency of spontaneous excitatory currents in layer V pyramidal (Pyr) cells, increased density of boutons on Pyr cell axons, as well as frequent electrographic and behavioral atypical absence seizures detected with video/EEG monitoring of chronically implanted mice (Chu et al., 2010). These findings raised the question of whether failure to prune excitatory synapses in the KO or ensuing epileptogenesis would be associated with postsynaptic abnormalities in dendrites and/or spines in KO mice, as occurs in other models of epilepsy (Wong, 2005) and developmental synaptic defects (Paolicelli et al., 2011). We hypothesized that a failure of synaptic pruning would result in higher spine density similar to the effect on axonal bouton density. Failure to prune synapses early in development might also result in persistence of less efficient spines (Kano & Hashimoto, 2009).

Dendritic spines are the primary recipient of excitatory inputs in Pyr cells (Harris & Kater, 1994) and highly specialized structures for regulation of synaptic functions (Harris & Kater, 1994; Bourne & Harris, 2008). Spines greatly vary in their morphologies, but typically consist of a stalk and a head whose membrane contains α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) postsynaptic glutamate receptors. The geometry of dendritic spines has direct impacts on synaptic transmission and local calcium signal processing (Koch & Zador, 1993; Noguchi et al., 2005). We classified the analyzed spines into three groups: thin, mushroom and stubby, based on the diameter of spine heads and relative length of spine necks (Methods below and Peters & Kaiserman-Abramof, 1970; Harris et al., 1992). Larger headed, mushroom-shaped spines may be more effective excitatory synaptic sites as they contain larger number of AMPA receptors; while synapses on smaller thin spines that contain a lower density of glutamate receptors are generally assumed to be functionally weaker (Irwin et al., 2000; Matsuzaki et al., 2001; Arellano et al., 2007; Bourne & Harris, 2008). This categorization is not absolute, because there may be a continuum of intermediate spine morphologies (Arellano et al., 2007) and ongoing plastic changes in spine density and structure (Yuste & Bonhoeffer, 2001).

Other aspects of dendritic structure such as length (Redmond et al., 2002) and branching pattern (Lohmann et al., 2002) can also be altered by the level of ongoing activity and innervations (Wong & Ghosh, 2002), observations that led us to examine gross dendritic morphology as well as spine density and types.

In the present experiments, we filled layer Vb Pyr cells of C1q KO and wild-type (WT) mice with biocytin and quantitated structural alterations in their basal dendritic length, spine density and spine types. Compared to WT controls, neurons of C1q KOs had longer basal

dendrites and a higher density of spines/ μm of dendritic length, resulting in a significantly larger number of spines per cell. Furthermore, there was a shift in spine type toward thinner and smaller spines and a trend toward decreased mushroom spine density. Results suggest that failure to prune synapses during development in the C1q KO is associated with longer basal dendrites and an increased density of thin, presumably functionally weaker spines on basal dendrites of layer V Pyr neurons.

Methods

All experiments were conducted in accordance with the policies and guidelines of the Stanford Institutional Animal Care and Use Committee. Each animal was coded by an independent observer and tissue processing and analysis of filled cells were performed by blinded investigators. In the course of whole-cell recordings, 9 layer Vb Pyr cells, 3 each from 3 WT male mice and 8 cells from C1q KO male mice (3 each from 2 mice and 2 from the third mouse) (all age P23) were filled with 0.2% biocytin. Slices containing filled cells were fixed overnight in 4% paraformaldehyde. Biocytin was visualized by reacting slices with Avidin D conjugated with Alexa Fluor 488.

Images were obtained with Zeiss LSM 510 confocal microscopy with a 40 $\times$ oil immersion lens for analysis of dendritic arborizations and a 100 $\times$ objective for examination of spine types. Alexa Fluor was excited by an Ar/Kr laser at 488 nm. Settings for pinhole size, gain and offset were kept constant throughout the different experiments. Image z stacks at 40 $\times$ were rendered to Neurolucida (MBF Bioscience) to trace the soma and basal dendrites completely. These stacks were analyzed using Neurolucida Explorer software to trace the soma and basal dendrites of each cell. The software was then used to convert these tracings into quantities of dendrite length etc.

