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Dendrite Growth and the Effect of Ectopic Rheb Expression on Cortical Neurons

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Abstract

Ras homology enriched in brain (Rheb) is a GTPase that activates the protein kinase mammalian Target of Rapamycin (mTOR). Rheb mutations cause intellectual delay and megalencephaly. mTOR hyperactivation causes a constellation of neurodevelopmental disorders called “mTOR-opathies” that are frequently accompanied by hyperexcitable cortical malformations. Cortical malformations within the anterior cingulate cortex (ACC) and somatosensory cortex (SSC) frequently colocalize with hyperexcitability. Although Rheb and mTOR are implicated in the formation of cortical lesions, seizure activity, and defects in neuronal migration, the contribution of Rheb to changes in neuron size and dendrite morphology is not well established. Here, *in utero* electroporation of the developing embryonic brain was used to assess soma and dendrite growth in ACC and SCC layer II/III neurons. We found that between P0 and P21, neuronal soma size increased by 50 and 122 percent in the ACC and SSC, respectively. The increased size was accompanied by an increase in the number of basal dendrites and enhanced dendrite complexity. As an indicator of the involvement of the mTOR pathway in neuron maturation, phosphorylation of the mammalian target of rapamycin (mTOR) substrate S6 was identified in migrating cortical neuroblasts and maturing neurons. Notably, ectopic expression of Rheb caused cortical malformations comprised of ectopically positioned cytomegalic neurons with dendrite hypertrophy. This study provides a direct comparison of neuron maturation across two cortical regions during development, provides evidence for mTOR pathway activity during neuron

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Ethics Approval and Consent to Participate.

Research protocols were approved by and performed according to the Clemson University Institutional Animal Care and Use Committee.

Consent for Publication. Not applicable

Availability of Data and Materials. The data generated or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Interest: none

Author's Contributions. A.M.S. and D.M.F. designed experiments, performed *in utero* electroporations, and wrote the paper. A.M.S., C.S., and M.C.M. performed immunohistochemistry and confocal microscopy. A.M.S. and C.S. performed morphological analysis. D.M.F. acquired funding. All authors read and approved the manuscript.

maturation, and demonstrates that ectopic Rheb expression without mutation is sufficient to induce cortical malformations with cytomegaly and dendrite hypertrophy.

Keywords

mTOR; Rheb; dendrite; focal cortical dysplasia

Introduction

Inactivating mutations in the Tuberous Sclerosis Complex (TSC) 1/2 genes cause the disorder TSC [1–3]. TSC patients have focal regions within the cerebral cortex called cortical tubers. Cortical tubers contain ectopically placed cytomegalic and dysmorphic neurons that have hypertrophic dendrites and hyperactive mTOR [4–9]. These features are shared with focal cortical dysplasias (FCDs) [10]. Mutations in mTOR, AKT3, DEP-domain containing 5 (DEPDC5), and NPRL3 also cause FCDs and other cortical irregularities [11–18]. Collectively, these disorders can be categorized as mTOR-opathies which share the pathognomonic feature of hyper-active mTOR. It is unclear which neuroanatomical, cellular, and biochemical alterations underlie the neuropathological manifestations seen in these patients.

mTOR is a protein kinase that partitions into two heteromeric complexes, mTORC1 and mTORC2, and has emerged as a potential pharmacological target in numerous disorders [19]. mTORC1 controls cell growth by phosphorylating p70S6 kinase (p70S6K) and 4EBP [20–22]. p70S6K subsequently phosphorylates the ribosomal S6 subunit (S6) [20,23–26]. mTORC1 is activated by the GTPase Rheb and is inhibited by the GTPase activating protein complex encoded for by TSC1/2 [27–29].

mTORC1 and mTORC2 are required for corticogenesis [30–32]. Knocking out mTOR in neural stem cells (NSCs) reduces NSC proliferation and cortical thickness [32]. mTORC1 and mTORC2 also regulate neuron morphology and dendrite complexity [33–38]. Effects on neuron morphology and dendrite complexity are phenocopied by expression of constitutively active Rheb or Tsc1 knockout [36,39–41]. Taken together, mTORC1 is required for the production and maturation of cortical neurons.

