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NEUROANATOMICAL CHARACTERIZATION OF THE CELLULAR AND AXONAL ARCHITECTURE OF SUBCORTICAL BAND HETEROTOPIA IN THE BXD29-Tlr4^{lps-2J}/J MOUSE CORTEX

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Abstract—Subcortical band heterotopia (SBH) are malformations of the human cerebral cortex typically associated with epilepsy and cognitive delay/disability. Rodent models of SBH have demonstrated strong face validity as they are accompanied by both cognitive deficits and spontaneous seizures or reduced seizure threshold. BXD29-Tlr4^{lps-2J}/J recombinant inbred mice display striking bilateral SBH, partial callosal agenesis, morphological changes in subcortical structures of the auditory pathway, and display sensory deficits in behavioral tests (Rosen et al., 2013; Truong et al., 2013, 2015). Surprisingly, these mice show no cognitive deficits and have a higher seizure threshold to chemiconvulsive treatment (Gabel et al., 2013) making them different than other rodent SBH models described previously. In the present report, we perform a detailed characterization of the cellular and axonal constituents of SBH in BXD29-Tlr4^{lps-2J}/J mice and demonstrate that various types of interneurons and glia as well as cortical and subcortical projections are found in SBH. In addition, the length of neuronal cilia was reduced in SBH compared to neurons in the overlying and adjacent normotopic cortex. Finally, we describe additional and novel malformations of the hippocampus and neocortex present in BXD29-Tlr4^{lps-2J}/J mice. Together, our findings in BXD29-Tlr4^{lps-2J}/J mice are discussed in the context of the known neuroanatomy and phenotype of other SBH rodent models.

Key words: neocortex, malformation, subcortical band heterotopia, neuronal migration.

INTRODUCTION

The birthplace of virtually all excitatory neocortical neurons occurs along the apical region of the fetal lateral ventricles, and therefore newly-generated cortical neurons must migrate past several other well-characterized regions before reaching the cortical plate. One such region includes the intermediate zone (IZ), where migrating neurons change from a bipolar morphology and adopt a multipolar morphology characterized by extension of thin processes in various directions (Tabata and Nakajima, 2003; Kriegstein and Noctor, 2004; LoTurco and Bai, 2006). Migrating neurons also change their movement patterns in the IZ from largely radial movement in regions below the IZ (i.e. ventricular zone, subventricular zone), to more of a dynamic and random pattern of movement which can be in any direction. Migration behavior in the IZ can also include periods when multipolar neurons appear to remain motionless (Tabata and Nakajima, 2003).

The importance of neuronal migration into and out of the IZ is best exemplified by the human condition known as Double-Cortex Syndrome which is characterized by the presence of heterotopia of neurons and glia in the neocortical white matter, also known as subcortical band heterotopia (SBH). In humans, SBH are linked to mutation of *DCX* or *LIS1*; however, a larger number of genes cause SBH in mice. In light of the fact that the fetal IZ is fated to become the neocortical white matter, a current model for SBH formation in humans posits that multipolar neurons in the fetal IZ fail to re-adopt a bipolar

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Abbreviations: BDA, biotinylated detran amine; ChAT, choline acetyltransferase; CR, calretinin; GABA, gamma amino-butyric acid; GAD, glutamic acid decarboxylase 67; GFAP, glial fibrillary acidic protein; IBA1, ionized calcium-binding adapter protein 1; IZ, intermediate zone; MLH, molecular layer heterotopia; NDS, normal donkey serum; NeuN, neuronal nuclear marker; NGS, normal goat serum; NPY, neuropeptide-Y; Olig2, oligodendrocyte transcription factor 2; PBS, phosphate-buffered saline; PV, parvalbumin; SBH, subcortical band heterotopia; TH, tyrosine hydroxylase.

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morphology and fail to initiate radial migration out of IZ and into the cortical plate (Bai et al., 2003; Loturco and Bai, 2006). As the IZ transitions to the neocortical white matter, neurons that have failed to leave the IZ form the SBH characteristic of Double-Cortex Syndrome. The observation that SBH in mice (Rosen et al., 2013; Kielar et al., 2014; Yamamoto et al., 2015) and rats (Goodman and Gilbert, 2007; Gilbert et al., 2014) have similar basic cytoarchitecture to those in humans demonstrates the important role of neuronal migration out of the IZ.

