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A critical and previously unsuspected role for doublecortin at the neuromuscular junction in mouse and human

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Abstract

Mutations in the microtubule-associated protein doublecortin (DCX) cause type I (X-linked or XLIS) lissencephaly in hemizygous males and subcortical band heterotopia (SBH) in females, with defects in neuron migration during development affecting cortical lamination. We found that besides its well-established expression in migrating neurons of the brain, doublecortin (Dcx in mice) is also expressed in motor neurons and skeletal muscle in embryonic neuromuscular junctions (NMJs), raising the possibility of a role in synaptogenesis. Studies with whole-mount preparations of embryonic mouse diaphragm revealed that loss of Dcx leads to abnormal presynaptic arborization and a significantly increased incidence of short axonal extensions beyond innervated acetylcholine receptor (AChR) clusters in the developing NMJ. This phenotype, albeit relatively mild, suggests that Dcx contributes to a stop/stabilizing signal at the synapse, which normally limits further axonal growth following establishment of synaptic contact with the postsynaptic element. Importantly, we also identified abnormal and denervated NMJs in a muscle biopsy from a 16-year-old female patient with SBH, showing both profound presynaptic and postsynaptic morphological defects. Overall, these combined results point to a critical role of doublecortin in the formation of the NMJ.

Keywords: Neuromuscular junction; Doublecortin; Lissencephaly type I; Synapse formation

1. Introduction

Type I lissencephaly corresponds to a spectrum of rare neurodevelopmental disorders associated with brain malformations. Most patients suffer from intellectual disability, intractable epilepsy and hypotonia [1,2]. At the cellular level, this disease is characterized by defects in neuronal migration, leading to a disorganization of the cortical layers, and abnormal neuronal differentiation. Doublecortin (DCX) was originally identified as one of the causative genes for this disorder [3,4]. The DCX gene is located on the human X chromosome and DCX mutations cause X-linked lissencephaly (XLIS) in hemizygous males and subcortical band heterotopia (SBH or Double Cortex) in females, with both familial and sporadic forms [5,6]. Males with XLIS are severely disabled, with minimal neurological development and refractory epilepsy [7]. Females with SBH present more heterogeneous and milder clinical features. Seizures in SBH patients appear within the first decade of life, and often evolve to multifocal and refractory epilepsy. In addition to these symptoms, delayed motor development as well as hypotonia can be observed [5,7,8], suggesting deficits in the neuromuscular system.

The Dcx gene encodes an intracellular 40 kDa protein that is expressed in migrating postmitotic neurons [3,4]. The protein is present in cell bodies and is enriched in the leading process as well as in differentiating axons and dendrites [9]. Dcx is a microtubule-associated protein (MAP) that stabilizes microtubules and favorizes their polymerization [9–13]. This protein contains two evolutionarily conserved DC domains that occur in tandem in its N-terminus [14], which is unique among

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MAPs and is responsible for the binding to microtubules. Most of the missense mutations identified in patients cluster in these DC domains whereas nonsense mutations are spread throughout the gene, mainly in female patients [5,14,15]. The same DC domain is found in other members of the Dcx family, such as Dcx-like kinases (Dclk1 and 2), and is followed in these proteins by a kinase domain [16,17]. Compared to humans, mice deficient for Dcx show a milder phenotype with an abnormal hippocampal pyramidal cell layer but normal neocortical lamination [18–20]. However, the Dclk1 and Dcx double knockout phenotype shows more severe cortical disorganization, resembling the human phenotype for DCX mutations, suggesting a functional redundancy between the two genes [21,22]. Since Dcx transcripts are present in the spinal cord, and abnormal neuromotor skills have been observed in patients with DCX mutations, we asked if the Dcx/DCX protein could play a role in neuromuscular junction (NMJ) formation.