Recently, pathogenic RHEB mutations were identified in patients with intellectual delay and megalencephaly [42]. The etiology of RHEB associated intellectual delay and of the mTOR-opathies is unclear. *In utero* electroporation of constitutively active Rheb has been used to generate focal cortical dysplasia-like lesions that induce spontaneous seizures in mice [40,41,43]. Wild-type Rheb overexpression also induces migratory defects, although whether wild-type Rheb alters cortical neuron morphology is not clear [44]. Here we examined neuron morphology in the ACC and SSC. We chose these regions because cortical tubers and FCDs are frequently localized to the ACC and SSC. In addition, hyperexcitability and altered functional connectivity in these regions are well documented in epilepsy and autism spectrum disorders [45–48]. We measured neuron soma and dendrite growth during development and tested the effect of Rheb overexpression on these outcomes. Remarkably, we found that ectopic wild-type Rheb overexpression was sufficient to increase dendrite

complexity across cortical regions by postnatal day 14. *In utero* Rheb electroporation caused ectopic neuron placement and enhanced cell growth. Taken together, the results of this study demonstrate that Rheb overexpression without mutation is sufficient to cause focal cortical dysplasia-like lesions across cortical regions and implies that altered Rheb expression without mutation may perturb development.

Materials and Methods

Animals

Research protocols were approved by the Clemson University Institutional Animal Care and Use Committee. Experiments were performed on CD1 mice.

In utero electroporation

High purity endotoxin free DNA (~1–1.5 µg/µl) was diluted in PBS with 0.1% fast green as a tracer. DNA solution (0.5–1.0 µl) was injected into the lateral ventricles of embryos at E14.5–16 using manual pressure with a pulled glass pipette. Glass pipettes were pulled with a P97 Sutter micropipette puller and irradiated in a sterile hood with UV light. After DNA injection, electrodes (model 520; BTX) were soaked in PBS and placed on the heads of the embryos, and five 30V square pulses of 50 ms duration with 950 ms intervals were applied using a pulse generator (ECM830; BTX). A combination of plasmids encoding GFP, tomato, or Rheb under the chicken β-actin promoter with CMV enhancer (CAG) was electroporated.

Slice preparation and immunohistochemistry

Mice were deeply anesthetized where appropriate with pentobarbital (50 mg/kg) or on ice (P0 pups) and decapitated. The brain was removed in PBS and placed in 4% paraformaldehyde in PBS and incubated for 4 hours at room temperature (e12-P0) or overnight at 4°C, then washed in PBS. The region of electroporation was imaged using a Nikon AZ100. The next day, 300-µm-thick slices were prepared using a vibratome (Leica VTS 1000). Immunostaining was performed in free-floating 300-µm-thick slices. Free-floating sections were blocked in PBS containing 0.1% Triton X-100, 0.1% Tween-20, and 2% BSA and incubated in primary antibodies (see below) overnight at 4°C. After three washes in PBS containing 0.1% Tween-20, slices were incubated with the appropriate secondary antibody (Alexa Fluor series at 1:500 [Invitrogen]) for 1 hour at room temperature. Primary antibodies were goat anti-DCX (1:500; Santa Cruz Biotechnology Inc.) and rabbit anti-pS6 (1:1,000; Cell Signaling; Ser240/244, 61H9, #4838). Each staining was replicated in at least 3 slices from 3–5 different mice. Z-section images were acquired on a spectral confocal microscope (Leica SPE) with a ×20 dry objective (N.A. 0.75) or ×63 oil immersion objective (N.A. 1.5). Low-magnification images were acquired with a ×5 dry objective. Images were analyzed and reconstructed using ImageJ 1.51r software or Photoshop CS6.

Sholl analysis and cell growth measurements

Images of the anterior cingulate cortex and the somatosensory cortex were uploaded to Fiji (ImageJ 1.51r/n). Dendrites were traced using the Simple Neurite Tracer plug-in. The marking tool was used to trace the projections from the soma to the projection terminus.

Sholl analysis was performed at 10 μm intervals to a maximum radius of 400 μm to quantify dendritic arborization using the sholl plug-in (http://fiji.sc/Sholl_Analysis, version 3.4.5). The longest dendrite was selected as the primary apical dendrite and length calculated by simple neurite tracer. Basal dendrites were quantified by Simple Neurite Tracer as dendrites localized to the bottom two thirds of the soma.

To measure cell size, Z-stack images of GFP+ cells coronal sections were acquired with a $\times 20$ dry objective (Numerical Aperture, 0.75) using high-low settings to minimize saturation. In Image J, the freehand tracing tool was used to outline cell somas. Cell size was represented relative to the indicated controls.

Statistics

Student's T tests were performed for comparisons. Experiments were performed on 3-8 mice for each condition.