A Sholl analysis was performed on each cell tracing by constructing concentric circles at 40, 80 and 120 μm from soma center and counting the number of basal dendritic branch crossings. For spine analysis, using Sholl data as a guide, four dendritic segments of $\sim 25 \mu\text{m}$ were selected from each cell, two centered at the 40 μm Sholl intersection and two distal at 80 μm from the soma center. These segments were viewed under the confocal microscope and Zstacks taken at 100 $\times$, with a zoom 4, optical distance 0.2 μm and resolution 1024 $\times$ 1024, to capture all of the spines present on the dendritic surface.

Using Neurolucida software, each of these dendritic segments was traced and each spine was categorized as one of three types: thin, stubby, or mushroom (Harris et al., 1992; Harris & Kater, 1994), according to measurements of stalk length and head width. Thin spines were classified as those with small heads (diameter $< 0.5 \mu\text{m}$) and a ratio of head diameter to neck length of less than 0.5; stubby spines were clear protrusions from the dendrites without identifiable stalks; the rest of the spines were considered as mushroom type (Fig. 3A). We did not find filopodia-like dendritic protrusions in the filled neurons (but see Mattila & Lappalainen, 2008). For each dendritic segment, the total number of imaged spines was counted as well as the number in each respective category. The spines of all 4 segments for each cell were pooled to calculate spine density of that cell. Total analyzed dendritic segment length for spine types was $103.8 \pm 5.5 \mu\text{m} / \text{neuron}$ ($n=7$) for KO cells and $101.1 \pm 4.7 \mu\text{m}$ for WT cells ($n=8$). Spine classification and counts were also made by the blinded observer. Statistical significance was determined by Student's t-Test at $p < 0.05$.

Dendrite segments were selected carefully using the following criteria: 1) complete biocytin-filling; 2) no overlap with adjacent dendrites; 3) within 60 μm from the surface of the section; 4) all confocal stacks extended 2 μm above to 2 μm below the imaged dendrite.

In order to assess the putative expression of GluR2 in dendritic thin spines, we also performed dual immunocytochemical staining for GluR2 and biocytin. KO slices with biocytin-filled cells were processed using the Avidin D – Alexa Fluor method, as described above. Next, slices were incubated with primary antibodies against GluR2 (rabbit polyclonal at 1:500, Millipore, Billerica, MA). After 48 hours, slices were reacted with fluorescent secondary antibodies (donkey anti-rabbit Cy3 at 1:100, Jackson ImmunoResearch, West Grove, PA). Confocal z-stack images were obtained from the dendritic segments of biocytin-labeled cells under a 100× oil objective with an optical distance of 0.2 μm and a zoom 4.

Results

We measured length and branching of basal dendrites as well as spine types and density from layer V Pyr cells from 3 KO and 3 WT mice. There was a significantly increased dendritic length in KO (2680 ± 159 μm, $n=8$ cells) compared to WT mice (2119 ± 108 μm, $n=9$ cells) ($p = 0.012$; Fig. 1). Sholl analysis from these cells showed that KO mice had a significantly greater number of dendritic intersections than control at 80 μm (22.6 ± 1.9 vs. 16.2 ± 1.3 , respectively, $p = 0.016$) and 120 μm (12.4 ± 1.4 vs. 8.2 ± 0.6 , respectively, $p = 0.025$), but not at the 40 μm Sholl distance (20.0 ± 1.1 vs. 18.1 ± 0.7 , $p = 0.162$) (Fig. 2).

