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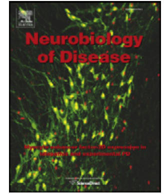

Normalization of Dyrk1A expression by AAV2/1-shDyrk1A attenuates hippocampal-dependent defects in the Ts65Dn mouse model of Down syndrome

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ABSTRACT

The cognitive dysfunctions of Down Syndrome (DS) individuals are the most disabling alterations caused by the trisomy of human chromosome 21 (HSA21). In trisomic Ts65Dn mice, a genetic model for DS, the overexpression of HSA21 homologous genes has been associated with strong visuo-spatial cognitive alterations, ascribed to hippocampal dysfunction. In the present study, we evaluated whether the normalization of the expression levels of *Dyrk1A* (Dual specificity tyrosine-phosphorylation-regulated kinase 1A), a candidate gene for DS, might correct hippocampal defects in Ts65Dn mice. In the hippocampus of 2 month-old Ts65Dn mice, such normalization was achieved through the stereotaxical injection of adeno-associated viruses containing a short hairpin RNA against Dyrk1A (AAV2/1-shDyrk1A) and a luciferase reporter gene. The injected hippocampi were efficiently transduced, as shown by bioluminescence in vivo imaging, luciferase activity quantification and immunohistochemical analysis. At the molecular level, viral infusion allowed the normalization of the targeted Dyrk1A expression, as well as of the key players of the MAPK/CREB pathway. The electrophysiological recordings of hippocampal slices from Ts65Dn mice injected with AAV2/1-shDyrk1A displayed attenuation of the synaptic plasticity defects of trisomic mice. In contrast, contralateral hippocampal injection with an AAV2/1 control virus containing a scrambled sequence, showed neither the normalization of Dyrk1A levels nor changes of synaptic plasticity. In the Morris water maze task, although long-term consolidation of the task was not achieved, treated Ts65Dn mice displayed initially a normalized thigmotactic behavior, similar to euploid littermates, indicating the partial improvement in their hippocampal-dependent search strategy. Taken together, these results show Dyrk1A as a critical player in the pathophysiology of DS and define Dyrk1A as a therapeutic target in adult trisomic mice.

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Introduction

Down Syndrome (DS, [OMIM 190685]) is a frequent genetic cause of mental retardation and is accompanied by a plethora of symptoms affecting almost all organs (Epstein, 2001). Individuals affected by the trisomy of human chromosome 21 (HSA21) display learning and memory alterations that lead to cognitive disabilities (Lott and Dierssen, 2010). Much effort has been directed towards the genetic dissection of DS, in particular focusing on the generation and characterization of murine models to correlate genotype and phenotype. Among the murine models generated, Ts65Dn mice are the best characterized and display cognitive alterations similar to those observed in DS individuals. Ts65Dn mice have a triplicated fragment of mouse chromosome 16 (MMU16) that is homologous to HSA21. The trisomic region expands from MRP139 to Znf295 and contains roughly 136 genes that are orthologous to human genes on HSA21 (Gardiner, 2004).

Phenotypic characterization of DS murine models has indicated the relevance of specific HSA21-orthologous genes that strongly contribute to the phenotypic alterations of DS murine models either directly and/or by altering downstream pathways. Based on the latter, recent reports have shown the possibility of transiently correcting the downstream effectors and pathways altered by the overexpression of HSA21-orthologous genes with pharmacological or dietetic compounds that attenuate the cognitive alterations exhibited by DS murine models (Fernandez et al., 2007; Costa et al., 2008; Rueda et al., 2010; Guedj et al., 2009). An alternative therapeutic strategy, based on genotype–phenotype studies, which support the strong contribution of specific

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genes in the DS murine model phenotype, consisted of directly targeting HSA21-orthologous candidate genes in order to normalize expression levels. One of these candidate genes is Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A gene product (*DYRK1A* [MIM 600855]) which interacts with and phosphorylates a variety of neuronal molecular substrates (Aranda et al., 2011). Since an excess dosage of Dyrk1A gene results in 1.5-fold overexpression in DS brains (Dowjat et al., 2007; Guimera et al., 1999) and leads to motor and cognitive alterations in TgDyrk1A mice (Altafaj et al., 2001), Dyrk1A has been proposed as a strong candidate gene for the cognitive alterations associated with DS. In a recent study, we reported the specific inhibition of Dyrk1A with a small interfering RNA (shRNA)-based approach, combined with a viral delivery system, which resulted in an amelioration of the motor deficits of TgDyrk1A mice (Ortiz-Abalia et al., 2008). Based on this proof-of-concept work, in the present study we questioned whether the selective knockdown of Dyrk1A in a trisomic genetic context would correct the hippocampus-related phenotypes present in Ts65Dn mice, the most well characterized trisomic mouse model of DS. Here we show that the efficient normalization of Dyrk1A in adult Ts65Dn mice is accompanied by the normalization of synaptic plasticity molecular markers and of the electrophysiological properties of the hippocampus. These data, together with the observed attenuation of the behavioral hippocampal deficits of treated mice, support a critical role of Dyrk1A in the hippocampal phenotype of Ts65Dn mice and highlight the possibility to compensate the cognitive deficits of adult trisomic mice by Dyrk1A-targeted therapies.

