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Developmental alterations of the septohippocampal cholinergic projection in a lissencephalic mouse model

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ABSTRACT

LIS1 is one of principal genes related with Type I lissencephaly, a severe human brain malformation characterized by abnormal neuronal migration in the cortex. The *LIS1* gene encodes a brain-specific 45 kDa non-catalytic subunit of platelet-activating factor (PAF) acetylhydrolase-1b (PAFAH1b), an enzyme that inactivates the PAF. We have studied the role of *Lis1* using a *Lis1/sLis1* murine model, which has deleted the first coding exon from *Lis1* gene. Homozygous mice are not viable but heterozygous have shown a delayed corticogenesis and neuronal dysplasia, with enhanced cortical excitability. *Lis1/sLis1* embryos also exhibited a delay of cortical innervation by the thalamocortical fibers. We have explored in *Lis1/sLis1* mice anomalies in forebrain cholinergic neuron development, which migrate from pallium to subpallium, and functionally represent the main cholinergic input to the cerebral cortex, modulating cortical activity and facilitating attention, learning, and memory. We hypothesized that primary migration anomalies and/or disorganized cortex could affect cholinergic projections from the basal forebrain and septum in *Lis1/sLis1* mouse. To accomplish our objective we have first studied basal forebrain neurons in *Lis1/sLis1* mice during development, and described structural and hodological differences between wild-type and *Lis1/sLis1* embryos. In addition, septohippocampal projections showed altered development in mutant embryos. Basal forebrain abnormalities could contribute to hippocampal excitability anomalies secondary to *Lis1* mutations and may explain the cognitive symptoms associated to cortical displasia-related mental diseases and epileptogenic syndromes.

1. Introduction

LIS1 is one of the principal genes related to Type I lissencephaly, a severe human brain malformation characterized by a smooth brain without the sulci and gyri that appear in the human brain (Reiner et al., 1993). Lissencephalic brain exhibits an aberrant cortical organization due to abnormal neuronal migration during embryonic development (Reiner et al., 1995; Kato and Dobyns, 2003; Barkovich et al., 2005; Wynshaw-Boris, 2007). This anomalous brain development in mouse models and humans produces clinical symptoms such as epilepsy, recurrent seizures, mental retardation, and predisposition to develop psychotic disorders (Dobyns et al., 1993; Nakatani et al., 2006; Tabares-Seisdedos et al., 2008).

Previous studies using murine genetic models with mutations in the *Lis1* gene showed that both increase and decrease in *Lis1* expression result in a disorganization of neural precursors in the ventricular layer and an abnormal cell migration of neuroblasts in the cerebral cortex (Hirotsune et al., 1998; Cahana et al., 2001; Gambello et al., 2003; Bi et al., 2009; Pramparo et al., 2011). In this work, our model is the *Lis1/sLis1* mutant mouse (Cahana et al., 2001), which represents a good model of humans' lissencephaly type I, since it is usually consequence of genetic aploinsufficiency of *LIS1* gene (Reiner et al., 1993; Dobyns et al., 1993). *Lis1/sLis1* homozygotes are not viable but studies in heterozygotes have shown abnormal cell morphology and neuronal organization in the cortical plate (Cahana et al., 2001), as well as cortical dysplasia and enhanced excitability of the cerebral cortex (Valdes-Sanchez et al., 2007). *Lis1/sLis1* embryos also exhibit a delay of neocortical innervations by the thalamic fibers (Cahana et al., 2001). It has been suggested that the maturation of neurons in the cortical plate plays an important function in the invasion of thalamocortical axons into the neuronal layers of the developing cortex (Ghosh et al., 1990).

Abbreviations: CA, cornu ammonis (three subdivisions CA1–CA3); DG, dentate gyrus; f, fimbria; hDB, horizontal limb of the diagonal band; MS, medial septum; sr, stratum radiatum; St, striatum; Th, thalamus; vDB, vertical limb of the diagonal band.

