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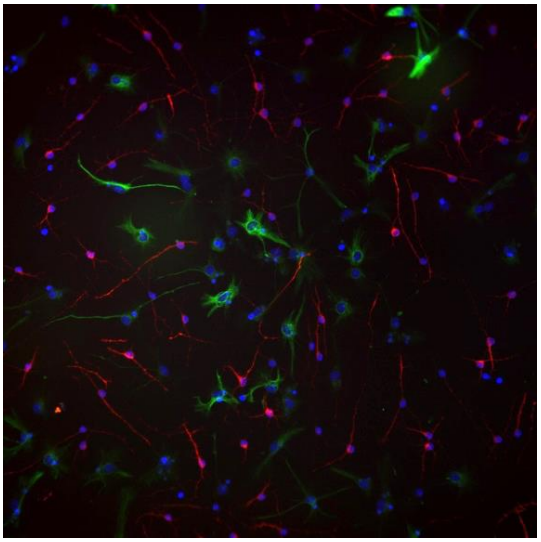
TRISOMIC NEURAL PROGENITOR CELLS AS NOVEL PHARMACOLOGICAL TARGETS IN DOWN SYNDROME



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Abbreviations

Aβ	Amyloid β
ACM	Astrocyte conditioned medium
AD	Alzheimer's disease
APP	Amyloid precursor protein
ARP 2/3	Actin-related protein complex 2/3
BDNF	Brain derived neurotrophic factor
BFCN	Basal forebrain cholinergic neurons
bFGF	Basic fibroblast growth factor
BrdU	5-bromo-2'-deoxyuridine
CA 1, 2	Cornu Ammonis field 1, 2
CFC	Contextual fear conditioning
CHD	Congenital heart disease
CNS	Central nervous system
CSA	Cyclosporine A
DPN	Day post natal
DCX	Doublecortin
DG	Dentate gyrus
DIV	Days *in vitro*
DS	Down syndrome
DSCR	Down syndrome critical region
DYRK1A	Dual specificity tyrosine phosphorylation regulated kinase 1A
ECD	Extracellular domain
EdU	5-ethynyl-2'-deoxyuridine
EGCG	Epigallocatechin-3-gallate
EGF	Epidermal growth factor
EU	Euploid
GABA	Gamma-aminobutyric acid
GAPs	GTPase-activating proteins
GBP	Gabapentin
GC	Glucocorticoids
GEFs	Guanine exchange factors
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
GSK3ß	Glycogen synthase kinase 3 beta
GW	Gestation week
Hsa21	Human chromosome 21
IHC	Immunohistochemistry
iPSCs	Induced pluripotent stem cells

IQ	Intelligence quotient
JAK-STAT	Janus kinase-signal transducer and activator
KO	Knockout
LiCl	Lithium chloride
LTD	Long-term depression
LTP	Long-term potentiation
LA	Linoleic acid
MAP2	Microtubule associated protein 2
Mmu 10,16,17	Mouse chromosome 10,16,17
MWM	Morris water maze
NFATc	Nuclear factor of activated T cell cytoplasmatic
NMDA	N-methyl-D-aspartic acid
NPC	Neural progenitor cells
NSC	Neural stem cells
OA	Oleic acid
OLIG1-2	Oligodendrocyte transcription factor 1 and 2
Ptch1	Patched 1
PGB	Pregabalin
PI3K	Phosphoinositide 3-kinase
PPARs	Peroxisome-proliferator activated receptors
Rac1	Ras-related C3 botulinum toxin substrate 1
RCAN1	Regulator of calcineurin 1
SGZ	Subgranular zone
Shh	Sonic hedgehog
SOD1	Superoxide dismutase 1
Smo	Smoothened
SSRI	Selective serotonin reuptake inhibitor
SVZ	Subventricular zone
S100ß	S100 calcium binding protein B
TM	Trans-membrane
TrkB	Tropomyosin receptor kinase B
TRKs	Tyrosine receptor kinases
TS	Trisomic
TSP-1	Thrombospondin-1
VWF-A	Von Willebrand factor A
VZ	Ventricular zone
WT	Wild type
5-HT	5-hydroxytryptamine
7,8-DHF	7,8-dihydroxyflavone
β2AR	β2 adrenergic receptor

Contents

Chapter 1

1.1 Down syndrome

Down syndrome (DS) is the most common genetic cause of intellectual disability. DS was discovered and, for the first time, clinically described by John Langdon Down in 1866 (Langdon-Down, 1866). Only a century later, in 1959, the triad Lejeune–Gautier–Turpin identified that trisomy 21 (T21) was the genomic abnormality underlying DS (Lejeune, Gautier, & Turpin, 1959). People with DS have experienced an increase in their life expectancy over the past few decades, at least in western countries, thanks to improvements in medical cares and social interaction, passing from 12 years (in 1940) to 60 years (Presson et al., 2013). Nowadays the most invalidating aspect of the disease remains intellectual disability. Thus, the need to identify therapies to improve intellectual disability is becoming urgent (Presson et al., 2013). Despite numerous efforts, at present no therapies are available to rescue brain developmental alterations in DS individuals (Bartesaghi et al., 2011; Kazemi et al., 2016). The following chapters summarize DS phenotypic abnormalities, focusing on neurodevelopmental alterations, preclinical models used in DS research, cell types involved in DS pathophysiology and potential therapeutic approaches.

1.1.1 Epidemiology

DS manifests itself in people of all races and socio-economic status, with a prevalence of 1/700-800 live births. Data showed that the probability of giving birth to a child with DS increases significantly with the mother age (above 35 years). At present the incidence of DS has not decreased despite several prenatal diagnosis, available since the middle of sixties, (Summers et al., 2007) and maternal serum screening (Smith & Visootsak, 2013). Indeed, studies have reported increasing trends in DS pregnancies in various parts of the world ascribed to increased lifespan and maternal age. As shown in Figure 1, Maternal

age is the biggest risk factor with an incidence of 1:1,500 births under age 25 and 1:100 births at age 40 (Fig. 1) (Herault et al., 2017; Loane et al., 2012; McKenzie et al., 2016).

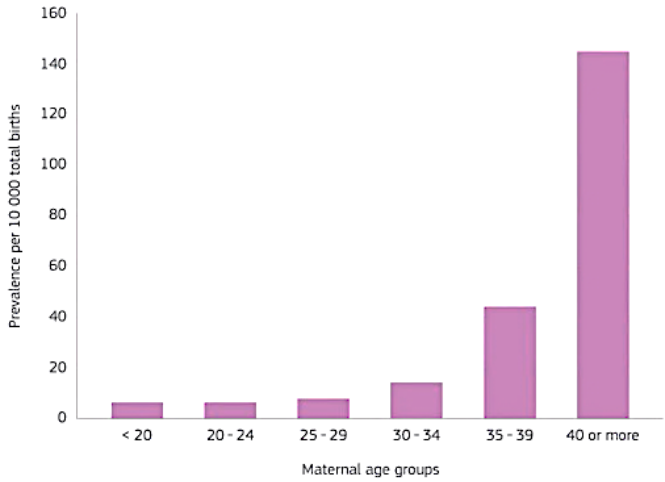

Figure 1. Graph from the European Union showing the prevalence of Down syndrome increasing with maternal age (Lanzoni et al., 2019).

1.1.2 Etiology and the genetics of Down syndrome

The triplication of human chromosome 21 (Hsa21) observed in DS is caused by abnormal cell division that results in an extra full or partial copy of chromosome 21. The most frequent form of DS is full Hsa21 trisomy: chromosome 21 is unable to separate during meiosis in a developing ovum, or less frequently, in sperm, culminating in an extra copy of the entire Hsa21 in all cell types (Antonarakis et al., 2004). Another cytogenetic form of DS is Robertsonian translocation. This condition is less prevalent than full trisomy 21 and occurs only in 2-4% of the cases, where a segment of the chromosome becomes fused to a different chromosome pair (such as chromosomes 13, 14, 15, 22 or 21) (Pelleri et al., 2016). Another genetic rearrangement that can occur in DS is mosaicism. This condition occurs in 3-4 % of DS population: in this case some

cells within a single tissue display a normal karyotype, while others show trisomy (Antonarakis et al., 2017; Rachidi & Lopes, 2011).

In 2000 a consortium performed the sequencing of chromosome 21 (Hsa21). [The most updated and revised version is available in the web, (www.ncbi.nlm.nih.gov/genome/gdv/) (Strippoli et al., 2019)]. Hsa21 is the smallest among human autosomes, consisting of about 46 million base pairs in its DNA, containing 325 non-protein encoding genes and 222 protein coding genes (Gupta et al., 2016). Since 1959 literature data have showed that DS is caused by an extra copy of chromosome 21 (Lejeune et al., 1959), however the mechanisms by which the trisomy disrupts development are still not well understood (El Hajj et al., 2016). Two hypothesis have been proposed to explain the effect of trisomy on brain phenotypes: the "gene-dosage effect" and the "amplified developmental instability". The first one proposes that increased dosage of several dosage-sensitive genes and their encoded proteins determined DS phenotypes (Bartesaghi et al., 2011; Delabar et al., 1993; Korenberg et al., 1994; Lyle et al., 2008). The second hypothesis asserts that trisomy 21 causes a general alteration in developmental homeostasis determining most manifestations of DS (Antonarakis et al., 2004; Bartesaghi et al., 2011; Roizen & Patterson, 2003). These two different theses could coexist in DS pathophysiology and the validity of one of the two hypothesis is still an open discussion (Strippoli et al., 2019).

Furthermore, literature data suggested that the triplication of some Hsa21 genes is sufficient to manifest DS (Aula et al., 1973; Ilbery et al., 1961; Wahlsten et al., 2019) supporting another hypothesis: the "Down syndrome critical region" (DSCR). The DSCR is a region of 3.8-6.5 Mb on 21q21.22, with approximately 33 genes responsible for the majority of DS phenotypes that will be summarized in the following chapters (Asim et al., 2015; Pritchard & Kola, 1999).

Studies showed that also epigenetic changes can occur both in fetal brains and blood from newborn DS infants and contribute to the pathology (Strippoli et al., 2019; Vacca et al., 2019).

1.1.3 Phenotypic traits and clinical features observed in Down syndrome

Down syndrome individuals are characterized by several phenotypic traits and clinical features that occur to some degree in every person with trisomy 21. A typical DS trait is the facial dysmorphology identified in microgenia, flat nasal bridge, oblique eye fissures, a bulging tongue and a short neck. In addition to these phenotypic traits, trisomy 21 is also a risk factor for other several diseases. One of the most invaliding is the congenital heart disease (CHD) which is the main cause of death for the two first years of life in individual with DS, whit a frequency of 40-50 % (Benhaourech et al., 2016; Ferencz et al., 1989; Roper & Reeves, 2006). Other phenotypic features that can affect DS individuals are malformations of the gastrointestinal tract, muscle hypotonia, leukemia, thyroid disorders, such as hypothyroidism, epilepsy and also Alzheimer's disease (AD) (Amr, 2018; Barca et al., 2014; Mateos et al., 2015; Noble, 1998).

In particular, a connection between AD and DS has been long suspected since 50-70 % of DS individuals develop dementia by the age of 40. For this reason trisomy 21 is considered as the most common genetic cause of a neurodegenerative disease (Ballard et al., 2016; Dekker et al., 2018; Herault et al., 2017). An important role for this connection is partially played by the overexpression of amyloid precursor protein, encoded by the gene APP, which increases the risk of early-onset of AD. Indeed, amyloid-β accumulates in the brain across the lifespan of people with DS. APP is not the only one AD-linked protein described in DS literature. Studies reported that the triplication of the gene that encodes for the Dual-specificity tyrosine-(Y) phosphorylation regulated

kinase (DYRK1A) may play an important role in this context. DYRK1A is a proline-directed serine/threonine kinase (Park et al., 2010) proposed as one of the most relevant contributors to the neurological abnormalities that will be summarized in the next paragraph (Dowjat et al., 2007).

1.1.4 The neurological phenotype of Down syndrome

Intellectual disability (ID) is the main feature of DS resulting in a gradually declining intelligence quotient (IQ) during childhood (between 6 months and 2 years). In child IQ varies from 35 (mild) to 70 (moderate) and DS adult individuals usually show a mental age from 8 to 9 years old (Weijerman & De Winter, 2010). Beginning at an early age, DS neurological phenotype seems to be due to impairments in several brain regions such as the cerebellum, the medial temporal lobe, hippocampus and prefrontal cortex (Fig. 2) (Bartesaghi et al., 2011; Nadel, 2003).

In particular, ID affects the area involved in speech (poor and slower), in spatial memory and in long-/short-term memory performances, especially in maintaining phonological information over a short delay. By contrast, DS individuals show a relative preserved visuo-spatial memory ("where" memory) (Bostelmann et al., 2018; Godfrey & Lee, 2018; Vicari, 2006). Implicit memory is preserved in DS children, while explicit memory is impaired. In detail, implicit memory requires low attention and is supported by automatic processes. Explicit memory requires high attention, conscious learning and strategies. Infants with DS show also motor skill impairments: these children are unable to roll until 5-6.4 months and to sit independently until 8.5-11.7 months (Bartesaghi et al., 2011). Cerebellar hypoplasia and motor dysfunctions can affect learning skills in DS patients (Vicari, 2006).

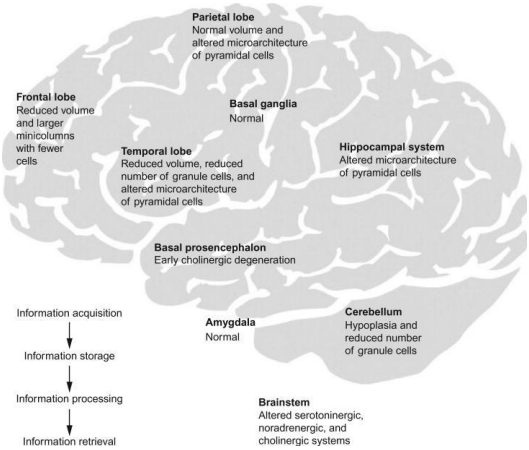

Figure 2. Brain map of structures involved in the learning circuit (acquisition, processing or information storage). Under each brain area the status in a Down syndrome brain is specified (Lott & Dierssen, 2010).

Furthermore, studies showed that both environment and genetic expression influence DS neurological phenotype that changes across the life span and, in most cases, culminate with dementia, as previously described (Bartesaghi et al., 2011).

1.2 Neurodevelopmental alterations in Down syndrome brain

Down syndrome is a neurodevelopmental disorder in which the brain develops differently from an euploid child and in particular is altered in configuration and reduced in size (Bartesaghi et al., 2011). The widespread brain hypoplasia and the consequent hypocellularity are considered to be one of the main cause of intellectual disability (Stagni et al., 2018).

1.2.1 Gross anatomy

DS Brain morphology is characterized by reduced size (20 % smaller than euploid developing brain) and weight. The reduction in size is detectable in 4-5 month fetuses and is maintained for the rest of the gestation (Engidawork &

Lubec, 2003; Lott, 2012). Numerous DS brain regions are smaller compared with control individuals (Pinter et al., 2001; Stagni et al., 2018) and volume reduction is pronounced for the hippocampus, cerebellum and brainstem (Guidi et al., 2014; Raz et al., 1995; Stagni et al., 2018).

1.2.2 Cytoarchitecture

Characteristic features of the DS brain are the diffused hypocellularity, astrocytic hypertrophy and decreased thickness of cortical layers (Bartesaghi et al., 2011). At early gestation stages cell density appears normal, but later (gestation week, GW, 19-23) less neurons are described and scarcity continues throughout early life compared to euploid fetuses (Golden & Hyman, 1994; Guidi et al., 2008). Compared to matched euploid fetuses at GW 17-21, DS hypocellularity is prominent in the dentate gyrus (DG) of hippocampus and in the parahippocampal gyrus (Guidi et al., 2008; Stagni et al., 2018). Also the granule cell density in the cerebellum is reduced in children and then adults with DS (Baxter, 2000; Guidi et al., 2008; Ross et al., 1984; Stagni et al., 2018).

In addition to neuronal alterations there are also defects of other cell types, including glial cells. Indeed, an increased astrocyte-neuron ratio has been described in hippocampal structures of DS fetuses compared to euploid ones (Bartesaghi et al., 2011; Guidi et al., 2008). An increased astrocyte number and astrocytic hypertrophy have been shown in both developing and adult DS brain (Griffin et al., 1998).

1.2.3 Neurogenesis alterations

During the development of fetal brain, neural stem cells (NSC) proliferate, maturate into neural progenitor cells (NPC) and differentiate into neurons in a process called neurogenesis (Kitamura et al., 2009). In detail, the neural tube

starts to differentiate into an outer zone and an inner proliferative layer since GW 6, at that time some neurons have already been born. At GW 7 the subventricular zone (SVZ) and the ventricular zone (VZ) appear. At GW 7-8 neurons migrate from these zones to form the cortical plate (Chan et al., 2002). By GW 24 a large reduction of VZ and SVZ occurs combined with a reduction in proliferation (Chan et al., 2002). The neurogenesis process in the dentate gyrus (DG) starts at GW 12 and is maintained within the first postnatal year (Rice & Barone Jr., 2000). Neurogenesis is mainly active during brain development, but this process is maintained also during adulthood in two specific neurogenic niches: the subventricular zone (SVZ) and the subgranular zone (SGZ) of hippocampus (Bortolotto et al., 2019; Cvijetic et al., 2017; Martínez-Cerdeño & Noctor, 2018).

The reduction of this process during early life stages is thought to be among the major neurodevelopmental defects leading to DS cognitive impairment (Stagni et al., 2018). Neurogenesis in the fetal DS brain has been little studied since the difficulties in obtaining fetal material. However, several studies showed that cell proliferation is impaired in different regions of the fetal DS brain, such as in the hippocampus germinal zones, DG and germinal matrix of the inferior horn of the lateral ventricle (Contestabile et al., 2007; Guidi et al., 2008; Stagni et al., 2018). This defective development determines the reduction in neuron number and impairment in cortical structures detected in DS fetuses and then in children (Contestabile et al., 2007; Pinter et al., 2001; Schmidt-Sidor et al., n.d.; Sylvester, 1983; Winter et al., 2000). These data have been further investigated in the chapter about neural progenitor cells in DS.

1.2.4 Gliogenesis alterations

In humans, the formation of the general architecture of brain regions and neurogenesis are mostly complete at birth, while maturation of the two major glial cell populations (astrocytes and oligodendrocytes), myelination, synaptogenesis and synapse pruning occur during postnatal brain development (Jiang & Nardelli, 2015; Lee et al., 2016), since neurogenesis precedes gliogenesis.

Several studies showed an increased neurogenic-to-gliogenic modulation in DS brain, but the mechanisms and the relative consequences of this shift are still unclear. Indeed, compared to matched euploid fetuses at GW 17-21, astrocytes are increased in the hippocampus (Guidi et al., 2008) and in the frontal lobe, where they appear also more mature than the euploid counterpart (Dossi et al., 2018)

1.2.5 Dendrite, spine, synapse and neurotrophin alterations in Down syndrome brain

Dendritic spines are structures, rich in actin, that constitutes the postsynaptic terminals of excitatory synapses (Lee, Zhang, & Webb, 2015). Beginning from infancy (3-4 months of age), typical DS hallmarks are the reduced neuronal complexity with atrophy of the dendritic tree, spine density reduction and alterations in spine shape (Bartesaghi et al., 2011). These abnormalities do not recover at subsequent life stages (Torres et al., 2018). 3-4-month-old DS children show dendritic hypotrophy in neurons of the parietal cortex (Schulz & Scholz, 1992), motor cortex (Prinz et al., 1997) and visual cortex (Becker, Armstrong & Chan, 1986). Another typical DS feature, not observed in fetuses, but appearing in newborns and older DS infants, is the reduction and alteration in spine number and morphology (Takashima et al., 1981). Indeed smaller spines, with short

stalks, have been reported in children with DS compared with age-matched euploid individuals (Marin-Padilla, 1976).
All together dendrite and spine alterations in DS imply a reduction of the surface available to exchange synaptic inputs. Indeed, a defective synaptic function and organization are typical features of DS brain, associated with alterations of transmitter systems (Bartesaghi et al., 2011; Chakrabarti et al., 2007; Kurt et al., 2000). Monoamines, such as serotonin and dopamine, has been demonstrated to be reduced in frontal cortex of DS fetuses compared to euploid one (Risser et al., 1997; Whittle et al., 2007)

Actin cytoskeleton rearrangements are important for the formation of dendritic spines and synapses (Risher & Eroglu, 2012). Different proteins involved in the formation of the neuronal cytoskeleton are downregulated in DS brain including the beta-tubulin (Pollak et al., 2003), the microtubule associated protein, MAP2 (Ohara et al., 1999), and the actin-related protein complex 2/3 (ARP 2/3). ARP2/3 is a complex that controls actin remodeling and is required, as a downstream effector, at various stages of brain development (Chou & Wang, 2016; Weitzdoerfer et al., 2002). Another protein downregulated in DS brain is moesin (Lubec et al., 2001). Moesin is involved in plasma membrane-actin cytoskeleton cross-linking and it is critical for the morphology of neurons and the formation of long-term memory (Freymuth et al., 2017).

Furthermore, neurotrophin levels are impaired during DS brain development (Bartesaghi et al., 2011). These molecules are important to support neuronal differentiation, migration, synaptic plasticity and survival (Campenot & MacInnis, 2004; Chao et al., 1998; Chao et al., 2006; Sofroniew et al., 2001). In particular the brain derived neurotrophic factor (BDNF), a neurotrophin that binds the surface tyrosine receptor kinases (TRKs), belongs to this family.

Reduced expression of BDNF is observed both in hippocampus (Guedj et al., 2009) and cerebral cortex of DS fetuses (Toiber et al., 2010).

1.2.6 Specific triplicated genes with a role in Down syndrome neuropathology

The sequencing of genes on chromosome 21 has been completed, although the functions of most of the encoded proteins is not still clear (Engidawork & Lubec, 2003). Recently, Roizer et al. showed that proteins encoded by specific triplicated genes play a role in DS neuropathology (Fig. 3) (Roizen & Patterson, 2003). Below there are several examples of genes that encode proteins involved in DS pathology.

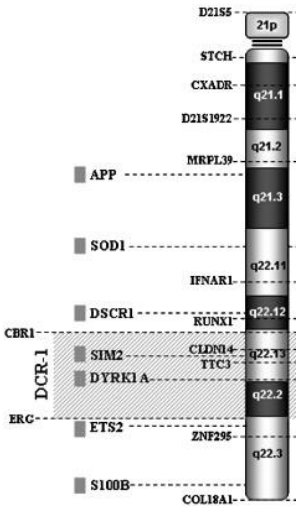

Figure 3. A cartoon in which specific genes on the human chromosome 21 (Hsa21) triplicated in DS fetal brain and involved in brain development are represented, including DYRK1A, APP, S100B, SOD1, DSCR-1 (also known as RCAN1). DCR-1: Down syndrome chromosomal region-1. Picture modified from Rachidi & Lopes, 2011.

DYRK1A (dual specificity tyrosine phosphorylation regulated kinase 1A) encodes a member of the dual-specificity tyrosine phosphorylation regulated kinase (DYRK) family. As previously introduced it plays an important role in the

neurological abnormalities associated with DS. Indeed, during brain development DYRK1A regulates neural progenitor cell proliferation and neuronal differentiation (Dowjat et al., 2007). In the young adult brain DYRK1A can hyperphosphorylate tau, determining progressively depolymerization of actin microfilament, dendritic hypotrophy and neurofibrillary tangles formation. This aspect affects several DS brain regions, such as hippocampus, prefrontal cortex, midbrain, thalamus, hypothalamus and basal ganglia (Lott & Head, 2019; Wisniewski et al.,1985).

APP (amyloid precursor protein) encodes the amyloid precursor protein (APP), a trans-membrane protein mostly expressed in neuronal synapses. This gene is reported to play a key role in DS neurodevelopmental alteration and, as previously mentioned, is involved in the development of Alzheimer-like pathology in DS adults. Furthermore, APP can participate to neuronal plasticity (Turner et al., 2003) and it can affect the formation and transmission of synapses in cultured hippocampal neurons (Priller et al., 2006).

S100β (S100 calcium binding protein B) encodes for a protein member of the S100 family released by astroglial cells. It is highly expressed during development and aging. Increased levels of S100β characterize both adult DS and AD individuals.

SOD1 (superoxide dismutase 1) encodes the superoxide dismutase [Cu-Zn], an enzyme that binds Cu and Zn to breakdown superoxide radicals and convert them to H_2O_2 to avoid cell damage. This is a constitutive enzyme which activity is increased by 50% in DS. Increased levels of SOD1 determine increased oxidative stress and lipid peroxidation in brain human DS cortical neurons (Bartesaghi et al., 2011).

RCAN1 (regulator of calcineurin 1) encodes the regulator of calcineurin factor and it is overexpressed especially in fetal DS brain. RCAN1 protein inhibits calcineurin A, a serine threonine phosphatase that activates the nuclear factor of

activated T cell cytoplasmatic (NFATc). NFAT is involved in the regulation of cell proliferation, neuronal migration and survival (Serrano-Pérez et al., 2015).

1.3 Rodent models in Down Syndrome research

Mouse models are able to mimic, as closely as possible, human pathologies. Mice are important tools that can be investigated to understand the mechanisms underpinning a disease. In particular, in DS they can be used to: 1) investigate genotype-phenotype relationship; 2) identify dosage-sensitive genes involved in DS pathophysiology, 3) test the effect of potential drugs. Till now, a large number of mouse models that recapitulate the phenotypic features of DS have been developed in order to exploit this complex genetic disorder (Bartesaghi et al., 2011; Herault et al., 2017). Several chromosomal rearrangements and modifications have occurred over the evolutionary time that separate mice and humans. Thus, the human chromosome 21 has three orthologous regions on mouse chromosomes 16, 17 and 10, as shown in Figure 4 (Herault et al., 2017). Most of the murine genes that are homologous to humans reside on Mmu16 (102), Mmu17 (19) and the rest on Mmu 10 (37) (Gupta et al., 2016).

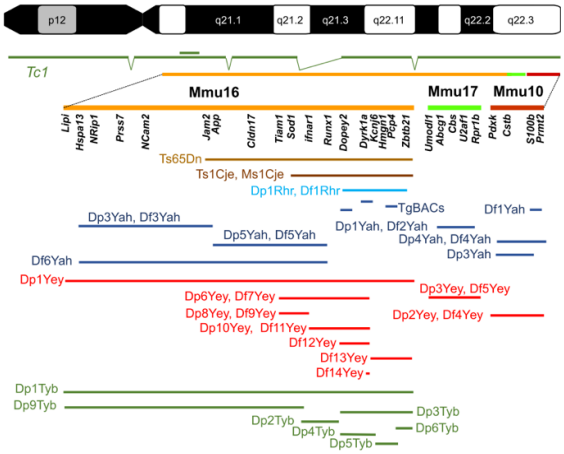

Figure 4. The human chromosome 21 (Hsa21) is represented at the top. Below the orthologous regions found on mouse chromosome 16 (Mmu16, orange), 10 (Mmu10, red) and 17 (Mmu 17,

green) are shown. Several genes homologous to Hsa21 in the Down syndrome critical region are indicated below each murine chromosome. The humanized Tc1 mouse model is represented in dark green below the Hsa21. Below the murine chromosomes there are several examples of DS mouse models. For each mouse model the part of Down syndrome critical region incorporated in each mouse model (different color) is reported (Herault et al., 2017).

The first effort in DS mouse modelling was attempted by Gropp et al. in 1975. Gropp et al. developed a mice with full trisomy of chr 16, **Ts16**, (Gropp, Kolbus & Giers, 1975). This model is limited due to its embryonic lethality and, importantly, they do not model DS phenotype because the bulk of triplicated genes derive from Mmu16 regions not homologous to Hsa21 (Webb, Brown & Anderson, 1998).

The field of DS preclinical research progressed by the discovery (1990) and phenotypic characterization (1995) of the **Ts65Dn** mouse (Davisson et al., 1990; Reeves et al., 1995). This model shows a segmental (partial) trisomy of Chr16 generated by a Robertsonian translocation (as a consequence of exposure to radiation). Ts65Dn mice bear the extra copy of chromosome 16 (Mmu 16) translocated onto a small Mmu17 segment. The triplicated Mmu16 includes 90 conserved protein-coding genes that are orthologous to Hsa21 (Choong et al., 2015; Gupta et al., 2016). Ts65Dn mice are trisomic for around the 55% of the orthologous genes on Hsa21, but are also trisomic for a large number of genes that are not orthologs of Hsa21 genes (Gupta et al., 2016). These mice recapitulate many features similar to DS individuals since embryonic life stages and then in adulthood (Stagni et al., 2018). For these reasons, in the last decades the Ts65Dn mouse line has been widely used to investigate DS and has provided many important understandings in this research field (Bartesaghi et al., 2015).

Ts65Dn mice phenotype is characterized by postnatal developmental delay starting from a reduced birth weight, skeletal malformations, muscular trembling, and, in adulthood, male sterility (Galdzicki & Siarey, 2003).

Similarly to humans, the Ts65Dn mouse line shows several neurodevelopmental alterations, the majority summarized in Figure 5 and described in the following paragraph. Indeed, Ts65Dn mice exhibit a reduced brain size and a widespread cell paucity immediately after birth (since the post-natal day 2, P2) in the hippocampus, DG, SVZ, striatum, thalamus, neocortex and cerebellum (Belichenko et al., 2004; Bianchi et al., 2010; Giacomini et al., 2015; Guidi et al., 2014; Lorenzi & Reeves, 2006).

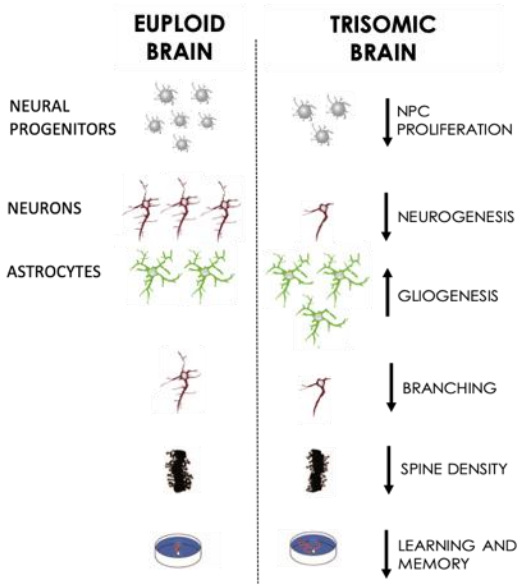

Figure 5. Major brain alterations in euploid and trisomic brain in the Ts65Dn animal model. These features recapitulate the human Down syndrome brain phenotype. Image modified from Bartesaghi et al., 2011.

As in humans, the hypocellularity that characterized Ts65Dn brain is associated with a widespread neurogenesis impairment ascribed to an impaired proliferation of neural progenitor cells (Stagni et al., 2018).

The neurogenic-to-gliogenic shift is observed also in Ts65Dn mice. Contestabile et al., showed an increased number of cells with an astrocytic phenotype in the DG of young Ts65Dn mice (P 30) compared to age-matched euploid mice (Contestabile et al., 2007; Lee et al., 2016). Furthermore, in these mice an increased inhibitory-excitatory neuron ratio is observed in the cortex and in the Cornu Ammonis field 1 (CA1) of Ts65Dn mice at P8 and P15 (Chakrabarti et al., 2010). Studies observed dendrite and spine abnormalities in density and shape in young Ts65Dn mice. 45-day-old Ts65Dn mice show hypotrophic dendritic trees in the DG granule cells compared to age-matched mice and 10-week-old Ts65Dn mice show fewer and shorter dendritic branches in pyramidal cells (Dierssen et al., 2003; Pollonini et al., 2008). In parallel, in adult Ts65Dn the synaptic density is significantly impaired in the DG, CA1 and CA2 of hippocampus if compared with euploid mice (Ayberk Kurt et al., 2004). As in humans, reduced BDNF levels were detected in the hippocampus of 15-day-old Ts65Dn mice (Bianchi et al., 2010).

Importantly, young adult Ts65Dn mice exhibit impairment in learning and memory. They show abnormalities in hippocampal synaptic plasticity and hyperactivity under certain experimental condition. These defects are associated with a defective long-term potentiation (LTP), ascribed to a decrease in the density of N-methyl-D-aspartate (NMDA) receptors, and depression (LTD) (Contestabile et al., 2010). Similar to DS child, studies showed sensor and motor alterations in DS mice since the birth, maintained during adulthood: 4-6-month-old Ts65Dn mice show impaired motor functions (Costa et al., 2010).

As in humans, APP protein expression is upregulation from embryonic day 15 in Ts65Dn mouse cerebral cortex. In adulthood these mice exhibit the signs that are considered the onset of Alzheimer's disease: degeneration of cholinergic basal forebrain neurons and impaired cholinergic system (Galdzicki & Siarey, 2003).

Despite its importance, Ts65Dn mice have some limitations. At first, as previously introduced, the animal bears the triplication of several genes that are non-DS-related (Reinholdt et al., 2011). Second, since Ts65Dn males are sterile (Moore et al., 2010) the colony is usually expanded only by using Ts65Dn dams. This aspect can affect pups determining developmental abnormalities that are independently from the trisomy (Bartesaghi et al., 2015, 2011; Herault et al., 2017; Stagni et al., 2018).

Another model of partial trisomy 16 is the Ts1Cje , created by Sago et al. **Ts1Cje** are mice trisomic for a small region of Mmu 16, containing 79 gene orthologous to Hsa21. As for Ts65Dn mice, this model exhibits learning and behavioral disabilities, but these deficits are less severe than those of Ts65Dn. Studies showed basal forebrain cholinergic neuron degeneration also in these mice (Bhattacharyya & Svendsen, 2003; Villar et al., 2005).

In the last two decades the field of DS mouse modelling changed significantly with the advent of two lines: the chromosome engineered and the transchromosomic mice, that are transgenic animals bearing a chromosome isolated from a different species. Indeed, in 2005 O'Doherty et al. published the first transchromosomic DS model, namely **Tc1** (formally called Tc(Hsa21)1TybEmcf) (O'Doherty et al., 2005). These animals contain 269 genes of the Hsa21, including those gene orthologous located on Mmu17, 16 and 10 that are not present in Ts65Dn and Ts1Cje (O'Doherty et al., 2005). However, the biggest disadvantage of Tc1 mice is that they develop mosaicism in different tissues (Herault et al., 2017).

1.4. Focus on cell types involved in Down syndrome pathophysiology

1.4.1 Neural progenitor cells

Stem cells are defined as "pluripotent cells able to self-renew and to differentiate into other cell types in tissues and organs" (Li & Zhao, 2008). The behavior and fate of stem cells can be affected by their location and by signals released in the niche, a specific microenvironment where stem cells can exist also as progenitor cells (Romito & Cobellis, 2016).

In the Central Nervous System (CNS) neural progenitor cells (NPC) are able to self-renew and to differentiate into neurons, astrocytes and oligodendrocytes. NPC can be found widespread in the developing fetal brain whereas in the neonatal, but especially in the mature adult brain, NPC are mainly restricted into the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus of hippocampus (Bernabeu-Zornoza et al., 2019; Bortolotto et al., 2017; Meneghini et al., 2010; Valente et al., 2012).

Several studies investigated whether trisomy 21 affects progenitor cells during fetal development. Liu et al. performed a systematic review of the literature, both in DS individuals and in DS mouse models. They showed that trisomy 21 impairs proliferation of different type of progenitor cells, in particular severely impaired are neural progenitor cells of trisomic individuals (Figure 6) (Liu et al., 2015).

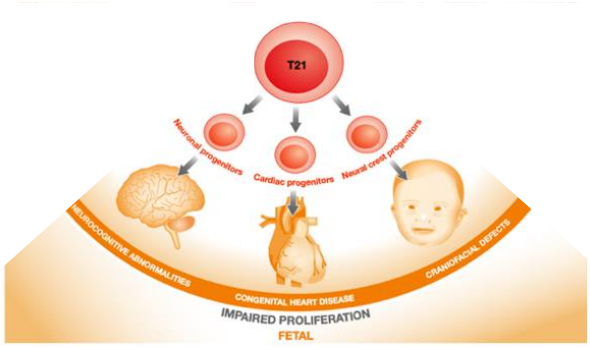

Figure 6. Impact of trisomy 21 on the proliferation of progenitor cells from different lineages during fetal development. As shown in the cartoon, different progenitors can be affected by the

trisomy 21 and impact on the development of craniofacial structures, hearth and, importantly, brain. Image adapted from Liu et al., 2015.

Literature data showed that DS brain hypocellularity, already mentioned in previous chapters, is associated with a widespread reduction in the NPC proliferation rate in several brain regions of DS fetuses (Stagni et al., 2018): the ventricular germinal matrix, cerebellum and hippocampus (Contestabile et al., 2007; Guidi et al., 2008). Studies performed on neural progenitor cells differentiated from trisomic induced pluripotent stem cells (iPSCs) derived from a DS individual exhibited a reduced proliferation compared to euploid (Murray et al., 2015). Moreover, Hibaoui et al. characterized iPSCs from monozygotic twins discordant for trisomy 21: also in this case a reduced number of NPC has been detected in trisomic cells compared to diploid ones (Hibaoui et al., 2014).

Mice models are helpful for investigating the distribution in space and time of the phenotypic defects observed in the trisomic brain. Indeed, similarly to human brain the bulk of neurogenesis in mice happens in the VZ and SVZ before birth, while in the SGZ it occurs in the two postnatal weeks and continues into young adulthood, then decreases with age (Altman & Bayer, 1975, 1990).
In particular, SVZ is an important postnatal niche that gives rise to granule neurons of the olfactory bulbs and cells of the neocortex in the first postnatal days (Brazel et al., 2003). In Ts65Dn pups a reduced proliferation rate have been detected both in the SVZ at the day post-natal 2 (P2), at P15 (representative picture in Figure 7B) (Bianchi et al., 2010; Guidi et al., 2014; Stagni et al., 2016) and in the DG at P2, P6 and P15 (representative picture in Figure 7A) (Contestabile et al., 2009; Giacomini et al., 2015; Stagni et al., 2018, 2019). Moreover, studies showed a strong decreased proliferation also in cerebellar precursor cells of P0, P2, P6 Ts65Dn pups (Contestabile et al., 2009; Roper et al., 2006).