Results

Plasmids encoding GFP were introduced into neural stem cells of embryonic day 14.5 mice by *in utero* electroporation (Figure 1A). By changing the directionality of the electrodes, selective targeting of the SSC or ACC could be achieved (Figure 1B). Neurons began to extend small basal cellular processes protruding from a small elliptical soma by P0, at which point the morphology of neurons in the ACC and SSC were similar (Figure 1E, F). Soma size increased in the ACC by more than 50% from P0 to P21 ($P_0=1.00 \pm 0.02745$, $N=31$ vs. $P_{21}=1.571 \pm 0.05132$, $N=58$; $P<0.0001$). By P21 the average size of cells in the SSC had increased by $>100\%$ ($P_0=1.00 \pm 0.02326$, $N=183$ vs. $P_{21}=2.222 \pm 0.04841$, $N=142$; $P<0.0001$) (Figure 1C, D). These results demonstrate that the soma size of cortical neurons increases between P0 and P21.

Cortical neuron dendrite complexity was assessed at P0 after e14.5 *in utero* electroporations of CAG-GFP. Dendrites were traced in coronal sections of the SSC and ACC. At P0, control neurons had few cellular processes across cortical regions having similar complexity (Figure 2A). P0 neurons consisted of a single long apical projecting process and 1-2 shorter basal dendrites and there was no significant difference between these regions (Figure 2B). The dendrite complexity at P21 was observed to be significantly increased proximal to the soma (10–100 μm), having on average a range of 2.5625 to 6.0 crossings compared to 0.0689655 to 1.82759 crossings (P_0 ; $N=29$ vs. P_{21} ; $N=16$; $P<0.0001$). On average, SSC neurons had 1.3 basal dendrites at P0, but this increased to 4.6 basal dendrites by P21 ($N=38$; 1.316 ± 0.1733 vs. $N=34$; 4.618 ± 0.2274 ; $P<0.0001$). These results confirm that between P0 and P21 dendrite arborization occurs in cortical neurons.

Previous studies have established that mTOR is active within the developing forebrain. To confirm these findings, we performed immunohistochemical analysis of coronal e14.5-16.5 brain sections for the phosphorylated form of S6 (240/244), an established mTOR substrate. pS6 staining at embryonic day 14-16.5 was predominantly localized to DCX positive neuroblasts and enriched in neuroblasts within the outer layers of the cortical plate (Figure 3A, B). pS6 positive neuroblasts were also occasionally found in the ventricular zone

(Figure 3C). pS6 levels remained elevated at P0 in cortical neurons labeled with GFP following e14.5 *in utero* electroporations (Figure 3D–F). pS6 could be detected in the ACC at P21 (Figure 3G, H), as well as in electroporated neurons in the SSC (Figure 3I). Taken together, the mTORC1 pathway is active in the developing brain in maturing neurons.

Abnormal dendrite patterning caused by mTOR hyper-activation is seen in FCD and TSC models [3,6,36,39,40,49,50]. Ectopic expression of constitutively active or wild-type Rheb has been used to model FCDs. To determine whether Rheb overexpression is sufficient to increase cell size, we performed *in utero* electroporations of wild-type Rheb. Soma size was examined at P14 in control and Rheb expressing neurons. Rheb expressing neurons had significantly larger and more complex dendrite arbors in the ACC and SSC (Figure 4A, B). Ectopic cells were found in lower layers of the cortex (Figure 4C, D). Moreover, Rheb overexpression significantly increased soma size (Figure 4E). The misplaced cells in combination with an increased size and hypertrophic dendritic arbors gave the appearance of a focal cortical dysplasia-like lesion (Figure 4F).

Discussion

Here we demonstrate the utility of *in utero* electroporation to measure and influence cortical neuron growth. Previous studies have manipulated the mTOR pathway in individual regions within the cortex to follow changes in growth [40,41]. *In utero* electroporation has been widely used to manipulate and label neurons of the developing brain to mimic neurodevelopmental disorders [51–53]. At P0, the data demonstrate that ACC and SSC neurons were similar in size. By P21 however, neurons grew significantly, with SSC neurons increasing in size by more than 50% and ACC neurons approximately doubling in size. During the same period, dendrite complexity and the number of basal dendrites also increased significantly. The study of dendrite arborization presented here focused predominantly on regions proximal to the soma.

