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Treatment with β-2 adrenergic receptor agonists: a tool for improving brain developmental alterations in Down syndrome?

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ABBREVIATION LIST

7,8-DHF = 7,8-dihydroxyflavone
Ach = Acetylcholine
AchE = Acetylcholine esterase
AD = Alzheimer's disease
AICD = Amiloyd intracellular domain
ANOVA = Analysis of variance
APP = Amyloid precursor protein
ASD = Autism spectrum disorder
ASM = Airway smooth muscle
Aβ = Amyloid β
BACE1 = Beta-secretase 1
BDNF = Brain derived neurotropic factor
BFCN = Basal forebrain cholinergic neurons
BrdU = 5-Bromo-2'-deoxyuridine
B1-AR = β1-adrenergic receptors
β2-AR = β2-adrenergic receptors
CA1 = Cornus Ammoni 1
CA3 = Cornus Ammoni 3
CDKs = Cyclin-dependent kinases
CFC = Contextual fear conditioning
CL = Clenbuterol
CNS = Central nervous system
COPD = Chronic obstructive pulmonary disease
CREB = c-AMP response element-binding protein
CSA= Clycosporine A
Cy3 = Cyanine 3
DCX = Doublecortin
DG = Dentate gyrus
DS = Down syndrome
DSCAM = Down syndrome cell adhesion molecule
DYRK1A = Dual-specific tyrosine-(Y)-phosphorylation regulated kinase 1A
E = Embryonic day
EGCG = Epigallo-catechin-3-gallate
ELN = ELND006
ERK1/2 = Extracellular signal–regulated kinases 1/2
EU = Euploid
FDA = Food and Drugs Administration
GABA = γ-Aminobutyric acid
GIRK = G-protein coupled inward rectifying K+ channel
Gli1/2 = Glioma-associated oncogene 1/2
GRIK1 = Glutamate ionotropic receptor kainate type subunit 1
GSK3β = Glycogen synthase kinase 3β
GW = Gestational week
HRP = Horse radish peroxydase
HSA21 = Human chromosome 21
ID = Intellectual disability
IFNs = Interleukins and interferons
IHC = Immunohistochemistry
IPSC = Inhibitory postsynaptic current
iPSC = Human pluripotent stem cell
IQ = Intelligence quotient

KCNJ6 = Potassium voltage-gated channel subfamily j member 6.
L/M = Learning and memory
LC = Locus coeruleus
LABAs = Long-Acting β Agonists
LSD = Least significance difference
LTD = Long term depression
LTP = Long term potentiation
LPS = Lipopolysaccharide
Mb = Megabases
mEPSC = Miniature excitatory postsynaptic current
MWM = Morris water maze
NA = Noradrenaline
NAP = NAPVSIPQ peptide
ncRNAs = Noncoding RNAs
NeuN = Feminizing locus on x-3 (neuronal marker)
Nf-kB = nuclear factor kappa-light-chain-enhancer of activated B cells
NGF = Nerve growth factor
NMDA = N-methyl-D-aspartate
NOR = Novel object recognition
NPCs = Neural progenitor cells
OD = Optical density
OLIG1/2 = Oligodendrocyte transcription factor 1 and 2
P = Postnatal day
PBS = Phosphate buffer saline
PSD-95 = Post-synaptic density protein 95
PTCH1 = Patched1
PTZ = Pentylentetrazole
RCAN1 = Regulator of calcineurin 1
ROS = Reactive oxygen species
S100β = S100 calcium binding protein β
SABAs = Short-Acting β Agonists
SALM = Salmeterol
sAPP = Soluble amyloid precursor protein
SAL = Saline
SE = Standard error
SGZ = Subgranular zone
SHH = Sonic hedgehog
shRNA = Short hairpin RNA
SIM2 = Single-minded homolog 2
SOD1 = Superoxide dismutase 1
SSRI = Selective serotonin reuptake inhibitor
SVZ = Subventricul zone
SYN = Synaptophysin
SYNJ1 = Synoptojanin 1
Thr = Threonine
TRKB = Tropomyosin receptor kinase
TSH = Thyroid-stimulating hormone
Ultra-LABAs = Ultra-Long Acting β Agonists
VGAT = Vesicular GABA transporter
VGLUT1 = Vesicular glutamate transporter 1
VZ = Ventricular zone
WRAM = Water radial arm maze

ABSTRACT

Down syndrome (DS) is a genetic condition (incidence: 800/1000) caused by triplication of human chromosome 21. The phenotype of DS has been linked to an extra copy of one or more of about 300 protein coding genes. Cognitive impairment is the unavoidable hallmark and the most disabling feature of DS. Accumulating evidence shows that DS is characterized by numerous neurodevelopmental alterations among which neurogenesis reduction appears to play a particularly prominent role. This defect is worsened by dendritic pathology and impairment of proper connectivity. At present, there are no therapies for the treatment of cognitive defects in DS. In the present study, we used the Ts65Dn mouse model of DS in order to establish whether it is possible to improve the trisomy-linked defects with new pharmacotherapies untested so far.

The screening of two libraries of FDA-approved drugs in neural progenitor cells (NPCs) derived from Ts65Dn mice for a drug-repurposing project revealed various new hits that were able to enhance neurogenesis. Among the new hits, Clenbuterol (CL) and Salmeterol (SALM), two β2-adrenergic agonists used for the treatment of asthma, resulted particularly effective. Based on this evidence, we carried out experiments in Ts65Dn mice in order to establish whether the effects elicited *in vitro* are replicated *in vivo*. To this purpose, Ts65Dn pups were daily treated with different doses of CL (0.01 mg/kg, 0.5 mg/kg, 1.0 mg/kg or 2.0 mg/kg) or SALM (0.01mg/g or 1.0 mg/kg) from postnatal day 3 (P3) to P15. At the end of treatment, we examined the effects of these compounds on the hippocampal dentate gyrus, a region fundamental for declarative memory that largely develops postnatally. We found that the lowest dose of CL was sufficient to fully restore dendritic spine density and dendritic size (total length and number of segments of dendritic tree) of the granule cells. The highest dose only, however, was able to moderately improve neurogenesis of hippocampal NPCs. While no dose of SALM was able to improve neurogenesis, treatment with both tested doses was sufficient to fully restore dendritic pathology. Although both CL and SALM restored spine density in Ts65Dn mice, a comparison of their effects showed that the spine density increase induced by CL was larger in comparison with that induced by SALM.

This study provides novel evidence that two β2-adrenergic agonists (CL and SALM) are able to fully restore the dendritic pattern in the hippocampus of a DS mouse model. Importantly, even the lowest dose tested here was sufficient to restore dendritic development. After a translation that takes into account species-specific metabolic differences, this dose is in the range of doses used in children and adults for the treatment of asthma. Thus, our study suggests that treatment with CL or SALM during an appropriate time window may represent a suitable therapy to correct dendritic pathology in children with DS.

INTRODUCTION

DOWN SYNDROME

Down syndrome (DS, OMIM 190685), is a genetic pathology caused by the triplication of human chromosome 21 (HSA21). It is the most frequent cause of mental retardation of genetic origin and accounts for nearly 30% of moderate-to-severe mental retardation. It is and is one of the best studied neurodevelopmental alterations of genetic origin. The behavioral phenotype associated with DS emerges in infancy and accumulates in early childhood, with impairment of speech, language, motor skills, cognition and adaptive behavior. It must be noted that the IQ in adults with DS may also be influenced by the increased risk of early onset dementia of the Alzheimer's type. The increased protein expressions of genes on HSA 21 leads to a cascade of effects in the development of fetal brain structures and subsequent structural and behavioral effects across the life span of individuals with DS. The precise mechanisms that govern specific gene dosage effects and their translation to behavior are not well understood. A small region of the distal part of the long arm of HSA 21 (21q22.1–22.3), the DS critical region, is associated with many of the physical features of DS, particularly facial features, congenital heart disease and duodenal stenosis, and some aspects of mental retardation, whereas loci outside this region appear to contribute to the full behavioral phenotype, including mental retardation, accelerated aging, and Alzheimer's disease (AD) (Korenberg et al., 1994).

HISTORY

The first description of what is now called DS was by Edouard Onesimus Seguin (1812–1880), a student of Itard, the founder in France and in the United States, of methods and systems for educating those with mental retardation. In 1846, he published his classical textbook on the subject.

In 1866, John H. Langdon Down gave the next description of DS. In his paper, Down attempted a classification of patients with mental retardation based on ethnic characteristics, recognizing four categories: The ''Ethiopian variety''; the "Malay variety"; the ''American continent variety'' and the "Mongolian variety".

Down mixes visionary concepts, such as that of rehabilitation, and old prejudice, such as that of parental degeneracy, and his language is all but politically correct in describing the different types of idiocy according to ethnic varieties. In 1923, T. Halbertsma of Haarlem, The Netherlands, argued that DS was of germinal origin. He did so on the basis of twin data, 15 cases of dizygotic twins always being discordantly affected, and the two pairs of presumed monozygotic twins being concordantly affected. In 1932, long before the discovery of the human karyotype, Waardenburg predicted that DS was probably caused by a chromosomal aberration. This thesis was sustained by two pediatricians, Adrien Bleyer of St. Louis and Guido Fanconi of Zurich. In 1939, Penrose demonstrated that it was maternal, not paternal age that correlated with the occurrence of DS. Then came 1959, when Lejeune et al. discovered that trisomy of HSA 21 is the cause of DS (Neri and Opitz, 2009).

ETIOLOGY

Genomic aneuploidy, defined as an abnormal number of copies of a genomic region, is a common cause of human genetic disorders. Classically, the term aneuploidy was restricted to the presence of supernumerary copies of whole chromosomes (trisomy), or absence of chromosomes (monosomy), but this definition has been extended to include deletions or duplications of subchromosomal regions.

Trisomy 21 is a model of all human disorders that are the result of supernumerary copies of a genomic region (Antonarakis et al., 2004). In DS, the extra copy of genes presents on HSA21 (Galdzicki et al., 2001, Papavassiliou et al., 2009) is caused by three types of genetic rearrangements:

Full trisomy 21 (Ts21). This trisomy is characterized by the presence of a third whole 21st chromosome which is usually of maternal origin. This is the most common cause of DS and results from meiotic or mitotic non-disjunction. The net result is that the total number of chromosomes in every somatic cell is 47 instead of the normal 46 (Galdzicki et al., 2001, Papavassiliou et al., 2009).

Robertsonian translocation (2–4% of cases). Partial (or segmental) trisomies that involve a genomic region of more than one chromosomal band (usually larger than 5 Mb) are much less frequent than whole-chromosome trisomies. They usually result from abnormal meiosis and segregation in individuals with balanced chromosomal rearrangements (Galdzicki et al., 2001, Papavassiliou et al., 2009).

Mosaicism (2–4% of cases)***.*** This abnormality may be carried by both parents and can increase the probability of DS in offspring. Cases of DS mosaicism occur when some somatic cells have 47 chromosomes and others have their normal complement of 46 (see (Galdzicki et al., 2001)). Mosaicism is caused by an error in mitotic cell division soon after conception. There also are cases of partial Ts21, where only the distal part of the long arm of chromosome 21 is duplicated (Papavassiliou et al., 2009).

EPIDEMIOLOGY

DS has been estimated to occur in approximately 1 in 800-1000 infants with an estimated number of 5400 infants with DS born each year in the United States. For children with DS, the chance of survival beyond the first year of life has improved in recent years (Shin et al., 2009), with 90% of children with DS now surviving beyond 5 years of age. Children with DS have an increased risk for endocrinologic, hematologic, respiratory, and neurologic sequelae, as well as psychiatric and social problems later in life (Irving et al., 2008). Infancy is characterized by a delay in cognitive development and the IQ declines from the first year to late childhood (Rachidi and Lopes, 2008).

Various screening strategies have been introduced over the years with the aim of offering prospective parents the ability to make informed decisions (see (Contestabile et al., 2010)). Other temporal trends, particularly changes in birth rate and maternal age profile, are also likely to have an influence on the live birth rate of infants with DS. Survival of infants with DS has improved because of better care (especially of cardiovascular malformations) and survival into mid or late adult life is now expected. Few adults with DS are

likely to be capable of unsupported independent existence. Any change in the birth prevalence and survival of DS will have implications for the provision of long-term care of children and adults with this condition.
Screening strategies varied in the framework of a study (Irving et al., 2008) carried out in the northern region of England: serum screening was first used in a pilot project in two health districts in 1988 and was introduced across the region in 1991. Maternity units responsible for about 50% of deliveries offered screening to all pregnant women, regardless of age, since 1992. The remaining units offered amniocentesis on an age-related basis. In 1997–2000, two of these units, responsible for about 8% of deliveries, began to offer serum screening to all mothers. Thus, by 2000, serum screening was available to nearly 60% of mothers in this region. In the study area, it would appear that where serum screening for trisomy 21 is offered regardless of age, it is taken up by about 50% of women (Irving et al., 2008). Prenatal screening techniques for DS include assessment of ultrasonographic markers, particularly measurement of nuchal translucency in the first trimester of pregnancy and maternal serum screening during both the first and second trimesters. Techniques used in making definitive prenatal diagnoses include amniocentesis and chorionic villus sampling. In addition, a noninvasive method of prenatal diagnosis involving molecular detection of fetal DNA in maternal blood is being developed (Palomaki et al., 2011).

THE HUMAN CHROMOSOME 21 (HSA21)

A useful approach to defining the genetic basis of DS phenotypes is to examine individuals bearing partial trisomies for HSA21. HSA21 is the smallest human chromosome, and its overall gene density is approximately 15 genes per Mb. Studying the overexpression of transcription factor genes on HSA21 will provide important information about the proportion of the entire transcriptome that is influenced by each of these genes, and by their combined action (Antonarakis, 2017). On the basis of such observations, a region (DCR-1) localized between CBR1 and ERG and containing 20 genes is suggested to be responsible for morphogenetic and cognitive features. The gene content of HSA21 has been almost completely determined by the publication of the DNA sequence of the long arm (21q) of HSA21 (Hattori et al., 2001). According to the latest published estimate, gene number for HSA21 is more than 500 genes and putative genes. In addition to protein-coding genes, bioinformatics annotation has established that the chromosome 21 harbors five MicroRNA (miRNA) genes: *miR-99a*, *let-7c*, *miR-125b-2*, *miR-155* and *miR-802.* Because of their role as post-transcriptional regulators of gene expression, miRNAs offer a new scenario in which their increased dosage and consequent overexpression may result in an apparently paradoxical decreased expression of certain proteins in trisomic organisms (Keck-Wherley et al., 2011).

THE MOST STUDIED TRIPLICATED GENES

Among the over-expressed genes of the HSA21, *SIM2, DYRK1A, DSCAM, APP, SOD1, S100β, SYNJ1, OLIG1/2* appear to play a prominent role in the DS brain phenotype (Roizen and Patterson, 2003).

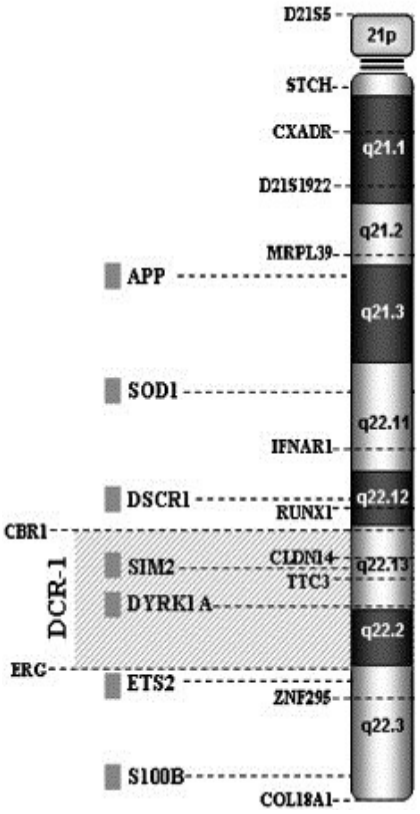


Fig.1. The human chromosome 21 (HSA21).
Principal genes located on HSA21, the regions 21p (short arm) and 21q (long arm) and their sub-regions, including the DS critical region (DSCR) are represented. From (Rachidi and Lopes, 2008).

SIM2. The single-minded (*SIM*) gene was originally identified in Drosophila as a mutation affecting neurogenesis and causing early lethality. Similarly to Drosophila *Sim*, the mammalian *SIM* genes, *SIM1* and *SIM2*, are characterized by restricted expression patterns, particularly in the developing Central Nervous System (CNS). SIM1 and SIM2 proteins interact with the ubiquitous partners ARNT and ARNT2, Tango orthologs, and migrate to the nucleus where they can activate or repress target genes. Human *SIM2* was first identified by exon trapping from a region of the human chromosome 21 (21q22.2) associated with many of the pathological features of DS (Rachidi et al., 2005). Study of chromosome 21 genes expressed in fetal CNS and potentially involved in the human brain development could greatly help the understanding of the pathogenesis of mental retardation and brain alterations in DS patients. Few data currently exist regarding the developmental role of *SIM2* and its expression during human fetal development and corresponding mouse stages during CNS development (Yamaki et al., 1996).

DYRK1A. Overexpression of dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1A (DYRK1A), encoded by a gene located in the DS critical region (21q22.13), is considered a major contributor to developmental abnormalities in DS. It encodes a protein kinase whose unique genetic and biochemical

property have been evolutionarily conserved from insects to humans (Tejedor et al., 1995). DYRK1A regulates numerous genes involved in neuronal commitment, differentiation, maturation, and apoptosis. Fibroblasts derived from individuals with DS exhibit impaired proliferation due to elongation of the G1 phase of the cell cycle, and the extended G1 duration is restored by knocking down DYRK1A (Chen et al., 2013). Moreover, DS iPSCs (Induced pluripotent stem cell) exhibit abnormal neural differentiation that is largely improved by targeting DYRK1A pharmacologically or by shRNA (Hibaoui et al., 2014). This evidence suggests a key role of DYRK1A in the regulation of proliferation and differentiation of trisomic neural precursor cells (NPCs). A study has demonstrated anomalous NMDA receptor-mediated LTP in the prefrontal cortex of mBACtg-Dyrk1A mice (Thomazeau et al., 2014), resulting in excessive inhibition. Given the role of GABA and glutamate transmission in neurogenesis, LTP and cognitive function, an imbalance between GABAergic and glutamatergic synapse activity may also profoundly impair cognition in DS. Finally, DYRK1A may contribute to AD-like neuropathological features in DS by modulating the formation of intracellular Tau inclusions and the production of β-amyloid (Park et al., 2010).

DSCAM. DSCAMs (Down syndrome cell adhesion molecules) are a group of immunoglobulin-like transmembrane proteins that contain fibronectin III domains. The founding member of the family was isolated in a positional cloning study that sought to identify genes located on HSA 21 at the locus 21q22.2-q22.3 that is implicated in the neurological and cardiac phenotypes associated with DS. In Drosophila, DSCAM proteins are involved in neuronal wiring. In vertebrates, the role of these cell adhesion molecules in neurogenesis, dendritogenesis, axonal outgrowth, synaptogenesis, and synaptic plasticity is only just beginning to be understood (Cvetkovska et al., 2013). The over-expression of this protein in DS patients may be related to cellular dysfunctions that affect the development of the CNS, and/or favor AD-related dementia in adulthood (Perez-Nunez et al., 2016).

APP. Amyloid beta (A4) Precursor Protein (APP) is a transmembrane protein involved in the regulation of synapse formation and neural plasticity (Turner et al., 2003, Priller et al., 2006). The gene is located in a region of the distal part of chromosome 21 (21q21.3). APP is expressed in both neuronal cells and extra-neuronal tissues and belongs to a larger evolutionarily conserved APP superfamily found in different organisms from nematode to man. Alzheimer's disease (AD) and related dementia constitute a spectrum of age-related neurodegenerative diseases leading to major cognitive and behavioral deficits. AD is a global problem affecting over 30 million people worldwide and some 10 million in Europe alone. The hypothesis of the causes of AD places the 40-42 amino acid amyloid β-peptide (Aβ), derived by proteolytic processing of APP, center stage in the cell death process. Recent reappraisals of the hypothesis. However. have highlighted that Aβ -independent factors may also contribute to the disease process and that oligomeric forms of Aβ may be the principal toxic agent (Larson and Lesne, 2012). In neuronal cells, APP is anterogradely transported in vesicles by kinesin-mediated fast transport to various cell compartments including synapses. There are three major isoforms of APP (APP695, APP751, APP770) generated as a result of alternative splicing of exons 7 and 8.

Until recently, no clear-cut functional differences have been ascribed to the different APP isoforms apart from the protease-inhibitory role of the KPI domain. However, it appears that the neuronal APP695 isoform is preferentially involved in regulation of gene expression (Nalivaeva and Turner, 2013). APP isoforms were shown to be differentially expressed during brain maturation and alternative splicing and processing of the APP gene was found to be regulated by various factors, including hormones, growth factors, phorbol esters and interleukins. The regulatory region of the *APP* gene contains consensus sites recognized by the transcription factor specificity protein 1 (SP1). Recently microRNAs, which represent small, non-coding RNAs interacting with target mRNA and mediating translational inhibition or transcript destabilization, were suggested to regulate APP gene expression and to play an important role in neurodegeneration (Salta and De Strooper, 2012).

SOD1. The superoxide dismutase 1 (*SOD1*) gene is located in a region of the distal part of HSA 21 (21q22 band) which is triplicated and known as the critical region of DS. SOD1 catalyzes the conversion of superoxide anion (O2 •−) into hydrogen peroxide (H_2O_2), two of the main reactive oxygen species (ROS) continuously generated mainly by the aerobic cells in the mitochondria (Gutteridge and Halliwell, 2010). H_2O_2 in turn is degraded by catalase (CAT) and glutathione peroxidase (GPx) into water and molecular oxygen. Hydrogen peroxide is produced in excess as a result of increased SOD1 activity without the concomitant increase of complementary antioxidant defense mechanisms, such as CAT and GPx activity (Gutteridge and Halliwell, 2010). The accumulation of endogenous H_2O_2, which is highly diffusible and relatively stable, is able to generate other deleterious ROS through the Haber–Weiss–Fenton reactions, thereby damaging important cellular components, oxidizing biomolecules such as amino acid residues, proteins, lipids and DNA (Gutteridge and Halliwell, 2010). Cortical neurons from individuals with DS exhibit increased levels of intracellular reactive oxygen species and lipid peroxidation, preceding neuronal death, which can be prevented by antioxidants. This evidence suggests that SOD1-induced enhancement of free radical levels may contribute to increased apoptosis in DS (see (Stagni et al., 2018a)).

S100β. S100 calcium binding protein beta(*S100β*) is located in a region of the distal part of chromosome 21 (21q22.3). S100β protein, which is mainly oxidized, becomes neurotoxic and induces apoptosis and neuroinflammatory processes marked by microglial activation when in a reduced state. S100β and its receptor RAGE (receptor for advanced glycation end products) have been found to be increased in AD and DS (Bialowas-McGoey et al., 2008).

SYNJ1. Synaptojanin 1. This gene encodes a phosphoinositide phosphatase that regulates levels of membrane phosphatidylinositol-4,5-bisphosphate, It is located in the 21q22.11 region of chromosome 21 and it is highly conserved throughout evolution (Cremona et al., 2000). The synaptojanin family comprises proteins that are present in the presynaptic nerve terminals and are key players in synaptic vesicle recovery. Thus, expression of synaptojanin may affect synaptic transmission and membrane trafficking. There is evidence that over-

expression of SYNJ1 in DS is functionally linked to the enlargement of early endosomes, that in turn provokes disruption of synaptic vesicle transportation (Cossec et al., 2012).

OLIG1/2. Oligodendrocyte transcription factor 1 (OLIG1) and oligodendrocyte transcription factor 2 (OLIG2) are basic helix-loop-helix (bHLH) transcription factors that are located in the 21q22.11 region of chromosome 21. OLIG1/2 promote formation and maturation of oligodendrocytes, especially within the brain, and cooperate during neural tube formation. OLIG2 mis-expression impairs cortical progenitor proliferation, causes precocious cell cycle exit, massive neuronal cell death, downregulation of neuronal specification factors including NGN1, NGN2 and PAX6, and a defect in cortical neurogenesis (see (Stagni et al., 2018a)).

COMMON HEALTH PROBLEMS IN DS

Patients with DS are characterized by numerous phenotypic defects and medical problems caused by the triplication of HSA21. The most invalidating aspect of the DS condition is intellectual disability that is the unavoidable hallmark of DS. In addition, individuals with DS have many other congenital abnormalities, such as gastrointestinal anomalies, hypotonia, craniofacial abnormalities, audio vestibular, visual impairment and hematopoietic disorders. Furthermore, people with DS tend to develop leukemia, thyroid disorders, and AD like pathology with age (see (Kazemi et al., 2016)).

Data on the extent of serious congenital abnormalities that are associated with DS are provided by two studies from population-based congenital malformation registries (Dublin study and Strasbourg study). In the Dublin study there were 389 live births and nine stillbirths affected with DS in a total of 225808 births between 1980 and 1989 (Hayes et al., 1997). In the Strasbourg study there were 398 cases of DS (265 live births, eight stillbirths, eight miscarriages, 117 induced abortions) in a total of 238942 births in Strasbourg from 1979 to 1996 (Stoll et al., 1998). Data on these congenital malformations obtained from these studies are reported below.

Congenital heart defects (CHD)

The incidence of CHD in newborn babies with DS is up to 50% (see (Asim et al., 2015)). The three most common types of congenital heart defects reported in the Dublin and Strasbourg study were atrioventricular canal defects (28% of those with heart defects in the Dublin study and 43% in the Strasbourg study), ventricular septal defects (28% and 32% respectively), and patent ductus arteriosus (21% and 5% respectively). In the Dublin study, 20% of the children born with congenital heart defects died in the first year of life compared with 4% of those without such defects. There is about a 60-fold increase in congenital heart disease in children with DS compared with the prevalence in the general population (see (Noble, 1998)).

Atrioventricular septal defects (AVSDs). Of all the heart defects, AVSDs are the most serious and the only ones, which have an adverse impact on survival. AVSDs are a grave heart malformation with particular clinical features. In the normal formation of the heart the endocardial cushions grow toward each other and leave openings between the atria and the ventricles. The mitral and tricuspid valves form in the openings. The

atrioventricular canal defect is caused by a failure of this process and results in the formation of a single valve structure with a septal defect above and below it. Depending on the location of this valve structure it can also present as an inlet ventricular septal defect (resulting in blood flow only between the ventricles) or an ostium primum atrial septal defect (leaving only a hole between the atria) (see (Noble, 1998)). Survival can be improved with surgery, but the prognosis is still poor because of the increased risk of heart failure. Atrioventricular septal defects are genetically linked to HSA21 and are about 420 times more common in people affected with DS than in the general population (see (Noble, 1998)).

Ventricular septal disease (VSD). VSD is a defect characterized by a hole between the top chambers (receiving chambers or atria) which provokes a backwash from the right heart side (pulmonary) to the left (arterial). Holes can be located in the middle of the central heart wall (most common), in the top septum (sinus venosus defects) or in the lower part of septum (partial atrioventricular septum disease).

Other rare congenital heart problems are: isolated secundum atrial septal defects (8%), isolated tetralogy of Fallot (4%) and isolated patent ductus arteriosus (Freeman et al., 1998). Importantly, most of the anomalies in infants with DS are suitable for complete surgical correction (see (Arumugam et al., 2016)).

Gastrointestinal defects. The second most common types of malformations in DS individuals are those of the gastrointestinal tract. These defects can affect up to 7-12% of children with DS (Freeman et al., 2009, Bull and Committee on, 2011). The three most common defects are duodenal stenosis/atresia (3.9%), anal stenosis/atresia (1.0%), esophageal atresia with or without tracheoesophageal fistula (0.4%), pyloric stenosis (0.3%) and Hirschsprung disease (HD; 0.8%) (see (Arumugam et al., 2016)). HD is a relatively rare condition in children with DS. It involves a long segment of the lower part of the large bowel wall, in which there is an absence of normal myenteric ganglion cells in a segment of the colon (Amiel et al., 2008). This encourages chronic constipation, poor weight gain, vomiting and swollen abdomen. There is a form of pathology in which only a short part of the bowel participates and the symptoms are less severe.

Other congenital abnormalities have been reported in association with DS. In the Strasbourg study these were urinary tract malformations (6%), limb defects (9%, half of which were minor), and congenital cataract (1%). The Dublin study reported 21% of the live births affected with various abnormalities other than those of the heart or gastrointestinal tract (see (Noble, 1998)).

Other medical problems associated with DS

There are several serious medical problems associated with DS that include leukaemia, epilepsy, hypertension, thyroid disorders and AD.

Leukaemia. DS increases the risk of developing acute megakaryoblastic Leukaemia (AMKL) and acute lymphoblastic leukaemia (ALL) in comparison with the general population. Hayes and colleagues reported in their study that 1.65% of the DS children developed leukaemia (see (Noble, 1998)). Leukaemia in DS is mainly of two types: acute non-lymphatic and acute lymphatic. Most cases of acute non lymphatic leukaemia are acute megakaryoblastic leukaemia that occurs during the first four years of life. In the general population

this type of leukaemia is rarely seen in young children. A transient form of megakaryoblastic leukaemia is also seen in infants with DS and although this condition disappears spontaneously, as many as 25% of infants with it will develop acute megakaryoblastic leukaemia between 1 and 4 years of age (Wiseman, 2009).

Thyroid disorders. The incidence of persistent primary congenital hypothyroidism detected in infants with DS by neonatal screening has been reported to be about 0.7%, 27 times more than in the general population. Symptoms of hypothyroidism might be mistaken for symptoms related to the natural course of DS. Therefore, it is important to monitor annually for thyroid function in view of the fact that neuro-endocrine changes occurring with aging play a main role not only in homeostasis but also in the occurrence and/or in the progression of metabolic, functional and cognitive alterations (Ferrari et al., 2004). Thyroid dysfunction, expressed as a high TSH concentration, is associated with growth retardation in children with DS who are younger than 4 years. Unfortunately, there are very few studies that have systematically examined the frequency of thyroid disease in infants with DS.

Epilepsy. The incidence of epilepsy in people with DS is 1.4-17% and is globally higher in comparison with the general population (~1%) (Barca et al., 2014). In a study reported by Noble (see (Noble, 1998)), about 15% of DS patients (16-18 years old) were affected by epilepsy.

Alzheimer's disease (AD). The *APP* gene is located on HSA21 and its protein product APP is cleaved by beta and gamma secretases to release neurotoxic Aβ peptides. Development of dementia in DS is though to result from the over-production of neurotoxic Aβ peptides as a direct consequence of the triplication of the APP gene (see (Noble, 1998, Lott, 2012, Hartley et al., 2015)). The prevalence of AD in people with DS increases from 8% between the ages of 35 and 49 years, to 55% in persons between 50 and 59 years and 75% in persons above the age of 60. Similarly to the general population, the presence of the E4 allele of the apolipoprotein E gene appears to be associated with an earlier onset of AD in the DS population (Wiseman, 2009). It has been suggested that an abnormal APP metabolism initiates AD pathogenesis by triggering a set of events that result in Aβ aggregation, particularly of the Aβ-42 peptide, in the extracellular plaques (Choong et al., 2015, Hartley et al., 2015). HSA21 genes other than APP may also contribute to the early onset of AD in people with DS. For example, DYRK1A can phosphorylate the tau protein at a key priming site that permits its hyperphosphorylation, followed by depolymerization of actin microfilaments, dendritic hypotrophy and neurofibrillary tangle formation. Aβ-42 fragment and tau affect several brain regions in DS, including the prefrontal cortex, hippocampus, basal ganglia, thalamus, hypothalamus and midbrain (Wisniewski et al., 1985).

BRAIN DEVELOPMENTAL ALTERATIONS IN DS

Several studies have focused on the neuroanatomical correlates of cognitive impairment in DS.

Brain size. There is strong evidence that individuals with DS have an overall reduced brain volume (reduction>20%), and numerous brain regions appear to be smaller in fetuses and adults in comparison with

controls. A pioneering study in fetuses aged 15-22 weeks found no gross differences in brain shape, weight, and fronto-occipital length. Neither were any differences in the volume of the cerebellum or hippocampus (Schmidt-Sidor et al., 1990). This is in contrast with the hypotrophy found by various other studies regarding the DS fetal brain. In fact, ultrasonographical data and analysis of autoptic specimens have revealed that the reduction in brain size appears already in 4–5 month old DS fetuses (Guihard-Costa et al., 2006) and size reduction has been detected in various hippocampal structures (Guidi et al., 2008), cerebellum (Guidi et al., 2011), subiculum (Stagni et al., 2018b), and inferior temporal region (Guidi et al., 2018) of fetuses with DS at GW 17-22. In addition to weight and volume differences, the fetal DS brain exhibits distinctive shape alterations. It is markedly brachycephalic due to a reduction in the length of the frontal lobe, with an increase in the transparietal length (Guihard-Costa et al., 2006). Consistently with prenatal measurements, neuroradiological studies with magnetic resonance imaging (MRI) have demonstrated that a 17% decrease of brain volume persists postnatally in 10–20-year old DS patients. Brains of infants and children with DS have a reduced volume in comparison with typical children due to a reduction in the volume of both the gray and white matter (see (Stagni et al., 2018a)). In addition, it has been reported that the frontal and occipital lobes and cerebellum have a reduced size in autoptic brain samples of DS children (Pinter et al., 2001, Kates et al., 2002, Menghini et al., 2011, Smigielska-Kuzia et al., 2011). MRI studies also showed selective decrease of the hippocampus and temporal lobe in children and young individuals with DS (Pinter et al., 2001).

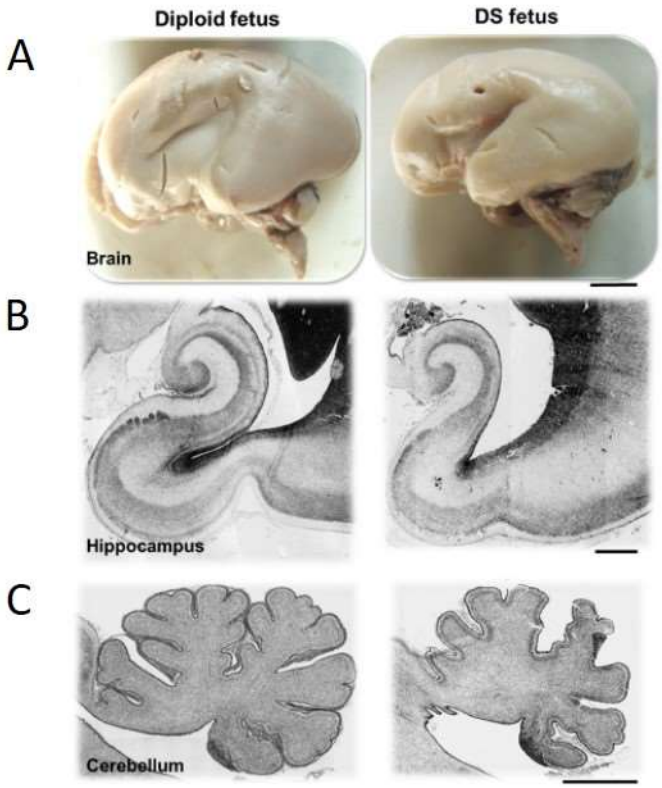


Fig.2. Brain hypotrophy in DS fetuses.
A-C: Examples of brain (scale bar 1 cm), hippocampus (scale bar 1 mm) and cerebellum (scale bar: 2 mm) of diploid and DS fetuses (17-21 GW). Images were taken from (Guidi et al., 2008, Guidi et al., 2011, Stagni et al., 2018a). Abbreviation: DS, Down syndrome.

Neuroradiological and neuropathological findings in brains of DS adults further documented the reduced volume of several areas, including the hippocampus, entorhinal, frontal, prefrontal and temporal cortices, amygdala, cerebellum, brain stem nuclei (e.g., locus coeruleus) and mammillary bodies of the hypothalamus. It has been suggested that the neurodevelopmental abnormalities of DS arise from a decreased generation of neurons during critical phases of brain development. Indeed, the number of neurons is reduced in the hippocampus, parahippocampal gyrus, neocortex, and subiculum of DS fetuses (Guidi et al., 2008, Stagni et al., 2018b) and in the granule cell layer and cortex of DS children (Baxter et al., 2000, Buxhoeveden et al., 2002). Moreover, during aging, brain atrophy superimposes on pre-existing developmental abnormalities. Indeed, MRI studies have reported atrophy of the medial temporal lobe, including hippocampus, amygdala, and neocortical regions such as the corpus callosum and the parietal, frontal, and occipital cortices in aged non-demented DS patients, suggesting prodromal stages of AD-like pathology (Teipel and Hampel, 2006).

