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Altered cholesterol biosynthesis causes precocious neurogenesis in the developing mouse forebrain

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ABSTRACT

We previously reported a mutation in the cholesterol biosynthesis gene, *hydroxysteroid (17-beta) dehydrogenase 7* (*Hsd17b7*^{rudolph}), that results in striking embryonic forebrain dysgenesis. Here we describe abnormal patterns of neuroprogenitor proliferation in the mutant forebrain, namely, a decrease in mitotic cells within the ventricular zone (VZ) and an increase through the remainder of the cortex by E11.5. Further evidence suggests mutant cells undergo abnormal interkinetic nuclear migration (IKNM). Furthermore, intermediate progenitors are increased at the expense of apical progenitors by E12.5, and post-mitotic neurons are expanded by E14.5. *In vitro* primary neuron culture further supports our model of accelerated cortical differentiation in the mutant. Combined administration of a statin and dietary cholesterol *in utero* achieved partial reversal of multiple developmental abnormalities in the *Hsd17b7*^{rudolph} embryo, including the forebrain. These results suggest that abnormally increased levels of specific cholesterol precursors in the *Hsd17b7*^{rudolph} embryo cause cortical dysgenesis by altering patterns of neurogenesis.

1. Introduction

Cholesterol homeostasis is critical for human health and is implicated in a number of disorders from cardiovascular disease in adults to severe developmental abnormalities in the embryo (Porter and Herman, 2011). The developing embryo is especially susceptible to disruptions in cholesterol homeostasis, because the placental and blood–brain barriers can limit the transport of cholesterol between physiologic compartments. Consistent with a high requirement for *in situ* synthesis of cholesterol in the brain (Jurevics et al., 1997; Orth and Bellosta, 2012; Tint et al., 2006), a common feature of genetic disorders of cholesterol biosynthesis are central nervous system (CNS) abnormalities. Because the brain is especially sensitive to perturbations in cholesterol metabolism, it is important to understand the mechanism(s) by which genetic or acquired (e.g., drug inhibition) deficiencies in enzymes of cholesterol biosynthesis affect cholesterol production and utilization during forebrain development.

Hsd17b7 (*hydroxysteroid 17-beta dehydrogenase 7*) encodes a 3-keto-steroid reductase that converts 4-methylzymosterone to 4α-methylzymosterol and zymosterone to zymosterol during post-squalene cholesterol biosynthesis (Marijanovic et al., 2003). Null mutations in mouse *Hsd17b7* lead to early embryonic death, precluding studies on the role of *Hsd17b7* in brain development (Jokela et al., 2010; Shehu et al., 2008). The *Rudolph* mutation is a hypomorphic allele of *Hsd17b7* (*Hsd17b7*^{rud}) which, when carried in the homozygous state, allows survival to mid-organogenesis with widespread CNS anomalies (Stottmann et al., 2011). The CNS phenotype of the *Hsd17b*^{rud} mutation is unique among known mutants in the cholesterol biosynthetic enzymatic pathway and provides a novel tool for studying the relationship between cholesterol metabolism and mammalian neural development.

Consistent with reduced activity of Hsd17b7, sterol analysis of the *Hsdb17b7*^{rud} mutant revealed increased levels of cholesterol intermediates upstream of the Hsd17b7 enzyme, decreased level of downstream intermediates, and an accumulation of unidentified sterols not detected in control embryos. There is also an approximate 20% decrease in cholesterol in *Hsd17b7*^{rud} brains (Stottmann et al., 2011). It is not entirely clear if the defects in mouse models of cholesterol metabolism result from a decreased level of cholesterol or increased levels of cholesterol precursors and related metabolites. In the case of the *Dhcr7* mouse, for

