



Long-term effects of neonatal treatment with fluoxetine on cognitive performance in Ts65Dn mice[☆]



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ABSTRACT

Individuals with Down syndrome (DS), a genetic condition caused by triplication of chromosome 21, are characterized by intellectual disability and are prone to develop Alzheimer's disease (AD), due to triplication of the amyloid precursor protein (APP) gene. Recent evidence in the Ts65Dn mouse model of DS shows that enhancement of serotonergic transmission with fluoxetine during the perinatal period rescues neurogenesis, dendritic pathology and behavior, indicating that cognitive impairment can be pharmacologically restored. A crucial question is whether the short-term effects of early treatments with fluoxetine disappear at adult life stages. In the current study we found that hippocampal neurogenesis, dendritic pathology and hippocampus/amygdala-dependent memory remained in their restored state when Ts65Dn mice, which had been neonatally treated with fluoxetine, reached adulthood. Additionally, we found that the increased levels of the APP-derived β CTF peptide in adult Ts65Dn mice were normalized following neonatal treatment with fluoxetine. This effect was accompanied by restoration of endosomal abnormalities, a β CTF-dependent feature of DS and AD. While untreated adult Ts65Dn mice had reduced hippocampal levels of the 5-HT1A receptor (5-HT1A-R) and methyl-CpG-binding protein (MeCP2), a protein that promotes 5-HT1A-R transcription, in neonatally-treated mice both 5-HT1A-R and MeCP2 were normalized. In view of the crucial role of serotonin in brain development, these findings suggest that the enduring outcome of neonatal treatment with fluoxetine may be due to MeCP2-dependent restoration of the 5-HT1A-R. Taken together, results provide new hope for the therapy of DS, showing that early treatment with fluoxetine enduringly restores cognitive impairment and prevents early signs of AD-like pathology.

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Introduction

Intellectual disability is the invariable hallmark and the most invalidating feature of Down syndrome (DS), a genetic disorder caused by triplication of chromosome 21. Moreover, individuals with DS are prone to develop Alzheimer's disease (AD) in adulthood, with consequent worsening of cognitive functions (Dierssen, 2012). Due to an improvement in medical care, life expectancy for individuals with DS has increased during the last decade and, consequently, individuals with DS may outlive their parents.

Intellectual impairment in DS is due to alterations of brain development that can be traced back to fetal life stages. These alterations

include widespread neurogenesis impairment, excessive number of astrocytes, dendritic atrophy, and connectivity impairment (Bartsaghi et al., 2011). These defects are distributed throughout the brain and, therefore, numerous brain functions are altered. Much effort has been taken during the past decade to identify the gene/s responsible for the aberrant brain development and cognitive profile of DS. The genetic culprits have not been unequivocally identified, most likely because these alterations do not depend on a single gene but rather, derive from a mixture of actions, to which various genes may contribute. Nevertheless, candidate genes that play an important role in both brain development and function can be used as targets for therapeutic interventions. In addition, therapies can be attempted by acting downstream, seeking to improve structural and neurochemical defects engendered by gene triplication.

Various pharmacological approaches have been attempted in mouse models of DS, showing that it is possible to improve some of the morpho-functional defects of the trisomic brain and related behavior (Bartsaghi et al., 2011; Costa and Scott-McKean, 2013). Most of these studies have been carried out in adult animals. It must be noted,

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however, that in spite of a certain degree of plasticity retained in adulthood, the overall organization of the brain mainly depends on events taking place during embryonic and perinatal life stages. Considering that one of the most severe defects of trisomy is early neurogenesis impairment (Guidi et al., 2008; Guidi et al., 2011), therapies to improve this defect should be started as soon as possible, otherwise the brain will have a permanently reduced asset of neurons. Likewise, the severe dendritic pathology due to trisomy should be restored as early as possible, in order to allow the establishment of appropriate neuronal connections. With this idea in mind, we attempted to rescue the morpho-functional defects of the trisomic brain with early pharmacotherapies. We decided to use fluoxetine, a selective serotonin reuptake inhibitor because: 1) the serotonergic system is altered in DS (Bar-Peled et al., 1991; Boullin and O'Brien, 1971; Lott et al., 1972a, 1972b; Whittle et al., 2007); 2) serotonin is fundamental for neurogenesis, dendritic development and synaptogenesis (Banar et al., 2004; Encinas et al., 2006; Mogha et al., 2012; Santarelli et al., 2003; Whitaker-Azmitia, 2001). We found that treatment with fluoxetine during the embryonic period rescued overall brain development and that neonatal treatment induced full recovery of hippocampal neurogenesis, dendritic development, connectivity and hippocampus-dependent memory. The effect of either the pre- or early postnatal treatments were retained up to adolescence (Bianchi et al., 2010; Guidi et al., 2014; Guidi et al., 2013).

