Neurobiology of Disease 103 (2017) 11–23

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Long-term effect of neonatal inhibition of APP gamma-secretase on hippocampal development in the Ts65Dn mouse model of Down syndrome

Fiorenza Stagni [a,1], Alessandra Raspanti [b,1], Andrea Giacomini [a,1], Sandra Guidi [a], Marco Emili [a], Elisabetta Ciani [a], Alessandro Giuliani [c], Andrea Bighinati [d], Laura Calzà [d], Jacopo Magistretti [b], Renata Bartesaghi [a,*]

[a] Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy
[b] Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Pavia, Italy
[c] Department of Veterinary Medical Sciences,University of Bologna, Bologna, Italy
[d] Health Sciences and Technologies - Interdepartmental Center for Industrial Research (HST-ICIR), University of Bologna, Bologna, Italy

ARTICLE INFO

Article history:
Received 5 September 2016
Revised 9 March 2017
Accepted 26 March 2017
Available online 28 March 2017

Keywords:
Down syndrome
Neonatal pharmacotherapy
APP
Gamma-secretase
Hippocampal development

ABSTRACT

Neurogenesis impairment is considered a major determinant of the intellectual disability that characterizes Down syndrome (DS), a genetic condition caused by triplication of chromosome 21. Previous evidence obtained in the Ts65Dn mouse model of DS showed that the triplicated gene APP (amyloid precursor protein) is critically involved in neurogenesis alterations. In particular, excessive levels of AICD (amyloid precursor protein intracellular domain) resulting from APP cleavage by gamma-secretase increase the transcription of Ptch1, a Sonic Hedgehog (Shh) receptor that keeps the mitogenic Shh pathway repressed. Previous evidence showed that neonatal treatment with ELND006, an inhibitor of gamma-secretase, reinstates the Shh pathway and fully restores neurogenesis in Ts65Dn pups. In the framework of potential therapies for DS, it is extremely important to establish whether the positive effects of early intervention are retained after treatment cessation. Therefore, the goal of the current study was to establish whether early treatment with ELND006 leaves an enduring trace in the brain of Ts65Dn mice. Ts65Dn and euploid pups were treated with ELND006 in the postnatal period P3-P15 and the outcome of treatment was examined at ~one month after treatment cessation. We found that in treated Ts65Dn mice the pool of proliferating cells in the hippocampal dentate gyrus (DG) and total number of granule neurons were still restored as was the number of pre- and postsynaptic terminals in the stratum lucidum of CA3, the site of termination of the mossy fibers from the DG. Accordingly, patch-clamp recording from field CA3 showed functional normalization of the input to CA3. Unlike in field CA3, the number of pre- and postsynaptic terminals in the DG of treated Ts65Dn mice was no longer fully restored. The finding that many of the positive effects of neonatal treatment were retained after treatment cessation provides proof of principle demonstration of the efficacy of early inhibition of gamma-secretase for the improvement of brain development in DS.

1. Introduction

During the last few years, there has been a growing interest in the field of pharmacotherapies for intellectual disability in Down syndrome (DS) (see (Costa and Scott-McKean, 2013, Gardiner, 2015)), a genetic condition due to triplication of chromosome 21. To this purpose, mouse models have been exploited among which the Ts65Dn mouse, a model that recapitulates numerous features of trisomy 21 (Reeves, 2006), is the most widely used. The developmental alterations in the brain of Ts65Dn mice are due to perturbation of numerous pathways. Accordingly, studies that have used quite different pharmacological approaches in the Ts65Dn mouse model show that it is possible to ameliorate or even rescue the structural and behavioral deficits that characterize the Ts65Dn model of DS (see(Costa and Scott-McKean, 2013, Gardiner, 2015)). Most of these studies have been carried out in adulthood, a time at which neurogenesis and neuron maturation are well off. Neurogenesis and connectivity alterations are the major structural defects of the DS brain (see (Bartesaghi et al., 2011, Dierssen, 2012). Since these processes take place prenatally and in the early postnatal period, these periods represent critical windows of opportunity for the rescue of brain development. Indeed, studies in the Ts65Dn model show that pre- and early postnatal pharmacotherapies may have a