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Although it is well established that the basal forebrain neurons have significant migrations from pallial and subpallial territories where they originate (Xu et al., 2008; Pombero et al., 2011), and play a crucial role in modulating cortical activity, by their projections to the frontal cortex and hippocampus, they have not been studied in *Lis1/sLis1* mice.

The term basal forebrain (BF) refers to structures located close to the medial and ventral surfaces of the cerebral hemispheres. Basal forebrain areas, including the medial septum/vertical limb of the diagonal band (MS/vDB), horizontal limb of the diagonal band (hDB), Meynert basal nucleus, sublenticular substantia innominata, and peripallidal regions, contain neurons showing different neurotransmitters, cellular morphologies and projection patterns (Brauer et al., 1991; Dinopoulos et al., 1988; Gritti et al., 1993; Walker et al., 1989; Zaborszky et al., 1986). BF neurons project to the frontal cortex and hippocampus and play an important role in modulating cortical activity and facilitating processes of attention, learning, and memory (Freund and Antal, 1988; Gritti et al., 1993; Kiss et al., 1990a,b; Buzsaki, 2002). This role is subserved by BF cholinergic neurons but also requires the participation of other non-cholinergic neurons. These neurons include γ-amino butyric acid (GABAergic) neurons, some of which project in parallel with the cholinergic cells to the cerebral cortex (Gritti et al., 1997). Forebrain cholinergic projection neurons are found in the medial septum (MS) (also known as Ch1), the vertical (Ch2) and horizontal limbs (Ch3) of the nucleus of the diagonal band, and the basal magnocellular complex (Ch4) (Mesulam et al., 1983). Ch4 includes cholinergic neurons in the ventral pallidum, the nucleus basalis of Meynert, the ansa lenticularis, the substantia innominata (SI), and the magnocellular preoptic nucleus; these Ch4 cholinergic neurons provide the major source of input to cortical areas (Mesulam et al., 1983). Neurons of the MS/vDB complex (Ch1) provide the major cholinergic innervation of the hippocampus (Teles-Grilo Ruivo and Mellor, 2013), and cholinergic cells of the hDB (Ch3) project to the olfactory bulb.

The septohippocampal projection has been the subject of numerous investigations on its topographical, cytochemical and electrophysiological characteristics (Daitz and Powell, 1954; Frotscher et al., 1996; Morin, 1950). Three different routes have been described as the septohippocampal pathway: the cingular bundle (or supra-callosal dorsal route, overlying the corpus callosum), the fornix (also known as infra-callosal dorsal route) and the ventral pathway (Gage et al., 1984; Milner and Amaral, 1984; Cassel et al., 1997). This ventral projection arises from the DB region and reaches the hippocampus by running over the surface of the amygdaloid complex. Conversely, dorsal fibers originated from MS/vDB cells and enter into the hippocampus via the fornix and fimbria, as well as the cingular bundle over the genu of corpus callosum.

These septohippocampal projections terminate in the CA1, CA3, and dentate regions of the hippocampus. Storm-Mathisen and Guldberg (1974) have reported that the largest part of cholinergic afferents was coursing through the fimbria–fornix pathways (approx. 75%), the supracallosal and ventral pathways contributing only a rather small part of the cholinergic hippocampal innervations (approx. 25%) (see also Cassel et al., 1997). The density of the cholinergic terminals is highest immediately underneath and above the granule cell layer in the dentate gyrus, and around the pyramidal cell layer in the CA1 and CA3 of Ammon's horn. Freund (1992) showed that the MS/vDB-GABAergic terminals in the hippocampus preferentially synapse with inhibitory interneurons (see also Freund and Antal, 1988). Experimental and clinical studies have shown that the integrity of septohippocampal connections is necessary for processes such as hippocampal synchronization and theta and gamma rhythm generation, as well as memory acquisition and consolidation (Buzsaki, 2002; revised in Teles-Grilo Ruivo and Mellor, 2013).