While these results clearly show that it is possible to rescue the major brain defects caused by gene triplication with early intervention and that this effect can outlast treatment cessation, there is a crucial question that still needs an answer. Do the restoring effects of an early therapy vanish in the long run or is the rescue of development a long-term outcome that extends to adult life stages? The question is not trivial because fluoxetine (or other drugs) do not reduce the gene burden but simply bypass it, by interfering with its effects. Consequently, the possibility exists that an initial restoration of brain development may go awry with time, once the therapy is interrupted. Therefore, the goal of the current study was to establish whether an early pharmacotherapy with fluoxetine has long-term effects on the brain that are retained at adult life stages. To this purpose, we treated neonate Ts65Dn mice with fluoxetine during the first two postnatal weeks and examined the outcome of treatment when the same mice reached adulthood.

Materials and methods

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/6JEl x C3H/HeSnJ (B6EiC3Sn) F1 males. Animals were karyotyped as previously described (Reinholdt et al., 2011). The day of birth was designated postnatal day zero. A total of 77 mice were used. 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.

Experimental protocol

Euploid ($n = 23$) and Ts65Dn ($n = 12$) mice received a daily subcutaneous injection (at 9–10 a.m.) of fluoxetine (Sigma-Aldrich) in 0.9% NaCl solution from postnatal day 3 (D3) to D15 (dose: 5 mg/kg from D3 to D7; 10 mg/kg from D8 to D15). Age-matched euploid ($n = 22$) and Ts65Dn ($n = 20$) mice were injected with the vehicle (Fig. 1). The total number of Ts65Dn mice used was smaller in

comparison with that of euploid mice because the number of trisomic pups in a litter is only approximately 20–40% (Roper et al., 2006). The smaller number of treated Ts65Dn mice in comparison with their untreated counterparts (see above) was not due to a treatment-induced high mortality rate but to the fact that, for unpredictable reasons, in the litters treated with fluoxetine the number of trisomic pups was smaller in comparison with that in untreated litters. Each treatment group had approximately the same composition of males and females. Animals were killed at the age of 3.0–3.5 months and body weight was recorded prior to sacrifice. After sacrifice, the brain was excised and weighed. Data on body and brain weight are reported in Supplementary results. Starting from eighteen days before reaching 3.0–3.5 months of age mice were behaviorally tested (Fig. 1). Some of these animals (5–6 animals for each condition) received an intraperitoneal injection (150 μ g/g body weight) of BrdU (5-bromo-2-deoxyuridine; Sigma), a marker of proliferating cells (Nowakowski et al., 1989) in 0.9% NaCl solution (at 11–12 a.m.) one month before being killed (Fig. 1). The brain of another group of animals (5–8 animals for each condition) was quickly removed, the hippocampal formation and a 2–3 mm-thick brain slice immediately rostral to the hippocampal formation that included the septal region (called here septal forebrain) were dissected, kept at -80°C and used for Western blotting. A summary of the overall study design is reported in Supplementary Table 1.

Histological procedures

Mice that had received BrdU were deeply anesthetized, the brain was removed and the left hemisphere was fixed by immersion in 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. These hemispheres were stored in the fixative for 72 h, kept in 20% sucrose in phosphate buffer for an additional 24 h, frozen and stored at -80°C . The right hemisphere was rinsed in PBS and Golgi-stained. The left hemisphere was cut with a freezing microtome into 30- μ m-thick coronal sections that were serially collected in antifreezing solution and used for the following procedures (see also Table 1): immunohistochemistry for i) Ki-67, ii) cleaved caspase-3, iii) DCX, iv) SYN, and v) Rab5 and double-fluorescence immunohistochemistry for BrdU and either a neuronal (NeuN) or an astrocytic (GFAP) marker. Details of histological procedures and related measurements are reported in Supplementary methods.

Western blotting

Total proteins were obtained as previously described (Trazzi et al., 2011). We analyzed the levels of p21, 5-HT1A receptor (5-HT1A-R), Erk1/2 and phospho-Erk1/2, methyl-CpG-binding protein (MeCP2), brain-derived neurotrophic factor (BDNF), amyloid precursor protein (APP), APP carboxyterminal fragment beta (β CTF) and beta site cleaving enzyme 1 (BACE1) in homogenates of the hippocampal formation and the levels of 5-HT1A-R and β CTF in homogenates of the septal forebrain. Details of the procedures are reported in Supplementary methods.

Behavioral testing

Mice were behaviorally tested using the Morris Water Maze (MWM) test, the Novel Object Recognition (NOR) test and the Passive Avoidance (PA) test as previously described (Bevins and Besheer, 2006; Gallagher et al., 1993; Loizzo et al., 2013). See Supplementary methods for more details.

Statistical analysis

Data from single animals represented the unity of analysis. Results are presented as mean $\pm$ standard error of the mean (SE). Statistical testing was performed using a two-way analysis of variance (ANOVA)

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