Neurogenesis. The early occurrence of brain abnormalities in DS points to neurogenesis impairment as the major determinant of intellectual disability. It has been proposed that aberrant copies of single chromosomes could alter the timing of the mitotic cell cycle and the rate of cell proliferation during development. Accordingly, it has been hypothesized that, in DS, the extra copy of chromosome 21 affects the cell cycle of neuronal precursors during the process of neurogenesis. There is evidence that in fetuses with DS (GW 17–23) the number of proliferating cells, detected with immunostaining for Ki-67 (a protein expressed during all phases of the cell cycle) is notably reduced in various brain regions. In particular neurogenesis impairment has been detected in the hippocampal dentate gyrus, the germinal zones of the hippocampus proper and parahippocampal gyrus, and in the germinal matrix of the inferior horn of the lateral ventricle (Contestabile et al., 2007, Guidi et al., 2008). In addition, a notably reduced number of proliferating cells has been detected in the external granular layer of the cerebellum, and in a region of the fifth cerebellar lobe that is the remnant of the cerebellar ventricular zone (Guidi et al., 2011). A notable reduction in cell proliferation, as assessed by the cell cycle marker Ki-67 and the M-phase marker phospho-histone H3 (PH3) immunostaining, was also found along the VZ and SVZ of the frontal cortex of DS fetuses (GW 18) (Lu et al., 2012). Analysis of proteins expressed in various stages of the cell cycle revealed an elongation of the G2 phase, possibly accounting for the reduced proliferation rate found during neurogenesis in the DS brain (Contestabile et al., 2007). Subsequent studies further demonstrated that the number of differentiated neurons is also reduced in DS developing brain, while astrocytes are virtually unaffected (Guidi et al., 2008). Indications of defective neurogenesis in DS also derives from *in vitro* studies showing that neuronal precursors isolated from DS fetal brains and cultured as neurospheres give rise to reduced numbers of neurons when differentiated (Lu et al., 2012). A recent study generated an isogenic iPSC model of DS by reprogramming primary human skin

fibroblasts from an adult individual with mosaic DS (Murray et al., 2015). This study shows that NPCs derived from neuroembryoid bodies exhibit a reduced proliferation rate in comparison with disomic NPCs. Another study has characterized iPSCs derived from monozygotic twins discordant for trisomy 21 (Hibaoui et al., 2014).

Apoptosis. In typical subjects, the number of neurons in the different structures of the central nervous system is relatively constant. This number is established during early developmental stages through complex interactions and fine-tuning of signaling processes which control cell proliferation, programmed cell death (apoptosis) and cell differentiation (Oppenheim, 1991, Haydar et al., 1999). Increased apoptosis is found in the dentate gyrus and subventricular zone of DS fetuses, indicating that alterations of both programmed cell death and neurogenesis concur to the hypocellularity of DS brains (Guidi et al., 2008). In contrast, no significant differences in cell death have been detected in human progenitor cells derived from fetal DS cortices (Bhattacharyya et al., 2009). Upregulation of pro-apoptotic factors, such as BCL-2-like protein 4 (BAX), the downregulation of anti-apoptotic factors, such as B-cell lymphoma 2 (BCL-2), and apoptosis-associated proteins have been found in different cortical and cerebellar regions of DS fetuses (Seidl et al., 2001, Helguera et al., 2005). However, unaltered expression of apoptosis-related proteins such as caspase-3, FAS, BCL-2 and Annexins I, II, V and VI has been reported in the cerebellum and cortex of DS fetuses (Engidawork et al., 2001, Gulesserian et al., 2001, Contestabile et al., 2007). Although some studies show an enhancement of apoptosis, the number of cells undergoing apoptosis is very low, suggesting that this process does not have a prominent role in reducing the final neuron number in the DS brain (Abraham et al., 2001, Guidi et al., 2008, Guidi et al., 2011).

Dendritic development. Dendrites represent the main receptive structures of neurons and dendritic spines host the majority of neuronal synapses. Altered development of dendritic structures is a hallmark of many forms of intellectual disability, including DS. In fact, the length and branching of dendrites and spine density are reduced in the hippocampus and cortex of the DS brain. Such dendritic abnormalities are progressively acquired during development (see (Kaufmann and Moser, 2000)). Indeed, normal or even increased dendritic branching in fetuses and neonates contrasts with degenerative changes in older children with DS. In fact, neuronal morphology and spine density are comparable in the visual cortex of DS and euploid fetuses. The dendritic atrophy seen in childhood progresses during adulthood, with marked reduction of dendritic branching, length, and spine density in elderly adults. Becker et al. showed that dendritic branching and length in both apical and basilar dendrites were greater in infants with DS younger than 6 months than in normal infants (Becker et al., 1986). Indeed, in normal subjects, cortical dendritic arborization and spine number raise from birth to 15 years of age and gradually decrease after 20 years of age. Conversely, dendritic arborization and spines only poorly increase in DS children and rapidly degenerate in adults with DS (Takashima et al., 1994). A reduced spine density has been found in the apical dendrites of pyramidal neurons of the hippocampus and cingulate and in both the apical and basilar dendritic arbors of CA1 and CA2-3

pyramidal neurons in patients with DS in comparison with age-matched controls (Suetsugu and Mehraein, 1980, Takashima et al., 1989). In addition, starting from infancy, dendritic spines are small, have short stalks and are intermingled with unusually long spines (Marin-Padilla, 1976). Consistently, the levels of drebrin, a protein involved in regulating spine morphology and synaptic plasticity, are reduced in the frontal and temporal cortex of DS patients (Weitzdoerfer et al., 2001). Given the role of dendritic spines as essential structures for brain connectivity and plasticity of synaptic circuits, it is straightforward to postulate that alterations in these neuronal microcompartments may affect neuronal network activity.

Neurotransmitter system. Neurochemical alterations of several transmitter-identified neuronal systems have been found in DS brains. Reduced levels of neurotransmitters important for brain development such as gamma–aminobutyric acid (GABA, the main inhibitory neurotransmitter), taurine, serotonin and dopamine, have been found in DS fetuses (Whittle et al., 2007). Decreased levels of excitatory neurotransmitters, monoamines, histamine and 5-hydroxytryptamine, as well as reduced activity of the histamine synthesizing enzyme histidine decarboxylase (HDC) have also been found in various areas of adult DS brains, suggesting the existence of profound alterations of neuronal network activity in DS.

Age-related neurodegeneration. The age-related deterioration of cognitive performance in DS may be related to degeneration of the cholinergic neurons in the nucleus basalis of Meynert, which precociously degenerate in AD. Indeed, deficits in the cholinergic system similar to those occurring in AD have been demonstrated in the DS brain (Contestabile et al., 2008). Degeneration of basal forebrain cholinergic neurons (BFCN) and decreased activity of choline acetyltransferase (ChAT) have been observed in both DS and AD. Notably, the basal forebrain cholinergic system is apparently normal in DS fetuses and infants in terms of both neuronal numbers and ChAT activity, but starts to degenerate during late adolescence and adulthood, further supporting the view that degenerative processes occur in DS subjects during aging. At the cellular level, DS degenerative and neurodevelopmental mechanisms concur to altering neuronal compartments such as dendrites.

NEUROLOGICAL DEFECTS IN DS

Intellectual capacity. DS is the most common cause of severe intellectual disability. Almost all people with DS have intellectual disability (ID) to some extent, which is the main clinical feature of the disorder. The intelligence quotient (IQ) score is the traditional method of assessing intellectual ability and is based on a set of questions that measure reasoning ability and power of deduction without relying on prior knowledge (Vicari, 2006). The IQ scores of the general population have a normal (Gaussian) distribution with a mean of 100 and a standard deviation of 15; 2.5% of the population have scores of less than 70 (that is, more than two standard deviations below the mean) and 2.5% have scores greater than 130 (that is, more than two standard deviations above the mean). ID is generally defined by an IQ score of less than 70; 50–70 being categorized as mild, 20–50 severe, and less than 20 profound. It is estimated that among children affected

with DS, 10% have profound intellectual disability, 70% severe, and 20% mild or none (Bartesaghi et al., 2011). In addition to intellectual disability, a significant proportion of DS subjects develop cognitive decline and Alzheimer's dementia with aging. Studies investigating the prevalence of AD in the DS population have reported divergent values varying from 8% to 100% (Nadel, 2003).

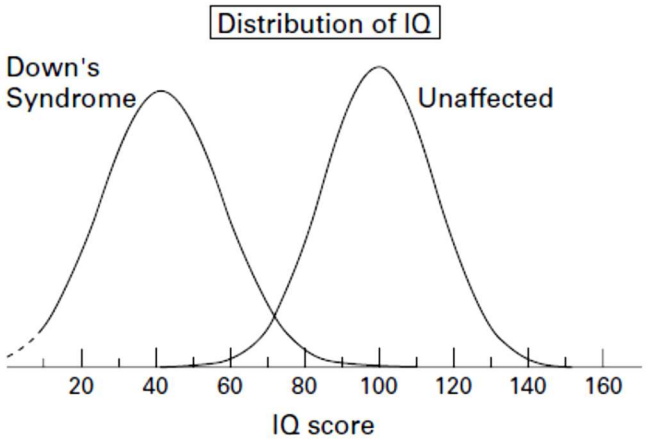


Fig.3. Natural history of Down's syndrome: a brief review for those involved in antenatal screening.
Distribution of IQ in 21-year old with and without Down's syndrome. From (Noble, 1998).

Autism spectrum disorder (ASD). In Children with DS the intellectual disability, together with the expression of other phenotypic social and behavioral differences over time, leads to a profile that carries increased risk for other comorbid developmental disorders such as autism spectrum disorder (ASD); (see (Channell et al., 2019)). ASD is a neurodevelopmental disorder that is clinically defined by the presence of two symptoms: (i) problems with social communication and social interaction, and (ii) restricted, repetitive patterns of behavior, interests, or activities. Current estimates suggest that approximately 7–19% of individuals with DS have comorbid ASD (DiGuiseppi et al., 2010). Despite the relatively high prevalence of ASD in DS, the nature of this comorbidity is still poorly understood. This has led to discrepant findings regarding the prevalence rate and, more importantly, how to identify ASD in individuals with DS who carry their own phenotypic profile with potentially overlapping features to ASD (see (Channell et al., 2019)).

Motor skills. Children with DS usually do not acquire motor skills at the same rate as their typically developing (TD) peers. However, children with DS may achieve rolling between 5 and 6.4 months, independent sitting between 8.5 and 11.7 months, thus suggesting that the emergence of motor milestones is only slightly retarded in DS. Nonetheless, the delay is greater for later developing motor skills: DS infants crawl on hands and knees between 12.2 and 17.3 months of age and walk between 15 and 74 months (Lott et al., 2012). Moreover, children with DS seem to follow the same sequence of motor milestones of typically developing infants albeit qualitative differences or atypical movement patterns in order to maintain postural stability are often reported. Unusual postures may be also connected to the presence of hypotonia frequently

associated with the syndrome. The lack of muscle tone is certainly related to the reduced strength in children and adolescents even if the exact influence of hypotonia on motor development remains to be determined (Vicari, 2006).

Linguistic abilities. Infants with DS show many of the normal features of prelanguage behavior, including babbling and imitation, although there are some subtle but possibly important differences between DS and normally developing infants in this regard (Chapman and Hesketh, 2000). In fact, children with DS show slowest rates of development in grammatical morphology. Children with DS usually have very poor linguistic capacities and a longer period of phonological errors and poorer intelligibility, although language comprehension is relatively more advanced than syntax (Chapman and Hesketh, 2000, Vicari, 2006). Impairment in language development of children with DS is evident when they are compared with typical developing peers of the same mental age.

Learning and memory. Learning deficits in DS children involve both short-term and long-term memory. DS children perform significantly worse than normal children on explicit memory tasks (Chapman and Hesketh, 2000). However, they show normal learning ability for tasks requiring implicit memory processing, indicating a functional dissociation between implicit and explicit memory. This is in agreement with the different mechanisms of these two types of memory processing. In fact, implicit memory is sustained by substantially automatic processes requiring low attention, while explicit memory deals with intentional conscious learning and require information coding, retrieval strategies and high degree of attention. Consistently, poor information encoding, impaired retrieval abilities and attention deficits have been demonstrated in DS, accounting for the selective impairment of explicit memory in infants and children. Consistently, tasks requiring high degree of information processing exacerbate verbal working memory deficits and unmask defective visuospatial skills in DS children and adults (see (Visu-Petra et al., 2007)). Both hippocampal- and prefrontal-related functions appear defective in individuals with DS.

Visuo–spatial abilities: Visuo-spatial short-term memory capacity is relatively spared in DS on low-control tasks or when the visual and spatial components are tested separately (Vicari, 2006). However, in recognition tasks, when memory load increases or when visual and spatial demands are combined, the performance of DS children is impaired compared with mental age-matched controls (see (Visu-Petra et al., 2007)).

MOUSE MODELS FOR THE STUDY OF DS

TRISOMIC MOUSE MODELS

The long arm of HSA21 contains 33.7 Mb and approximately include ~160 encoding diverse protein functions, a large family (~45 members) encoding apparently redundant keratin associated proteins, and a number of provisionally annotated microRNAs (Gardiner, 2015). The homologous gene are present to syntenic regions of mouse chromosomes 16, 17, and 10 (Gardiner, 2003) (Fig. 4). Most investigations have focused on the MMU16 syntenic region. The distal end of mouse chromosome 16 (Mmu16) contains ~100 HSA21 orthologous protein-coding genes, the internal part of Mmu 17 contains ~20 protein-coding genes and the internal part of Mmu 10 contains ~40 protein-coding genes (Gardiner, 2015). The triplication of a syntenic chromosomal region with HSA21 make mouse models very useful to tackle gene-dosage imbalance and mimic the possible interactions between different genes present in three copies. Mouse models are very useful tools in dissecting clinical phenotypes, in identifying candidate genes and generating cDNA chips for investigating gene expression profile in trisomic mice versus DS patients.

Numerous mouse models of DS have been generated during the last 30 years. Their features are reported in the excellent review by Herault (Herault et al., 2017) to which the reader is referred for details. We describe below some of these models, with particular emphasis on the Ts65Dn mouse, because this is the model that has been more largely used so far.

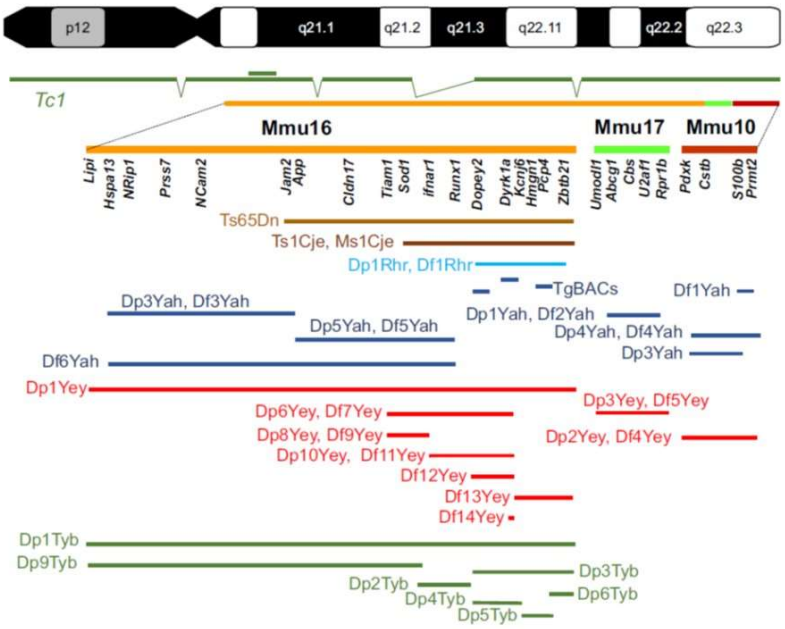


Fig.4. Trisomic and transgenic mouse models of Down syndrome, their syntenic regions and genes on HSA 21. Human chromosome 21 (p and q arms; G-banding) is depicted at the top of the figure, with the mouse genome orthologous region found on chromosome 16 (Mmu16), Mmu10 and Mmu17 shown respectively in orange, light green and red. A few known genes that are homologous to HSA21 genes in the DS critical region are listed below each chromosome. The transchromosomic Tc1 mouse model is shown in dark green, with deletions and a duplication (double bar) relative to HSA21 depicted. Below, the segment of the DS critical region encompassed in different mouse models for DS is illustrated. The original Ts65Dn and Ts1Cje models (shown in brown) originated by accidental translocation of Mmu16 segments respectively on Mmu17 and Mmu12, with some additional changes. Olson et al. (2004) published the

first engineered duplication (Dp) and deletion [deficiency (Df)] for the DS critical region (light blue). New models have been developed in the last 10 years by the authors of this Review, as shown in dark blue, red and green. TgBACs, a few models for BAC or PAC (P1-derived artificial chromosome) transgenic lines. From (Herault et al., 2017).

Ts16 mice, trisomic for the entire MMU16, provided the first model for DS. Their main limitation was embryonic or early postnatal lethality, presumably due to triplication of genes located on the proximal portion of MMU16, not syntenic with HSA21 (Rachidi and Lopes, 2008). In contrast, mice with partial translocations of MMU16 (Ts65Dn, Ts1Cje and Ms1Ts65) live until adulthood and show many clinical phenotypes similar to those seen in DS patients. Ts16 embryos showed small body and brain size (Lacey-Casem and Oster-Granite, 1994) and delayed development of the precursors of the sensory organs and skull ossification (Ludwig et al., 1997), abnormal neuronal migration and evidence of oxidative stress (Behar and Colton, 2003). Unfortunately, Ts16 embryos die in utero, making it impossible to test phenotypes in young and adult mice, thus restricting the face and predictive validities of this model (Rueda et al., 2012).

Ts65Dn mice are currently the most widely used mouse model for DS. This strain is the chromosomally unbalanced progeny of a mouse mutant carrying a balanced translocation; the genetic alteration was randomly induced by irradiation at Muriel Davisson's laboratory (Davisson et al., 1990). The unbalanced derivative chromosome in Ts65Dn consists of a genomic fragment of approximately 13 Mb extending from *Mrpl39* to the telomere on Mmu16 with approximately 49.2% of the syntenic regions and approximately 55% of the HSA21 gene orthologs triplicated (Davisson et al., 1993). The Ts65Dn mouse shows developmental delay and abnormalities in different organs mimicking those found in DS, particularly in the brain. It must be noted that the Ts65Dn mouse carries three copies of an extra segment (arising from Mmu17) with non-DS-related genes, including ∼35 protein-coding genes, 15 non-protein-coding genes and 10 pseudogenes (Herault et al., 2017). Thus, some of these genes may contribute to impairment of brain development. For instance, the Mmu17 triplicated gene Tiam2 (T Cell Lymphoma Invasion and Metastasis 2), similarly to Tiam1, plays a critical role in neurite outgrowth and remodeling of synaptic connections. Synj2 belongs to the synaptojanin family of proteins that are key players in the synaptic vesicle recovery at the synapse (Duchon et al., 2011).

Brain size. Inconsistent information is available regarding differences in brain weight between euploid and Ts65Dn mice in the early postnatal period. According to some studies, the Ts65Dn mouse has a reduced brain weight at P2 and P15, but other studies do not report differences at P8, P15, or P22. Some studies show that the Ts65Dn mouse has no volume defects in the dentate gyrus of the hippocampus, cortex, striatum and cerebellum at P2, P6, P10, and P22. In contrast, other studies report differences in the volume of the dentate gyrus at P2 and P15, in the size of the cerebellum at P2, P6 and P14, and in the volume of the subiculum at P2 (see (Stagni et al., 2018a)) (Stagni et al., 2018b).

Cellularity. Ts65Dn mice exhibit a reduced cellularity in the neocortex, dentate gyrus, hippocampus, striatum, thalamus, hypothalamus mesencephalon, and cerebellum at P2 (Guidi et al., 2014). Hypocellularity in the

dentate gyrus has also been documented at P6 and P15 (see (Stagni et al., 2018a)) and in the perirhinal cortex in 1.5–3.5 month-old mice (Roncace et al., 2017).

Neurogenesis. A reduction of neurogenesis has been demonstrated in the neocortex of Ts65Dn embryos at E13.5 (Chakrabarti et al., 2007). In contrast, during embryonic development neural precursors in the medial ganglionic eminence (MGE) of the Ts65Dn mouse exhibit a faster proliferation rate. These precursors will give origin to inhibitory neurons, which may account for the large inhibitory weight observed in Ts65Dn mice (Chakrabarti et al., 2010). A reduced number of proliferating cells has been found in the thalamus, hypothalamus, mesencephalon and pons of P2 mice (Guidi et al., 2014) and in the neocortex and striatum of P2 and P15 Ts65Dn mice (Bianchi et al., 2010b, Guidi et al., 2014). In addition, proliferation defects have been found in the dentate gyrus at P2, P6 and P15 and in the subventricular zone at P2 and P15 (see (Stagni et al., 2018a)) (Stagni et al., 2017a). Moreover, the Ts65Dn mouse shows a strong decrease in the proliferation rate of cerebellar granule cell precursors at P0 and at P2 and this defect is retained at P6 (see (Stagni et al., 2018a)). In addition, neural progenitor cells of Ts65Dn mice show differentiation abnormalities similarly to individuals with DS. In particular, a reduction in the number of cells differentiated into neurons and an increase in the number of cells with an astrocytic phenotype has been found in NPCs derived from the subventricular zone of neonate Ts65Dn mice (Trazzi et al., 2011, Trazzi et al., 2013). Consistently with this evidence, in the dentate gyrus and cerebellum of young adult Ts65Dn mice the total number of new cells with a neuronal phenotype is significantly reduced and the number of new cells with an astrocytic phenotype is increased in comparison with euploid mice (see (Stagni et al., 2018a)).

Apoptosis. Conflicting results have been reported regarding apoptosis in trisomic mouse models. An increased apoptotic cell death has been found in the cortex of P0 Ts65Dn mice (Lu et al., 2011) and in the external granular layer of the cerebellum of P2 Ts65Dn mice (Contestabile et al., 2009). In contrast, no difference in the number of apoptotic cells was found in the neocortex of the Ts65Dn mouse at prenatal and early postnatal stages (Chakrabarti et al., 2007), in the hippocampus of young Ts65Dn mice, in the cerebellum of P6 Ts65Dn mice, and in the subventricular zone of P15 Ts65Dn mice (see (Stagni et al., 2018a)).

Dendritic development. Neurons of Ts65Dn mice show differences in spine density in comparison with euploid mice in several brain regions. The most consistent changes were noted in the dentate gyrus, where there was a significant decrease in spine density on dendrites of Ts65Dn granule cells at P21 and 3 months in comparison with euploid mice (Belichenko et al., 2004). In addition to defects in spine density, the granule cells in the dentate gyrus of P45 Ts65Dn mice have a shorter dendritic length (-36%) and a reduced number of segments (-45%) than euploid mice (Guidi et al., 2013). A striking difference between euploid and Ts65Dn mice was the absence of branches of higher order in Ts65Dn mice (Guidi et al., 2013).

Connectivity. The optical density of levels of a presynaptic (synaptophysin; SYN) and a postsynaptic (PSD-95) marker has been shown to be significantly lower in the Ts65Dn mice in comparison with euploid mice in layers II–III and IV–VI of the neocortex, in the dentate gyrus, field CA3, and field CA1. Accordingly, an

evaluation of the number of SYN and PSD-95 puncta showed that Ts65Dn mice had fewer SYN and PSD-95 immunoreactive puncta in all the examined regions, indicating defects in the number of presynaptic and postsynaptic terminals (Stagni et al., 2016). In addition, it has been shown that Ts65Dn mice exhibit a marked increase in the colocalization of synaptophysin with the inhibitory presynaptic proteins GAD65 and VGAT, suggesting an increase in the inhibitory weight (Belichenko et al., 2007). In addition, at the electron microscopic level, the synaptic apposition length of inhibitory synapses was significantly increased, with no change in excitatory synapses (Belichenko et al., 2009b). In agreement with these synaptic alterations, Ts65Dn mice exhibit severe impairment of long-term potentiation (LTP) in the dentate gyrus (Kleschevnikov et al., 2004) and perirhinal cortex (Roncace et al., 2017). In addition to these neurodevelopmental defects, Ts65Dn mice undergo age-related neurodegeneration associated with reduced levels of choline acetyltransferase (ChAT), nerve growth factor (NGF), and neurotrophic tyrosine kinase receptor type 1 (TRKA) in the basal forebrain cholinergic neurons (BFCN), and loss of tyrosine hydroxylase (TH) in the locus coeruleus (LC). These effects are relevant to premature cognitive decline and development of AD (Roubertoux and Carlier, 2010, Rueda et al., 2012).

Behavior. In addition to developmental delay and brain abnormalities, the Ts65Dn mouse also exhibits behavioral alterations. This mouse model shows significant hyperactivity in the dark and in other settings that provoke caution and lack of movement in normal animals, such as in open-field and plus-maze tests. In addition, Ts65Dn mice display notably reduced levels of performance in tasks that require the integrity of the hippocampal system, such as spontaneous alternation (T-maze task), contextual memory (CFC test), spatial learning, long-term memory and cognitive flexibility (MWM test; RAM test), non-spatial short- and long-term declarative memory (novel object recognition; NOR). Ts65Dn mice also show deficits in learning an operant conditioning paradigm. In contrast, the Ts65Dn model exhibits no deficits in sensory capabilities and coordinated behaviors such as olfactory sensitivity, visual abilities, orienting reactions, forelimb strength, postural skills, coordination, climbing, locomotion, and motor coordination and balance deficits (rotarod test) (see (Bartesaghi et al., 2011)), (Rueda et al., 2012).

Ts1Cje mice are the unbalanced progeny of a mouse mutant carrying a balanced translocation, t(12; 16), which was induced by gene-targeting in Charles Epstein's laboratory (Sago et al., 2000). The unbalanced derivative chromosome in Ts1Cje consists of a genomic fragment of approximately 8.1 Mb from Sod1 to the telomere on Mmu16, with the Sod1 gene inactivated (Sago et al., 2000). Ts1Cje mice harbor 3 copies of approximately 67% of the HSA21 gene orthologs triplicated in Ts65Dn mice (Davisson et al., 1990). This model shows brain developmental alteration at E14.5 when compared with the euploid counterpart (Ishihara et al., 2010). Moreover, Ts1Cje mice exhibit a reduction in cortical thickness at E14.5 and an overall reduction in cortical neurogenesis in the period E13.5–E14.5 in comparison with euploid mice (Ishihara et al., 2010). At P12, Ts1Cje mice show no reduction in brain weight and no modifications of overall brain volume compared with euploid mice. However, they exhibit an enlargement of the lateral ventricle that is twice as large as that

of euploid mice (Ishihara et al., 2010). Cultures of NPCs from the subventricular zone of Ts1Cje mice exhibit a reduction in the number of cells differentiated into neurons and an increase in new astrocytes with no differences in the number of new oligodendrocytes (Moldrich et al., 2009, Hewitt et al., 2010). Impairment of neurogenesis has also been found in the subventricular zone and dentate gyrus of Ts1Cje mice at 3 months age (Ishihara et al., 2010). In addition to these neuroanatomical defects, Ts1Cje mice show behavioral abnormalities in the MWM test (Sago et al., 1998).

Ms1Rhr mice were generated by crossing Ts65Dn mice with a mutant carrying a deletion in the *Cbr1* - *Fam3b* region (Olson et al., 2004), thus resulting in the reduction of the *Cbr1* - *Fam3b* segment to 2 copies in Ts65Dn mice. A reported phenotype of Ms1Ts1Rhr mice was an approximately 18% reduction in the brain volume (Aldridge et al., 2007).

Ts1Rhr mice are trisomic for the *Cbr1* - *Fam3b* region (Olson et al., 2004) and syntenic to the so-called DS critical chromosomal region on HSA21 (Delabar et al., 1993). Ts1Rhr mice show abnormalities in the synaptic structure and a significant alterations in the open field test, novel object recognition test and T-maze task (Belichenko et al., 2009a).

Ms1Ts65Dn mice are produced by crossing Ts65Dn mice with t(12; 16)1Cje mice. These mice are segmentally trisomic for the segment from *Mrpl39* to *Sod1*. No significant cognitive impairment was found in the Morris water maze test for this mutant (Sago et al., 2000).

Ts1Yah mice that carry a 0.59-Mb duplication between *Abcg1* and *U2af1* in the HSA21 syntenic region on Mmu17 were generated in Yann Herault's laboratory (Pereira et al., 2009). Interestingly, this duplication apparently led to increased hippocampal LTP in the mutant mice, providing the first evidence of possible genetic interaction between different mouse syntenic regions underlying altered synaptic plasticity associated with DS. Moreover, Ts1Yah mice showed defects in novel object recognition, open-field and Y-maze tests, similarly to other DS models, but an enhancement of hippocampal-dependent spatial learning in the Morris water maze (Pereira et al., 2009).

Tc1 mice are a model of trisomy 21 that transmits a freely segregating, almost complete human chromosome 21 with only an approximately 4.9-Mb deletion that contains approximately 19 genes (O'Doherty et al., 2005). The Tc1 "transchromosomic" mouse line has been characterized by a number of laboratories. Several major DS-related phenotypes were observed in this model, including abnormal developmental cognitive phenotypes, despite a subset of cells in Tc1 mice that did not carry the transchromosome (O'Doherty et al., 2005). The behavioral experiments, including novel object recognition (short- but not long-term object recognition memory) and the Morris water maze test, showed that Tc1 mice are impaired in learning and memory (O'Doherty et al., 2005, Morice et al., 2008, Hall et al., 2016). In addition, electrophysiological experiments in hippocampal slices showed decreased in LTP in the dentate gyrus (O'Doherty et al., 2005, Morice et al., 2008). However, additional behavioral experiments in freely moving mice showed that long-term memory and synaptic plasticity are preserved in Tc1 mice (Morice et al., 2008). Ultrastructural analyses

of the proximal dendrites of CA3 pyramidal cells in both fixed tissue and living cells of Tc1 mice revealed synapse loss and altered spine morphology (Witton et al., 2015).

Dp(10)1Yey /+, Dp(16)1Yey /+ and Dp(17)1Yey/+ mice carry the duplications spanning the entire HSA21 syntenic regions on Mmu10, Mmu16 and Mmu17, respectively (Yu et al., 2010). These mice were generated by crossing the mutants carrying individual duplications, which represent all the evolutionarily conserved genetic alterations and interactions of DS in mice. These mutant mice exhibit DS-related heart defects and abnormal cognitively relevant phenotypes such as a significant decrease in hippocampal LTP and a significant impairment in cognitive behaviors including the Morris water maze and contextual fear conditioning tests (Yu et al., 2010, Liu et al., 2018). Unlike the Ts65Dn and Tc1 models, Dp(1)1Yey /+, Dp(16)1Yey /+ and Dp(17)1Yey /+ mice are viable and fertile after backcrossing to C57BL/6J mice for five generations. Compound mutants can also be generated in these backgrounds. Therefore, these models can be used to alleviate the effects of heterogeneous strain backgrounds. In addition, the inbred and congenic backgrounds of the models can facilitate the analysis to identify the genetic modifiers for mutant phenotypes, including cognitively relevant phenotypes.

TRANSGENIC MICE

Transgenic mouse models have been generated in order to study the effect of cell specific and stage-specific overexpression of a unique gene. These include models for overexpression of the Cu–Zn superoxide dismutase 1 (*SOD1*), the neurotrophic factor *S100β*, the beta amyloid peptide (*App*), the transcription factor *Ets2*, the Down syndrome critical region (*Dscr1*), the Drosophila minibrain homolog (*Dyrk1A*), and the transcription factor single minded (*Sim2*).

Models	Human syntenic region and genes	Neurological dysfunctions similar to DS
Tg SOD1	SOD1 (21q22.11), superoxide dismutase, key enzyme in the metabolism of oxygen-derived free radicals	Neuromuscular junction defects, decreased serotonin level, neuronal degeneration in brainstem, learning defects
Tg APP	APP (21q21.3), β-amyloid precursor protein involved in senile plaque formation in DS and AD	Dystrophic neuritis associated with congophilic plaques, learning defects
Tg Ets2	ETS2 (21q22.2), erythroblastosis virus E26 Transformation-specific transcription factor	Neuonal cell apoptosis, brachycephaly, neurocranial and cervical skeletal defects
Tg S100β	S100β (21q22.3), calcium-binding protein beta neurotrophic factor released by astrocytes	Abnormal dendritic development, astrocytosis, learning and memory deficits
Tg Dyrk1A and Tg YAC152F7	DYRK1A (21q22.13), Cbr1-Cldn14 (21q22.12-q22.13)-containing Dyrk1A dual-specificity tyrosine-(Y)-phosphorylation regulated Kinase 1A	Abnormal brain structure increased brain weight and neuronal size, learning deficits
Tg Sim2	SIM2 (21q22.13), single-minded, transcription factor/helix-loop–helix, master regulator in CNS cell fate	Altered behavior and learning deficits
Tg DSCR1	DSCR1 (21q22-12), Down syndrome critical region 1	Neurological phenotype, impaired working memory in null mice
Tg YAC230E8	TTC3-DYRK1A (21q22.13) containing DOPEY2	Increased cortical neuronal density, learning deficits

Table 1. Transgenic mouse models for Down syndrome, their human syntenic genes and their neurological dysfunctions. From (Rachidi and Lopes, 2008).

SOD1 mRNA and protein have been found at increased level in human and mouse trisomic tissues (Mao et al., 2005). Transgenic mice containing human *SOD1* have 1.6–6-fold increased enzyme activity as compared to controls, associated with decreased plasma serotonin levels and serotonin accumulation rate in transgenic mouse platelets, a phenomenon similar to that reported in DS. Over-expression of SOD1 in transgenic mice leads to an apparently specific defect in distal motor neuron terminals, indicating that this gene can selectively affect motor neurons. Moreover, these transgenic mice show decreased cell number in several brain areas and decreased LTP in hippocampal field CA1 (Zang et al., 2004). The enzyme SOD1 catalyzes the conversion of superoxide anion (O2 •−) into hydrogen peroxide (H_2O_2), two of the main reactive oxygen

species (ROS) continuously generated mainly by the aerobic cells in the mitochondria (Gutteridge and Halliwell, 2010). Premature aging, one of the characteristics of DS, may involve oxidative stress and impairment of proteasome activity due to increased activity of SOD1.

S100β RNA and protein are over expressed in DS and AD brains. The transgenic mouse TgS100β shows dendritic abnormalities similar to those seen in fetal DS brains and astrocytosis. Transgenic mice over expressing human *S100β* show changes in cytoskeletal markers (Shapiro and Whitaker-Azmitia, 2004), suggesting involvement of S100β overexpression in glial–neuronal interactions, aging and neuronal growth pathology, in DS and AD. Early overexpression of S100β may, therefore, indicate a potential role of this protein in dendritic abnormalities and mental retardation (Rachidi and Lopes, 2008).

APP is over expressed in DS, at more than the expected 1.5- fold (Lyle et al., 2004). Transgenic mice TgAPP exhibit overexpression of APP in the neocortex and hippocampal region, mimicking features of DS. This transgenic model shows dystrophic neurites associated with plaques and mild learning defects (Lamb et al., 1993, Sturchler-Pierrat et al., 1997). When tested in the MWM, APP transgenic mice show impairment in the probe test and in the reverse probe test (Janus, 2004).

ETS2. Transgenic mice over expressing ETS2 develop neurocranial and cervical skeletal abnormalities, similarly to trisomy 16 mouse models and humans with DS, in which the gene dosage of ETS2 is increased. This over-expression of ETS2 induces also neuronal apoptosis, suggesting that overexpression of ETS2 may contribute to the increased rate of neuronal apoptosis in DS (Wolvetang et al., 2003a). Fibroblasts over expressing ETS2 display molecular abnormalities seen in DS, such as elevated expression of APP and an increase in APP protein level (Wolvetang et al., 2003b), suggesting that ETS2 overexpression in DS determines overexpression of APP and that it may play a role in the pathogenesis of the brain abnormalities seen in DS and AD.