* Corresponding author at: Department of Biomedical and Neuromotor Sciences, Physiology Building, Piazza di Porta San Donato 2, I-40126 Bologna, BO, Italy.
E-mail address: renata.bartesaghi@unibo.it (R. Bartesaghi).
[1] Contributed equally to the work.
Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2017.03.012

very large effect on neurogenesis, connectivity and behavior, being able, in some instances, to fully restore brain development (see (Stagni et al., 2015)). The advantage of preclinical studies is that mouse models provide the unique possibility to test a variety of different compounds. Identification of a panel of effective compounds in mouse models will increase the spectrum of possibilities to discover a therapy that is effective in individuals with DS.

Various studies have clearly shown that the Sonic Hedgehog pathway, a crucial pathway for brain morphogenesis and neural precursor proliferation, is altered in DS (Roper et al., 2006; Trazzi et al., 2011; Trazzi et al., 2013). This alteration can be ascribed to a cascade of events stemming from amyloid precursor protein (APP) triplication and culminating into overexpression of Patched 1 (Ptch1), the repressor of this pathway (Trazzi et al., 2011; Trazzi et al., 2013). In particular, cleavage of the APP-derived carboxy-terminal fragments (CTFs) by the enzyme gamma-secretase gives origin to the amyloid precursor protein intracellular domain (AICD) and either p3 or beta-amyloid (Abeta). In the trisomic brain, due to excessive APP expression there are excessive AICD levels that, in turn, increase the transcription of Ptch1 (Trazzi et al., 2011; Trazzi et al., 2013). It is important to observe that Ptch1 over-expression is present in the DS brain starting from fetal life stages (Trazzi et al., 2011), which prospects the possibility that derangement of the Shh pathway may be an important determinant of neurogenesis reduction and brain hypocellularity. During the last few years, various selective APP gamma-secretase inhibitors have been developed by ELAN Inc. (Basi et al., 2010), as strategic tools to reduce Abeta levels in Alzheimer's disease. Among these, ELND006 (ELN) is a gamma-secretase inhibitor which retains selectivity and incorporates improved drug-like properties (Basi et al., 2010; Probst et al., 2013). In a previous study we obtained evidence that neonatal treatment with this inhibitor of gamma-secretase was able to reinstate the functionality of the Shh pathway in the Ts65Dn mouse model of DS and that this effect led to restoration of neurogenesis and cellularity in the hippocampal dentate gyrus (Giacomini et al., 2015). These animals were treated from postnatal day 3 (P3) to postnatal day 15 (P15), *i.e.* the period of maximum neurogenesis in the hippocampal dentate gyrus, and were examined at the end of treatment. Since treatment was administered during the most critical time window for hippocampal development, it is possible that the dentate gyrus would remain in its restored state after treatment cessation. Yet, granule neurons are continuously added to the dentate gyrus and maturation of granule neurons requires 2–3 months. Unless treatment has effects that span beyond its cessation, its beneficial effects may tend to extinguish with time. The half-life of the effects of ELN has been shown to be in the range of 12–24 h (Brigham et al., 2010). Therefore, its direct effect on APP processing and, thus, Ptch1 levels, are expected to extinguish in a relatively short time. However, a treatment schedule of 13 days may induce secondary effects on signaling pathways that, if retained with time, may cause long-term effects even after treatment cessation.

In the framework of studies aimed at identifying therapies for intellectual disability in DS it is of paramount importance to establish not only the efficacy of the tested therapies but also the duration of their effects. This knowledge may help to choose the compound/s most suitable to be tested in clinical trials. Therefore, the goal of the current study was to establish whether neonatal treatment with ELN leads to enduring restoration of hippocampal development.