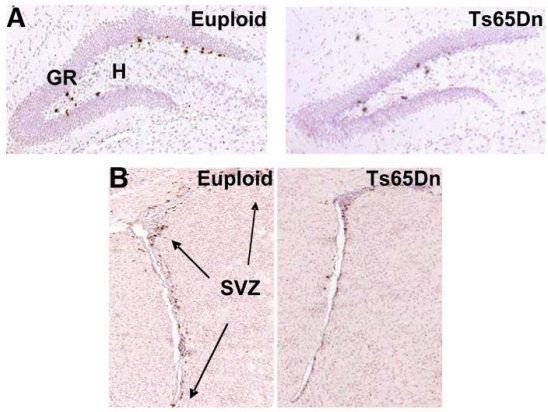

Figure 7. Representative pictures of proliferation impaired in the dentate gyrus (DG, **A**) and subventricular zone (SVZ, **B**) of euploid and Ts65Dn mice at the post-natal day 15 (P15). **A** and **B** are immunostained sections for Bromodeoxyuridine (BrdU) (brown), commonly used to detect proliferating cells. Sections were counterstained with hematoxylin (pink). Euploid and Ts65Dn mice received an injection of BrdU at the day fifteen after birth and then were sacrificed after 2 h. GR=granular cell layer; H=hilus, SVZ=subventricular zone (from Bianchi et al., 2010).

1.4.1.1 Mechanisms underlying impaired neural progenitor proliferation

The mechanisms underlying a reduction in the number of neural progenitor cells in DS are not totally understood (Liu et al., 2015). Evidence in literature shows that cell cycle alterations in DS brain are strictly involved in the impaired NPC proliferation (Najas et al., 2015; Salomoni & Calegari, 2010). Cell cycle is controlled by cyclin-dependent kinases (CDK), their inhibitors, cyclins and it is composed by four different phases: G1, G2, S and M phase. Impaired phases in the cell cycle can affect the balance between undifferentiated NPC and NPC addressed to a specific phenotype acquisition (Smith & Calegari, 2015). Literature data showed an extended G1 phase in DS human fibroblasts (Chen et al., 2013). Other studies detected an elongated G2 phase in DG and in ventricular germinal layer of DS fetuses compared to age-matched euploid fetuses (Contestabile et al., 2007).

In mice, NPC isolated from the hippocampus and lateral ventricle of Ts65Dn embryos exhibit an elongated S phase as well as of the entire cycle (Chakrabarti et al., 2007). G1 phase is elongated also in granule precursor cells of Ts65Dn neonates if compared with the euploid counterpart (Lorenzi & Reeves, 2006).

Several data mainly ascribe these mechanisms to the triplicated genes RCAN1, DYRK1A, APP and the oligodendrocyte transcription factor 1 and 2 (OLIG1-2) (Chakrabarti et al., 2010; Najas et al., 2015; Stagni et al., 2018, 2019). As already mentioned, RCAN1 encodes for a protein able to interact with calcineurin A. This interaction inhibits the pathway calcineurin-NFATc, axis hypothesized to regulate SVZ-derived NPC proliferation and differentiation (Bianchi et al., 2010). NFATc and the related transcripted genes can be regulated also by DYRK1A (Arron et al., 2006; Jung et al., 2011). Interestingly DYRK1A can phosphorylate RCAN1 and this event primes RCAN1 phosphorylation mediated by GSK3β (Glycogen synthase kinase 3 beta), resulting in RCAN1 increased activity (Coronel et al., 2019). Since GSK3β is overactivated in DS brain, in conjugation with DYRK1A and RCAN1, it may inhibit NFAT activity (Stagni et al., 2018).

Moreover, at GW 14 and GW 18 OLIG2 is overexpressed in DS frontal cortex. Studies showed OLIG2 overexpression also in human NPC differentiated from iPSCs derived from a DS patient compared with euploid NPC (Chakrabarti et al., 2010; Lu et al., 2012). In particular, in human trisomic NPC an impaired proliferation rate matches with increased OLIG2 expression (Lu et al., 2012). To make this picture more complex, the increased APP levels detected in DS brain negatively affect cell proliferation. Data suggested that this mechanism is due to increase in APP intracellular fragment (AICD), obtained by APP processing. Excessive levels of AICD may interfere with GSK3β signaling and Sonic Hedgehog (SHH) pathway, involved in stem cell proliferation. Thus,

increased AICD levels determine the consequent increase of the protein patched homolog 1 (PTCH1), a repressor of the mitogenic SHH (Trazzi et al., 2011).

1.4.1.2 Mechanisms underlying impaired neural progenitor phenotype acquisition

Neurons, astrocytes and oligodendrocytes are cells that compose the human forebrain, derived from the VZ and SVZ, and, at first, neurons are generated, followed by astrocytes and oligodendrocytes (Sauvageot & Stiles, 2002).

As previously mentioned, in Down syndrome neuronal phenotype acquisition is defective, and trisomic NPC show a shift toward an astroglial phenotype, observed since the earliest stages of life. The quantification of the number of astrocytes ($GFAP^+$) and of the mature neurons ($NeuN^+$) in DS fetal hippocampi showed fewer neurons and more astrocytes compared to euploid fetuses (Guidi et al., 2008). Also *in vitro*, trisomic NPC differentiated from iPSCs spontaneously give rise to more astrocytes ($S100\ \beta^+$) and fewer neurons ($betaIII\text{-}Tubulin^+$). Moreover, these neurons exhibit a decreased neurite length (Chen et al., 2014a; Hibaoui et al., 2014).

Even in DS mouse models the NPC phenotype acquisition is altered. Indeed, an imbalance between neurons and astrocytes is observed in cultures of SVZ-derived NPC from Ts65Dn pups (Stagni et al., 2019; Trazzi et al., 2011) and in the DG and cerebellum of young adult Ts65Dn mice (Ishihara et al., 2010; Stagni et al., 2016).

Different intracellular pathways may play a role in the DS dysregulated phenotype acquisition. In particular, a key role seems to be played by the Janus kinase-signal transducer and activator (JAK-STAT). JAK-STAT activator ligands and receptors are overexpressed in DS brain (Bonni et al., 1997; Trazzi et al., 2013). Studies suggested that the overstimulation of this pathway is linked to the triplicated genes DYRK1A and APP: APP enhances the activity of JAK-

STAT and increases GFAP levels in Ts65Dn NPC derived from P2 pups (Trazzi et al., 2013). Further studies showed that gliogenesis may be affected by the interaction between JAK-STAT signaling and NOTCH (Taylor et al., 2007) which is activated in DS and affected by APP (Fischer et al., 2005).

1.4.2 Astrocytes

Astrocytes display several functions such as modulation of neuronal plasticity, synaptic maturation and pruning, ionic homeostasis and, importantly, a trophic function to support neuronal activity (Dossi et al., 2018; Sidoryk-Wegrzynowicz et al., 2011; Vasile et al., 2017). In recent years novel insights have been gained about the role of astrocytes in normal brain and in pathological condition, as summarized in Figure 8. Indeed, astrocytes in intellectual disabilities show an altered astrocytic morphology (increased GFAP levels), a defective secretion of trophic signals, impaired synaptic pruning, ionic homeostasis and an altered glutamate sensing and clearance (Figure 8) (Chen et al., 2014b; Cresto et al., 2019; Vacca et al., 2019)

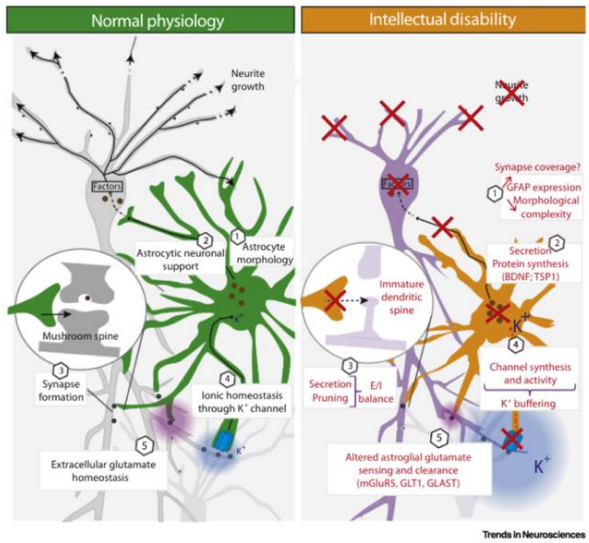

Figure 8. Astrocytes in physiology (left, green) and in intellectual disabilities (right, orange) (Cresto et al., 2019)

In particular, DS astrocytes are not only more abundant, proliferative and mature in their morphology (Zdaniuk et al., 2011), but their functions are altered. Chen et. al studied both DS astrocytes and DS neurons obtained from iPSCs isolated from DS individuals, compared with euploid cells. They demonstrated that DS astrocytes exhibit lower levels of synaptogenic molecules and higher levels of reactive oxygen species (ROS). This reactive state has been hypothesized to disrupt astrocyte homeostasis hampering their ability to promote maturation and support to neurons (Chen et al., 2014). Furthermore, evidence in literature showed an aberrant calcium signaling (more frequent) and increased Ca^{2+} fluctuations both in astrocytes derived from DS iPSCs and in DS mouse models. Elevated Ca^{2+} levels are hypothesized to reduce neuronal excitability in DS (Cresto et al., 2019; Mizuno et al., 2018).

1.4.2.1 Non-cell autonomous regulation of neural progenitor cells

Astrocytes are secretory cells able to secrete a wide array of hormones, neurotransmitters and metabolic, trophic factors. Release occurs through several distinct pathways that include diffusion through channels, controlled exocytosis and transporter translocation (Verkhratsky et al., 2016). These molecules exhibit important functions such as the formation of functional synapses (Araujo et al., 2018) or neurogenesis, mediated, for example by BDNF, S100B and thrombospondins (Clarke & Barres, 2013; Cvijetic et al., 2017). Cvijetic et al. recently revealed first evidence of the complex signaling system implicated in the cross-talk between NPC and astrocytes, identifying novel pathways that can affect progenitors in a non-cell autonomous way (Cvijetic et al., 2017).

Taking into account this information, an important aspect that recently emerged is that DS astroglia presents functional alterations (Chen et al., 2014b) that may affect NPC and their progeny. To support this hypothesis Chen et al. showed that the media collected from human trisomic astrocytes (astrocyte conditioned

media-ACM) reduces human trisomic NPC neuronal differentiation (reduced levels of ßIII^{+} cells) and neurite length of trisomic neurons compared with trisomic cells treated with control media (Chen et al., 2013; Cresto et al., 2019). Furthermore, Garcia et al., demonstrated that a key astrocyte secreted molecule, thrombospondin-1 (TSP-1), is defective both in the secretome of DS human fetal astrocytes and in DS fetal brain (Garcia et al., 2010; Torres et al., 2018). Moreover, defective TSP-1 levels impair the development and morphology of dendritic spine in neurons from newborns rat. Indeed, addition of exogenous TSP-1 on neurons positively modulated astrocyte-mediated spine and corrected synaptic alterations (Garcia et al., 2010). All together these data highlight a potential role of TSP-1 in DS spine pathology (Torres et al., 2018).

TSP-1 is a calcium-binding protein that participates in cellular responses to injury, cytokines and growth factors (Chen et al., 2000). This protein plays a role in adult NPC proliferation and differentiation (Lu & Kipnis, 2010), synaptogenesis and spine formation (Eroglu et al., 2009; Garcia et al., 2010; Risher & Eroglu, 2012).

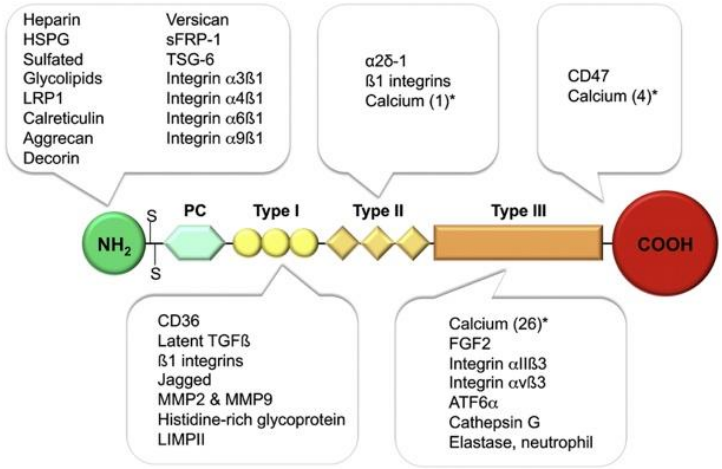

Figure 9. Representative structure of Thrombospondin 1. Type I, II, III are different binding sites. For each domain are indicated the TSP-1 interactors. The asterisk (*) indicates the number of bounded calcium ions (Resovi et al., 2014).

As shown in Figure 9, TSP-1 contains different domains involved in the interaction with a multiplicity of receptors and ligands (Chen et al., 2000; Resovi et al., 2014). Worthy of attention is the α2δ-1 subunit of neuronal voltage-sensitive calcium channels (Dolphin, 2013). Recent evidence show that astrocytes control excitatory synaptogenesis by TSP-1 which functions via the α2δ-1 subunit independently from calcium channels (Dolphin, 2018; Eroglu et al., 2009).

Furthermore, this subunit is important since the antiepileptic and neuropathic pain drugs pregabalin (PGB) and gabapentin (GBP) bind α2δ-1 (Eroglu et al., 2009; Valente et al., 2012). A recent study showed that α2δ-1 is expressed on the surface of neural progenitor cells isolated from wild type mice and, through its binding, PGB and GBP are able to promote adult hippocampal neurogenesis, both *in vitro* and *in vivo* (Valente et al., 2012). Thus, this study gave the first evidence of a proneurogenic effect mediated via α2δ-1 (Valente et al., 2012).

TSPs are known as molecules able to regulate the actin cytoskeleton that, as already mentioned, is involved in formation and remodeling of dendrites and synapses (Risher & Eroglu, 2012). Importantly, recent evidence showed that TSP-1/α2δ-1 interaction control synaptogenesis postsynaptically via Rac1. Rac1 is a small GTPase that belongs to the Rho family GTPases, proteins that control spine morphology and number (Risher & Eroglu, 2012). As shown in Figure 10, Rac1 exists in two forms: an inactive form (GDP-bound) and an active form (GTP-bound). The transition between the two forms is mediated by two catalytic factors, the GTPase-activating proteins (GAPs) and the guanine exchange factors (GEFs) (Tejada-Simon, 2015). The relationship between Rac1 inactivated and activated state is important for proper interaction of Rac1 with other targets downstream the signaling pathway. As summarized in Figure 11, Rac1 is important for several brain functions, including learning and memory and

dendritic spine development. Indeed, Rac1 alterations are described in different neurodegenerative diseases, including Alzheimer's disease (Désiré et al., 2005; Kikuchi et al., 2019; Risher et al., 2018).

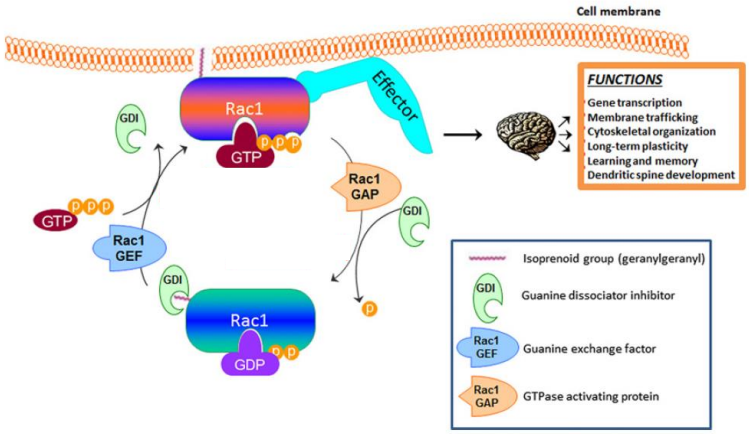

Figure 10. Graphical representation of key players in activation and membrane translocation for the small GTPase Rac1. In the orange field are listed several function that are ascribed to Rac1 activity in the brain. (Image modified from Tejada-Simon, 2015).

1.5. Potential therapeutic approaches proposed in Down syndrome

As already introduced, DS brain, both human and mouse, shows a constellation of defects involved in the cognitive impairment. The investigation of these defects is the starting point to find the rational basis to devise therapies that may correct DS brain developmental defects. Indeed, in the last decades, intense efforts have been carried out to identify pharmacotherapies and/or neurobiological factors known to positively affect brain of DS individuals and to potentially improve one or more DS-linked brain phenotype (Stagni et al., 2015). Mostly of these studies have been performed using the Ts65Dn mouse model (Gupta et al., 2016).

A key issue in the field is the optimum timing for drug administration in DS. Indeed, most of the attempts to pharmacologically correct DS-brain linked

defects have been made in adult mice. However, neurodevelopmental defects, both in human and in mice, are already detectable at fetal life stages. As represented in Figure 11 the bulk of neurogenesis occurs before birth, both in humans and mice. DG and cerebellum represent two exceptions: while in the DG neurogenesis continues throughout life, in the cerebellum stops after the first post-natal period. In this picture, adult therapies may relatively affect brain (hippocampal neurogenesis and circuitry), while perinatal (neonatal and prenatal) therapies may potential affect and correct overall brain development (Guidi et al., 2014; Stagni et al., 2015) (see chapter about NPC alterations in DS). Overall these data suggest that perinatal therapies that target neural progenitor cell alterations may be the potential optimal interventions in DS. Indeed, proof of concept studies showed that DS brain defects can be pharmacologically corrected in the animal model, if the therapy is administered in the perinatal period (Bianchi et al., 2010; Guidi et al., 2014; Nakano-Kobayashi et al., 2017; Stagni et al., 2015). These corrections can be maintained through individual life span. In the following chapters, some examples of drugs that have been tested in DS animal model will be summarized.

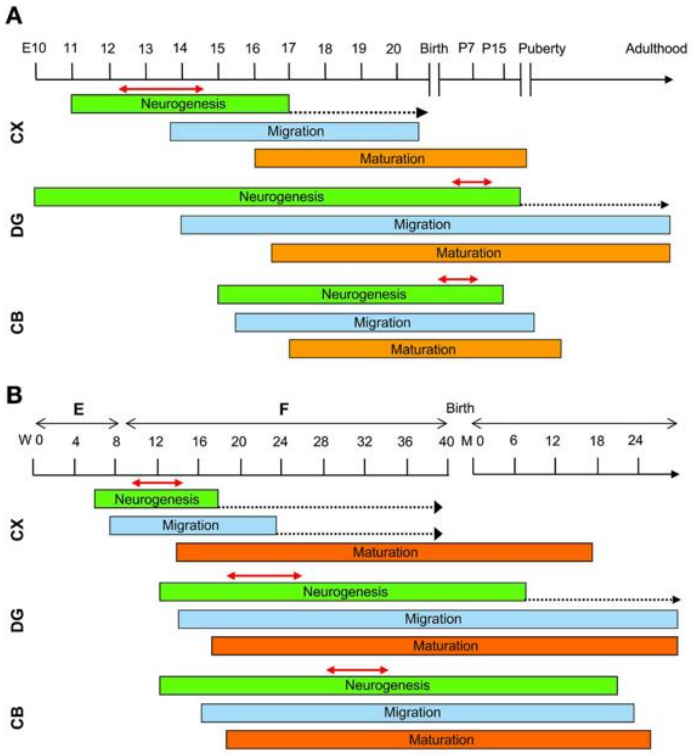

Figure 11. Timeline of brain development in mouse (A) and human (B). CB, cerebellum; CX, neocortex; DG, dentate gyrus; E, embryonic; F, fetal; M, month; P, post-natal; W, week (Stagni et al., 2015)

1.5.1 Lithium

Lithium chloride (LiCl) is the first-line treatment for bipolar disorder. The molecular mechanisms of LiCl are not known in detail, studies demonstrated that lithium acts in part inhibiting the activity of GSK3β and modulating Wnt/β-catenin pathway (Pasquali et al., 2010). GSK3β antagonizes the Wnt signaling pathway and is upregulated in DS brain (Granno et al., 2019; Stagni et al., 2018; Zhang et al., 2019).

Contestabile et al. showed that administration of a diet containing $LiCO_3$ (2.4 g/kg) for 1 or 4 weeks in 5-6-month-old Ts65Dn mice promotes the proliferation of NPC through the activation of the Wnt/β-catenin pathway. The treatment is

able to restore neurogenesis in the DG of these mice to physiological levels, completely rescuing the synaptic plasticity of newborn neurons and recovering mice cognitive performances (Contestabile et al., 2013). In agreement with these findings, Bianchi et al., demonstrated that administration for 1 month of lithium (2.4 g/kg of $LiCO_3$ in the food pellets) fully restores SVZ and DG proliferation in 12-month-old Ts65Dn mice (Bianchi et al., 2010b). Despite these interesting data in adult trisomic mice, in clinic the treatment with lithium is associated with many side effects [including gastrointestinal side effects, polyuria, tremor, weight gain, dermatological effects (Gitlin, 2016)]. Bartesaghi et al. tried a neonatal treatment with LiCl in Ts65Dn pups. In detail, they gave lithium to trisomic mothers in order that pups should receive the drug through the milk, but lithium had a lethal effect on the offspring (Bartesaghi et al., 2011).
Despite its interesting effects in preclinical models, overall experimental data exclude the possibility of using lithium in patients.

1.5.2 Fluoxetine

Fluoxetine is an antidepressant that acts as a selective serotonin reuptake inhibitor (SSRI). This drug is largely prescribed in clinic for adult patients, but also for children and adolescents (Boylan et al., 2007).
5-hydroxytryptamine (5-HT, also named serotonin) is one of the major neurotransmitters of the CNS. Serotonin is essential for brain development since the earliest fetal stages and the serotoninergic system is shown to be altered both in DS fetuses and mice (Whittle et al., 2007).
Guidi et al. demonstrated that a prenatal treatment with fluoxetine in pregnant Ts65Dn mice (10 mg/kg, daily subcutaneous injection, from E10 to birth) is able to fully rescue DS brain abnormalities and behavioral deficits (Guidi et al., 2014). Furthermore, Bianchi et al. showed that fluoxetine modulates cell survival,

increases neurogenesis and dendritic development in the DG (Bianchi et al., 2010), restores functional connectivity of hippocampal synapses and hippocampus-dependent learning in Ts65Dn pups treated only during the two postnatal weeks (5 mg/kg from P3 to P7; 10 mg/kg from P8 to P15, daily subcutaneous injection) (Bianchi et al., 2010; Guidi et al., 2014). In addition, Stagni et al. observed that the beneficial effects of a neonatal treatment with fluoxetine endure in 45-day-old Ts65Dn mice, rescuing cognitive impairment in adulthood (Stagni et al., 2015).

These results represented a breakthrough in DS preclinical research, since they showed that DS neurodevelopmental impairments can be pharmacologically corrected by a perinatal intervention. These corrections rescue cognitive impairment in DS mice. Based on these findings, fluoxetine has been suggested as a potential prenatal therapy in rescuing fetal DS brain defects and dysfunctions (Kuehn, 2016). In 2014 the University of Texas Southwestern Medical Center approved a pilot study in pregnant mothers to investigate whether fluoxetine is effective in rescue cognitive impairments in DS fetuses, no information about this trial is yet available (Stagni et al., 2015).

Despite the really encouraging evidence obtained in the animal model, several adverse effects have been reported both in patients and mouse model of depression after long term treatment with this drug, including dizziness, nausea, headache and lipid metabolism abnormalities (Pan et al., 2018; Riediger et al., 2017). Furthermore, treatment with fluoxetine during pregnancy can affect the normal heart development of fetuses (Daud et al., 2016).

1.5.3 Epigallocathechin-3-gallate

In the past few years a lot of interest has been raised up in polyphenols. Polyphenols are phytochemicals produced by plants as secondary metabolites in

response to stress conditions. Epigallocatechin-3-gallate (EGCG) is a flavonoid of green tea extracts. The antioxidant activity and the inhibition of DYRK1A seems to be the potential mechanisms of action of EGCG in DS (Pons-Espinal et al., 2013; Thomazeau et al., 2014). Indeed, Valenti et al. showed that EGCG (20 µM) restores mitochondrial energy deficits observed in peripheral cells isolated from DS individuals (Valenti et al., 2013).

Stagni et al. demonstrated that neonatal administration of ECGC (25 mg/kg, daily subcutaneous injection from P3 to P15) corrects many DS-associated brain alterations at P15, but does not elicit durable effects in the hippocampus when measured at P45 (Stagni et al., 2016).

Catuara-Solarz et al. reported that green tea extracts containing EGCG (green tea extract, 45% EGCG) in combination with environmental enrichment for 30 days in 1-2-month-old Ts65Dn mice, enhances dendritic spine density in Cornu Ammonis field 1 (CA1) and stabilizes the proportion between excitatory and inhibitory synaptic markers in the DG and CA (Catuara-Solarz et al., 2016). In 3-month-old Ts65Dn mice EGCG (2-3 mg/day, 30 day treatment) normalizes DYRK1A activity, restores brain plasticity and partially rescues learning and memory (De la Torre et al., 2014). EGCG (20 µM) improves proliferation of adult hippocampal NPC isolated from 6/8-week-old Ts65Dn mice and restores the defective mitochondrial biogenesis (Valenti et al., 2016).

Despite this evidence, other scientific reports suggested that the administration of 10 mg/kg/day of pure stabilize ECGC failed to improve learning and memory in Ts65Dn young mice (treatment from P24 to 3-7 weeks after birth) (Stringer et al., 2015; Vacca et al., 2019b).

However, in the recent years EGCG has been proposed as a drug candidate for treatment of DS (Valenti et al., 2016). This treatment appears suitable for clinical applications due to the positive effects detected in the animal models and the low toxicity measured after long term treatments (Isbrucker et al., 2006). Based on

these data, recently, De la Torre et al. proved that the treatment with EGCG in young DS individuals (9 mg/kg/day, 6 month treatment) improves adaptive behavior and some specific memory skills compared to placebo-treated patients (De la Torre et al., 2014; De la Torre & Dierssen, 2012). At present, a new clinical trial in young DS patients (10 mg/kg/day, 6-12 years old DS and fragile X patients), started in January 2018, is ongoing. This trial aims at evaluating EGCG safety and tolerability in DS children and young DS adolescents and investigate whether EGCG affects cognitive performances. Trial information are available at clinicaltrials.gov/show/NTC03624556 (PERSEUS).

1.5.4 Diet supplementation

In the past years diet supplementations raised up interest in the scientific community since several studies indicated that DS individual are deficient of micronutrients such as vitamins, amino acids and enzymes.

As already mentioned, a common feature between DS individuals and DS mice is the degeneration of basal forebrain cholinergic neurons (BFCN) (Bartesaghi et al., 2011; Sago et al., 1998). Cholinergic neurons provide acetylcholine, a key neurotransmitter in brain (Stagni et al., 2015). Studies showed that the administration of choline in Ts65Dn mice during pregnancy (25 mM in drinking water, starting at the embryonic day, E1) and continued during lactation (up to P21) increases BFCN and cognitive performances in the offspring (evaluated at the age of 6 months) (Moon et al., 2010). Moon et al. suggested that choline mechanism of action should be mediated via epigenetic regulation or targeting the phospholipid composition of membranes (Moon et al., 2010)

However, despite clinical trials conducted in early infants using diet supplementations are available, at present no one showed an improvement either in cognitive functions or in psychomotor development (Vacca et al., 2019).

1.6. Phenotypic drug screening and the drug repurposing strategy

At present drug discovery in DS is mainly focused in early pharmaceutical interventions and development of appropriate outcome measures (Hart et al., 2017), but as already introduced, there are no approved pharmacotherapies to treat DS brain alterations so far (Kazemi et al., 2016).

The goal of drug discovery is to develop efficacious and safe therapeutics to treat human diseases, but develop new human therapeutics is a lengthy, costly process with an attrition rate > 90%. Drug screening is one of the mostly used process that allows the identification and the optimization of potential drug candidates to progress into clinical trials (Croston, 2017). In particular, there are two main ways to perform a screening campaign, through a target based or a phenotypic-based approach. In the past 25 years, molecular target-based drug screening has become the most commonly used technology in pharmaceutical industry and academia. This approach is based on the knowledge of a dysfunctional molecular target and/or mechanism of action (Zheng et al., 2013). However, recently the interest in phenotypic screening has been renewed. In detail, a phenotype is defined as any type of observation or biochemical/physical characteristic of an organism, such as the heart rate in a zebrafish (Williams & Hong, 2016) or in a cell system, the cell proliferation rate (Yin et al., 2017). These phenotypes can be used as read out in the process of drug discovery. Thus, phenotypic drug screening, also called ‘forward pharmacology’ or ‘classical pharmacology’ (Takenaka, 2008; Vogt & Lazo, 2005), is an appealing strategy because it does not need an *a priori* knowledge of a target or a molecular mechanism of action but it is usually associated with features of the disease, that may be exploited to develop a cell-based assay (Aulner et al., 2019). In particular, primary cell cultures can be used in phenotypic drug screening campaigns in order to achieve more physiologically relevant results (Yin et al., 2017; Zheng et al., 2013).

In the past years, the pharmaceutical industry invested a lot of money in the search for new molecular entities, but, as mentioned before, the process of identification of new drugs is costly, time consuming and with high attrition rate (Pushpakom et al., 2018). Since 2000, some industries changed their strategies investing energies also in drug repurposing. Drug repurposing or repositioning consists in finding new pharmacological indications for approved drugs. This approach is useful to bypass the long, risky and really expensive preclinical phase. In such regard drug repurposing is a potentially successful strategy that may help discovering effective therapies in orphan diseases, since relying on approved drugs with established bioavailability/safety profiles in humans (Clout et al., 2019).

This approach is particularly attractive and it could be a winning strategy when, in preclinical research, it is associated with a phenotypic drug screening, especially in the CNS therapeutic area that exhibits the lowest success rates in research and development (Clout et al., 2019).

The most famous example of drug repositioned was Sildenafil in 1998, approved for hypertension, then repositioned for erectile dysfunction (Kim, 2015). In the nervous system pharmacotherapies one other example is the anticonvulsant drug gabapentin repositioned as analgesic in neuropathic pain (Reaume, 2011).

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Chapter 2

Thesis outline

Since several years, in the laboratory of Neuroplasticity at University of Piemonte Orientale where this thesis has been undertaken, my colleagues have been interested in studying adult neurogenesis and in the investigation of the cellular and molecular mechanisms that activate and regulate neural progenitor cells (NPC) in response to clinically relevant drugs (Bortolotto et al., 2017, 2019; Cuccurazzu et al., 2013; Meneghini et al., 2014; Valente et al., 2012). In more recent years the laboratory has also become interested in studying the regulation of neonatal neurogenesis in Down syndrome (DS), a neurodevelopmental disorder.

DS is the main genetic cause of intellectual disability. Decreased proliferation of NPC, widespread neurogenesis impairment and increased astrogliogenesis are considered among the major determinants of brain atrophy and intellectual disability in DS individuals (Stagni et al., 2018). The best characterized and studied animal model for DS is the Ts65Dn mouse line which recapitulates most of the features of the human pathology, including cognitive impairment (Gupta et al., 2016). In the recent years it has been suggested that perinatal (prenatal and neonatal) therapies that target and correct NPC alterations may represent potential interventions in DS. Indeed, proof of concept studies showed that DS brain defects, including cognitive impairment, can be pharmacologically corrected in the Ts65Dn animal model, if the therapy is administered in the perinatal period (Guidi et al., 2014; Nakano-Kobayashi et al., 2017; Stagni et al., 2015). However, at present, drugs effective in mouse models pose some limitations due to their side effects, reducing their possibility to be translated in the clinical setting. Altogether these data highlight the need for better elucidating the mechanisms underlying DS pathophysiology and identifying novel

pharmacological targets and approaches to counteract intellectual disability in DS patients.
Thus, my entire PhD project was based on the idea that trisomic NPC can be a key pharmacological target to correct DS brain abnormalities during early life stages.
Based on this hypothesis the overall goal of my PhD was to unravel novel mechanisms underlying trisomic NPC dysfunctions and investigate whether and how it was possible to identify new pharmacological approaches targeting NPC abnormalities during DS brain development.

In detail, chapter 3 describes the results obtained from my first aim that consisted in the investigation of whether FDA/EMA approved drugs could correct the defective proliferation of trisomic (TS) NPC, profiting from a drug repurposing strategy. We phenotypically characterized NPC *in vitro* in order to confirm the same phenotype as *in vivo*. Then, through a miniaturized, reproducible and sensitive assay we had set up, we were able to identify 30 drugs which increased TS NPC proliferation. These drugs belonged to three pharmacological classes: glucocorticoids, β2 adrenergic agonists, immunosuppressant drugs. Among the most potent hits we identified the immunosuppressant cyclosporine A (CSA) which was further characterized *in vitro* and, based on its pharmacological properties, then tested *in vivo*, in collaboration with the group of Prof. Renata Bartesaghi at the University of Bologna, in the Ts65Dn mouse line (see chapter 4).

Chapter 4 describes the phenotypic characterization of CSA *in vitro* and *in vivo*. In detail, my specific aim was to investigate whether CSA promoted TS NPC proliferation and evaluate whether CSA affected neurogenesis, gliogenesis and neuronal maturation of TS NPC. Since the promising *in vitro* results, we then

tested CSA effects *in vivo*. The treatment in neonatal Ts65Dn and euploid pups with 15 mg/kg/day of drug from post-natal day 3, P3, to P15 significantly i) increased the number of proliferating NPC in the SGZ and SVZ, ii) positively impacted on neuronal density in the dentate gyrus and iii) largely increased spine density in granule cells of Ts65Dn-treated mice.

Chapters 5 and 6 describe how we contributed to dissect the role of NPC exposed to specific treatments tested *in vivo* in Ts65Dn and euploid mice. These activities were done in collaboration with the group of Prof. Bartesaghi and, in Novara, we contributed with *in vitro* studies using trisomic and euploid NPC as a cellular model.

In more details, as described in chapter 5, my goal was to investigate whether a flavone derivative, 7,8-dihydroxyflavone (7,8-DHF), that activates the tropomyosin-related kinase B (TrkB) receptor of the brain-derived neurotrophic factor (BDNF), was able to affect proliferation and neuronal differentiation of TS NPC (0.3-10 µM). *In vivo* neonatal treatment with 7,8-DHF (5 mg/kg/day, P3-P15): i) increased the number of NPC in the dentate gyrus, ii) restored the number of granule cells and iii) increased dendritic spine density. Importantly, 7,8-DHF (5 mg/kg/day, P3-P45) improved memory and learning performance in treated Ts65Dn mice.

In chapter 6 we investigated the *in vitro* and *in vivo* effects of corn oil. At first, we showed that *in vivo* 4-month-old mice treated with corn oil (10 µl/g) for 1 month exhibited i) increased neurogenesis; ii) dendritic development and, in parallel, iii) increased learning and memory, exclusively in Ts65Dn mice. Based on the interesting *in vivo* results, my aim in this study was to investigate whether two main components of corn oil, linoleic acid (LA) and oleic acid (OA) affected

TS NPC proliferation *in vitro* (100 µM). Secondarily, we investigated whether LA affected NPC proliferation through the activation of the peroxisome-proliferator activated receptors (PPARs).

In the past, the laboratory of Neuroplasticity identified novel signaling pathways that, if disrupted, can affect NPC and their communication with niche astrocytes in a cell-autonomous and non-cell autonomous manner (Cvijetic et al., 2017). It is currently hypothesized that NPC dysfunctions and their communication with other cell types, including astrocytes, may also contribute to DS pathophysiology. (Chen et al., 2014; Mizuno et al., 2018). Indeed DS astroglia exhibit functional alterations that can affect NPC and their progeny, including defective release of soluble signals like thrombospondin-1 (TSP-1) (Garcia et al., 2010; Torres et al., 2018), a key astrocyte-derived signal involved in neurogenesis (Lu & Kipnis, 2010), synaptogenesis (Eroglu et al., 2009) and spine formation (Risher et al., 2018). Despite these data, the role of TSP-1 in DS non-neuronal cells is largely unexplored.

In the last period of my PhD activities I was also involved in the investigation of TSP-1 signaling in trisomic murine NPC. In chapter 7 I have summarized some unpublished and still preliminary results on this specific topic.

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Chapter 3

Unpublished data

A drug repurposing strategy results in the identification of novel drug classes correcting defective properties of trisomic neural progenitor cells

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KEYWORDS: Down syndrome; Neural Progenitor Cells; Pharmacotherapy; Drug repurposing; drug screening

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ABSTRACT

Down syndrome (DS) is a neurodevelopmental disorder caused by triplication of chromosome 21 in which decreased Neural Progenitor Cell (NPC) proliferation is associated with a widespread neurogenesis impairment. NPC alterations are considered as major determinants of brain atrophy and intellectual disability in DS pathology. A well-studied preclinical model for DS is the Ts65Dn mouse line that recapitulates several features of the human disorder, including cognitive impairment. Recent findings suggest that early pharmacological treatments may potentially correct these defects in Ts65Dn mice, resulting in amelioration of cognitive performance. Unfortunately at present none of the drugs effective in DS animal models appear suitable for clinical applications.

The goal of this study was to identify novel drugs able to correct proliferative deficits of Ts65Dn-derived trisomic NPC, which could be then tested in DS animal models. In detail, we aimed at developing an *in vitro* cell-based assay to screen 1,590 FDA/EMA approved drugs according to a drug repurposing strategy. We established all the critical assay conditions so to obtain a miniaturize, sensitive, reproducible and ready-to-use proliferative assay in trisomic NPC, using lithium chloride (LiCl) as reference drug. As result of this effort, we identified 30 molecules more effective than LiCl in promoting trisomic NPC proliferation. Among these drugs, glucocorticoids, β2 adrenergic agonists and the immunosuppressant CSA were identified and further characterized.

The assay we have developed allows not only the identification of new drug candidates, but holds, in the future, the potential to unravel novel signaling pathways involved in DS pathophysiology.

INTRODUCTION

Down syndrome (DS), a neurodevelopmental disorder resulting from the triplication of chromosome 21, represents the most common genetic cause of intellectual disability (Bartesaghi et al., 2011; Vacca et al., 2019). Despite huge progress has been achieved in improving life expectancy and quality in DS individuals, intellectual disability still remains the most invaliding aspect of the human disorder.

A well characterized DS animal model is the Ts65Dn mouse line. These mice closely recapitulate key features of DS human brain, such as reduced proliferation of neural progenitor cells (NPC), impaired neurogenesis, defective dendrite branching and spine density, and, importantly, cognitive impairment (Bartesaghi et al., 2011; Gupta et al., 2016; Herault et al., 2017; Vacca et al., 2019). Reduction in NPC proliferation and neuronal differentiation, starting from fetal period, are currently considered major neurodevelopmental defects leading to DS associated cognitive impairment (Chen et al., 2014a; Hibaoui et al., 2014; Stagni et al., 2018). Based on this working hypothesis, NPC may represent targets of novel pharmacological approaches to the human disorder.