Total spine density and distinct subtypes of spines were measured in 7 KO and 8 WT Pyr cells. There were no significant differences in occurrence of different types of spines between 40 and 80 μm Sholl distances (data not shown), a result consistent with previous reports showing that dendritic spine morphology does not depend on distance from soma (Konur et al., 2003; Ballesteros-Yanez et al., 2006; Arellano et al., 2007). Therefore, we combined data from segments at 40 and 80 μm in our analysis. The KO cells had a greater total spine density ($0.60 \pm 0.03/\mu\text{m}$ dendritic length; $n=7$ cells) than WT ones ($0.49 \pm 0.03/\mu\text{m}$ dendritic length; $n=8$ cells) ($p = 0.034$) (Fig. 3D1). There was a remarkable increase in the occurrence of thin spine subtype in KO mice ($0.26 \pm 0.02/\mu\text{m}$ vs. $0.14 \pm 0.02/\mu\text{m}$ dendritic length in WT; $p = 0.002$) (Fig. 3B, C, D2). There were non-significant increases in stubby spines (KO: 0.20 ± 0.02 vs. control: $0.15 \pm 0.02/\mu\text{m}$ dendritic length) ($p = 0.058$), and decreases in mushroom (KO: 0.14 ± 0.02 vs. control: $0.20 \pm 0.01/\mu\text{m}$ dendritic length) ($p = 0.051$) (Fig. 3D2). Thin spines accounted for 39.3% of total spines in KO and 27.6% in WT, while mushroom spines accounted for 23.2% of all spines in KO and 41.5% of WT. These results indicated a larger proportion of thin spines on the basal dendrites of KO compared to WT cells.

In order to assess whether the thin spines in the C1q KO cortex possessed GluRs, we performed immunocytochemical staining for GluR2 in biocytin-filled layer V KO Pyr cells. We found that some of thin spines showed colocalization with GluR2 (Fig. 4), suggesting thin spines may be at least partially functional.

Discussion

These results show that there are three structural modifications in basal dendrites of C1q KO mice as compared with WT controls: 1) higher density of dendritic spines; 2) markedly higher density of thin spines; and 3) longer dendritic length and increased branching. These dendritic alterations are likely postsynaptic structural consequences of failure of synaptic pruning in the C1q KO mice.

Higher spine density

There is increasing evidence for pruning functions of the complement proteins (Stevens et al., 2007; Rosen & Stevens, 2010; Schafer et al., 2012; Stephan et al., 2012). C1q and other

complement proteins are localized in synaptic regions where they tag weak or redundant synaptic structures that are removed through phagocytosis by microglia (Stephan et al., 2012). There are also a number of other reports of involvement of microglia in synaptic pruning. For example, Trapp and coworkers (2007) showed that microglial activation by inflammation mediates synaptic stripping in the cerebral cortex. A transiently reduced number of microglia delays synaptic pruning, and is associated with occurrence of excessive dendritic spines in developing hippocampus (Paolicelli et al., 2011). The increased spine density in the present study can thus result from pruning defects in the C1q KO mice. The group of Pyr cells we analyzed was obtained from P23 KO mice in which frequent epileptiform discharges and behavioral seizures are known to occur (Chu et al., 2010). This raises the possibility that the seizure activity *per se* might contribute to the dendritic abnormalities described above in the KO mice. Dendritic abnormalities in models of partial and generalized epilepsy usually involve spine loss (Muller et al., 1993; Drakew et al., 1996; Jiang et al., 1998; Zha et al., 2005 & 2009; Ampuero et al., 2007; Zeng et al., 2007; Santos et al., 2011; Kitaura et al., 2011; Guo et al., 2012; reviewed in Wong & Guo, 2012), rather than the increases reported here. However, increases in spine density associated with seizures are also reported (Aliashkevich et al., 2003; Freiman et al., 2011; Zhao et al., 2012). Dendritic length may be increased in both pyramidal cells and some classes of interneurons in some epilepsy models (Teskey et al., 2006; Zhang et al., 2009; Halabisky et al., 2010). Data from experiments focused on the timing of onset and extent of seizure activity relative to the dendritic abnormalities might determine whether alterations in activity, as well as the established pruning failure, contribute to epileptogenesis in C1q KO mice.