2. Methods

2.1. Colony

Female Ts65Dn mice carrying a partial trisomy of chromosome 16 (Reeves et al., 1995) were obtained from Jackson Laboratories (Bar Harbour, ME, USA) and the original genetic background was maintained by mating them with C57BL/6JEi x C3SnHeSnJ (B6EiC3) F1 males. Animals were genotyped as previously described (Reinholdt et al., 2011). The day of birth was designated postnatal day zero (P0). The animals' health and comfort were controlled by the veterinary service. The animals had access to water and food *ad libitum* and lived in a room with a 12:12 h dark/light cycle. Experiments were performed in accordance with the Italian and European Community law for the use of experimental animals and were approved by Bologna University Bioethical Committee. In this study all efforts were made to minimize animal suffering and to keep the number of animals used to a minimum.

2.2. Experimental protocol

We previously found that a dose of 30 mg/kg of ELND006 (ELN; gift by ELAN Inc., USA) from postnatal day 3 (P3) to postnatal day 15 (P15) had no acute effect on mice viability (Giacomini et al., 2015). However, we found here that euploid and Ts65Dn mice treated with this dose exhibited a higher mortality rate (death rate = 30–40%) after weaning. For this reason, we decided to reduce the dose of ELN. In a pilot experiment we found that a 20 mg/kg dose did not increase the mortality rate and was able to reinstate cell proliferation in the dentate gyrus of Ts65Dn mice ($n = 4$–5 mice for each experimental group; data not shown), similarly to the dose of 30 mg/kg. Therefore, in the current study we treated mice with a dose of 20 mg/kg. Euploid ($n = 20$) and Ts65Dn ($n = 11$) mice received a daily subcutaneous injection of ELN (dose 20 mg/kg) dissolved in the vehicle (25% PEG300, 25% ethylen glycol, 25% cremophor, 15% ethanol, 10% propanol) from P3 to P15. Age-matched euploid ($n = 19$) and Ts65Dn ($n = 10$) mice were injected with the vehicle. These mice will be called here untreated mice. Each treatment group had approximately the same composition of males and females. On P45-P50 mice were weighed, sacrificed, the brain was quickly removed and weighed. These mice will be called here P45 mice (Fig. 1A). The left hemisphere was fixed by immersion in PFA 4%, frozen and used for immunohistochemistry. The right hemisphere was kept at −80 °C and used for western blotting. The number of animals used for each of the experimental procedures described below is specified in the figure legends. Other groups of mice were treated from P3 to P15 with either ELN (20 mg/kg) (euploid: $n = 8$; Ts65Dn: $n = 4$) or vehicle (euploid: $n = 9$; Ts65Dn: $n = 7$) and at 30–45 days of age were used for electrophysiological recordings from field CA3 (Fig. 1A).

2.3. Histological procedures

The left hemisphere was cut with a freezing microtome in 30-μm-thick coronal sections that were serially collected in anti-freezing solution (30% glycerol; 30% ethylen-glycol; 10% PBS10X; 0.02% sodium azide; MilliQ to volume) and used for immunohistochemistry for Ki-67, synaptophysin (SYN) and postsynaptic density protein-95 (PSD-95).

2.3.1. Ki-67 immunohistochemistry

One out of 6 sections were taken starting from the beginning to the end of the hippocampal formation ($n = 16$–20 sections). Sections were incubated overnight at 4 °C with rabbit monoclonal anti-Ki-67 antibody (1:100; Thermo Scientific). Section were then incubated for 2 h with a Cy3 conjugated anti-rabbit IgG (1:200; Jackson Immunoresearch). Sections were counterstained with Hoechst 33342 in order to label cell nuclei.

2.3.2. SYN and PSD-95 immunohistochemistry

Free-floating sections ($n = 4$–6 per animal) from the hippocampal formation were submitted to fluorescence immunohistochemistry for SYN and PSD-95. Sections were incubated for 24 h at 4 °C with mouse monoclonal anti-SYN (SY38) antibody (Millipore-Biomanufacturing and Life Science Research, Billerica, MA, USA) or rabbit polyclonal anti-PSD-95 antibody (Abcam) both diluted 1:1000. Sections were then incubated for 2 h with a FITC-conjugated anti-mouse antibody or with a CY3-conjugated anti-rabbit (Jackson Laboratory) antibody both diluted 1: 200.