DYRK1A. Two transgenic mouse models over expressing DYRK1A have been generated. The first one, carried a human YAC 152F7, containing *Dyrk1A*; the second, carried the full-length *Dyrk1A* cDNA (Altafaj et al., 2001). *DYRK1A* gene is crucial for various brain functions including learning and memory (Smith et al., 1997). Accordingly, both models show a significant impairment in spatial learning and memory, indicating hippocampal and prefrontal cortex functional alteration. In addition, these transgenic mice show increased brain weight and neuronal size, dysfunction of reference memory (Smith et al., 1997, Altafaj et al., 2001), delayed craniocaudal maturation and motor dysfunction (Altafaj et al., 2001). All these alterations are comparable with those found in murine models of DS with trisomy of chromosome 16 and suggest a causative role of DYRK1A in mental retardation and motor anomalies in DS patients.

SIM2 has been shown to be over expressed in Ts1Cje fetuses and in trisomic tissues. Variations of *Sim2* expression level were observed in cortical layers, hippocampus and cerebellum, key brain regions involved in learning and memory (Rachidi et al., 2005). Transgenic mice over expressing *Sim2* (TgSim2) display reduced

sensitivity to pain and mild impairment of learning. These behavioral anomalies resemble some phenotypes observed in Ts65Dn and Ts1Cje mice (Martinez-Cue et al., 1999) and in DS patients.

DSCR1 RNA and protein have been shown to be over expressed in the brain of DS fetuses (Lyle et al., 2004). Transgenic mice over expressing DSCR1 have been shown to exhibit heart malformations (Sanna et al., 2006) but no information is available regarding their neurological phenotypes (Sanna et al., 2006).

THERAPIES FOR DS

DS is a disorder of gene expression, as the basic genetic abnormality is the presence of an additional copy of the 45Mb acrocentric HSA21. The overexpression of protein-coding genes and ncRNAs is likely to disturb several cellular functions and developmental processes. In addition, the supernumerary HSA21 may result in a global disturbance of the transcriptome owing to the additional chromosomal material, regardless of its genic content or regulatory repertoire.

In the 16 years following HSA21 genome sequencing (Hattori et al., 2001) there has been considerable progress in understanding the protein-coding gene content of HSA21, the content of other functional genomic elements and the individual variability of the sequences of HSA21. There has also been substantial progress in understanding the effect of DS on the cellular transcriptome and on cellular differentiation. The use of human induced pluripotent stem cells (iPSCs) and of several mouse models of DS have greatly enhanced our understanding of DS (Antonarakis, 2017). During the past 15 years, numerous efforts have been done in order to find interventions that are able to improve the defects that characterize the trisomic brain. To this purpose, mouse models of DS have been used. A few clinical trials have been also attempted in individuals with DS.

THERAPIES IN THE TS65DN MOUSE MODEL

The Ts65Dn was the first viable mouse model of DS (Davisson et al., 1993, Reeves et al., 1995) and remains the most popular model (Fig.5). We will focus here on therapies attempted in the Ts65Dn mouse, because most of the studies aimed at correcting trisomy-linked brain alterations and learning and memory (L/M) have used this model. Most of these studies were conducted in adult mice, while fewer therapies were explored at earlier life stages (neonatally or prenatally). The interventions attempted so far have been selected based on different rationales, as summarized below.

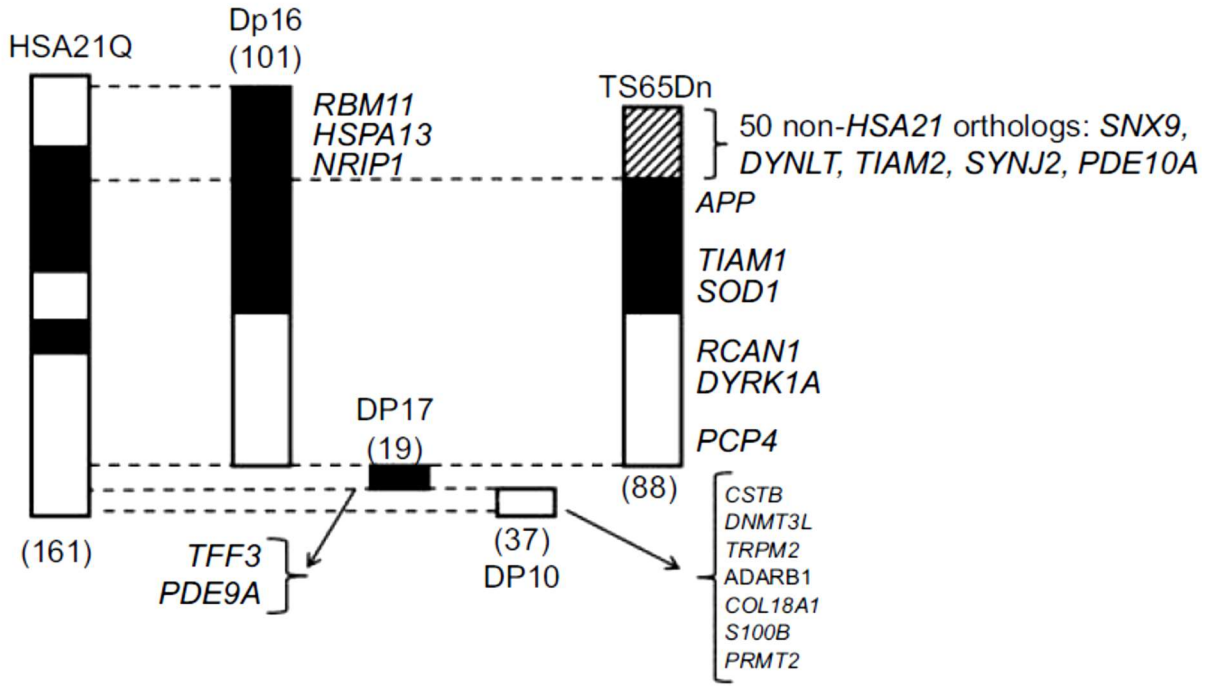


Fig.5. Distribution of HSA21genes on mouse chromosomes 16, 17, and 10. From Gardiner (Gardiner, 2015).

THERAPIES TARGETED TO TRANSMITTER SYSTEMS

Acetylcholinesterase inhibitors

Acethylcoline (Ach) is a key neurotransmitter in the peripheral and central nervous system. Ach is synthesized in cholinergic neurons by the enzyme choline acetyl-transferase and is converted in the inactive form by Acethylcoline esterase (AchE). DS has been associated with abnormalities in peripheral and central cholinergic functions (see (Hart et al., 2017)) and with reduction in the number of cholinergic neurons, which may affect cortical neuronal connectivity and maturation during early development (Becker, 1991).

The use of acetylcholinesterase inhibitors to treat Ts65Dn mice has been motivated by the potential to prevent or reverse the loss of functional markers in the BFCN. In a first study, physostigmine was used to treat male Ts65Dn mice at three different ages: 4 months; 10 months; and 16 months. Mice were tested for the rescue of deficits in the four-arm spontaneous alternation task. Performance in this task is known to deteriorate with age in rodents, and this deterioration can be alleviated in rats with cholinesterase inhibitors. There is evidence that people with DS have impaired olfaction, even at young ages, and that this defect increases with age. In a second study, the effects of galantamine were examined with an olfactory test of L/M. When young Ts65Dn mice (3–6 months of age) were treated for 10 days with galantamine the deficits were completely rescued (see (Gardiner, 2015)).

Choline

The effects of choline supplementation were examined following both prenatal and perinatal treatment. Dams were maintained on an enriched choline diet (4.5-fold higher than standard chow) throughout

pregnancy and until litters were weaned. Offspring were maintained on standard choline diets until testing, at the age of 13–17 months. The Ts65Dn offspring of choline-treated dams exhibited complete L/M rescue in the water radial arm maze (WRAM) test. In addition, treated Ts65Dn mice had a larger number of DCX-positive cells in the dentate gyrus, in comparison with untreated Ts65Dn mice, although their number was still reduced in comparison with euploid mice. Finally, treated Ts65dn mice showed an increase in the levels of ChAT, TrkA and number of BFCN (Moon et al., 2010, Velazquez et al., 2013, Ash et al., 2014).

$GABA_A$ receptor antagonists

Coordinated regulation of LTP and Long-Term Depression (LTD) is considered a cellular model of L/M. An imbalance between excitatory and inhibitory neurotransmission in the Ts65Dn mouse is therefore proposed as a mechanism that contributes to impaired L/M. Based on these observations, the $GABA_A$ receptor (GABAAR) antagonists picrotoxin, bilobalide, and pentylenetetrazole (PTZ) were tested for their efficacy in rescuing the performance of 3-4-month-old Ts65Dn mice in novel object recognition (NOR). Results show that chronic treatment consisting in 2 weeks of daily injection was sufficient for the rescue of learning in the NOR. In the T-maze test, the performance of the spontaneous alternation was normalized after PTZ treatment (see (Gardiner, 2015)). In 4-month-old Ts65Dn mice, four weeks of treatment with PTZ rescued performance in the MWM test, but caused some side effects (Rueda et al., 2008). The proposal of PTZ for clinical trials in DS has been controversial because it is a known convulsant agent. Although seizures in rodents have been seen only at doses significantly higher than those used in the Ts65Dn mouse, children with DS are at increased risk of seizures and therefore could be more sensitive to PTZ. PTZ is not currently approved by US Food and Drug Administration (FDA). GABAAR exhibit considerable heterogeneity in terms of their composition and functional properties because 19 different subunits are assembled as heteropentamers (Deidda et al., 2014). To lessen the probability of negative side effects in people with DS, other more specific GABAAR antagonists are currently being investigated.

$GABA_B$ receptor antagonist

The HSA21 gene, *KCNJ6*, encodes a G protein-coupled inwardly-rectifying potassium channel (GIRK)2, that couples to GABAB receptors. The levels of GIRK2 in Ts65Dn are elevated in the hippocampus, frontal cortex, substantia nigra, and perirhinal cortex (Harashima et al., 2006, Roncace et al., 2017). Elevated GIRK2 levels have been proposed to contribute to abnormal synaptic plasticity in DS. GABAB-induced potassium currents are elevated in the hippocampus of the Ts65Dn mouse, thus suggesting that they likely contribute to the observed imbalance between excitatory and inhibitory neurotransmission. Building on this work, inhibition of the $GABA_B$ receptor was examined using CGP55845, a $GABA_B$ receptor antagonist. CGP55845 was administered to 2-3-month-old Ts65Dn mice for 3 weeks or acutely (2-3 h before testing). CGP55845 rescued memory deficits as assessed with the TM, NOR and CFC behavioral tests and restored LTP in hippocampal slices from Ts65Dn mice (Kleschevnikov et al., 2012).

Memantine

Memantine is an uncompetitive antagonist of the N-methyl-d-aspartate (NMDA) receptor. Memantine has been shown to rescue L/M in mouse models of AD (Minkeviciene et al., 2004) and is currently approved by the US FDA for use in moderate to severe AD. Interestingly, the mechanism of action of memantine is related with proteins encoded by HSA21. The activity of the N-methyl-d-aspartate receptor (NMDAR), and therefore of excitatory neurotransmission, is regulated by the phosphorylation/dephosphorylation of receptor subunits, with the protein phosphatase calcineurin (CaN) playing a major role. There are nine HSA21-encoded proteins, all trisomic in the Ts65Dn mouse, that directly or indirectly interact with the NMDAR. Among these, there is the regulator of CaN, RCAN1. While it was first shown that the elevated expression of RCAN1 inhibited CaN activity, it is now clear that the precise regulation of CaN activity is complex and governed by both the relative proportions of RCAN1 splice variants and the phosphorylation state of RCAN1, which is affected by the HSA21 kinase DYRK1A. RCAN1 activity is further modulated by levels of reactive oxygen species that are affected by the HSA21 proteins superoxide dismutase (SOD1), amyloid precursor protein (APP), and the transcription factor BACH1. Given these many contributions, it is not obvious how NMDARs are affected either during baseline activity or in dynamic responses to stimulation. Nevertheless, LTP, which is dependent on NMDA receptors, is inhibited in the Ts65Dn mouse, where all these HSA21 genes are present in triplicate. Acute injection of memantine has been shown to rescue L/M deficits in CFC in male Ts65Dn at both 4–6 months and 10–14 months of age (Costa et al., 2008). Subsequently, it was shown that 9 weeks of chronic memantine treatment of 9-month-old male Ts65Dn mice partially rescued impaired performance on the MWM (Rueda et al., 2010). The effects of treatment with memantine, however, are no longer present at one week after treatment discontinuation (see (Gardiner, 2015)).

Fluoxetine

Fluoxetine, also known by the trade name Prozac, is an antidepressant belonging to the selective serotonin reuptake inhibitor (SSRI) class. Fluoxetine works by delaying the reuptake of 5- HT through the inhibition of the serotonin transporter. This mechanism allows 5-HT to persist longer in the inter-synaptic cleft and, thus, to enhance its action on postsynaptic neurons.

Studies in rodents show that chronic treatment with fluoxetine, and other antidepressants, stimulates the production of new neurons and their incorporation into functional networks (Malberg et al., 2000). The first study with fluoxetine in Ts65Dn mice examined the effect of treatment in adult male mice (2–5 months of age). Results demonstrated that 2 weeks of fluoxetine injections rescued the reduced proliferation rate of neural progenitors in the dentate gyrus (Clark et al., 2006). A study in neonate Ts65Dn mice showed that neonatal treatment in the postnatal period P3-P15 restored the number of proliferating cells in the dentate gyrus, the total number of granule cells, the dendritic pattern and hippocampal connectivity. Importantly, one month after treatment cessation (at P45) Ts65Dn showed normal learning as assessed with in CFC test (Bianchi et al., 2010b, Guidi et al., 2013, Stagni et al., 2013). In a subsequent study, it has been found that

hippocampal neurogenesis, dendritic pathology and hippocampus-dependent memory remained in their restored state when Ts65Dn mice, which had been neonatally treated with fluoxetine, reached adulthood (3-month-old) (Stagni et al., 2015). In a further study, fluoxetine was administered during pregnancy in order to establish whether prenatal treatment had an impact on overall brain development. Pregnant Ts65Dn female mice were injected daily with fluoxetine from embryonic day 10 to birth and the effects of treatment were examined in the progeny at P2 and at P45. Results showed that in P2 Ts65Dn pups prenatally treated with fluoxetine exhibited restored neurogenesis and cellularity throughout the forebrain (subventricular zone, dentate gyrus, neocortex, striatum, thalamus, hypothalamus), midbrain (mesencephalon) and hindbrain (cerebellum and pons). Moreover, prenatal treatment with fluoxetine had enduring effects on Ts65Dn mice aged 45 days. Indeed, neural precursor proliferation was still restored, the dendritic development of the granule neurons and the number of and pre- and post-synaptic terminals were normalized and there was restoration of cognitive performance in the NOR and CFC tests (Guidi et al., 2014).

THERAPIES WITH NEUROPROTECTIVE AGENTS, ANTIOXIDANTS, FREE RADICAL SCAVENGERS AND IMMUNOSUPPRESSANT

Neuropeptides

Neuropeptides are small proteinaceous substances produced and released by neurons through the regulated secretory route and acting on neural substrates. Two peptide sequences (NAPVSIPQ and SALLRSIPA, abbreviated NAP and SAL, respectively) derived from the neurotrophic factors activity-dependent neuroprotective protein (ADNP) and activity-dependent neurotrophic factor (ADNF) have been shown to protect from Aβ toxicity, Tau hyperphosphorylation, and fetal alcohol exposure. In a first study, 10-month-old Ts65Dn mice were treated with NAP/SAL for 9 days. During days 4-9 mice were subjected to the MWM test and showed rescue of L/M (Incerti et al., 2011). However, after a further 10 days with no additional treatment, treated Ts65Dn mice no longer remembered the platform location (Incerti et al., 2011). In a second study, pregnant Ts65Dn females were injected with NAP/SAL from E8 to E12. At 8-10 months of age the treated offspring were evaluated in the MWM test. Treated Ts65Dn offspring exhibited improvement in learning abilities. Unfortunately, data regarding the probe test that assesses memory are lacking (Incerti et al., 2012).

Estrogen

The choice of estrogen was based on the observation of premature menopause and early onset cognitive decline in women with DS. On this basis, 11–14 month-old female Ts65Dn mice were treated with estrogen for 2 months, and then tested in the discriminating water T-maze. Estrogen significantly improved initial learning, although not to the levels of control mice. Estrogen also reversed the age-related loss of ChAT and NGF, two BFCN functional markers (Granholm et al., 2002). In 6-month-old male Ts65Dn mice treatment with estrogen for 3 weeks failed to rescue the learning deficit in the WRAM (Hunter et al., 2004b). These results suggest sex differences in estrogen responses.

Minocycline

Minocycline is a derivative of tetracycline, an antibiotic compound. In addition to antibiotic properties, minocycline is of interest because it is considered to have neuroprotective effects, modulating the activity of interleukin 1 beta (inflammation) and inhibiting caspases (apoptosis). In 10-month-old male Ts65Dn mice, 3 months of treatment with minocycline improved performance in the WRAM, although not to the levels of controls (see (Gardiner, 2015)) and rescued age-related loss of ChAT in the BFCN (Hunter et al., 2004a).

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is an indole amine mainly synthesized and secreted by the pineal gland. It is well studied for its role in the regulation of circadian rhythms and, in particular, sleep patterns. Recent experiments treating mouse models of AD have indicated that melatonin has many beneficial effects as an antioxidant, an antiapoptotic, and an anti-inflammatory agent that offers protection from neurodegeneration and cognitive deficits (Galano et al., 2011). Melatonin has also been used in clinical trials for AD (Brusco et al., 1998, Wu and Swaab, 2005). A recent study shows that melatonin has a positive impact on cognitive performance in Ts65dn mice. 5-month-old male Ts65Dn mice treated with melatonin for 4 months plus another one-month (5 months in total) during behavioral testing exhibit improved abilities in the MWM test. In addition, melatonin increases the levels of ChAT in BFCNs (Corrales et al., 2013). In subsequent studies the same authors demonstrated that treatment with melatonin rescued impairment of LTP and neurogenesis, reduced oxidative stress and decreased hippocampal senescence in the Ts65Dn model (Corrales et al., 2014, Parisotto et al., 2016).

Cyclosporine A

In 1997, the US Food and Drug Administration (FDA) officially approved cyclosporine A (CSA) for the treatment in patients. CSA is an immunosuppressant and its clinical use is limited by side effects that include nephrotoxicity, neurotoxicity and hepatotoxicity. Recent evidence shows that treatment with CSA restores proliferation, acquisition of a neuronal phenotype, and maturation in cultures of NPCs derived from the SVZ of the lateral ventricle of Ts65Dn mice (Stagni et al., 2019). Experiments *in vivo* showed that Ts65Dn mice treated with CSA in the postnatal period P3-P15 underwent restoration of NPC proliferation in the SVZ and in the subgranular zone of the hippocampal dentate gyrus, total number of hippocampal granule cells and spine density in the dendritic arbor of the granule cells (Stagni et al., 2019).

THERAPIES TARGETED TO PERTURBED SIGNALING PATHWAYS

Lithium

Lithium is a common treatment for bipolar depression since the 1970s. While the molecular mechanisms underlying its effects are not known in detail, it has been shown that lithium acts in part by inhibiting the activity of GSK3β and modulating signaling in the Wnt/β-catenin pathway. In addition, it inhibits the activity of inositol phosphatases. Lithium has been shown to increase adult neurogenesis in the dentate gyrus of rodents (see (Bartesaghi et al., 2011)). A study in 12-month-old female Ts65Dn mice showed that after 1

month of treatment with lithium there was a neurogenesis increase in the SVZ, rostral migratory stream and olfactory bulb and restoration of the size of the pool of precursor cells in the SVZ. These effects were accompanied by restoration of olfactory functions. In contrast, no neurogenesis enhancement was found in the SGZ of the dentate gyrus (Bianchi et al., 2010a, Guidi et al., 2017). A study in younger Ts65Dn mice (~5-month-old) showed that treatment with lithium for 1 month rescued neurogenesis in the dentate gyrus, LTP in the dentate gyrus, and performance in CFC, NOR, and OL but not in the T-maze test (Contestabile et al., 2013).

SAG

Brains from individuals with DS and the Ts65Dn mice have small cerebella and reduced density of cerebellar granule cells. These defects have been attributed to derangement of the Sonic Hedgehog (SHH) pathway, a mitogenic pathway crucially involved in brain development. **SAG 1.1**, is an agonist of smoothened (SMO), a component of the Shh pathway, that relieves the inhibitory effect of PTCH1 (the inhibitor of SHH pathway) on SMO. A single injection of SAG 1.1, has been shown to rescue decreased cell numbers in the granule cell layer of the cerebellum in pups of Ts65Dn mice (Roper et al., 2006a). Importantly, the positive effects of SAG1.1 on cerebellar cellularity remained until 4 months of age. In addition, this treatment restored the MWM test performance and hippocampal LTP but did not improve cerebellar LTD and performance in the Y-test (Das et al., 2013).

DAPT

Elevated levels of the Aβ peptide are present in the DS brain from young ages and are postulated to cause, or at least contribute to, the common occurrence of AD in older individuals with DS. Levels of the parent protein APP and the α- and β-secretase APP products were shown to be elevated in brains of 4-month-old female Ts65Dn mice compared with controls, although neither Aβ accumulation nor plaques are seen in the Ts65Dn mice. FemaleTs65Dn mice injected with a γ-secretase inhibitor, N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-Butyl Ester (DAPT), undergo a decrease in Aβ fragments to the levels seen in control brains. Moreover, this treatment rescued performance in both acquisition and retention in the MWM test (Netzer et al., 2010).

ELND006

ELND006, is a selective inhibitor of the enzyme γ-secretase and thereby reduces the formation of AICD (amyloid precursor protein intracellular domain), a small peptide derived from the processing of APP. Excessive levels of AICD have been shown to up-regulate the transcription of Ptch1 (Patched1), the Shh receptor that keeps the pathway repressed. Treatment with ELND006 has been shown to normalize AICD levels and to restore proliferation in cultures of NPCs derived from the SVZ of the Ts65Dn mouse (Giacomini et al., 2015). Experiments *in vivo* showed that Ts65Dn mice treated with ELND006 in the postnatal period P3-P15 underwent restoration of neurogenesis in the SVZ and hippocampal dentate gyrus, restoration of granule cell number, synapse development and reduction in the expression levels of various genes that are

transcriptionally regulated by AICD (Giacomini et al., 2015). At one month after treatment cessation, the pool of proliferating cells in the hippocampal dentate gyrus and total number of granule neurons were still restored as was the number of pre- and postsynaptic terminals in the stratum lucidum of CA3, indicating that this treatment has long-lasting effects (Stagni et al., 2017b).

THERAPIES WITH NATURAL COMPOUNDS

Epigallocatechin-3-gallate (EGCG)

EGCG is a flavonoid present in green tea extracts. In a review of the activity of commercially available protein kinase inhibitors against a panel of 28 kinases, EGCG was shown to be highly specific for two of these kinases – the HSA21-encoded kinase, DYRK1A, and the MAPKAPK5 kinase (aka PRAK), inhibiting each by ~90% (Bain et al., 2003). EGCG also inhibited ERK2, ROCK2, and PDK1, although to a lesser extent (~50%). The triplicated gene DYRK1A is thought to be an important contributor to the neurological phenotype of DS. Thus, its inhibition by EGCG provides a potential means for the rescue of the phenotypic features of DS. The effects of EGCG in Ts65Dn mice, initially studied at the electrophysiological levels, demonstrated normalization of LTP at the Schaffer collateral-CA1 synapse in hippocampal slices exposed to EGCG (Xie et al., 2008). It was subsequently demonstrated that 3-month-old male Ts65Dn mice treated with EGCG for 30 days underwent an improvement in their performance in the MWM test to the level of euploid mice (De la Torre et al., 2014). However, another study demonstrated that pure EGCG administered to Ts65Dn mice from weaning for either three or 7 weeks did not improve performance in a battery of behavioral tasks (Stringer et al., 2015). A subsequent study demonstrated that treatment with EGCG from P3 to P15 restored neurogenesis, total hippocampal granule cell number and levels of pre- and post-synaptic proteins in the hippocampus and neocortex of Ts65Dn mice. At one month after treatment cessation, however, these effects were no longer present and there was no improvement in hippocampus dependent memory and learning, indicating that this treatment does not have long-lasting effects (Stagni et al., 2016). A recent study has explored the effects of EGCG in combination with environmental enrichment for 30 days in 1-2-month-old female Ts65Dn mice. Co-treatment restored cortico-hippocampal-dependent L/M (MWM, NOR tests), rescued dendritic spine density in field CA1 and normalized the proportion of excitatory and inhibitory synaptic markers in field CA1 and dentate gyrus (Catuara-Solarz et al., 2016). No information is available regarding the duration of these effects. A study in NPCs isolated from the hippocampus of Ts65Dn mice confirms that exposure to EGCG exerts pro-proliferative effects. Interestingly, this effect was accompanied by restoration of mitochondrial biogenesis, suggesting that the positive effects of EGCG on the brain may also be mediated by an antioxidant action (Valenti et al., 2016).

7,8-dihydroxyflavone (7,8-DHF)

The brain-derived neurotrophic factor (BDNF) is a neurotrophin that plays a key role in brain development by specifically binding to tropomyosin-related kinase receptor B (TrkB). BDNF administration is impracticable because BDNF does not cross the blood-brain barrier. The 7,8-dihydroxyflavone (7,8-DHF), a natural flavone

present in several plants, is a mimetic of BDNF that is able to cross the blood-brain barrier and bind with high affinity and specificity the TrkB receptor (Liu et al., 2010, Liu et al., 2013). It has been shown that administration of 7,8-DHF enhances the activation of the TRKB receptor and increases spine density in several brain regions (Zeng et al., 2012), promotes neurogenesis in the dentate gyrus (Liu et al., 2010), and fosters neurite outgrowth (Tsai et al., 2013) in various animal disease models (Liu et al., 2016). A recent study in Ts65Dn mice shows that neonatal treatment with 7,8-DHF from P3 to P15 increases the number of NPCs in the dentate gyrus and restores granule cell number and spine density (Stagni et al., 2017a). Importantly, treatment with 7,8-DHF from P3 to adolescence (P50) is able to restore the L/M defects of Ts65Dn mice, without causing adverse effects (Stagni et al., 2017a). In 4-5-month-old male Ts65Dn mice, a combination of aerobic exercise and treatment with 7,8-DHF for 4 weeks rescues synaptic plasticity and memory deficits (Parrini et al., 2017). In contrast Ts65Dn mice treated with 7,8-DHF for about 40 days starting from 4 months of age did not show any improvement in L/M (Giacomini et al., 2019). This discrepancy may be due to the lower dose used in the study by Giacomini et al. in comparison with the dose used in the study by Parrini et al.

Luteolin

Luteolin (3,4,5,7-tetrahydroxyflavone), a natural flavonoid, is widely distributed in different types of plants. It has antioxidant, anti-inflammatory, and other pharmacological effects. Moreover, Luteolin appears to protect dopaminergic neurons from Lipopolysaccharide (LPS)-induced injury, perhaps by inhibiting microglial activation (Chen et al., 2008). Luteolin is clinically used to treat tumors, hepatitis, hypertension, and amyotrophic lateral sclerosis (Lin et al., 2008, Jiang et al., 2013). A recent study examined the effects of injections of luteolin (10 mg/kg/day) for 4 weeks in 12-weeks-old Ts65Dn mice. The results showed that Luteolin enhanced the proliferation of neurons in the hippocampal DG, increased the number of DCX+ cells in the granular layer and NeuN+ cells in the subgranular region of the DG and increased the protein levels of BDNF and p-ERK1/2 in the hippocampus. Moreover, luteolin improved L/M abilities in the MWM test as well as the ability in the NOR test (Zhou et al., 2019).

Corn Oil

There is evidence that in addition to their role in metabolism, fatty acids or derivatives can serve as signaling molecules by affecting intra- and extracellular receptor systems (Georgiadi and Kersten, 2012). Linoleic acid (LA) has been shown to have profound effects on the proliferation of different stem cell types (Kang et al., 2014), including neural stem cells (Beltz et al., 2007, Tokuda et al., 2014). Oleic acid (OA) which is the primary fatty acid in the white matter of the mammalian brain (O'Brien and Sampson, 1965), has been shown to promote axonogenesis in the striatum during brain development (Polo-Hernandez et al., 2010) and to favor dendritic differentiation (Rodriguez-Rodriguez et al., 2004). Corn oil, which is extracted from the germ of corn, contains a high percentage of both LA and OA. In a recent study, four-month-old Ts65Dn and euploid mice were treated with corn oil for 30 days. In the MWM test Ts65Dn treated with corn oil showed a large

improvement in hippocampus-dependent L/M. In addition, treated Ts65Dn mice showed an increase in neurogenesis and dendritogenesis in the dentate gyrus and the number of new granule cells and their dendritic pattern became similar to those of euploid mice (Giacomini et al., 2018).

CLINICAL TRIALS

The development of clinical trials for people with DS has progressed from being primarily focused on AD to targeting pediatric populations and investigating early pharmacological interventions. Clinical trials in individuals with DS were based on condition-specific aspects of neurobiology, neurochemistry, and neuroplasticity or connectivity within the brain (Lott, 2012, Esbensen et al., 2017). These studies began in the 1960s and continued throughout the 1980s with trials investigating vitamins and supplements (Table 2). These early studies were often based on anecdotal case reports with no clear mechanistic rationale and were typically small, single-center trials, sometimes open-label, making it difficult to draw valid conclusions regarding efficacy. Beginning in the 1990s and into the 2000s, pre-clinical research using the Ts65Dn mouse model and other translational research made it possible to target molecular mechanisms in the brain in order to address the cognitive and functional deficits associated with the trisomic condition (Hart et al., 2017).

Before 1989	1990s	2000s	2010s	Ongoing
Vitamins and supplements	Cognition			
Pituitary extract (Berg, Kerman, Stern, & Mittwoch, 1961)	Donepezil (Kishnani et al., 1999)	Piracetam (Lobaugh et al., 2001)	Folinic acid (Blehaut et al., 2010)	Folinic acid and thyroid hormone (NCT01576705, Jerome Lejeune Inst.)
Niacin (Heaton-Ward, 1962)		Donepezil (Heller et al., 2004)	Donepezil (Kishnani et al., 2010)	PTZ (COMPOSE study, Balance therapeutics)
U series vitamin (Bumbalo, 1964)		Thyroxine (van Trotsenburg et al., 2005)	Rivastigmine (Heller et al., 2010)	Memantine (NCT02304302, University Hospitals Cleveland Medical Center)
Vitamin B6, 5-hydroxytryptophan (Pueschel, Reed, Cronk, & Goldstein, 1980)		Rivastigmine (Heller, Spiridigliozzi, Crissman, Sullivan, Eells, et al., 2006)	Donepezil (Kondoh et al., 2011)	Intranasal glulisine (NCT02432716, HealthPartners Institute)
Megavitamins and thyroid hormone (Harrell et al.,1981)		Vitamins, minerals and folinic acid (Ellis et al., 2008)	Memantine (Boada et al., 2012)	ACI-24 (NCT02738450, AC Immune)
Vitamins and minerals (Bennett, McClelland, Kriegsmann, Andrus, & Sells, 1983)		Donepezil (Kishnani et al., 2009)	EGCG (De la Torre et al., 2014)	
Vitamins and minerals (Weathers, 1983)			Rivastigmine (Spiridigliozzi et al., 2016)	
Megavitamins (Smith, Spiker, Peterson, Cicchetti, & Justine, 1984)			EGCG and cognitive training (de la Torre et al., 2016)	
Vasopressin (Eisenberg, Hamburger-Bar, & Belmaker, 1984)			ELND005 (Rafii et al., 2017)	
Vitamin B6 (Coleman et al., 1985)			Basmisanil (NCT01436955, Hoffmann-La Roche)	
Thiamine (Lonsdale & Kissling, 1986)			Basmisanil (NCT02024789, Hoffmann-La Roche)	
Vitamins and minerals (Bidder, Gray, Newcombe, Evans, & Hughes, 1989)			Basmisanil NCT02484703, Hoffmann-La Roche)	
			Donepezil (NCT02094053, Eisai Inc.)	
	Dementia in DS			
			Antioxidants (Lott et al., 2011)	Nicotine (NCT01778946, Vanderbilt)
			Memantine (Hanney et al., 2012)	
			Vitamin E (NCT01594346/NCT00056329, New York State Institute for Basic Research)	

EGCG, epigallocatechin gallate; PTZ, pentylenetetrazole.

Table 2. History of clinical trial in Down syndrome (image from (Hart et al., 2017)).

Anticholinesterase therapy

Acetylcholine (Ach) is a neurotransmitter that has an important role in memory, attention, reasoning, and language. Ach is synthesized in cholinergic neurons by the enzyme choline acetyl-transferase and is converted in the inactive form by Acetylcholine esterase (AchE). In DS, Ach has been associated with abnormalities in peripheral and central cholinergic functions (see (Hart et al., 2017)) with reduction in the number of cholinergic neurons, which may effect cortical neuronal connectivity and maturation during early development (Becker, 1991).

Donepezil is a reversible inhibitor of acetylcholinesterase, which is thought to maintain levels of acetylcholine, and has been reported to have some benefits for people with AD in the general population. A study headed by a research group at Duke University Medical Center, examined the efficacy and tolerability of donepezil in young adults (18–35 years of age) with DS. In this first large-scale, multicenter trial of a

pharmacological agent for DS, donepezil appeared safe but did not demonstrate any efficacy on improving scores associated with the primary or secondary measures (Kishnani et al., 2009). The same research group also reported findings from the first multicenter, randomized, clinical trial to measure the efficacy and tolerability of donepezil hydrochloride in children and adolescents (10–17 years) with DS (Kishnani et al., 2010). This trial also failed to demonstrate any benefit for donepezil versus placebo in children with DS, although donepezil again appeared to be well tolerated. A recent review (Livingstone et al., 2015) concluded that in adults there was no difference in cognitive functioning or behavior between individuals with DS treated with donepezil and placebo, although the probability to undergo an adverse event was significantly higher for subjects with DS on donepezil (see (Hart et al., 2017)).

Rivastigmine is an inhibitor of AchE and butyrylcholinesterase (BChE), approved for the treatment of mild to moderate AD and dementia due to Parkinson's disease. Rivastigmine has been shown to have benefit on the cognitive, functional and behavioral problems commonly associated with AD (Finkel, 2004)) and Parkinson's disease dementias (Emre et al., 2004). A recent randomized double-blind, placebo-controlled trial of rivastigmine in children and adolescents with DS suggested potential improvement in a subset of participants for expressive language, but overall there were no significant effects on adaptive function, executive function and language or memory measures (Spiridigliozzi et al., 2016).

Piracetam therapy

Piracetam is a member of the class of drugs known as nootropics, which are generally thought to enhance cognitive function by influencing vascular and neuronal functions in instances of brain dysfunction (Winblad, 2005). A Phase II study placebo-controlled, 2-period crossover (Lobaugh et al., 2001) in children with DS (ages 6–13) evaluated the effect of piracetam on a range of cognitive functions, including attention, memory, and learning. The study concluded that piracetam therapy did not significantly improve cognitive performance and in 7 out of the 18 children who completed the study treatment was associated with adverse events of the central nervous system (Lobaugh et al., 2001).

Memantine therapy

Memantine is a low-affinity uncompetitive antagonist of glutamatergic NMDA receptors, This drug, which is approved for treatment of moderate-to-severe AD, was tested in different clinical trials in individuals with DS (Boada et al., 2012, Hanney et al., 2012). In a pilot clinical trial study, the main aim was to assess whether a relatively short drug regimen of memantine could be efficacious in improving at least one subdomain of cognition (i.e. hippocampus-dependent tasks) in adults with DS. The authors of this study compared the effects of 16-weeks of treatment with either memantine or placebo on cognitive and adaptive functions of 40 young adults (aged 18-32 years) with DS. Although no significant differences were observed between the memantine and placebo groups on the two primary measures, a significant improvement was seen in hippocampus-dependent function (Boada et al., 2012). In a study in adults with DS over age 40, a 1-year treatment with memantine (at a dose of 10 mg/day) caused no improvements in primary or secondary

measures of cognition or adaptive function (Hanney et al., 2012). Memantine was well tolerated, with only infrequent and mild adverse events noted. A randomized, double-blind, placebo-controlled phase II trial in young adults with DS aged 15–32 is trying to assess whether a 16-week treatment with memantine will have an effect on L/M (see (Hart et al., 2017)).

$GABA_A$ receptor antagonist therapy

Excessive GABA-mediated neurotransmission has been proposed as one of the underlying causes of the cognitive deficits in Ts65Dn mice (Belichenko et al., 2004, Fernandez et al., 2007). In line with this idea, a pre-clinical study in Ts65Dn mice demonstrated that treatment with a $GABA_A$ antagonist improves L/M (Fernandez et al., 2007).

