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DYRK1A-mediated Cyclin D1 Degradation in Neural Stem Cells Contributes to the Neurogenic Cortical Defects in Down Syndrome

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ABSTRACT

Alterations in cerebral cortex connectivity lead to intellectual disability and in Down syndrome, this is associated with a deficit in cortical neurons that arises during prenatal development. However, the pathogenic mechanisms that cause this deficit have not yet been defined. Here we show that the human DYRK1A kinase on chromosome 21 tightly regulates the nuclear levels of Cyclin D1 in embryonic cortical stem (radial glia) cells, and that a modest increase in DYRK1A protein in transgenic embryos lengthens the G1 phase in these progenitors. These alterations promote asymmetric proliferative divisions at the expense of neurogenic divisions, producing a deficit in cortical projection neurons that persists in postnatal stages. Moreover, radial glial progenitors in the Ts65Dn mouse model of Down syndrome have less Cyclin D1, and *Dyrk1a* is the triplicated gene that causes both early cortical neurogenic defects and decreased nuclear Cyclin D1 levels in this model. These data provide insights into the mechanisms that couple cell cycle regulation and neuron production in cortical neural stem cells, emphasizing that the deleterious effect of *DYRK1A* triplication in the formation of the cerebral cortex begins at the onset of neurogenesis, which is relevant to the search for early therapeutic interventions in Down syndrome.

1. Introduction

The mammalian neocortex is the brain region responsible for cognitive function, sensory perception and consciousness. It is formed by many types of neurons and glial cells, all of which are distributed across six histologically defined layers that are generated in a spatially and temporally-regulated manner thanks to the interplay of intrinsic molecular programs and extracellular cues (Tiberi et al., 2012). Impaired development of this brain structure has been associated with mental deficiency and other major neurological disorders (Lewis and Sweet, 2009; Rubenstein, 2010; Sun and Hevner, 2014).

Around 80% of neocortical neurons are excitatory projection neurons that extend axons to distant intracortical targets and to subcortical regions, whilst the remainder are inhibitory interneurons involved in local circuits (DeFelipe et al., 2013; Greig et al., 2013). The distinct types of projection neurons are produced in the dorsolateral telencephalon (*pallium*) of the embryo from multipotent neural stem cells (NSCs) known as radial glia (RG), and from more restricted progenitors, the intermediate progenitors (IPs). These neurons are generated in an inside–outside pattern, first generating the neurons that form the layer closest to the ventricle (layer VI) and lastly those that form the most superficial layers (Layers II–III). Projection neurons within a layer have common molecular characteristics and connectivity patterns, which they acquire at their birth (Greig et al., 2013; Molyneaux et al., 2007).

During neurogenesis, RG progenitors divide asymmetrically in the ventricular zone (VZ) producing another RG cell and either a neuron or an IP (Noctor et al., 2004; Haubensak et al., 2004; Miyata et al., 2004). This progenitor moves to a more basal proliferative layer, the subventricular zone (SVZ), where it divides symmetrically to produce a pair of neurons directly or it does so after 1 to 3 rounds of symmetric amplifying divisions (Noctor et al., 2004; Kowalczyk et al., 2009). Consequently, as neocortical development progresses and the cellularity in the SVZ increases, IPs become the major source of projection neurons (Breunig et al., 2011; Kowalczyk et al., 2009). According to this model, the number and proportion of projection neuron subtypes in a cortical radial column are related to the number of RG progenitors that are

Abbreviations: DS, Down syndrome; IP, intermediate progenitor; NSC, neural stem cell; RG, radial glia; SVZ, subventricular zone; VZ, ventricular zone

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present at the onset of neurogenesis, as well as to the number of VZ (apical) and SVZ (basal) proliferative and neurogenic divisions (Huttner and Kosodo, 2005; Noctor et al., 2001, 2007).