Recent studies have suggested that it may be possible to pharmacologically correct NPC proliferation, neurogenesis and, in parallel, improve cognitive performance in DS animal models, by administering perinatal (neonatal and prenatal) drug treatments (Guidi et al., 2014; Stagni et al., 2015; Stagni et al., 2017). Unfortunately, despite this very important proof of concept achievement, at present none of the drugs which are effective in DS animal models appear suitable for clinical applications, mainly due to unfavorable tolerability and/or safety issues (Kazemi et al., 2016). Based on these premises, there is an urgent need of identifying novel drugs that may be tested in animal models, and eventually in patients.

Unfortunately, identifying and developing new human therapeutics is a lengthy and costly process, with an attrition rate higher than the 90%. In particular, the Central Nervous System (CNS) therapeutic area exhibits the lowest success rate in drug discovery (Gribkoff & Kaczmarek, 2017; Zheng et al., 2013). In the past, the most frequently pursued strategy in drug discovery was "target-based" (Croston, 2017): drug screening for different therapeutic areas was mainly performed by using cellular assays, often generated in cell lines, (over)expressing the target of interest in search for selective agonists/antagonists/inhibitors. In recent years interest in phenotypic assays has been exponentially growing in drug discovery (Zheng et al., 2013). Phenotypic drug screening is appealing because it does not require *a priori* knowledge of a specific target or a molecular mechanism implicated in a given disease (Aulner et al., 2019). According to this paradigm, screening campaigns aim at identifying drugs that can correct or attenuate a cellular phenotype which is regarded to be relevant for disease pathophysiology, regardless of the mechanism(s) targeted by the drugs. Although difficult, primary cell cultures can also be used in phenotypic drug screening campaigns in order to achieve more physiologically relevant results (Yin et al., 2017; Zheng et al., 2013). Moreover phenotypic cell-based screening assays can also be combined with strategies of drug repurposing, the process of finding new applications for clinically approved drugs. As opposed to *de novo* drug discovery, when approved drugs are identified in a screening campaign, they have the advantage of well-established features of bioavailability, tolerability and safety in humans so to potentially reduce time for human translation (Clout et al., 2019). Several successful examples of drug repurposing in high medical need human disorders are available (Kim, 2015; Reaume, 2011)

Based on these premises, the goal of this study was to develop a reliable and reproducible *in vitro* phenotypic cell-based assay which could be used to screen and identify, among clinically approved drugs, compounds which are able to

correct the proliferative defects of trisomic (TS) neural progenitor cells isolated from the subventricular zone of Ts65Dn mouse pups. Combined with appropriate secondary assays, our screening efforts confirmed the activity of several drug classes, some of which have been also successfully used *in vitro* to assess their effects on neuronal differentiation of TS NPC and, *in vivo*, to correct hypocellularity and neurogenesis impairment in Ts65Dn mice. Herein we describe in detail the set up of the phenotypic assay, its use for the screening of two commercial libraries of approved drugs, as well as the activities for confirmation and validation of identified hit molecules. Last but not least, we discuss the implications of these findings for ongoing and future *in vivo* studies in Ts65Dn mice.

MATERIALS AND METHODS

Mouse colony

Ts65Dn mice were provided by Jackson Laboratories (Bar Harbor, ME, USA) and generated by mating B6EiC3Sn a/A-Ts(17^16)65Dn females with C57BL/6JEiJ x C3H/HeSnJ (B6EiC3Sn) F1 hybrid males (Reeves et al. 1995). Only first generation litters were used. Pup genotyping was performed as previously described (Rheinold et al., 2011). Animals had *ad libitum* access to water and food in a room with a 12:12 h light/dark cycle. Experiments were performed in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) for the use of experimental animals and were approved by Italian Ministry of Public Health (1033/2015-PR and 47/2019).

Isolation and Culture of SVZ-derived neural progenitor cells

Cells were isolated from the subventricular zone (SVZ) of the lateral ventricles of newborn (postnatal days 1-2, P 1-2) euploid (EU) and trisomic (TS) pups.

NPC cultures were obtained by pooling 3-6 pups and cultured as free floating neurospheres according to established protocols (Cvijetic et al., 2017; Meneghini et al., 2014). Briefly, cells were cultured in complete medium containing DMEM/F-12 supplemented with B27, Glutamax™ (2 mM, Life Technologies), heparin sodium salt (4 µg/ml; ACROS Organics), hFGF (10 ng/ml, Peprotech), hEGF (20 ng/ml; Peprotech) and 100 U/100 µg/ml Penicillin/Streptomycin (Life Technologies). Primary (Passage 1, P1) neurospheres were dissociated using Stempro Accutase (Life Technologies) after 6 days *in vitro* (DIV). Thereafter, neurospheres were passaged every 5 DIV. At least seven NPC preparations from P2 to P12 were used for this study.

Phenotypic drug screening

Briefly, two commercial libraries were used: the Prestwick chemical library® (Prestwick Chemical), a recognized screening drug collection composed by 1,120 approved drugs (Kanvatirth et al., 2019; Torres et al., 2018), and the Screen-Well® FDA Approved Drug Library V2 composed by 770 compounds (Enzo Life Sciences) (Corsello et al., 2017). We developed a method based on trisomic neural progenitor cell proliferation (Stagni et al., 2019). Cells were seeded in a low proliferative condition (10 ng/ml hFGF) and incubated for 30 minutes at 37°C, 5 % CO_2. Then, the compounds were added to each well, in quadruplicates, at a final concentration of 1 µM with 0.05% DMSO. As pro-proliferative controls hEGF (20 ng/ml, Peprotech) and LiCl (2 mM, Sigma-Aldrich) were added to each plate in quadruplicates. Plates were incubated for 96 h in the humidity box and then measured using the Cell Titer Glo assay (Promega) following manufacturer's instructions. The humidity chamber (18x26x8 cm) contained a water reservoir and a plastic layer where could be located up to six 96 well plates. At the bottom of the chamber there was holes (∅ 5 mm) where the air composed by 5 % CO_2 could flow into the box. The drug activity was calculated as

percentage of change compared to basal conditions (cells in presence of 10 ng/ml hFGF and 0.05 % DMSO).

In order to validate potential hits, we performed drug concentration response curves from 0.1 to 1000 nM (DMSO 0.05 % as vehicle), using the same proliferation assay. Desonide, clobetasol, betamethasone, terbutaline, formoterol and isoproterenol powder were supplied by MedChemExpress.

Z prime calculation:

The quality of the assay is represented by the Z-prime value, a value between 0.5 and 1 assesses an excellent high-throughput assay (Zhang, Chung & Oldenburg, 1999). It was calculated as previously described by Zhang et al. (Zhang et al., 1999). Briefly, for each screened plate the Z prime were determined using the following formula:

$$Z = 1 - \frac{3SD\ of\ positive\ control + 3SD\ of\ negative\ control}{|mean\ of\ positive\ control - mean\ of\ negative\ control|}$$

ATP-based cell proliferation measurement

For assessing proliferation, trisomic SVZ-derived NPC were dissociated in a single cell suspension and plated onto Nunclon™ Delta Surface 96-well plate (Thermo Fisher Scientific) in number of 4×10^3 cells/well in standard medium, composed by DMEM/F-12 medium supplemented with B27, Glutamax™ (2 mM, Life Technologies), heparin sodium salt (4 µg/ml; ACROS Organics), hFGF (10 ng/ml, Peprotech) and 100 U/100 µg/ml Penicillin/Streptomycin (Life Technologies). Plates were then incubated for 96 h at 37°C, 5% CO_2.

In order to detect proliferation differences in NPC we used a simple, fast and commercial luciferase-based method, the Cell Titer Glo assay (Promega) which biochemically quantified the levels of ATP as relative luminescence units (RLU) in each well, value directly proportional to the number of viable cells.

Luminescence was read on a Victor^3-V plate reader (PerkinElmer). Data were expressed as difference over euploid NPC in basal condition (hFGF 10 ng/ml + 0.05 % DMSO).

EdU incorporation based proliferation assay

EdU (5-ethynyl-2-deoxyuridine) incorporation was performed using the Click-iT® EdU Alexa Fluor® 488 HCS Assay Kit (Thermo Fisher Scientific), according to manufacturers' instructions. Briefly, NPC neurospheres were dissociated in a single cell suspension and plated onto laminin-coated 96-well plate (Falcon) in number of 4×10^3 cells/well in standard medium for 48-96 h. In the last 12 h period, EdU was added to each well at a final concentration of 10 μM. After that, cells were fixed by 4% paraformaldehyde solution in phosphate-buffered saline (PBS, pH 7.4) for 20 min at RT. EdU detection was performed using an InCell Analyzer 2200 (GE). In each experiment, 37 fields/well (corresponding to about 50 % of the total well surface) were counted. Data were expressed as difference over euploid basal condition (10 ng/ml hFGF + 0.05 % DMSO).

Statistical analysis

Results were presented as mean ± standard deviation of experiments run in triplicate. Data were analyzed with GraphPad Prism 7.0®. The statistical analysis were performed using either a one-way ANOVA or a two-way ANOVA. Post hoc multiple comparison were carried out using a Tukey's test or a Fisher's least significant difference (LSD) test. Results are considered statistically relevant with $p<0.05$.

RESULTS

Proliferative defects in murine neonatal trisomic neural progenitor cells: pharmacological correction by LiCl.

We isolated NPC from the SVZ of Ts65Dn (TS) and EU pups and cells were cultured in suspension, as neurospheres, in hEGF/hFGF containing medium. Initially, proliferation was measured by an ATP-based commercial kit, the Cell Titer Glo assay (Promega). Cells were seeded in presence of low (10 ng/ml) hFGF-mediated proliferative conditions and after 96 h in presence of vehicle (0.05 % DMSO), we detected significantly reduced proliferation in vehicle-treated TS NPC when compared with vehicle-treated EU NPC (mean percentage decrease ± S.D. over vehicle-treated EU NPC: 34 ± 21; $p<0.05$) (Fig. 1A). Reduced proliferation of TS NPC was also confirmed by a distinct method based on incorporation of 5-ethynyl-2'-deoxyuridine (EdU; Fig. 1B), which established that TS NPC proliferation was significantly reduced compared with vehicle-treated EU NPC (mean percentage decrease ± S.D. over vehicle-treated EU NPC: 21 ± 7.2 ; $p<0.001$).

Previous studies demonstrated that defective proliferation of TS NPC can be pharmacologically corrected by lithium chloride (LiCl) both *in vitro* (Trazzi et al., 2014) and *in vivo* (Bianchi et al., 2010, Contestabile et al., 2013). Based on such previous evidence, we initially tested the effect of a previously characterized drug concentration (2 mM). As expected, using the ATP based assay, we observed that LiCl promoted proliferation in EU NPC (mean percentage increase ± S.D. over vehicle-treated EU NPC: 52 ± 43 ; $p<0.01$ vs EU veh) and in TS NPC (mean percentage increase ± S.D. over vehicle-treated TS NPC: 45 ± 34 ; $p<0.05$ vs TS veh) (Fig. 1A). The effect of LiCl on TS NPC was significantly different compared with EU NPC ($p<0.01$ TS LiCl vs EU LiCl). Comparable results were

obtained using the EdU incorporation assay (Fig. 1B). In detail, LiCl, compared with vehicle promoted proliferation both in EU (mean percentage increase over vehicle-treated EU NPC: 35 ± 5.9; $p<0.001$ vs EU veh) and TS NPC (34 ± 1.7; $p<0.001$ vs TS veh).

Although both biochemical assays detected a pro-proliferative effect of LiCl on EU and TS NPC, for further experiments and for the screening activities we decided to use the ATP-based assay, which is simpler and faster than the EdU incorporation assay.

In the same experimental conditions, we also evaluated the effects of a wide range of LiCl concentrations (0.375-3.5 mM) in both EU and TS NPC and proliferation rate was tested by ATP intracellular content (Fig. 2A). Cells grown in presence of hFGF 10 ng/ml were exposed to LiCl or vehicle for a 96 h incubation period. Starting at 0.75 mM concentration, LiCl produced a concentration-dependent increase in proliferation of both EU and TS NPC ($p<0.001$ vs veh) with a maximal increase elicited at 2 mM both in EU NPC (mean percentage ± S.D. increase over vehicle-treated EU NPC: 101 ± 12 , $p<0.001$ vs EU veh) and TS NPC (mean percentage increase ± S.D. over vehicle-treated TS NPC: 132 ± 14 ; $p<0.001$ vs TS veh) (Fig. 2A). Based on these results, 2 mM LiCl was chosen as positive control in subsequent experiments.

Then, in order to evaluate the time-dependency of drug effects, 2 mM LiCl was also tested at different time points in cultures of both EU and TS NPC. In these experiments a further positive control was added, namely human epidermal growth factor (hEGF), at a 20 ng/ml concentration (Meneghini et al., 2014; Schwindt et al., 2009). We observed an increased proliferation of both EU and TS NPC exposed to vehicle after 48, 72 and 96 h, if compared with 24 h ($p<0.001$ vs 24 h). A multiple comparison between genotypes in presence of vehicle, at different time points, revealed that proliferation of TS NPC was significantly reduced after 48, 72 and 96 h if compared to vehicle-treated EU NPC ($p<0.01$,

$p<0.05$ and $p<0.01$, respectively). In the same experimental setting after 72 h LiCl significantly increased proliferation of both EU ($p<0.001$ vs veh) and TS NPC ($p<0.05$ vs veh), if compared with vehicle-treated cells after 24 h. After 96 h we confirmed the pro-proliferative effect of LiCl on both genotypes ($p<0.001$ vs veh 24 h) (Fig. 2B). In parallel, the positive control hEGF increased proliferation of both EU and TS NPC after 48, 72 and 96 h, if compared with vehicle 24 h ($p<0.001$ vs veh) (Fig. 2B).

When drug screening is performed in cell-based assays, especially when primary cultures are used, reproducibility over culture passages represent a crucial and critical issue. In order to assess whether the proliferative effects elicited by 2 mM LiCl were reproducible in cultures at different passages, we exposed different TS NPC preparations (n=4) from passages 1-14 to the drug or vehicle. As shown in Fig. 2C, we observed no statistically significant difference in LiCl response from P2-12, when compared with response obtained at P1. Conversely, LiCl effect was significantly reduced starting from P13 and at P14 (mean percentage ± S.D. decrease over passage 1: 33 ± 6.1 and 44 ± 9.5 , respectively; $p<0.05$ vs passage 1). Based on these results, library screening activities were performed with NPC derived from different cell preparations at P1-12.

Altogether these experiments confirm the difference in proliferation between TS and EU NPC and the pro-proliferative effect of LiCl, with optimal and reproducible effects at 2 mM and at a 96 h time point.

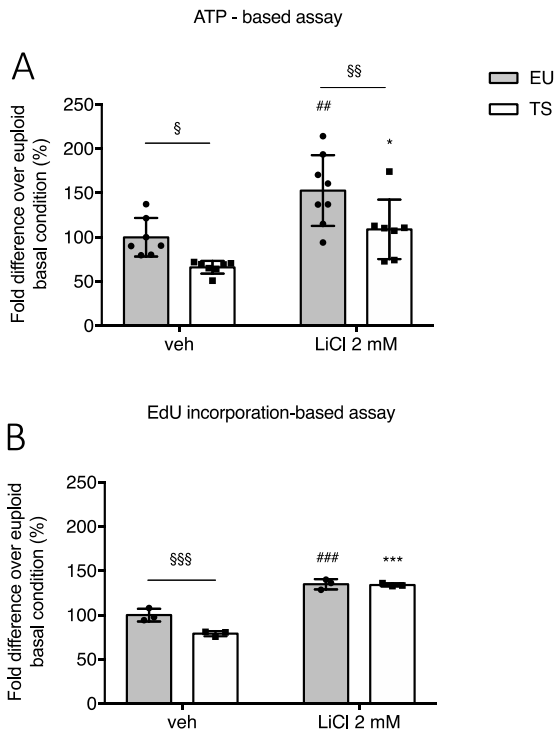

Figure 1. Reduced cell proliferation in trisomic SVZ-derived NPC can be corrected by LiCl

Proliferation experiments are performed on euploid (EU, grey bar) and trisomic (TS, white bar) SVZ-derived NPC. **A.** ATP based assay. EU/TS NPC are seeded in standard conditions (hFGF 10 ng/ml) and in presence of vehicle (DMSO 0.05 %) or LiCl (2 mM). Proliferation is measured after 96 h and data are represented as difference in comparison with euploid NPC basal condition. Error bars represent the standard deviation of n=7 replicates, run in seven different experiments. Each dot is the mean of three replicates. **B.** Edu incorporation-based assay. EU/TS NPC are seeded in standard conditions (hFGF 10 ng/ml) and in presence of vehicle (DMSO 0.05 %) or LiCl (2 mM) for 48 h, EdU is added in the last 8 h both in EU and TS NPC. Data are represented as difference in comparison with euploid NPC basal condition. Error bars represent the standard deviation of n=3 replicates, run in one experiment. Each dot represents a replicate. Results are considered statistically relevant with $p<0.05$ (## $p<0.01$, ### $p<0.001$ vs. vehicle-treated EU cells; *$p<0.05$, ***$p<0.001$ vs. vehicle-treated TS cells, Two way Anova, followed by Tukey's *post hoc*). The § indicate a difference in proliferation between genotypes (§ $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ vs EU, Two way Anova followed by Fisher's LSD *post hoc*).

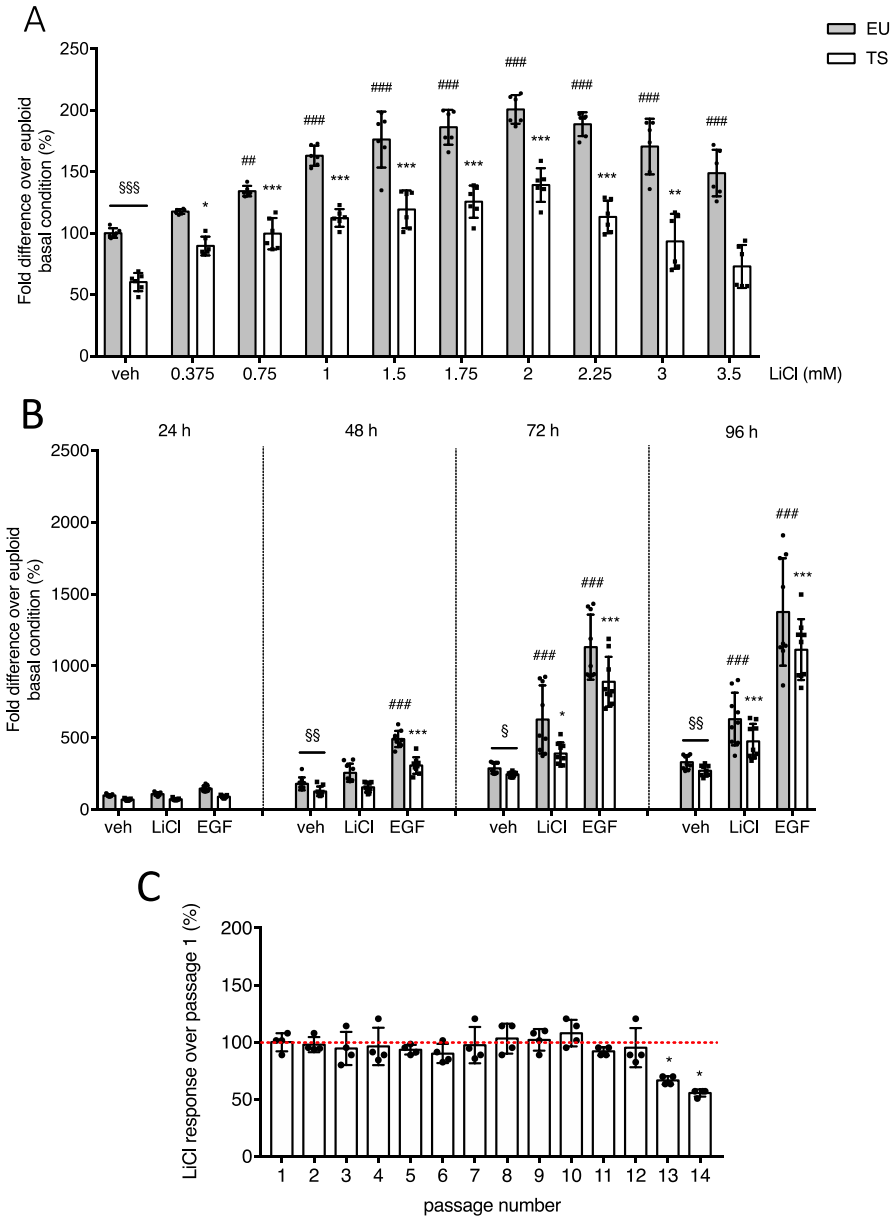

Figure 2. Characterization of LiCl response on SVZ-derived neonatal NPC

Proliferation experiments are performed on euploid (EU, grey bar) and trisomic (TS, white bar) SVZ-derived NPC. **A.** EU/TS NPC are seeded in medium containing 10 ng/ml hFGF-mediated proliferative conditions in presence of vehicle (veh) or different concentrations of LiCl (mM) for 96 h. Error bars represent the standard deviation of n=6 replicates, run in two different experiments. Each dot represents a replicate. Data are represented as difference in comparison

with euploid NPC basal condition. **B.** EU/TS NPC are seeded in medium containing 10 ng/ml hFGF in presence of vehicle, LiCl (2 mM) or hEGF (20 ng/ml) for 24, 48, 72 an 96 h. Data are represented as difference in comparison with euploid NPC basal condition. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a replicate. **C.** Lithium response over NPC culture passages. TS NPC are seeded in medium containing 10 ng/ml hFGF in presence of LiCl (2 mM) or vehicle for 96 h. Proliferation is represented as difference in comparison with LiCl response at passage one. The red dotted line represented the effect of LiCl at passage 1. Error bars represent the standard deviation of n=4 replicates, run in four different experiments. Each dot represents the mean of three replicates. For each preparation an average of 3-6 Ts65Dn and euploid pups are pooled together. Results are considered statistically relevant with $p<0.05$ (# $p<0.05$; ## $p<0.01$; ### $p<0.001$ vs. vehicle-treated EU cells; * $p<0.05$; ** $p<0.01$;*** $p<0.001$ vs. vehicle-treated TS cells, Two way Anova, followed by Tukey's *post hoc*). The § indicate a difference in NPC proliferation between genotypes (§ $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ vs EU cells, Two way Anova followed by Tukey's *post hoc*).

Further validation and reproducibility of the phenotypic-NPC based assay

A 96 hour incubation period appeared as a suitable time point for a screening assay development, with 2 mM LiCl and 20 ng/ml hEGF as positive controls, but we had to take into account evaporation as an important variable. Indeed edge effect caused by evaporation could negatively affect drug final concentrations and assay reproducibility (Maddox et al., 2008). In order to quantify edge effect in our experimental conditions, hypothesizing a 100 µl final working volume, we calculated evaporation at the edge and center of 96 well-plates hosted in the CO_2 incubator inside or outside a home-made humidity chamber, as previously described (Walzl et al., 2012). After 96 h incubation, medium volume was minimally or less reduced in edge wells of plates incubated in the humidity chamber (mean µl/well ± S.D.: 95 ± 0.89, 99.8 ± 0.3, in edge and central wells respectively), compared with wells of plates left outside the humidity chamber (mean µl/well ± S.D.: 89 ± 2.3 and 95 ± 1.3, in edge and central wells,

respectively). Since no evaporation was observed in central wells of plates kept in the humidity chamber, we decided to perform the drug screening campaign under this experimental condition and using only the center of 96 w-plates.

Compounds in most drug libraries are conveniently dissolved in DMSO. This commonly used solvent has many effects on cellular models, including NPC (Pal et al. 2012), and can give rise to confounding, false negative/positive results. For these reasons, we tested DMSO effects on TS (and EU) NPC proliferation under basal conditions (hFGF 10 ng/ml) over a wide range of concentrations of the solvent (0.01-2 %). We detected a statistically significant reduction in the percentage of proliferating NPC only at the highest concentration of DMSO. In particular, we observed a 71 % decrease of EU NPC treated with 2 % DMSO and a 63 % decrease of TS NPC treated with 2 % DMSO (Fig. 3A). Based on the fact that drug compounds were dissolved in 100% DMSO and that we planned to test them at 1 μM final concentration, we chose 0.05% DMSO as vehicle for further experiments.

In order to investigate the quality of the screening, we calculated the Z-prime value (Z') for each screened drug plate (see methods). We considered four parameters, the means and the standard deviations of the positive (2 mM LiCl and DMSO 0.05 %) and the negative control (10 ng/ml hFGF and DMSO 0.05 %). We obtained a score between 0.46 and 0.7, with a mean score of 0.55 (Fig. 3B), supporting a reliable assay and a good separation between the positive and the negative controls.

Altogether these results suggest that the best condition to reduce intra- and inter-plates variability and the quality of the assay was to use the condition humidity chamber, central wells, and DMSO 0.05 %.

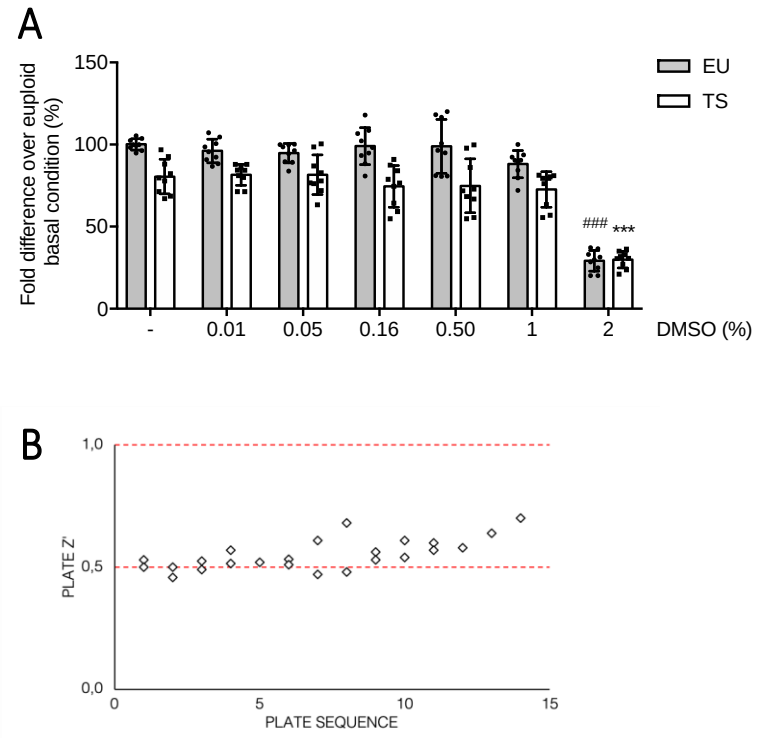

Figure 3. DMSO effect on SVZ-derived NPC and graphical representation of Z-prime value

A. Proliferation experiments are performed on euploid (EU, grey bar) and trisomic (TS, white bar) SVZ-derived NPC. EU/TS NPC are seeded in medium containing 10 ng/ml hFGF and a range of DMSO concentrations (0.01-2%) for 96 h. Data are represented as difference in comparison with euploid NPC basal condition. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a replicate. Results are considered statistically relevant with $p<0.05$ (### $p<0.001$ vs. vehicle-treated EU cells (-); ***$p<0.001$ vs. vehicle-treated TS cells (-), One way Anova, followed by Tukey's *post hoc*). **B.** Graphical representation of the Z prime value calculated for each screening plate. Calculations were performed as described in the method section and on the x-axis is represented the plate number, on the y-axis the Z' value for each drug plate. Red dotted lines represent the range for an excellent assay.

Drug screening and hit identification

Two distinct commercial drug libraries were used for the screening campaign: the Prestwick chemical library® (Prestwick Chemical), a well-recognized drug collection composed by 1,120 FDA/EMA approved drugs (Kanvatirth et al., 2019; Torres et al., 2018), and the Screen-Well® FDA Approved Drug Library V2, composed by 770 compounds (Enzo Life Sciences) (Corsello et al., 2017). Although a total of 1,890 drugs were tested, since the libraries are partially overlapping, altogether 1,590 chemically distinct compounds were screened. The complete screening workflow is schematized in Figure 4A. Trisomic SVZ-derived NPC (P3-P12) were seeded in 96 well-plates in presence of low (10 ng/ml) hFGF-mediated proliferative conditions and incubated at 37 °C, 5 % CO_2. After 30 minutes, vehicle (0.05 % DMSO), positive controls (2 mM LiCl and 20 ng/ml hEGF, both in 0.05 % DMSO) and library drugs (1 μM, 0.05 % DMSO) were added to wells in quadruplicates. Plates were then incubated for 96 h in the humidity box. At the end of the incubation period, proliferation was measured using the Cell Titer Glo assay kit (Promega), according to manufacturers' conditions. TS NPC from P2-P12 of n=3 different preparations were used for the entire campaign. Data were calculated as mean percentage change over standard, vehicle-treated conditions (10 ng/ml hFGF, 0.05 % DMSO). The results of the screening are summarized in Figure 4B as a dot plot, where each dot corresponds to a single drug, tested in quadruplicates. The effect of LiCl (+ 39%, red dotted line, $p<0.001$ vs vehicle-treated cells) was used as threshold to identify equally or more effective drugs (hits). In more details, we identified 30 FDA-EMA approved drugs more potent than LiCl in increasing TS NPC proliferation (red dots, Fig. 4B). When analyzed, potential hits belonged to three pharmacological classes: glucocorticoids, β2-adrenergic agonists and immunosuppressant drugs. We recognized also a category defined as others composed by α-β mixed adrenergic ligands (dobutamine HCl, etilefrine hydrochloride,), β mixed

adrenergic ligands (isoproterenol HCl; isosuxprine HCl,), β-adrenergic antagonists (pindolol) and a phosphodiesterase type 4 (PDE IV) inhibitor (glycopyrrolate iodide). All the potential hits and their relative percentage of increase over vehicle-treated cells are listed in table 1.

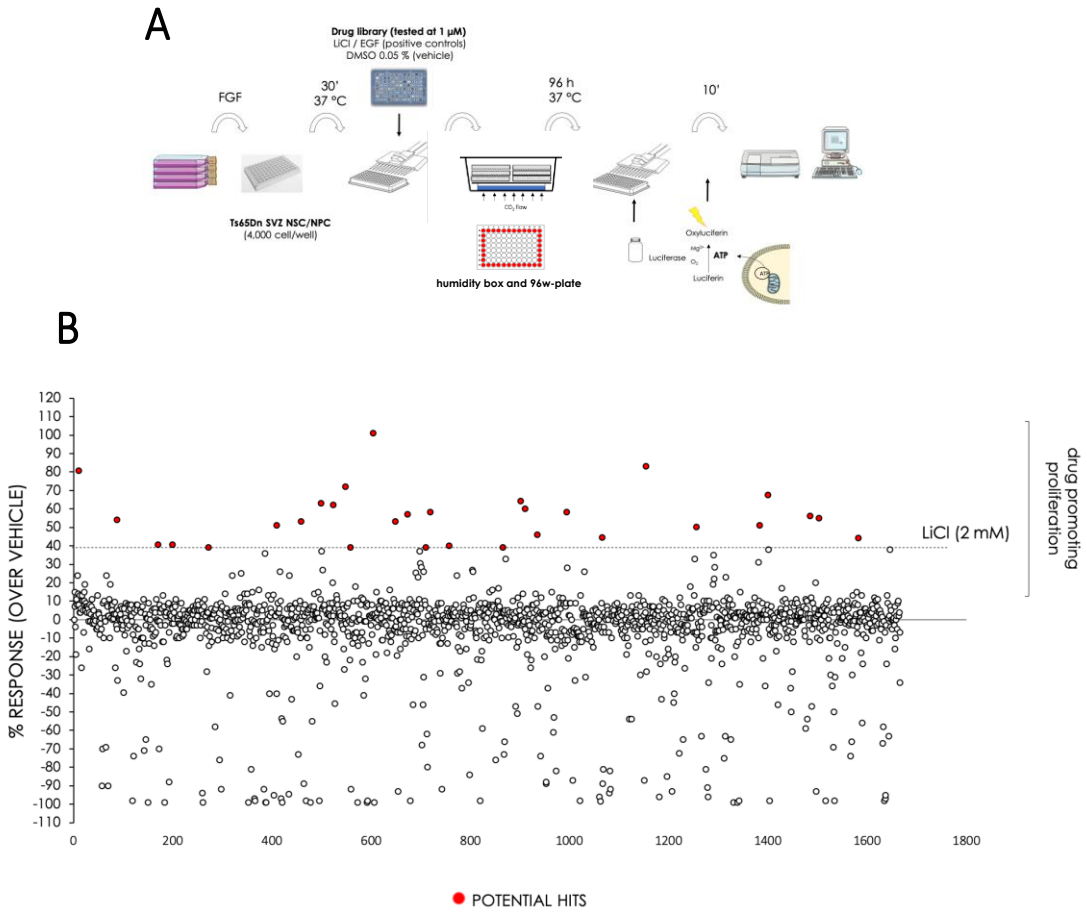

Figure 4. Results of the drug library screening in trisomic neonatal SVZ-derived NPC

A. Representative cartoon of the screening workflow. In the first step trisomic NPC are seeded in medium containing hFGF 10 ng/ml in 96 w-plates, after 30 minutes vehicle (0.05 % DMSO), positive controls (2 mM LiCl and 20 ng/ml hEGF, both in 0.05 % DMSO) and library drugs (1 µM, 0.05 % DMSO) are added. Only central wells (white wells) of a 96 w-plate are used. The edge wells (red one) are filled with 300 µl of distilled sterile water. After 96 h in the humidity box plates are removed and measured using the Cell Titer Glo assay (Promega) (see methods).

The reaction needs ten minutes and consists in cell lysis and ATP release into the media. Luciferin is transformed in oxyluciferin by luciferase in presence of ATP and luminescence is measured. The obtained value expressed as relative luminescence unit (RLU) is directly proportional to the quantity of ATP contained into each well. **B.** Scatter plot of the screened compounds represented as percentage of change over vehicle-treated TS NPC (hFGF 10 ng/ml and DMSO 0.05%). In the x-axis is represented the number of compound tested. In the y-axis the percentage of change over vehicle-treated cells. Each dot is a drug screened in quadruplicates at the final concentration of 1 μM. The dotted line represents the effect of LiCl (2 mM; + 39 %), fixed as a threshold to define a potential hit. Drugs more potent than LiCl are represented as red dots.

Hit confirmation and characterization

In order to confirm and validate potential hits, each drug was retested in the ATP-based proliferation assay using fresh powders from commercial suppliers. Concentration response curves from 0.1 to 1000 nM (DMSO 0.05 % as vehicle) were performed in different TS NPC preparations.

At first, in order to confirm previous results, we investigated whether selected glucocorticoids promoted proliferation in EU and TS NPC. We treated both EU and TS NPC in a concentration range of desonide, betamethasone and clobetasol (0.1-1000 nM). We detected a decreased proliferation of TS NPC in presence of vehicle, if compared with the euploid counterpart ($p<0.001$; Fig. 5 A, B) ($p<0.05$; Fig.5 C).

When EU NPC were incubated with desonide, we observed a concentration-dependent increase in proliferation of EU NPC in presence of 1, 10, 100 and 1000 nM desonide (mean percentage increase ± S.D. over vehicle-treated EU NPC: 17 ± 4 ; 38 ± 4.5 ; 46 ± 16 ; 51 ± 3.4 ; $p<0.001$ vs EU veh). In the same experimental setting, starting at 10 nM concentration, desonide produced an increase in proliferation of TS NPC at the concentrations of 10, 100, 1000 nM if compared with vehicle (mean percentage increase ± S.D. over vehicle-treated TS NPC: 39 ± 1.8 ; 39 ± 6.3 ; 42 ± 6.3 ; $p<0.001$ vs TS veh) (Fig. 5A).

When EU NPC were exposed to betamethasone we detected an increase in proliferation at the concentrations of 10, 100 and 1000 nM if compared with vehicle (mean percentage increase ± S.D. over vehicle-treated EU NPC: 29 ± 7.3 ; 28 ± 6.1 ; 35 ± 2.1 ; $p<0.001$). Comparably, betamethasone produced an increase in proliferation of TS NPC at the concentrations of 10, 100, 1000 nM if compared with vehicle (mean percentage increase ± S.D. over vehicle-treated TS NPC: 61 ± 2.6 ; 49 ± 1 ; 58 ± 6.1 ; $p<0.001$ vs TS veh) (Fig. 5B).

When clobetasol propionate was tested on NPC, we observed at all the tested concentrations (0.1-1000 nM) an increase in proliferation of both EU NPC (mean percentage increase ± S.D. over vehicle-treated EU NPC: 40 ± 4.3 ; 49 ± 4.5 ; 49 ± 6.4 ; 46 ± 10 ; 50 ± 4.1 ; $p<0.001$ vs EU veh) and TS NPC (mean percentage increase ± S.D. over vehicle-treated TS NPC: 38 ± 7.3 ; 37 ± 7.5 ; 44 ± 3.3 ; 41 ± 4.3 ; 39 ± 2.6 ; $p<0.001$ vs TS veh) (Fig. 5C).
As expected, LiCl (2 mM) significantly promoted proliferation of both genotypes if compared with vehicle-treated cells ($p<0.001$ vs veh) (Fig. 5 A-C).
Taken together these results confirm the pro-proliferative effect of desonide, betamethasone and clobetasol on the proliferation rate of TS NPC. We showed that these compounds were effective also on EU NPC.

In order to confirm whether β2-adrenergic agonists promote proliferation, we treated both EU and TS NPC with formoterol and terbutaline (0.1-1000 nM).
We observed a decreased proliferation of TS NPC in presence of vehicle, if compared with the euploid counterpart ($p<0.001$ vs EU veh) (Fig. 6A-C).
Starting at 1 nM concentration, formoterol produced a concentration-dependent increase in proliferation of EU NPC with a maximal effect elicited at 100 nM (mean percentage increase ± S.D. over vehicle-treated EU NPC: 63 ± 7; $p<0.001$ vs EU veh). Comparably, in the same conditions, formoterol enhanced the

proliferation of TS NPC at 10, 100 and 1000 nM concentrations (mean percentage increase ± S.D. over vehicle-treated TS NPC: 19 ± 3.2 ; 41 ± 3.7 ; 33 ± 2.5 ; $p<0.001$ vs TS veh) if compared with vehicle-treated TS cells (Fig. 6A).