Pentylenetetrazole (PTZ) is a $GABA_A$ antagonist that was previously approved by the FDA for treatment of various cognitive impairments. PTZ is currently under investigation for cognitive enhancement in individuals with DS. A placebo-controlled study with PTZ up to 12 weeks in adolescents and young adults (age 13–35) with DS has shown a pro-cognitive effect in the domains of language, executive function, and adaptive behavior. Although the study has completed enrollment and follow-up assessments, results have not been published yet (see (Hart et al., 2017)).

Basmisanil (Hoffmann-La Roche Pharmaceuticals) is a selective $GABA_A$ α5 negative allosteric modulator (NAM). Two multi-center, phase II, randomized, double-blind, placebo-controlled studies have tested the effect of basmisanil (26-weeks of treatment) on cognition in DS in adolescents/adults (age 12–30 years) and a pediatric population (age 6-11 years). Unpublished results from the CLEMATIS study showed that basmisanil was not associated with a significant impact on cognition or adaptive behavior in young adults and adolescents with DS, leading to early discontinuation of the study in the pediatric population (see (Hart et al., 2017)).

Therapy targeting Aβ

ELND005 is scyllo-inositol that is thought to neutralize Aβ oligomers and prevent them from aggregating. ELND005 has been proposed as therapy in individuals with DS because it may prevent the accumulation of plaques contributing to AD-like pathology and improve working memory and cognitive functions by regulating brain myo-inositol levels. A clinical trial with ELND005 in the general population with AD did not demonstrate significant effects on cognition or adaptive function (see (Hart et al., 2017)). A phase II clinical study in young adults with DS and without dementia has shown that ELND005 was safe and well-tolerated and that there were no serious adverse effects (Rafii et al., 2017). Results of the clinical study revealed improvements in neuropsychiatric symptoms at baseline, improvements in 7 of 8 subjects receiving 250 mg twice daily of ELND005. There were, however, no significant overall treatment group-related trends on cognitive or behavioral measures.

Antioxidant therapy

Mitochondria are recognized as the most susceptible target of ROS formation and somatic mitochondrial DNA (mtDNA) control region (CR) mutations occur in demented individuals with DS as well as those with AD in the general population (Lott et al., 2011). A clinical trial with antioxidants was designed to test the hypothesis that a combination of antioxidants with a mitochondrial cofactor could lead to either stabilization or improvement in cognitive functioning for demented adults with DS. This 2-year randomized, double-blind, placebo-controlled trial assessed whether daily oral antioxidant supplementation of alpha-tocopherol, ascorbic acid, and alpha-lipoic acid was effective, safe, and tolerable for 53 individuals with DS and AD-like dementia (mean age of approximately 50 years). However, the study determined that the antioxidant supplement was ineffective as a treatment for dementia in individuals with DS and AD (Lott et al., 2011). A similar lack of efficacy was noted in a study on 156 infants (under 7 months) with DS treated with antioxidants and folinic acid (0.1 mg/day) (Ellis et al., 2008). Integration began in 4 months old infants and this study examined the participants with the Griffiths developmental quotient and an adapted MacArthur communicative development inventory at 18 months after starting supplementation. Both clinical trials provided no evidence to support the use of **antioxidant or folinic acid** supplements in DS (see (Hart et al., 2017)). In contrast to these data, a double-blind, placebo-controlled study in a pediatric DS population (3–30 month-old infants) has found a significant improvement in the psychomotor development for infants taking 1 ± 0.3 mg/kg folinic acid daily in comparison with the placebo group (Blehaut et al., 2010). This effect was larger in a sub-analysis of subjects taking concomitantly thyroid hormone. Folinic acid and thyroid hormone are currently investigated in combination in a 4-arm, placebo-controlled trial for improvement of psychomotor development in young children with DS, ages 6–18 months (see (Hart et al., 2017)).

Green tea extract therapy

EGCG, is a compound found in green tea leaves, and a well-known DYRK1A inhibitor. EGCG has been investigated in preclinical studies and human clinical trials for DS. De la Torre and colleagues tested the effects of green tea extracts containing EGCG in a randomized, placebo-controlled pilot study. The aims of the trial were to investigate the clinical benefits and safety of EGCG administration for 3 months in young adults with DS. This pilot study showed an improvement in memory recognition, working memory and quality of life (De la Torre et al., 2014). The same group has completed a Phase II clinical study in a larger group of young adults with DS. Results show that a combination of EGCG and cognitive training for 12 months had positive effects on visual recognition memory, inhibitory control, and adaptive behavior (de la Torre et al., 2016). A phase III clinical trials with a larger population of individuals with DS will be needed to assess and confirm the long-term efficacy of EGCG and cognitive training (see (Hart et al., 2017)).

BACKGROUND OF THE CURRENT STUDY: DRUG REPURPOSING FOR DS

Drug repurposing is a strategy for identifying new uses for approved or investigational drugs that are outside the scope of the original medical indication. This strategy offers various advantages over developing an entirely new drug for a given indication. First, and perhaps most importantly, the risk of failure is lower because the repurposed drug has already been found to be sufficiently safe in preclinical models and humans, if early-stage trials have been completed. Thus, it is less likely to fail at least from a safety point of view in subsequent efficacy trials. Second, the time frame for drug development can be reduced, because most of the preclinical testing, safety assessment and, in some cases, formulation development have already been completed. Third, less investment is needed, although this will vary greatly depending on the stage and process of development of the repurposing candidate. Historically, drug repurposing has been largely opportunistic and serendipitous; once a drug was found to have an off-target effect or a newly recognized on-target effect, it was taken forward for commercial exploitation (Pushpakom et al., 2018).

DRUGS SCREENING CAMPAIGN IN NEURONAL PROGENITOR CELLS FROM THE TS65DN MOUSE

In the framework of an international project funded by Assicurazioni e Fondazione Generali spa ("New avenues for the rescue of intellectual disability in Down syndrome") and lead by Prof. Renata Bartesaghi (Dipartimento di Scienze Biomediche e Neuromotorie, Alma Mater Studiorum-Università di Bologna, Italy) a drug screening campaign was carried out by one of the partners (Prof.ssa Mariagrazia Grilli, University of Novara, Italy). Two chemical libraries of FDA approved drugs, the Prestwick library composed by 1,120 compounds and the ENZO library composed by 775 compounds were used in order to identify new drugs (hits) which could increase the neurogenic potential of trisomic neural precursor cells and their differentiation. The general idea was to test then the more promising hits *in vivo*, in the Ts65Dn mouse. Since all the molecules of the library are approved drugs, the one/s that will result effective in the DS mouse model might be readily tested in clinical trials in individuals with DS. Among chemical structures that increased NPC proliferation and resulted as or even more effective than controls (lithium and noradrenaline) the screening identified: 1) some immunosuppressant drugs; 2) several catecholaminergic drugs; 3) glucocorticoid drugs (GC). The results of the drug screening campaign have been presented at the 3rd Meeting of T21RS, Barcelona, June 6-10, 2019 (Salvalai, 2019). The finding that catecholaminergic drugs increase the proliferation rate in trisomic cells appears particularly interesting because literature data point to the importance of noradrenergic deficits and/or failure of β-adrenergic signaling in DS and DS-associated cognitive deficits. Among the catecholaminergic drugs identified as neurogenesis enhancers in the drug screening campaign on trisomic cells various β2 adrenergic receptor agonists emerged as very effective. In particular, clenbuterol hydrochloride (CL) and salmeterol xinafoate (SALM) had the largest effects on the proliferation rate of

trisomic NPCs. It is important to note that these two compounds are used as medication in children and adults for the treatment of asthma. Thus, given that these compounds are effective in the Ts65Dn mouse, results may be readily transferred in clinical trials in children with DS. Based on all these considerations, in the current study we have examined the effects of CL and SALM on hippocampal development in Ts65Dn pups.

BRIEF OUTLINE OF THE NORADRENERGIC SYSTEM

ANATOMY

The noradrenergic (NA) system has various functions throughout the central and peripheral nervous system. It plays a dominant role in the regulation of the circulation, not only during emergencies but in the activities of daily living such as standing up, exercise, adjustments to meal ingestion, and thermoregulation. Cardiac noradrenergic stimulation increases the force and rate of heart contraction and increases the rate of spontaneous depolarization. Sympathetic noradrenergic nerves enmesh blood vessels-especially arterioles-throughout the body (Goldstein, 2013). The NA system has been posited to be involved in stress responding (Ma and Morilak, 2004, Arnsten and Li, 2005, Kvetnansky et al., 2009), arousal (Aston-Jones et al., 2001), signal detection (Berridge and Waterhouse, 2003), decision making (Aston-Jones and Cohen, 2005), memory retrieval (Bouret and Sara, 2005), learning (Harley, 1987), psychomotor and cognitive activation ((Schildkraut, 1965), adaptation and trophic processes (Feeney et al., 1993), depression (Karolewicz et al., 2005), and behavioral inhibition and non-reward (Murrough et al., 2000).

The Locus coeruleus (LC) is the principal site for brain synthesis of noradrenaline (Sara et al., 1994, Kitchigina et al., 1997). The LC is formed by a group of neurons located in the brainstem within close proximity to the fourth ventricle (Aston-Jones et al., 1994). Although the LC contains a relatively small number of neurons (<50,000 in humans) it exerts immense influence over forebrain circuits, as a result of extensive collateralization and divergence. LC neurons receive inputs from different parts of brain: medulla oblongata (Aston-Jones et al., 1986), prefrontal and cingulate cortices, amygdala, posterior hypothalamus, and spinal cord (Arnsten and Goldman-Rakic, 1984). The LC neurons, in turn, project to many brain regions including the thalamus, frontal and entorhinal cortices, basolateral amygdala, and hippocampus (Loughlin et al., 1986) (Fig.6).

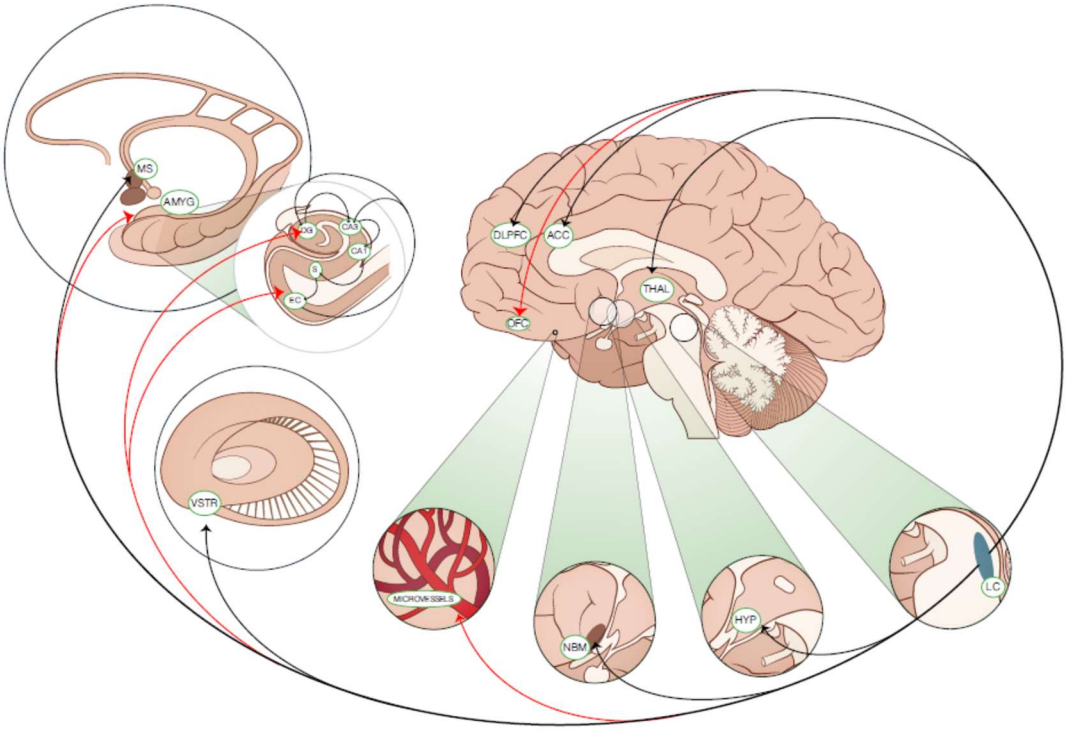


Fig.6. Schematic representation of major ascending noradrenergic (Na-ergic) neurons originating in the locus coeruleus (LC). From (Phillips et al., 2016).

The number of neurons of the LC steadily declines with aging until central NA levels are depleted by 50% by the 5th decade of life (see (Phillips et al., 2016)). This reduction is problematic because abnormalities of the NA system have been associated with a number of conditions in the CNS including neurodegenerative disorders such as Huntington's and Parkinson's disease, AD, and DS (see (Phillips et al., 2016)). The role that LC degeneration and reduced levels of NA play in clinical manifestation of various disorders remains unknown. In AD condition, it has been shown that NA neurons of the LC undergo degeneration (Zarow et al., 2003). Indeed, post-mortem studies reveal an approximately 60% reduction in LC neurons in persons with AD as compared with controls (German et al., 1992). Similarly to AD, lower levels of NA have been detected in cortical and subcortical regions of post-mortem brain samples of individuals with DS (Yates et al., 1981, Godridge et al., 1987).

OUTLINE OF THE ADRENERIGIC RECEPTORS

The noradrenergic receptors are of two major classes, named α and β. The α class comprises the α1 and α2 subgroups and the β class comprises the β1, β2 and β3 subgroups. The α and β receptors are widely distributed in the body, where they play a role in a number of functions. Table 3 summarizes the distribution of the α and β receptors in various body organs.

Receptor Type	Tissue Distribution	Mechanism of Action	Agonist Potency	Physiological Effects	Agonist	Antagonist
α1	Vascular Smooth Muscles, Visceral smooth Muscles	Gq-protein coupled activates Phospholipase C, IP3+DAG	Epi ≥ NE >> Iso	Smooth muscle contractions, Gluconeogenesis, Vasoconstriction	Norepinephrine, Phenylephrine, Methoxamine	Doxazosin, Phentolamine, Prazosin
α2	Pre-synaptic terminals, pancreas, platelets, Ciliary epithelium, Salivary Glands	Gi-protein coupled inhibits Adenyl cyclase	Epi ≥ NE >>Iso	Inhibits release of Neurotransmitter	Clonidine, Monoxidine	Yohimbine, Idazoxan, Tolazoline
β1	Heart, Kidney, some pre-synaptic terminals	Gs-protein coupled activates Adenyl cyclase +PKA	Iso > Epi ≥ NE	Increase heart rate and Renin secretion	Isoproterenol, Norepinephrine, Dobutamine	Propranolol, Metoprolol, Atenolol
β2	Visceral smooth muscles, Bronchioles, Liver, Skeletal Muscles	Gs-protein coupled activates Adenyl cyclase +PKA, Ca-channels	Iso > Epi >> NE	Vasodilation, Bronchodilation, Inhibits insulin secretion	Isoproterenol, Salbutamol, Salmeterol, Albuterol, Formoterol, Terbutaline, Levalbuterol	Propranolol, ICI-118,551, Nadolol, Butoxamine
β3	Adipose Tissue	Gs-protein coupled activates Adenyl cyclase +PKA	Iso = NE > Epi	Increase lipolysis	Isoproterenol, Amibegron, Solabegron	SR59230A

NE: Norepinephrine, **Epi:** Epinephrine and **Iso:** Isoproterenol

Table 3. Distribution of the α- and β-receptors in various body organs. From (Flood, 2018)

B ADRENERIGIC RECEPTORS

Since in the current study we have tested the effects of two β2-receptor agonists on hippocampal development, we will focus here on the β-adrenergic receptors, with particular emphasis on the β2-receptor.

Structure

The human β-adrenoceptor gene is situated on the long arm of chromosome 5 and codes for an intronless gene product of approximately 1,200 base pairs (Kobilka et al., 1987). The β-AR is composed of 413 amino-acid residues of approximately 46,500 Daltons (Johnson, 2006). β-AR have been subdivided into at least three distinct groups: β1, β2, and β3, classically identified in cardiac, airway smooth muscle, and adipose tissue, respectively (Frielle et al., 1988). There is a 65% to 70% homology between β1, β2, and β3-receptors. Like all G protein-coupled receptors, the β2-receptor has 7 transmembrane-spanning a-helices. There are three extracellular loops, with one being the amino-terminus, and three intracellular loops, with a carboxy-terminus. The receptor is N-glycosylated at amino acids 6, 15, and 187; these are important for insertion into the cell membrane and for agonist-induced receptor trafficking (Johnson, 2006).

Distribution

The β-AR are widely distributed in human body organs and regulate physiologic functions such as bronchodilation, vasodilation, glycogenolysis in the liver, and relaxation of uterine and bladder muscles. The human β-AR are widely expressed not only in airway smooth muscles, but also in a wide variety of cells such as skeletal muscle cells, cardiac cells, epithelial cells, endothelial cells, brain cells, and immune cells including mast cells, macrophages, eosinophils and microglial cells (Johnson, 1998, Peterson et al., 2014, Flood, 2018). Position emission tomography (PET) has now made possible the noninvasive quantification of β-AR *in vivo* using the radioligand (IIC) CGP12177 (Ueki et al., 1993). Serial measurements have shown pulmonary β-AR

density to be 10.9 ± 1.0 picomole (pmol)/g tissue, compared with 8.8 ± 2.3 pmol/g for cardiac tissue (Ueki et al., 1993). There was no difference in β-AR density between normal subjects and patients with asthma (see (Johnson, 1998)), but an inverse relationship was reported between FEV1 (% predicted) and lung β-AR density (see (Johnson, 1998)). Radioligand binding studies on lobectomy specimens have shown β2-receptor density to increase with increasing airway generation, with high levels in the alveolar region (Spina et al., 1989).

Signaling Cascades

Distinct subtypes of β-AR exist and have important pharmacological consequences. β1-AR predominate in the heart and the cerebral cortex, whereas β2-AR predominate in the lung and cerebellum. However, in many cases, β1- and β2-AR coexist in the same tissue, sometimes mediating the same physiological effect. The brain contains both β1-and β2-AR, which cannot be differentiated in terms of their physiological functions (Michael J Kuhar, 1999). Ligand binding to β1- or β2-AR leads to activation of guanine nucleotide-binding regulatory Gs-proteins. This, in turn, activates adenylate cyclase (AC), followed by an increase of intracellular cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A (PKA) ((Seeds and Gilman, 1971, Maguire et al., 1977, Strulovici et al., 1984, Hausdorff et al., 1989). The coupling of the β-AR to adenylate cyclase is mediated by a trimeric Gs protein, which consists of α, β, and γ subunits (Robison et al., 1967). There is evidence that β-AR exist in two forms, activated and inactivated, and that under resting conditions these two forms are in equilibrium but with the inactivated state being predominant (Onaran et al., 1993). The β2-AR is in the activated form when it is associated with a subunit of the G protein, together with a molecule of guanosine triphosphate (GTP), and it is through this subunit that the receptor is coupled to adenylate cyclase. The replacement of the GTP by guanosine diphosphate (GDP) both catalyzes the conversion of ATP to cAMP by the enzyme and dramatically reduces the affinity of a subunit for the receptor, causing dissociation and the receptor to return to its low-energy, inactivated form (Onaran et al., 1993). Depending on which receptor (β1- or β2-AR) is activated by ligands, different effects are observed based on the activation of downstream cascades (Fig.7).

In addition to the activation of Gs-proteins, ligand binding to β2-AR can also activate extracellular signal-regulated kinases (ERK), MAPKs, Akt, and tyrosine kinase transactivation, a pathway mediated by Gi/Go proteins. ERK/MAPK kinase protein is a key step in the activation of the cAMP response element-binding protein (CREB) that mediates the transcription of many proteins (Fig.7) (Abramson et al., 1988, Xiao et al., 1995, Daaka et al., 1997, Maudsley et al., 2000, Zhu et al., 2001, Ahmed and Frey, 2005).

There is evidence that β2-AR–activated MAPK phosphorylates the glucocorticoid receptor (GR), leading to a priming event, rendering the receptor more sensitive to steroid-dependent activation. β2-AR activation also increases the translocation of the GR from the cell cytosol to the nucleus, a fundamental step in the anti-inflammatory activity of corticosteroids. These mechanisms provide a molecular basis for synergy between the β2-receptor and the GR. (see (Johnson, 2006)).

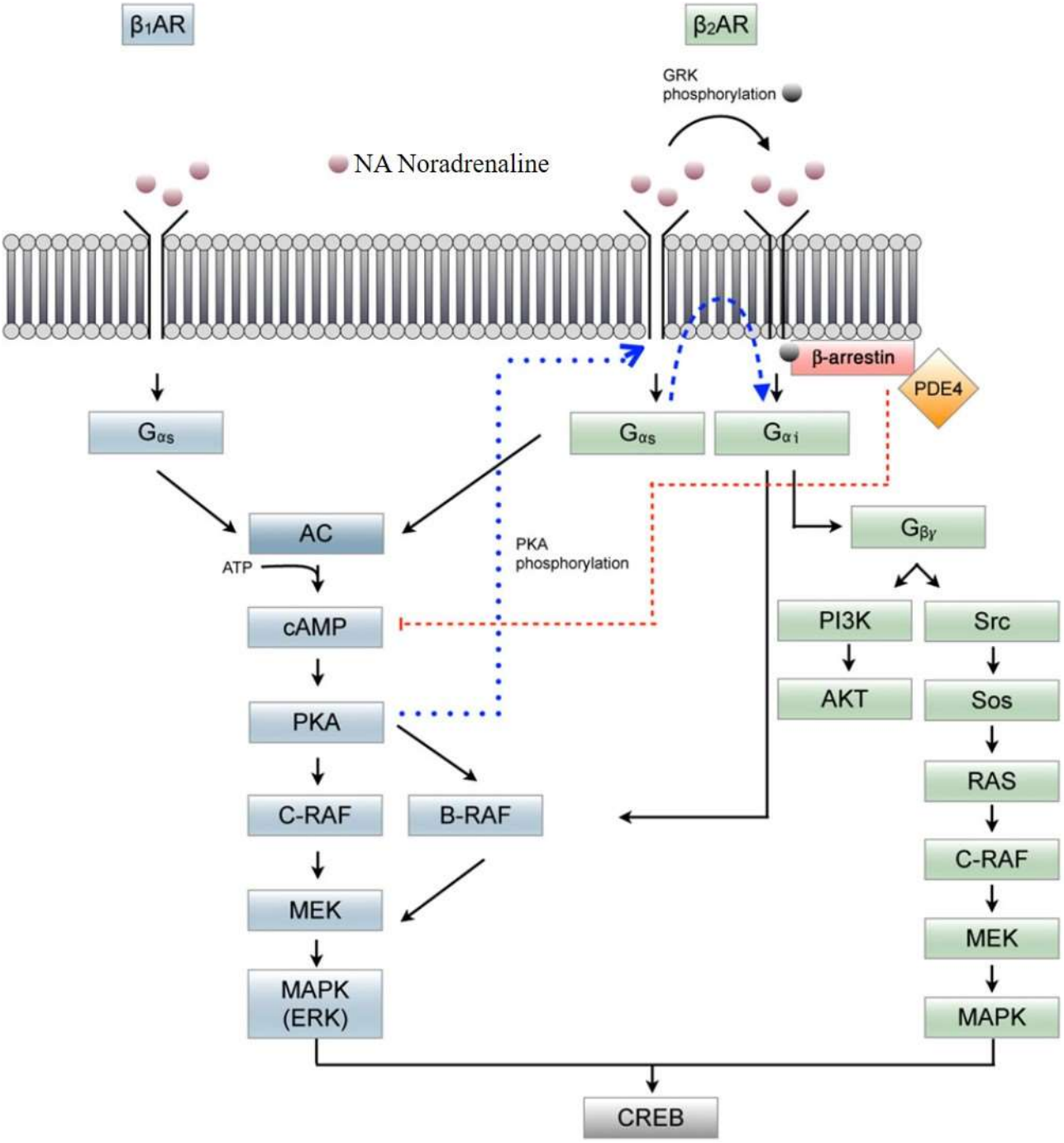


Fig.7 Summary of the different signaling pathways that respond to activation of β1AR (blue) and β2AR (green). From (Hagena et al., 2016).

The β2-AR have been shown to interact with members of the arrestin protein family. The β-arrestins not only participate in attenuation of β2-AR signaling and receptor internalization, but also function as signal transducers, connecting β2-ARs to multiple signaling pathways, such as p38 and ERK MAPKs and NF-κB. Additionally, it was suggested that β-arrestins have a nuclear function linked to the regulation of transcription (Lefkowitz et al., 2006, Ma and Pei, 2007). The β2-AR activation induces a suppressive effect on nuclear factor

kappa-light-chain-enhancer of activated B cells (NF-κβ) and on nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), the inhibitor of NF-κβ (Safi et al., 2018). The transcription factor NF-kB is a master regulator of immunity and inflammation (Hayden and Ghosh, 2012). NF-kB deregulation is associated with a variety of human diseases, including chronic inflammatory diseases and cancer (Baker et al., 2011, Hayden and Ghosh, 2011, DiDonato et al., 2012). It has been shown (Wang et al., 2009) that fenoterol, a selective β2-AR agonist, causes translocation of β -arrestin-2 to the cell membrane in THP-1 monocytes. β -arrestin-2, via a non-defined mechanism, subsequently mediates redistribution of TLR4/CD14 complexes in the cell membrane and downregulates plasma membrane display of CD14 costimulatory molecules, hampering NF-κB-dependent gene expression. These findings suggest β2-AR activation can disrupt NF-κB activity already at an early step in the TLR4 signaling cascade.

The autoregulatory process of receptor desensitization is associated with β2-AR activation. This process operates as a safety device to prevent overstimulation of receptors in the face of excessive β2-agonist exposure. Desensitization occurs in response to the association of the receptor with the agonist molecule. The mechanisms consist in three main processes: (i) uncoupling of the receptors from adenylate cyclase; (ii) internalization of uncoupled receptors; and (iii) phosphorylation of internalized receptors. The extent of desensitization depends on the degree and duration of the β2-adrenoceptor/ β2-agonist response (Johnson, 2006). The process of desensitization can differ markedly from cell to cell and tissue to tissue.

B RECEPTORS IN THE BRAIN AND HIPPOCAMPUS

A quantitative autoradiography with [125I] iodopindolol examined β2-AR in post-mortem human brain samples. Results showed that the concentration of β-receptors was highest in all subfields of the hippocampus, followed by cerebellum, and then thalamic nuclei, basal ganglia, midbrain, and cerebral cortex. Low levels were found in the white matter and hypothalamus (Reznikoff et al., 1986).

Regarding the hippocampus, both β1 and β2-AR receptors are expressed in the pyramidal cells in hippocampal fields CA1, CA3 and dentate gyrus (Booze et al., 1993, Milner et al., 2000, Guo and Li, 2007, Cox et al., 2008), although relatively low levels appear to be expressed in CA3 (Booze et al., 1993). In the CA1 and CA3 regions, β1- and β2-AR are preferentially expressed in neurons as opposed to astrocytes. In the rat, β2-AR in the CA1 and CA3 regions have been shown in the membranes, cytoplasm and nucleus, whereas β1-AR are expressed only in membranes and cytoplasm (Guo and Li, 2007). In the dentate gyrus, β-AR are distributed within the granule cells at postsynaptic sites (Milner et al., 2000). β1 and β2-AR receptors are also expressed by hippocampal interneurons. Interneurons that express β1-AR are more prevalent in the CA1 and CA3 regions than in the dentate gyrus, where they are relatively sparse. Interneurons that express β2-AR are more uniformly distributed in the different regions of the hippocampus (Cox et al., 2008). In the hippocampus, β3-AR are almost absent, with the exception of a subset of neural precursor cells in the subgranular zone of the dentate gyrus (Jhaveri et al., 2010).

In the hippocampus, β-AR appear to play a central role in LTP and LTD - two cellular processes underlying memory - by driving the direction of changes in synaptic strength (Lemon et al., 2009, Hansen and Manahan-Vaughan, 2015) and grading the persistency of synaptic plasticity (Kemp and Manahan-Vaughan, 2008, Hagena and Manahan-Vaughan, 2012, Hansen and Manahan-Vaughan, 2015). In the dentate gyrus, application of a β-AR agonist or NA causes LTP of the population spike (Chaulk and Harley, 1998, Swanson-Park et al., 1999, Lethbridge et al., 2014). LTP of both the population spike and dendritic excitatory postsynaptic potential resulting from NA application has been found in the dentate gyrus in experiments *in vitro* (Stanton and Sejnowski, 1989). In experiments *in vivo*, LTP in the dentate gyrus appears to be reinforced by β-AR agonists or NA application (Hansen and Manahan-Vaughan, 2015). This effect of β-AR in the dentate gyrus can be attributed to the fact that noradrenergic innervation originating from the LC is very dense in this region and the NA content may be higher compared with the CA1 and CA3 regions (Loy et al., 1980). *In vivo*, application of a β-AR agonist in the dentate gyrus can induce LTD (Lethbridge et al., 2014), but at higher agonist concentrations LTP occurs, suggesting that the degree of β-AR activation may be decisive for the type of synaptic plasticity induced. The different hippocampal subfields are thought to play specific roles in the encoding of different aspects of memory, such as working memory (Nakao et al., 2002, Bikbaev et al., 2008, Kesner and Warthen, 2010), pattern completion and separation (Leutgeb and Leutgeb, 2007, Kesner and Warthen, 2010, Hagena and Manahan-Vaughan, 2011, Neunuebel and Knierim, 2014), mismatch detection (Lee et al., 2005, Kumaran and Maguire, 2007, Duncan et al., 2012), and holistic completion of spatial representation (Kemp and Manahan-Vaughan, 2007). In view of their role in LTP and LTD, the hippocampal β-AR appear to be key players in the regulation of the content and persistency of synaptic information and memory.

B2 ADRENERGIC AGONIST (SABA; LABA; ULTRA LABAS)

The first pharmacological use of β agonists took place around 5000 years ago in Chinese medicine when the ephedrine-containing plant ma huang was used to alleviate respiratory conditions (see (Billington et al., 2017)). In the twentieth century, further research and enlightenment led to increased use of β agonists in respiratory disease, particularly following the introduction of the first pure (but nonselective between β1-AR and β2-AR) β agonist, isoprenaline, in the 1940s. The first β2-AR-selective agonist, salbutamol, was synthesized in 1968. In addition to reduction of the side effects associated with the non-selective β agonist isoprenaline, it was also superior in terms of duration of the effect (see (Billington et al., 2017)). Today the β agonists constitute the frontline treatment for both asthma and Chronic obstructive pulmonary disease (COPD). They exert their bronchodilatory effects via β2-AR located on airway smooth muscle (ASM) cells. In addition to the receptors expressed on ASM cells, β2AR are also found on a number of other cell types within the lungs including epithelial cells, submucosal glands, vascular endothelium, vascular smooth muscle and inflammatory cells including mast cells, macrophages and eosinophils (Barnes, 2004).

The β agonists are grouped into three classes: Short-Acting β Agonists (SABAs), Long-Acting β Agonists (LABAs) and Ultra-Long Acting β Agonists (ultra-LABAs). As suggested by the names SABAs have short half-lives and are used as rapid relievers whereas LABAs and ultra-LABAs provide sustained symptomatic relief due to their longer duration of action (see (Billington et al., 2017)).

The **SABAs**, have a bronchoprotective effect that is evident in minutes and remains for 4-6 hours. They are used via metered dose or dry powder inhalers, provide almost instant symptomatic relief and are the frontline therapy in asthma to combat bronchoconstriction and acute exacerbations.

The **LABAs** have a bronchodilatory effect for up to 12 hours. The prolonged duration of action of the LABAs currently used in clinical practice is not thought to be due to a difference in receptor kinetics but rather retention within the cell membrane and hence a continued presence of the drug near the receptor (see (Billington et al., 2017)). LABAs are a frontline treatment for COPD. Unfortunately, monotherapy with a LABA in asthma is contraindicated. This is due both to LABA monotherapy proving less clinically effective but mainly due to the adverse effects. Although the reasons for such adverse effects are multiple, with some still unknown, the majority of adverse effects can be attributed to either: i) a lack of selectivity for the β2-AR, resulting in "off-target" effects mediated by either alpha or β1 adrenoceptors; or ii) ill-defined β2-AR-mediated effects that appear to involve either β2-AR desensitization or exacerbation of airway inflammation and its consequences (see (Billington et al., 2017)).

The **ultra-LABAs** produce an effect for 12h and more. The ultra-LABAs were given approval by the European Medicines Agency (EMA) in 2009 and by the FDA in 2011 for the maintenance treatment of patients with COPD. The ultra-LABAs have not yet been approved for use in the treatment of asthma. However, clinical trials to ascertain their suitability for asthma therapy are ongoing.

β-agonists have a long history of "side" effects. In fact, chronic or high-dose exposure to β2-AR agonists may have pro-inflammatory effects. Experiments *in vitro* have shown that β2-AR agonists can enhance the Th2 inflammatory pathway by inhibiting interleukin (IL)-12 and interferon (IFN)-γ (Agarwal and Marshall, 2000). *In vivo*, pretreatment with β2-AR agonists increases the severity of the late asthmatic reaction (Lai et al., 1989) and continuous treatment is associated with an increase in sputum eosinophils (Lazarus et al., 2001).

THE B2 AGONISTS USED IN THE CURRENT STUDY

CLENBUTEROL

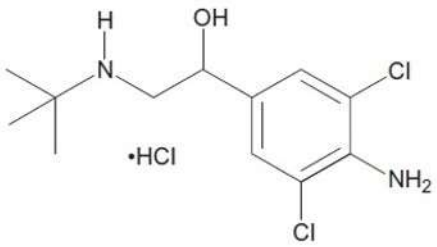


Fig.8. Structure of clenbuterol hydrochloride. From (Al-Majed et al., 2017).

Clenbuterol (CL) (Fig.8) is a small (molecular weight (MW) 277.20) long-acting β-agonist (LABA). It was patented in 1967 and came into medical use in 1977. CL is a bronchodilator used in the management of reversible airways obstruction, in asthma and in certain patients with chronic obstructive pulmonary disease. ***Actions.*** CL is a direct-acting sympathomimetic agent with mainly β-adrenergic activity and a selective action on β2-AR (β2 agonist). It is clinically used as a bronchodilator in the management of reversible airways obstruction, as in asthma and in certain patients with COPD. In patients with asthma, as required β agonist therapy is preferable to regular use. An increased need for, or decreased duration of effect of CL indicates deterioration of asthma control and the need for review of therapy. The most important action of CL and other β2-agonists in the lung is relaxation of airway smooth muscle. For this reason, such drugs are widely used for relief of bronchospasm in human asthma and similar diseases in animals. When these drugs bind to β2-AR, they activate adenylyl cyclase, which leads to an increase in the intracellular concentration of the second messenger cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA). In the tracheobronchial tree and in the uterus, β2-agonists, cAMP, and PKA inhibit smooth muscle contraction by opening K+ channels and by downregulation of myosin light-chain kinase activity (see (Al-Majed et al., 2017)). CL has been used illicitly in animal feeds (especially bovine) in an attempt to promote weight gain and to increase muscle to lipid mass. CL has been abused by sportsmen for its anabolic effects, although it is doubtful as to whether it enhances performance. Myocardial infarction was described in an otherwise healthy 17-year-old bodybuilder after abuse of CL. Coronary artery spasm and temporary thrombosis were suggested as possible explanations for this adverse effect (see (Al-Majed et al., 2017)). β2-AR agonists used in the treatment of respiratory disorders have also been shown to have neuroprotective properties, by reducing apoptosis induced by the excitotoxin kainic acid (Semkova et al., 1996, Gleeson et al., 2010). In rats, pre-treatment with CL (0.5 mg/kg i.p.) attenuates expression of the proinflammatory molecules IL-1b, IFN-γ, iNOS and ameliorates apoptosis in the kainic (10 mg/kg) model of excitotoxicity. These effects are accompanied

by increases in BDNF and NGF expression in the hippocampus (Gleeson et al., 2010). CL (0.5 mg/kg i.p.) administration to rats suppresses Nf-kB activity and ameliorates expression of the Nf-kB inducible genes TNF-α and ICAM-1 in response to bacterial lipopolysaccharide (LPS), while concurrently elevating the temporal expression of the Nf-kB inhibitory protein IkBα (Ryan et al., 2013). The β2-AR stimulation is thought to drive an anti-inflammatory phenotype in the CNS that may be of benefit in conditions where inflammation contributes to neuropathology (Griffin et al., 2018). In APP/PS1 mice intraperitoneal injection of 2.0 mg/kg of clenbuterol enhances hippocampal neurogenesis, increases dendritic branching, the density of dendritic spines, and increases the levels of the synaptic proteins PSD-95, synapsin 1 and synaptophysin. Furthermore, clenbuterol decreases cerebral amyloid plaques by decreasing APP phosphorylation at Thr668 (Chai et al., 2016).