Studies have demonstrated that the basal dendrites of PTEN and TSC1 knockouts or Rheb over-expression resulted in basal dendrite hypertrophy [6,36,40,41,49]. Since these proteins impinge upon the mTOR pathway, and because mTOR regulates a wide range of cellular processes within the developing cortex, mTOR pathway activity was also assessed. Using antibodies directed to the mTOR substrate pS6 240/244, we confirmed that mTOR activity is elevated during the perinatal period. The role of the mTOR pathway in neuron growth and dendritogenesis has been described for the hippocampus, olfactory bulb, and cortex. The results of this study demonstrate an enrichment of pS6 within maturing neuroblasts, highlighting the importance of mTOR signaling in newborn neurons.

mTOR pathway hyperactivity in the developing cerebral cortex contributes to a wide range of neurodevelopmental disorders [54]. To increase the activity of the mTOR pathway, we electroporated wild-type Rheb into the developing cerebral cortex. Indeed, wild-type Rheb increased neuron soma size. Moreover, we found that Rheb electroporated neurons had increased dendrite complexity. Abnormal dendrite arborization is a shared characteristic in a range of models of neurodevelopmental disorders. These patients often present with neurological manifestations, including epilepsy, that are indicative of hyperconnectivity.

Since mTOR is required for dendrite arborization under normal physiological conditions, it can be expected that overexpression is sufficient to drive dendrite hypertrophy.

Conclusions

The results of this study support a model wherein Rheb overexpression, even in the absence of a mutation, stimulates abnormal neuron growth and dendrite arborization. These results highlight that uncontrolled Rheb signaling to mTOR causes cellular phenotypes that are associated with neurodevelopmental disorders.

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Highlights

- Neuron soma and dendrite growth rates differ across cortical regions
- mTOR activity as assessed by pS6 is abundant in neuroblasts and maturing neurons
- Rheb overexpression is sufficient to induce cortical focal cortical dysplasia-like lesions comprised of cytomegalic neurons with hypertrophic dendrites

Figure 1. Growth of Cerebral Cortical Neurons

A) Schematic diagram of *in utero* electroporation B) Electroporated cerebral cortex outlined (upper) and schematic of electrode directionality in relation to plasmid injection. C) Quantification of relative area of soma in the ACC at P0 and P21. D) Quantification of relative area of soma in the SSC at P0 and P21. E) Representative image of GFP positive neurons at P0 and P21 in the ACC. Scale bar = 50 μ m. F) Representative image of GFP positive neurons at P0 and P21 in the SSC. Scale bar = 50 μ m. ACC=Anterior Cingulate Cortex, SSC=Somatosensory Cortex, ****= $P < 0.0001$

Figure 2. Dendrite Arborization in Electroporated Cerebral Cortical Neurons

A) Sholl analysis of P0 neurons in the ACC (red) and SSC (green). B) Quantification of the average number of basal dendrites at P0 in the ACC and SSC. C) Sholl analysis of P0 (black) and P21 (green) neurons in the somatosensory cortex. D) Quantification of the number of basal dendrites in P0 and P21 neurons. ACC=Anterior Cingulate Cortex, SSC=Somatosensory Cortex, ****= $P < 0.0001$

Figure 3. mTOR Pathway Activity in Maturing Cortical Neurons

A) Coronal section of e16 brain stained for pS6 (red), DCX (green), Topro-3 (blue). Scale bar = 150 μ m. B) Cortical plate demonstrating pS6 (red) cells are DCX (green) positive neuroblasts. Scale bar = 50 μ m. C) Image of ventricular zone demonstrating that most pS6 (red) positive cells are DCX positive (green) neuroblasts. Scale bar = 50 μ m. D) Coronal section of e14.5 electroporated brain at P0 showing GFP positive cells. Scale bar = 100 μ m. E) P0 coronal section shown in E showing pS6. Scale bar = 100 μ m. F) Merge of GFP (green), pS6 (red), and Topro-3 (blue) showing co-localization of mTOR activity in neurons. Scale bar = 100 μ m. G) Coronal section of P21 brain showing pS6 staining in ACC. Scale bar = 50 μ m. H) 5 $\times$ image of G from P21 brain showing GFP (green) and pS6 (red) in ACC. Scale bar = 150 μ m. I) Coronal section of P21 brain showing GFP (green), pS6 (red), and Topro-3 (blue) in SSC. Scale bar = 50 μ m. ACC=Anterior Cingulate Cortex, SSC=Somatosensory Cortex

Figure 4. Ectopic Rheb Expression Drives Abnormal Cortical Neuron Growth and Dendrite Arborization

A) Sholl analysis of control and Rheb ACC neurons at P14. B) Sholl analysis of control and Rheb SSC neurons at P14. C) Representative image of P14 control electroporated SSC neurons. Scale bar = 50 μm . D) Representative image of P14 Rheb electroporated SSC neuron. Scale bar = 50 μm . E) Quantification of SSC soma size. F) P14 Rheb electroporated neurons. Scale bar = 50 μm . ACC=Anterior Cingulate Cortex, SSC=Somatosensory Cortex
 *= $P<0.05$ ****= $P<0.0001$