When NPC where incubated with terbutaline we observed an increase in proliferation of EU NPC only at 100 and 1000 nM concentrations (mean percentage increase ± S.D. over vehicle treated EU NPC: 21 ± 6.1 ; 41 ± 8.9 ; $p<0.001$ vs EU veh). In the same experimental conditions, terbutaline increased the proliferation of TS NPC only at the concentration of 1000 nM (mean percentage increase ± S.D. over vehicle-treated TS NPC: 39 ± 4.5 ; $p<0.001$ vs TS veh) (Fig.6B).

We also investigated whether the non-selective β-agonist isoproterenol affected NPC proliferation, by treating both EU and TS NPC in presence of a drug concentration range (0.1-1000 nM). Isoproterenol at the concentration of 10, 100, 1000 nM produced an increase in proliferation of both EU NPC (mean percentage increase ± S.D. over vehicle-treated EU NPC: 47 ± 5 ; 68 ± 3 ; 52 ± 1.5 ; $p<0.001$ vs EU veh) and TS NPC (mean percentage increase ± S.D. over vehicle-treated TS NPC: 29 ± 2.6 ; 51 ± 7.2 ; 45 ± 8.6 ; $p<0.001$ vs TS veh) (Fig. 6C).

Taken together these results confirm that formoterol, terbutaline and isoproterenol enhance TS NPC proliferation. These drugs were effective also in EU NPC.

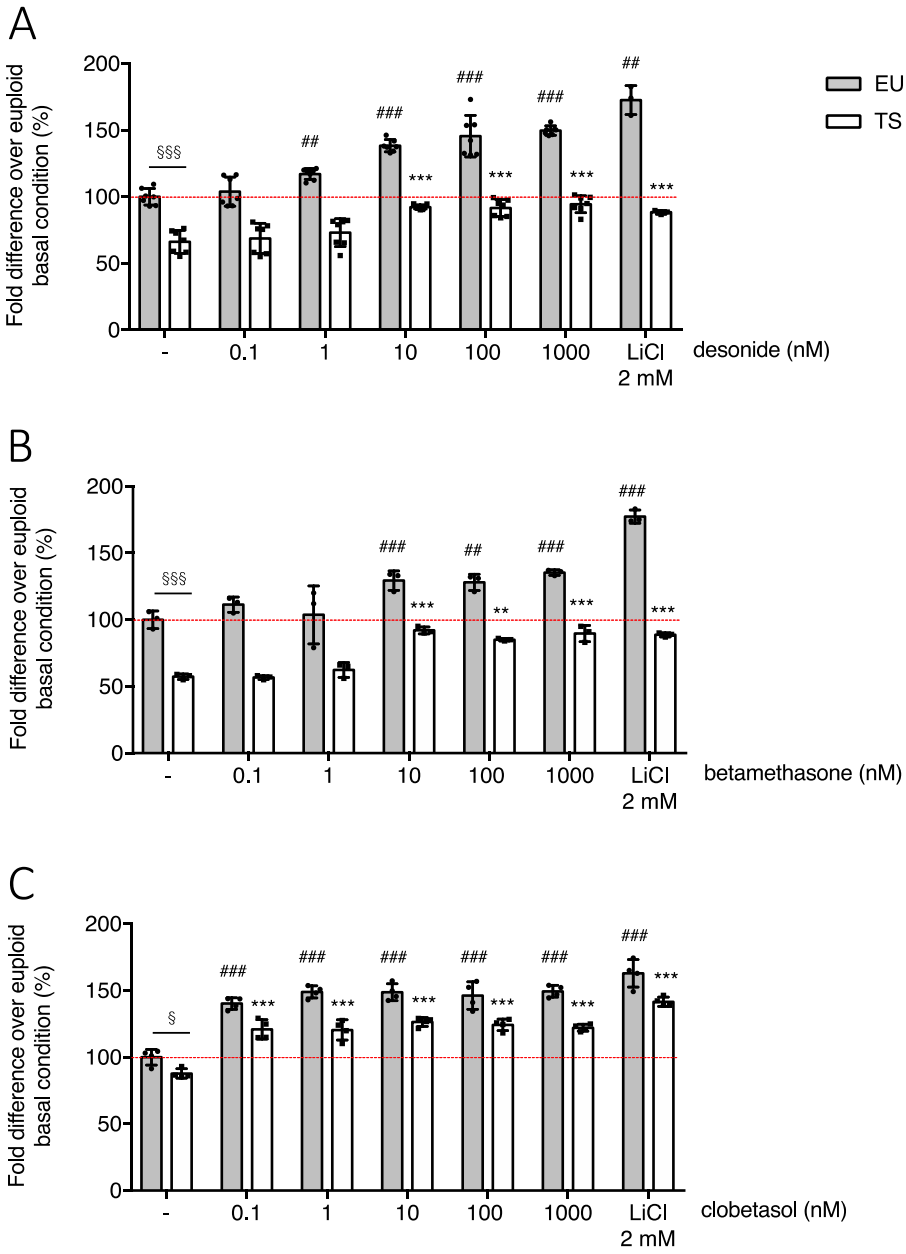

Figure 5. Desonide, betamethasone and clobetasol promote proliferation in euploid and trisomic neonatal SVZ NPC

Proliferation experiments are performed on euploid (EU, grey bar) and trisomic (TS, white bar) SVZ-derived NPC. **A.** EU/TS NPC are seeded in medium containing 10 ng/ml hFGF in presence of vehicle (-, DMSO 0.05 %), a range of desonide concentrations (0.1-1000 nM) or LiCl (2 mM)

for 96 h. Error bars represent the standard deviation of n=6 replicates, run in two different experiments and each dot represents a replicate. **B.** EU/TS NPC are seeded in medium containing 10 ng/ml hFGF in presence of vehicle (-; DMSO 0.05 %), a range of betamethasone concentrations (0.1-1000 nM) or LiCl (2 mM) for 96 h. Error bars represent the standard deviation of n=3 replicates, run in one experiment and each dot represents a replicate. **C.** EU/TS NPC are seeded in medium containing 10 ng/ml hFGF in presence of vehicle (-; DMSO 0.05 %), a range of clobetasol propionate concentrations (0.1-1000 nM) and LiCl (2 mM) for 96 h. Error bars represent the standard deviation of n=4 replicates, run in one experiment and each dot represents a replicate. Data are represented as difference in comparison with euploid NPC basal condition and the red dotted line represents the euploid NPC basal condition. Results are considered statistically relevant with $p<0.05$ (## $p<0.01$, ### $p<0.001$ vs. vehicle-treated EU cells; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. vehicle-treated TS cells, Two way Anova, followed by Tukey's *post hoc*). The § indicate a difference between genotypes in basal condition (§ $p<0.05$ vs. vehicle-treated EU NPC, Two way Anova, followed by Tukey's *post hoc*).

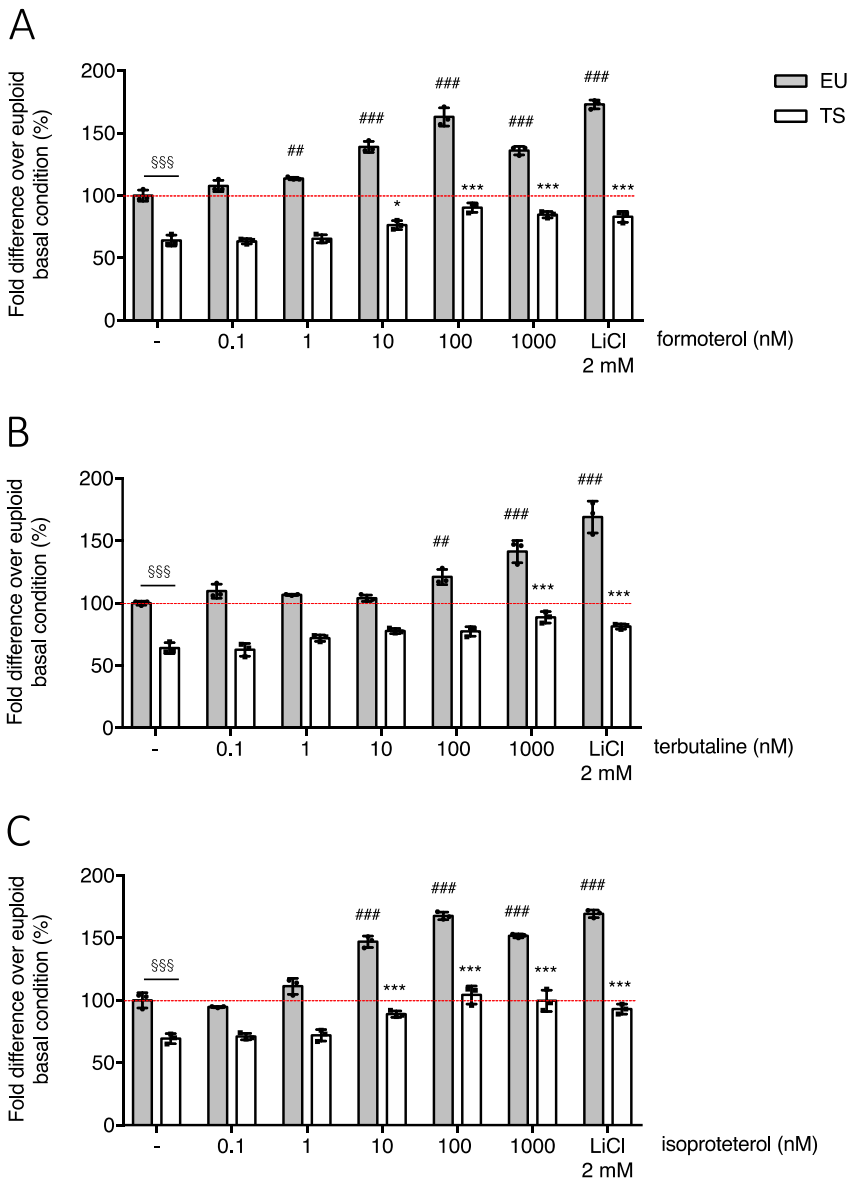

Figure 6. Formoterol, terbutaline and isoproterenol promote proliferation in euploid and trisomic neonatal SVZ-derived NPC

Proliferation experiments are performed on euploid (EU, grey bar) and trisomic (TS, white bar) SVZ-derived NPC**. A.** EU/TS NPC are seeded in 10 ng/ml hFGF in presence of vehicle (-; DMSO 0.05 %), a range of formoterol concentration (0.1-1000 nM) or LiCl (2 mM) for 96 h. Error bars represent the standard deviation of n=3 replicates, run in one experiment and each dot represents a replicate. **B.** EU/TS NPC are seeded in 10 ng/ml hFGF in presence of vehicle (-; DMSO 0.05

%), a range of terbutaline concentrations (0.1-1000 nM) or LiCl (2 mM) for 96 h. Error bars represent the standard deviation of n=3 replicates, run in one experiment and each dot represents a replicate. **C.** EU/TS NPC are seeded in 10 ng/ml hFGF in presence of vehicle (-; DMSO 0.05 %), a range of isoproterenol concentrations (0.1-1000 nM) or LiCl (2 mM) for 96 h. Error bars represent the standard deviation of n=3 replicates, run in one experiment and each dot represents a replicate. Data are expressed as difference in comparison with euploid NPC basal condition and the red dotted line represents euploid NPC basal condition. Results are considered statistically relevant with $p<0.05$ (## $p<0.01$, ### $p<0.001$ vs. vehicle-treated EU cells; *$p<0.05$, ***$p<0.001$ vs. vehicle-treated TS cells, Two way Anova, followed by Tukey's *post hoc*). The § indicate a difference between genotypes in basal condition (§ $p<0.05$ vs. vehicle-treated EU NPC, Two way Anova, followed by Tukey's *post hoc*).

DISCUSSION

DS brain shows several defects potentially contributing to cognitive impairment (Bartesaghi et al., 2011; Lott & Dierssen, 2010). One of them is a remarkable hypocellularity which is evident since early fetal stages. Several past and current lines of research in DS propose a key pathophysiological role for reduced proliferation of trisomic NPC (Guidi et al., 2008; Lorenzi & Reeves, 2006; Murray et al., 2015; Stagni et al., 2018), which is also associated with an altered differentiation program toward neuronal lineages (Chen et al., 2013, 2014b; Hibaoui et al., 2014). A few years ago a pioneer study in Ts65Dn animal model showed that a pharmacological perinatal (prenatal and neonatal) treatment targeting the serotoninergic system could correct trisomic NPC phenotypes (Stagni et al., 2015) and result in partial correction of cognitive impairment. Since then, other preclinical DS research programs have proposed potential therapies that may correct DS phenotype in animal models (Herault et al., 2017; De la Torre & Dierssen 2012; Vacca et al., 2019), but so far these efforts have failed to translate into clinical applications (Kazemi et al., 2016).

In this study, we set up a phenotypic cell-based assay using reduced proliferation of murine trisomic neural progenitor cells as a primary read out to screen, for drug repurposing, FDA-EMA approved drugs. In principle, this strategy may dramatically reduce the possibility to move a drug candidate into the clinical setting (Clout et al., 2019; Reaume, 2011). To our knowledge, this study represents the first attempt to identify potential DS therapies using a drug repurposing strategy, and a NPC-based primary assay.

Based on these premises, we first developed and validated a phenotypic assay able to detect changes in the proliferation rate of neonatal trisomic, compared to euploid, NPC. We identified LiCl (2 mM) as a positive control drug able to promote NPC proliferation. LiCl has been chosen since its proneurogenic effect was demonstrated not only *in vitro,* but also *in vivo* in adult Ts65Dn mice (Bianchi et al., 2010; Contestabile et al., 2013). LiCl is a first line drug for bipolar disorders and it has been proposed to promote NPC proliferation through the inhibition of glycogen synthase kinase-3β (GSK-3β) (Pasquali et al., 2010; Zhang et al., 2019). Unfortunately its toxicity prevents its clinical use in DS children. The overall goal of our study was to identify, among approved drugs, molecules able to increase proliferation in trisomic NPC. We optimized assay quality and reproducibility in order to be able to use the assay for a screening campaign where read out for proliferation was quantification of the intracellular ATP content. These data have been further confirmed by another biochemical proliferation assay based on EdU incorporation. Primary NPC showed a reproducible response to 2 mM LiCl from passage 1 to 12. This was a critical aspect of the assay since the screening campaign lasted months and we utilized several distinct NPC preparations. All drugs were screened at a final concentration of 1 μM, in agreement with other drug screening protocols (Hughes et al., 2011) and in presence of DMSO 0.05 % which did not perturbe the assay. As a source of drugs, we used two well-established FDA-EMA approved libraries

characterized by high pharmacological and chemical diversity (Corsello et al., 2017; Kanvatirth et al., 2019; Torres et al., 2018). At the end of the efforts we obtained about 30 potential hits, selected based on their ability to be equally or more effective than LiCl. Hits belonged to several pharmacological classes and we focused our attention on some of them for confirmation and validation activities. Drugs that were reproducibly effective on proliferation in subsequent studies were also tested for their effects on neuronal differentiation, which is also defective in trisomic NPC (*data not shown*).

Among different pharmacological classes identified through the screening campaign, glucocorticoids (GC) were the most unexpected ones. Usually, they are associated to a stress response which negatively affect NPC proliferation and, in general, neurogenesis (Saaltink & Vreugdenhil, 2014). During pregnancy, maternal stress has been proposed to affect embryonic neurogenesis and the risk to develop neurological disorders maybe due to excessive glucocorticoid transfer from the mother to the fetus (Odaka et al., 2017). Sunberg et al. showed that dexamethasone (1 µM, the same concentration we used) reduces neural stem cell proliferation (Sundberg et al., 2006). Another study demonstrated a decreased hippocampal volume, number of proliferating cells in SVZ and SGZ in animal treated with dexamethasone (0.5 mg/kg/day from P4 to P7) (Kanagawa et al., 2006). Although the majority of the studies suggest negative effects of GC on NPC, at least another group reported that GC (0.005-50 µM) may increase proliferation of NPC derived from human induced pluripotent stem cells (iPSCs), reprogrammed from fetal lung fibroblasts, in a concentration-dependent manner (Ninomiya et al., 2014). Another study showed that NPC obtained from hiPSCs could synthetize glucocorticoids, promoting their proliferation and inhibiting their neuronal differentiation (Nürnberg et al., 2018). Glucocorticoids bind the glucocorticoid receptor (GR), but questions are open about GR isoforms

expressed in NPC and target genes that are inhibited or activated by these receptors (Gündisch et al., 2012; Najm et al., 2015; Saaltink & Vreugdenhil, 2014). It should not disregarded that non-transcriptionally mediated effects of GC are also well known. Indeed, the response mediated by GR may be much more complex than expected, involving multiple parallel mechanisms which integrate signals from other receptors (Hapgood et al., 2016). It has been shown that glucocorticoids may negatively affect NPC proliferation and differentiation through intracellular signaling pathways such as phosphoinositide 3-kinase (PI3K)/Akt, sonic hedgehog (Shh) and Wnt (Nelson et al., 2001; Odaka et al., 2017). These pathways are well known to be impaired in DS (Granno et al., 2019; Perluigi et al., 2014), but this appears not relevant since we observed a proliferative effect mediated by GC both in trisomic and euploid NPC. For example, in DS animal models, a reduced proliferation of cerebellar granule cell precursors has been associated with attenuated Shh response. This effect was tentatively explained by hyperactivation of the transmembrane receptor Patched1 (Ptch1) and inhibition of the Smoothened (Smo) receptor (Giacomini et al., 2015; Trazzi et al., 2011), which is involved in the regulation of stem cell proliferation (Vicario et al., 2019). In agreement with these findings, it has been proposed that some glucocorticoid molecules promote NPC proliferation acting as Smo agonists (Wang et al., 2010). Nevertheless, none of the GC identified as hits in our screening activities were smo agonists. On the other hand clobetasol propionate, a smo agonist, was present in the library and, although less effective than LiCl, it promoted TS NPC proliferation. We tested clobetasol in a concentration response curve and we observed that the drug increased NPC proliferation both in trisomic and euploid cells, once again leaving us clueless about the mechanism behind these effects. Future studies should be undertaken to elucidate the pro-proliferative effect of GC on neonatal NPC.

Through the screening activities several β2 adrenergic agonists were also identified as hits. Like GC, β2AR agonists were effective both in TS and EU NPC. When validated in concentration response curves, formoterol was more potent than terbutaline, in line with its higher affinity for β2AR (Lemoine et al., 1992; Molimard et al., 1998). Although administration of the long acting β2AR formoterol (2 mg/kg, i.p.) in 4-6-month-old Ts65Dn mice resulted in improved cognitive functions and promoted dendritic complexity (Dang et al., 2014), NPC were not proposed as cellular targets for mediating such effects in the DS animal model. Recently our group proposed a novel pharmacological property of salmeterol and formoterol, namely promotion of adult hippocampal neurogenesis both *in vitro* and *in vivo* in wild type mice (Bortolotto et al., 2019). At least *in vitro* the drugs acted by directly promoting neuronal differentiation of adult NPC, as proven by a lack of response in β2AR KO NPC. Interestingly, in adult hippocampal progenitors β2AR agonists were devoid of proliferative effects, suggesting a different sensitivity of neonatal versus adult NPC. Other adrenergic compounds were identified in the screening campaign, for example the non-selective beta agonist isoproterenol (see table 1). Jhaveri et al. demonstrated that *in vitro* isoproterenol and norepinephrine (NE) exhibit a proliferative effect in the neurosphere assay via activation of the β3AR (Jhaveri et al., 2010), so at present we cannot exclude that this receptor subtype may also mediate the effects of isoprotenerol in EU/TS NPC. Interestingly, in adult wt mice β2AR drugs were also very effective in promoting a significant increase in dendritic length and complexity of DCX^+ hippocampal neuroblasts (Bortolotto et al., 2019). Based on these results and our *in vitro* screening data, a currently ongoing study at the University of Bologna (in the Laboratory of Prof. Bartesaghi) is evaluating the effects of subchronic administration of β2AR agonists that pass the blood brain

barrier in neonatal TS and EU pups with encouraging preliminary results (*data not shown*).

Although not characterized in this work, other interesting hits identified during our screening campaign were the immunosuppressants cyclosporine A (CSA) and tacrolimus. Based on the results of the screening and the *in vitro* characterization of its activities (Stagni et al., 2019), CSA was then tested *in vivo*. A daily treatment of Ts65Dn mice from P3 to P15 with CSA (15 mg/kg/day, s.c.) increased NPC proliferation in both SVZ and SGZ regions, increased neuronal density and dendritic arborizations in the dentate gyrus of treated mice examined at P45 (Stagni et al., 2019). Studies are ongoing to assess whether amelioration of cognitive performance also correlate with neuroanatomical effects. In the future, it may be important to evaluate the long-term effects of CSA treatment on cognitive performances of Ts65Dn mice. Unfortunately, CSA toxicity is likely to prevent its use in the DS clinical setting, but since the remarkable effects of the drug in the murine model, we believe it will be very important in the near future to unravel its underlying mechanism(s) of action. These type of studies may ultimately provide key information on novel pharmacological targets in DS pathophysiology.

In conclusion, we were able to set up a reproducible and sensitive trisomic NPC-based phenotypic assay. Combined with a drug repurposing strategy, this assay was instrumental for performing a screening campaign of 1590 approved drugs. The efforts allowed the identification of novel classes of compounds which could correct reduced proliferation of trisomic NPC. At least in one case, CSA, these results prompted us to test the drug *in vivo* with very encouraging results: the drug corrected brain atrophy, neurogenesis and spine defects in Ts65Dn mice (Stagni et al., 2019), proving once again the concept that NPC may represent pharmacological targets in DS. Other identified hits are currently under testing

in vivo in Ts65Dn mice. Since the phenotypic nature of the assay, in the future we may need to unravel the underlying mechanism(s) of action of the identified hits that are also effective *in vivo*. These mechanisms may also be different from the one(s) involved in the primary action of approved drugs. In such case, our experimental strategy may ultimately result in identifying novel signaling pathways involved in the pathophysiology of DS.

β2 ADRENERGIC / ANTIASTHMATIC DRUGS	
NAME	**% RESPONSE (OVER STD)**
Clenbuterol hydrochloride	51
Ritodrine hydrochloride	51
Terbutaline hemisulfate	55
Fenoterol hydrobromide	57
Formoterol	58
Metaproterenol Hemisulfate (Orciprenaline)	58
Levalbuterol-HCl	64
Salbutamol	67,5

GLUCOCORTICOIDS	
NAME	**% RESPONSE (OVER STD)**
Budesonide	39
Beclomethasone dipropionate	40,5
Betamethasone	40,5
Triamcinolone Acetonide	44
Mometasone furoate	44,5
Loteprednol Etabonate	46
Amcinonide	54
Difluprednate	62
Desonide	63

IMMUNOSUPPRESSANTS	
NAME	**% RESPONSE (OVER STD)**
Cyclosporin A	53
Tacrolimus (Fk506)	56

OTHERS	
NAME	**% RESPONSE (OVER STD)**
Dobutamine hydrochloride	39
Isoxsuprine hydrochloride	39
Glycopyrrolate Iodide	40
Pindolol	50
Etilefrine hydrochloride	53
Levonordefrin	60
Dipivefrin hydrochloride	72
(±) Isoproterenol-HCl	80,5
Norepinephrine Bitartrate Monohydrate	83
Epinephrine (L-(-)-Epinephrine-(+)-Bitartrate)	101

Table 1. List of drugs promoting proliferation more than lithium chloride (2 mM LiCl) classified in pharmacological classes.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 4

Neonatal treatment with cyclosporine A restores neurogenesis and spinogenesis in the Ts65Dn model of Down syndrome

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ABSTRACT

Down syndrome (DS), a genetic condition due to triplication of chromosome 21, is characterized by reduced proliferation of neural progenitor cells (NPCs) starting from early life stages. This defect is worsened by a reduction of neurogenesis (accompanied by an increase in astrogliogenesis) and dendritic spine atrophy. Since this triad of defects underlies intellectual disability, it appears of importance to establish whether it is possible to pharmacologically correct these alterations. In this study, we exploited the Ts65Dn mouse model of DS in order to obtain an answer to this question. In the framework of an *in vitro* drug-screening campaign of FDA-approved drugs, we found that the immunosuppressant cyclosporine A (CSA) restored proliferation, phenotype acquisition and maturation of neural progenitor cells (NPCs) from the subventricular zone (SVZ) of the lateral ventricle of Ts65Dn mice. Based on these findings, we treated Ts65Dn mice with CSA in the postnatal period P3-P15. We found that treatment fully restored NPCs proliferation in the SVZ and in the hippocampal subgranular zone (SGZ) and total number of hippocampal granule cells. Moreover, CSA enhanced development of dendritic spines on the dendritic arbor of the granule cells whose density even surpassed that of euploid mice. In hippocampal homogenates from Ts65Dn mice we found that CSA normalized the excessive levels of p21 and APP, two key determinants of proliferation and differentiation impairment. Results show that neonatal treatment with CSA restores the whole triad of defects of the trisomic brain. The use of a therapy with CSA for DS may pose caveats because it is an immunosuppressant that may cause adverse effects. However, CSA analogues that mimic its effect without eliciting immunosuppression may represent practicable tools for ameliorating brain development in individuals with DS.

Key words: Down syndrome; Neurogenesis; Spinogenesis; Pharmacotherapy; Cyclosporine A

INTRODUCTION

Intellectual disability is one of the most serious problems in Down syndrome (DS), a genetic condition caused by triplication of chromosome 21. The impairment of brain function, which is already detectable in infants with DS, is attributable to severe impairment of key neurodevelopmental processes. In particular, evidence in fetuses/infants with DS and in DS mouse models shows that trisomic neural precursor cells have a reduced proliferation rate and exhibit an altered differentiation program that causes a reduction in the number of cells that differentiate into neurons and an increase in the number of cells that differentiate into astrocytes (Dierssen, 2012; Stagni et al., 2018). Neuronal maturation is also altered in DS, which leads to neurons with a reduced dendritic arborization and a reduced density of dendritic spines. The unavoidable outcome of these defects is the impairment of overall brain wiring, explaining the alteration within a constellation of cognitive domains that characterizes DS. Intense efforts are currently underway in order to establish whether it is possible to pharmacologically ameliorate intellectual disability in DS. In this connection, there are two possible and not mutually exclusive approaches. One possibility is to use drugs that specifically target cellular pathways that are altered in the DS brain and that are known to regulate one or more of the neurodevelopmental processes mentioned above. This approach requires preliminary knowledge regarding the molecular alterations of the DS brain, the choice of the molecular pathway to be targeted, and the choice of the drug that putatively acts on this pathway. A second possibility is the repositioning of drugs/compounds that were designed and approved for other pathologies. Various studies show that drugs

designed for quite different purposes may actually exert some benefits in various brain disorders. Thus, this strategy is not simply based on an *a priori* assumption. This approach requires i) the development of a reproducible and sensitive phenotypic assay, based on relevant defective properties of trisomic neural progenitor cells (NPCs); ii) a subsequent *in vitro* screening of libraries of clinically approved drugs in the search for those that may revert that phenotype. In principle, cell-based screening combined with strategies of drug repurposing offers the opportunity to significantly reduce risks and costs associated with developing new therapeutics and, more importantly, may dramatically reduce time for human translation. By following this strategy, we aim to identify clinically approved drugs that are able to restore proliferative and differentiative defects of NPCs derived from the Ts65Dn mouse, a widely-used model of DS. After *in vitro* identification of effective molecules, our final goal is to test their effects *in vivo* in the Ts65Dn mouse. Herein we show the identification of the immunosuppressant and clinically relevant cyclosporine A (CSA) as a drug that can not only restore the proliferation rate of NPCs and their differentiation into neurons *in vitro*, but that is also effective *in vivo* in the Ts65Dn mouse. According to recent work, a dose of 15 mg/kg/day of CSA increases the pool of actively dividing cells in the dentate gyrus of wild type mice and also favors the generation of new granule neurons (Chow & Morshead, 2016). Herein we show that Ts65Dn neonatal treatment with 15 mg/kg/day of CSA for a short period of time (13 days) restores proliferation of NPCs in the two major brain neurogenic niches (the subventricular zone and the subgranular zone of the hippocampal dentate gyrus) and reinstates hippocampal development, in terms of granule cell number and spinogenesis. This provides evidence that CSA treatment can rescue the major neurodevelopmental defects of the DS brain.

MATERIALS AND METHODS

Mouse colony

Ts65Dn mice were generated by mating B6EiC3Sn a/A-Ts(17^16)65Dn females with C57BL/6JEiJ x C3H/HeSnJ (B6EiC3Sn) F1 hybrid males. This parental generation was provided by Jackson Laboratories (Bar Harbor, ME, USA). To maintain the original genetic background, the mice used were of the first generation of this breeding. Animals were genotyped as previously described (Reinholdt et al., 2011). The day of birth was designated postnatal day zero (P0). The animals' health and comfort were controlled by the veterinary service. The animals had access to water and food *ad libitum* and lived in a room with a 12:12 h light/dark cycle. Experiments were performed in accordance with the European Community 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.

IN VITRO EXPERIMENTS

Isolation and culture of SVZ neural progenitor cells

Cells were isolated from the subventricular zone (SVZ) of the lateral ventricle of newborn (age 1-2 days) euploid and Ts65Dn mice, as previously described (Stagni et al., 2017). Briefly, brains were removed, the SVZ region was isolated and collected in ice-cold PIPES buffer pH 7.4. After centrifugation, tissue was digested for 10 min at 37°C using Trypsin/EDTA 0.25% (Life Technologies) aided by gentle mechanical dissociation. Cell suspensions from individual mice were pooled and plated onto 25 cm^2 cell-culture flask (Thermo Fisher Scientific) and cultured as floating neurospheres in medium containing basic fibroblast growth factor (bFGF, 10 ng/ml; Peprotech) and epidermal growth factor (EGF,

20 ng/ml; Peprotech) using an established protocol (Meneghini et al., 2014). Primary (Passage 1, P1) neurospheres were dissociated using Stempro Accutase (Life Technologies) after 7 days *in vitro* (DIV); thereafter neurospheres were passaged every 5 DIV. For further *in vitro* studies cells from P3 to P12 were used.

Phenotypic drug screening

For the drug screening, two different commercial libraries (Prestwick chemical library®, Prestwick Chemical, and Screen-Well® FDA Approved Drug Library V2, Enzo Life Sciences), containing a total of 1,887 FDA/EMA-approved drugs were used. These libraries were chosen for their chemical and pharmacological diversity. Trisomic SVZ NPCs (P3-P12) pooled from at least 3-5 pups were dissociated in a single cell suspension and plated onto Nunclon™ Delta Surface 96-well plate (Thermo Fisher Scientific) at a density of 4×10^3 cells per well in DMEM/F-12 medium supplemented with B27, Glutamax™ (2 mM, Life Technologies), heparin sodium salt (4 µg/ml; ACROS Organics), bFGF (10 ng/ml, Peprotech) and 100 U/100 µg/ml Penicillin/Streptomycin (Life Technologies) for 30 min, at 37 °C. Compounds were added to each well in quadruplicates (1 µM final concentration, in 0.05% DMSO). In parallel, EGF (20 ng/ml, Peprotech) and LiCl (2 mM, Sigma-Aldrich), that have been shown to restore proliferation of NPCs of Ts65Dn mice *in vivo* (Bianchi et al., 2010a, Contestabile et al., 2013) and *in vitro* (Trazzi et al., 2014), were added to each plate in quadruplicates as pro-proliferative controls. Lithium concentration was chosen based on previous evidence (Trazzi et al., 2014). Cell proliferation was quantified after 96 h incubation in a humidity chamber (to minimize evaporation) and quantified as relative luminescence units (RLU) values using a CellTiter-Glo ATP-based assay kit (Promega) on a Victor^3-V plate reader (PerkinElmer) (Stagni et al., 2017). Drug activity was calculated as percentage of change compared to basal conditions (cells grown in presence of 10 ng/ml FGF and 0.05

% DMSO). The same proliferation assay was performed to assess hit concentration response curves (0.1-1000 nM).

Neural Progenitor Cell Proliferation and Differentiation

In order to evaluate cell proliferation in secondary assays, EdU (5-ethynyl-2-deoxyuridine) incorporation was performed using the Click-iT® EdU Alexa Fluor® 488 HCS Assay Kit (Thermo Fisher Scientific). Briefly, neurospheres (P3-P12) were dissociated in a single cell suspension and plated onto laminin-coated 96-well plate (Falcon) at a density of 4×10^3 cells per well in DMEM/F-12 medium supplemented with B27, Glutamax™, heparin sodium salt (4 μg/ml; ACROS Organics), bFGF (10 ng/ml) and 100 U/100 μg/ml Penicillin/Streptomycin (Life Technologies) in presence of CSA (1000 nM; MedChem Express) or its vehicle (DMSO 0.05%) for 72 h. In the last 12 h period, EdU was added to each well at a final concentration of 10 μM. After that, cells were fixed for 20 min at room temperature using 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). EdU detection was performed according to manufacturer's instructions. In each experiment, 37 fields/well (corresponding to about 50 % of the total well surface) were counted using an InCell Analyzer 2200 (GE). Cell death was evaluated using the CytoTox-Glo™ Cytotoxicity Assay (Promega) according to the manufacturer's instructions. Cells were exposed to CSA (30-1000 nM, MedChem Express) or its vehicle (DMSO 0.05%) for 96 h. Cytotoxicity was quantified as relative luminescence unit (RLU) on a Victor^3-V plate reader (PerkinElmer) and expressed as percentage over the total number of cells. For differentiation experiments neurospheres from the SVZ were dissociated into single cells and plated onto laminin-coated Lab-Tek 8-well permanox chamber slides (Thermo Fisher Scientific) at a density of 35×10^3 per well in differentiation medium (DMEM-F12 supplemented with B27, 2 mM Glutamax and 100 U/100 mg/ml

penicillin/streptomycin). NPCs were treated in presence of CSA (3–1000 nM) or vehicle (DMSO 0.05 %) for 96 h. After that, cells were fixed for 20 min at room temperature using 4% paraformaldehyde. Phenotypic characterization of NPC-derived cells was carried out by immunolocalization for MAP2 (rabbit polyclonal, 1:50,000; Abcam) and Nestin (chicken monoclonal, 1:2,500; Neuromics). Secondary antibodies were as follows: AlexaFluor555-conjugated goat anti rabbit (1:1,400; MolecularProbes), and AlexaFluor488-conjugated goat anti chicken (1:1,400; Molecular Probes). In additional experiments, in which NPCs were exposed to selected concentrations of CSA (100–1000 nM) or vehicle for 96 h, in parallel to MAP2/nestin, GFAP immunoreactivity was evaluated using a mouse anti-GFAP monoclonal antibody (1:600, Millipore), and a secondary AlexaFluor555-conjugated goat anti mouse antibody (1:1600; Molecular Probes). Nuclei were counterstained with 0.8 ng/ml Hoechst (Thermo Fisher Scientific) diluted in PBS. In each experiment, five fields/well (corresponding to about 150–200 cells/well) were counted with a 60X objective by a Leica DMIRB inverted fluorescence microscope. Immunoreactive cells were counted and their percentage over total viable cells was calculated. In differentiating cultures exposed to CSA (3-1000 nM) for 96 h, the number of $MAP2^+$ cells exhibiting neuritic processes was counted at random locations in three fields/well and their number was expressed as the percentage over total cell number in each sampled location. All experiments were run in triplicate.

IN VIVO EXPERIMENTS

Experimental protocol

According to recent work, a dose of 15 mg/kg/day of CSA has a pro-neurogenic effect in the dentate gyrus of adult wild type mice (Chow & Morshead, 2016). Based on this evidence, we treated euploid and Ts65Dn mice with CSA (MedChem Express, 15 mg/kg/day in vehicle; s.c. injection) or vehicle (PBS with

2.5% DMSO) from postnatal day 3 (P3) to P15. Mice that received CSA will hereafter be called “treated mice” (treated euploid mice: n=15; treated Ts65Dn mice: n=14). Mice that received the vehicle will be called “untreated mice” (untreated euploid mice: n=21; untreated Ts65Dn mice: n=21). Each experimental group was composed of a similar number of males and females (treated euploid mice: 8 males, 7 females; treated Ts65Dn mice: 8 males, 6 females; untreated euploid mice: 10 males, 11 females; untreated Ts65Dn mice: 11 males, 10 females). On P15, mice received a subcutaneous injection (150 μg/g body weight) of BrdU in TrisHCl 50 mM 2h before being killed. The brains were excised and cut along the midline. The left hemispheres of a group of mice were fixed by immersion in PFA 4% and frozen, and the right hemispheres were used for Golgi staining. The right hemispheres of other mice were kept at –80°C and used for western blotting. The body weight of mice of all groups was recorded prior to sacrifice and the brain weight was recorded immediately after brain removal. The number of animals used for each experimental procedure is specified in the figure legends. In order to establish whether lower doses of CSA have a pro-neurogenic effect in Ts65Dn mice similar to that elicited by the 15.0 mg/kg dose (see Results), we also tested the effects of 1.5 mg/kg (n=6 mice: 3 males, 3 females) or 7.5 mg/kg (n=4 mice: 3 males, 1 female) of CSA (same protocol as above).

Histological procedures

The frozen brains 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 10X; sodium azide 0.02%; MilliQ to volume).

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. 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 hippocampal formation was incubated with 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. Brains were Golgi stained using the FD Rapid Golgi Stain ™ Kit (FD Neuro 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).

Image acquisition

Immunofluorescence images were taken with a Nikon Eclipse TE 2000-S inverted microscope (Nikon Corp., Kawasaki, Japan), equipped with a Nikon digital camera DS-Qi2. Bright field images were taken on a light microscope (Leitz) equipped with a motorized stage and focus control system and a Coolsnap-Pro color digital camera (Media Cybernetics, Silver Spring, MD, USA). Measurements were carried out using the software Image Pro Plus (Media Cybernetics, Silver Spring, MD 20910, USA).

Measurements

Number of BrdU-positive cells. BrdU-positive cells in the dentate gyrus and the region of the SVZ that spans along the whole rostro-caudal extent of the hippocampal formation were detected using a fluorescence microscope (Eclipse; objective: x 20, 0.5 NA). Quantification of BrdU-labeled nuclei was conducted in every 6^{th} section using a modified unbiased stereology protocol that has previously been reported to successfully quantify BrdU labeling (Malberg et al., 2000; Kempermann & Gage, 2002; Tozuka et al., 2005). All BrdU-labeled cells located in the granule cell layer and subgranular zone and in the SVZ were counted in their entire z axis (1 µm steps) in each section. To avoid oversampling errors, nuclei intersecting the uppermost focal plane were excluded. The total number of BrdU-labeled cells per animal was determined and multiplied by six to obtain the total estimated number of cells per dentate gyrus and per SVZ.