Rodents models of SBH such as the *tish* rat (Lee et al., 1997) and *Eml1* mutant mice (Kielar et al., 2014) have demonstrated strong face validity as they are accompanied by both cognitive deficits and spontaneous seizures or reduced seizure threshold. Recently, Rosen and colleagues (2013) demonstrated that the recombinant inbred strain of mice BXD29-Tlr4^{lps-2J}/J (BXD29 mice), exhibit bilateral SBH over the hippocampus as well as partial callosal agenesis in 100% of mice. Morphological changes in the thalamic and brainstem auditory nuclei have also been demonstrated in these mice and are consistent with observed deficits in auditory behavioral tests (Truong et al., 2013). Despite the large size of SBH, surprisingly higher doses of chemiconvulsant are needed to induce seizures in BXD29 mice compared to coisogenic controls (Gabel et al., 2013). In addition, longer latency until seizure onset is observed in BXD29 mice. Equally surprising, BXD29 mice show no deficits in spatial and non-spatial versions of the Morris water maze (Rosen et al., 2013; Truong et al., 2013). Thus, BXD29 mice are different from all other rodent models of SBH described to date.

The neuroanatomy of SBH in mouse models can provide valuable information regarding how cells and circuits reorganize in neocortical malformations in mice and potentially in humans. For example, it is unclear to what extent the same diversity of neurons and glia that normally exists in the neocortex is also present in SBH. In addition, an emerging literature has linked neuronal cilia development with neuronal migration (Baudoin et al., 2012; Higginbotham et al., 2012; Guo et al., 2015) suggesting changes in neuronal cilia development may be associated with neurons in SBH. With this in mind, here we report anatomical studies demonstrating cellular and axonal diversity in SBH in BXD29 mice. First, we demonstrate that diverse gamma amino-butyric acid (GABA)-containing interneurons are present in SBH. Second, we demonstrate that three major glia cell types are present in SBH. Third, we provide evidence of diverse afferent and efferent projections in SBH including those from the brainstem. Fourth, we demonstrate that loss of polarity and defective cilia growth are a characteristic of neurons in SBH. Finally, we show that additional neocortical and hippocampal malformations are present in BXD29 mice indicating widespread neuronal migration defects in this model. These data increase our understanding of *Double-Cortex Syndrome* and SBH in animal models and provide important clues to similar cellular and axonal diversity that may be present in human SBH.

EXPERIMENTAL PROCEDURES

All mice were bred in academic vivaria from BXD29 founders obtained from The Jackson Laboratory (stock No. 000029). Mice were housed in temperature-controlled facilities, maintained between 20 and 22.5 °C, with 12 h light/dark cycles, in standard plastic cages (11.75 × 7.5 × 5; in inches) with no more than five mice per cage, and with food and water available *ad libitum*. Mice of either sex aged between 14 and 120 days were used; note that SBH are present at birth (Rosen et al., 2013). All measures were taken to minimize pain or discomfort in mice and experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All procedures were approved by the Institutional Animal Care and Use Committee at the New York Institute of Technology.

We followed histological methods identical to that described previously (Ramos et al., 2008, 2013, 2014). Following perfusion and fixation (0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer), brains were cryo-preserved in 30% sucrose and then sectioned on a cryostat (35–40 μm) and collected in 0.1 M phosphate-buffered saline (PBS). Immunocytochemistry was used to reveal the presence of different cellular and axonal phenotypes in heterotopia using methods previously described (Ramos et al., 2008, 2013). Briefly, free-floating sections were collected into different wells and washed with PBS (three times). Sections were permeabilized and blocked in 5% normal goat serum (NGS) and 0.2% Triton X-100 for 1 h. Sections were incubated in primary antibodies with 2.5% NGS in PBS at 4 °C overnight.

In the present study the following primary antibodies were used: serotonin transporter (5-HTT; 1:1000; Immunostar, Hudson, WI, USA), tyrosine hydroxylase (TH; 1:1000; Millipore, Billerica, MA, USA); choline acetyl-transferase (ChAT; 1:1000; Millipore); calretinin (CR; 1:1000; Millipore), parvalbumin (PV; 1:1000, Swant, Switzerland); neuropeptide-Y (NPY; 1:1000; Bachem Americas; Torrence, CA, USA); glutamic acid decarboxylase 67 (GAD; 1:1000; Millipore); glial fibrillary acidic protein (GFAP; 1:1000; Sigma, St. Louis, MO, USA); oligodendrocyte transcription factor 2 (Olig2; 1:1000; Millipore); ionized calcium-binding adapter protein 1 (IBA1; 1:1000, Wako USA Richmond, VA, USA); neuronal nuclear marker (NeuN; 1:1000; Millipore); Cux1 (1:1000; Santa Cruz Biotechnology); DCX (1:250, Santa Cruz Biotechnology).

Following incubation in primary antibodies, sections were rinsed several times with PBS and incubated in biotinylated secondary antibodies (goat anti-mouse, goat anti-rabbit, horse anti-goat; 1:200, Vector Labs, Burlingame, CA, USA) for 2 h at room temperature. Sections were rinsed three times with PBS and then incubated for 1 h in an avidin–horseradish peroxidase mixture. Sections were rinsed in PBS three times and then reacted with 0.05% diaminobenzidine in the presence of 0.0015% H_2O_2. For epifluorescence imaging, Alexa 488 or Alexa 568-conjugated secondary