Regulation of the cell cycle, and particularly of the G1 phase of the cell cycle, is important for the normal expansion of the neocortex in both rodents and primates (Dehay and Kennedy, 2007). G1 is a critical phase, integrating extracellular signals that induce either commitment to a further round of cell division, or withdrawal from the cell cycle and differentiation (Cunningham and Roussel, 2001; Dehay and Kennedy, 2007; Salomoni and Calegari, 2010; Zetterberg et al., 1995). Pioneering cumulative S-phase labelling experiments performed in the mouse embryo showed that as neurogenesis progresses the cell cycle of neocortical progenitors extends due to a progressive lengthening of the G1 phase (Takahashi et al., 1995). Moreover, there is evidence of a correlation between cell cycle length and neurogenesis, which has led to the formulation of the cell cycle length hypothesis (Gotz and Huttner, 2005). According to this hypothesis, the time that a progenitor spends in G1 determines the final effect of a particular cell fate determinant, which could be equivalent (symmetric divisions) or distinct (asymmetric divisions) in the two daughter cells (Dehay and Kennedy, 2007; Gotz and Huttner, 2005; Salomoni and Calegari, 2010). Indeed, it was more recently shown that manipulating the duration of the G1 phase in neocortical apical progenitors alters the production of IPs and neurons (Lange et al., 2009; Pilaz et al., 2009).

Down syndrome (DS), the most common genetic cause of intellectual disability, is caused by trisomy of chromosome 21. DS brains are smaller than normal brains and they exhibit neuronal deficits in several regions, including the cerebral cortex (Ross et al., 1984). Infants with DS also present hypocellularity in this brain structure (Schmidt-Sidor et al., 1990; Wisniewski, 1990), indicating that defects in prenatal development are a major determinant of the deficit in adults. Indeed, fewer cells (Larsen et al., 2008) and disorganized laminas are evident in the cerebral cortex of DS foetuses from as early as the second trimester of gestation (Golden and Hyman, 1994).

The availability of DS mouse models in which different regions of chromosome 21 are in trisomy (Haydar and Reeves, 2012; Liu et al., 2011) has allowed the effect of trisomic genes on prenatal development to be assessed, assigning phenotypic aspects of the syndrome to a region of chromosome 21. In the best studied model of DS, the Ts65Dn mouse (Reeves et al., 1995), the growth of the neocortical wall is delayed due to the impaired production of neurons early in neurogenesis that is concomitant with a lengthening of the cell cycle in the ventricular germinal layer (Chakrabarti et al., 2007). The Ts1Cje mouse is a DS model with a smaller trisomic region than the Ts65Dn mouse (Haydar and Reeves, 2012), yet it also develops an abnormally thin neocortex and slower cell cycle exit is observed during embryogenesis (Ishihara et al., 2010). Importantly, the proliferation markers expressed in the neocortical germinal matrix of DS foetuses also suggest cell cycle defects which underpin the reduced neuron production (Contestabile et al., 2007). There are around 80 genes in the triplicated segment common to Ts65Dn and Ts1Cje mice, which contains the DS critical region (*DSCR*) of chromosome 21 (Delabar et al., 1993; Toyoda et al., 2002). Thus, it is likely that dosage imbalance of one or a few genes in this region contributes to the deficit of cortical neurons in DS.

In this study we have assessed the possibility that triplication of *DYRK1A*, a *DSCR* gene, contributes to the hypocellularity of the cerebral cortex associated with DS. DYRK1A (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A) encodes a constitutively active kinase that phosphorylates serine and threonine residues in a variety of substrates (Becker and Sippl, 2011). In humans, truncating mutations in the *DYRK1A* gene cause primary microcephaly (Courcet et al., 2012) and autism (O'Roak et al., 2012). Moreover, mice and flies with haploinsufficiency of the *Dyrk1a*/*minibrain* genes have smaller brains (Fotaki et al., 2002; Tejedor et al., 1995), indicating that the role of DYRK1A in brain growth is conserved across evolution. Experiments on neural progenitors derived from induced pluripotent stem cells from monozygotic twins discordant for trisomy 21 highlight *DYRK1A* as one of the chromosome 21 genes important for the proliferation and differentiation defects associated with DS (Hibaoui et al., 2014). However, despite the evidence from different model systems showing that DYRK1A regulates neural proliferation and differentiation (Tejedor and Hammerle, 2011), the pathogenic effects of DYRK1A overexpression in the formation of brain circuits in DS remain unclear (Haydar and Reeves, 2012).