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example, there is a 67% reduction of brain cholesterol content but no prenatal lethality or forebrain abnormalities in homozygous null mutants (Fitzky et al., 2001). However, a common feature among all mouse mutants with CNS defects is the accumulation of intermediates upstream of each targeted enzyme. In a study by Cunningham et al. (2015), a neural progenitor-specific (GFAP-Cre) ablation of the cholesterol biosynthesis enzyme Nsdhl, (NAD(P) dependent steroid dehydrogenase-like), a component of the sterol demethylase complex that also includes Hsd17b7, resulted in progressive loss of cortical and hippocampal neurons, marked cortical neuronal apoptosis, and postnatal death with increased levels of several sterol intermediates. In vitro assays provided evidence that reducing the levels of 4-methylsterols improved neural development (Cunningham et al., 2015). We therefore hypothesized that cholesterol deficiency is not the primary cause of the *Hsdb17b7*^{rud} mutant phenotype and that reducing the high levels of cholesterol intermediates upstream of the mutant Hsd17b7 enzyme would improve neural development in the *Hsd17b7*^{rud} mouse.

In the present study, we determined with *in vivo* and *in vitro* studies that the distinctive phenotype of the *Hsdb17b7*^{rud} mutant forebrain is caused by precocious differentiation and migration of neuronal progenitors. We also demonstrate that manipulation of the cholesterol biosynthesis pathway through combined pharmacological (simvastatin) and dietary (cholesterol) treatments can ameliorate multiple abnormalities in the *Hsd17b7*^{rud} mutants, including improving forebrain development.

2. Results

2.1. Apical polarity is disrupted in the *Hsd17b7*^{rud} cortex

Our previous study of the *Hsd17b7*^{rud} mutant cortex found disorganized cortical tissue and cellular rosettes at embryonic day (E) 15.5 (Stottmann et al., 2011). To address our hypothesis that the cortical defects were caused by disrupted tissue polarity, we examined markers of apical polarity in *Hsd17b7*^{rud} mutants from E10.5 to E14.5. At E10.5 through E12.5, both phalloidin and β-catenin, markers for apical adherens junctions, were present along the ventricular zone (VZ) in the appropriate apically-restricted domains in *Hsd17b7*^{rud} and control embryos at E10.5 through E12.5 (Fig. 1A–F, K–P). However, by E13.5, while both apical markers appeared continuous along the ventricular surface of control embryos, there are minor disruptions in phalloidin staining at the ventricular surface of the mutant (Fig. 1G, H, asterisk) and β-catenin is completely lost along the VZ, failing to form a continuous line of adherens junctions (Fig. 1G, H, Q, R). By E14.5, large sections of the mutant ventricular surface are entirely devoid of both markers (Fig. 1I, J, S, T). In addition to phalloidin and β-catenin staining, staining for the marker of apical junctions, ZO-1, was measured. At E12.5, ZO-1 was restricted to the ventricular surface in both the *Hsd17b7*^{rud} and control forebrain (Fig. 1U, V). However, altered ZO-1 immunoreactivity was evident by E13.5 and continued through E14.5, while proper alignment of the tight junctions was lost in the *Hsd17b7*^{rud} cortex (Fig. 1W–Z). Hsd17b7 is not absolutely required to maintain epithelial polarity, however, as siRNA knockdown in an IMCD cell 3-D spheroid assay did not alter spheroid formation (Fig. S1). Thus, the displaced neurons we observe in mutant cortical tissue likely arise through another mechanism.