Pharmacokinetics and metabolism. CL is able to penetrate into the brain through the blood brain barrier (BBB) in different species (e.g. mouse, rat, horses and primates). In rats, autoradiography demonstrated high levels of radioactivity in the placenta with lower levels in the fetuses after 30 min administration. The bioavailability of this compound is very high (more than 80%) (see web site link1). The pharmacokinetics of CL therapeutic doses (20, 40, and 80 μg/man) following oral administration of CL hydrochloride was determined in healthy volunteers. The plasma levels of CL reached maximum values of 0.1, 0.2, and 0.35 ng/mL, respectively, in a dose-dependent manner within 2.5 h, which lasted for over 6 h after administration. The half-life of CL in plasma was estimated to be about 35 h (Yamamoto et al., 1985). The cumulative urinary excretion of unchanged compound corresponded to about 20% of the administered dose as measured at 72 h following a single oral administration. In rats, after oral administration of CL hydrochloride at a dose of 2 μg/kg, the plasma level of CL reached their maximum value at about 1 h after administration. In rabbits, the plasma concentrations of CL reached the maximum values of about 0.2 and 0.8 ng/mL within 2 h following administration of CL hydrochloride at doses of 0.5 and 2 μg/kg, respectively. The half-life of CL in plasma was about 30 h in rats and about 9 h in rabbits (see (Al-Majed et al., 2017)). The metabolic processing of CL remains unknown in the main target species (e.g. bovine), and only limited data have been published concerning its biotransformation in laboratory animals. The metabolic pathway leading to the formation of CL hydroxylamine prevails at high dosages. CL hydroxylamine (but not 4-nitroclenbuterol) was also formed extensively when the drug was incubated with rat liver microsomal fractions in aerobic conditions. It is concluded that oxido-reduction during urine preparation have previously impaired the identification of this toxicologically important CL metabolic route (Zalko et al., 1997). Excretion of CL is mainly via the kidneys for intravenous administration, and biotransformation for oral administration. No accumulation has been observed (Couet et al., 1989). When administered at the recommended dose of 0.8 mg/kg per 12 h, CL reaches plasma levels that should relax airway smooth muscle. Urinary elimination is prolonged and irregular (see (Al-Majed et al., 2017)).

Toxicity and side effects. CL was moderately toxic in mice and rats after oral administration, the lethal dose 50 (LD50 values) was in the range of 80-175 mg/kg bw. Intravenous administration causes more toxicity with LD50 values in the range of 30-85 mg/kg bw. The main signs of toxicity include lethargy, tachycardia in rats, dogs, cats and a variety of farm animals, accompanied by reductions in systolic, diastolic blood pressure and tonic-clonic convulsions after oral administration. In mice, it causes decreases in spontaneous activity and also leads to increases in barbiturate-induced sleeping time.

No evidence of carcinogenicity was noted in a two-year oral study in mice with doses of up to 25 mg/kg bw per day. In a rats, a reproductive toxicity study was carried out using oral doses of 1-50 mg/kg bw per day from 10 weeks prior to mating in males and two weeks prior to mating in females. Although CL showed no side effects on fertility, doses of 50 mg/kg bw per day resulted in the deaths of pups soon after birth. Unfortunately, the mechanism involved in this lethal effect is unknown. In teratogenicity studies in rats, oral doses of 10 and 100 mg/kg bw per day produced teratogenic effects that included hydrocephalus, anasarca, umbilical hernia, anophthalmia, rib variations and splintering of vertebrae. These effects were accompanied by signs of maternal toxicity. In humans, 10 µg (0.167 µg/kg bw) of CL given by the inhalation route induced no signs of tachycardia as determined by ECG. In patients with cardiac arrhythmia this dose of CL produced no changes attributable to the drug. Furthermore, more than 100 patients that received CL at the doses of 20-60 µg/day (0.3-1.0 µg/kg bw per day) for 1 year showed no adverse effects except for slight tremor and occasional, mild tachycardia.

SALMETEROL

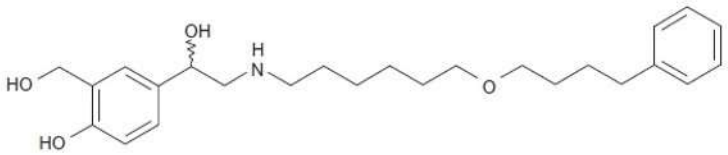


Fig.9. Structure of Salmeterol xinafoate. From (Anwar et al., 2015).

Salmeterol (SALM) (Fig.9) is a small (MW 415.57) long-acting β-agonist (LABA) that was patented in 1983 and came into medical use in 1990 for the treatment of asthma and chronic obstructive pulmonary disease (Johnson et al., 1993, Johnson, 2001). The principal action of SALM, like other β2-agonists, is to relax airway smooth muscle by stimulating β2 adrenergic receptors (see (Anwar et al., 2015)).

Actions. SALM showed positive effects in asthma (Shrewsbury et al., 2000), providing improvements in bronchodilator efficacy and patient outcomes compared with short-acting β-agonist (SABA), which have duration of action of 4–6 h only (Boyd et al., 1997). The principal action of SALM, like other β2 agonists, is to relax airway smooth muscle by stimulating β2-AR. This increases the intracellular messenger cyclic AMP that

is responsible for the control of smooth muscle tone (Johnson, 1998). Thus, activation of the β2-AR results directly in bronchodilation. Initial improvement in asthma control may occur within 30 min following oral inhalation of SALM and the maximum benefit may not be achieved for 1 week or longer after initiating treatment with SALM (see (Anwar et al., 2015)). Clinically important improvements are maintained for up to 12 h in most patients receiving SALM oral inhalation powder. In the prevention of exercise-induced bronchospasm in adolescents and adults, SALM oral inhalation powder provides protection for up to 9 h and up to 12 h in children 4–11 years of age (see (Anwar et al., 2015)). Pretreatment with SALM in human monocytic cell line THP-1 and murine macrophage cell line RAW264 significantly inhibited production of proinflammatory mediators. SALM downregulated PgLPS-mediated phosphorylation of the ERK1/2 and c-Jun but not p38 mitogen-activated protein-kinases (MAPKs). SALM also attenuated activation of NF-kB via inhibition of nuclear translocation ofp65-NFkB, the transcriptional activity of NF-kB and IkBa phosphorylation (Sharma et al., 2017).

Pharmacokinetics and metabolism. SALM is able to cross the BBB in trace amounts (Fitzpatrick et al., 1990, Manchee et al., 1993), but there is no information whether the drug and/or its metabolites cross the placenta. Analysis in rats showed a presence of this compound in the milk, but there is no information regarding its distribution into human milk. (see (Anwar et al., 2015)). SALM is only partially metabolized in humans and the swallowed fraction contributes to 28–36% of the total systemic bioavailability (Clearie et al., 2010). It has a half-life of 5.5 h after oral administration (see (Anwar et al., 2015)). The bronchodilatory effect of SALM lasts for more than 12 hours. As the effect begins slowly and reaches its maximum within 2–3 hours after a single dose, it is not suitable for the treatment of acute asthma attack. (Kirjavainen et al., 2018). SALM is 94 to 98% bound to human plasma proteins, both albumin and α1-acid glycoprotein. Inhalation of SALM at therapeutic doses produces low plasma concentrations that are detectable for only a short period of time immediately after administration. This makes a full characterization of SALM pharmacokinetic profile at therapeutic doses difficult. In addition, the therapeutic doses of SALM are associated with minimal systemic pharmacodynamic effects (Kempsford et al., 2005). Plasma SALM concentrations of 0.1 to 0.2 and 1 to 2 µg/L were attained in healthy volunteers about 5 to 15 minutes after inhalation of single doses of 50 and 400 µg, respectively. In patients who inhaled SALM 50 µg twice a day for 10 months, a second C_{max} of 0.07 to 0.2 µg/L occurred 45 to 90 minutes after inhalation, probably due to gastrointestinal absorption of swallowed drug. Oral administration of 1 mg of radiolabeled [14C] salmeterol (as salmeterol xinafoate) to two healthy individuals gave peak plasma SALM concentrations of about 650 µg/L at about 45 minutes (see (Cazzola et al., 2002). The dose-normalized C_{max} values in comparative animal studies suggest that the systemic absorption and bioavailability in humans is greater (about 15 times greater) then that in rodents (Brogden and Faulds, 1991). It is eliminated in the feces (60%) and urine (25%), principally as metabolites (see (Anwar et al., 2015)).

Toxicity. Inhaled SALM, like other β-ARs agonist drugs, can produce dose-related cardiovascular effects and effects on blood glucose and/or serum potassium. The cardiovascular effects (heart rate, blood pressure) associated with SALM inhalation aerosol occur with similar frequency, and are of similar type and severity, as those noted following albuterol administration. No tumors were seen after treatment with SALM 0.2 mg/kg in children and adults. Moreover, no effects on fertility were identified in rats treated with SALM at oral doses up to 2 mg/kg.

RATIONALE AND GOAL OF STUDY

A major challenge in drug screening is the possibility that molecules that are effective *in vitro* do not replicate the same effects *in vivo*. In a drug screening campaign, we found that two β2-AR agonists, Clenbuterol and Salmeterol, were able to increase the neurogenesis defects that characterize trisomic neural precursor cells. Based on these premises, the general goal of the current work was to establish whether these two hits – untested so far in DS - are able to improve/restore the major developmental defects of the trisomic brain. To this purpose, we have exploited the Ts65Dn mouse, a widely used model of DS because it recapitulates numerous aspects of the human pathology. Since drugs that are able to increase neurogenesis may also foster neural maturation, the specific goals of the current study were to establish whether neonatal treatment with these β2 agonists is able to increase neurogenesis and neuronal maturation in the hippocampus, a brain region that largely develops in the early postnatal period in rodents.

SECTION 1: EFFECTS OF NEONATAL TREATMENT WITH CLENBUTEROL

MATERIALS AND METHODS SECTION 1

COLONY

Female Ts65Dn mice (strain 1924) carrying a partial trisomy of chromosome 16 (Reeves et al., 1995) were obtained from Jackson Laboratories (Bar Harbor, 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). A total of 126 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 light/dark cycle. Experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the use of experimental animals and were approved by Italian Ministry of Public Health. 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=67) and Ts65Dn (n=26) mice, received a daily subcutaneous injection (at 9-10 am) of different doses of clenbuterol (0.01; 0.5; 1.0; and 2.0 mg/kg) in 0.9% NaCl solution from postnatal day 3 (P3) to P15 (Fig.1). Age-matched euploid (n=22) and Ts65Dn (n=11) mice were injected with saline (hereafter referred to as "untreated mice"). Table 1 summarizes the number and sex of mice of the different experimental groups.

	Male	Female	Total
Eu Sal	16	6	22
Ts65Dn Sal	6	5	11
Eu + CL 0.01 mg/kg	6	3	9
Ts65Dn + CL 0.01 mg/kg	6	2	8
Eu + CL 0.5 mg/kg	11	7	18
Ts65Dn + CL 0.5 mg/kg	4	3	7
Eu + CL 1.0 mg/kg	11	2	13
Ts65Dn + CL 1.0 mg/kg	2	1	3
Eu + CL 2.0 mg/kg	15	12	27
Ts65Dn + CL 2.0 mg/kg	4	4	8

Table 1. The table reports the number of male and female mice used in this study. Abbreviations: CL, Clenbuterol; Sal, Saline.

Mice were killed at the age of 15 days (P15 mice). Mice received a subcutaneous injection of BrdU (5-bromo-2-deoxyuridine; Sigma; 150 μg/g body weight) in TrisHCl 50 mM 2h before being killed, in order to label proliferating cells (Fig.1). The brains were excised and cut along the midline. The left hemispheres of all groups of mice were fixed by immersion in PFA 4% and frozen, and the right hemispheres were immersed in a Golgi impregnation solution. The body weight of mice of all group was recorded prior to sacrifice. The number of animals used for each experimental procedure is specified in the figure legends.

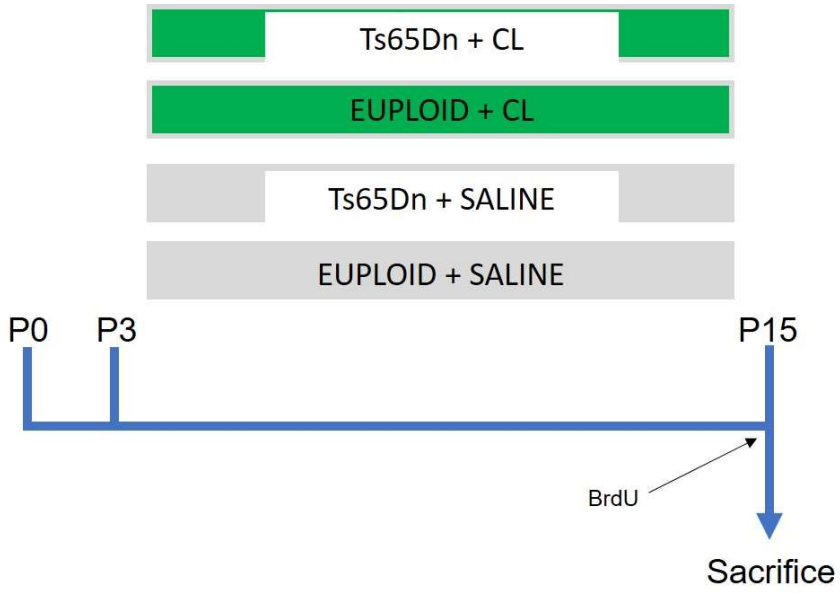


Fig. 1. Experimental protocol.
Euploid and Ts65Dn mice received a daily injection of either saline (EUPLOID + SALINE; Ts65Dn + SALINE) or Clenbuterol dissolved in saline 0.01, 0.5, 1.0 and 2.0 mg/kg (EUPLOID + CL; Ts65Dn + CL) from P3 to P15. At P15, mice received one injection of BrdU, and were killed after 2 h in order to evaluate the number of cells in the S-phase of the cell cycle. The brains of these mice were used for immunohistochemistry and Golgi-staining. Abbreviations: BrdU, bromodeoxyuridine; CL: clenbuterol; P: postnatal day.

HISTOLOGICAL PROCEDURES

The left hemispheres were cut with a freezing microtome into 30-µm-thick coronal sections that were serially collected in anti-freezing solution (30% glycerol; 30% ethylen-glycol; 10% PBS 10×; sodium azide 0.02%; MilliQ to volume) and used for Hoechst staining and BrdU immunohistochemistry. The right hemispheres were used for Golgi staining, as described below.

HOECHST-STAINING

One out of six free-floating sections taken from the beginning to the end of the hippocampal formation (n=15–19 sections) were incubated for 2 min in Hoechst nuclear dye (2 mg/ml in PBS) and mounted on slides.

BrdU IMMUNOHISTOCHEMISTRY

Fluorescence immunohistochemistry was carried out as previously described (Contestabile et al., 2007, Bianchi et al., 2010b, Guidi et al., 2013, Giacomini et al., 2015). One out of six free-floating sections (n = 15–19 sections) from the beginning to the end of the hippocampal formation was incubated with a rat anti-BrdU antibody (diluted 1:200; Biorad) and detection was performed with a Cy3-conjugated anti rat-secondary antibody (diluted 1:200; Jackson Immunoresearch). Sections were then mounted on slides.

GOLGI STAINING

The right hemispheres were Golgi stained using the FD Rapid Golgi Stain™Kit (FDNeuro Technologies, Inc.). Brains were immersed in the impregnation solution containing mercuric chloride, potassium dichromate and potassium chromate (the impregnation solution was prepared by mixing equal volumes of Solutions A and B

of the FD Rapid Golgi Stain™Kit) and stored at room temperature in the dark for 2 weeks. Then, brains were transferred into Solution C (FD Rapid Golgi Stain™Kit) and stored at room temperature in the dark for at least 72 h. After these steps, hemispheres were cut with a microtome into 90-µm-thick coronal sections that were mounted on gelatin-coated slides and were air dried at room temperature in the dark for at least one day. After drying, sections were rinsed with distilled water and subsequently stained in a developing solution (FD Rapid Golgi Stain Kit).

MEASUREMENTS

Number of BrdU-positive cells

Immunofluorescence images were taken with a Nikon Eclipse TE2000-S inverted microscope (Nikon Corp., Kawasaki, Japan; objective: 20x, 0.5 NA), equipped with a Nikon digital camera DS-Qi2. Quantification of BrdU-labeled nuclei was conducted in every 6th section using a modified unbiased stereology protocol that has previously been reported to successfully quantify BrdU labeling (Malberg et al., 2000, Kempermann and Gage, 2002, Tozuka et al., 2005). In each section, all BrdU-labeled cells located in the subgranular zone and granule cell layer of the dentate gyrus were counted along the entire z axis (1 µm steps). To avoid oversampling errors, nuclei intersecting the uppermost focal plane were excluded. The total number of BrdU-labeled cells per dentate gyrus was estimated by multiplying by six (the section sampling frequency: ssf) the sum of the cells counted in all sampled sections.

Stereology of the dentate gyrus

Unbiased stereology was performed on Hoechst-stained sections. The optical dissector method was used to obtain granule cell density, and the Cavalieri principle was used to estimate volume of the dentate gyrus, as previously described (Stagni et al., 2017a).

Spine density

In Golgi-stained sections, the spines of the granule cells of the dentate gyrus were counted using a 100× oil immersion objective lens (1.4 NA). Spine density values were evaluated in dendritic segments located in the inner (proximal dendrites) and outer (distal dendrites) half of the molecular layer. For each neuron, 3 proximal and 3 distal segments were analyzed. For each animal, spines were counted in at least 4/5 neurons. The length of each sampled dendritic segment was determined by tracing its profile and the number of spines was counted manually. The linear spine density was calculated by dividing the total number of spines by the length of the dendritic segment. Spine density was expressed as number of spines per 20 µm dendrite.

Measurement of the dendritic tree

For dendritic reconstruction, the following system was used: (i) light microscope (Leitz, Hexagon Metrology GmbH, Wetzlar, Germany, Europe) equipped with a motorized stage and focus control system; (ii) color digital video camera attached to the microscope; and (iii) dedicated software, custom-designed for dendritic reconstruction (Immagini Computer, Milan, Italy). This software was interfaced with Image Pro Plus (Media

Cybernetics, Silver Spring, MD 20910, USA), with the Stage Pro module for controlling the motorized stage in the x, y and z directions. The dendritic tree of Golgi-stained granule cells was traced live, at a final magnification of 500x, by focusing into the depth of the section. The operator starts with branches emerging from the cell soma and after having drawn the first parent branch goes on with all daughter branches of the next order in a centrifugal direction (Fig. 2). At the end of tracing, the program reconstructs the number and length of individual branches, the mean length of branches of each order, and total dendritic length.

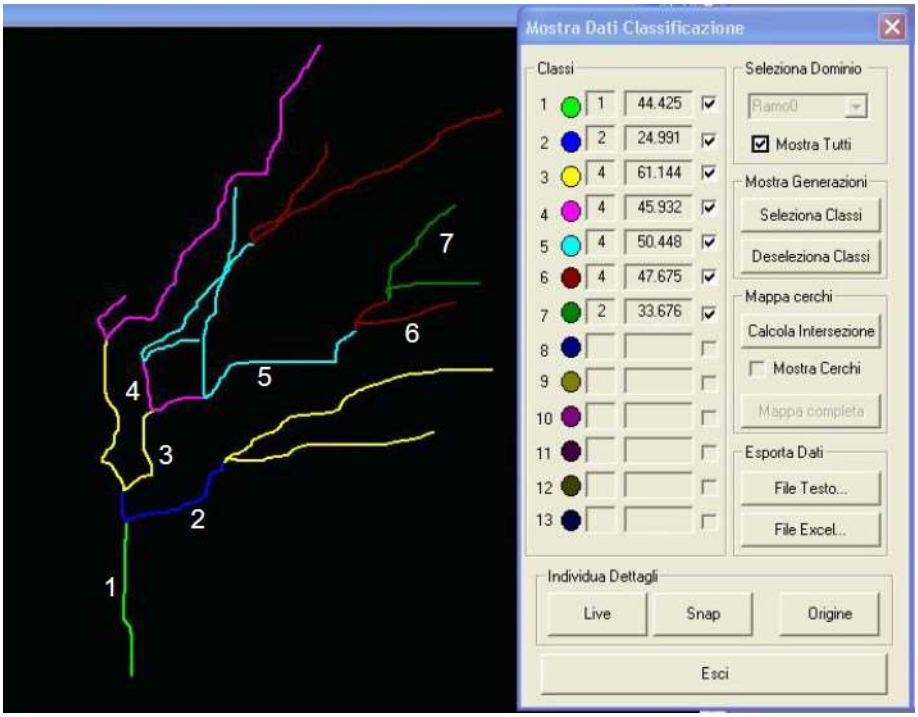


Fig. 2. Computerized reconstruction of granule cell dendrites.
Dendrites were traced in a centrifugal direction. Numbers indicate the different dendritic orders (marked by the software with different colors).

STATISTICAL ANALYSIS

Results are presented as mean ± standard error of the mean (SE). Data were analyzed with the IBM SPSS 22.0 software. Before running statistical analyses, we checked data distribution and homogeneity of variances for each variable using the Shapiro-Wilk test and Levene's test, respectively. If the data were normally distributed statistical analysis was carried out using either a one-way ANOVA or a two-way ANOVA with genotype (euploid, Ts65Dn) and treatment (saline, CL), as factors. Post hoc multiple comparisons were then carried out using Fisher's least significant difference (LSD) test. If the data were not normally distributed, transformations were made to achieve normality. If the transformed data did not achieve normality, statistical analysis was carried out using the Kruskal-Wallis test followed by the Mann–Whitney U test. Based on the "Box plot" tool available in SPSS Descriptive Statistics, in each analysis we excluded the extremes, i.e., values that were larger than 3 times the IQ range [$x \geq Q3 + 3 * (IQ)$; $x \leq Q1 - 3*(IQ)$].

RESULTS SECTION 1

EFFECT OF NEONATAL TREATMENT WITH CLENBUTEROL ON NEURAL PRECURSOR PROLIFERATION IN THE DENTATE GYRUS OF TS65DN MICE

The early postnatal period is a critical time window for neurogenesis in the hippocampal dentate gyrus, a region that is fundamental for long-term declarative memory. In the current study we examined the impact of CL on the proliferation rate of neural progenitor cells (NPCs) in the dentate gyrus of euploid and Ts65Dn mice. We have used here the same timing protocol as in previous studies in which we tested the effects of various pharmacological agents on brain development (Bianchi et al., 2010b, Giacomini et al., 2015, Stagni et al., 2016, Stagni et al., 2017a). Since there are no data regarding pro-neurogenic effects exerted by CL in Ts65Dn mice, we first tested the effects of different doses of CL on the proliferation rate in the SGZ of Ts65Dn mice. Mice received a daily subcutaneous injection of saline or CL (0.01, 0.5, 1.0, and 2.0 mg/kg) from P3 to P15. On P15, mice received an intraperitoneal injection of BrdU, a marker of cells in the S-phase of the cell cycle (Nowakowski et al., 1989), 2h before being killed. The NPCs giving origin to granule cells of the dentate gyrus are mainly located in the subgranular zone (SGZ), but NPCs can also be found in the granule cell layer. In serial sections immmunostained for BrdU we evaluated the total number of BrdU-positive cells in the SGZ and granular layer of the dentate gyrus. A one-way ANOVA on the number of BrdU-positiive cells in treated Ts65Dn mice showed no significant effect of treatment [$F(4,13) = 1.622$, $p = .228$]. *Post hoc* LSD test showed that in mice treated with the dose of 2.0 mg/kg the number of BrdU-positive cells was larger in comparison with mice treated with the doses of 0.01, 0.5, and 1.0 mg/kg) (Fig. 3B).

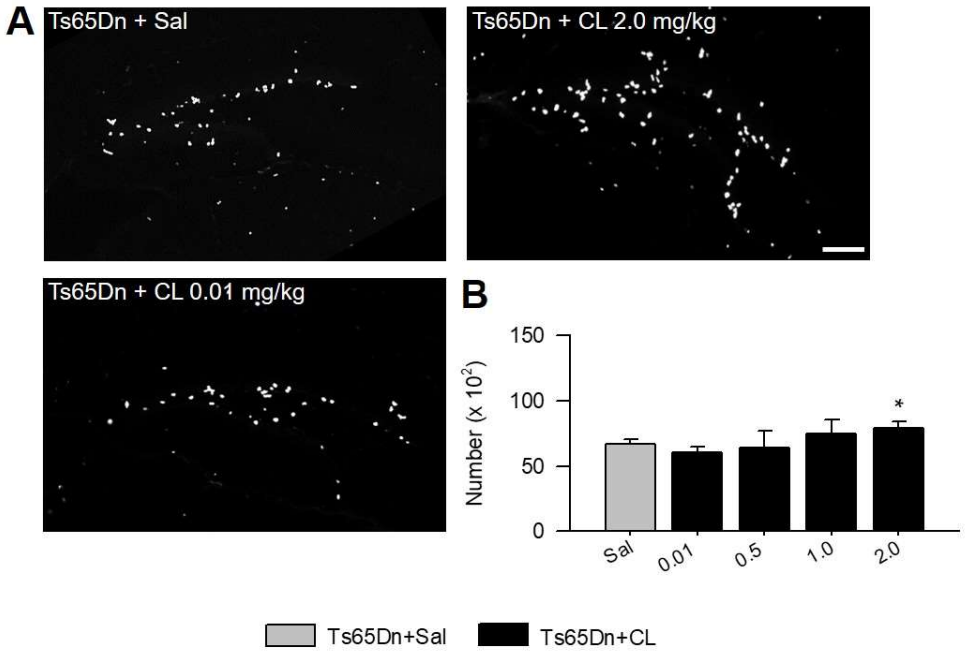


Fig. 3. Effects of neonatal treatment with different doses of CL on the number of cells in the S-phase of the cell cycle in the dentate gyrus of P15 Ts65Dn mice.
A: Representative images of sections immunostained for BrdU from the dentate gyrus of a saline-treated Ts65Dn mouse and Ts65Dn mice that were treated with either 0.01 or 2.0 mg/kg of Clenbuterol. Calibration bar=100 μm. B: Total number of BrdU-positive cells in the dentate gyrus of Ts65Dn mice that were treated with saline (n=5), 0.01 mg/kg CL (n=3), 0.5 mg/kg CL (n=3), 1.0 mg/kg CL (n=3), and 2.0 mg/kg CL (n=3) in the period P3-P15. Values (mean ± standard error (SE)) in (B) refer to one dentate gyrus. * $p < 0.05$. The asterisk indicates a difference in comparison with saline-treated Ts65Dn mice. (Fisher's LSD test after one-way ANOVA). Abbreviations: CL, clenbuterol; Sal, saline.

We tested then the effect of the highest dose of CL (2.0 mg/kg) in euploid mice and found no significant increase in the number of BrdU-positive cells in in comparison with untreated euploid mice ($p = .176$; two-tailed t-test) (Fig. 4C). A two-way ANOVA on total number of BrdU-positive cells in untreated euploid and Ts65Dn mice and euploid and Ts65Dn mice treated with the 2.0 mg/kg of CL showed no genotype x treatment interaction, but revealed a main effect of genotype [$F(1,13) = 17.893$, $p = .001$] and a main effect of treatment [$F(1,13) = 5.921$, $p = .030$]. A *post hoc* Fisher's LSD test showed that untreated Ts65Dn mice had fewer BrdU-positive cells in comparison with untreated euploid mice (Fig.4D). Treated Ts65Dn mice underwent an increase in the number of BrdU-positive cells that became significantly larger in comparison with their untreated counterparts and similar to that of untreated euploid mice (Fig. 4D). This evidence indicates that the pro-neurogenic effects elicited by CL on trisomic NPCs *in vitro* (Salvalai, 2019) are replicated *in vivo*, but with the highest tested dose of CL only.

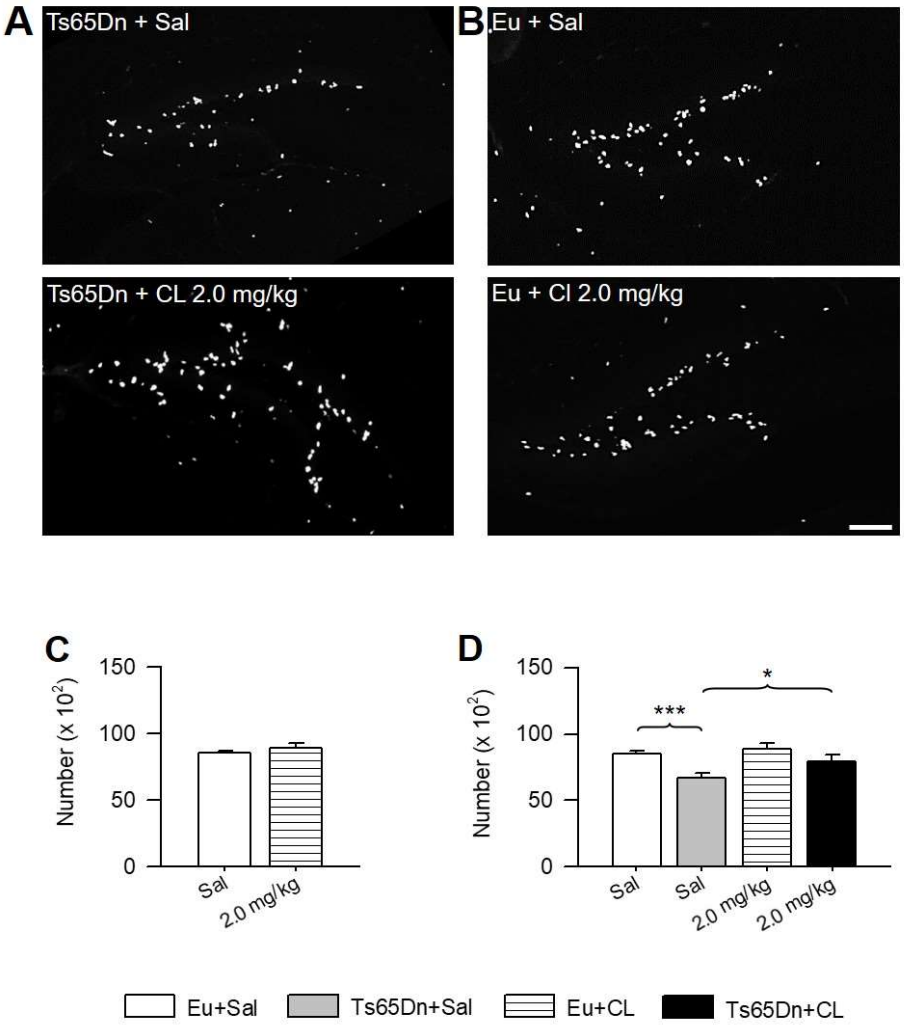


Fig. 4. Effects of neonatal treatment with CL on the number of cells in the S-phase of the cell cycle in the dentate gyrus of P15 Ts65Dn and euploid mice.

A, B: Representative images of sections immunostained for BrdU from the dentate gyrus of a saline-treated Ts65Dn mouse and a Ts65Dn mouse treated with 2.0 mg/kg of CLEN (A) and of a saline-treated euploid mouse and a euploid mouse treated with 2.0 mg/kg of CL (B). Calibration bar=100 μm. C: Total number of BrdU-positive cells in the dentate gyrus of euploid mice that were treated with saline (n=5) or 2.0 mg/kg CL (n=3). D: Comparison of the effect of treatment with saline or CL 2.0 mg/kg in euploid and Ts65Dn mice. Values (mean ± SE) in (C-D) refer to one hemisphere. * $p < 0.05$; *** $p < 0.001$ (Fisher's LSD test after two-way ANOVA). Abbreviation: CL, clenbuterol; Eu, euploid; Sal, saline.

EFFECT OF CLENBUTEROL ON THE STEREOLOGY OF THE DENTATE GYRUS OF TS65DN MICE

In the hippocampal dentate gyrus, the production of granule cells mainly takes place in the first two postnatal weeks (Altman and Bayer, 1975). Thus, in view of the increase in the proliferation potency of NPCs in the SGZ of Ts65Dn mice treated with 2.0 mg/kg of CL, we wondered whether this effect was followed by an improvement/restoration of the defective cellularity that characterizes the dentate gyrus of trisomic mice. To clarify this issue, we stereologically evaluated the total number of granule cells in treated and untreated mice. We additionally evaluated the area of Hoechst-stained nuclei of the granule cells. The Kruskal-Wallis test on the volume of the granule cell layer showed significant differences across groups [$\chi 2$ (3) = 9.096, p =

.028]. The Mann-Whitney test showed a reduced volume of the granule cell layer in Ts65Dn mice compared to euploid mice (U = 1.000, p = .010) and demonstrated that treatment did not increase the volume of the granule cell layer (Fig. 5B). A two-way ANOVA on the granule cell density showed a genotype x treatment interaction [F(1,18) = 17.924, p < .001], a main effect of genotype [F(1,18) = 27.736, p < .001], and of treatment [F(1,18) = 12.314, p = .003]. Fisher's LSD test, carried out *post hoc*, showed that the density of the granule cells of untreated Ts65Dn mice was reduced (Fig. 5C) in comparison with that of euploid mice and that treatment fully restored cell density. A two-way ANOVA on total number of granule cells showed no genotype x treatment interaction, no effect of treatment but revealed a main effect of genotype [F(1,18) = 21.749, p < .001]. A *post hoc* Fisher's LSD test showed that the granule cell number of untreated Ts65Dn mice was reduced in comparison with untreated euploid mice and that treatment did not increase total granule cell number (Fig. 5D). The Kruskal-Wallis test on the area of Hoechst-stained nuclei showed no significant effect and the Mann-Whitney test showed no difference among groups (Fig. 5E).

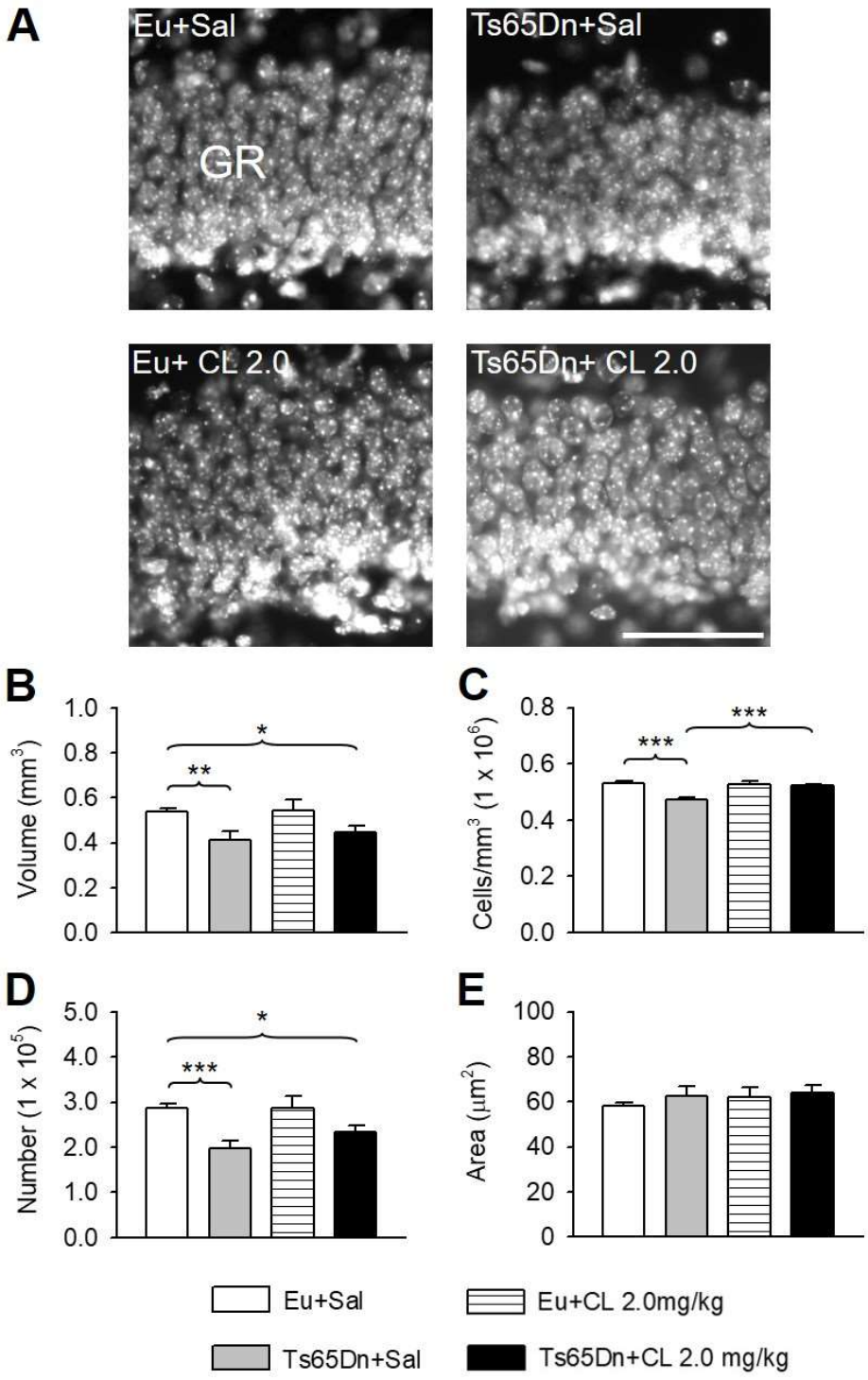


Fig. 5. Effects of neonatal treatment with CL on granule cell number in the dentate gyrus of P15 Ts65Dn and euploid mice.

