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# NEURONAL PRODUCTION AND PRECURSOR PROLIFERATION DEFECTS IN THE NEOCORTEX OF MICE WITH LOSS OF FUNCTION IN THE CANONICAL Wnt SIGNALING PATHWAY

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**Abstract—To better understand the function of the Wnt pathway in the developing telencephalon, we analyzed neocortical development in low density lipoprotein receptor-related protein (LRP) 6 mutants. LRP6 mutant mice are hypomorphic for the canonical Wnt signaling pathway and have hypoplasia of the developing neocortex. While early telencephalic morphogenesis is largely intact in these mice, probably due to compensation by LRP5, the mutant mice develop a dramatically thinner cortical plate. There is a prominent reduction of neurogenesis leading to a thin cortical plate. Reduced proliferation late in gestation probably also contributes to the hypoplasia. Although there are marked decreases in the numbers of layer 6 and layers 2–4 neurons all laminar identities are generated and there is no evidence of compensatory increases in layer 5 neurons. In addition, LRP6 mutants have partial penetrance of a complex of cortical dysmorphologies resembling those found in patients with developmental forms of epilepsy and mental retardation. These include ventricular and marginal zone heterotopias and cobblestone lissencephaly. This analysis demonstrates that canonical Wnt signaling is required for a diverse array of developmental processes in the neocortex in addition to the previously known roles in regulating precursor proliferation and patterning.** 

**Key words: lissencephaly, neurogenesis, neural precursor, heterotopia, cortical lamination, neocortex.**

Several Wnt ligands are expressed at the caudomedial margin of the developing cortex in a specialized neuroepithelial structure called the cortical hem (Grove et al., 1998). The localized expression of Wnts (and bone morphogenic proteins, BMPs) in the cortical hem (Parr et al., 1993; Furuta et al., 1997; Grove et al., 1998) has led to the idea that the cortical hem is a signaling center acting to control development of the adjacent cortex (O'Leary and Nakagawa, 2002; Ragsdale and Grove, 2001). Functionally and developmentally the cortical hem is thought to be analogous to the roofplate of the dorsal spinal cord (Monuki et al., 2001). In distinction to the Wnts localized in the hem, other Wnt ligands are expressed widely throughout the cortical ventricular zone (VZ) or throughout the cortical plate (Grove et al., 1998). Despite evidence for functions of Wnts in the development of the hippocampus (Galceran et al., 2000; Lee et al., 2000; Zhou et al., 2004a), there remain questions about the roles of Wnt signaling in neocortical development.

There is suggestive evidence that Wnt signaling may regulate neocortical development. Mice expressing dominant active β-catenin throughout the neuroepithelium have major alterations in neocortical VZ architecture and precursor cell proliferation (Chenn and Walsh, 2002). However, this study is based on ectopic over-expression rather than loss-of-function analysis and shows that disruption of Wnt signaling is sufficient to perturb cortical VZ development but not whether Wnt/β-catenin signaling is required. Recently, cortex specific conditional β-catenin loss-of-function mutant mice showed potential functions for Wnt signaling in early patterning of the cortex and precursor proliferation (Machon et al., 2003; Backman et al., 2005). However, since β-catenin also regulates cadherin dependent cell–cell adhesion and asymmetric division, it is unlikely that the effects seen are solely due to changes in Wnt signaling. In fact, there are suggestions that the most prominent effect of loss of β-catenin in the developing cortex are due to loss of cell adhesion rather than loss of Wnt signaling tone (Junghans et al., 2005).

Low density lipoprotein receptor-related protein (LRP) 6 is a required signaling co-receptor for the canonical Wnt pathway (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). In this pathway, Wnts bind to a Frizzled cooperatively with LRP6 (or its homolog LRP5). Signaling is achieved by stabilization of β-catenin, which then translocates to the nucleus activating transcription with the members of the Lef/Tcf family. Wnt, Frizzled and Lef/Tcf mutants each have developmental effects in some tissues, however, it is likely that the high redundancy masks significant aspects of their functions, especially in the brain, which is particularly rich in the expression of members of each of these families. LRP6 is a bottleneck in the pathway and the phenotypes in other organs are hypomorphic phenotypes for many Wnt dependent processes. It is likely that there is some compensation by the ubiquitously expressed LRP5 but LRP6 is the developmentally more important family member since LRP6 mutant mice show dramatic

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*Abbreviations:* BrdU, bromodeoxyuridine; LRP, low density lipoprotein receptor-related protein; pHH3, phosphohistone-H3; SVZ, subventricular zone; VZ, ventricular zone.

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decreases in Wnt pathway signaling throughout the embryo (Maretto et al., 2003). In this study we decided to utilize the LRP6 mutant mice as a model of diminished canonical Wnt signaling to address potential physiologic *in vivo* functions of the Wnt signaling pathway in corticogenesis. Since previous studies have not analyzed mice that have specific defects in Wnt signaling, this question remains unresolved.

## EXPERIMENTAL PROCEDURES

### Mating and genotyping of LRP6 and Lef1 mutant mice

LRP6 +/− mice (Pinson et al., 2000) were crossed with Lef1 +/− mice (Galceran et al., 2000) to produce LRP6 +/−;Lef1 +/− offspring. Double heterozygotes were mated to each other or mated to LRP6 +/− or to Lef1 +/− females. Inheritance of the LRP6 mutant allele was determined by X-gal staining intensity and by axial morphology (Pinson et al., 2000). Lef1 mutants were genotyped by PCR of genomic DNA prepared from tail or limb biopsies using primers described previously (Galceran et al., 2000). All animal experiments conformed with institutional guidelines for ethical animal research designed to minimize the number of animals and their suffering.