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

Spine density. In Golgi-stained sections from the dentate gyrus, spines of granule cells were counted using a 100x 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, 3-4 proximal and 3-4 distal segments were analyzed. For each animal, spines were counted in at least 4 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 100 µm dendrite.

Western blotting

In homogenates of the hippocampal formation, total proteins were obtained as previously described (Trazzi et al., 2011) and the levels of p21 (1:200, Santa Cruz Biotechnology; catalog number: sc-271532) were evaluated. Densitometric analysis of digitized images with ChemiDoc XRS$^+$ was performed with Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA) and intensity for each band was normalized to the intensity of the Ponceau S staining. This evaluation has the advantage that it does not rely on a single protein for normalization, thereby circumventing the possibility that the chosen ‘‘housekeeping” proteins may vary in some conditions (Romero-Calvo et al., 2010).

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. Data were normally distributed with the exception of granule cell density. In this case, statistical analysis was carried out using the Kruskal-Wallis test followed by the Mann–Whitney U test. For all other examined variables statistical analysis was carried out using either a one-way ANOVA or a two-way ANOVA with genotype (euploid, Ts65Dn) and treatment (vehicle, CSA), as factors. *Post hoc* multiple comparisons were carried out using Fisher’s least significant difference (LSD) 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 [$x \geq Q3 + 3 * (IQ)$; $x \leq Q1 - 3 * (IQ)$]. The number of mice included in (and excluded from, if any) individual analyses is reported in the legends of figures and Table 1. A probability level of $p \leq 0.05$ was considered to be statistically significant.

		n.	Mean	SE		n.	Mean	SE	
Body									*p*
	Euploid+Veh	21	7.58	± 0.20	Euploid+CSA	15	8.72	± 0.38	0.015
	Ts65Dn+Veh	21	6.65	± 0.37	Ts65Dn+CSA	14	6.09	± 0.38	0.228
	p		0.031				0.001		
Brain									*p*
	Euploid+Veh	20	0.40	± 0.01	Euploid+CSA	14	0.39	± 0.01	0.054
	Ts65Dn+Veh	20	0.39	± 0.01	Ts65Dn+CSA	14	0.37	± 0.01	0.049
	p		0.005				0.016		

Table 1. Effect of treatment with CSA on body and brain weight

Body weight and brain weight (mean ± SE) in grams, of euploid and Ts65Dn mice that received either vehicle (Veh) or cyclosporine A (CSA; 15.0 mg/kg) in the period P3-P15, measured on P15. The *p* value in the row below each variable refers to the comparison between untreated euploid (Euploid+Veh) and Ts65Dn (Ts65Dn+Veh) mice and treated euploid (Euploid+CSA) and Ts65Dn (Ts65Dn+CSA) mice. The column "n." reports the number of animals included in the statistical analysis. For the brain weight analysis we excluded one untreated euploid mouse, one untreated Ts65Dn mouse and one treated euploid mouse. The *p* value in the column on the right refers to the comparison between untreated and treated mice of the same genotype (Fisher LSD test after two-way ANOVA).

RESULTS

Identification and characterization of CSA effects in trisomic NPC phenotypic assays

NPCs from the SVZ of neonate Ts65Dn mice exhibit impairment of proliferation rate, similarly to the *in vivo* condition (Trazzi et al., 2011; Trazzi et al., 2013; Stagni et al., 2017). As result of a screening effort, among 1,887 tested FDA-EMA drugs the immunosuppressant CSA was identified as a drug promoting proliferation of trisomic NPCs (% increase of proliferation: +53% at 1000 nM vs. basal conditions) [$F(2,15) = 56.10$, $p < 0.001$] (Fig. 1A). We used as a positive control lithium chloride, a well-established *in vivo* neurogenesis enhancer in DS (Bianchi et al., 2010a; Contestabile et al., 2013; Trazzi et al., 2014; Stagni et al., 2017), and found that lithium chloride at 2 mM enhanced proliferation by +39% vs. basal conditions [$F(2,15) = 56.10$, $p < 0.001$] (Fig. 1A).

CSA was then tested under a wide range of concentrations (0.1-1000 nM). A one-way ANOVA showed a significant effect of treatment [$F(5,42) = 10.855$, $p < 0.001$]. A *post hoc* Fisher's LSD test showed that CSA concentrations of 0.1-100 nM had no significant effect on proliferation, while a concentration of 1000 nM increased the number of trisomic NPCs (Fig. 1B). By using a cytotoxicity assay, we could exclude that CSA-mediated effect on the number of NPCs was due to increased cell viability. Indeed, none of the tested CSA concentrations affected cell death rate (Fig. 1C). In order to obtain more direct evidence on the pro-proliferative effect of CSA, we evaluated the proliferation rate based on incorporation of the thymidine analogue EdU (5-ethynyl-2-deoxyuridine) in trisomic and euploid cultures exposed to CSA (1000 nM) or vehicle. A two-way ANOVA showed a genotype X treatment interaction [$F(1,8) = 72.531$, $p < 0.001$], a main effect of genotype [$F(1,8) = 277.386$, $p < 0.001$] and a main treatment [$F(1,8) = 262.037$, $p < 0.001$]. A post hoc Fisher's LSD test showed that trisomic

and euploid cells exposed to CSA 1000 nM underwent a proliferation increase in comparison with their untreated counterparts (Fig. 1D). Importantly, in response to CSA treatment, trisomic NPCs displayed a proliferation rate similar to untreated euploid NPCs.

In addition to proliferation impairment, trisomic NPCs exhibit impairment in the acquisition of a neuronal phenotype and in neuronal maturation, i.e., development of neuritic processes (Trazzi et al., 2011; Trazzi et al., 2013; Stagni et al., 2017). In cultures of NPCs under differentiating conditions we evaluated the percentage of cells that were i) immunopositive to MAP2 (a marker of cells with a neuronal phenotype) and immunonegative to Nestin (a marker of undifferentiated NPCs); ii) double immunopositive to MAP2 and Nestin, i.e., neuroblasts; iii) immunonegative to MAP2 and to Nestin (putative glial cells). A one-way ANOVA on the percentage of cells that were MAP2-positive and Nestin-negative ($MAP2^+/Nestin^-$) showed a significant effect of treatment [$F(6,14) = 7.735$, $p < 0.001$]. Fisher's LSD test, carried out *post hoc*, showed that drug concentrations of 100-1000 nM caused a significant increase in the percentage of $MAP2^+/Nestin^-$ cells in comparison with cultures treated with vehicle (Fig. 1E), suggesting that CSA favors the acquisition of a neuronal phenotype. A one-way ANOVA on the percentage of cells that were MAP2-positive and Nestin-positive ($MAP2^+/Nestin^+$) showed no effect of treatment [$F(6,14) = 2.182$, $p < 0.108$] (Fig. 1F) suggesting that treatment does not affect the population of neuroblasts. An evaluation of the percentage of cells that were negative to both MAP2 and Nestin ($MAP2^-/Nestin^-$) showed a significant effect of treatment [$F(6,14) = 5.389$, $p < 0.004$]. A *post hoc* Fisher's LSD test showed that concentrations of 100-1000 nM caused a reduction in the percentage of $MAP2^-/Nestin^-$ cells (Fig. 1G). Since cells that are immunonegative to both MAP2 and Nestin mainly represent cells committed to glia (Cvijetic et al., 2017), these results suggest that CSA treatment promotes neuronal differentiation of

trisomic NPCs and that this effect takes place at the expense of their commitment toward non-neuronal lineages (glia).

In order to obtain a more direct evidence of the glial nature of $MAP2^-/Nestin^-$ cells, we carried out additional experiments in which we evaluated, in parallel to the percentage of cells that were $MAP2^+/Nestin^-$ or $MAP2^+/Nestin^+$, the percentage of cells expressing the astrocytic marker GFAP. A one-way ANOVA on the percentage of $MAP2^+/Nestin^-$ cells confirmed a significant effect of drug treatment [$F(3,11) = 14.94$, $p = 0.001$]. Fisher's LSD test, carried out *post hoc*, confirmed that CSA 100-1000 nM caused a significant increase in the percentage of $MAP2^+/Nestin^-$ cells in comparison with vehicle-treated trisomic cells (Fig. 1H). A one-way ANOVA on the percentage of $MAP2^+/Nestin^+$ cells showed again no effect of treatment (Fig. 1I). An evaluation of the percentage of $GFAP^+$ cells showed a significant effect of treatment [$F(3,23) = 8.860$, $p = 0.001$]. A *post hoc* Fisher's LSD test showed that CSA concentrations 100-1000 nM reduced the percentage of $GFAP^+$ (Fig, 1J). These results confirm our hypothesis that CSA promotes neuronal differentiation of trisomic NPCs at the expense of their commitment toward an astrocytic phenotype. A similar effect was also observed in euploid cultures (data shown in Supplementary Fig. 1, not shown in this thesis).

In order to establish the effect of CSA on neuron maturation we evaluated the percentage of cells exhibiting neuritic processes in differentiating cultures of trisomic NPCs exposed to different concentrations of CSA. A one-way ANOVA on the percentage of NPCs that exhibited neuritic processes showed a significant effect of treatment [$F(5,12) = 8.025$, $p = 0.002$]. Fisher's LSD test, carried out *post hoc*, showed that all tested drug concentrations increased the percentage of cells with neuritic processes in comparison with cultures in presence of vehicle (Fig. 1L). Taken together these data show that CSA increases the proliferation rate and fosters the process of neurogenesis and neuron maturation of trisomic

NPCs.

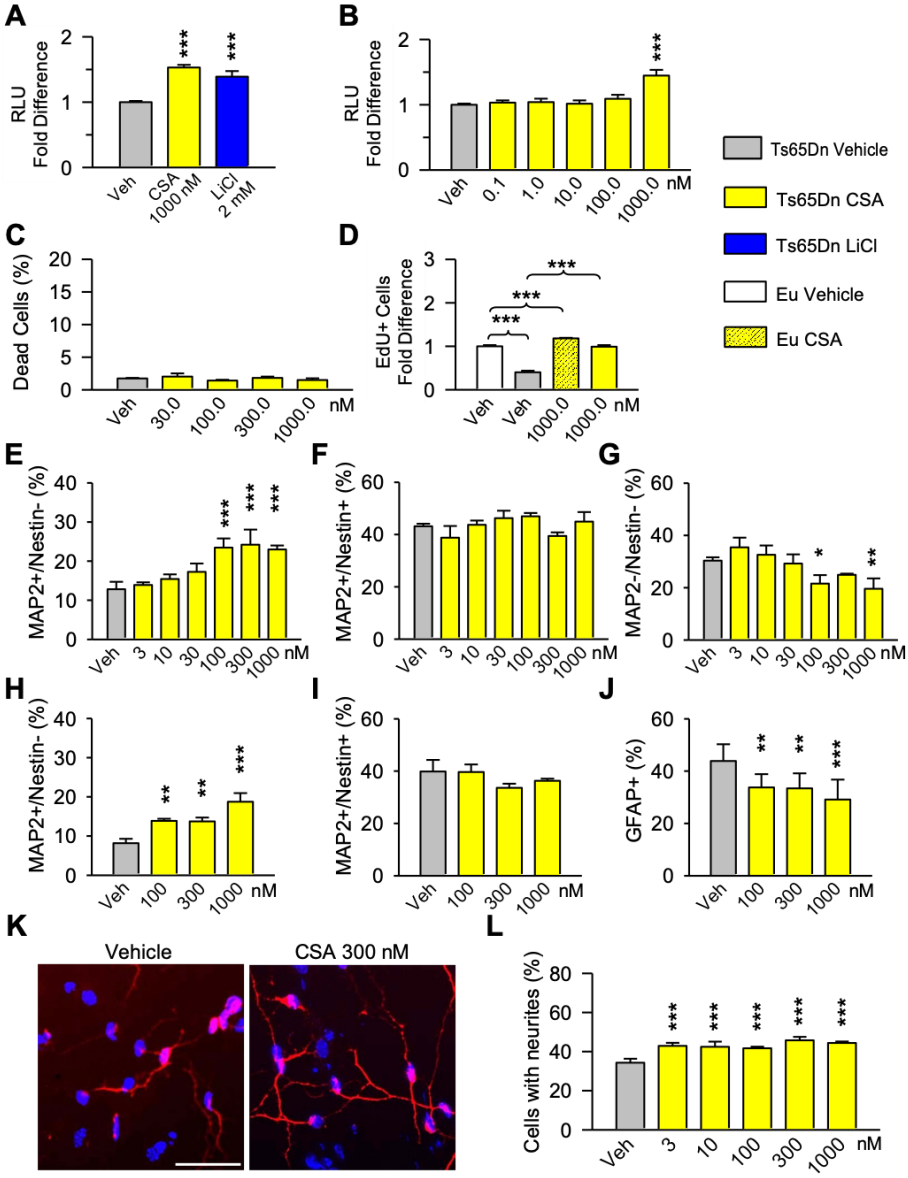

Fig. 1. Effect of CSA on proliferation, differentiation and maturation of trisomic NPCs. A, B: Effect of CSA 1000 nM or LiCl 2.0mM **(A)** and of different concentrations of CSA **(B)** on the proliferation rate (evaluated as relative luminescence units, RLU; see Methods) of NPCs of Ts65Dn mice at 96 h in culture. Data are expressed as fold change in comparison with NPCs

exposed to vehicle alone (DMSO 0.05%). **C:** Percentage of dead cells in trisomic cultures exposed to different concentrations of CSA for 96 h. **D:** EdU-positive cells in cultures of euploid and trisomic NPC exposed to vehicle or to CSA 1000 nM for 72 h. Data are expressed as fold change in comparison with euploid NPCs exposed to vehicle alone. **E-J:** Percentage of $MAP2^+/Nestin^-$ cells **(E, H),** $MAP2^+/Nestin^+$ cells **(F, I)**, $MAP2^-/Nestin^-$ cells **(G)**, and $GFAP^+$ cells **(J)** in cultures of trisomic NPC under differentiating conditions and exposed to the indicated concentrations of CSA for 96 h. **K, L**: Representative confocal microscope image **(K)** and percentage **(L)** of $MAP2^+$ cells (red) exhibiting neuritic processes in cultures of NPCs from the SVZ of Ts65Dn mice grown under differentiating conditions and exposed to the indicated concentrations of CSA for 96 h. Images in **(K)** show $MAP2^+$ cells that were exposed to either vehicle (DMSO 0.05%) or CSA 1000 nM. Nuclei were counterstained with Hoechst (blue). Scale bar=50 μm. Data derive from pooled (3–5) mice. The asterisks in A, B, E, G, H, J, and L indicate a difference in comparison with vehicle-treated cultures: * $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$ (Fisher's LSD test after ANOVA). Abbreviations: CSA, cyclosporine A; EdU, 5-ethynyl-2-deoxyuridine; Eu, euploid; GFAP, glial fibrillary acidic protein; LiCl, Lithium chloride; MAP2, microtubule associated protein 2; Veh, vehicle.

Effect of neonatal treatment with CSA on neural precursor proliferation in the dentate gyrus and SVZ of Ts65Dn mice

The early postnatal period is a critical time window for neurogenesis in the SGZ of the hippocampal dentate gyrus. In addition, in neonate mice a prominent proliferation rate is present in the SVZ of the lateral ventricle. This is a neurogenic niche that gives origin to the neurons of the forebrain, prenatally, and thereafter produces granule cells destined to the olfactory bulb, glial cells and, possibly, generates neurons destined to the neocortex (Brazel et al., 2003). In view of the relevance of these two neurogenic niches, in the current study we examined the impact of CSA on the proliferation rate of NPCs both in the dentate gyrus and SVZ of euploid and Ts65Dn mice. To this purpose, we treated mice with 15 mg/kg/day of CSA, because this dose has a pro-neurogenic effect in the dentate gyrus of adult wild type mice (Chow & Morshead, 2016).

A two-way ANOVA on the total number of BrdU-positive cells in the dentate gyrus showed a genotype x treatment interaction [$F(1,16) = 8.995$, $p = 0.008$], but no main effect of either genotype or treatment. A *post hoc* Fisher's LSD test showed that, in agreement with previous evidence (Bianchi et al., 2010b, Giacomini et al., 2015, Stagni et al., 2016, Stagni et al., 2017), untreated Ts65Dn mice had notably fewer proliferating cells in comparison with untreated euploid mice. The number of proliferating cells in treated Ts65Dn mice underwent a large increase (+26%) and became similar to that of untreated euploid mice (Fig. 2A,B). Treatment had no effect on the number of NPCs in the dentate gyrus of euploid mice (Fig. 2A,B). In order to establish whether doses of CSA lower than 15 mg/kg positively affect cell proliferation, we treated Ts65Dn pups with 1.5 mg/kg or 7.5 mg/kg of CSA in the period P3-P15 and examined the effects of treatment in the dentate gyrus. A one-way ANOVA on the number of BrdU-positive cells in the dentate gyrus of Ts65Dn mice that had received vehicle or 1.5 mg/kg, 7.5 mg/kg, and 15.0 mg/kg of CSA showed a significant effect of treatment [$F(3,17) = 6.374$, $p = 0.004$]. *Post hoc* LSD test showed that, unlike the dose of 15.0 mg/kg, the doses of 1.5 mg/kg and 7.5 mg/kg did not increase the number of BrdU-positive cells (Fig. 2C).

A two-way ANOVA on the total number of BrdU-positive cells in the SVZ showed no genotype x treatment interaction, whereas a main effect of genotype [$F(1,16) = 21.764$, $p < 0.001$] and a main effect of treatment [$F(1,16) = 38.978$, $p < 0.001$] appeared. A *post hoc* Fisher's LSD test showed that Ts65Dn mice had fewer cells in comparison with untreated euploid mice. After treatment with CSA, Ts65Dn mice underwent an increase in the number of proliferating cells (+25%) that became similar to that of untreated euploid mice (Fig. 3B). In the SVZ of euploid mice, treatment caused an increase in the number of proliferating cells that became larger (+21%) in comparison with that of their untreated counterparts (Fig. 3B).

These results show that neonatal treatment with CSA is able to restore the proliferation rate of NPCs in both the dentate gyrus and SVZ of Ts65Dn mice.

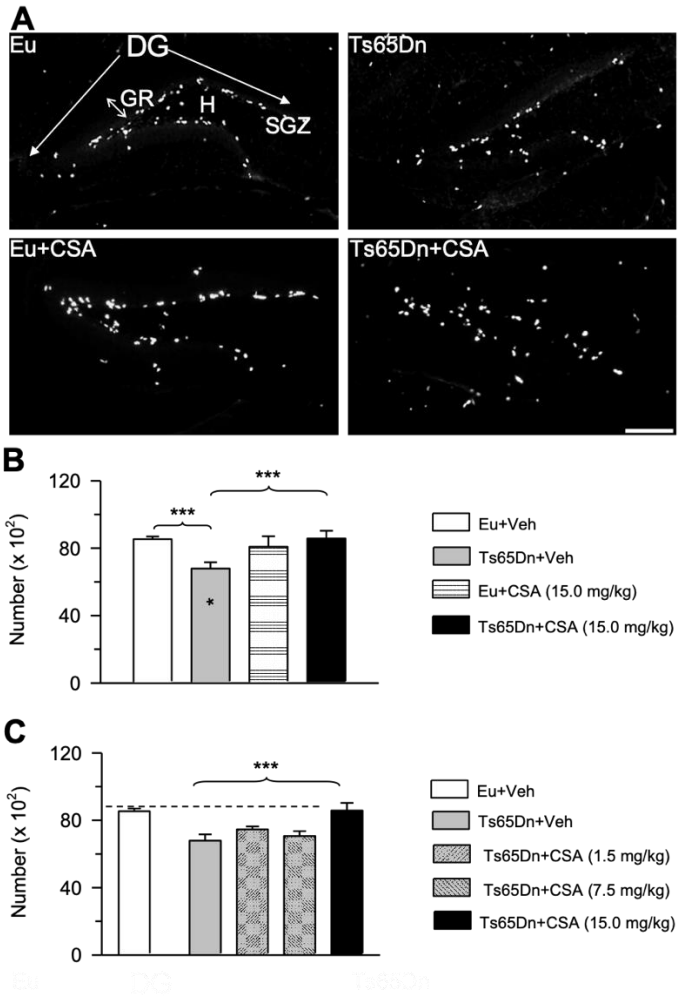

Fig. 2. Effects of neonatal treatment with CSA on the size of the population of cells in the S-phase of the cell cycle in the dentate gyrus of Ts65Dn and euploid mice.

A: Representative images of sections immunostained for BrdU from the dentate gyrus of untreated euploid and Ts65Dn mice, and of euploid and Ts65Dn mice that were treated daily with 15.0 mg/kg of CSA in the period P3-P15. Calibration bar=200 μm. **B:** Total number of BrdU-positive cells in the dentate gyrus of untreated euploid (n=5) and Ts65Dn (n=6) mice, and of treated euploid (n=4) and Ts65Dn (n=5) mice.

C: Number of BrdU-positive cells in the dentate gyrus of Ts65Dn mice that received a daily

injection of vehicle (n=6; same mice as in B) or 1.5 mg/kg (n= 6), 7.5 mg/kg (n=4), and 15.0 mg/kg (n=5; same mice as in B) of CSA in the period P3-P15. The number of BrdU-positive cells in euploid mice that received the vehicle reported in (B) is shown for comparison. Values (mean ± SE) refer to one hemisphere. * $p \leq 0.05$; *** $p \leq 0.001$ (Fisher's LSD test after two-way ANOVA). Black asterisks in the gray bar indicate a difference between untreated Ts65Dn mice and treated euploid mice. Abbreviation: CSA, cyclosporine A; Eu, euploid; GR, granule cell layer; H, hilus; SGZ, subgranular zone; Veh, vehicle.

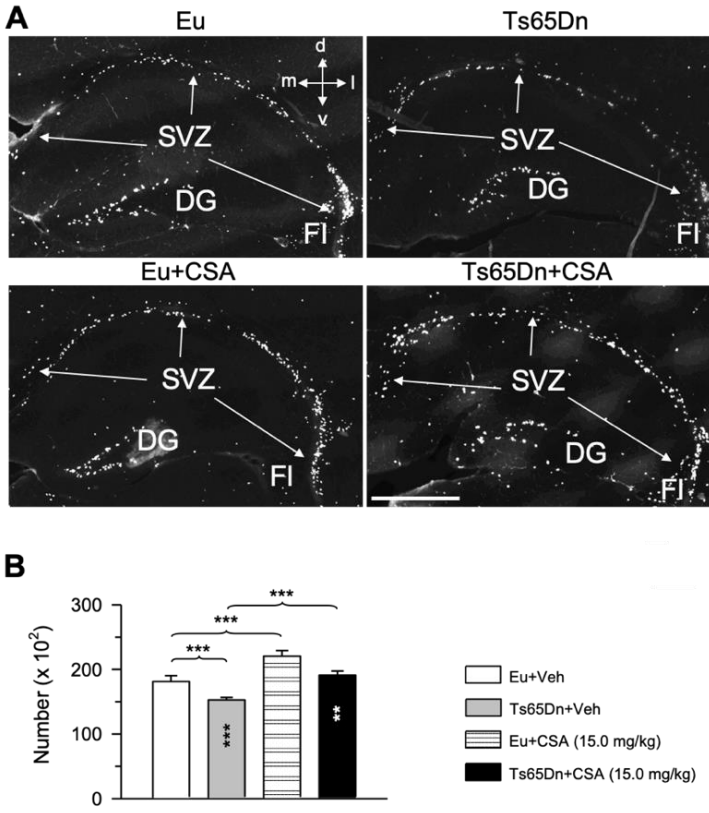

Fig. 3. Effects of neonatal treatment with CSA on the size of the population of cells in the S-phase of the cell cycle in the SVZ zone of Ts65Dn and euploid mice.

A: Representative images of sections immunostained for BrdU from the SVZ of untreated euploid and Ts65Dn mice, and of euploid and Ts65Dn mice that were treated daily with CSA in the period P3-P15. Calibration bar=500 μm. **B:** Total number of BrdU-positive cells in the SVZ of untreated euploid (n=5) and Ts65Dn (n=6) mice, and of euploid (n=4) and Ts65Dn (n=5) mice treated with CSA. Values (mean ± SE) refer to one hemisphere. ** $p \leq 0.01$; *** $p \leq 0.001$ (Fisher's LSD test after two-way ANOVA). Black asterisks in the gray bar indicate a difference between untreated

Ts65Dn mice and treated euploid mice. White asterisks in the black bar indicate a difference between treated Ts65Dn mice and treated euploid mice. Abbreviations: CSA, cyclosporine A; d, dorsal; Eu, euploid; DG, dentate gyrus; FI, fimbria; l, lateral; m, medial; SVZ, subventricular zone; v, ventral; Veh, vehicle.

Effect of CSA 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 & Bayer, 1975). Thus, in view of the treatment-induced increase in the proliferation potency of NPCs of the SGZ, we expected this effect to lead to 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. A two-way ANOVA on the volume of the dentate gyrus showed no genotype x treatment interaction, but a main effect of genotype [$F(1,15) = 8.705$, $p = 0.010$], and of treatment [$F(1,15) = 8.887$, $p = 0.009$]. Fisher's LSD test, carried out *post hoc*, showed that the volume of the granule cell layer of untreated Ts65Dn mice was reduced (Fig. 4B) in comparison with that of euploid mice and that treatment restored the volume of the granule cell layer. The Kruskal-Wallis test showed a significant effect of treatment on granule cell density [χ^2 (3) = 12.149, $p < 0.007$]. The Mann-Whitney test showed a reduced granule cell density in Ts65Dn mice compared to euploid mice ($U = 0.001$, $p = 0.004$) and demonstrated that treatment caused an increase in granule cell density ($U = 0.001$, $p = 0.006$) (Fig. 4C). A two-way ANOVA on total number of granule cells showed no genotype x treatment interaction, but did demonstrate a main effect of genotype [$F(1,17) = 19.301$, $p = 0.001$], and of treatment [$F(1,17) = 15.844$, $p = 0.0.001$]. A *post hoc* Fisher's LSD test showed that untreated Ts65Dn mice had a reduced number of granule cells in comparison with euploid mice and that treatment restored granule cell number (Fig. 4D). A two-way ANOVA on the thickness of the granule cell layer showed no genotype

x treatment interaction, whereas a main effect of genotype [$F(1,16) = 4.742$, $p = 0.045$] was present, as was a main effect of treatment [$F(1,16) = 6.039$, $p = 0.026$]. A *post hoc* Fisher's LSD test showed that the granule cell layer of untreated Ts65Dn mice had a reduced thickness in comparison with that of euploid mice and that this reduction was restored by treatment (Fig. 4E).

At variance with the neocortex, the granule cell layer develops according to an outside-inside pattern. Therefore, the older neurons occupy the superficial part of the granule cell layer and the younger neurons occupy its lower portion. In Hoechst-stained sections, younger neurons are recognizable due to their smaller size and a more translucent pattern in comparison with older neurons (Fig. 4A). We deemed it of interest to evaluate the thickness of the region of the granule cell layer that was occupied by younger neurons (see the double-headed black arrow in Fig. 4A). A two-way ANOVA on the thickness of the inner granule cell layer showed a genotype x treatment interaction [$F(1,17) = 7.477$, $p = 0.014$] and a main effect of genotype [$F(1,17) = .5.791$, $p = 0.028$], but no main effect of treatment. Fisher's LSD test, carried out *post hoc*, showed that the inner part of the granule cell layer of untreated Ts65Dn mice was reduced in thickness compared to that of euploid mice and that this reduction was restored by treatment (Fig. 4F). The expansion of the inner part of the granule cell layer in treated Ts65Dn mice indicates that the treatment-induced increase in the proliferation rate of granule cells precursors in the SGZ of Ts65Dn mice (see above) translates into an increase in the number of new granule neurons that are added to the inner part of the granule cell layer and, hence, in total granule cell number. Unlike in Ts65Dn mice, in euploid mice drug treatment had no effect on the stereology of the granule cell layer (Fig. 4B-F), which is consistent with the absence of effects on the proliferation rate of the granule cell precursors.

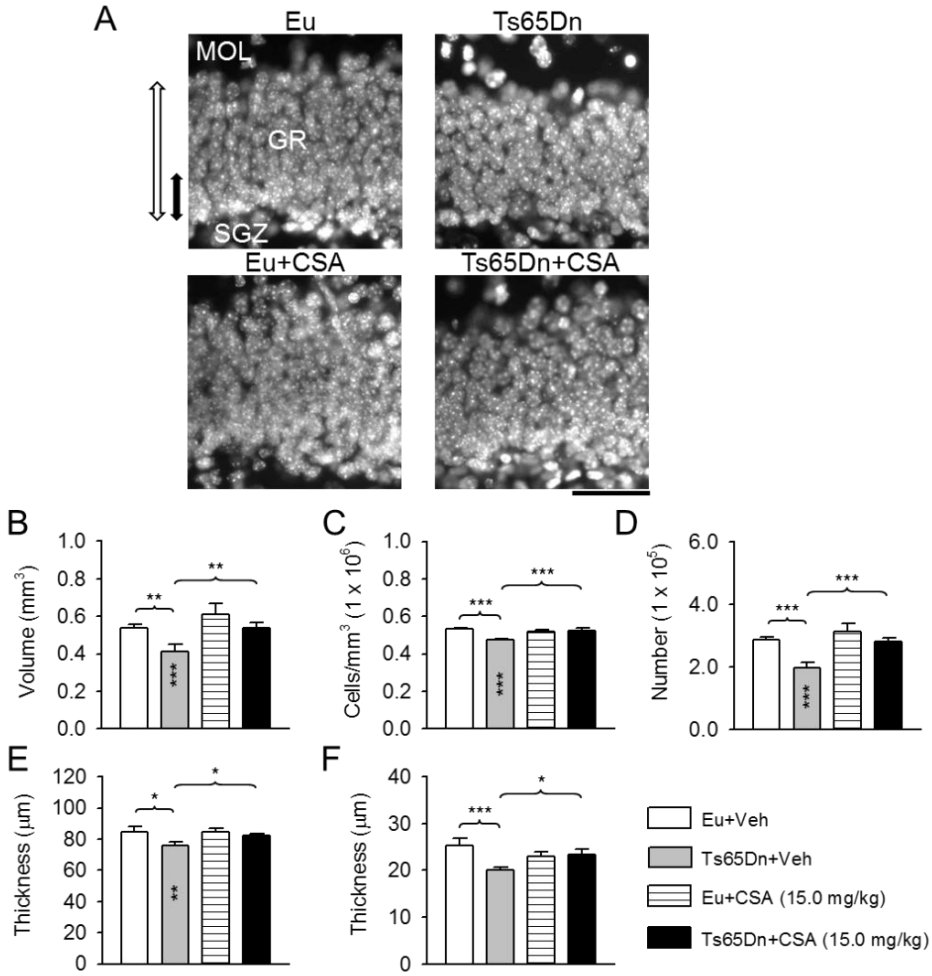

Fig. 4. Effects of neonatal treatment with CSA on the stereology of the granule cell layer of Ts65Dn and euploid mice.

A: Representative images of Hoechst-stained sections showing the granule cell layer of an animal from each experimental group. The double-headed white arrow indicates the thickness of the granule cell layer, while the double-headed black arrow indicates the thickness of the innermost part of the granule cell layer. Calibration bar=50 µm. **B-F:** Volume of the granule cell layer **(B)**, granule cell density **(C)**, total number of granule cells **(D)**, thickness of the granule cell layer **(E)**, and thickness of the innermost part of the granule cell layer **(F)** of untreated euploid (n=6) and Ts65Dn (n=6) mice, and of euploid (n=4) and Ts65Dn mice (n=5) treated with CSA. Values (mean ± SE) refer to one dentate gyrus. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ (Fisher's LSD test after two-way ANOVA for data reported in B and D-F; Mann-Whitney test after Kruskal-Wallis test for data reported in C). Black asterisks in the gray bar indicate a difference between untreated

Ts65Dn mice and treated euploid mice. Abbreviations: CSA, cyclosporine A; Eu, euploid; GR, granule cell layer; MOL, molecular layer; SGZ, subgranular zone; Veh, vehicle.

Effect of CSA 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 CSA 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. The examples of Golgi-stained dendritic branches reported in Fig. 5A clearly show that treatment causes a patent increase in spine density both in euploid and Ts65Dn mice. A two-way ANOVA on spine density in the proximal dendrites showed a genotype x treatment interaction [$F(1,17) = 20.174$, $p = 0.001$], a main effect of genotype [$F(1,17) = 6.803$, $p = 0.018$], and a main effect of treatment [$F(1,17) = 180.800$, $p < 0.001$]. A *post hoc* Fisher's LSD test showed that the spine density of untreated Ts65Dn mice was significantly reduced (-24%) in comparison with that of untreated euploid mice (Fig. 5B,C). After treatment with CSA the number of spines of Ts65Dn mice underwent a notable increment (+78% vs. untreated Ts65Dn mice) and became larger (+36%) than that of untreated euploid mice (Fig. 5B,C). A large increase in spine density (+30%) also took place in treated euploid mice (Fig. 5B,C). A two-way ANOVA on spine density in the distal dendrites showed a genotype x treatment interaction [$F(1,17) = 18.574$, $p = 0.001$], a main effect of genotype [$F(1,17) = 4.748$, $p = 0.044$], and a main effect of treatment [$F(1,17) = 136.054$, $p < 0.001$]. A *post hoc* Fisher's LSD test showed a significantly reduced spine density (-21%) in untreated Ts65Dn mice compared

to untreated euploid mice (Fig. 5B,C). After treatment with CSA the number of spines of Ts65Dn mice underwent a notable increment (+64% vs. untreated Ts65Dn mice) and became larger (+30%) than that of euploid mice (Fig. 5B,C). A large increase in spine density (+23%) also took place in treated euploid mice (Fig. 5B,C).

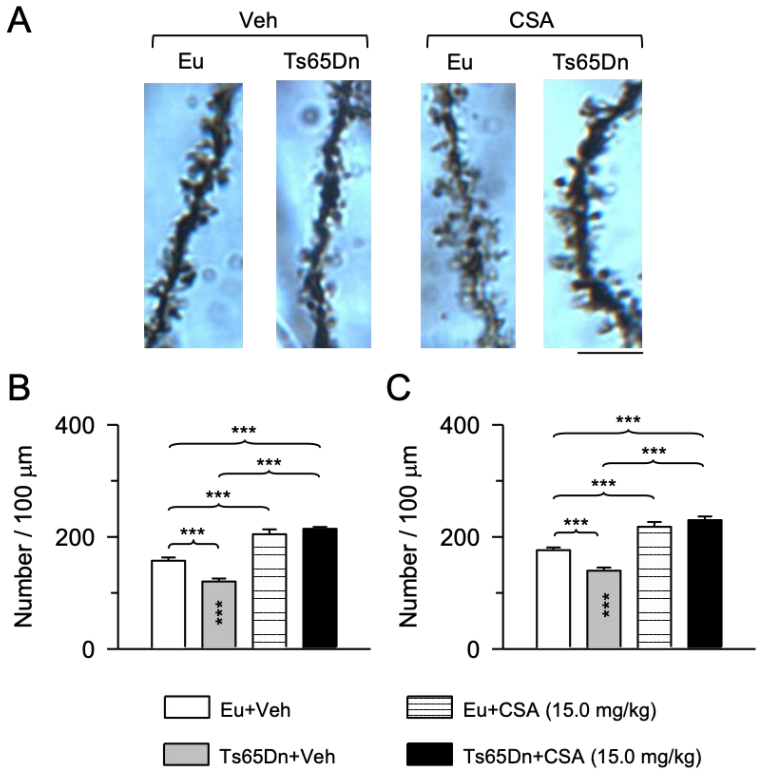

Fig. 5. Effects of neonatal treatment with CSA on dendritic spine density in the dentate gyrus of Ts65Dn and euploid mice.

A: Photomicrographs of Golgi-stained granule cell dendrites showing spines on distal dendritic branches in an animal from each experimental group. Calibration bar=5 µm. **B,C:** Spine density on dendritic branches in the inner **(B)** and outer **(C)** half of the dendritic tree of the granule cells of untreated euploid (n=6) and Ts65Dn mice (n=6) and euploid (n=4) and Ts65Dn (n=5) mice treated with CSA. Values in (B,C) are mean ± SE. *** $p \leq 0.001$ (Fisher's LSD test after two-way ANOVA). Black asterisks in the gray bar indicate a difference between untreated Ts65Dn

mice and treated euploid mice. Abbreviations: CSA, cyclosporine A; Eu, euploid; Veh, Vehicle.

Effect of CSA on p21 levels in the hippocampal formation of Ts65Dn mice

Elongation of the cell cycle and a precocious exit from the cell cycle appear to be key mechanisms underlying the typical impairment of neurogenesis that characterizes DS. Overexpression of p21 in the trisomic brain appears to be an important determinant involved in cell cycle alteration and, hence, in the reduction in proliferation rate (see (Stagni et al., 2018)). A two-way ANOVA on the p21 levels in the hippocampal formation showed no genotype x treatment interaction, and no main effect of genotype or of treatment. A *post hoc* Fisher's LSD test showed that untreated Ts65Dn mice had higher levels of p21 in comparison with euploid mice and that treatment with CSA reduced p21 levels to an extent that they became similar to those of euploid mice (Fig. 6B). In euploid mice, treatment had no effect on p21 levels (Fig. 6B).

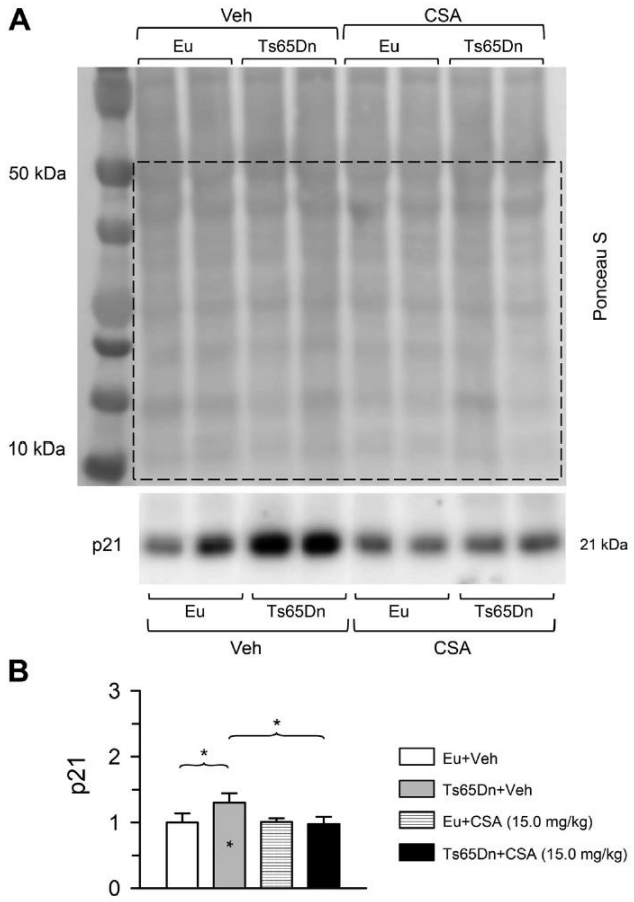

Fig. 6. Effects of neonatal treatment with CSA on p21 protein levels in the hippocampal formation of Ts65Dn and euploid mice.