A: Representative images of Hoechst-stained sections showing the granule cell layer of an animal from each experimental group. Calibration bar=50 μm. B-E: Volume of the dentate gyrus (B), granule cell density (C), total number of granule cells (D), and area of Hoechst-stained granule cell nuclei (E) of saline-treated euploid (n=6) and Ts65Dn (n=6) mice, and euploid (n=5) and Ts65Dn mice (n=5) treated with 2.0 mg/kg of CL. Values (mean ± SE) refer to one dentate gyrus. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Fisher LSD test after two-way ANOVA for data reported in C and D; Mann-Whitney test after Kruskal-Wallis test for data reported in B). Abbreviations: CL, clenbuterol; Eu, euploid; GR, granule cell layer; Sal, saline.

EFFECT OF CLENBUTEROL ON DENDRITIC SPINE DENSITY IN THE DENTATE GYRUS OF TS65DN MICE

Spine density reduction is a typical feature of the trisomic brain (Benavides-Piccione et al., 2004, Guidi et al., 2013) that, in conjunction with hypocellularity, is thought to be a critical determinant of intellectual disability. In order to establish whether CL improves spine density, in Golgi-stained brains we evaluated spine density in the dendritic arbor of the granule neurons. Since the inputs to the dendritic tree of granule cells are organized in a laminar manner, we deemed it of interest to separately evaluate spine density in dendritic branches harbored in the outer half and inner half of the molecular layer.

In order to establish whether treatment with CL ameliorates spine density in Ts65Dn mice and, if so, in a dose-dependent manner, we evaluated spine density in mice treated with different doses of CL (0.01, 0.5, 1.0, and 2.0 mg/kg). A one-way ANOVA on spine density in the proximal dendrites of Ts65Dn mice revealed a significant effect of treatment [$F(4,13) = 34.510$, $p < .001$]. A *post hoc* Fisher's LSD test showed that all doses of CL (0.01, 0.5, 1.0, and 2.0 mg/kg) caused a significant increase in spine density in comparison with saline-treated Ts65Dn mice (Fig. 6B; Table 2). The spine density increase ranged between +49% and +60%. A one-way ANOVA on spines in the distal dendrites of Ts65Dn mice revealed a significant effect of treatment [$F(4,13) = 26.117$, $p < .001$]. A *post hoc* Fisher's LSD test showed that all doses (0.01, 0.5, 1.0 and 2.0 mg/kg) caused a significant increase in spine density in comparison with saline-treated Ts65Dn mice (Fig. 6C; Table 2). The spine density increase ranged between +35% and +50%. No difference was found in the magnitude of the effect elicited by the different tested doses of CL, with the exception of the dose of 0.5 mg/kg that had a slightly smaller effect on the distal spines in comparison with the dose of 0.01 mg/kg. (Table 2).

		Proximal Spines / 20 µm	Distal Spines / 20 µm
		p	p
Ts65Dn + Saline	Ts65Dn + CL 0.01 mg/kg	**< .001**	**< .001**
	Ts65Dn + CL 0.5 mg/kg	**< .001**	**< .001**
	Ts65Dn + CL 1.0 mg/kg	**< .001**	**< .001**
	Ts65Dn + CL 2.0 mg/kg	**< .001**	**< .001**
Ts65Dn + CL 0.01 mg/kg	Ts65Dn + CL 0.5 mg/kg	.226	**.043**
	Ts65Dn + CL 1.0 mg/kg	.868	.067
	Ts65Dn + CL 2.0 mg/kg	.935	.252
Ts65Dn + CL 0.5 mg/kg	Ts65Dn + CL 1.0 mg/kg	.291	.809
	Ts65Dn + CL 2.0 mg/kg	.199	.314
Ts65Dn + CL 1.0 mg/kg	Ts65Dn + CL 2.0 mg/kg	.804	.438

Table 2. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of different concentrations of CL on spine density on proximal and distal dendritic branches of the granule cells in the dentate gyrus of Ts65Dn mice.

In order to establish whether treatment favors spinogenesis in euploid mice too, we examined spine density in euploid mice treated with the doses of 0.01, 0.5, 1.0 and 2.0 mg/kg. A one-way ANOVA on spine density in the proximal dendrites of euploid mice revealed a significant effect of treatment [$F(4,13) = 6.582$, $p = .004$]. A *post hoc* Fisher's LSD test showed that euploid mice treated with the doses of 0.01, 1.0 and 2.0 mg/kg underwent a significant increase in spine density in comparison with saline-treated euploid mice (Fig.6E; Table 3). The spine density increase ranged between +9%-+27%. A one-way ANOVA on spine density in the distal dendrites of euploid mice revealed a significant effect of treatment [$F(4,13) = 11.596$, $p < .001$]. A *post hoc* Fisher's LSD test showed that all doses (0.01, 0.5, 1.0 and 2.0 mg/kg) caused a significant increase in spine density in comparison with the vehicle (Fig. 6F; Table 3). The spine density increase ranged between +15%-+29%.

		Proximal Spines / 20 µm	Distal Spines / 20 µm
		p	p
Euploid + Saline	Euploid + CL 0.01 mg/kg	**.001**	**< .001**
	Euploid + CL 0.5 mg/kg	.162	**.011**
	Euploid + CL 1.0 mg/kg	**.027**	**< .001**
	Euploid + CL 2.0 mg/kg	**.003**	**.001**
Euploid + CL 0.01 mg/kg	Euploid + CL 0.5 mg/kg	**.022**	**.027**
	Euploid + CL 1.0 mg/kg	.108	.352
	Euploid + CL 2.0 mg/kg	.491	.259
Euploid + CL 0.5 mg/kg	Euploid + CL 1.0 mg/kg	.394	.152
	Euploid + CL 2.0 mg/kg	.080	.213
Euploid + CL 1.0 mg/kg	Euploid + CL 2.0 mg/kg	.327	.833

Table 3. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of different concentrations of CL on spine density on proximal and distal dendritic branches of the granule cells in the dentate gyrus of euploid mice.

We next compared the effects of the different doses of CL in euploid and Ts65Dn mice. To this purpose, we carried out a two-way ANOVA on spine density with genotype (euploid and Ts65Dn mice) and treatment (saline, 0.01, 0.5, 1.0 and 2.0 mg/kg) as factors. The two-way ANOVA on spine density in the proximal dendrites showed a genotype x treatment interaction [$F(4,26) = 5.465$, $p = .002$], no main effect of genotype, but a main effect of treatment [$F(4,26) = 32.113$, $p < .001$]. A *post hoc* Fisher's LSD test showed that the spine density of saline-treated Ts65Dn mice was significantly reduced (-24%) in comparison with that of saline-treated euploid mice (Fig. 6B). After treatment with any dose of CL the number of spines of Ts65Dn mice underwent a notable increase and became even larger (+14%-+22%) than that of saline-treated euploid mice (Fig. 6B). A two-way ANOVA on spine density in the distal dendrites showed no genotype x treatment interaction, no main effect of genotype, but a main effect of treatment [$F(4,26) = 35.868$, $p < .001$]. A *post hoc* Fisher's LSD test showed that the spine density of saline-treated Ts65Dn mice was significantly reduced

(-15%) in comparison with that of saline-treated euploid mice (Fig. 6C). After treatment with any dose of CL the number of spines of Ts65Dn mice underwent a notable increase and became larger (+15%-+28%) than that of saline-treated euploid mice (Fig. 6C).

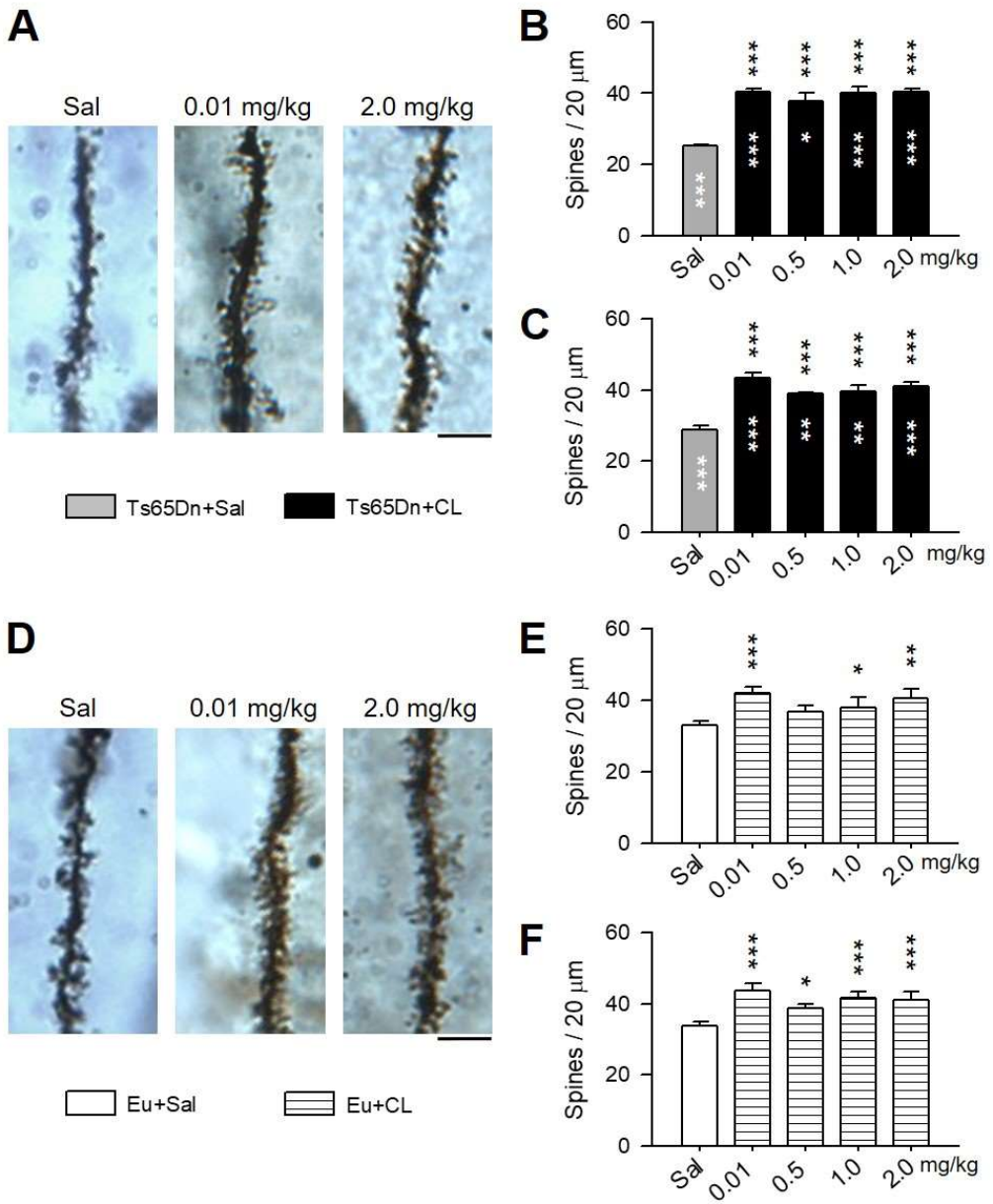


Fig. 6. Effects of neonatal treatment with CL on dendritic spine density in the dentate gyrus of P15 Ts65Dn and euploid mice.

A, D: Photomicrographs of Golgi-stained granule cell dendrites showing spines on distal dendritic branches in Ts65Dn (A) and euploid (D) mice. Calibration bar=5 µm. B, E: Spine density (mean ± SE) on the proximal dendrites of the granule cells of Ts65Dn (B) and euploid (E) mice and on the distal dendrites of Ts65Dn (C) and euploid (F) mice that were treated with saline (Ts65Dn mice: n=6; euploid mice: n=6), 0.01 mg/kg CL (Ts65Dn mice: n=3; euploid mice: n=3), 0.5 mg/kg CL

(Ts65Dn mice: n=3; euploid mice: n=3), 1.0 mg/kg CL (Ts65Dn mice: n=3; euploid mice: n=3), and 2.0 mg/kg CL (Ts65Dn mice: n=3; euploid mice: n=3) in period P3-P15. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Fisher LSD test after one-way or two-way ANOVA). Black asterisks above the bars indicate an effect of treatment in each genotype (Fisher LSD test after one-way ANOVA), and white asterisks in the bars indicate a difference between Ts65Dn mice and saline-treated euploid mice (Fisher's LSD test after two-way ANOVA). Abbreviations: CL, clenbuterol; Eu, euploid; Sal, saline.

EFFECT OF CLENBUTEROL ON THE DENDRITIC TREE OF THE GRANULE CELL OF TS65DN MICE

Dendritic development is a process that largely takes place postnatally, in particular in the dentate gyrus, where a large number of neurons are generated after birth. In view of the positive effects of all tested doses of CL on dendritic spine density in Ts65Dn mice, we wondered whether the lowest tested dose (0.01 mg/kg) was able to foster dendritic development. To this purpose, we examined Golgi-stained granule neurons located in the outer portion of the granule cell layer A one-way ANOVA on the total length of dendritic tree showed a genotype x treatment interaction [$F(2,9) = 7.527$, $p = .012$]. A *post hoc* Fisher's LSD test showed that Ts65Dn mice had a shorter dendritic length (-25%) (Fig. 7B; Table 4) than saline-treated euploid mice. In Ts65Dn mice treated with CL 0.01 mg/kg the dendritic length (Fig. 7B; Table 4) underwent an increase (+22%) and became similar to that of saline-treated euploid mice (Fig. 7B; Table 4).

	Total length
	p
Eu + Sal/Ts65Dn + Sal	**.004**
Eu + Sal/ Ts65Dn+ CL 0.01 mg/kg	.225
Ts65Dn + Sal/ Ts65Dn+ CL 0.01 mg/kg	**.06**

Table 4. The table reports the p values of Fisher's LSD test after one-way ANOVA on the effect of 0.01mg/kg of CL on the total length of dendritic tree of the granule cells in the dentate gyrus of Ts65Dn mice.

The Kruskal-Wallis test on the number of segments of dendritic tree revealed a difference between the groups [$\chi2(2) = 8.244$, $p= .016$]. The Mann-Whitney tests on the number of dendritic segments showed a reduced number of segments in saline-treated Ts65Dn mice (-34%) (Fig.7C; Table 5) in comparison with saline-treated euploid mice. In Ts65Dn mice treated with CL 0.01 mg/kg the number of segments underwent an increase (+72%) and became similar to that of saline-treated euploid mice (Fig. 7C; Table 5).

	Number of segments	
	U	p
Eu + Sal/Ts65Dn + Sal	.000	**.014**
Eu + Sal/ Ts65Dn+ CL 0.01 mg/kg	3.000	.177
Ts65Dn + Sal/ Ts65Dn+ CL 0.01 mg/kg	.000	**.034**

Table 5. The table reports the U and p values of the Mann-Whitney test after the Kruskal-Wallis test on the number of segments of the granule cells of saline treated euploid and Ts65Dn mice, and Ts65Dn mice treated with 0.01 mg/kg CL.

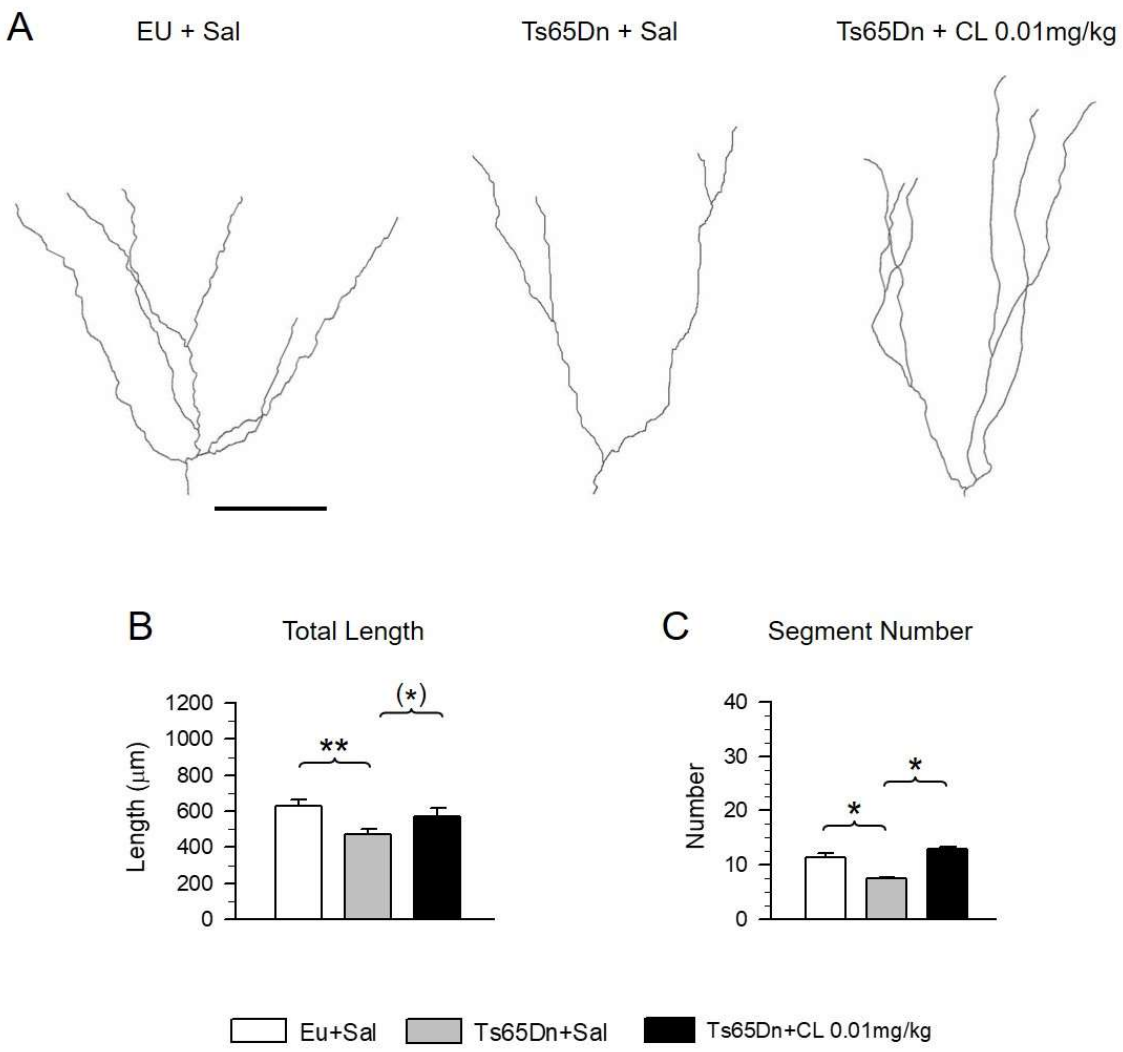


Fig. 7. Effect of clenbuterol on the dendritic size of the granule cells of Ts65Dn mice.
A: Examples of the reconstructed dendritic tree of granule cells from an animal of each of the following experimental groups: EU + Sal, Ts65Dn + Sal and Ts65Dn + CL 0.01 mg/kg. Calibration bar 50 µm. B: Mean total dendritic length of the dendritic tree in EU + SAL mice (n=5), Ts65Dn + SAL mice (n=4) and Ts65Dn + CL 0.01 mg/kg mice (n=3). C: Mean number of dendritic segments of the same mice. Values in B, C represent mean ± SE. (*) $p = 0.06$; * $p < 0.05$; ** $p < 0.01$; (Fisher LSD test after one-way ANOVA or Mann-Whitney test after Kruskal-Wallis test). Abbreviations: CL, clenbuterol; Eu, euploid; Sal, saline.

EFFECT OF CLENBUTEROL ON THE BODY WEIGHT

The Ts65Dn strain is characterized by a high mortality rate during gestation (Roper et al., 2006b). In view of the fragility of this strain, we deemed it important to establish whether treatment with CL has adverse effects on the viability and growth of Ts65Dn mice. In this study, we evaluated the body weight of P15 mice that received saline or different doses of CL (0.01, 0.5, 1.0 and 2.0 mg/kg) in order to establish the outcome of treatment on growth. A one-way ANOVA on the body weight of Ts65Dn mice revealed no significant effect

of treatment. However, a *post hoc* Fisher's LSD test showed that the dose of 2.0 mg/kg caused a significant reduction (-24%) in body weight in comparison with saline-treated Ts65Dn mice (Fig. 8A, Table 6).

		Body Weight
		p
Ts65Dn + Saline	Ts65Dn + CL 0.01 mg/kg	.808
	Ts65Dn + CL 0.5 mg/kg	.642
	Ts65Dn + CL 1.0 mg/kg	.241
	Ts65Dn + CL 2.0 mg/kg	**.034**
Ts65Dn + CL 0.01 mg/kg	Ts65Dn + CL 0.5 mg/kg	.829
	Ts65Dn + CL 1.0 mg/kg	.333
	Ts65Dn + CL 2.0 mg/kg	.077
Ts65Dn + CL 0.5 mg/kg	Ts65Dn + CL 1.0 mg/kg	.429
	Ts65Dn + CL 2.0 mg/kg	.131
Ts65Dn + CL 1.0 mg/kg	Ts65Dn + CL 2.0 mg/kg	.715

Table 6. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of different concentrations of CL on the body weight of Ts65Dn mice.

A one-way ANOVA on the body weight of euploid mice revealed no significant effect of treatment with all doses in comparison with saline-treated euploid mice (Fig. 8B, Table 7).

		Body Weight
		p
Euploid + Saline	Euploid + CL 0.01 mg/kg	.642
	Euploid + CL 0.5 mg/kg	.991
	Euploid + CL 1.0 mg/kg	.518
	Euploid + CL 2.0 mg/kg	.388
Euploid + CL 0.01 mg/kg	Euploid + CL 0.5 mg/kg	.659
	Euploid + CL 1.0 mg/kg	.345
	Euploid + CL 2.0 mg/kg	.263
Euploid + CL 0.5 mg/kg	Euploid + CL 1.0 mg/kg	.528
	Euploid + CL 2.0 mg/kg	.408
Euploid + CL 1.0 mg/kg	Euploid + CL 2.0 mg/kg	.948

Table 7. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of different concentrations of CL on the bodyweight of euploid mice.

In order to compare the effect of treatment across groups, we next carried out a two-way ANOVA on the body weight with genotype (euploid and Ts65Dn mice) and treatment (saline, 0.01, 0.5, 1.0 and 2.0 mg/kg) as factors. The two-way ANOVA showed no genotype x treatment interaction, no main effect of treatment, but revealed a main effect of genotype [$F(1,116) = 38.374$, $p < .001$]. A *post hoc* Fisher's LSD test showed that the body weight of saline-treated Ts65Dn mice and of Ts65Dn mice treated with different doses of CL was significantly reduced in comparison with that of saline-treated euploid mice (Fig. 8A,B).

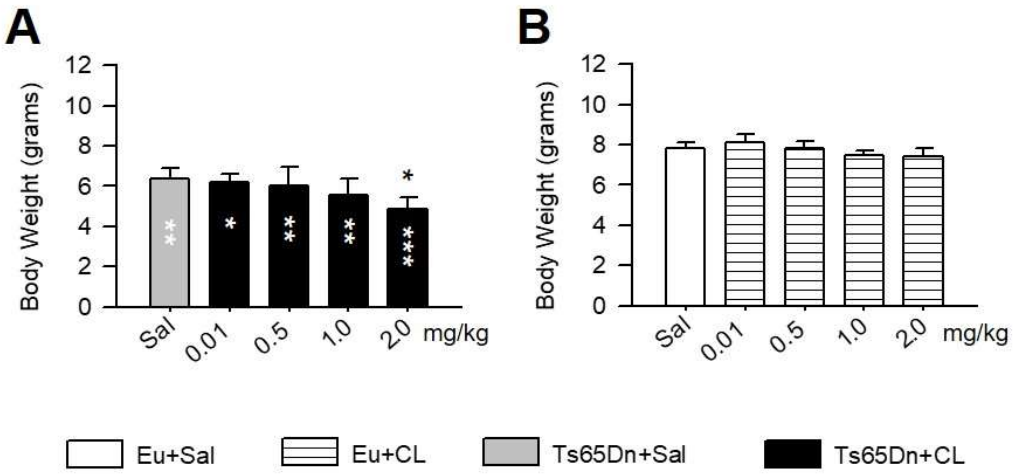


Fig. 8. Effect of neonatal treatment with CL on the body of P15 Ts65Dn and euploid mice.
A-B: Body weight of Ts65Dn mice (A) and of euploid mice (B) that were treated with saline (Ts65Dn mice: n=11; euploid mice: n=22), 0.01 mg/kg CL (Ts65Dn mice: n=8; euploid mice: n=9), 0.5 mg/kg CL (Ts65Dn mice: n=7; euploid mice: n=18), 1.0 mg/kg CL (Ts65Dn mice: n=3; euploid mice: n=13), and 2.0 mg/kg CL (Ts65Dn mice: n=8; euploid mice: n=27) in the period P3-P15, measured on P15. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Fisher LSD test after one-way or two-way ANOVA). Black asterisks above the bars indicate an effect of treatment in each genotype (Fisher LSD test after one-way ANOVA) and white asterisks in the bars indicate a difference between Ts65Dn mice and saline-treated euploid mice (Fisher LSD test after two-way ANOVA). Abbreviations: CL, clenbuterol; Eu, euploid; Sal, saline.

DISCUSSION SECTION 1

NEONATAL TREATMENT WITH CL DOES NOT RESTORE HIPPOCAMPAL NEUROGENESIS AND CELLULARITY IN THE TS65DN MOUSE

Neurogenesis impairment is one of the key determinants of brain malfunctioning in DS. Hence, it is of importance to find treatments that counteract this defect. The current study shows that low doses of the β2-AR agonist CL did not change the proliferation rate of the NPCs in the dentate gyrus of Ts65Dn mice. Only the highest dose tested here (2.0 mg/kg) was able to produce an increase in the proliferation rate, although this effect was of relatively small magnitude. In view of this modest effect, there was no restoration of total granule cell number and treated Ts65Dn still had fewer granule cells than untreated euploid mice. In euploid mice, the dose of 2.0 mg/kg produced no effect on the number of proliferating cells and total number of granule cells. Consistently with our current findings that a dose of CL of 2.0 mg/kg/day can increase neurogenesis Ts65Dn mice, a study in the APP/PS1 model of AD shows that this dose is able to restore neurogenesis in the dentate gyrus (Chai et al., 2016). Likewise, administration of 2.0 mg/kg/day of another the β2-AR agonist, Formoterol, restores neurogenesis in the dentate gyrus of adult Ts65Dn mice (Dang et al., 2014). While we found here that CL did not increase the number of proliferating cells in euploid mice, Formoterol also increases the number of proliferating cells in euploid mice (Dang et al., 2014).

Evidence in the rat shows that the dentate gyrus mainly expresses β2-AR (Nicholas et al., 1993). A study focusing on progenitor cells in the adult dentate gyrus examined the mRNA expression of all kinds of receptors in neural progenitor cells using RT-PCR. Results show that mRNAs of α1A, α1B, α1D, α2C, β1 and β2-ARs were present in these cells, although the mRNAs of α2A, α2B, and β3-ARs were not (Masuda et al., 2012). The role of the noradrenergic system on the proliferation rate of the granule cells has been established by several studies. For instance, it has been shown that depletion of noradrenalin decrease the proliferation of granule cell precursors in the dentate gyrus of adult rats (Kulkarni et al., 2002) and that stimulation of the β-receptors with the agonist isoproterenol (2.0 mg/kg/day) activates the pool of quiescent precursor cells and increases their proliferation in the hippocampus of adult mice (Jhaveri et al., 2014). Furthermore, in cell cultures from the adult rat dentate gyrus, noradrenaline promotes proliferation of the pool of the 2a early progenitor cells through the β2-AR (Masuda et al., 2012), while the β1-AR selective agonist Xamoterol does not, indicating a key role of the β2-AR in the promotion of neurogenesis mediated by the noradrenergic system.

The current findings that a relatively high dose of CL was necessary in order to elicit an increase in the proliferation rat of NPCs is somewhat surprising considering the large pro-neurogenic effects with a wide spectrum of doses of CL observed in the drug screening campaign carried out in cultures of trisomic NPCs (Salvalai, 2019). It must be noted, however, that in some instances results of experiments carried out *in vitro* are not replicated *in vivo*. One of the reasons for these discrepancies may be related to the pharmacokinetic

of the drug. In the *in vivo* condition, drugs must cross the BBB and their permeability across the BBB determines their final concentration in the brain. Thus, drugs that are effective *in vitro* may not yield the same pro-neurogenic effects in vivo because they do not reach a sufficiently high concentration in the brain. Yet, CL has a good BBB permeability (Geyer and Frampton, 1988) and, consistently with this evidence, we found that a dose of CL of 0.01 mg/kg was sufficient to restore spine density and dendritic length in Ts65Dn mice (see also below). The latter finding indicates that CL crosses the BBB in an amount sufficient to exert biological actions. Taken together these findings show that the dose that was necessary to increase the proliferation rate of NPCs was 200 times larger than the dose that was sufficient to increase spine density. A possible interpretation of these results is that the cellular machinery underlying dendritic development is more sensitive to the action of the β2-AR than the machinery that regulates the cell cycle. An alternative possibility is that, unlike postmitotic granule cells, the progenitors of the granule cells in the neonatal hippocampus have a relatively low expression of the β2-AR and that a high concentration of the agonist (in our case, CL) is needed in order to affects the pathways that regulate the cell cycle. A low concentration of receptors may not be an obstacle *in vitro*, because continuous exposure to a constant concentration of CL may overcome a low receptor expression. Unfortunately, very few studies have examined the ontogeny of the β2-AR in the brain. The finding that in the cortex of rats, the number of β-AR undergoes a large increase from postnatal day 2 and 8 (Keshles and Levitzki, 1984) suggests that a similar timeline may be exhibited by the hippocampal progenitors. Specific studies are needed in order to establish the ontogeny and cellular distribution of the β2-AR in postmitotic and mitotic cells in the dentate gyrus of mice during early phases of postnatal development. β3 receptors, which are typically expressed by adipocytes, have been shown to be largely expressed in the hippocampus of the rat (Summers et al., 1995). Interestingly, an *in vivo* and *in vitro* study in mice show that β3 receptors, are expressed in a subpopulation of hippocampal precursor cells and that the proliferation of these precursors is enhanced by noradrenaline through specific activation of β3 receptors (Jhaveri et al., 2010). It is possible that in the neonate hippocampus unlike in the adult hippocampus the pool of progenitor cells expresses more β3 than β2-AR. This may also explain the necessity of high doses of CL in order to stimulate their proliferation.

As described in the Introduction, activation of β2-AR activates signaling cascades (see Fig. 7 in the Introduction) that culminate into an increase in the expression of the transcription factor cAMP response element binding protein (CREB). While it is well-established that CREB signaling represent a key mechanism in the regulation of survival and maturation of neurons, its role in the regulation of neurogenesis appears to be less prominent. This, in conjunction with a possible low expression of the β2-AR in hippocampal progenitors, may concur to explain the relatively moderate pro-proliferative response elicited by CL in Ts65Dn mice.

The basic fibroblast growth factor (bFGF) is a trophic factor that enhances neurogenesis and whose expression is reduced in Ts65Dn mice (Dang et al., 2014). Activation of the β2-AR through CL (10 mg/kg, i.p.)

has been shown to increase the expression of bFGF in the cortex, hippocampus and cerebellum by its central action on β2-AR (Follesa and Mocchetti, 1993). Interestingly, bFGF has been shown to cause a CREB phosphorylation that is not mediated by any of previously known signaling pathways that lead to CREB phosphorylation (Sung et al., 2001). Thus, it is possible that an increase in bFGF participates in the pro-proliferative effects of CL observed here in Ts65Dn mice. Noteworthy, the dose that was effective in our study (2.0 mg/kg) is much lower than that used by Follesa. It is possible that a larger dose is needed in order to elicit sufficiently larger increase in bFGF and, thus, larger pro-proliferative effects.

NEONATAL TREATMENT WITH CL RESTORES THE DENDRITIC DEVELOPMENT OF GRANULE CELLS IN THE TS65DN MOUSE

In addition to neurogenesis impairment, dendritic pathology is a second major developmental defects of DS. The dendritic tree of trisomic neurons has a reduced number of branches, lacks higher order branches and has a notably reduced density of dendritic spines (Guidi et al., 2013). Therefore, in the current study, we were interested in establishing whether treatment with CL can ameliorate these defects in the granule cells of the dentate gyrus of Ts65Dn mice. We found that CL fully restored dendritic development. In particular, Ts65Dn mice treated with CL underwent a large increase in spine density that even surpassed that of euploid mice. The effect took place along the whole extent of the dendritic tree of the granule cells. Regarding the dendritic size, treatment restored the total length and the number of segments of dendritic tree of the granule cells. These findings suggest that the β2-system may be a key actor involved in the regulation of dendritic development in the trisomic brain. The positive effects of CL on dendritic branching and spine density in Ts65Dn mice are in agreement with similar findings in the APP/PS1 model of AD (Chai et al., 2017). Importantly, we found that even the lowest dose of CL tested here was sufficient to elicit these effects. This not trivial aspect, that may have an impact for the translation of our results, will be discussed in the General Discussion section. Our results in euploid mice show that while CL had no effect on the proliferation rate of the granule cell precursor, it caused an increase in the dendritic length and spine density of the granule cells. This strengthens the conclusion that β2-AR agonists are more powerful in fostering neuron maturation rather than proliferation. There is evidence that exposure of neurons to conditioned media from noradrenaline-stimulated glial cells increase dendritic complexity and that conditioned media from glial cells treated with the β2-AR agonists SALM and CL, but not the β1-AR agonist xamoterol, mimicked the ability of noradrenaline to increase neuronal complexity. In addition, noradrenaline induced the expression of a range of growth factors, including BDNF (Day et al., 2014). This suggests that the effects of β2-AR agonists on dendritic complexity may be both cell-autonomous and mediated by soluble factors released by astrocytes. This may concur to explain the powerful effects of CL on the dendritic pattern of Ts65Dn and euploid mice.

BDNF is one of the master regulators of dendritic development and spine density production/maturation during development and in the adult brain (De Vincenti et al., 2019). There is evidence that the levels of BDNF are reduced both in fetuses with DS and in the Ts65Dn model (see (Rueda et al., 2012)). Thus, among the

molecular mechanisms underlying dendritic pattern impairment in DS, the BDNF-TrKB system may be a major candidate. As shown in Fig. 7, the β2-AR activate extracellular signal-regulated kinases (ERK/, MAPKs), that represent key steps in the activation of CREB. While the question regarding the effects of CREB on neurogenesis is not completely settled, it is clear that CREB plays a key role in the process of neuron maturation (Merz et al., 2011). This effect is mediated by the transcription of many proteins, including BDNF (Ahmed and Frey, 2005). Thus, the effects of treatment with CL on dendritic development may be largely mediated by BDNF. This conclusion is supported by demonstration that CL increases the levels of BDNF in the kainic model of excitotoxicity (Gleeson et al., 2010). The cellular effects of the BDNF-TrkB system are mediated by three major pathways, among which the RAS/MEK/ERK pathway appears to play a paramount role (Arevalo and Wu, 2006). Indeed, it has been shown that the BDNF-TrkB signaling-induced increase in spine density of hippocampal pyramidal neurons requires ERK1/2 activation (Alonso et al., 2004).

