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Engineering *DYRK1A* overdosage yields Down syndrome-characteristic cortical splicing aberrations

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ABSTRACT

Down syndrome (DS) associates with impaired brain functions, but the underlying mechanism(s) are yet unclear. The "gene dosage" hypothesis predicts that in DS, overexpression of a single gene can impair multiple brain functions through a signal amplification effect due to impaired regulatory mechanism(s). Here, we report findings attributing to impairments in the splicing process such a regulatory role. We have used DS fetal brain samples in search for initial evidence and employed engineered mice with MMU16 partial trisomy (Ts65Dn) or direct excess of the splicing-associated nuclear kinase Dyrk1A, overdosed in DS for further analyses. We present specific albeit modest changes in the DS brain's splicing machinery with subsequently amplified effects in target transcripts; and we demonstrate that engineered excess of *Dyrk1A* can largely recapitulate these changes. Specifically, in both the fetal DS brains and the *Dyrk1A* overdose models, we found ample modestly modified splicing-associated transcripts which apparently induced secondary enhancement in exon inclusion of key synaptic transcripts. Thus, DS-reduced levels of the dominant-negative TRKBT1 transcript, but not other TRKB mRNA transcripts, were accompanied by corresponding decreases in BDNF. In addition, the DS brains and *Dyrk1A* overdosage models showed selective changes in the transcripts composition of neuroligin mRNAs as well as reductions in the "synaptic" acetylcholinesterase variant *AChE-S* mRNA and corresponding increases in the stress-inducible *AChE-R* mRNA variant, yielding key synaptic proteins with unusual features. In cotransfected cells, *Dyrk1A* overdosage caused parallel changes in the splicing pattern of an *AChE* mini-gene, suggesting that *Dyrk1A* overdosage is both essential and sufficient to induce the observed change in the composition of *AChE* mRNA variants. Furthermore, the *Dyrk1A* overdosage animal models showed pronounced changes in the structure of neuronal nuclear speckles, where splicing events take place and in SR proteins phosphorylation known to be required for the splicing process. Together, our findings demonstrate DS-like brain splicing machinery malfunctioning in *Dyrk1A* overexpressing mice. Since individual splicing choices may alter cell fate determination, axon guidance, and synaptogenesis, these findings suggest the retrieval of balanced splicing as a goal for DS therapeutic manipulations early in DS development.

Introduction

Down syndrome (DS; OMIM 190685), the most common genetic disorder leading to mental retardation, is caused by the presence of all or part of an extra copy of chromosome 21 (*HSA21*, for *Homo sapiens*) (Antonarakis et al., 2004). The neurodevelopmental DS phenotype includes brain abnormalities and moderate mental retardation (Antonarakis and Epstein, 2006; Deutsch et al., 2005). Studies of patients with partial trisomy 21 suggested that the 2-Mb region around locus D21S55 in *HSA21*, DCR-1 (Ahlbom et al., 1996), can be crucial in the pathogenesis of DS (Guimera et al., 1996). Among the genes harbored in this region, those with dosage sensitivity may be of great interest for understanding the neurobiological alterations in DS patients. However, gene copy numbers do not always correspond to protein expression levels (Engidawork and Lubec, 2001; Ferrando-Miguel et al., 2004), suggesting seminal contributions for impaired regulatory mechanisms in DS (Dierssen et al., 2009). A common gene regulatory process, especially relevant for genes that are expressed in the nervous system is pre-mRNA splicing, which affects important regulatory decisions in nearly every step in neuronal development, from neuroblast commitment to synaptic

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specialization, modulating proteins ranging from transcription factors to cell adhesion molecules (Li et al., 2007).

Many human disorders are generated by misregulation of alternative splicing (Licatalosi and Darnell, 2006; Yamada and Nabeshima, 2004). Even if small, changes in the level of splicing machinery components can perturb the composition of splicing complexes and thus induce many changes in downstream processed transcripts. In particular, alternative splicing plays a critical role in the nervous system, where more than 75% of the transcripts are alternatively spliced (Licatalosi and Darnell, 2006; Stamm et al., 2005). The different variants play essential and sometimes inverse roles in ion channel function, receptor specificity, neuronal cell recognition, neurotransmission, and learning and memory (Li et al., 2007). Misregulated alternative splicing is responsible for many nervous system diseases such as Alzheimer's, Parkinson's, Pick's, and Huntington's diseases (Licatalosi and Darnell, 2006) but has not yet been explored in other disorders affecting cognition, such as the mental retardation in DS.

Splicing aberrations can directly cause disease, modify the severity of the disease phenotype or be linked with disease susceptibility (Wang and Cooper, 2007). Among the proteins that are involved in the splicing machinery, the members of the SR protein (SRp) family of splicing factors, which contain Ser/Arg-rich domains are primarily involved in mRNA maturation (Hanamura et al., 1998). SRps act as splicing regulatory proteins, binding to exonic splicing enhancer elements and stimulating exon inclusion. SRp family members are mainly named by their sizes (e.g., SRp20, SRp50, SRp55, etc.) and are influenced by protein interactions and posttranslational modifications. Different SRp have different roles depending on combinatorial contributions of cell type, signaling, and posttranslational modifications. For example, ASF/SF2 (alternative splicing factor/splicing factor 2) affects 5′ splice site selection, whereas SC35 (splicing component, 35 kDa) plays an opposite role in the splicing of AChE (Meshorer et al., 2005).