A: Representative western blots showing immunoreactivity for p21 and the Ponceau S. Protein levels of p21 were normalized to all proteins with molecular weight between 10 and 50 kDa, as indicated by the dashed rectangle. **B:** Levels of p21 were examined in untreated euploid mice (n=11), untreated Ts65Dn mice (n=12), treated euploid mice (n=10), and treated Ts65Dn mice (n=8). One untreated euploid mouse (yielding 10) and one untreated Ts65Dn mouse (yielding 11) were excluded from p21 analysis. Protein levels (mean ± SE) are expressed as fold difference in comparison with untreated euploid mice. * $p \leq 0.05$ (Fisher's LSD test after two-way ANOVA). Black asterisks in the gray bar indicate a difference between untreated Ts65Dn mice and treated euploid mice. Abbreviations: CSA, cyclosporine A; Eu, euploid; Veh, vehicle.

General effects of CSA

The Ts65Dn strain is characterized by a high mortality rate during gestation and before weaning (Roper et al., 2006). The total number of mice used in the *in vivo* study was 81 (vehicle-treated mice: n=42; CSA-treated mice n=39). Three vehicle-treated (7.1%) and three CSA-treated (7.7%) mice died in the P3-P15 period. The similarity in the mortality rate across groups suggests that treatment has no adverse effects on the health of mice. We evaluated the body and brain weight of P15 mice that received vehicle or CSA (15.0 mg/kg) in order to establish the outcome of treatment on growth. A two-way ANOVA on body weight showed a genotype x treatment interaction [$F(1,64) = 6.826$, $p = 0.011$] and a main effect of genotype [$F(1,64) = 29.560$, $p < 0.001$], but no main effect of treatment. A *post hoc* Fisher's LSD test showed that untreated and treated Ts65Dn mice had a lower body weight compared to their euploid counterparts (Table 1). A comparison of the body weight of treated and untreated mice showed that treated Ts65Dn mice had a similar body weight in comparison with their untreated counterparts and that treated euploid mice had a larger body weight in comparison with their untreated counterparts (Table 1). These findings indicate that treatment has no adverse effects on somatic growth. A two-way ANOVA on brain weight showed no genotype x treatment interaction, while a main effect of genotype [$F(1,64) = 14.117$, $p < 0.001$] and a main effect of treatment [$F(1,64) = 7.899$, $p = 0.007$] were present. A *post hoc* Fisher's LSD test showed that untreated and treated Ts65Dn mice had a lower brain weight compared to their euploid counterparts (Table 1). A comparison of the brain weight of treated and untreated mice showed that treated euploid and Ts65Dn mice had a reduced brain weight in comparison with their untreated counterparts (Table 1). Observation of the values reported in Table 1 shows that the brain weight reduction was 4% in treated vs. untreated euploid mice and 5% in treated vs. untreated Ts65Dn mice. This evidence shows that treatment exerts a moderately negative effect on brain

growth.

DISCUSSION

CSA positively impacts on proliferation, differentiation and maturation of trisomic NPCs

By exploiting cultures of NPCs from the SVZ, we found that CSA i) restores the reduced proliferation rate that characterizes trisomic NPCs; ii) rescues the aberrant differentiation program of trisomic NPCs, because it increases the number of cells that differentiate into neurons and, concomitantly, reduces the number of cells that differentiate into astrocytes; iii) restores the development of neuritic processes; iv) does not affect cell death. The pro-proliferative and pro-neuronogenic effects of CSA found here in trisomic NPCs are consistent with evidence obtained in neurospheres from the dentate gyrus of wild type mice, showing that CSA increases both the number of neurospheres and the frequency of neuron-containing neurospheres relative to those containing glia (Chow & Morshead, 2016) at the same concentrations as those used here. It has been shown that CSA increases neurite outgrowth of cultured dorsal root gangliar cells with an EC_{50} of 50 nM (see (Hamilton & Steiner, 1998)). This is in line with the current findings that CSA fosters neurite outgrowth of trisomic NPCs and that concentrations as low as 3 nM are sufficient to elicit this effect.

Neonatal treatment with CSA restores neurogenesis and spinogenesis in the Ts65Dn mouse

In view of potential pharmacotherapies for DS, it is of obvious importance to demonstrate that the effects observed *in vitro* also take place in the greater complexity of the *in vivo* condition. Our results show that in Ts65Dn pups treated with CSA for 13 days there was full restoration of the number of BrdU-positive

cells in the dentate gyrus and SVZ, indicating that treatment positively impacts on the two major forebrain neurogenic niches. An evaluation of the pro-neurogenic effects of three different doses of CSA showed that a dose of 15 mg/kg/day (but not lower doses) was able to fully rescue cell proliferation in Ts65Dn mice. It is worthy to note, that the 15.0 mg/kg dose translates into approximately 1.2 mg/kg/day in the human setting (Reagan-Shaw et al., 2008). The finding that the lower doses tested here did not increase NPC proliferation suggests a threshold for the pro-neurogenic effects of CSA.

The NPCs of the SGZ give origin to granule neurons and astrocytes destined to the dentate gyrus. In agreement with the pro-*neuronogenic* effect observed *in vitro*, in treated Ts65Dn mice there was an increase in the size of the innermost part of the granule cell layer, which harbors the newly-generated granule cells. This effect was accompanied by a large increase in the volume and thickness of the granule cell layer and total number of granule cells. The NPCs of the SVZ give origin to granule cells destined to the olfactory bulb and to astrocytes and oligodendrocytes destined to the cortex (Brazel et al., 2003). This suggests that the CSA-induced increase in the proliferation potency of NPCs in the SVZ may positively impact on postnatal development of the olfactory bulb and neocortex.

In the current study, we were interested in establishing whether treatment with CSA can ameliorate the severe spine density reduction that characterizes the granule cells of the dentate gyrus of Ts65Dn mice. We found that CSA largely enhanced the process of spinogenesis and that Ts65Dn mice treated with CSA 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. The major extrinsic input to the hippocampal formation is constituted by the performant pathway, which takes its origin from the medial

and lateral divisions of the entorhinal cortex (Amaral & Witter, 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 treatment-induced increase in spine density on proximal and distal dendrites suggests that this effect may lead to restoration of the memory functions mediated by both divisions of the entorhinal cortex.

There are several candidates for the molecular mechanisms underlying NPC proliferation impairment in DS, among which p21 may be a particularly critical one. The protein p21, also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1, is overexpressed in the brain of fetuses with DS and in the Ts65Dn model (Engidawork et al., 2001; Stagni et al., 2015). Since p21 inhibits the transition from the G1 to the S-phase of the cell cycle, its overexpression may be a key determinant of proliferation impairment in DS. We found here that treatment with CSA normalized the levels of p21 in the hippocampal formation of Ts65Dn mice, suggesting that this effect may underlie restoration of proliferation. CSA is classically known to inhibit in T-lymphocytes the activity of the calcineurin-NFAT pathway and this effect is mediated by its binding to cyclophilin A. For this reason, it is used in a clinical setting in order to prevent graft rejection in organ transplantation. CSA, however, has a high affinity for other cyclophilins (B, C, D) (Hamilton & Steiner, 1998) and can therefore modulate various signaling pathways and exert calcineurin-independent effects (Sachewsky et al., 2014). It is worth noting that CSA appears to block the activity of the p38 signaling pathway (Matsuda & Koyasu, 2000), one of the three subgroups of the mitogen-activated protein kinase superfamily. Activation of p38 increases the mRNA and protein levels of the transcription factor p53 which, in

turn, promotes the transcription of various genes, including p21 (Saha et al., 2014). In the brain of individuals with DS there is an increased activation of p38 and p53 (Swatton et al., 2004; Tramutola et al., 2016), and increased activation of p53 has also been detected in the brain of the Ts65Dn model (Tramutola et al., 2016). Thus, the inhibitory effect exerted by CSA on the p38 pathway may account for the normalization of p21 levels found here in treated Ts65Dn mice and, hence, restoration of proliferation.

Several protein kinases, including p38, are essential factors in spine growth (Tada & Sheng, 2006). It has been shown that inhibition of p38 activity increases the size and number of dendritic spines (Fernandez et al., 2012) and that in p38 heterozygous knockdown mice there is an increase in dendritic spine density (Dai et al., 2016). In view of the inhibitory role of p38 in spine morphogenesis, it seems reasonable to hypothesize that the spine density increase observed here in CSA-treated mice may be attributable to a CSA-mediated inhibition of p38.

Conclusions and future perspectives

DS is characterized by impairment of NPC proliferation, acquisition of a neuronal phenotype, and dendritic development. An obvious question regards the possibility of pharmacologically restoring this whole triad of defects. While the gene burden is the *primum movens* of overall brain and somatic alterations in DS, it is likely that the triad of DS neurodevelopmental defects is attributable to the alteration of specific pathways. Therefore, it may be necessary to use co-treatment with different drugs, in order to fully correct brain development. Importantly, the current study shows that treatment with a single drug, CSA, is able to restore the entire triad of defects of the trisomic brain.

In view of its extensive effects, CSA may prove an ideal drug for DS. However, caution must be exercised because CSA is an immunosuppressant and its clinical use is limited by side effects that include nephrotoxicity, neurotoxicity and

hepatotoxicity (Matsuda & Koyasu, 2000; Bartynski et al., 2001). Regarding side effects of CSA, we did not find a reduction of mice viability after 13 days of treatment, suggesting a lack of patent adverse effects on the general health of Ts65Dn (and euploid) mice. A recent study shows that three children with DS treated with CSA (approximately 6 mg/kg/day) as therapy for idiopathic aplastic anemia did not experience severe or unexpected adverse events during treatment (Suzuki et al., 2016). Another report describes the case of a girl with DS treated with prednisone and CSA (about 4 mg/kg twice a day) for the treatment of alopecia. This report does not describe adverse effects of treatment either (Gensure, 2013). In both studies, the treatment lasted for months. Although these studies show that CSA is a safe and reasonable treatment even for individuals with DS, the possibility of side effects of treatment must be taken into account. Indeed, in mice treated with CSA we found a small (4-5%) reduction in overall brain weight, which is in line with similar evidence obtained in rats (Setkowicz and Kadulski, 2007). The causes of this brain weight reduction remain to be elucidated. Recent evidence shows brain protein turnover is much higher than previously assumed (3-4% day) which makes the brain prone to undergo considerable remodeling (Smeets et al., 2018). It has been shown that CSA inhibits protein synthesis in rat liver (Backman et al., 1988). If a similar effect takes place in the brain, this may explain the brain weight reduction found here in CSA-treated mice. There is evidence that while CSA does not change the overall density of Nissl-stained neurons in rats, it reduces the number of calretinin-and parvalbumin-positive neurons (Setkowicz and Kadulski, 2007), the number of glioma-infiltrating microglial cells (Gabrusiewicz et al., 2011), and the survival of reactive astrocytes in culture (Pyrzynska et al., 2001). It cannot be ruled out that the brain weight reduction observed here after treatment with CSA may be due to a reduction in the number of some cells populating the brain. An important issue that needs to be addressed in further studies will be to

establish whether a shorter treatment schedule can restore the neurodevelopmental defects of DS without affecting overall brain weight.

The toxicity of CSA appears to be directly tied to its calcineurin-based mechanism of action. It must be remarked that various studies have shown that the immunosuppressive and neurotrophic actions of immunosuppressants are separable and that the neurotrophic properties of immunosuppressant drugs are not mechanistically linked to their immunosuppressive actions but operate by separate pathways (see(Hamilton & Steiner, 1998; Nigro et al., 2013)). Non-immunosuppressant analogues of CSA (and of other immunosuppressant drugs such as FK506) have been shown to bind to their respective immunophilins and inhibit their activity, but they lack the ability to interact with calcineurin. For instance, a non-immunosuppressive analogue of CSA (MeAla-6-CsA) stimulates neurite outgrowth of PC12 cells, similarly to the action of CSA (Steiner et al., 1997), and the non-immunosuppressive analogue of CSANIM811 mimics the pro-survival effects of CSA on NPCs *in vitro* (Sachewsky et al., 2014). The immunosuppressive effect of CSA may represent a serious liability in the context of treatment for DS. However, by exploiting non-immunosuppressive analogues of CSA it may be possible to obtain the same positive effects on brain development as those elicited by CSA, with none of the unwanted effects due to immunosuppression. We hope that our study may prompt further work to clarify this important issue. If CSA analogues prove to have the same positive impact as CSA on neurogenesis and spinogenesis they may be considered as practicable tools for ameliorating brain development in individuals with DS.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Chapter 5

A flavonoid agonist of the TrkB receptor for BDNF improves hippocampal neurogenesis and hippocampus-dependent memory in the Ts65Dn mouse model of DS

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Running Title: Therapy with 7,8-DHF in a Down syndrome model

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ABSTRACT

Intellectual disability is the unavoidable hallmark of Down syndrome (DS), with a heavy impact on public health. Reduced neurogenesis and impaired neuron maturation are considered major determinants of altered brain function in DS. Since the DS brain starts at a disadvantage, attempts to rescue neurogenesis and neuron maturation should take place as soon as possible. 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). Systemic BDNF administration is impracticable because BDNF has a poor blood-brain barrier penetration. Recent screening of a chemical library has identified a flavone derivative, 7,8-dihydroxyflavone (7,8-DHF), a small-molecule that crosses the blood-brain barrier and binds with high affinity and specificity to the TrkB receptor. The therapeutic potential of TrkB agonists for neurogenesis improvement in DS has never been examined. The goal of our study was to establish whether it is possible to restore brain development in the Ts65Dn mouse model of DS by targeting the TrkB receptor with 7,8-DHF. Ts65Dn mice subcutaneously injected with 7,8-DHF in the neonatal period P3-P15 exhibited a large increase in the number of neural precursor cells in the dentate gyrus and restoration of granule cell number, density of dendritic spines and levels of the presynaptic proteins synaptophysin. In order to establish the functional outcome of treatment, mice were treated with 7,8-DHF from P3 to adolescence (P45-50) and were tested with the Morris Water Maze. Treated Ts65Dn mice exhibited improvement of learning and memory, indicating that the recovery of the hippocampal anatomy translated into a functional rescue. Our study in a mouse model of DS provides novel evidence that treatment with 7,8-DHF during the early postnatal period restores the major trisomy-linked neurodevelopmental defects, suggesting that therapy with 7,8-DHF may represent a possible breakthrough for Down syndrome.

Key Words: Down syndrome; pharmacotherapy; BDNF/TrkB system; hippocampus; neurogenesis; memory

INTRODUCTION

Down syndrome (DS) is a relatively common genetic condition (1:750-1000) caused by the triplication of human chromosome 21. One of the most important consequences of trisomy 21 is a delay in neurological development, which manifests progressively as microcephaly and intellectual disability (reviewed by (Delabar et al., 2006; Bartesaghi et al., 2011; Dierssen, 2012; Haydar & Reeves, 2012)). Neurogenesis reduction and impaired dendritic morphogenesis are the major neurodevelopmental defects of DS and are thought to underlie cognitive disability. The molecular mechanisms underlying brain development alterations are likely to be manifold, due to the complexity of gene imbalance in DS, and no therapies currently exist for the rescue of neurocognitive impairment in DS.

Most of the brain neurons are produced in the prenatal period, with the notable exception of those involved in the formation of the hippocampus, where neurogenesis continues postnatally and throughout life (Seress et al., 2001; Rice & Barone, 2010; Stiles & Jernigan, 2010; Spalding et al., 2013). Unlike neurogenesis, neuron maturation and the establishment of brain wiring largely take place in the perinatal period. After the critical periods of neurogenesis and synaptogenesis the brain can undergo relatively limited plastic changes. Thus, the perinatal period represents a window of opportunity for therapies aimed at improving the neurodevelopmental alterations of DS. Since the DS brain starts at a disadvantage, attempts to rescue neurogenesis and neuron maturation should take place as soon as possible. Previous studies have shown that perinatal treatment with fluoxetine, a selective serotonin reuptake inhibitor (SSRI), fully

restores brain development and cognitive performance in the Ts65Dn mouse model of DS (Bianchi et al., 2010b; Guidi et al., 2014). This discovery provides the first evidence that brain defects due to the trisomic condition are reversible and gives new hope for therapeutic interventions in individuals with DS. Although fluoxetine is a widely-used antidepressant, that may also be prescribed in children, its use during pregnancy may cause alterations in heart development (Reefhuis et al., 2015). Thus, we deem it extremely important to find molecules that have the same positive impact as fluoxetine in the trisomic brain but that may pose fewer caveats for clinical application.

The brain-derived neurotrophic factor (BDNF) is a neurotrophin that plays a key role in brain plasticity by specifically binding to tropomyosin-related kinase receptor B (TrkB) (Haniu et al., 1997). This binding causes dimerization and autophosphorylation of the TrkB receptor, which triggers the activity of several intracellular pathways, thereby favoring neurogenesis, neuritogenesis and spine growth ((see (Vilar & Mira, 2016)). In the DS brain, BDNF levels are already reduced at fetal life stages (Guedj et al., 2009; Toiber et al., 2010) and reduced BDNF levels have been shown in various brain regions of the Ts65Dn mouse (Bimonte-Nelson et al., 2003; Bianchi et al., 2010b; Fukuda et al., 2010, Begenisic et al., 2015). Interestingly, SSRI administration increases BDNF levels (Malberg and Blendy, 2005), and a triangulation between neurogenesis, the serotonergic system, and BDNF signaling is supported by several findings, suggesting that BDNF-TrkB signaling may be involved in the pro-neurogenic effects of SSRI antidepressants, such as fluoxetine (Gardier et al., 2009; Foltran & Diaz, 2016). Indeed, TrkB ablation in hippocampal neural precursor cells blocks the increase in proliferation and neurogenesis that occurs in response to antidepressants (Vilar & Mira, 2016). Consistently with this evidence, fluoxetine increases BDNF levels in Ts65Dn mice (Bianchi et al., 2010b; Stagni et al.,

2013), suggesting that BDNF may be involved in the beneficial effects of fluoxetine treatment.

In view of the role of the BDNF-TrkB system in neurogenesis and dendritic morphogenesis, it is conceivable that interventions targeted to the BDNF-TrkB system may be exploited in order to improve these defects. Systemic administration of BDNF is impracticable because BDNF has a poor blood-brain barrier penetration. This obstacle could be circumvented by using TrkB agonists that can enter the brain. Recent screening of a chemical library has identified a flavone derivative, 7,8-dihydroxyflavone (7,8-DHF), as the first small-molecule compound that binds with high affinity and specificity to the TrkB receptor, activates its downstream signaling cascade (Liu et al., 2010), and penetrates the blood brain barrier (Liu et al., 2013). Administration of 7,8-DHF enhances the activation of phosphorylated TrkB and increases spine density in several brain regions (Zeng et al., 2012), promotes neurogenesis in the dentate gyrus (Liu et al., 2010), fosters neurite outgrowth (Tsai et al., 2013) and exerts therapeutic efficacy in various animal disease models that are related to deficient BDNF signaling (Liu et al., 2016).

Various pharmacotherapies have been attempted in DS mouse models (Bartesaghi et al., 2011; Costa & Scott-McKean, 2013; Gardiner, 2015). Some of these studies have prompted clinical trials in children and young adults with DS (De la Torre et al., 2014, de la Torre et al., 2016) which emphasizes the potential translational impact of preclinical research. A comparison of the effects of different therapies in mouse models shows that many of them were effective (Stagni et al., 2015). It should be noted, however, that some of the used drugs may be not devoid of side effects, and/or have ephemeral effects, which diminishes their translational impact. For instance, lithium may impair the renal function, inhibitors of GABAA receptors may have pro-convulsant effects, melatonin may affect puberty in children. The therapeutic potential of TrkB

agonists for neurogenesis improvement in DS has never been examined. Considering the important role of the BDNF-TrkB receptor system on neurogenesis, we expect that by acting upon this system by exploiting the flavonoid 7,8-DHF it may be possible to positively impact the DS brain. Moreover, considering that flavonoids are natural substances it seems likely that their administration at appropriate doses may be devoid of side effects. Based on these premises, we deemed it important to investigate whether treatment with 7,8-DHF is able to restore trisomy-linked neurogenesis defects. To this purpose, we exploited the Ts65Dn mouse, a widely-used model of DS. We focused on the hippocampal formation, one of the most important brain regions involved in pattern separation/completion and memory formation (Rolls, 2016). The hippocampal dentate gyrus mainly develops in the early postnatal period in rodents and continues to produce new neurons throughout life in all species examined including human beings (Altman & Bayer, 1975; Altman and Bayer, 1990a, b; Spalding et al., 2013; Workman et al., 2013). We show here that early treatment with 7,8-DHF restores cellularity and neuron maturation in the hippocampal dentate gyrus of the Ts65Dn model of DS and that these effects are accompanied by restoration of hippocampus-dependent memory.

MATERIALS AND METHODS

Mouse colony

Ts65Dn mice were generated by mating B6EiC3Sn a/A-Ts(17^16)65Dn females with C57BL/6JEiJ x C3H/HeSnJ (B6EiC3Sn) F1 hybrid males. This parental generation was provided by Jackson Laboratories (Bar Harbor, ME, USA). To maintain the original genetic background, the mice used were of the first generation of this breeding. Animals were genotyped as previously described (Reinholdt et al., 2011). Because C3H/HeSnJ mice carry a recessive mutation

that leads to retinal degeneration, animals used for the behavioral study were genotyped by standard PCR to screen out all mice carrying this gene. The day of birth was designated postnatal day zero (P0). The animals' health and comfort were controlled by the veterinary service. The animals had access to water and food ad libitum and lived in a room with a 12:12 h light/dark cycle. Experiments were performed in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) for the use of experimental animals and were approved by Italian Ministry of Public Health (813/2016-PR). In this study, all efforts were made to minimize animal suffering and to keep the number of animals used to a minimum.

IN VITRO EXPERIMENTS

Cultures of SVZ or SGZ neural progenitor cells

Cells were isolated from the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) of newborn (P1-P2) euploid and Ts65Dn mice. Briefly, brains were removed, the SVZ and SGZ regions were isolated and individually collected in ice-cold PIPES buffer pH 7.4. After centrifugation, tissue was digested for 10 min at 37°C using Trypsin/EDTA 0.25% (Life Technologies) aided by gentle mechanical dissociation. Cell suspension from each individual mouse was plated onto 25 cm^2 cell-culture flask (Thermo Fisher Scientific) and cultured as floating neurospheres in medium containing basic fibroblast growth factor (bFGF, 10 ng/ml; Peprotech) and epidermal growth factor (EGF, 20 ng/ml; Peprotech) using an established protocol (Meneghini et al., 2014). Primary (Passage 1, P1) neurospheres were dissociated using StemproAccutase (Life Technologies) after 7 days in vitro (DIV), thereafter neurospheres were passaged every 5 DIV. For proliferation studies neurospheres (P3-P12) from the SVZ were dissociated in a single cell suspension and plated onto Nunclon^{TM} Delta Surface 96-well plate

(Thermo Fisher Scientific) at a density of 4×10^3 cells per well in DMEM/F-12 medium supplemented with B27, Glutamax™, heparin sodium salt (4 μg/ml; ACROS Organics), bFGF (10 ng/ml) and 100 U/100 μg/ml Penicillin/Streptomycin (Life Technologies) in presence of 7,8-Dihydroxyflavone (7,8-DHF; 0.3-10.0 M, Sigma Aldrich) or its vehicle (DMSO 0.06%) for 96 h. Since lithium has been shown to restore proliferation of NPCs of Ts65Dn mice in vivo (Bianchi et al., 2010a; Contestabile et al., 2013) and in vitro (Trazzi et al., 2014), the effect of exposure to lithium 2 mM was also examined, as positive control. This dose was chosen based on previous evidence (Trazzi et al., 2014). Cell proliferation was quantified as relative luminescence units (RLU) values using CellTiter-Glo viability assay reagent (Promega) on a Victor^3-V plate reader (PerkinElmer). For differentiation experiments neurospheres from the SVZ and SGZ were dissociated into single cells and plated onto laminin-coated Lab-Tek 8-well permanox chamber slides (Thermo Fisher Scientific) at a density of 35×10^3 per well in differentiation medium (DMEM-F12 supplemented with B27, 2 mM Glutamax and 100 U/100 mg/ml penicillin/streptomycin). NPCs were treated in presence of 7,8-DHF (0.3–10.0 M, Sigma Aldrich) or vehicle (DMSO 0.02 %) for 96 h. Phenotypic characterization of NPC-derived cells was carried out by immunolocalization for MAP2 (rabbit polyclonal,1:600; Millipore) and Nestin (chicken monoclonal, 1: 1,500; Neuromics). Secondary antibodies were as follows: AlexaFluor555-conjugated goat anti rabbit (1:1,400; MolecularProbes); AlexaFluor488-conjugated goat anti chicken (1: 1,400; Molecular Probes). Nuclei were counterstained with 0.8 ng/ml Hoechst (Thermo Fisher Scientific) diluted in PBS. In each experiment, five fields/well (corresponding to about 150–200 cells/well) were counted with a 60X objective by a Leica DMIRB inverted fluorescence microscope. Immunoreactive cells were counted and their percentage over total viable cells was calculated. The number of MAP2 cells exhibiting neuritic

processes was counted at random locations in five fields/well and their number was expressed as percentage over total cell number in each sampled location. All experiments were run in triplicate.

IN VIVO EXPERIMENTS

In the current study, treatment with either 7,8-DHF or vehicle began on postnatal day 3 (P3). All mice that survived in the P0 to P3 period entered this study, with no specific selection criteria. A total of 185 mice entered the study (96 males and 89 females). The number of vehicle-treated and 7,8-DHF treated mice was 96 and 89, respectively. Seven vehicle-treated (7.3%) and five 7,8-DHF-treated (5.6%) mice died before weaning, in the P6-P22 period. The similarity in the mortality rate across groups suggests that treatment has no adverse effects on the health of mice.

Experimental protocol

Pilot experiment. In a pilot study we tested the effects of different doses of 7,8-DHF on the proliferation rate in the SGZ of Ts65Dn mice. Mice received a daily subcutaneous injection of 7,8-DHF (2.5, 5.0, or 10.0 mg/kg in PBS with 1-2% DMSO) from postnatal day 3 (P3) to P15. On P15, mice received an intraperitoneal injection (150 g/g body weight) of BrdU (5-bromo-2-deoxyuridine; Sigma), a marker of cells in the S-phase of the cell cycle (Nowakowski et al., 1989) in TrisHCl 50 mM 2h before being killed and the number of BrdU positive cells in the SGZ was evaluated. We found that the optimum dose was 5.0 mg/kg (see Fig. 3A). Therefore, this study (Experiment 1 and Experiment 2) was carried out using a 5.0 mg/kg dose.

Experiment 1. Euploid (n=25) and Ts65Dn (n=15) mice received a daily subcutaneous injection (at 9-10am) of 7,8-DHF (5.0 mg/kg in vehicle: PBS with 1% DMSO) from P3 to P15. This timing was chosen because it corresponds to

that previously used to test the effects of fluoxetine and other pharmacotherapies in the neonate Ts65Dn mouse (Bianchi et al., 2010b; Giacomini et al., 2015; Stagni et al., 2016). Age-matched euploid (n=35) and Ts65Dn (n=21) mice were injected with the vehicle (these mice will be named here "untreated mice"). On P15, mice received an intraperitoneal injection (150 g/g body weight) of BrdU in TrisHCl 50 mM 2h before being killed (Fig. 2A). The brains were excised and cut along the midline. The left hemisphere of a group of mice was fixed by immersion in PFA 4% and frozen, and the left hemisphere of another group of mice was used for Golgi staining. The right hemispheres of all mice was kept at –80°C and used for western blotting.

Experiment 2. Euploid (n=17) and Ts65Dn (n=16) mice received a daily subcutaneous injection (at 9-10am) of 7,8-DHF (5.0 mg/kg in vehicle) from postnatal day 3 (P3) to postnatal day P40-P55. Age-matched euploid (n=19) and Ts65Dn (n=14) mice were injected with the vehicle. These mice will be called here P45 mice. Mice were behaviorally tested in the 6 days that preceded the day of sacrifice (Fig. 2B).

The body weight of mice of all groups was recorded prior to sacrifice and the brain weight was recorded immediately after brain removal. The number of animals used for each experimental procedure is specified in the figure legends and in Supplementary Table 2.

Histological procedures

The frozen brains 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% PBS10X; sodium azide 0.02%; MilliQ to volume).

Immunohistochemistry

Immunohistochemistry (IHC) was carried out as previously described (Contestabile et al., 2007; Bianchi et al., 2010b; Guidi et al., 2013; Giacomini et al., 2015).

BrdU immunohistochemistry. One out of six free-floating sections (n=15-18 sections) from the hippocampal formation of P15 mice was incubated with rat anti-BrdU antibody. Detection was performed with a Cy3-conjugated anti rat-secondary antibody as indicated in Table 1.

Golgi staining

Brains of P15 mice were Golgi stained using the FD Rapid Golgi Stain^{TM} Kit (FD NeuroTechnologies, Inc.). Brains were immersed in the impregnation solution containing mercuric chloride, potassium dichromate and potassium chromate and stored at room temperature in darkness for 3 weeks. Hemispheres were cut with a microtome in 90-μm-thick coronal sections that were mounted on gelatin-coated slides and were air dried at room temperature in the dark for one day. After drying, sections were rinsed with distilled water and subsequently stained in a developing solution (FD Rapid Golgi Stain Kit).

Measurements

Image acquisition

Immunofluorescence images were taken with a Nikon Eclipse TE 2000-S inverted microscope (Nikon Corp., Kawasaki, Japan), equipped with a Nikon digital camera DS 2MBWc. Measurements were carried out using the software Image Pro Plus (Media Cybernetics, Silver Spring, MD 20910, USA). Bright field images were taken on a light microscope (Leitz) equipped with a motorized stage and focus control system and a Coolsnap-Pro color digital camera (Media Cybernetics, Silver Spring, MD, USA).

Table 1
Antibodies used for immunohistochemistry and Western blotting.

Antigen	Application	Antibody dilution-manufactures
α-Tubulin	WB	Primary: mouse monoclonal 1:1000 (Clone B-5-1-2) (Sigma-Aldrich, T5168) Secondary: HRP-conjugated anti-mouse 1:20000 (Jackson Immunoresearch, 115-035-003)
Brain-derived neurotrophic factor (BDNF)	WB	Primary: rabbit polyclonal 1:500 (N-20) (Santa Cruz Biotechnology, cs-546) Secondary: HRP-conjugated anti-rabbit 1:10000 (Jackson Immunoresearch, 111-035-003)
5-Bromo-2-deoxyuridine (BrdU)	IHC	Primary: rat monoclonal 1:200 (BioRad, OBT0030) Secondary: Cy3-conjugated anti-rat IgG 1:200 (Jackson Immunoresearch, 112-165-143)
Extracellular signal-regulated kinase (ERK1/2)	WB	Primary: mouse monoclonal 1:1000 (3A7) (Cell Signaling Technology, 9107) Secondary: HRP-conjugated anti¦mouse 1:10000 (Jackson Immunoresearch, 115-035-003)
phosphorylated ERK (p-ERK1/2)	WB	Primary: rabbit polyclonal 1:1000 (Cell Signaling Technology, 9101) Secondary: HRP-conjugated anti-rabbit 1:10000 (Jackson Immunoresearch, 111-035-003)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	WB	Primary: rabbit polyclonal 1:5000 (Sigma-Aldrich, G9545) Secondary: HRP-conjugated anti-rabbit 1:10000 (Jackson Immunoresearch, 111-035-003)
Synaptophysin (SYN)	WB	Primary: rabbit polyclonal 1:1000 (Abcam, ab 14692) Secondary: HRP-conjugated anti-rabbit 1:10000 (Jackson Immunoresearch, 111-035-003)
Tropomyosin receptor kinase (Trk) B Full Length (FL) and TrkB truncated (T1)	WB	Primary: rabbit monoclonal 1:1000 (Cell Signaling Technology, 80E3) Secondary: HRP-conjugated anti-rabbit 1:10000 (Jackson Immunoresearch, 111-035-003)
Phosphorylated TrkB-FL (p-TrkB-FL)	WB	Primary: rabbit polyclonal 1:1000 (Millipore, ABN1381) Secondary: HRP-conjugated anti-rabbit 1:10000 (Jackson Immunoresearch, 111-035-003)

BrdU-positive cells

BrdU-positive cells in the DG of P15 mice were detected using a fluorescence microscope (Eclipse; objective: x 40, 0.75 NA; final magnification: x 400). Quantification of BrdU-labeled nuclei was conducted in every 6th section using a modified unbiased stereology protocol that has previously been reported as successfully quantifying BrdU labeling (Malberg et al., 2000; Kempermann and Gage, 2002; Tozuka et al., 2005). All BrdU labeled cells located in the granule cell and subgranular layers were counted in their entire z axis (1 µm steps) in each section. To avoid oversampling errors, nuclei intersecting the uppermost focal plane were excluded. The total number of BrdU labeled cells per animal was determined and multiplied by six to obtain the total estimated number of cells per DG.

Stereology of the DG

Unbiased stereology was performed on Hoechst-stained sections from P15 mice. The optical dissector method was used to obtain density, and Cavalieri principle was used to estimate volume (West & Gundersen, 1990). To include 15-20 sections, one every 6th section was selected, beginning at a random position

within the first 6 sections. In order to obtain granule cell numerical density, counting frames (disectors) with a side length of 30 μm and a height of 10 μm spaced in a 100 μm square grid were systematically used. Granule cell nuclei were counted with a x 64 oil objective (1.4 NA). Granule cell nuclei intersecting the uppermost focal plane and intersecting the exclusion lines of the count frame were not counted. The neuron density (Nv) is given by NV = (Q/dis)/Vdis where Q is the number of particles counted in the disectors, dis is the number of disectors and Vdis is the volume of the disector. For calculation of Vdis the disector height was corrected for section shrinkage in the z-plane (Dorph Petersen et al., 2001) according to the formula: h=counting thickness x (original thickness/measured thickness). The section thickness was measured during neuron counting at different random locations. In the analyzed sections, the mean section thickness was 16 μm (range: 12-18 μm). For volume (Vref) estimation with the Cavalieri principle, in each sampled section, the area of the granule cell layer was measured by tracing its contours. The volume of the granule cell layer (Vref) was estimated (West & Gundersen, 1990) by multiplying the sum of the cross sectional areas by the spacing T between sampled sections (180 μm). The total number (N) of granule cells was estimated as the product of Vref and the numerical density (Nv).

N= NV x Vref

Spine density

In Golgi-stained sections from the DG of P15 mice, spines of granule cells were counted using a 100x oil immersion objective lens (1.4 NA). Spine density values were obtained from dendritic segments in the inner and outer half of the molecular layer. For each neuron, 2-3 segments were analyzed in the outer and inner half of the molecular layer, respectively. For each animal, spines were counted in at least 8 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 100 μm dendrite.

Western blotting

In homogenates of the hippocampal formation of P15 mice, total proteins were obtained as previously described (Trazzi et al., 2011) and the levels of the following proteins were evaluated: BDNF, TrkB full length (TrkB-FL), phosphorylated TrkB (p-TrkB), the truncated form 1 of the TrkB receptor (TrkB-T1), phosphorylated ERK1 (p-ERK1), phosphorylated ERK2 (p-ERK2), ERK1, ERK2, synaptophysin (SYN), GAPDH and α-Tubulin using the antibodies reported in Supplementary Table 1. Densitometric analysis of digitized images with ChemiDoc XRS+ was performed with Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA) and intensity for each band was normalized to the intensity of the corresponding GAPDH or α-Tubulin band.

Behavioral testing

Morris Water Maze (MWM). Mice were trained in the MWM task to locate a hidden escape platform in a circular pool, using a previously used protocol (Stagni et al., 2016), that was altogether based on a published protocol (Vorhees, 2006). The apparatus consisted of a large circular water tank (1.00 m diameter, 50 cm height) with a transparent round escape platform (10 cm^2). The pool was virtually divided into four equal quadrants identified as northeast, northwest, southeast, and southwest. The tank was filled with tap water at a temperature of 22±1.0ºC. Mice are more prone to undergo hypothermia than rats and in the MWM hypothermia may cause a reduction in swimming speed (Iivonen et al., 2003). Evaluation of the swimming speed showed no speed differences across consecutive trials, suggesting that at this temperature mice did not undergo

hypothermia (Supplementary Fig. 1, not shown in this thesis). This is consistent with evidence that a temperature of 22°C is "high enough" to reduce stress and potential hypothermia and "low enough" to maintain the animals' motivation to escape the pool (Costa et al., 2010). The tank was filled with water up to 0.5 cm above the top of the platform and the water was made opaque with milk. The platform was placed in the tank in a fixed position (in the middle of the south-west quadrant). The pool was placed in a large room with various intra- (squares, triangles, circles and stars) and extra-maze visual cues. Each mouse was tested in one session of 4 trial on the first day and in two sessions of 4 trials in the following 4 days with an inter-session interval of 45 min. A video camera was placed above the center of the pool and connected to a videotracking system (Ethovision 3.1; Noldus Information Technology B.V., Wageningen, Netherlands). Mice were released facing the wall of the pool from one of the following starting points: North, East, South, or West and allowed to search for up to 60 s for the platform. If a mouse did not find the platform, it was gently guided to it and allowed to remain there for 15 s. During the inter-trail time (10 s) mice were placed in an empty cage. For the learning phase, we evaluated the latency to find the hidden platform, time in periphery, percentage of time in periphery, path length, proximity to the platform, and swimming speed. Retention was assessed with one trial (probe trial), on the sixth day, 24 h after the last acquisition trial, using the same starting point for all mice. Mice were allowed to search for up to 60 s for the platform. For the probe trial, the latency of the first entrance in the former platform zone, the frequency of entrances in the former quadrant, the proximity to the former platform position (Gallagher's test), the percentage of time spent at the periphery (thigmotaxis), the swimming speed and the percentage of time spent in each quadrant were employed as measures of retention of acquired spatial preference. All experimental sessions were carried out between 9.00am and 5.00pm. The mice used for the MWM did not carry a

recessive mutation that leads to retinal degeneration. The following number of mice were tested. Untreated euploid mice: n=19; untreated Ts65Dn mice: n=14; 7,8-DHF-treated euploid mice: n=17; 7,8-DHF-treated Ts65Dn mice: n=16. Three untreated euploid mice (yielding n=16), one 7,8-DHF-treated euploid mouse (yielding n=16) and one 7,8-DHF-treated Ts65Dn mouse (yielding n=15) were excluded from MWM analysis due to thigmotaxis for a whole recording session.