There may be a relationship between the previously-reported increase in bouton density of layer V Pyr cell axons of C1q KO mice (Chu et al., 2010) and the present data showing an increase in spine density in these mice. However, because axons of layer V Pyr cells innervate targets outside of layer V, and the filled neurons receive excitatory inputs from other laminae and regions, such a correlation between pre- and postsynaptic structures will be difficult to establish. Results of immunolabeling of GluR2 suggest that at least some of the thin spines may be innervated and functional. Moreover, because layer V neurons receive a significant recurrent excitatory input from other layer V Pyr cells (Salin et al., 1995) it is likely that a proportion of the inputs onto the spines are from the small boutons of KO layer V Pyr cells. However, since precise verification of the putative structural correlation would require labeling of connected pre- and postsynaptic neurons, or orthodromic labeling of inputs to the filled cells from other sites, a precise correlation between the pre- and postsynaptic anatomical abnormalities cannot be derived from the present data.

Thin spines

During developmental pruning, weaker synapses are preferentially targeted and removed, while stronger ones are kept and strengthened (Kano & Hashimoto, 2009). In the C1q KO brains, the thin spines are most likely associated with weaker synaptic connections that are preserved due to loss of pruning function mediated by C1q protein (Stevens et al., 2007; Stephan et al., 2012). The relatively small bouton size (Chu et al., 2010) and the smaller head of thin spines in the KO mice are consistent with known correlations between size of presynaptic (e.g. the active zone area of boutons) and postsynaptic structures (e.g. postsynaptic density (PSD)) (Schikorski & Stevens, 1997). Filopodia are thin finger-like processes that may be precursors of dendritic spines (Mattila & Lappalainen, 2008). The thin spines identified in our experiments could be distinguished from filopodia by the presence of spine heads. The absence of filopodial protrusions in our preparations may be due to their occurrence at very early developmental stages in mammalian cortex (Portera-Cailliau et al., 2003) and subsequent removal during maturation (Fiala et al., 1998). It is also possible that

genes other than C1q, such as plexins (Liu et al., 2005) or the fragile X mental retardation protein (FMRP) (Comery et al., 1997) might be involved in the pruning long thin dendritic processes.

Longer dendritic length

Increase in dendritic branching and length in layer V Pyr cells have also been observed previously in the kindling model (Teskey et al., 2006) and in dendrites of interneurons in pilocarpine-treated epileptic mice (Zhang et al., 2009; Halabisky et al., 2010). However, the mechanisms underlying these abnormalities are unclear. One possibility is that seizure activity up-regulates the expression of brain-derived neurotrophic factor (BDNF) (Binder et al., 2001) that in turn enhances dendritic growth in the immature cortex (McAllister et al., 1995; Danzer et al., 2002; Tolwani et al., 2002). This mechanism may also account for the hypertrophy of dendrites of hilar interneurons in epileptic mice following pilocarpine-induced status epilepticus (see Figs.1 and S1 in Halabisky et al., 2010). Increased calcium influx through NMDA receptors activated by excessive glutamate release during seizure activity might further promote both dendritic growth (Redmond et al., 2002) and branching (Lohmann et al., 2002).

Functional correlations

Impacts of a long and slender spine neck may be bi-directional. On one hand, the slender neck can act as an electrical filter to reduce the amplitudes of excitatory postsynaptic potentials (EPSPs) received by parent dendrites and somata (Araya et al., 2006). However, as shown by a recent simulation modeling study, amplitudes of EPSPs in spine heads are heavily dependent on spine neck resistance rather than synaptic conductances or spine head diameters (Gulledge et al., 2012). Thus, higher spine neck resistance associated with thin spine necks in KO mice might generate larger EPSP amplitudes within spine heads and subsequently evoke NMDA-spikes (Gulledge et al., 2012). The NMDA spikes constitute a dominant amplification mechanism in distal dendrites and facilitate neuronal firing in Pyr cells (Larkum et al., 2009). Moreover, the presumed enhanced activation of NMDA receptors in heads of thin spines may induce stronger long-term potentiation in KO mice (Madison et al., 1991; Noguchi et al., 2005). The thin spines may limit the amplitudes of EPSPs received by dendritic shafts and somata, but may also increase the excitability of neuronal circuits through strong activation of NMDA receptors in KO mice. Excitatory synaptic signaling via the increased numbers of thin spines in the C1q KO cortex does not apparently affect the mean amplitude of synaptic currents, which is neither enhanced nor attenuated (Chu et al., 2010). Although smaller spines may have a decreased number of AMPA receptors (Matsuzaki et al., 2001), this may not affect the EPSP/C amplitude if the postsynaptic receptors are not saturated by the released glutamate (Liu et al., 1999; McAllister & Stevens, 2000; Nimchinsky et al., 2004).