Phosphorylation of SRp is required at the onset of spliceosome assembly (Stamm, 2008). Splicing factors are organized in the highly compartmentalized mammalian nucleus, concentrated in the splicing speckles. These structures (about 25/nucleus) are enriched in splicing factors, pre-messenger RNA, as well as transcription, export, and nuclear structural machinery. They are located in the interchromatin regions, close to highly active transcription sites. The speckles are thought to be a “storage” or assembly place for the splicing machinery. Upon signaling, their components can possibly be mobilized to the target sites. Several kinases and phosphatases are able to modify the splicing machinery, regulating the accessibility of these factors to the splicing events (for a detailed review, see Lamond and Spector, 2003). A recent example involves the phosphorylation of ASF/SF2, which was suggested to regulate the alternative splicing of tau in DS (Shi et al., 2008).

Expanding this concept, we predicted that splicing efficiency and accuracy at large may be a significant contributor to the DS phenotype and its variability. To challenge this prediction, we comparatively analyzed SRp expression and phosphorylation and the splicing variant profiles of key target genes involved in neurodevelopment and cognition in fetal cerebral cortices from DS carriers and in engineered Dyrk1A overexpression systems.

The dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), a member of an evolutionarily conserved protein kinase family (Becker et al., 1998), has been proposed as an exceptional dosage-sensitive candidate gene for DS. DYRK1A is a proline-directed serine/threonine-specific protein kinase whose activity depends on tyrosine autophosphorylation in the catalytic domain. It maps to the DCR-1 and is highly expressed in fetal and adult brains (Guimera et al., 1999). Multiple reports show involvement of DYRK1A in many different pathways, spanning transcription, translation, and signal transduction (Arron et al., 2006; Gwack et al., 2006; Sitz et al., 2004; Yang et al., 2001). In neurons, DYRK1A localizes to both the nucleus and cytoplasm, including neuronal processes and synapses. In the nucleus, it localizes to splicing speckles and overexpression of active DYRK1A in cultured cells associates with speckles disassembly (Alvarez et al., 2003). Transgenic mice overexpressing *Dyrk1A* (TgDyrk1A) exhibit hyperactivity and impaired neuromotor development as well as spatial learning and memory (Altafaj et al., 2001; Martinez de Lagran et al., 2004), a phenotype which is reversible by targeting *Dyrk1A* with AAVshRNA (Ortiz-Abalia et al., 2008). Comparable alterations are found in the MMU16 chromosome 16 partial trisomy murine model of DS, Ts65Dn (Galdzicki et al., 2001), suggesting that *DYRK1A* overexpression in trisomy 21 carriers may contribute to the DS mental retardation and motor anomalies phenotype. The long list of DYRK1A substrates includes cyclin L2 (de Graaf et al., 2004), SF3b1 (de Graaf et al., 2006), and ASF (Shi et al., 2008), suggesting its possible involvement in the regulation of the splicing machinery (Alvarez et al., 2003; de Graaf et al., 2004; Dean and Dresbach, 2006; Dierssen and de Lagran, 2006). Therefore, we decided to preliminarily investigate the splicing changes induced in the DS brain and compare our findings to those in mice with enforced *Dyrk1A* overexpression. Specifically, we analyzed the splicing machinery at the RNA, protein, and phosphorylation levels with a particular focus on genes involved with the molecular basis of learning.

Materials and methods

Human brain samples

Cerebral cortices of fetal brains with or without DS (12 males of each) at 16–19 weeks gestation (Supplementary Table I) were obtained from the fetal tissue bank of Hospital Vall d'Hebron (Barcelona, Spain) and Hospital La Fe (Sabadell, Spain). Samples were obtained between 6 and 12 h postmortem and were frozen or embedded in paraffin. Diagnosis of all cases was genetically and histopathologically confirmed (Dr. N. Torán and Dr. JC Farreras). The inclusion criterion for control samples was that fetal demise was not due to a known genetic cause. Fetal samples were stored at −80 °C until use. DS fetal samples showed elevated DYRK1A protein levels (Supplementary Fig. 1). All experimental procedures were approved by the local ethical committee (CEE-Vall d'Hebrón and CEE-PRBB) and met the guidelines of the local and European regulations.

Animals

The production of transgenic mice overexpressing *Dyrk1A* (TgDyrk1A) has been previously described (Altafaj et al., 2001). Briefly, the original founder was obtained by insertion of the transgene into C57BL/6JXSJL (Charles River, Barcelona, Spain) embryos, and the stock is maintained by crossing C57BL/6JXSJL wild type females and transgenic males derived from the original founder. MMU16 Ts65Dn segmental trisomic mice, free from the Rd mutation, were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Nontransgenic and disomic littermates served as controls. Both TgDyrk1A and Ts65Dn mice showed elevated protein levels of Dyrk1A compared to strain-matched wild type mice (Supplementary Fig. 1). Animals were housed under 12:12-h light–dark cycle (lights on at 8:00 a.m.) in controlled environmental conditions, 60% of humidity, and at 22 ± 2 °C. All animal procedures were approved by the local ethical committee (CEEA-IMIM and CEEA-PRBB) and met the guidelines of the local (law 32/2007) and European regulations (EU directive no. 86/609, EU decree 2001-486) and the Standards for Use of Laboratory Animals no. A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14).