### Immunohistochemistry

Embryo heads or brains were fixed by immersion in 4% paraformaldehyde and sectioned on a cryostat at 7 $\mu$m. Rabbit antibodies to Tbr1 (1:1000; gift of R. Hevner, University of Washington, Seattle, WA, USA), phospho-histone-H3 (pHH3, 1:2000; Upstate Biotechnology, Charlottesville, VA, USA), TLE4 (1:1000; gift of S. Stifani, McGill University, Montreal, QC, Canada), BLBP (1:500; Chemicon, Temecula, CA, USA), GFAP (1:1000; Chemicon) and Nestin (1:100; gift of R. McKay, National Institutes of Health, Bethesda, MD, USA), and mouse antibodies to MAP2 (1:5; gift of V. Lee, University of Pennsylvania, Philadelphia, PA, USA), Pax6 (1:1000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) were used according to standard immunohistologic protocols and detected with secondary antibodies conjugated to Alexa fluorochromes (Molecular Probes, Invitrogen, Carlsbad, CA, USA), followed by nuclei-counterstaining with Hoechst 33258 (Sigma, St. Louis, MO, USA).

### Acute bromodeoxyuridine (BrdU) labeling and birth dating

Acute BrdU labeling was carried out in embryos from pregnant mice that were injected by BrdU intraperitoneally (100 $\mu$g/g body weight) 1 h prior to kill. For birth dating experiments, timed-pregnant mice (at E12.5 and E15.5) were injected with BrdU intraperitoneally at 40 $\mu$g/g body weight. The embryo brains were sampled at E18.5. The cryostat sections were pretreated with 2 N HCl for 30 min at 37 °C followed by staining with anti-BrdU antibody (1:1000; Roche, Nutley, NJ, USA).

### Statistical analysis

At least three animals of each genotype were used for statistical analysis by Student's *t*-test to determine significance in each case (Figs. 4, 5). The early born cortical neuronal production was determined by counting BrdU+ cells in deep layers (layer 6 and subplate) defined by Tbr1 immunostaining from the E12.5-BrdU-injection birth dating (Fig. 4a). The late born cortical neuronal production was examined by counting BrdU+ cells in upper cortical layers (future layers 2/3) beneath the marginal zone from the E15.5-BrdU-injection birth dating (Fig. 4b). Actively dividing cells in the neocortical proliferative zones were determined by counting of pHH3-labeling cells in the ventricular and subventricular zone (SVZ) of embryonic neocortex at E12.5, E15.5 and E18.5 (Fig. 5). Several (three to five/animal) sections from around the same (anterior hippocampal) level for each animal were selected and all the pHH3-labeled cells were counted in all these sections to derive an average number of stained cells per animal. These means were used to compare across genotypes. For E12.5 and E18.5 animals we counted the entire complement of pHH3-labeled cells while in E15.5 animals we boxed (using Photoshop; Adobe Systems, San Jose, CA, USA) 0.5 mm length of ventricular surface to include the full thickness of the cortical wall and counted all pHH3-labeled cells per box.

### *In situ* hybridization

Nonradioactive *in situ* hybridization histochemistry was performed using digoxigenin-labeled riboprobes for Cux2, ER81, Fezl, BF1, Reelin, Sfrp1, Sfrp2, Tbr1, Wnt2b and Wnt7b as described previously (Kim et al., 2001a).

## RESULTS

### LRP6 mutants have a smaller, thinner cortex

LRP6 mutant mice were isolated in a secretory gene trap screen that generates fusion proteins with LacZ, thus the distribution of LacZ recapitulates the expression of LRP6 protein (Pinson et al., 2000). In LRP6 +/− embryos stained with Xgal to completion virtually every cell in the animal was stained including throughout the brain (Zhou et al., 2004b). Gross inspection of the whole brain of LRP6 mutants near birth at E18.5 showed that the cortex and olfactory bulb were smaller in size while the midbrain was more normal in size (Fig. 1a). The cerebellum was also much smaller, as described previously (Pinson et al., 2000). Since LRP6 −/− mice die at birth it was not possible to examine postnatal stages.

The formation of neocortical layers follows a stereotyped radial plan so that the earliest born cortical neurons form a transient structure (the preplate) made up of two developmentally crucial populations of pioneer neurons: Cajal-Retzius cells and subplate cells. Some of these early cells (the Cajal-Retzius cells in particular) are now known to originate at the cortical margins and to migrate tangentially to cover the neocortex (Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Muzio and Mallamaci, 2005). As other neurons are born they migrate radially using radial glial processes to split the preplate into layer 1 (also called the marginal zone and the layer that contains the Cajal-Retzius cells) superficially and the subplate below. Between layer 1 and the subplate is the cortical plate, where new neurons are added in a characteristic inside out gradient; the latest born neocortical neurons are in layer 2 and the earliest born (other than subplate and layer 1) are in layer 6 (Rakic, 1988). To begin to assess the integrity of this process, we sectioned brains from wildtype and mutant mice and analyzed the structure of the cortex using a general cell stain to label cell nuclei (Hoechst 33258) and immunohistochemistry with an antibody against neuron specific $\beta$-tubulin (TuJ1 antibody). At E11.5 the preplate and VZ appeared normal (Fig. 1b). However, by E14.5,