The effect of DYRK1A overexpression on cortical neurogenesis has been assessed in the mouse embryo through electroporation, although the results obtained were inconclusive. The ectopic overexpression of DYRK1A in progenitors of the dorsal telencephalon induced proliferation arrest (Hammerle et al., 2011; Yabut et al., 2010), provoking premature neuronal differentiation (Yabut et al., 2010), a phenotype that is quite opposite to the growth delay of the cortical wall observed in the Ts65Dn embryos (Chakrabarti et al., 2007). These studies involved electroporation at mid-corticogenesis stages and the levels of DYRK1A overexpression were not controlled. More recent experiments showed that modest DYRK1A overexpression does not disturb the birth of cortical neurons when induced at the onset of neurogenesis (Kurabayashi and Sanada, 2013). Thus, the effect of DYRK1A on cortical neurogenesis seems to depend on the time and/or the level of overexpression.

Using mouse models that overexpress *Dyrk1a* under its endogenous regulatory sequences, mimicking the situation in DS, we now demonstrate that trisomy of *Dyrk1a* is sufficient to lengthen the G1 phase of the cell cycle and to bias the production of RG-derived neurons and IPs during the early phase of corticogenesis, and that the triplication of the *Dyrk1a* gene is necessary for dampened early neurogenesis in the developing neocortex of Ts65Dn embryos.

2. Materials and Methods

2.1. Animals

In this study we have used embryos and postnatal *Dyrk1a*$^{+/-}$ mice, mBACTg*Dyrk1a* mice, Ts65Dn mice and their respective wild-type littermates, as well as the mice resulting from crosses between Ts65Dn females and *Dyrk1a*$^{+/-}$ males. The day of the vaginal plug was defined as E0.5, and the day of birth was defined as P0.

The generation of Ts65Dn mice, *Dyrk1a*$^{+/-}$ mice and mBACTg*Dyrk1a* mice was described elsewhere (Davisson et al., 1993; Fotaki et al., 2002; Guedj et al., 2012). Mice were maintained in their original genetic backgrounds: *Dyrk1a*$^{+/-}$ mice by repeated backcrossing of *Dyrk1a*$^{+/-}$ males to C57BL/6Jx129S2/SvHsd F1 females (Harlan Laboratories); mBACTg*Dyrk1a* mice by repeated backcrossing of transgenic males to C57BL6/J females (Charles River Laboratories); and Ts65Dn mice by repeated backcrossing of parental Ts65Dn females (Jackson Laboratory, USA) to B6EiC3 males (Harlan laboratories). *Dyrk1a*$^{+/-}$ and mBACTg*Dyrk1a* mice were genotyped by PCR (Fotaki et al., 2002; Guedj et al., 2012) and Ts65Dn mice by PCR (Reinholdt et al., 2011) or by quantitative PCR (http://www.jax.org/cyto/quanpcr.html).

All the experimental procedures were carried out in accordance with the European Union guidelines (Directive 2010/63/EU) and the followed protocols were approved by the ethics committee of the Parc Científic de Barcelona (PCB).

2.2. Tissue Preparation for Histology

To obtain embryonic tissue, whole heads were fixed by immersion in 4% paraformaldehyde (PFA) for 24 h at 4 °C, cryoprotected with 30% sucrose in PBS, embedded in Tissue-Tek O.C.T. (Sakura Finetek), frozen in isopentane at −30 °C and sectioned on a cryostat. Cryosections (14 μm) were collected on Starfrost precoated slides (Knittel Glasser) and distributed serially. Postnatal P0 and P7 mice were deeply anaesthetized in a CO_2 chamber and transcardially perfused with 4% PFA.