Statistical analysis

Results are presented as mean ± standard error of the mean (SE). Data were analyzed with the IBM SPSS 22.0 software. Statistical analysis was carried out using either a one-way ANOVA or a two-way ANOVA with genotype (euploid, Ts65Dn) and treatment (vehicle, 7,8-DHF), as factors. Post hoc multiple comparisons were carried out using the Fisher least significant difference (LSD) test. For the learning phase of the MWM test, statistical analysis was performed using a three-way mixed ANOVA, with genotype and treatment as grouping factors and days as a repeated measure. For the probe test of MWM, we used a two-way ANOVA with genotype and treatment as factors followed by the Fisher LSD post hoc test. Based on the "Box plot" tool available in SPSS Descriptive Statistics we excluded from each analysis the extremes, i.e. values that were larger than 3 times the IQ range [$x \geq Q3 + 3 * (IQ)$; $x \leq Q1 - 3 * (IQ)$]. The number of mice included and excluded in individual analyses is reported in Supplementary Table 2. Figure legends report the number of mice used for statistical analysis. Supplementery Tables 3-8 report the p values of the post-hoc LSD test for each analysis. A probability level of $p \leq 0.05$ was considered to be statistically significant.

RESULTS

Effect of treatment with 7,8-DHF on proliferation, differentiation and maturation of trisomic NPCs

Confirming previous evidence (Trazzi et al., 2011; Trazzi et al., 2013), neural progenitor cells (NPCs) from the subventricular zone (SVZ) of neonate Ts65Dn mice exhibit impairment of proliferation rate (Fig. 1A). Cultures of trisomic NPCs were exposed to standard medium (vehicle) or standard medium plus different concentrations of 7,8-DHF, in order to establish whether treatment increases their proliferation rate. A one-way ANOVA showed a significant effect of treatment [$F(5,12) = 81.364$, $p \leq 0.001$]. A post hoc LSD test showed that none of the tested concentrations was able to increase the proliferation rate of trisomic NPCs and that the highest concentration (10.0 μM) even reduced proliferation (Fig. 1B). In contrast, cells were highly responsive to the pro-proliferative action of 2 mM lithium (Fig. 1B). In addition to proliferation impairment, trisomic NPCs exhibit impairment in the acquisition of a neuronal phenotype and maturation, i.e. development of neuritic processes (Trazzi et al., 2011; Trazzi et al., 2013). In order to establish whether treatment favors neurogenesis, in cultures of NPCs under differentiating conditions we evaluated the percentage of cells positive to MAP2, a marker of cells with a neuronal phenotype, and of cells positive to Nestin, a marker of neural stem cells. A one-way ANOVA on the percentage of cells that were MAP2 positive and Nestin negative ($MAP2^+/Nestin^-$) showed a significant effect of treatment [$F(5,12) = 9.354$, $p \leq 0.001$]. A post-hoc LSD test showed that all drug concentrations caused a large increase in the percentage of $MAP2^+/Nestin^-$ cells in comparison with cultures in the standard medium (Fig. 1C). Consistently with the lack of effect of treatment on the proliferation rate of NPCs (Fig. 1B) we found no change in the percentage of cells that were Nestin positive and MAP2 negative ($Nestin^+/MAP2^-$; Fig. 1D).

This evidence suggests that treatment does not affect the proliferation rate of neural stem cells but enhances the differentiation of their progeny into neurons. In order to establish the effect of 7,8-DHF on neuron maturation we evaluated the percentage of cells exhibiting neuritic processes in differentiating cultures of trisomic NPCs from the SVZ and from the subgranular zone (SGZ) of the dentate gyrus (DG), exposed to different concentrations of 7,8-DHF. A one-way ANOVA on the percentage of NPCs from the SGZ that exhibited neuritic processes showed a significant effect of treatment [$F(5,12) = 4.336$, $p = 0.017$]. A post-hoc LSD test showed that concentrations higher than 1.0 μM increased the percentage of differentiating cells in comparison with cultures in the standard medium (Fig. 1E,F). A one-way ANOVA on the percentage of NPCs from the SVZ that exhibited neuritic processes showed a significant effect of treatment [$F(5,12) = 12.364$, $p \leq 0.001$]. A post-hoc LSD test showed that doses higher than 0.3 μM increased the percentage of differentiating cells in comparison with cultures in the standard medium (Fig. 1G,H). Observation of Fig. 1F,H shows that effect of treatment on neuron maturation increased in a concentration-dependent manner. Evaluation of the number of apoptotic cells showed no effect of treatment (data not shown). Taken together these data show that 7,8-DHF does not increase the proliferation rate but fosters the process of neurogenesis and neuron maturation in cultures of NPCs.

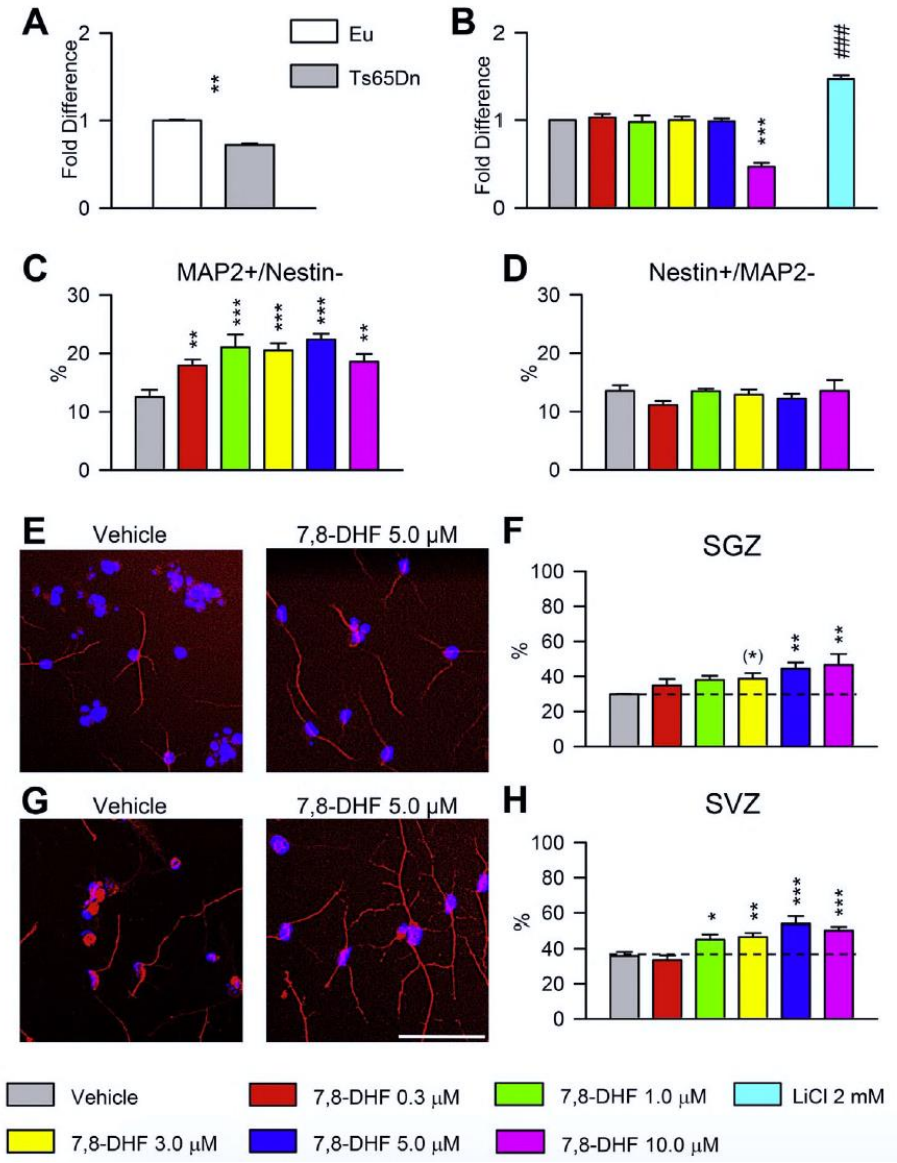

Fig. 1. Effect of 7,8-DHF on proliferation, differentiation and maturation of trisomic NPCs. **A:** Number of proliferating cells in cultures of neural progenitor cells (NPCs) from the SVZ of euploid and Ts65Dn mice. Data are expressed as fold change in comparison with euploid NPCs. *** $p \leq 0.001$, two-tailed t-test. **B:** Effect of different concentrations of 7,8-DHF or LiCl 2.0 mM on the proliferation rate of NPCs from the SVZ of Ts65Dn mice. Data are expressed as fold change in comparison with NPCs exposed to vehicle alone (DMSO 0.06%). **C,D:** Percentage of $MAP2^+/Nestin^-$ cells (C) and of $Nestin^+/MAP2^-$ cells (D) in cultures of NPCs from the SVZ of

Ts65Dn mice grown under differentiating conditions and exposed to different concentrations of 7,8-DHF for 96 h. **E-H:** Percentage of cells exhibiting neuritic processes (red) in cultures of NPCs from the SGZ (F) and SVZ (H) of Ts65Dn mice grown under differentiating conditions and exposed to different doses of 7,8-DHF for 96 h. Images in (E,G) show cells from the SGZ (E) and SVZ (G) of Ts65Dn mice that were exposed to either vehicle (DMSO 0.02%) or 7,8-DHF 5.0 µM. Scale bar=50 µm. Data in A-H were obtained in pooled cultures from euploid (n=4) and Ts65Dn (n=3) mice. The asterisks in (A, B, C, F, H) indicate a difference in comparison with untreated cultures exposed to DMSO alone [(*) $p \leq 0.06$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ (Fisher LSD test after ANOVA)]. The symbol # in (B) indicates a difference between cultures exposed to lithium and cultures exposed to vehicle alone (### $p \leq 0.001$, two-tailed t test). Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; Eu, euploid ; LiCl, Lithium chloride; MAP2, microtubule associated protein 2; SGZ, subgranular zone; SVZ, subventricular zone.

Effect of treatment with 7,8-DHF in vivo: general results

The Ts65Dn strain is characterized by a high mortality rate during gestation (Roper et al., 2006). For this reason, the number of Ts65Dn pups in a litter results approximately 30% instead of the theoretical value of 50%. Moreover, Ts65Dn mice exhibit a high mortality rate before weaning (Roper et al., 2006). This means that numerous litters are needed in order to obtain a sufficiently large number of Ts65Dn mice. In view of the fragility of this strain, we deemed it important to establish whether treatment with 7,8-DHF has adverse effects on the viability and growth of Ts65Dn mice. In the current study, treatment with either 7,8-DHF or vehicle began on postnatal day 3 (P3). All mice that survived in the P0 to P3 period entered this study, with no specific selection criteria. A total of 185 mice entered the study (96 males and 89 females). The number of vehicle-treated and 7,8-DHF treated mice was 96 and 89, respectively. Seven vehicle-treated (7.3%) and five 7,8-DHF-treated (5.6%) mice died before weaning, in the P6-P22 period. The similarity in the mortality rate across groups suggests that treatment has no adverse effects on the health of mice.

We evaluated the body and brain weight of P15 and P45 mice in order to establish the effect treatment on gross growth parameters. A two-way ANOVA on the body weight of P15 mice showed no genotype x treatment interaction [$F(1,92) = 0.63$, $p = 0.431$], no main effect of treatment but a main effect of genotype [$F(1,92) = 14.78$, $p \leq 0.001$]. Post hoc LSD test confirmed well established evidence that Ts65Dn mice have a reduced body weight in comparison with euploid mice and showed that treatment did not further reduce the body weight of Ts65Dn mice (Fig. 2C). A two-way ANOVA on the brain weight of P15 mice showed no genotype x treatment interaction [$F(1,92) = 1.09$, $p = 0.300$], a main effect of genotype [$F(1,92) = 7.73$, $p = 0.007$] and a main effect of treatment [$F(1,92) = 6.18$, $p = 0.015$]. Post hoc LSD test showed that Ts65Dn mice had a reduced brain weight in comparison with euploid mice and that treatment did not cause a further brain weight reduction (Fig. 2D). On the contrary, treated euploid mice underwent a slight but significant brain weight reduction in comparison with their untreated counterparts (Fig. 2D).

A two-way ANOVA on the body weight of P45 mice showed no genotype x treatment interaction [$F(1,62) = 1.57$, $p = 0.215$], no main effect of treatment but a main effect of genotype [$F(1,62) = 4.98$, $p = 0.029$]. Post hoc LSD test showed that Ts65Dn mice retained a reduced body weight in comparison with euploid mice and that treatment did not further reduce their body weight (Fig. 2E). In contrast, treated euploid mice underwent a body weight reduction in comparison with their untreated counterparts (Fig. 2E). A two-way ANOVA on the brain weight of P45 mice showed no genotype x treatment interaction [$F(1,62) = 2.06$, $p = 0.156$], no main effect of either genotype or treatment. Post hoc LSD test showed that untreated Ts65Dn mice had a reduced brain weight in comparison with untreated euploid mice and that this difference disappeared in Ts65Dn mice treated with 7,8-DHF (Fig. 2F). Taken together these findings show that

treatment with 7,8-DHF has no adverse effects on viability and body weight of Ts65Dn mice and that it has a positive impact on their brain weight .

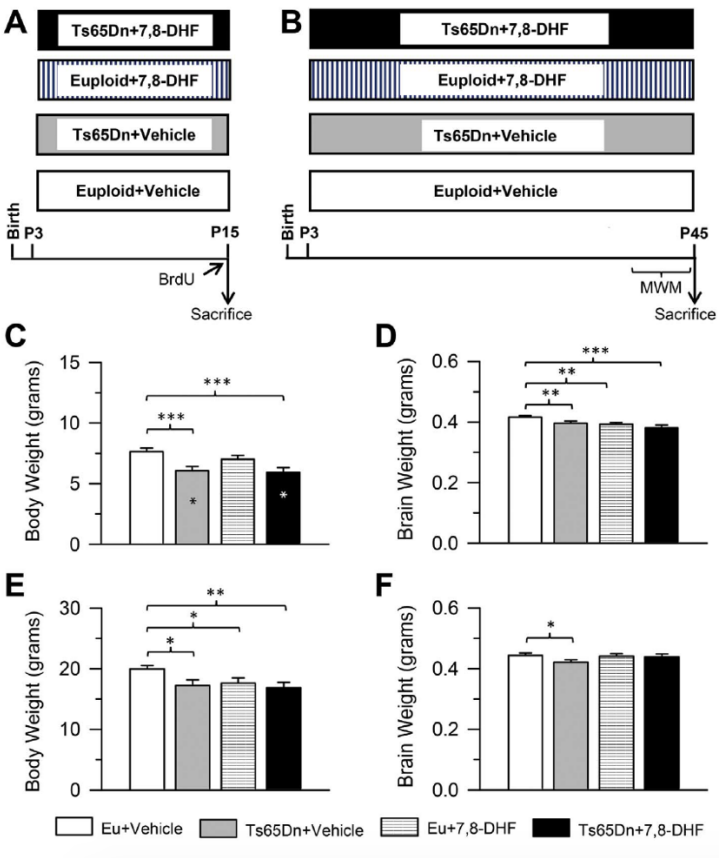

Fig. 2. Experimental protocol and general results of the in vivo experiments.

A: Euploid and Ts65Dn pups received one daily injection of either vehicle or 7,8-DHF from postnatal day 3 (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. **B:** Euploid and Ts65Dn mice received one daily injection of either vehicle or 7,8-DHF from postnatal day P3 to P45-50. These mice were tested with the Morris Water Maze test 6 days before being killed. **C,D:** Body (C) and brain (D) weight (mean SE) in grams of P15 euploid (n=35) and Ts65Dn (n=21) mice that received vehicle and euploid (n=25) and Ts65Dn (n=15) mice that received 7,8-DHF (5 mg/kg) in the period P3-P15. **E,F:** Body (E) and brain (F) weight (mean SE) in grams of P45 euploid (n=19) and Ts65Dn (n=14) mice that received vehicle and euploid (n=17) and Ts65Dn (n=16) mice that received 7,8-DHF (5.0 mg/kg) in the period P3-P45. * $p \leq 0.05$; ** $p \leq 0.01$; ***

$p \leq 0.001$ (Fisher LSD test after two-way ANOVA). Black asterisk in the gray bar indicate a difference between untreated Ts65Dn mice and treated euploid mice. White asterisks in the black bar indicate a difference between treated Ts65Dn mice and treated euploid mice. Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; BrdU, bromodeoxyuridine; Eu, euploid; MWM, Morris Water Maze, P, postnatal.

Effect of neonatal treatment with 7,8-DHF on neural precursor proliferation in the hippocampal dentate gyrus of Ts65Dn mice

Recent work has examined the effect of 7,8-DHF in models of Alzheimer disease. A dose of 5.0 mg/kg has been shown to have no toxic effects and to restore cognitive performance (Liu et al., 2010). In addition, this dose increases the proliferation rate of neural precursor cells of the DG (Liu et al., 2010). In order to establish whether this is the optimal dose for proliferation enhancement in Ts65Dn mice, we treated pups with vehicle, 2.5 mg/kg, 5.0 mg/kg or 10.0 mg/kg of 7,8-DHF in the period P3-P15. At the end of treatment, mice received one injection of BrdU and were killed after 2 h in order to examine the effect of treatment on proliferation rate. A one-way ANOVA on the number of BrdU-positive cells in the DG of Ts65Dn pups showed a significant effect of treatment [$F(3,20) = 4.15$, $p = 0.019$]. Post hoc LSD test showed that the lowest dose had no effect in comparison with vehicle-treated mice and that both the 5.0 mg/kg and the 10.0 mg/kg doses increased the number of BrdU-positive cells in Ts65Dn mice. In absolute terms, the 5.0 mg/kg dose had a higher pro-proliferative effect than the 10.0 mg/kg dose (Fig. 3A).

Based on the results reported above, all following experiments in vivo were carried out using a 5.0 mg/kg dose. In order to establish the effects of 7,8-DHF on proliferation rate of NPCs of the DG, Ts65Dn mice and their euploid littermates were daily injected with 5 mg/kg of 7,8-DHF in the period P3-P15. At the end of treatment, mice were injected with BrdU and the number of BrdU-positive cells in the SGZ of the DG was evaluated. A two-way ANOVA on the

total number of BrdU positive cells showed a genotype x treatment interaction [$F(1,19) = 8.53$, $p = 0.009$], a main effect of genotype [$F(1,19) = 21.25$, $p \leq 0.001$], but no effect of treatment. A post hoc Fisher LSD test showed that, in agreement with previous evidence (Bianchi et al., 2010b), untreated Ts65Dn mice had notably fewer proliferating cells in comparison with untreated euploid mice (total number per DG in Ts65Dn mice: n=7166±337, in euploid mice: n=10281±111). The number of proliferating cells in treated Ts65Dn mice underwent an increase (n=8963±449) and became statistically greater than that of their untreated counterparts, although it remained slightly lower in comparison with untreated euploid mice (Fig. 3B,C). Treatment had no effect on the number of NPCs in euploid mice (Fig. 3B,C). These results show that treatment in vivo, unlike in vitro, greatly enhances cell proliferation in trisomic mice, although the number of proliferating cells does not reach the same value as euploid mice.

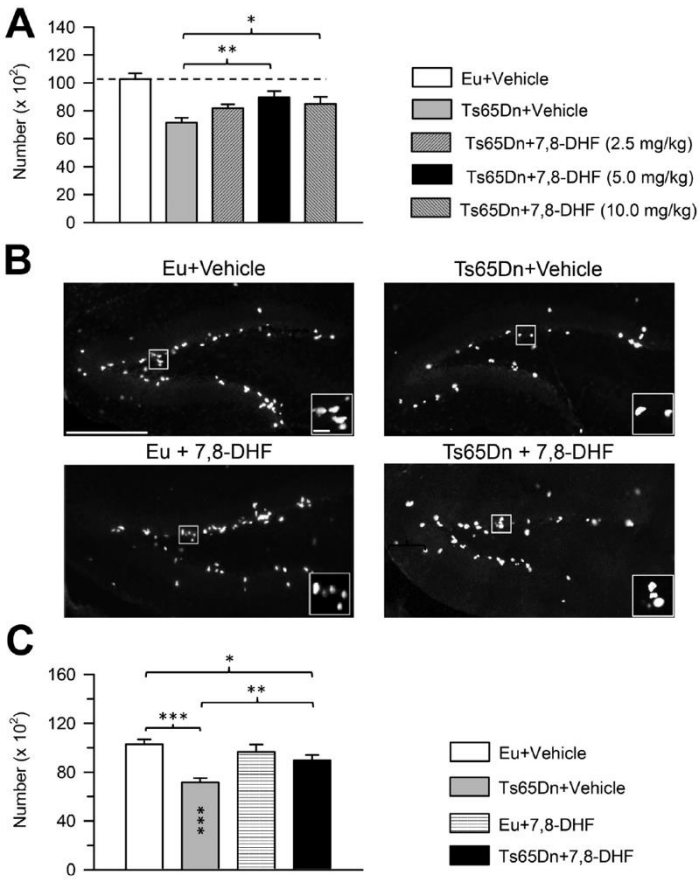

Fig. 3. Effects of neonatal treatment with 7,8-DHF on the size of the population of cells in the S-phase of the cell cycle in the dentate gyrus of P15 Ts65Dn and euploid mice.

A: In pilot experiments Ts65Dn mice received a daily injection of vehicle (n=8) or 7,8-DHF (2.5 mg/kg, n=4; 5.0 mg/kg, n=5; 10.0 mg/kg, n=7) in the period P3-P15. At P15, they were injected with BrdU and killed after 2 h. The histograms show the number of BrdU-positive cells in the DG of Ts65Dn mice treated with either vehicle or the indicated doses of 7,8-DHF. The number of BrdU-positive cells in euploid mice reported in (C) that received the vehicle is shown for comparison. **B:** Representative images of sections immunostained for BrdU from the DG of untreated euploid and Ts65Dn mice and euploid and Ts65Dn mice that were daily treated with 5.0 mg/kg of 7,8-DHF in the period P3-P15. Calibration bar=200 μm. The insets show zoomed images of the boxed area with examples of individual BrdU-positive cells. Calibration bar=20 μm. **C:** Total number of BrdU-positive cells in the DG of untreated euploid (n=7) and Ts65Dn (n=8) mice and euploid (n=3) and Ts65Dn (n=5) mice treated with 5.0 mg/kg of 7,8-DHF. Values (mean SE) in (A) and (C) refer to one hemisphere. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ (Fisher

LSD test after two-way ANOVA). Black asterisks in the gray bar indicate a difference between untreated Ts65Dn mice and treated euploid mice. Abbreviation: 7,8-DHF, 7,8-dihydroxyflavone; Eu, euploid.

Effect of 7,8-DHF on the number of granule neurons in the dentate gyrus of Ts65Dn mice

In view of the treatment-induced increase in the proliferation potency of neural precursor cells of the DG, we expected this effect to lead to improvement/restoration of the defective cellularity that characterizes the DG of trisomic mice (Bianchi et al., 2010b). To clarify this issue, we stereologically evaluated the total number of granule cells in treated and untreated mice. A two-way ANOVA on total number of granule cells showed a genotype x treatment interaction [$F(1,13) = 6.71$, $p = 0.022$], but no main effect of either genotype or treatment. A post hoc Fisher LSD test showed that untreated Ts65Dn mice had fewer granule neurons in comparison with euploid mice and that treatment caused a large increase in their number. Consequently, in treated Ts65Dn mice the number of granule cells became similar to that of untreated euploid mice (Fig. 4A,B). Unlike in Ts65Dn mice, in euploid mice treatment had no effect on total number of granule cells (Fig. 4A,B). These results show that neonatal treatment with 7,8-DHF restores the typical hypocellularity that characterizes the DG of trisomic mice.

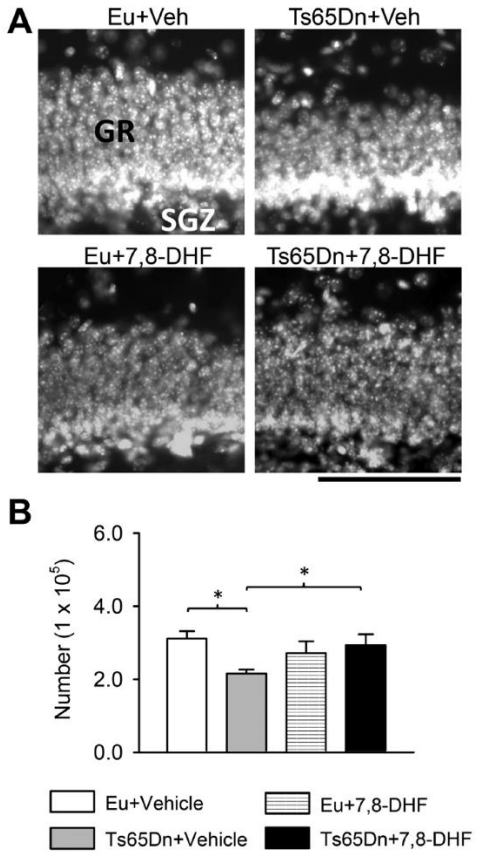

Fig. 4. Effects of neonatal treatment with 7,8-DHF on granule cell number in the dentate gyrus of P15 Ts65Dn and euploid mice.

A: Representative images of Hoechst-stained sections showing the granule cell layer of an animal from each experimental group. Calibration bar=100 µm. **B:** Total number of granule cells of untreated euploid (n=4) and Ts65Dn (n=4) mice and euploid (n=4) and Ts65Dn mice (n=5) treated with 5.0 mg/kg 7,8-DHF. Values (mean SE) refer to one DG. * $p \leq 0.05$ (Fisher LSD test after two-way ANOVA). Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; Eu, euploid; GR, granule cell layer; SGZ, subgranular zone.

Effect of 7,8-DHF 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 7,8-DHF improves this defect, in Golgi stained brains we evaluated spine density in the dendritic arbor of granule neurons. Since no differences between spine density on proximal and distal dendritic branches were found data were pooled together. A two-way ANOVA on spine density showed a genotype x treatment interaction [$F(1,12) = 13.23$, $p = 0.003$], a main effects of genotype [$F(1,12) = 19.93$, $p = 0.001$] and a main effects of treatment [$F(1,12) = 42.30$, $p \leq 0.001$]. A post hoc Fisher LSD test showed that untreated Ts65Dn had a considerably reduced spine density in comparison with untreated euploid mice (Fig. 5C). After treatment with 7,8-DHF the number of spines of Ts65Dn mice underwent a notable increment and became similar to that of euploid mice (Fig. 5C), indicating that treatment fully rescues spine development. In euploid mice treatment had no effect on spine density (Fig. 5C).

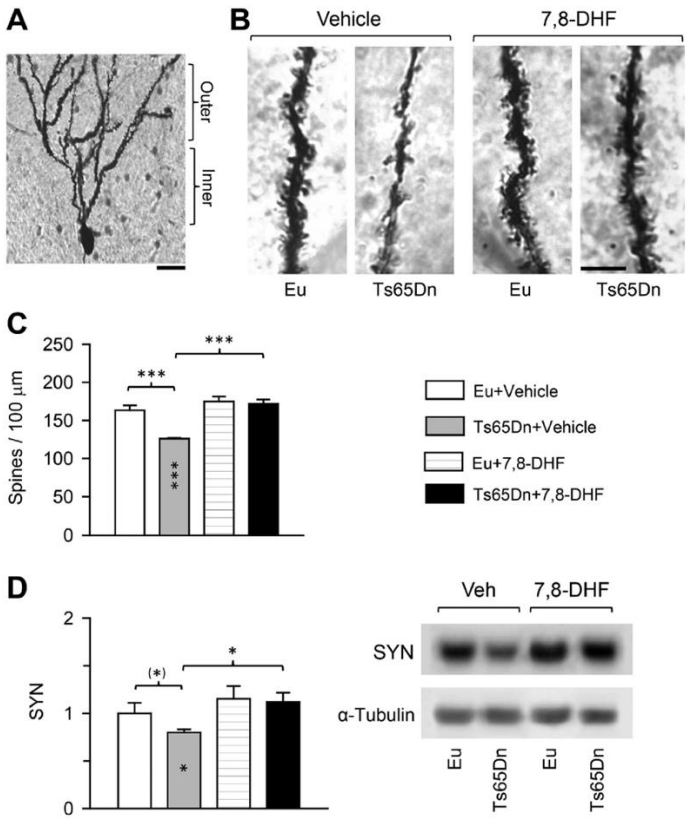

Fig. 5. Effects of neonatal treatment with 7,8-DHF on dendritic spine density and synaptophysin levels in the dentate gyrus of P15 Ts65Dn and euploid mice.

A: The photomicrograph shows a Golgi-stained granule cell. Dendritic spines were counted in the inner and outer half of the dendritic arbor of the granule cells. Calibration bar=10 µm. **B:** Photomicrograph of Golgi-stained granule cell dendrites showing spines on distal dendritic branches in an animal from each experimental groups. Calibration bar=5 µm. **C:** Spine density on the dendritic arbor of the granule cells of untreated euploid (n=4) and Ts65Dn mice (n=4) and euploid (n=4) and Ts65Dn (n=4) mice treated with 7,8-DHF. **D:** Western blot analysis of the expression levels of synaptophysin (SYN) in hippocampal homogenates of untreated euploid (n=10) and Ts65Dn (n=10) mice and treated euploid (n=5) and Ts65Dn (n=6) mice. SYN levels were normalized to GAPDH and expressed as fold difference in comparison with untreated euploid mice. Representative western blots are shown on the right. Values in (C,D) are mean SE. (*) $p \leq 0.06$; * $p \leq 0.05$; *** $p \leq 0.001$ (Fisher LSD test after two-way ANOVA). Black asterisks in the gray bar indicate a difference between untreated Ts65Dn mice and treated euploid mice. Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; Eu, euploid; Veh, Vehicle.

Effect of 7,8-DHF on synaptophysin levels in the hippocampal formation of Ts65Dn mice

Circuit formation is critically shaped in the early postnatal period throughout the brain. The trisomic brain is characterized by altered synaptic connectivity that, in conjunction with hypocellularity and dendritic pathology, largely contributes to impairment of signal processing (Bartesaghi et al., 2011). Synaptophysin (SYN) is a protein of the synaptic vesicles and is, therefore, a marker of synaptic terminals. To establish whether treatment with 7,8-DHF had an effect on synapse development, we examined the expression levels of SYN in the hippocampus of P15 mice.

A two-way ANOVA on the levels of SYN showed no interaction between genotype and treatment [$F(1,27) = 0.82$, $p = 0.372$], no main effect of genotype, but a main effect of treatment [$F(1,27) = 6.62$, $p = 0.016$]. Confirming previous evidence (Stagni et al., 2013), a post hoc Fisher LSD test showed that untreated Ts65Dn mice had reduced SYN levels in comparison with untreated euploid mice, although the difference was marginally significant, and that treatment with 7,8-DHF increased SYN levels that became similar to those of untreated euploid mice (Fig. 5D). An increase in SYN levels also took place in treated euploid mice in comparison with their untreated counterparts (Fig. 5D). These findings suggest that treatment with 7,8-DHF restores development of hippocampal synapses in Ts65Dn mice and enhances synaptic development in euploid mice.

Effect of 7,8-DHF on the BDNF-TrkB receptor system in the hippocampal formation of Ts65Dn mice

BDNF signaling is elicited when it binds to TrkB, resulting in the receptor dimerization and autophosphorylation. TrkB, the high affinity receptor of BDNF, and BDNF are essential for normal brain function (Bibel et al., 1999). The TrkB full-length receptor (TrkB-FL) possesses an intracellular tyrosine kinase domain

and is considered to mediate the crucial effects of BDNF. By contrast, the truncated form 1 of the TrkB receptor (TrkB-T1) lacks tyrosine kinase activity. It mediates inositol-1,4,5-trisphosphate-dependent calcium release (Rose et al., 2003). We examined the protein levels of BDNF and TrkB receptors in the hippocampus of P15 euploid and Ts65Dn mice in order to establish the effect of genotype and treatment on the BDNF/TrkB system.

A two-way ANOVA on the BDNF levels showed no genotype x treatment interaction [$F(1,48) = 0.86$, $p = 0.359$], a main effect of treatment [$F(1,48) = 8.76$, $p = 0.005$], but no effect of genotype. A post hoc Fisher LSD test showed that Ts65Dn mice had similar BDNF protein levels as euploid mice (Fig. 6B). Treatment with 7,8-DHF caused a reduction in BDNF levels both in euploid and Ts65Dn mice although the difference was statistically significant for the latter only (Fig. 6B). A two-way ANOVA on the levels of TrkB-FL receptor showed no genotype x treatment interaction [$F(1,45) = 2.17$, $p = 0.148$] , a main effect of genotype [$F(1,45) = 5.71$, $p = 0.021$] and no effect of treatment. A post hoc Fisher LSD test showed no difference between untreated euploid and Ts65Dn mice in the levels of TrkB-FL (Fig, 6A,C). In Ts65Dn, but not in euploid mice, treatment with 7,8-DHF caused a reduction in the levels of TrkB-FL (Fig. 6A,C). A two-way ANOVA on the levels of the phosphorylated form of TrkB receptor (p-TrkB-FL) showed no genotype x treatment interaction [$F(1,39) = 0.03$, $p = 0.865$], a main effect of treatment [$F(1,39) = 10.88$, $p = 0.002$] but no main effect of genotype. A post hoc Fisher LSD test showed that in untreated Ts65Dn mice the levels of p-TrkB-FL were similar to those of euploid mice. In both genotypes, treatment with 7,8-DHF caused an increase in the levels of p-TrkB-FL (Fig. 6A,D). A two-way ANOVA on the levels of the TrkB-T1 receptor showed a genotype x treatment interaction [$F(1,47) = 6.04$, $p = 0.018$], but no main effect of either treatment or genotype. A post hoc Fisher LSD test showed that untreated Ts65Dn mice has similar levels of TrkB-T1 as untreated euploid mice. Treated

Ts65Dn mice underwent a reduction in the levels of TrkB-T1 in comparison with their untreated counterparts and untreated euploid mice (Fig. 6A,E).

The activation of the TrkB-FL receptor allows its interaction with molecules that further interact and modify downstream targets, including the RAS/ERK signaling pathway. Since RAS/ERK signaling is involved in cell proliferation and differentiation, we examined the effects of treatment on the activation of ERK1/2 in hippocampal homogenates of Ts65Dn and euploid mice. A two-way ANOVA on p-ERK1 levels showed no genotype x treatment interaction [$F(1,29) = 0.78$, $p = 0.385$], but a main effect of genotype [$F(1,29) = 7.21$, $p = 0.012$] and of treatment [$F(1,29) = 4.64$, $p = 0.040$]. Post-hoc LSD test showed that treated Ts65Dn mice underwent an increase in p-ERK1 levels in comparison with untreated Ts65Dn mice as well as untreated euploid mice (Fig. 6F). A two-way ANOVA on p-ERK2 levels showed no genotype x treatment interaction [$F(1,29) = 1.73$, $p = 0.199$], a main effect of genotype [$F(1,29) = 8.92$, $p = 0.006$] but no main effect of treatment. Post-hoc Fisher LSD test showed that treated Ts65Dn mice underwent an increase in p-ERK2 levels in comparison with untreated euploid mice (Fig. 6F). A two-way ANOVA on the levels of ERK1 showed no genotype x treatment interaction [$F(1,28) = 0.815$, $p = 0.374$] and no main effect of either treatment or genotype. Post-hoc Fisher LSD test showed that treated Ts65Dn mice underwent an increase in ERK1 levels in comparison with untreated Ts65Dn mice (Fig. 6G). A two-way ANOVA on the levels of ERK2 showed no genotype x treatment interaction [$F(1,30) = 0.065$, $p = 0.801$], no main effect of genotype, but a main effect of treatment [$F(1,30) = 13.76$, $p = 0.001$]. Post-hoc Fisher LSD test showed that Ts65Dn mice treated with 7,8-DHF underwent an increase in ERK2 levels in comparison with their untreated counterparts and untreated euploid mice (Fig. 6G). An increase in ERK2 levels also took place in treated euploid mice in comparison with their untreated counterparts (Fig. 6G). There is evidence that ERK2 is approximately four time

more abundant than ERK1 in various brain regions and that alteration of the stoichiometry of the two isoform of ERK may have adverse effects (Lefloch et al., 2008). Therefore, we examined the relative abundance of ERK1/ERK2 and p-ERK1/ERK2 in treated and untreated mice. We found that in the hippocampal region of untreated euploid and Ts65Dn mice the ratio between ERK2 and ERK1 was approximately 3:1 and the ratio between p-ERK2 and p-ERK1 was approximately 2:1 (Supplementary Table 8, not shown here). Although in absolute terms treatment increased the levels of ERK1/2 and p-ERK1/2 in Ts65Dn mice (Fig. 6F,G), it did not affect their stoichiometry (Supplementary Table 8, not shown here).