Results

Stereotaxic injection of AAV2/1-shDyrk1A particles efficiently transduces the hippocampi of adult Ts65Dn mice

Normalization of Dyrk1A expression levels in the adult hippocampus of Ts65Dn mice was achieved employing a viral gene therapy approach, with a genetic construct which encodes for a short hairpin RNA (shRNA) that efficiently knocks-down Dyrk1A ("SH" particles, described in Ortiz-Abalia et al., 2008). The construct contains a luciferase reporter gene to facilitate the further tracking of viral infectivity and analysis of in vivo biodistribution. Recombinant viral particles were pseudotyped with viral capsid proteins from an AAV1 serotype, based on the reported high tropism of the latter for the hippocampus (Burger et al., 2004). The viral infectivity of chimeric AAV2/1 particles was evaluated in vitro using HEK293 cell line and mouse primary neuronal cortical cultures. Measurement of luciferase activity in HEK293 cells infected at different viral doses of either AAV2/1-shDyrk1A targeting Dyrk1A ("SH" particles) or viral particles carrying a scrambled sequence AAV2/1-scDyrk1A ("SC") showed similar infectivity of both viruses (Fig. 1A). Western blot analysis of Dyrk1A expression in transiently transfected HEK293 cells showed the ability of SH viral particles to down-regulate Dyrk1A expression levels (Fig. 1B). Infection studies of primary cortical cultures indicated the ability of AAV2/1 particles containing shDyrk1A (SH) or scDyrk1A (SC) sequences to infect glial (stained with anti-glial fibrillary acidic protein antibody) and neuronal cells (stained with anti-Tuj-1 antibody) (Fig 1C). Consequently, chimeric viral particles (2×10^9 vg) were stereotaxically delivered monolaterally in the CA3 layer of the left hippocampus (AP = −2.0 mm, L = 1.5 mm, DV = 2.0 mm) of 2 month-old Ts65Dn mice, as previously described (Ortiz-Abalia et al., 2008). Viral infectivity and biodistribution were then monitored by measuring luciferase activity in living animals (in vivo bioluminescence imaging) and in *post-mortem* dissected areas of the brain. Ten days after injection, and long after (up to four months), luciferase activity was detected in a restricted area of the brain, corresponding to the injected left hippocampus (Fig. 2A). In order to quantify relative biodistribution and putative viral diffusion, a group of Ts65Dn mice were sacrificed 6 weeks after surgery (time-point selected for physiological and behavioral experiments) and the regions of the brain dissected. Measurement of luciferase activity showed the viral transduction area to be restricted to the injected ipsilateral hippocampus, with negligible luciferase activity in the contralateral hippocampus or in other areas of the brain (Fig. 2A). To test whether viral infection diffused to the different areas of the injected hippocampus, immunohistochemical analysis was performed on 4 μm-thick paraffin embedded sections of paraformaldehyde-fixed brains with specific anti-luciferase antibody. The luciferase immunopattern was broadly distributed in the hippocampus, with a strong immunostaining of the CA3 area (where the viral particles were delivered stereotaxically), as well as immunosignal in the CA1 area and the dentate gyrus (DG) (Fig. 2B). Thus, immunohistochemical analysis indicated the ability of the viral particles to diffuse within the ipsilateral hippocampus and to infect the DG-CA3-CA1 circuitry.

Normalization of Dyrk1A expression levels in the hippocampi of adult Ts65Dn mice

To evaluate the ability of shDyrk1A viral particles to specifically knockdown Dyrk1A, total proteins were extracted from the hippocampi of euploid mice and Ts65Dn (non-treated and injected 6 weeks earlier with SH or SC sequences). Western blot analysis with anti-Dyrk1A antibody showed that Dyrk1A overexpression in the hippocampi of Ts65Dn mice was normalized only in those mice that received the AAV2/1-shDyrk1A particles (Fig. 3A). This data demonstrates the ability of this genetic tool to specifically knockdown Dyrk1A overexpression and to restore euploid-like Dyrk1A levels in the hippocampus of adult Ts65Dn mice. In order to evaluate whether this normalization was generalized in the hippocampus or restrained to specific areas, immunofluorescence analysis was performed. Analysis of Dyrk1A immunoreactivity showed that the global increase of Dyrk1A expression levels observed in Ts65Dn mouse hippocampi was broadly corrected in the hippocampus of trisomic mice injected with AAV2/1-shDyrk1A viral particles, showing normalized Dyrk1A expression in CA3, CA1 and DG hippocampal areas (Fig. 3B). After confirmation of viral infectivity and inhibitory efficiency, the functional consequences of Dyrk1A normalization in the hippocampus of 6 week-treated adult Ts65Dn mice were assessed. The characterization of putative hippocampal function recovery was performed at multiple levels by studying the molecular events underlying synaptic plasticity, evaluating electrophysiological activity and analyzing the cognitive behavioral phenotype of treated Ts65Dn mice.

Molecular changes associated with Dyrk1A normalization in adult Ts65Dn mice

Dyrk1A has been described as a protein kinase phosphorylating CREB and other players of the cytoskeleton and endocytic pathway, such as dynamin-1, synaptojanin-1 and amphiphysin-1 (Yang et al., 2011; Chen-Hwang et al., 2002; Adayev et al., 2006; Murakami et al., 2006). Said phosphorylation targets are involved in the biochemical and structural neuronal changes that underlie long-term potentiation (LTP), a compelling model for the synaptic mechanism that triggers certain forms of learning and memory. Therefore we hypothesized that the resulting Dyrk1A normalization could modify the phosphorylation pattern of the proteins that participate in the Erk/CREB signaling cascade, a major pathway that participates in the LTP process (Sweatt, 2004; Thomas and Huganir, 2004). In order to stimulate hippocampal activity, the different experimental groups of mice were submitted to contextual fear conditioning. It has been described that during contextual fear conditioning, mice display a transient increase of hippocampal Erk and CREB activity that can be monitored by Western blot analysis for dually phosphorylated Erk1/Erk2 and phosphorylated CREB (Sindreu et al., 2007). Therefore, euploid and trisomic mice (naive, injected with AAV2/1-shDyrk1A or with AAV2/1-scDyrk1A) were submitted to a contextual fear-conditioning paradigm. Ten

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