In the present study, were therefore sought to test the hypothesis that deleting the first coding exon from the *Lis1* gene would result in abnormal development of septal cholinergic and noncholinergic neurons that project to the hippocampus. Since data document that dosage reduction the *LIS1* gene severely obstructs cortical and hippocampal organization (Hirotsune et al., 1998), we postulated that *Lis1/sLis1* mutant mouse would have alteration in septohippocampal projection. To accomplish this goal, *Lis1/sLis1* mutant mice and their wt littermates were compared using the Allen Brain Atlas as a guide to count medial septal cholinergic and noncholinergic neuronal populations, and image analysis methods were used to measure the density and distribution of cholinergic neurites in several regions of the hippocampus, labeled for AChE, a reliable marker of cholinergic axons (Hedreen et al., 1985), and DiI projection experiments. Also, we studied *netrin 1* and its receptor DCC (deleted in colorectal cancer), a signal that is involved in septohippocampal axons chemo-attraction (Barallobre et al., 2000).

In relation to modeling human diseases and specially in the cases of phenotypes where *LIS1* mutations may imply predisposition to develop a mental diseases or primary epilepsy, the mild structural anomalies of *Lis1/sLis1* mouse phenotype in the hippocampus may better recapitulate clinical observations in humans than extreme hippocampal phenotypes of other mouse models with severe neuronal dysplasia and severe epilepsy (Hunt et al., 2012; revised by Belvindrah et al., 2014); ended, the absence of evident structural alterations in the brain of patients is highly common.

2. Materials and methods

2.1. Animals

The strain of *Lis1/sLis1* mutant mice was maintained on ICR genetic background, generated and genotyped as previously described (Cahana et al., 2001). The strain 67-green fluorescent protein (*GAD67-GFP*) knock-in mice was generated and maintained as described by Tamamaki et al. (2003). These animals express GFP in GABAergic neurons. Heterozygous *Lis1/sLis1* progeny were crossed with *GAD67-GFP* to obtain *Lis1/sLis1-GAD67-GFP* mice. All animal experimental assays were performed in compliance with the Spanish and European Union laws on animal care in experimentation (Council Directive 86/609/EEC) and approved by the Animal Experimentation Committee of our University.

For the purposes of staging embryos, the day of the vaginal plug formation was taken to be embryonic day 0.5 (E0.5). Embryos at stage E18.5 were anesthetized on ice and fixed by immersion for 3 h in 4% paraformaldehyde (PFA) at 4 °C, washed several times in PBS, and then the brain was dissected and postfixed overnight in 4% PFA at 4 °C. To fix brains from postnatal day 0 (P0) pups, P7, P15 and P21 mice, animals were anesthetized, perfused through the left ventricle of the heart with PBS, followed by 4% PFA in PBS. Brains were removed subsequently and postfixed overnight at 4 °C in the same fixative.

2.2. Immunohistochemistry

Brains were dehydrated progressively in ethanol, embedded in paraffin and finally 8 μm-thick coronal sections were obtained and mounted in four parallel series. The sections were dewaxed and rehydrated. The tissue was incubated with 0.09% hydrogen peroxide (H_2O_2) for 30 min, rinsed with phosphate buffer solution with 0.075% Triton X-100 (PBS-T), and then boiled in sodium citrate solution (0.01 M). Next, the tissue was first incubated with 10% lysine 1 M for 1 h and then with proper primary antibody overnight at room temperature. The antibodies used were: anti-choline acetyltransferase (ChAT) polyclonal antibody from goat (1:100; Millipore, Temecula, CA), anti-calbindin D-28k (CB) polyclonal antibody from rabbit (1:2000; Swant, Bellizona, Switzerland), anti-calretinin (CR) polyclonal antibody from rabbit (1:2000; Swant, Bellizona, Switzerland), anti-Ki67 monoclonal antibody from rabbit (1:200; Thermo Scientific, Freemont, CA), anti-NeuN monoclonal antibody from mouse (1:500; Millipore, Temecula, CA), anti-green fluorescent protein (GFP) polyclonal antibody from chicken (1:500; Aves Labs, Inc., Tigard, Oregon) and anti-deleted in