The NMJ is a synapse between a motor neuron and a skeletal muscle fiber that forms in several consecutive steps in mammals. This developmental process starts with the differentiation of a postsynaptic domain in the middle of the muscle, pre-patterning the muscle to attract the motor axon. Axons grow toward this postsynaptic domain and once their target is reached, neurite extension stops. Precise apposition of pre- and postsynaptic elements proceeds while further differentiation occurs in both elements. In a final step, synapse elimination occurs, refining synapse innervation and functioning. A number of key molecules have been described that play an instructive role in NMJ formation, although the critical biochemical pathways have not been fully elucidated. The major players characterized in this process are the neural isoform of agrin, a heparan sulfate proteoglycan secreted by the motor neuron, its transmembrane muscle receptor LRP4, and MuSK, a tyrosine kinase co-receptor that transduces agrin activity and its downstream cascades that lead to AChR clustering [23]. However, the mechanisms that control the pattern of presynaptic arborization and neurite extension are still largely unknown.

Here, we show that Dcx is expressed both by the motor neuron and the muscle during development. Analysis of the NMJ phenotype in Dcx knockout (Dcx-/Y) mutant mice during embryogenesis reveals significant defects in presynaptic arborization and neurites bypassing the NMJ, suggesting a defect in a stop signal for neuritic extension. Moreover, in a muscle biopsy from a female patient with double cortex, we show that NMJs are also severely disorganized, giving clinical relevance to this phenotype. We show therefore that DCX/Dcx is not only required for brain development, but also critical for correct NMJ formation.

2. Subject and methods

2.1. Clinical history of the patient

The patient is a 19-year-old girl without familial history, carrying a DCX mutation (c.176 G>A p.R59H). She came to medical attention at 6 months with infantile spasms. Neurological examination was normal. EEG showed atypical hypsarrhythmia with a high voltage activity suggestive of a cortical malformation. Infantile spasms were controlled with vigabatrin, but subsequent focal seizures starting at the age of 7 months were only partially controlled with sodium valproate and lamotrigine. Brain imaging (CT scan and MRI) showed thick SBH, with normal overlying cortex, and ventriculomegaly. Her motor development seemed normal, but she developed intellectual disability (estimated IQ 60), with frontal syndrome (frontal inhibition) and poor social interaction. During adolescence, she also developed scoliosis that required surgical treatment at the age of 15.

2.2. Human biopsies

Patient muscle biopsies were obtained following scoliosis surgery from Necker-Enfants malades Hospital (Paris, France) and from Bretonneau Hospital (Tours, France) according to local ethical Institutional Review Boards. Consents of the parents were obtained via protocols approved by the Hospital ethics board committees. Control patients and double cortex patient biopsies were treated in exactly the same conditions. Immediately after sampling, they were fixed with paraformaldehyde (PAF) 4% in PBS at 4 °C overnight, cryoprotected in 30% sucrose-PBS for a few hours and then stored frozen at −80 °C until use for immunofluorescence experiments.

2.3. Mice and cells

Dcx knockout mice were generated and characterized previously [24]. Housing conditions and experiments with animals were performed according to the ethical guidelines of French and European legislations (86/809/EEC and 00984.02). We used mutant Dcx-/Y and wild-type (WT) male mice littermates at E14, E16.5 and P8 stages. Embryonic mutant and WT mice from the same litter showed no major differences in weight, size and gross morphology regardless of the stage used. Animals from three different litters were used for each of the stages. The myogenic cell line MLCL was generated as described in [25]. Myoblasts were 50% confluent, and myotubes were analyzed at three different times of differentiation (T1, T2 and T3), which have been characterized previously in [26].

2.4. RNA preparation, microarray and RT-PCR

Total RNA was extracted from muscle cells at T1, T2 and T3 or from embryonic mouse muscle using the RNeasy Mini Kit (Qiagen). For microarray studies, cDNAs generated from 500 ng of RNA were converted into cRNA that were labeled with Digoxigenin-UTP using the Applied Biosystems Chemiluminescent RT-IVT labeling kit and hybridized to a mouse microarray following manufacturer's instructions (Applied Biosystems). Microarray experiments were performed on 3 replicates from 3 independent MLCL muscle cell cultures at T1, T2 and T3. Primers used for PCR were the following: Dcx F: 5′-TACGTTTCTACCGCAATGGGG-3′, Dcx R: 5′-CTGCTTTCCATCAAGGGTGTA-3′, Dclk1 F: 5′-TGTCGTTCGGCAGAGATATG-3′, Dclk1 R: 5′-TCGAACCTTCTTGGC