2.2. Patterns of proliferation and interkinetic nuclear migration are abnormal in the *Hsd17b7*^{rud} cortex

We previously observed decreased proliferation in the *Hsd17b7*^{rud} cortex at E14.5, which could explain the severe loss of cortical tissue during embryonic brain development (Stottmann et al., 2011). We therefore performed a detailed analysis of neurogenesis in the forebrain to further evaluate this phenomenon. We first examined phospho-Histone H3 (pHH3) localization with immunohistochemistry (IHC) to mark mitotic cells and found that, at E10.5, both the restricted localization of mitotic cells to the VZ and the total number of pHH3-positive cells appeared to be similar in control and mutant cortices (Fig. 2A–D). At E11.5, however, there was a significant reduction of pHH3-positive cells at the VZ in the *Hsd17b7*^{rud} mutants (96% in control vs. 80% in mutant), with a significant number of cells dividing distant from the VZ in the mutant cortex (4% in control vs. 20% in mutant; Fig. 2F–I). These patterns continued at E12.5, with an overall reduction in proliferation within the mutant cortex (Fig. 2K–N). By E13.5 to 14.5, there were significantly fewer mitotic cells left at the ventricular surface (98% control vs. 86% mutant at E13.5, and 98% control vs. 44% mutant at E14.5; Fig. 2P–S, U–X). In addition to changes in proliferation patterns, there was also a significant reduction in overall proliferation from E12.5 to E14.5 in the mutant relative to the control (Fig. 2O, T, Y). Thus, abnormal patterns and levels of proliferation are evident well before changes in polarity are seen.

The ectopic proliferation we see in *Hsd17b7*^{rud} mutants during the neurogenic phase of development could be due to either increased division of basal progenitors (to produce neurons) or displacement of dividing apical progenitors (radial glia). During early forebrain development, apical progenitor nuclei move up and down radial fibers in coordination with the cell cycle in a process called “interkinetic nuclear migration” (IKNM Spear and Erickson, 2012), in which nuclei move basally during G1-S phases and apically during S-G2 phase (Kosodo et al., 2011). During M-phase, apical progenitors align along the VZ and either divide “symmetrically” to maintain the progenitor pool or “asymmetrically” to generate daughter progenitor cells and neurons (Noctor et al., 2004). In contrast, basal progenitor cells (found in the subventricular zone) are unable to self-renew in the mouse and divide to generate two post-mitotic neurons (Haubensak et al., 2004). Because phosphorylated Vimentin (pVim) is an intermediate filament protein specific to mitotically active radial glia (Kamei et al., 1998), we used IHC for pVim to determine if the ectopic proliferation in the *Hsd17b7*^{rud} mutants is due to displaced apical progenitors, and we found that, whereas the pVim staining was restricted to cells along the VZ surface in the control forebrain, as expected, staining for pVim in the *Hsd17b7*^{rud} mutants identified the ectopic cells as dividing radial glia (Fig. 2Z, AA). These results indicate that the apical progenitor cells are undergoing improper IKNM and, as a result, dividing and differentiating in locations removed from the VZ instead of renewing the stem cell pool, as would true apical progenitors.

To directly assess IKNM, we tracked the migratory patterns of apical progenitor nuclei in the E11.5 cortex via pulse-chase analysis using EdU, which labels cells in S-phase at the height of IKNM (Buck et al., 2008). We tracked the progress of IKNM by labeling cells at S-phase and analyzing at; 1) 30 min, to show the initial locations of the progenitor population; 2) 4 h, to track basal to apical progress as cells migrate to the VZ; and 3) 8 h, when nuclei should be resuming apical to basal movement during the IKNM cycle. At 30-min post-injection, we found a distinct band of EdU-positive cells in the mid-cortex of the control, as expected (Fig. 3A), with the EdU-positive cells in the mutant appearing only slightly more dispersed (Fig. 3A–C). By 4-hour post injection, most wild-type cells were near the VZ, reflecting the expected gradient of cells moving apically towards the VZ. In the mutant cortex most EdU-positive cells were at the extreme edges of the cortex, with a significant reduction in the number of EdU-positive cells in the mid-cortex ($P < 0.05$, Fig. 3D–F, bin 2,4). At 8 h, most wild-type cells were at mid-cortex, indicating apical to basal progression (Fig. 3G–I), whereas, in the mutant cortex, most EdU-positive cells were located in the lower cortical bins, with significantly fewer cells present in the two outermost regions (bins 3,4). Similar to the previous finding that cells with improper IKNM showing a more rounded shape (Pacary et al., 2013), we found EdU-positive cells in the *Hsd17b7*^{rud} cortex to be rounded and less densely packed than cells in control cortex. These results suggest that loss of Hsd17b7 activity induces abnormal neural progenitor IKNM in the neurogenic forebrain, leading to abnormal patterns of differentiation.