NEONATAL TREATMENT WITH CL AFFECTS THE BODY MASS OF THE TS65DN MOUSE

Results showed that all tested dose of CL did not affect the body weight of euploid mice. In contrast, in Ts65Dn mice the highest tested dose (2.0 mg/kg) caused a reduction in the body weight. There is evidence that CL increases the skeletal muscle mass but concomitantly decreases the body fat content. For this reason body builders and athletes use CL in order to improve the muscle mass, although this use is illegal (Carter et al., 1991, Spiller et al., 2013). In the rat, the effects of CL intake on the body weigh have been shown to have a time-course related to the duration of treatment. During the first 6-8 days of treatment there is a weight loss that is followed by an increase in body weight above the initial levels (Carter et al., 1991). This effect appears to be due to a reduction in the food intake during the first week of treatment. Ts65Dn mice are constitutively smaller and more fragile in comparison with euploid mice (Reeves et al., 1995). This may explain the body weight reduction taking place in Ts65Dn but not euploid mice with the highest tested dose of CL.

It must be emphasized that the dose of 2.0 mg/kg was the only one that produced a neurogenesis increase in the dentate gyrus of Ts65Dn mice. The outcome of this dose on body weight must be taken into account in the evaluation of the benefits/risks of a therapy with CL for the rescue of neurogenesis. Importantly, a dose that was 200 times smaller (0.01 mg/kg) was sufficient to fully restore both spine density and dendritic length in Ts65Dn mice with no adverse effect on the body weight, suggesting a potential therapeutic use of CL for DS specifically for dendritic development.

SECTION 2: EFFECTS OF NEONATAL TREATMENT WITH SALMETEROL

MATERIALS AND METHODS SECTION 2

COLONY

Female Ts65Dn mice (strain 5252) carrying a partial trisomy of chromosome 16 (Reeves et al., 1995) were obtained from Jackson Laboratories (Bar Harbor, 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). A total of 83 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 light/dark cycle. Experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the use of experimental animals. 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=32) and Ts65Dn (n=18) mice, received a daily subcutaneous injection (at 9-10 am) with different doses of salmeterol (0.01 and 1.0 mg/Kg) in 0.9% NaCl solution from postnatal day 3 (P3) to P15 (Fig.1). Age-matched euploid (n=24) and Ts65Dn (n=9) mice were injected with the saline (hereafter referred to as "untreated mice"). Table 1 summarizes the number and sex of mice of the different experimental groups.

	Male	Female	Total
Eu Sal	16	8	24
Ts65Dn Sal	6	3	9
Eu + SALM 0.01mg/kg	6	10	16
Ts65Dn + SALM 0.01mg/kg	7	2	9
Eu + SALM 1.0mg/kg	10	6	16
Ts65Dn + SALM 1.0mg/kg	4	5	9

Table 1. The table reports the number of male and female mice used in this study.

Mice were killed at the age of 15 days (P15 mice). All mice received a subcutaneous injection of BrdU (5-bromo-2-deoxyuridine; Sigma; 150 mg/g body weight) in TrisHCl 50 mM 2h before being killed, in order to label proliferating cells (Fig. 1). The brains were excised and cut along the midline. The left hemispheres of all groups of mice were fixed by immersion in PFA 4% and frozen, and the right hemispheres were immersed in a Golgi impregnation solution. The body weight of mice of all group was recorded prior to sacrifice. The number of animals used for each experimental procedure is specified in the figure legends.

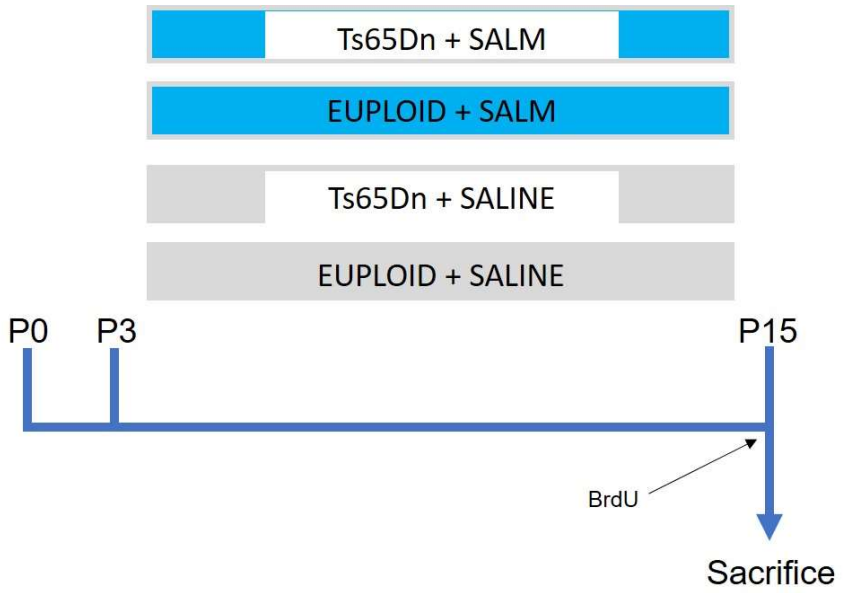


Fig. 1. Experimental protocol.
Euploid and Ts65Dn mice received a daily injection of either saline (EUPLOID + SALINE; Ts65Dn + SALINE) or treatment with SALM 0.01 and 1.0 mg/kg (EUPLOID + SALM; Ts65Dn + SALM) from P3 to P15. At P15, mice received one injection of BrdU, and were killed after 2 h in order to evaluate the number of cells in the S-phase of the cell cycle. The brains of these mice were used for immunohistochemistry and Golgi-staining. Abbreviations: BrdU, bromodeoxyuridine; P: postnatal day; SALM: salmeterol.

HISTOLOGICAL PROCEDURES

See Histological Procedures of SECTION 1

BrdU IMMUNOHISTOCHEMISTRY

Immunohistochemistry was carried out as previously described (Contestabile et al., 2007, Bianchi et al., 2010b, Guidi et al., 2013). One out of six free-floating sections (n = 13–15 sections) from the beginning to the end of the hippocampal formation was incubated with a rat anti-BrdU antibody (diluted 1:200; Biorad) and detection was performed with a HRP-conjugated anti-rat secondary antibody (dilution 1:200; Jackson Immunoresearch) and DAB kit (Vector Laboratories). Sections were counterstained with hematoxylin and then mounted on slides.

GOLGI STAINING

See Golgi-Staining of SECTION 1

MEASUREMENTS

Number of BrdU-positive cells

BrdU-positive cells in the dentate gyrus were detected using a Bright field microscope (HAMAMATSU Nanozoomer 2.0 RS objective: 20x, 0.75 NA). See Methods in SECTION 1 (Number of BrdU-positive cells).

Spine density:

See Methods in SECTION 1 (spine density).

Measurement of the dendritic tree:

See Methods in SECTION 1 (Measurement of the dendritic tree).

STATISTICAL ANALYSIS

See Methods in SECTION 1 (Statistical analysis).

RESULTS SECTION 2

EFFECT OF NEONATAL TREATMENT WITH SALMETEROL ON NEURAL PRECURSOR PROLIFERATION IN THE DENTATE GYRUS OF TS65DN MICE

A dose of 2.0 mg/kg of CL, unlike lower doses (0.01, 0.5 and 1.0 mg/kg) was able to increase the number of proliferating cells in the dentate gyrus of Ts65Dn mice but had side effects on the body weight (see Section 1). For this reason, we decided not to treat mice with a dose of 2.0 mg/kg of SALM. In order to establish whether a dose of 1.0 mg/kg of SALM was sufficient to improve neurogenesis, we treated Ts65Dn and euploid mice with 1.0 mg/kg of SALM from P3 to P15. A two-way ANOVA on the total number of BrdU-positive cells in the dentate gyrus of mice treated with SALM or saline showed no genotype x treatment interaction, a main effect of genotype [$F(1,16) = 33.937$, $p < .001$], and no main effect of treatment. A *post hoc* Fisher's LSD test showed that untreated Ts65Dn mice had fewer proliferating cells in comparison with untreated euploid mice (Fig.2B). In treated Ts65Dn mice the number of proliferating cells did not undergo any increase and remained similar that of untreated Ts65Dn mice. These findings show that the pro-neurogenic effects of SALM observed *in vitro* in cultures of trisomic cells from Ts65Dn mice are not replicated *in vivo.*

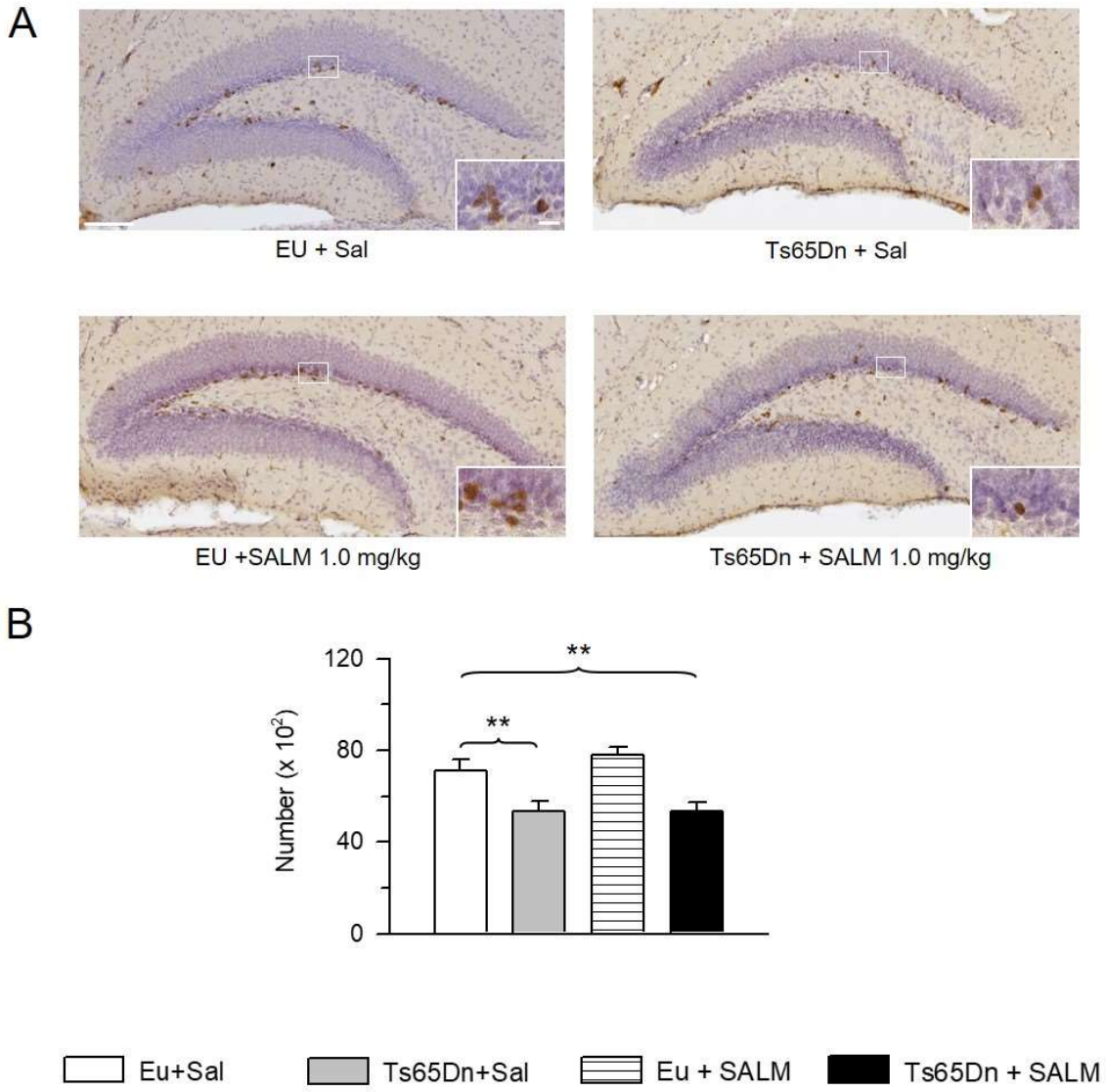


Fig. 2. Effects of neonatal treatment with salmeterol on the number of cells in the S-phase of the cell cycle in the dentate gyrus (DG) of P15 Ts65Dn and euploid mice.

A: Representative images of sections immunostained for BrdU from the dentate gyrus of untreated euploid and Ts65Dn mice and euploid and Ts65Dn mice that were daily treated with 1.0 mg/kg of SALM in the period P3-P15. Calibration bar = 100 µm. The insets show zoomed images of the boxed area with examples of individual BrdU-positive cells. Calibration bar = 10 µm. B: Total number of BrdU-positive cells in the dentate gyrus of untreated euploid (n = 5) and Ts65Dn (n = 4) mice and euploid (n = 5) and Ts65Dn (n = 6) mice treated with 1.0 mg/kg of SALM. Values (mean ± SE) in (B) refer to one dentate gyrus. **p≤0.01 (Fisher LSD test after two-way ANOVA). Abbreviations: Eu, euploid; Sal, Saline; SALM, salmeterol.

EFFECT OF SALMETEROL ON DENDRITIC SPINE DENSITY IN THE DENTATE GYRUS OF TS65DN MICE

The results of Section 1 showed that all tested doses of CL increased the density of dendritic spines in Ts65Dn mice and that the lowest dose (0.01 mg/kg) had the same effects has higher doses. Based on this evidence, we examined the effects of a low (0.01 mg/kg) and high dose (1.0 mg/kg) of SALM on spine density in the dendritic tree of the granule cells of Ts65Dn and euploid mice.

A one-way ANOVA on spine density in the proximal dendrites of Ts65Dn mice revealed a significant effect of treatment [$F(2,12) = 89.057$, $p < .001$]. A *post hoc* Fisher's LSD test showed that both doses of SALM (0.01 and 1.0 mg/kg) caused a significant increase in spine density in comparison with saline-treated Ts65Dn mice (Fig. 3B; Table 2). The spine density increase was about +25%. A one-way ANOVA on spine density in the distal dendrites of Ts65Dn mice revealed a significant effect of treatment [$F(2,12) = 148.745$, $p < .001$]. A *post hoc* Fisher's LSD test showed that both doses (0.01 and 1.0 mg/kg) caused a significant increase in spine density in comparison with saline-treated Ts65Dn mice (Fig. 3D; Table 2). The spine density increase ranged between +27% and +29%. No difference was found in the magnitude of the effect elicited by the two doses of SALM (Table 2).

		Proximal Spines / 20 µm	Distal Spines / 20 µm
		p	p
Ts65Dn + Saline	Ts65Dn + SALM 0.01 mg/kg Ts65Dn + SALM 1.0 mg/kg	**< .001** **< .001**	**< .001** **< .001**
Ts65Dn + SALM 0.01 mg/kg	Ts65Dn + SALM 1.0 mg/kg	.869	.319

Table 2. The table reports the p values of Fisher's LSD test after one-way ANOVA on the effect of two different doses of Salmeterol on spine density in proximal and distal dendritic branches of the granule cells in the dentate gyrus of Ts65Dn mice.

In order to establish whether treatment favors spinogenesis in euploid mice too, we examined spine density in euploid mice treated with the doses of 0.01 and 1.0 mg/kg. A one-way ANOVA on the dendritic spines in the proximal dendrites of euploid mice revealed no effect of treatment. A *post hoc* Fisher's LSD test showed that only the dose of 1.0 caused a significant increase in spine density in comparison with saline-treated euploid mice (Fig. 3C; Table. 3). A one-way ANOVA on the dendritic spines in the distal dendrites of euploid mice revealed no effect of treatment. A *post hoc* Fisher's LSD test showed that both doses (0.01 and 1.0 mg/kg) did not increase the number of spines in comparison with saline-treated euploid mice (Fig. 3E; Table 3). No difference was found in the magnitude of the effect elicited by the tested doses of SALM (Fig. 3E; Table 3).

		Proximal Spines / 20 µm	Distal Spines / 20 µm
		p	p
Euploid + Saline	Euploid + SALM 0.01 mg/kg Euploid+ SALM 1.0 mg/kg	.112 **.032**	.247 .309
Euploid + SALM 0.01 mg/kg	Euploid + SALM 1.0 mg/kg	.493	.881

Table 3. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of different doses of Salmeterol on spine density in proximal and distal dendritic branches of the granule cells in the dentate gyrus of euploid mice.

We next carried out a two-way ANOVA on spine density with genotype (euploid and Ts65Dn mice) and treatment (saline, SALM 0.01 and SALM 1.0 mg/kg) as factors. A two-way ANOVA on spine density in the proximal dendrites showed a genotype x treatment interaction [$F(2,24) = 46.863$, $p < .001$], a main effect of genotype [$F(1,24) = 101.481$, $p < .001$], and a main effect of treatment [$F(2,24) = 76.629$, $p < .001$]. A *post hoc* Fisher's LSD test showed that the spine density of untreated Ts65Dn mice was significantly reduced (-20%) (Fig. 3B) in comparison with that of untreated euploid mice. Both doses of SALM were able to restore the number of spines in Ts65Dn mice and their density became similar to that untreated euploid mice (Fig. 3B,C). Treatment had no effect on spine density in the proximal dendrites of euploid mice.

A two-way ANOVA on spine density in the distal dendrites showed a genotype x treatment interaction [$F(2,24) = 74.371$, $p .< .001$], a main effect of genotype [$F(1,24) = 117.266$, $p < .001$], and a main effect of treatment [$F(2,24) = 96.938$, $p .< .001$]. A *post hoc* Fisher's LSD test showed that the spine density of untreated Ts65Dn mice was significantly reduced (-22%) in comparison with that of untreated euploid mice (Fig. 3D). Both doses of SALM were able to restore the number of spines of Ts65Dn mice and their density became similar to untreated euploid mice (Fig. 3D,E). In euploid mice, the dose of 1.0 mg/Kg of SALM caused a very slight increase in spine density in the proximal dendrites in comparison with saline-treated euploid mice, while the lower dose (0.01 mg/kg) was ineffective (Fig. 3C). No effect of treatment was found on spine density in the distal dendrites of euploid mice.

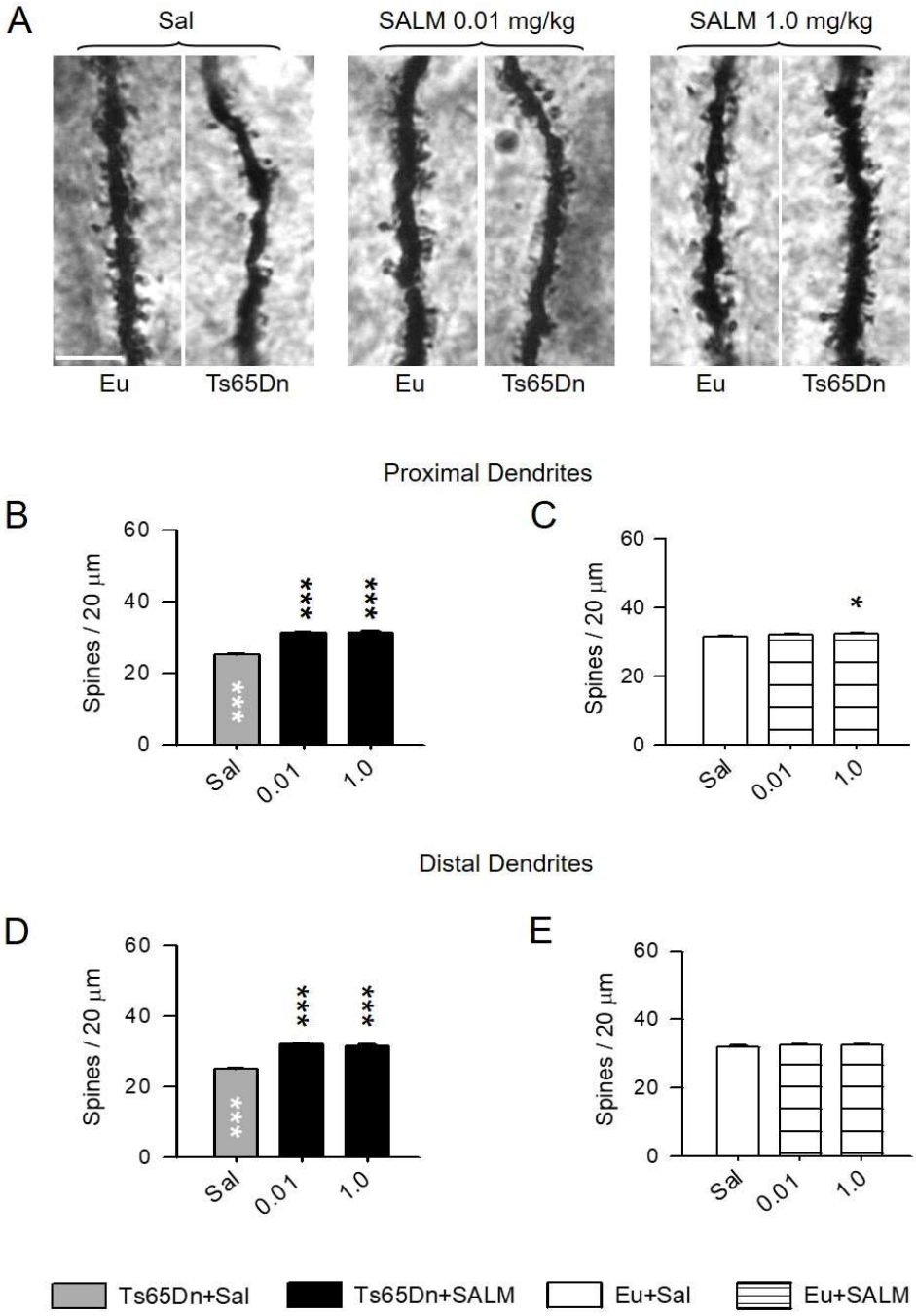


Fig. 3. Effects of neonatal treatment with SALM on dendritic spine density in the dentate gyrus of P15 Ts65Dn and euploid mice.

A: Photomicrographs of Golgi-stained granule cell dendrites showing spines on proximal and distal dendritic branches in Ts65Dn and euploid mice. Calibration bar=5 µm. B, E: Spine density (mean ± SE) on the proximal dendrites of the granule cells of Ts65Dn (B) and euploid (C) mice and on the distal dendrites of Ts65Dn (D) and euploid (E) mice that were treated with saline (Ts65Dn mice: n=5; euploid mice: n=5), 0.01 mg/kg SALM (Ts65Dn mice: n=5; euploid mice: n=5) and 1.0 mg/kg SALM (Ts65Dn mice: n=5; euploid mice: n=5) in period P3-P15. * $p < 0.05$; *** $p < 0.001$ (Fisher LSD test after one-way or two-way ANOVA). Black asterisks above the bars indicate an effect of treatment in each genotype (Fisher's LSD test after one-way ANOVA), and white asterisks in the bars indicate a difference between Ts65Dn mice and saline-treated euploid mice (Fisher's LSD test after two-way ANOVA). Abbreviations: Eu, euploid; Sal, saline; SALM, salmeterol.

EFFECT OF SALMETEROL ON THE DENDRITIC TREE OF THE GRANULE CELL OF TS65DN MICE

The results of the Section 1 showed that 0.01 mg/kg of CL increased the dendritic size in Ts65Dn mice. Based on this evidence, we examined the effects of the same dose (0.01 mg/kg) of SALM on the dendritic size of the granule cells of Ts65Dn and euploid mice. A one-way ANOVA on the total length of dendritic tree showed a genotype x treatment interaction [$F(2,10) = 9.181$, $p = .005$]. A *post hoc* Fisher's LSD test showed that Ts65Dn mice had a shorter dendritic length (-25%) (Fig.4B; Table 4) than saline-treated euploid mice. In treated Ts65Dn mice with SALM 0.01 mg/kg, the dendritic length underwent an increase (+31%) and became similar to those of saline-treated euploid mice (Fig.4B; Table 4).

	Total length
	p
Eu + Sal/Ts65Dn + Sal	**.003**
Eu + Sal/ Ts65Dn+ SALM 0.01 mg/kg	.695
Ts65Dn + Sal/ Ts65Dn+ SALM 0.01 mg/kg	**.007**

Table 4. The table reports the p values of Fisher's LSD test after one-way ANOVA on the effect of SALM 0.01 mg/kg on the total length of dendritic tree of the granule cells in the dentate gyrus of Ts65Dn mice.

A one-way ANOVA on the number of dendritic segments showed a genotype x treatment interaction [$F(2,10) = 12.429$, $p = .002$]. A *post hoc* Fisher's LSD test showed that Ts65Dn mice had a reduced number of segments (-34%) (Fig.4C; Table 5) in comparison with saline-treated euploid mice. In Ts65Dn mice treated with SALM 0.01 mg/kg the number of segments underwent an increase (+50%) and became similar to that of saline-treated euploid mice (Fig.4C; Table 5).

	Number of segments
	p
Eu + Sal/Ts65Dn + Sal	**.001**
Eu + Sal/ Ts65Dn+ SALM 0.01 mg/kg	.972
Ts65Dn + Sal/ Ts65Dn+ SALM 0.01 mg/kg	**.002**

Table 5. The table reports the p values of Fisher's LSD test after one-way ANOVA on the effect of SALM 0.01mg/kg on the number of segments of the dendritic tree of the granule cells in the dentate gyrus of Ts65Dn mice.

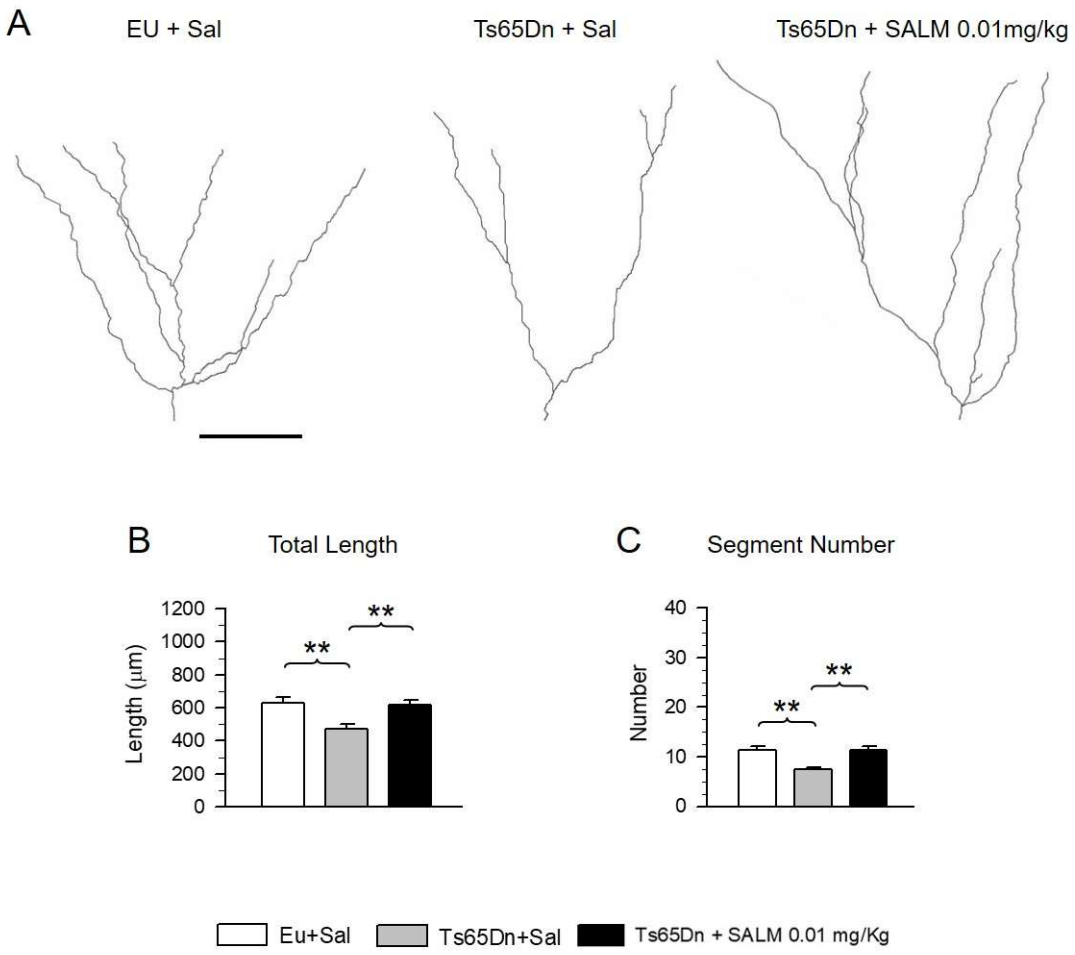


Fig. 4. Effect of SALM 0.01 mg/kg on the dendritic size of the granule cells of Ts65Dn and euploid mice.
A: Examples of the reconstructed dendritic tree of granule cells from animals of each of the following experimental groups: EU + Sal, Ts65Dn + Sal and Ts65Dn + SALM 0.01 mg/kg. Calibration bar 50 µm. B: mean total dendritic length of EU + SAL n=5, Ts65Dn + SAL n=4 and Ts65Dn + SALM 0.01 mg/kg n=4. C: mean number of dendritic segments of the same mice. Values in B,C represent mean ± SE. ** $p < 0.01$ (Fisher's LSD test after one-way ANOVA). Abbreviations: Eu, euploid; Sal, saline; SALM, salmeterol.

EFFECT OF SALMETEROL ON THE BODY WEIGHT

We evaluated the body weight of P15 mice that received saline or SALM (0.01 and 1.0 mg/kg) in order to establish the outcome of neonatal treatment on growth. A one-way ANOVA on the body weight of Ts65Dn mice revealed a significant effect of treatment [$F(2,24) = 5.695$, p .009]. A *post hoc* Fisher's LSD test showed that the dose of 0.01 mg/kg caused a significant increase (+29%) in the body weight in comparison with saline-treated Ts65Dn mice, while the higher dose (1.0 mg/kg) had no effect on the body weight (Fig. 5A, table 6).

		Body Weight
		p
Ts65Dn + Saline	Ts65Dn + SALM 0.01 mg/kg	**.003**
	Ts65Dn + SALM 1.0 mg/kg	.256
Ts65Dn + SALM 0.01 mg/kg	Ts65Dn + SALM 1.0 mg/kg	**.041**

Table 6. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of different concentrations of SALM on the body weight of Ts65Dn mice.

The Kruskal-Wallis test on the body weight of euploid mice revealed a significant effect of treatment [$\chi2(2) = 23.502$, $p< .001$]. The Mann-Whitney tests showed an increase in the body weight with both doses of SALM compared to saline-treated euploid mice (Fig. 5B, Table 7). No difference was found in the magnitude of the effect elicited by the two doses of SALM. The results of the Mann-Whitney test (U, p) are summarized in the Table 7.

		Body Weight	
	p	U	p
Euploid + Saline	Euploid + SALM 0.01 mg/kg	61.500	**<.001**
Euploid + Saline	Euploid + SALM 1.0 mg/kg	31.000	**<.001**
Euploid + SALM 0.01 mg/kg	Euploid + SALM 1.0 mg/kg	93.000	.187

Table 7. The table reports the U and p values of the Mann-Whitney test after the Kruskal-Wallis test on the effect of different concentrations of SALM on the body weight of Euploid mice.

The Kruskal-Wallis test on the body weight of euploid and Ts65Dn mice revealed a significant effect of treatment [$\chi2(1) = 41.623$, $p < .001$]. The Mann-Whitney test showed that the body weight of saline-treated Ts65Dn mice and of Ts65Dn mice treated with different doses of SALM was significantly reduced in comparison with that of saline-treated euploid mice (Fig. 5A,B). The results of the Mann-Whitney test (U, p) are summarized in Table 8.

		Body Weight	
		U	p
Euploid + Saline	Ts65Dn + Saline	1.000	**<.001**
	Ts65Dn + SALM 0.01 mg/kg	50.500	**.020**
	Ts65Dn + SALM 1.0 mg/kg	9.500	**<.001**
Euploid + SALM 0.01 mg/kg	Ts65Dn + Saline	1.000	**<.001**
	Ts65Dn + SALM 0.01 mg/kg	16.000	**.002**
	Ts65Dn + SALM 1.0 mg/kg	8.000	**<.001**
Euploid + SALM 1 mg/kg	Ts65Dn + Saline	0.000	**<.001**
	Ts65Dn + SALM 0.01 mg/kg	6.500	**<.001**
	Ts65Dn + SALM 1.0 mg/kg	0.000	**<.001**

Table 8. The table reports the U and p values of the Mann-Whitney test after the Kruskal-Wallis test on the effect of different concentrations of SALM on the body weight of Euploid and Ts65Dn mice.

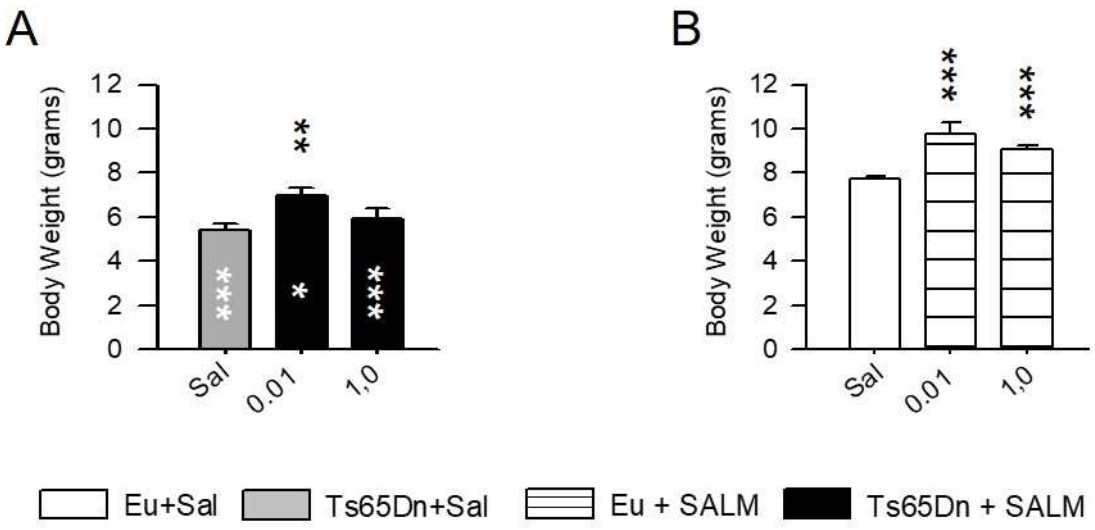


Fig. 5. Effect of neonatal treatment with SALM on the body weight of P15 Ts65Dn and euploid mice.
A-B: Body weight of Ts65Dn (A) and euploid (B) mice that were treated with saline (Ts65Dn mice: n=9; euploid mice: n=24), 0.01 mg/kg SALM (Ts65Dn mice: n=9; euploid mice: n=16) and 1.0 mg/kg SALM (Ts65Dn mice: n=9; euploid mice: n=16), in the period P3-P15, measured on P15. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney test after Kruskal-Wallis test). Black asterisks above the bars indicate an effect of treatment in each genotype (Fisher LSD test after one-way ANOVA or Mann-Whitney test after Kruskal-Wallis test) and white asterisks in the bars indicate a difference between Ts65Dn mice and saline-treated euploid mice (Mann-Whitney test after Kruskal-Wallis test). Abbreviations: Eu, euploid; Sal, saline; SALM, salmeterol.

DISCUSSION OF SECTION 2

NEONATAL TREATMENT WITH SALM DOES NOT RESTORE HIPPOCAMPAL NEUROGENESIS

Results show that a dose of 1.0 mg/kg of SALM does not increase the number of NPCs in the dentate gyrus of Ts65Dn and euploid mice. This result is consistent with the absence of effects observed here following treatment with the same dose of CL. We did not examine the effect of a dose of SALM of 2.0 mg/kg based on the finding that treatment with 2.0 mg/kg of CL had negative effects on the body weight of Ts65Dn mice. We found that a dose of 1.0 mg SALM does not affect the body weight of Ts65Dn mice. It remains to be established whether a dose of 2.0 mg/kg of SALM favors the proliferation of NPCs in the dentate gyrus of Ts65Dn mice and whether this dose has a negative effect on the body mass, similarly to CL. A perusal of the literature shows that the effects of SALM on neurogenesis have been less extensively explored than those of CL. The available evidence shows that SALM (1–100 nM) significantly increased the proliferation of neural precursor cells of the dentate gyrus in a dose-dependent manner (Masuda et al., 2012), indicating that SALM has pro-neurogenic effects similarly to CL. The absence of effects on neurogenesis in the Ts65Dn mouse *in vivo* can be explained following the same lines of reasoning reported in the Discussion of Section 1, regarding CL.