The higher spine density and longer dendritic length would result in a marked increase in the total number of spines on basal dendrites of individual C1q KO Pyr cells. This, together with the increased bouton density reported previously (Chu et al., 2010), suggests that there is a significantly larger number of excitatory synapses in KO compared to WT mice. This could, in part, account for the increased frequency of spontaneous and miniature EPSCs (Chu et al., 2010). The smaller spine heads and axonal bouton size are likely correlated with other structural synaptic parameters such as decreases in vesicle number, active zones and postsynaptic densities (Yeow & Peterson, 1991; Pierce & Lewin, 1994; Harris & Sultan, 1995) that would potentially affect excitatory synaptic transmission, particularly during periods of high frequency discharges, as may occur during seizures.

Overall, these results provide anatomical evidence to suggest that failure to prune presynaptic structures through activation of the classical complement cascade in C1q KO mice (Chu et al., 2010), may also result in abnormalities in postsynaptic elements, i.e. dendritic spines, and affect gross dendritic structure. Further research is required to assess potential contributions from epileptiform activity *per se*, as well as the functional consequences of the altered dendritic morphologies, such as changes in excitatory transmission and synaptic plasticity during high frequency repetitive activation of neocortical circuits.

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Figure 1. Increased dendritic length in C1q KO vs. WT mice

A: Confocal projection images of dendritic arbors of biocytin (green) – filled Pyr cells from WT (left) and KO (right) mice. **B:** Neuroexplorer projection images of 3-D reconstructed basal dendrites shown in A. **C:** Bar graph of the dendritic length of KO (n=8) and control (n=9) Pyr cells. *: p<0.05.

Figure 2. Increased dendritic branching in KO vs. WT mice

A: Concentric circle lines (red) overlapping 3-D reconstructed basal dendrites. The increment between Sholl lines was 40 μm . **B:** Bar graph of the numbers of intersections between dendrites and Sholl lines at three distances from the soma center: 40, 80, and 120 μm .

Figure 3. Increased density of total dendritic spines and thin spines in KO vs. WT mice
A: Three types of spines: thin (left), mushroom (middle), and stubby (right). Arrows indicate spine heads. **B:** Confocal images of dendritic segments from a WT (B1) and a KO (B2) Pyr cells. **C:** Reconstructed images of three distinct types of spines along corresponding dendritic segments shown in B (thin: red, mushroom: blue, stubby: green). For visual clarity, stubby spines are indicated by green arrows. **D:** Bar graph of the density of total spines (D1) and individual types of spines (D2) from 7 KO and 8 WT Pyr cells. **: $p < 0.01$.

Figure 4. Localization of glutamate receptor 2 (GluR2) in dendritic thin spines in a biocytin-filled C1q KO Pyr cell

A: Confocal image of a dendritic segment with dual immunolabeling for biocytin (green) and GluR2 (red), representative of findings in 6 biocytin-filled Pyr cells. Three representative sites of colocalization on thin spines (1, 2 and 3) were enlarged on right and indicated by arrows. Colocalization of the two immunoreactivities (yellow) is present on spines indicating that they express GluR2. Calibration = 1 μm for A and 0.5 μm for images 1–3.