NEONATAL TREATMENT WITH SALM RESTORES THE DENDRITIC DEVELOPMENT OF GRANULE CELLS IN THE TS65DN MOUSE

We found that SALM fully restored dendritic development. In particular, Ts65Dn mice treated with SALM underwent an increase in spine density that became similar to that of euploid mice. This effect took place along the whole extent of the dendritic tree of the granule cells. We have tested here the effects of a dose of 0.01 and 1.0 mg/kg of SALM and found no difference in the magnitude of the effects on spine density. This finding appears of relevance because it shows that a dose of 0.01 mg/kg of SALM is sufficient to fully restore spine density in Ts65Dn mice. In euploid mice SALM did not increase spine density, save for a very small increase elicited by the dose of 1.0 mg/kg in the distal dendrites of the granule cells. Regarding the dendritic size, treatment restored the total length and the number of segments of dendritic tree of the granule cells of TS65Dn mice, that became similar to those of euploid mice.

Regarding the mechanisms underlying the effects of SALM on the dendritic arbor of the granule cells, the same line of reasoning of the Discussion of Section 1 can be used in order to explain the effects of SALM. It is worthwhile noting that conditioned media from glial cells treated with SALM has effects on neurite complexity similar to those elicited by CL and also elicits an increase in glial expression of various growth factors (Day et al., 2014). This suggests that, in addition to cell autonomous effects, soluble factors released by astrocytes may concur to mediate the effects of SALM on the dendritic arbor of the granule cells.

NEONATAL TREATMENT WITH SALM INCREASES THE BODY WEIGHT OF TS65DN AND EUPLOID MICE

Results showed that euploid mice treated with SALM underwent a body weight increase and that the same effect took place in Ts65Dn mice treated with 0.01 mg/kg SALM. This is consistent with evidence that SALM has anabolic effects, although these effects were seen with very high doses of SALM (Moore et al., 1994). In our study, in contrast, the body weight increase took place with much lower doses. As mentioned above, Ts65Dn mice are constitutively smaller and more fragile than the euploid counterparts (Reeves et al., 1995). The finding that treatment with 0.01 mg/kg SALM caused a large increase in the body weight of Ts65Dn mice suggests that this treatment not only does exert a benefit on brain development, but that it also ameliorates the somatic growth. This effect may be related to activation of peripheral β2-AR in the muscle cells and/or other body organs with a consequent enhanced anabolic response. It is also possible that this effect is due to activation of β2-AR in the hypothalamic centers that regulate the metabolic rate and the food intake.

SECTION 3: COMPARISON OF THE EFFECTS OF NEONATAL TREATMENT WITH CLENBUTEROL OR SALMETEROL ON DENDRITIC SPINE DENSITY

COMPARISON OF THE EFFECTS OF A LOW DOSE OF CLENBUTEROL AND SALMETEROL ON DENDRITIC SPINE DENSITY

The previous sections showed that CL and SALM had almost no effects on neural precursor cell proliferation and that only a very high dose of CL was able to partially increase the number of proliferating cells in Ts65Dn mice. In contrast, all doses of CL and SALM were able to increase dendritic spine density in Ts65Dn mice. In order to establish which treatment has greater impact on spinogenesis and dendritic complexity, we first compared the effects of a low dose (0.01 mg/kg) of CL and SALM. A one-way ANOVA on spine density in the proximal dendrites of Ts65Dn mice revealed a significant effect of treatment [$F(2,16) = 310.584$, $p < .001$]. A *post hoc* Fisher's LSD test showed that a dose of CL of 0.01mg/kg had a larger effect in comparison with the same dose of SALM on spine density (Fig. 1A; Table 1). A Kruskal-Wallis test on the distal spines of Ts65Dn mice revealed a significant effect of treatment [$\chi2(2) = 14.147$, $p = .001$]. The Mann-Whitney test showed the lowest dose of CL had a larger effect in comparison with the same dose of SALM on spine density (Fig. 1C; Table 1).

		Proximal Spines / 20 µm	Distal Spines / 20 µm	
		p	U	p
Ts65Dn + Saline	Ts65Dn + CL 0.01 mg/kg	**<.000**	**.000**	**.010**
	Ts65Dn + SALM 0.01 mg/kg	**<.000**	**.000**	**.002**
Ts65Dn + CL 0.01 mg/kg	Ts65Dn + SALM 0.01 mg/kg	**<.000**	**.000**	**.025**

Table 1. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of a low dose (0.01 mg/kg) of CL and SALM on spine density in proximal dendritic branches and the U and p values of the Mann-Whitney test after the Kruskal-Wallis test on spine density in distal dendritic branches of the granule cells of Ts65Dnmice.

A one-way ANOVA on the proximal spines of euploid mice revealed a significant effect of treatment [$F(2,16) = 38.768$, $p < .001$]. A *post hoc* Fisher's LSD test showed that while a dose of CL of 0.01 mg/kg caused an increase in spine density in comparison with saline-treated euploid mice, the same dose of SALM was ineffective (Fig.1B; Table 2). A one-way ANOVA on the distal spines of euploid mice revealed a significant effect of treatment [$F(2,16) = 34.991$, $p < .001$]. A *post hoc* Fisher's LSD test showed that while a dose of CL of 0.01mg/kg caused an increase in spine density in comparison with saline-treated euploid mice, the same dose of SALM was ineffective (Fig1D; Table2).

		Proximal Spines / 20 µm	Distal Spines / 20 µm
		p	p
Euploid + Saline	Euploid + CL 0.01 mg/kg	**<.000**	**.000**
	Euploid + SALM 0.01 mg/kg	.841	.710
Euploid + CL 0.01 mg/kg	Euploid + SALM 0.01 mg/kg	**<.000**	**.000**

Table 2. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of a low dose (0.01 mg/kg) of CL and SALM on spine density in proximal and distal dendritic branches of the granule cells of euploid mice.

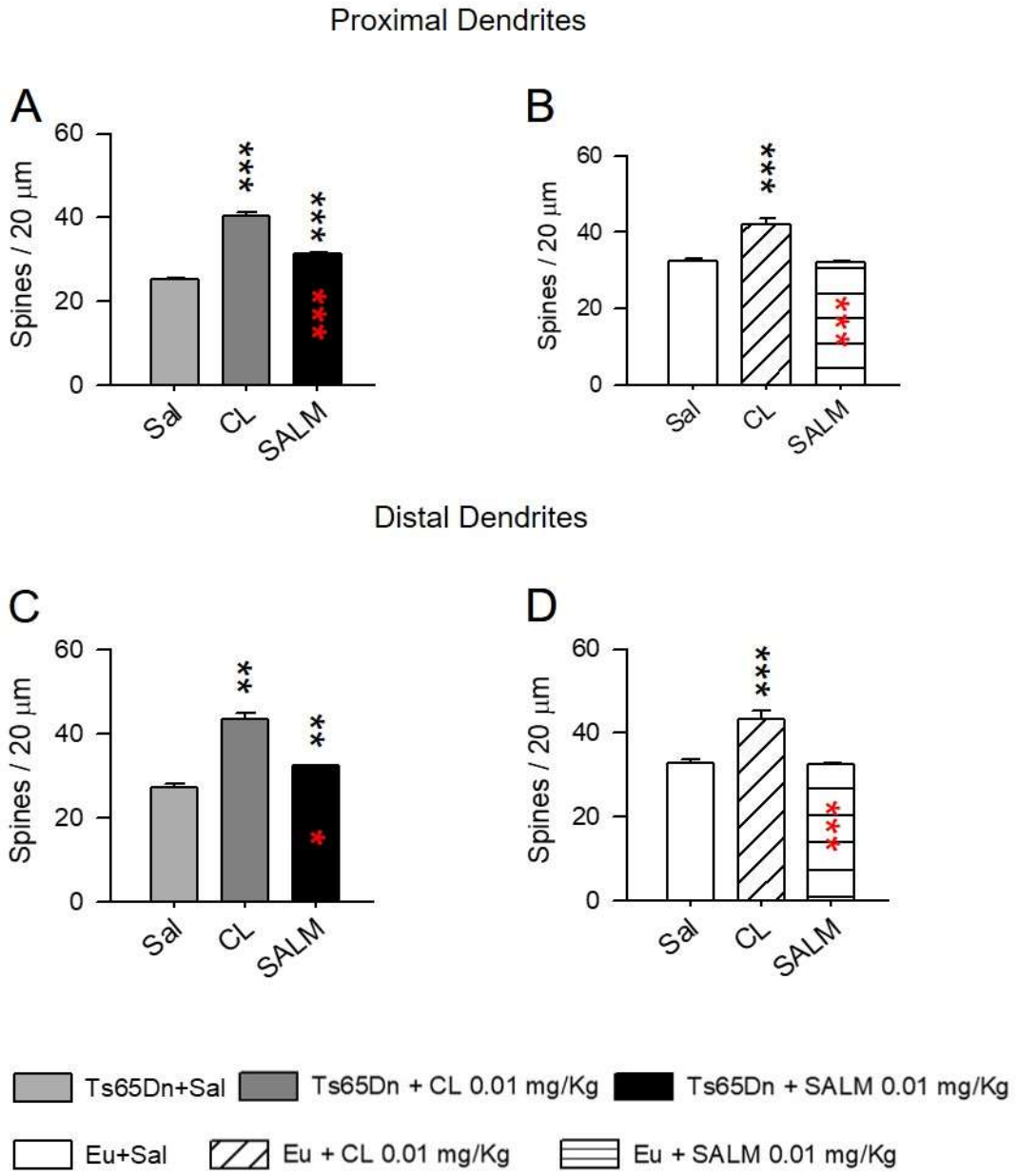


Fig. 1. Comparison of the effects of neonatal treatments with clenbuterol or salmeterol at 0.01mg/kg on dendritic spine density of P15 Ts65Dn and euploid mice.

A, D: Spine density (mean ± SE) in the proximal dendrites of the granule cells of Ts65Dn (A) and euploid (B) mice and in the distal dendrites of Ts65Dn (C) and euploid (D) mice that were treated with saline (Ts65Dn mice: n=5; euploid mice: n=5), 0.01 mg/kg CL (Ts65Dn mice: n=3; euploid mice: n=3) and 0.01 mg/kg SALM (Ts65Dn mice: n=5; euploid mice: n=5) in the period P3-P15. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Fisher's LSD test after one-way ANOVA or Mann-Whitney test after Kruskal-Wallis test). Black asterisks above the bars indicate an effect of treatment in each genotype (Fisher's LSD test after one-way ANOVA or Mann-Whitney test after Kruskal-Wallis test), and red asterisks in the bars indicate a difference between the effects of treatment with CL and SALM in Ts65Dn and euploid mice (Fisher's LSD test after one-way ANOVA or Mann-Whitney test after Kruskal-Wallis test). Abbreviations: CL, clenbuterol; Eu, euploid; Sal, saline; SALM, salmeterol.

COMPARISON OF THE EFFECTS OF A HIGH DOSE OF CLENBUTEROL AND SALMETEROL ON DENDRITIC SPINE DENSITY

We next compared the effects of a high dose of CL and SALM. A one-way ANOVA on spine density in the proximal dendrites of Ts65Dn mice revealed a significant effect of treatment [$F(2,16) = 156.291$, $p < .001$]. A *post hoc* Fisher's LSD test showed that a dose of CL of 1.0 mg/kg had a larger effect in comparison with the same dose of SALM on spine density (Fig. 2A; Table 3). A Kruskal-Wallis test on spine density in the distal dendrites of Ts65Dn mice revealed a significant effect of treatment [$\chi2(2) = 13.185$, $p = .001$]. The Mann-Whitney test showed that CL had a larger effect in comparison with the same dose of SALM (Fig. 2C; Table 3).

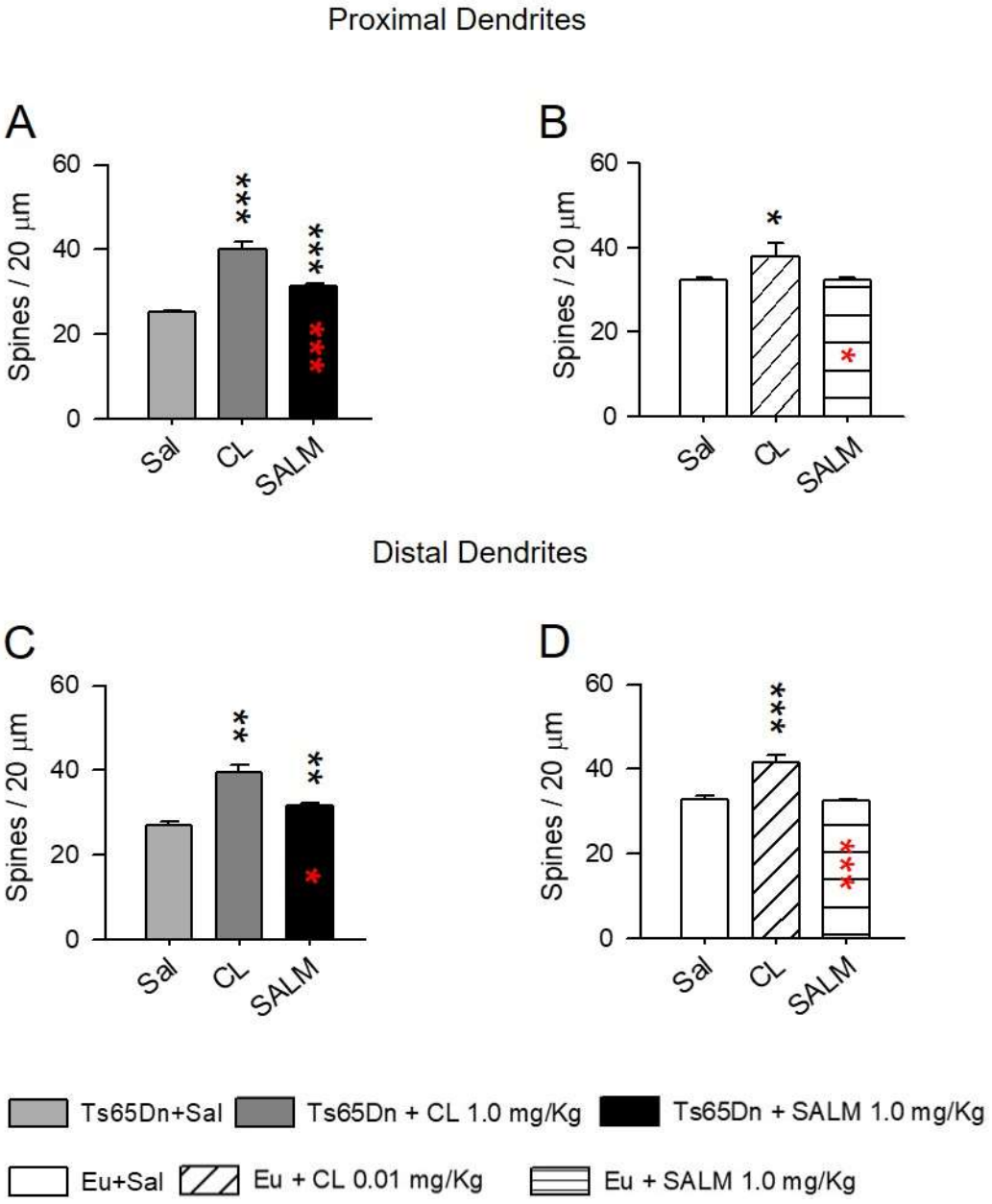
		Proximal Spines / 20 µm	Distal Spines / 20 µm	
		p	U	p
Ts65Dn + Saline	Ts65Dn + CL 1.0 mg/kg	**<.000**	**.000**	**.010**
	Ts65Dn + SALM 1.0 mg/kg	**<.000**	**2.000**	**.004**
Ts65Dn + CL 1.0 mg/kg	Ts65Dn + SALM 1.0 mg/kg	**<.000**	**.000**	**.024**

Table 3. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of a high dose (1.0 mg/kg) of CL and SALM on spine density on proximal branches of the granule cells in the dentate gyrus of Ts65Dn mice and the U and p values of the Mann-Whitney test after the Kruskal-Wallis test on spine density on distal branches of the granule cells of Ts65Dn mice.

A Kruskal-Wallis test on spine density in the proximal dendrites of euploid mice revealed a significant effect of treatment [$\chi2(2) = 7.412$, $p = .025$]. The Mann-Whitney test showed that CL had a larger effect on spine density in comparison with the same dose of SALM on euploid mice (Fig. 2B; Table 4). A one-way ANOVA on spine density in the distal dendrites of euploid mice revealed a significant effect of treatment [$F(2,16) = 25.512$, $p < .001$]. A *post hoc* Fisher's LSD test showed that CL had a larger effect in comparison with the same dose of SALM (Fig. 2D; Table 4).

		Proximal Spines / 20 µm		Distal Spines / 20 µm
		U	p	p
Euploid + Saline	Euploid + CL 1.0 mg/kg	.000	**.010**	**<.001**
	Euploid + SALM 1.0 mg/kg	23.500	.648	.660
Euploid + CL 1.0 mg/kg	Euploid + SALM 1.0 mg/kg	.000	**.025**	**<.001**

Table 4. The table reports the U and p values of the Mann-Whitney test after the Kruskal-Wallis test on the effect of a high dose (1.0 mg/kg) of CL and SALM on spine density on proximal branches of the granule cells in the dentate gyrus of Ts65Dn mice and the p values of the Fisher's LSD test after one-way ANOVA on the effect of the higher dose of CL and SALM on spine density on distal dendritic branches.



A, D: Spine density (mean ± SE) on the proximal dendrites of the granule cells of Ts65Dn (A) and euploid (B) mice and on the distal dendrites of Ts65Dn (C) and euploid (D) mice that were treated with saline (Ts65Dn mice: n=5; euploid mice: n=5), 1.0 mg/kg CL (Ts65Dn mice: n=3; euploid mice: n=3) and 1.0 mg/kg SALM (Ts65Dn mice: n=5; euploid mice: n=5) in period P3-P15. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Fisher LSD test after one-way ANOVA or Mann-Whitney test after Kruskal-Wallis test). Black asterisks above the bars indicate an effect of treatment in each genotype (Fisher's

LSD test after one-way ANOVA or Mann-Whitney test after Kruskal-Wallis test), and red asterisks in the bars indicate a difference between the effect of CL and SALM in Ts65Dn and euploid mice (Fisher's LSD test after one-way ANOVA or Mann-Whitney test after Kruskal-Wallis test). Abbreviations: CL, clenbuterol; Eu, euploid; Sal, saline; SALM, salmeterol.

COMPARISON OF THE EFFECTS OF A LOW DOSE OF CLENBUTEROL AND SALMETEROL ON THE DENDRITIC TREE

We compared the effects of a low dose of CL and SALM on the dendritic complexity of the granule cells. A one-way ANOVA on the total length of the dendritic tree of Ts65Dn mice revealed a significant effect of treatment [$F(2,8) = 7.140$, $p = .017$]. A *post hoc* Fisher's LSD test showed that treatments with both doses (CL 0.01 mg/kg and SALM 0.01 mg/kg) had the same effect on the dendritic length (Fig. 3B; Table 5).

	Total length
	p
Ts65Dn + Sal/ Ts65Dn+ CL 0.01 mg/kg	**.043**
Ts65Dn + Sal/ Ts65Dn+ SALM 0.01 mg/kg	**.006**
Ts65Dn + CL 0.01 mg/kg/ Ts65Dn+ SALM 0.01 mg/kg	.341

Table 5. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the effect of a low dose (0.01 mg/kg) of CL and SALM on the total length of dendritic tree of the granule cells in the dentate gyrus of Ts65Dn mice.

A one-way ANOVA on the number of dendritic segments of the granule cells in Ts65Dn mice revealed a significant effect of treatment [$F(2,8) = 33.744$, $p < .001$]. A *post hoc* Fisher's LSD test showed that a dose of CL 0.01 mg/kg had a larger effect in comparison with the same dose of SALM on the number of dendritic segments (Fig. 3C; Table 6).

	Number of segments
	p
Ts65Dn + Sal/ Ts65Dn+ CL 0.01 mg/kg	**<.001**
Ts65Dn + Sal/ Ts65Dn+ SALM 0.01 mg/kg	**<.001**
Ts65Dn + CL 0.01 mg/kg/ Ts65Dn+ SALM 0.01 mg/kg	**.050**

Table 6. The table reports the p values of the Fisher's LSD test after one-way ANOVA on the number of dendritic segments of the granule cells in Ts65dn mice treated with a low dose (0.01 mg/kg) of CL and SALM.

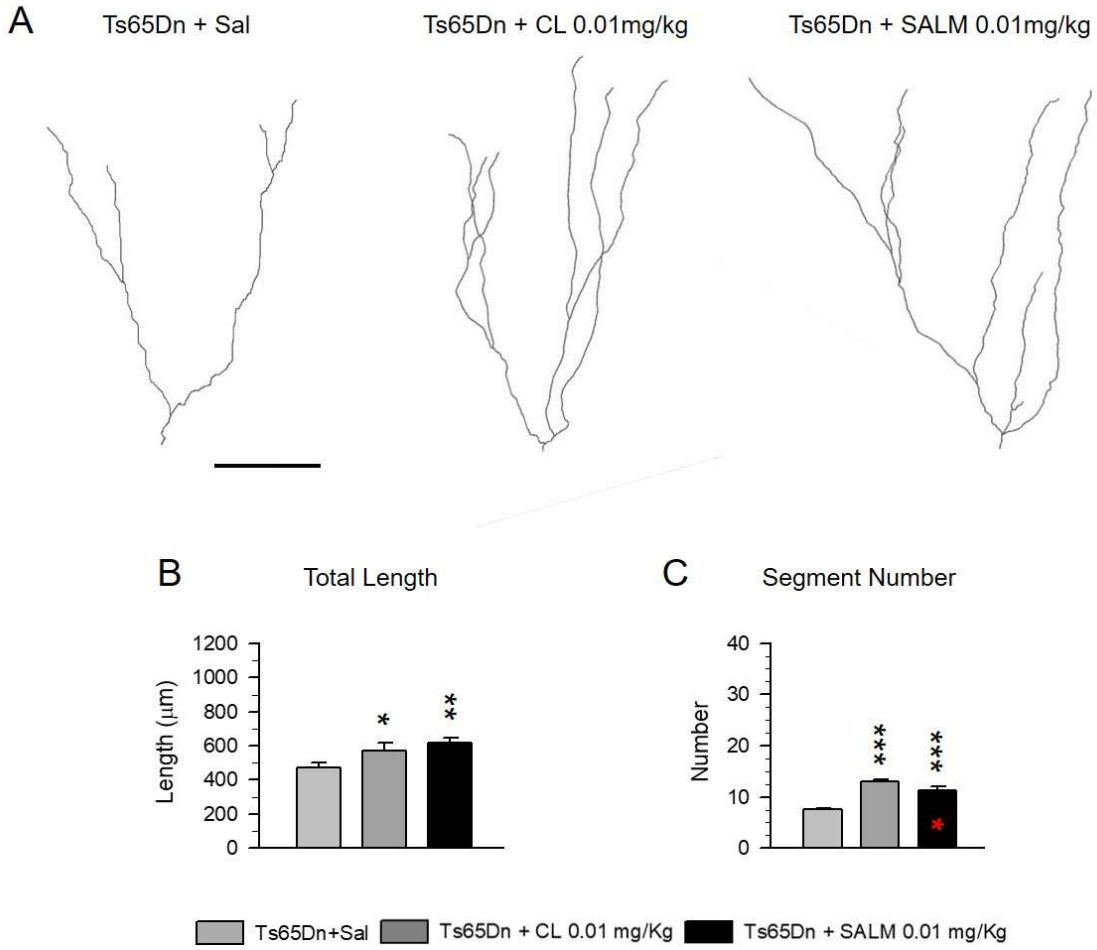


Fig. 3. Comparison of the effects of neonatal treatments with clenbuterol or salmeterol 0.01 mg/kg on the dendritic tree of P15 Ts65Dn mice.

A: Examples of reconstructed dendritic tree of granule cells from animals of each of the following experimental groups: Ts65Dn + Sal, Ts65Dn + CL 0.01 mg/kg and Ts65Dn + SALM 0.01 mg kg. Calibration bar 50 µm. B: mean total dendritic length of Ts65Dn + SAL n=4, Ts65Dn + CL 0.01 mg/kg n=3 and Ts65Dn + SALM 0.01 mg kg n=4. C: mean number of dendritic segments of the dendritic tree of the same mice. Values in B,C represent mean ± SE. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Fisher LSD test after one-way ANOVA test). Black asterisks above the bars indicate an effect of treatment in each genotype (Fisher LSD test after one-way ANOVA test), and red asterisks in the bars indicate a difference between the effect of CL and SALM in Ts65Dn mice (Fisher LSD test after one-way ANOVA test). Abbreviations: CL, clenbuterol; Eu, euploid; Sal, saline; SALM, salmeterol.

DISCUSSION OF SECTION 3

CLENBUTEROL EXERTS LARGER EFFECTS ON THE DENDRITIC TREE OF THE GRANULE CELLS IN COMPARISON WITH SALMETEROL

Results show that both CL and SALM enhance dendritic development of the granule cells, although some differences must be underlined. 1) The doses of 0.01 and 1.0 mg/kg of SALM enhance spine density in the proximal and distal dendrites of Ts65Dn mice, but their effect is significantly smaller in comparison with the effects elicited by the same doses of CL. 2) The doses of 0.01 and 1.0 mg/kg of SALM do not enhance spine density in the proximal and distal dendrites of the granule cells of euploid mice. In contrast, the same doses of CL largely enhance spine density in euploid mice. 3) The dose of 0.01 mg/kg of SALM increases the number of dendritic branches of the granule cells of Ts65Dn mice, but this effect is smaller in comparison with that elicited by the same dose of CL.

Although all β2-AR agonists have as final target the β2-AR, their efficacy may largely vary in relation to their pharmacokinetics. The latter, in turn, depends on the absorption in the gastrointestinal tract (in case of oral intake), metabolic processing and renal excretion. In addition, the effects of β2-AR agonists on the brain depend on their BBB permeability. Based on the duration of their effects, the β2-AR have been classified as SABAs, LABAs and ultra LABAs. Both CL and SALM belong to the class of LABAs. Among the LABAs, CL is readily absorbed (70–80%) from the gastrointestinal tract and has a greater potency in comparison with other members of the same class (Spiller et al., 2013). A comparison of CL and SALM shows that CL has a greater permeability across the BBB and a longer half-life (25–40 hours), in comparison with SALM (5.5 hours) (see the Section “The β2 agonists used in the current study”). The large BBB permeability of CL in conjunction with its long half-life implies that CL reaches larger brain levels than SALM and for a more prolonged time. This, in turn, means that CL can exert larger and more prolonged biological effects on the brain in comparison with SALM. Thus, the pharmacokinetics of CL can account for its larger effects on the development of the dendritic tree of the granule cells observed in the current study.

GENERAL DISCUSSION

B2-AR AGONISTS RESCUE DENDRITIC PATHOLOGY IN TS65DN MICE

The current study provides novel evidence that a brief pharmacological therapy (P3-P15) with two β2-AR agonists, CL or SALM, in the early postnatal period fully rescues dendritic pathology in the hippocampal dentate gyrus of the Ts65Dn mouse model of DS. In particular, treatment was able to restore both the length and number of branches of the dendritic tree as well as dendritic spine density. The latter effect took place at all levels of the dendritic tree and spine density was restored in proximal and distal dendritic branches.

The effects of CL and SALM on the whole dendritic tree of the granule cells is of relevance considering that the inputs to the granule cell dendrites are laminarly organized. The major extrinsic input to the hippocampal formation is represented by the perforant pathway, which takes its origin from the medial and lateral divisions of the entorhinal cortex (Amaral, 1995). Both inputs are fundamental for the participation of the hippocampal formation in long-term memory functions. The medial perforant pathway terminates on the middle third of the dendritic tree of the granule cells, while the lateral perforant pathway terminates on the outer third. The entorhinal cortex plays a crucial role in the memory systems of the temporal lobe because it receives signals from polymodal association cortices and transmits these signals to the hippocampal formation. The granule cells of the dentate gyrus are the first element of the so called “trisinaptic circuit” which is formed by the granule cells of the dentate gyrus, field CA3 pyramidal neurons and field CA1 pyramidal neurons. Signals from the entorhinal cortex are processed by this circuit and then sent back to the entorhinal cortex, which in turn, transmits these processed signals to polymodal association cortices. The treatment-induced increase in spine density on proximal and distal dendrites of the granule cells suggests that this effect may lead to restoration of connectivity from both divisions of the entorhinal cortex. The restoration of connectivity, in turn, implies restoration of the processing of signals that are fundamental for memory encoding/retrieval. Thus, treatment with CL or SALM may lead to improvement/restoration of the learning and memory defects that characterize the trisomic brain.

Our results show that treatment with doses that were sufficient to rescue dendritic pathology did not enhance the proliferation rate of the progenitors of the granule cells. We found here that a high dose of CL (2.0 mg/kg) was able to induce an increase in the proliferation rate of the hippocampal NPCs but this dose is well above the doses usable in humans (see below). Our results are in agreement with evidence that adult (5–6-month) Ts65Dn mice treated with the β2-AR agonist formoterol (2.0 mg/kg/day) did not undergo an increase in the number of new granule neurons (DCX-positive cells) in the DG. Formoterol-treated Ts65Dn mice, however, showed a significant increase in the number of dendritic branches (Dang et al., 2014). To the best of our knowledge, no other studies have examined the effects of other β2-AR in Ts65Dn mice. A study has explored the effect of xamoterol, a partial agonist of β1-AR on the behavior of adult Ts65Dn mice (Salehi et al., 2009, Faizi et al., 2011). Results show that treatment restores some of the behavioral deficits of Ts65Dn

mice, which emphasizes the importance of the noradrenergic system in the modulation of hippocampal function.

Neurogenesis impairment and dendritic pathology (hypotrophy of the dendritic tree accompanied by reduction in spine density) are the major developmental defects of DS. The current results suggest that treatment with β2-AR agonists may be a therapy of choice in order to correct one of the two major developmental defects of DS. It can be envisaged that combined therapy with CL or SALM and a drug than restores neurogenesis may represent a potential strategy for the overall restoration of brain development in DS.

POTENTIAL USE OF CL OR SALM AS THERAPY FOR DS?

A previous study showed that treatment with the β2-AR agonist formoterol restores neurogenesis, dendritic complexity and behavior in adult Ts65Dn mice (Dang et al., 2014). Since a high dose of formoterol was used (2.0 mg/kg/day), mice were co-treated with an antagonist of the β2-AR that does not cross the BBB in order to prevent peripheric effects of treatment. Thus, the study by Dang et al. remains at the proof of principle level because such a therapy may result impracticable in individuals with DS.

We found here that both CL and SALM increase the dendritic length and the density of dendritic spine in neonate Ts65Dn mice and that this effect was elicited even with the smallest dose tested here (0.01 mg/kg). While a dose of SALM of 0.01 mg/kg normalized spine density, the same dose of CL was able to enhance spine density to values that were even larger in comparison with euploid mice. The finding that the dose of 0.01 mg/kg of CL caused an increase in spine density in Ts65Dn mice to levels larger than spine density in saline- treated euploid mice suggests that a dose of CL lower than 0.01 mg/kg may be sufficient to induce restoration of dendritic pathology. This is of obvious relevance considering that a low dose of a given drug reduces the probability of unwanted side effects. Indeed, although CL and SALM are specific β2-AR agonist, the fact that high doses might also activate β1 receptors, thereby affecting the heart function, cannot be overlooked. Moreover, in the heart in addition to predominant β1 receptors, there is a small population of b β2-AR (Bernstein et al., 2011), which must be taken into account with treatment with high doses of β2-AR agonists.

A critical issue in animal studies aimed at exploring the effects of different drugs to combat different diseases regards the translation of the tested doses from animals to humans. The formula reported in Fig.1 is considered appropriate for this translation (Reagan-Shaw et al., 2008)

Species	Weight (kg)	BSA (m^2)	K_m factor
Human			
Adult	60	1.6	37
Child	20	0.8	25
Baboon	12	0.6	20
Dog	10	0.5	20
Monkey	3	0.24	12
Rabbit	1.8	0.15	12
Guinea pig	0.4	0.05	8
Rat	0.15	0.025	6
Hamster	0.08	0.02	5
Mouse	0.02	0.007	3

Formula for Dose Translation Based on BSA

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \text{ multiplied by } \frac{\text{Animal } Km}{\text{Human } Km}$$

Fig. 1 Formula for dose translation based on BSA. From (Reagan-Shaw et al., 2008).

Base on this formula, in Table 1 we report the human doses equivalent to the CL and SALM doses used here. It can be noted that a dose of 0.01 mg/kg of either agonist in mice corresponds to a dose of 0.0012 mg/kg in children and a dose of 2.0 mg/kg in mice corresponds to a dose of 0.24 mg/kg, in children. CL and SALM are used as therapeutic treatment for asthma (Inhalation powder) in children and adults at doses of 0.04 mg/kg twice daily for CL (Spiller et al., 2013) and 0.05 mg/day twice daily for SALM (Chapman et al., 1999). Considering the average weight of a child about 30 kg, these doses correspond to be 0.0027 mg/kg/day for CL and 0.0033 mg/kg/day for SALM. These values indicate that the doses of CL and SALM that are sufficient to restore the defects of the dendritic tree of the granule cells in Ts65Dn mice are in the range of the therapeutic doses.

Mouse	Adult	Child
Doses of CL or SALM		
0.01 mg/kg	0.00081 mg/kg	0.0012 mg/kg
0.5 mg/kg	0.04 mg/kg	0.06 mg/kg
1.0 mg/kg	0.081 mg/kg	0.12 mg/kg
2.0 mg/kg	0.16 mg/kg	0.24 mg/kg

Table 1. reported the concentrations we used in mice converted into human doses.

A study has evaluated the bronchodilating activity of CL in asthmatic children after single administration of different oral doses (0.5, 1.0, and 1.5 µg/kg), in order to assess possible adverse effects (Boner et al., 1987). The results of this study show that the dose of 1.0 µg/kg (i.e. 0.001 mg/kg) is the most advisable for children and has no effects on heart rate. Again, this dose (after translation) is in the range of the dose of CL and SALM that is sufficient to restore dendritic development in Ts65Dn mice.

We treated Ts65Dn mice in the neonatal period because this is a critical time window for hippocampal development. Further preclinical studies are needed in order to establish the long-term effect of neonatal treatment with CL or SALM on cognitive performance when mice reach adult life stages. In addition, it is important to establish whether treatment has positive effects even when administered at more advanced

life stages. Based on results obtained here with doses of CL and SALM that are practicable in children, we believe it may not be unreasonable to envisage a therapy with either CL or SALM for the improvement of brain development in children with DS. In the current study, we treated mice during the early postnatal period (P3-P15). Translation of the developmental time across mice and humans shows that the first two postnatal weeks of mice roughly correspond to the third trimester of gestation in humans (Clancy et al., 2001). This implies that treatment during the last period of pregnancy may be particularly suitable in order to rescue dendritic development and, possibly, other DS-related brain phenotypes. CL has been used in domestic animals (e.g. sheep and cattle) in order to increase their body mass (Moore et al., 1994). However, the use of their meats can cause adverse effects in humans. Therefore, the use of CL is no longer approved by FDA since the year 2006 (see web site link 2). The approved use of CL in the United States is currently limited to equine use as a bronchodilator. An additional reason for the exclusion of CL for humans is its misuse and abuse for weight loss and body building (Spiller et al., 2013). CL, however, is still available in Europe and Latin America as a bronchodilator in humans (Spiller et al., 2013); see web site link 3). SALM, however, is currently an approved drug by both FDA, and EMA. Thus, although CL has larger effects than SALM a therapy with CL for DS may be proposed in some countries only. Although SALM is less powerful than CL, a therapy with SALM has the advantage that it may be proposed for clinical trial worldwide. We hope that our study will prompt additional investigations aimed at better characterizing the effects of CL and SALM in the Ts65Dn mouse model and that the gained knowledge may pave the way to clinical trials in children/adults with DS.

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WEB SITES

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LINK3: https://www.ema.europa.eu/en/documents/psusa/clenbuterol-list-nationally-authorised-medicinal-products-psusa/00000794/201809_en.pdf