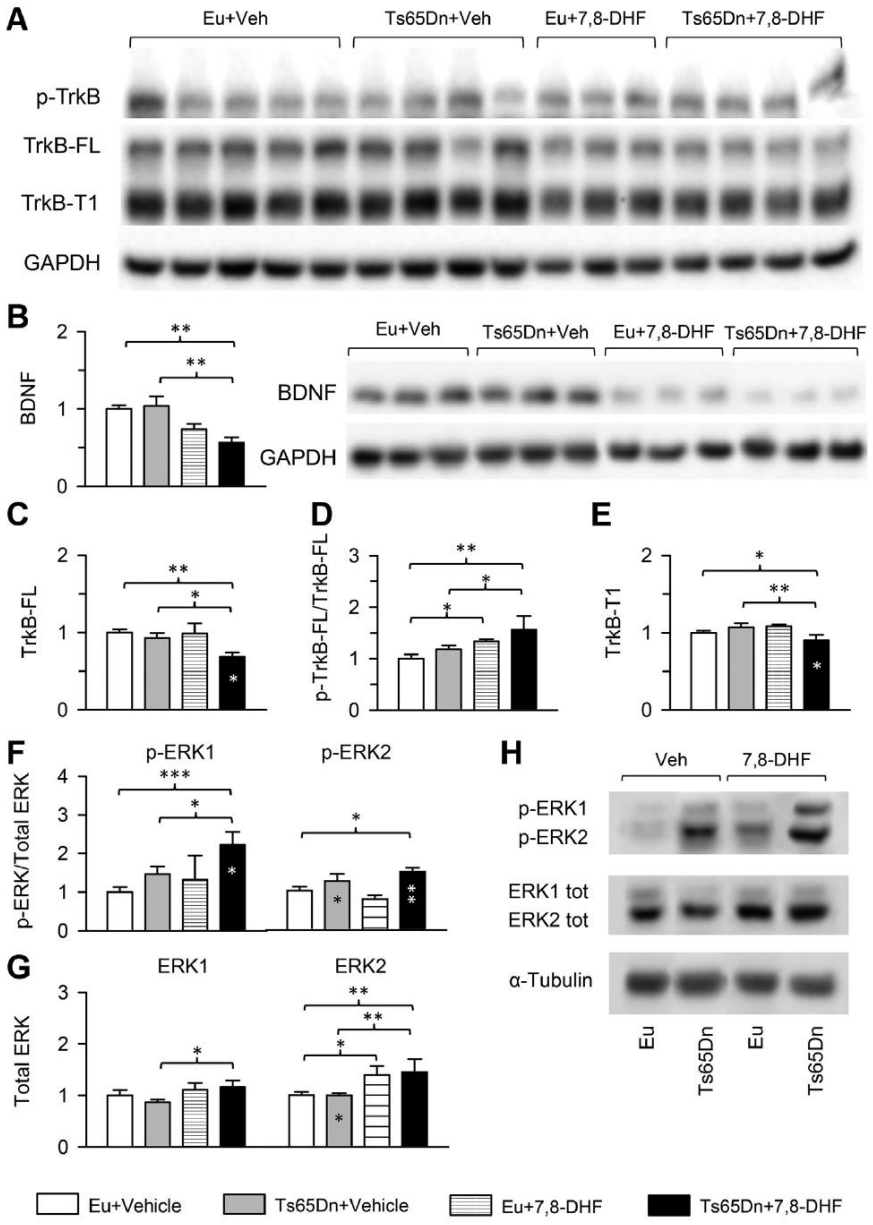

Fig. 6. Effects of neonatal treatment with 7,8-DHF on the BDNF/TrkB receptor system in the hippocampal formation of P15 Ts65Dn and euploid mice.

Western blot analysis of the BDNF/TrkB receptor system in the hippocampal formation of P15 Ts65Dn and euploid mice that received either vehicle or 7,8-DHF in the postnatal period P3-P15. **A:** representative western blots showing immunoreactivity for the phosphorylated TrkB receptor (p-TrkB-FL), the full length TrkB receptor (TrkB-FL), the truncated TrkB receptor (TrkB-T1),

and the housekeeping gene GAPDH. **B:** Levels of BDNF (untreated euploid mice: n=20; untreated Ts65Dn mice: n=21; treated euploid mice: n=5; treated Ts65Dn mice: n=6) and representative western blots showing immunoreactivity for BDNF and the housekeeping gene GAPDH. **C:** Levels of TrkB-FL (untreated euploid mice: n=19; untreated Ts65Dn mice: n=19; treated euploid mice: n=5; treated Ts65Dn mice: n=6). **D:** Levels of p-TrkB-FL (untreated euploid mice: n=15; untreated Ts65Dn mice: n=16; treated euploid mice: n=5; treated Ts65Dn mice: n=6). **E:** levels of TrkB-T1 (untreated euploid mice: n=19; untreated Ts65Dn mice: n=21; treated euploid mice: n=5; treated Ts65Dn mice: n=6). **F-H:** Western blot analysis of p-ERK1/p-ERK2 (untreated euploid mice: n=10; untreated Ts65Dn mice: n=12; treated euploid mice: n=5; treated Ts65Dn mice: n=6) (F) and total ERK1/ERK2 levels (untreated euploid mice: n=11; untreated Ts65Dn mice: n=11; treated euploid mice: n=5; treated Ts65Dn mice: n=6) (G) and representative western blots (H) showing immunoreactivity for p-ERK1, p-ERK2, ERK1, ERK2 and for the housekeeping protein α-Tubulin. Data in (B, C, E) were normalized to GAPDH; data in (G) were normalized to α-Tubulin; data in (D) were normalized to TrkB-FL, and data in (F) were normalized to total ERK1 and total ERK2, respectively. Protein levels (mean SE) are expressed as fold difference in comparison with untreated euploid mice. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ (Fisher LSD test after two-way ANOVA). Black asterisks in the gray bar indicate a difference between untreated Ts65Dn mice and treated euploid mice. White asterisks in the black bar indicate a difference between treated Ts65Dn mice and treated euploid mice. Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; Eu, euploid; Veh, vehicle.

Effect of 7,8-DHF on hippocampus-dependent learning and memory

At the age of P45 (an age approximately corresponding to adolescence), mice can be behaviorally tested with tasks that explore hippocampus-dependent learning and memory (Stagni et al., 2016). In order to establish whether the neuroanatomical effects of 7,8-DHF are functionally effective, we treated euploid and Ts65Dn mice from P3 to P45-50 and examined their behavior with the Morris Water Maze (MWM) test, a test that is classically used in trisomic mice to assess the effects of genotype and/or treatment on memory.

The learning phase of the test lasted 5 days and on day six mice were subjected to the probe test in order to evaluate spatial memory. For the learning phase, the

following variables were evaluated: escape latency, time in periphery, percentage of time in periphery, path length, proximity, and swimming speed. We carried out a three-way mixed ANOVA for all variables followed by post-hoc Fisher LSD test. Results of ANOVA are reported hereafter and results of the post-hoc test are summarized in Table 1.

A three-way mixed ANOVA on escape latency, with genotype and treatment as grouping factors and day as a repeated measure revealed no effect of genotype x treatment x day [$F(4,228) = 1.52$, $p = 0.196$]. We found a genotype x day interaction [$F(4,228) = 3.10$, $p = 0.016$], a treatment x day interaction [$F(4,228) = 2.77$, $p = 0.028$], no genotype x treatment interaction [$F(1,57) = 0.03$, $p = 0.874$], a main effect of genotype [$F(1,57) = 42.58$, $p \leq 0.001$], a main effect of treatment [$F(1,57) = 10.14$, $p = 0.002$], and a main effect of day [$F(4,228) = 21.75$, $p \leq 0.001$]. While euploid mice exhibited a fast learning improvement with time, untreated Ts65Dn mice exhibited a very scarce learning improvement and the latency to reach the platform did not decrease throughout the test (Fig. 7A, Table 1). In contrast, Ts65Dn mice treated with 7,8-DHF showed a learning improvement and, save for day 3, their performance was not statistically different from that of untreated euploid mice (Fig. 7A, Table 1). In euploid mice treated with 7,8-DHF the latency was reduced in comparison with that of untreated euploid mice (Fig. 7A), although the difference was statistically significant on day 2 only (Table 1).

A three-way mixed ANOVA on the time spent at the periphery zone (thigmotaxis), with genotype and treatment as grouping factors and day as a repeated measure revealed an effect of genotype x treatment x day [$F(4,228) = 2.88$, $p = 0.023$]. We found no genotype x day interaction [$F(4,228) = 0.99$, $p = 0.412$], a treatment x day interaction [$F(4,228) = 3.31$, $p = 0.012$], no genotype x treatment interaction [$F(1,57) = 0.001$, $p = 0.992$], a main effect of genotype [$F(1,57) = 19.63$, $p \leq 0.001$], a main effect of treatment [$F(1,57) = 8.07$, $p =$

0.006], and a main effect of day [$F(4,228) = 27.72$, $p \leq 0.001$]. A post-hoc Fisher LSD test showed that while untreated Ts65Dn mice spent more time at the periphery thigmotaxis than untreated euploid mice, Ts65Dn mice treated with 7,8-DHF spent a similar time as euploid mice (Fig. 7B, Table 1), suggesting an improvement in searching strategy. A reduction in thigmotaxis was also shown by euploid mice treated with 7,8-DHF.

A three-way mixed ANOVA on the percentage of time spent at the periphery, with genotype and treatment as grouping factors and day as a repeated measure revealed an effect of genotype x treatment x day [$F(4,228) = 3.01$, $p = 0.019$]. We found a genotype x day interaction [$F(4,228) = 2.47$, $p = 0.045$], a treatment x day interaction [$F(4,228) = 7.76$, $p \leq 0.001$], no genotype x treatment interaction [$F(1,57) = 1.48$, $p = 0.229$], a main effect of genotype [$F(1,57) = 11.71$, $p = 0.001$], a main effect of treatment [$F(1,57) = 8.04$, $p = 0.006$], and a main effect of day [$F(4,228) = 23.88$, $p \leq 0.001$]. Post-hoc Fisher LSD test showed that the time spent at the periphery by untreated Ts65Dn mice, expressed as percentage of the total latency, was similar to that of untreated euploid mice (Fig. 7C, Table 1). This means that the proportion of time spent at the periphery and outside the periphery was similar in euploid and Ts65Dn mice. Since in Ts65Dn mice the total latency to reach the platform was longer than in euploid mice, this means that Ts65Dn mice spent more time at the periphery as well as swimming outside the periphery, which implies that their longer escape latency can be attributed to both higher thigmotaxis levels and poorer spatial learning. In treated Ts65Dn mice the percentage of time in thigmotaxis underwent a reduction in comparison with their untreated counterparts (Fig. 7C, Table 1), suggesting an improvement in spatial learning.

A three-way mixed ANOVA on path length, with genotype and treatment as grouping factors and day as a repeated measure revealed no effect of genotype x treatment x day [$F(4,228) = 2.09$, $p = 0.082$]. We found a genotype x day

interaction [$F(4,228) = 7.80$, $p \leq 0.001$], no treatment x day interaction [$F(4,228) = 0.54$ $p = 0.707$], no genotype x treatment interaction [$F(1,57) = 0.05$, $p = 0.819$], no main effect of genotype, no main effect of treatment but a main effect of day [$F(4,228) = 43.74$, $p \leq 0.001$]. In all groups, the path length decreased from day 1 to day 5 (Fig. 7D). In untreated Ts65Dn mice, the reduction was smaller than in untreated euploid mice and on day 5 their path length was significantly larger in comparison with untreated euploid mice (Fig. 7D, Table 1). In contrast, on day 5 the path length of treated Ts65Dn mice was shorter in comparison with their untreated counterparts and equal to that of treated and untreated euploid mice, suggesting an improvement in searching strategy.

A three-way mixed ANOVA on proximity to the former platform position (Gallagher's test; proximity), with genotype and treatment as grouping factors and day as a repeated measure revealed an effect of genotype x treatment x day [$F(4,228) = 2.59$, $p = 0.038$]. We found a genotype x day interaction [$F(4,228) = 3.93$, $p = 0.004$], a treatment x day interaction [$F(4,228) = 4.79$, $p \leq 0.001$], no genotype x treatment interaction [$F(1,57) = 1.12$, $p = 0.295$], a main effect of genotype [$F(1,57) = 9.66$, $p = 0.003$], a main effect of treatment [$F(1,57) = 12.91$, $p = 0.001$], and a main effect of day [$F(4,228) = 13.39$, $p \leq 0.001$]. Fig. 7E shows that while in untreated euploid mice the proximity to the platform position increased from day 1 to day 5, untreated Ts65Dn mice underwent no improvement. In contrast treated Ts65Dn mice underwent an improvement and on day 5 their proximity was significantly larger than their untreated counterparts and similar to that of untreated and treated euploid mice (Fig. 7E, Table 1).

A three-way mixed ANOVA on swimming speed, with genotype and treatment as grouping factors and day as a repeated measure revealed an effect of genotype x treatment x day [$F(4,228) = 3.20$, $p = 0.014$]. We found no genotype x day interaction [$F(4,228) = 0.71$, $p = 0.584$] , no treatment x day interaction [$F(4,228) = 1.98$, $p = 0.098$], no genotype x treatment interaction [$F(1,57) = 0.09$, $p =$

0.760], a main effect of genotype [$F(1,57) = 5.27$, $p = 0.025$], no main effect of treatment, but a main effect of day [$F(4,228) = 20.05$, $p \leq 0.001$]. A post-hoc Fisher LSD test showed that in untreated Ts65Dn mice the swimming speed was similar to that of untreated euploid mice and treated euploid and Ts65Dn mice throughout the learning phase (Fig. 7F, Table 1), suggesting that their longer escape latency was not due to speed reduction. Treated Ts65Dn mice had a reduced speed in comparison with untreated euploid mice on day 1, 2, and 3 but similar to that of euploid mice in days 4 and 5, suggesting that their reduced escape latency was not due to an improvement in swimming speed. Treated euploid mice had a reduced speed in comparison with untreated euploid mice on day 1, but a similar speed on days 2-5 (Fig. 7F, Table 1).

In the probe test, we considered the following parameters as an index of spatial memory: i) latency to enter the former platform zone (latency), ii) frequency of entrances in the former quadrant (frequency), iii) proximity to the former platform position (Gallagher's test; proximity), iv) percentage of time spent at the periphery (thigmotaxis); v) swimming speed; vi) percentage of time spent in each quadrant. A two-way ANOVA on the latency showed no genotype x treatment interaction [$F(1,57) = 0.87$, $p = 0.356$], but a main effect of genotype [$F(1,57) = 10.24$, $p = 0.002$] and a main effect of treatment [$F(1,57) = 4.60$, $p = 0.036$]. Post-hoc Fisher LSD test showed that untreated Ts65Dn mice exhibited a larger latency than euploid mice and that treatment caused a notable reduction in their latency that became similar to that of untreated euploid mice (Fig. 8A). A two-way ANOVA on the frequency showed no genotype x treatment interaction [$F(1,57) = 0.001$, $p = 0.992$], but a main effect of genotype [$F(1,57) = 10.06$, $p = 0.002$] and a main effect of treatment [$F(1,57) = 7.46$, $p = 0.008$]. Post-hoc Fisher LSD test showed that untreated Ts65Dn mice exhibited a reduced frequency of entrances than euploid mice, although the difference was only marginally significant. In treated Ts65Dn mice there was a notable increase in

the frequency that became similar to that of untreated euploid mice (Fig. 8B), although this effects was only marginally significant (Supplementary Table 7). A large increase in the frequency of entrances took place in treated euploid mice (Fig. 8B). This effect is in line with the reduction in the percentage of time they spent at the periphery (Fig. 8F). A two-way ANOVA on the proximity showed no genotype x treatment interaction [$F(1,57) = 1.60$, $p = 0.211$], but a main effect of genotype [$F(1,57) = 4.81$, $p = 0.032$] and a main effect of treatment [$F(1,57) = 7.05$, $p = 0.010$]. Post-hoc Fisher LSD test showed that untreated Ts65Dn mice swam at a larger distance from the former platform zone in comparison with untreated euploid mice (Fig. 8C). Treated Ts65Dn mice swam closer to the former platform zone and their performance became similar to that of untreated euploid mice (Fig. 8C).

A two-way ANOVA on the percentage of time spent at the periphery showed no genotype x treatment interaction [$F(1,57) = 0.62$, $p = 0.436$], no main effect of genotype but a main effect of treatment [$F(1,57) = 12.03$, $p = 0.001$]. Post-hoc Fisher LSD test showed that in untreated Ts65Dn mice the percentage of time spent at the periphery was similar to that of untreated euploid mice (Fig. 8D). This indicates that Ts65Dn mice spent the same proportion of time in and outside the periphery as euploid mice and that their longer escape latency (Fig. 8A) can be attributed both to higher thigmotaxis levels and poorer spatial memory. In treated Ts65Dn mice the percentage of time spent in the periphery was reduced in comparison with their untreated counterparts mice (Fig. 8D), suggesting that improvement in thigmotaxis contributes to the shorter latency to reach the former platform zone. A reduction in the percentage of time in the periphery was also exhibited by treated vs. untreated euploid mice (Fig. 8D).

A two-way ANOVA on the swimming speed showed no genotype x treatment interaction [$F(1,57) = 0.44$, $p = 0.511$], no main effect of genotype and no main

effect of treatment and post-hoc Fisher LSD test showed no differences between groups (Fig. 8E).

A one-way ANOVA on the percentage of time spent in each quadrant for each experimental group showed a significant effect of quadrant in untreated euploid mice [$F(3,60) = 4.99$, $p = 0.004$], treated euploid mice [$F(3,60) = 12.20$, $p \leq 0.001$], and treated Ts65Dn mice [$F(3,56) = 4.15$, $p = 0.010$], but no effect of quadrant in untreated Ts65Dn mice. Post-hoc Fisher LSD test showed that untreated Ts65Dn mice exhibited no differences in the time spent in the former platform quadrant in comparison with the other quadrants (Fig. 8F). In contrast, treated Ts65Dn mice spent more time in the former platform quadrant, similarly to untreated and treated euploid mice (Fig.8F), suggesting a positive impact of treatment on spatial memory.

Taken together, these results are in agreement with a number of studies showing that Ts65Dn mice are impaired in spatial learning and memory. In treated Ts65Dn mice, the parameters of the learning phase tended to ameliorate day by day, although not to a significant level, but at day 5 the performance of Ts65Dn mice underwent a significant improvement in comparison with their untreated counterparts and was similar to that of untreated euploid mice. Importantly, in the probe test the behavior of treated Ts65Dn mice was similar to that of untreated euploid mice suggesting memory restoration.

Table 2. Learning phase of the Morris Water Maze. p values of the Fisher LSD test for the indicated variables.

		Latency					Time periphery (sec)					Time periphery (%)				
		D1	D2	D3	D4	D5	D1	D2	D3	D4	D5	D1	D2	D3	D4	D5
Eu + Veh	Ts + Veh	0.054	**0.041**	**0.001**	**0.006**	**< 0.001**	0.081	0.140	**0.028**	**0.011**	**0.001**	0.258	0.472	0.242	0.123	0.094
	Eu + 7,8-DHF	0.415	**0.013**	0.083	0.068	0.171	0.837	**0.002**	**0.041**	0.152	0.177	0.784	**< 0.001**	**0.007**	**0.013**	**0.009**
	Ts + 7,8-DHF	0.127	0.162	**0.045**	0.078	0.412	0.131	0.215	0.532	0.273	0.939	0.152	0.189	0.799	0.983	0.634
Ts + Veh	Eu + 7,8-DHF	0.246	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.120	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.384	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	Ts + 7,8-DHF	0.665	0.491	0.181	0.284	**0.002**	0.787	0.796	0.111	0.133	**0.001**	0.783	0.569	0.363	0.124	**0.036**
Eu + 7,8-DHF	Ts + 7,8-DHF	0.461	**< 0.001**	**< 0.001**	**0.001**	**0.033**	0.190	**< 0.001**	**0.009**	**0.014**	0.210	0.243	**< 0.001**	**0.004**	**0.016**	**0.034**

		Path length					Proximity					Swim speed				
		D1	D2	D3	D4	D5	D1	D2	D3	D4	D5	D1	D2	D3	D4	D5
Eu + Veh	Ts + Veh	**0.022**	0.473	0.129	0.449	**0.028**	0.382	0.620	0.325	**0.050**	**0.012**	0.055	0.297	0.474	0.402	0.140
	Eu + 7,8-DHF	0.269	0.103	0.905	0.111	0.660	0.180	**0.003**	**0.006**	**0.027**	**0.017**	**0.010**	0.514	0.776	0.344	0.705
	Ts + 7,8-DHF	**0.020**	0.342	0.413	0.712	0.685	0.293	0.719	0.877	0.557	0.245	**0.003**	**0.003**	**0.006**	0.056	0.051
Ts + Veh	Eu + 7,8-DHF	0.208	0.383	0.103	**0.024**	**0.010**	0.669	**0.014**	**< 0.001**	**< 0.001**	**< 0.001**	0.550	0.677	0.328	0.939	0.263
	Ts + 7,8-DHF	0.996	0.830	0.475	0.697	**0.011**	0.874	0.404	0.411	0.162	**0.001**	0.299	**0.048**	**0.042**	0.296	0.652
Eu + 7,8-DHF	Ts + 7,8-DHF	0.198	0.504	0.350	0.054	0.978	0.786	**0.001**	**0.005**	**0.009**	0.215	0.639	**0.015**	**0.003**	0.315	0.111

The numbers in bold correspond to statistically significant differences. Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; D, day; Eu, euploid; sec, seconds; Ts, Ts65Dn; Veh, vehicle

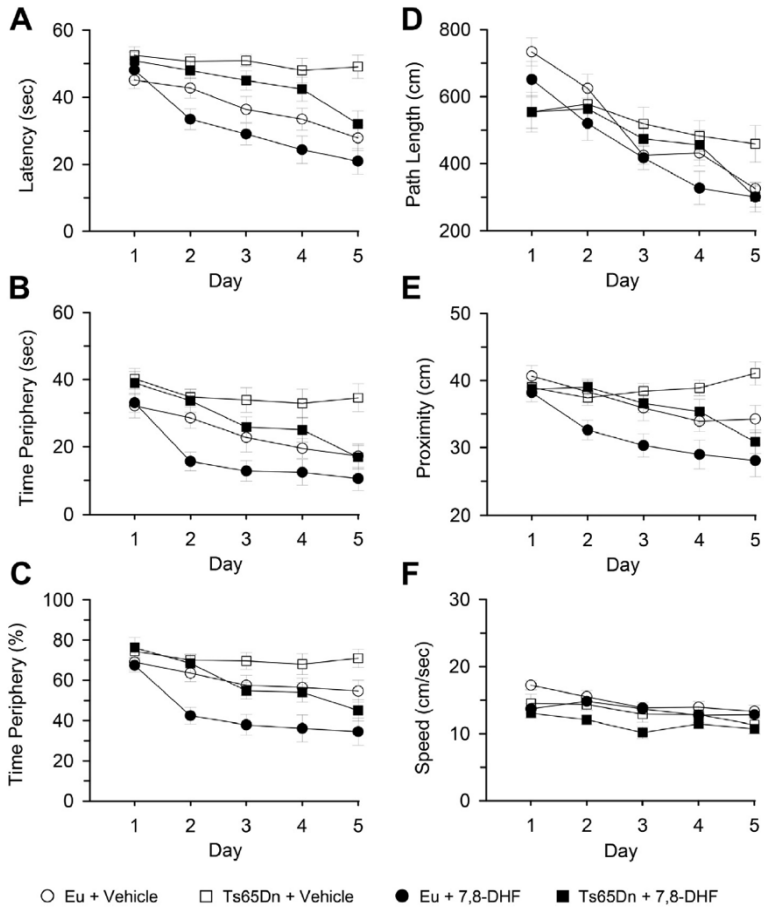

Fig. 7. Effect of treatment with 7,8-DHF on spatial learning in Ts65Dn and euploid mice. Mice received either vehicle or 7,8-DHF in the period P3-P45-50 and were behaviorally tested with the MWM starting from 6 days before reaching 45-50 days of age (untreated euploid mice: n=16; untreated Ts65Dn mice: n=14; treated euploid mice: n=16; treated Ts65Dn mice: n=15). The curves in (A-F) report data of euploid mice that received either vehicle (empty circle) or 7,8-DHF (filled circle) and Ts65Dn mice that received either vehicle (empty square) or 7,8-DHF (filled square). **A-E:** Learning phase of the MWM evaluated as latency to reach the platform (A), time spent at the periphery (thigmotaxis) (B), percentage of time spent at the periphery (C), path length (D), and proximity to the platform zone (E). **F:** Swimming speed. **B-D:** Values represent mean SE. Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; Eu, euploid; sec, seconds.

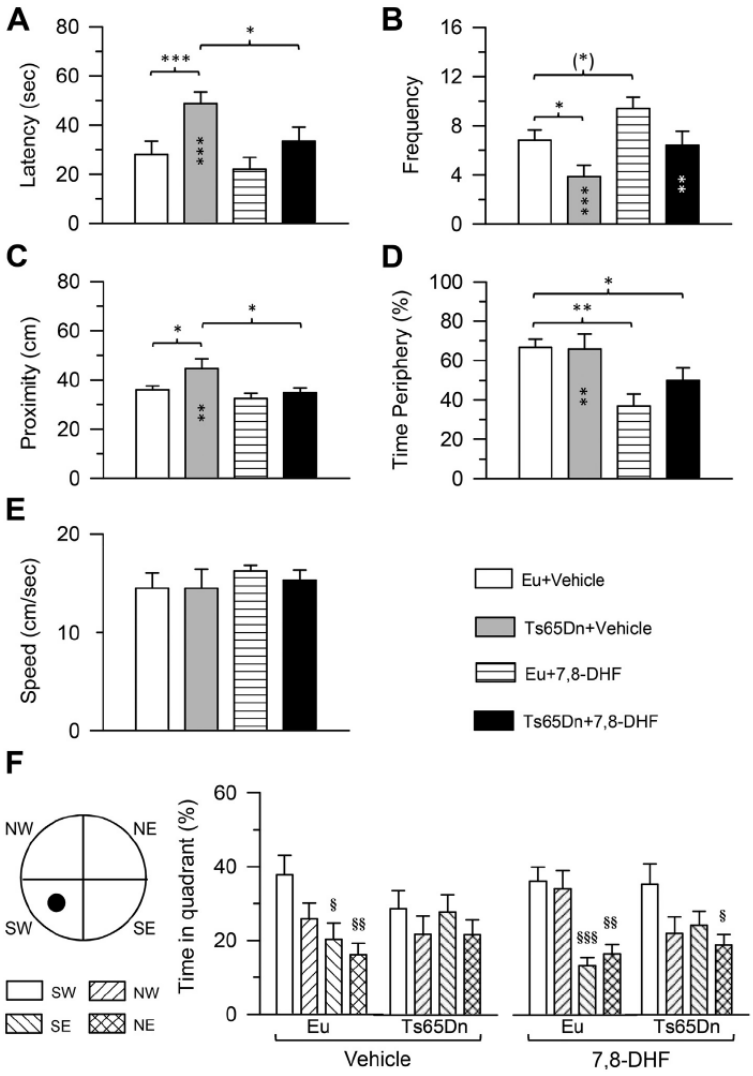

Fig. 8. Effect of treatment with 7,8-DHF on spatial memory in Ts65Dn and euploid mice. Spatial memory was assessed in the probe test after spatial learning (same mice as in Fig. 7). In the probe test, memory was assessed as latency to reach the former platform zone (A), number of crossings (frequency) over the former platform quadrant (B), proximity to the former platform zone (C), percentage of time spent at the periphery (D), percentage of time spent in quadrants (F). E: Swimming speed during the probe test. Values represent mean SE. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; (Fisher LSD test after ANOVA). Black asterisks in the gray bar indicate a difference between untreated Ts65Dn mice and treated euploid mice; white asterisks in the black bar indicate a difference between treated Ts65Dn mice and treated euploid mice. The symbol §

in (F) indicates a difference in comparison with the former platform quadrant. § $p \leq 0.05$; §§ $p \leq 0.01$; §§§ $p \leq 0.001$; (Fisher LSD test after One Way ANOVA). Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; Eu, euploid; sec, seconds.

DISCUSSION

This study shows that treatment with a BDNF mimetic restores hippocampal neurogenesis, dendritic spine density and largely improves behavior. It must be observed that the Ts65Dn mouse is trisomic for at least 55% of HSA21 orthologous protein-coding genes, but it lacks the remaining ~45%. Moreover, it bears 50 protein-coding genes that are not orthologs for HSA21 genes, a segment that is an artifact of the method used in its construction (Duchon et al., 2011; Reinholdt et al., 2011). Although the Ts65Dn mouse shows genetic limitations, it is still the most popular choice among DS models because it recapitulates many aspects of the human condition, including cytoarchitectural abnormalities in many brain regions and deficits in learning and memory (see (Bartesaghi et al., 2011; Rueda et al., 2012; Gardiner, 2015)). Moreover, the Ts65Dn mouse is the only DS model that has been used in preclinical evaluation of drugs for learning and memory (see (Gardiner, 2015)). Unfortunately, a perfect mouse model of DS does not presently exist although intense efforts are currently underway (Antonarakis, 2017). We hope that current results in the Ts65Dn mouse model may prompt further studies in other models of DS.

Treatment with the BDNF mimetic 7,8-DHF positively impacts the major defects of hippocampal development in Ts65Dn mice

At variance with adult Ts65Dn mice (Bimonte-Nelson et al., 2003; Fukuda et al., 2010; Begenisic et al., 2015), we found here no reduction in BDNF protein levels in the hippocampus of P15 Ts65Dn mice, suggesting that BDNF expression may be developmentally regulated. Although neonate Ts65Dn mice exhibited similar

BDNF levels as those of euploid mice, treatment with the BDNF mimetic 7,8-DHF resulted in the recovery of the major trisomy-linked developmental defects, i.e. neurogenesis reduction and dendritic pathology, which is in line with the key role played by BDNF in brain development.

In particular, we found that treatment with 7,8-DHF increased the number of dividing cells in the SGZ of Ts65Dn mice. While in untreated Ts65Dn mice the number of proliferating cells was -30% in comparison with untreated euploid mice, in treated Ts65Dn mice their number became -13% (see Fig. 3C), indicating that, although 7,8-DHF does not fully rescue NPC proliferation, it causes a large improvement. It is of interest to observe that in cultures of NPCs 7,8-DHF failed to increase cell proliferation (Fig. 1B), although it induced a robust effect on differentiation and maturation. This suggests that 7,8-DHF does not directly induce pro-proliferative signals in NPCs but that its pro-proliferative effects require the presence of other elements of the neurogenic niche (non-cell autonomous effect). Importantly, although the number of dividing cells in the SGZ of Ts65Dn mice was not fully rescued, total granule cell number was fully restored. This result may be explained by an effect of treatment on the process of phenotype acquisition, with a shift in the relative number of cells destined to become neurons. This conclusion is in line with the observation that in cultures of trisomic NPCs treatment largely increased the number of trisomic cells that differentiated into neurons (Fig. 1C).

We additionally found that 7,8-DHF favors the process of neurite elongation in trisomic neurons of both the SVZ and SGZ, indicating a positive impact of treatment on the process of neuron maturation. The granule neurons of Ts65Dn mice aged 15 days exhibited spine density reduction, indicating impairment in the process of spinogenesis from the earliest phases of hippocampal development. As each dendritic spine receives at least one excitatory input, a reduction in the number of spines of granule neurons implies a reduction in the

number of excitatory terminals and, consequently, reduced complexity of hippocampal circuitry. Evaluation of the levels of the presynaptic protein SYN in Ts65Dn mice showed that the counterpart of the spine density reduction was a reduction in SYN levels. Treatment with 7,8-DHF fully restored the reduced number of dendritic spines of Ts65Dn mice as well as SYN levels, suggesting a positive impact on the overall connectivity of the DG.

Conflicting results are reported in the literature regarding the pro-proliferative effect of the BDNF/TrkB system in different species and cellular systems (Foltran and Diaz, 2016; Vilar & Mira, 2016). Many studies suggest that BDNF fosters neurogenesis and neuron maturation but not proliferation of NPCs. Our results suggest that in Ts65Dn mice activation of the TrkB receptor enhances NPCs proliferation, in addition to neurogenesis and neuron maturation. Although the effect on proliferation was less prominent than the effect on neurogenesis and neuron maturation, the outcome was restoration of the defective cellularity in the granule layer of the DG. It is of interest to observe that some of the neurogenesis-enhancing therapies attempted so far in mouse models of DS may present caveats for human use due to the risk of uncontrolled proliferation in peripheral tissues and, thus, a cancerogenic effect (Bartesaghi et al., 2011; Gardiner, 2015). The finding that 7,8-DHF, in spite of its relatively moderate pro-proliferative activity, is able to restore the final number of granule neurons may render this molecule a good candidate for therapy in DS.

Treatment with 7,8-DHF rescues hippocampus-dependent behavior in Ts65Dn mice

Hippocampus-dependent learning and memory impairment is a consistent feature of DS and the Ts65Dn mouse model (Demas et al., 1996; Carlesimo et al., 1997; Vicari et al., 2000; Belichenko et al., 2007; Salehi et al., 2009). This defect is attributable to hippocampal hypocellularity, altered neuronal maturation and

altered connectivity. The granule cells of the DG are the first element of the hippocampal trisynaptic circuit, a circuit whose function is fundamental for long-term memory. The dendrites of the granule cells receive their major input from the entorhinal cortex that represents an interface between the hippocampal formation and the rest of the brain. Signals from polymodal association cortices sent by the entorhinal cortex to the DG are processed by the trisynaptic circuit and then sent back to the entorhinal cortex. We found here that in Ts65Dn mice hippocampus-dependent learning and memory were rescued after treatment with 7,8-DHF, indicating that the effects of treatment on the hippocampal defects that characterize the trisomic condition translate into a behavioral benefit. It remains to be established whether after treatment cessation these effects are retained at further life stages.

Activation of the TrkB receptor by 7,8-DHF enhances the activity of TrkB receptor-dependent signaling

In the hippocampus of P15 Ts65Dn mice we found normal levels of BDNF and of TrkB-FL, and TrkB-T1 receptors. Results showed reduced levels of BDNF and TrkB-FL receptor in Ts65Dn mice after thirteen days of treatment with 7,8-DHF, suggesting a compensatory reduction of their transcription and/or an increase in their degradation. There is evidence that treatment with BDNF or the BDNF mimetic 7,8-DHF elicits TrkB receptor ubiquitination and degradation (Liu et al., 2016). This mechanism may account for the reduction in the protein levels of the TrkB receptor observed here in treated Ts65Dn mice. The absence of a similar reduction in treated euploid mice suggests that the mechanisms underlying degradation of the TrkB receptor may be more powerful in the trisomic brain. It must be noted that, although treatment induced an overall reduction in TrkB receptor levels, its phosphorylation increased, indicating that treatment enhances TrkB-dependent signaling.

The cellular effects of the BDNF/TrkB system are mediated by three major pathways, among which the RAS/MEK/ERK pathway appears to be involved in key developmental processes such as differentiation and survival (Arevalo & Wu, 2006). We found that in treated Ts65Dn mice there was an increase in the levels of p-ERK1 and p-ERK2, which is consistent with the treatment-induced phosphorylation increase of the TrkB receptor. ERK activity is required for cell proliferation (Lefloch et al., 2008), and there is evidence that the BDNF/TrkB signaling-induced increase in spine density of hippocampal pyramidal neurons requires ERK1/2 activation (Alonso et al., 2004). This evidence strongly suggests that the increased activity of ERK1/2 following treatment with 7,8-DHF may represent a key contributor to the rescue of the key processes of hippocampal development in Ts65Dn mice.

Although much is now known regarding the role of ERK1/2, the mechanisms underlying their expression still need to be elucidated (Busca et al., 2016). We found here that treatment with 7,8-DHF increased both ERK1 and ERK2 levels. A recent study shows that the ratio between total ERK1 and ERK2 protein levels in different mouse brain regions is about 1:4, that the same ratio holds for p-ERK1/2 and that derangement of these ratios has adverse effects on the brain (Lefloch et al., 2008). Importantly, in Ts65Dn mice, treatment caused an increase in ERK1/2 and p-ERK1/2 but their ratios remained similar to those of their untreated counterparts. This indicates that treatment enhances the activity of ERK1/2 without disrupting the important balance between the two ERK isoforms.

Treatment with 7,8-DHF has no adverse effects on viability and growth of Ts65Dn mice

Previous evidence showed that chronic treatment with 7,8-DHF has no toxic effects (Liu et al., 2016). In agreement with this evidence, we found no effect of

treatment on mice viability. There is evidence that in rodents treatment with BDNF causes a reduction in food intake and that activation of muscular TrkB by 7,8-DHF regulates energy metabolism in muscles (Gray et al., 2006; Chan et al., 2015). Conversely, rodent models with a reduction in BDNF/TrkB signaling exhibit hyperphagia and obesity (Gray et al., 2006). We found that a relatively short treatment with 7,8-DHF (13 days: from P3 to P15) as well as a more prolonged treatment (42-47 days: from P3 to P45-50) did not cause a body weight reduction in Ts65Dn mice. In addition, we did not found an adverse effect of treatment on the brain weight of Ts65Dn mice, but rather, a positive effect on brain growth. From these findings it appears that a chronic treatment with 7,8-DHF has a safe profile on the general health of Ts65Dn mice.

Conclusions

7,8-DHF is a molecule that binds to the TrkB receptor and causes its dimerization and autophosphorylation, thereby mimicking the actions of BDNF. This binding replicates many actions of BDNF such as those on neurogenesis, neuron survival, learning and memory, and synaptogenesis. In view of these effects the use of 7,8-DHF in models of various brain disorders results in therapeutic efficacy (Liu et al., 2016). Our study provides novel evidence that treatment with 7,8-DHF during the neonatal period restores the major trisomy-linked neurodevelopmental defects in the hippocampus of a mouse model of DS.

Neurogenesis in the hippocampal formation, one of the most important brain regions involved in pattern separation/completion and memory formation (Rolls, 2016), is severely disrupted in fetuses with DS (Contestabile et al., 2007; Guidi et al., 2008). Neurogenesis impairment is likely to account for hippocampal hypotrophy and for impairment of hippocampus-dependent memory functions in children with DS (Vicari et al., 2000). Reduced BDNF levels have been reported in the brains of fetuses with DS (Guedj et al., 2009; Toiber et al., 2010). In view

of the crucial role played by this neurotrophin in key developmental processes (neurogenesis and neuron maturation), it is likely that impairment of BDNF/TrkB receptor signaling plays a significant role in the neurodevelopmental alterations that characterize DS.

The current results suggest that therapy with the BDNF mimetic 7,8-DHF may represent a possible strategy for improving brain development and memory in children, and possibly in adults, with DS. In spite of the intrinsic limitations of mouse models, our work suggests that it is possible to restore trisomy-linked developmental deficits by pharmacologically targeting the TrkB receptor with a naturally occurring flavonoid. As pointed out in the Introduction, the problem of pharmacological interventions is that they are, in most cases, Janus-faced. The positive effects of many of the drugs used so far in DS models are the "good face" of Janus but the "bad face" deals with the far-from-irrelevant issue of safety. Flavonoids are compounds naturally present in vegetables and fruits (Rendeiro et al., 2015) and exert beneficial effects on the brain in health and disease (Spencer, 2008; Williams & Spencer, 2012). Considering that treatment with 7,8-DHF has no toxic effects in wild type mice (Liu et al., 2013) and in Ts65Dn mice (current study), our results suggest that early treatment with 7,8-DHF may represent a therapeutic strategy alternative to other drugs and with a good translational potential for improving brain development in DS.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Chapter 6

Treatment with corn oil improves neurogenesis and cognitive performance in the Ts65Dn mouse model of Down syndrome

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ABSTRACT

Individuals with Down syndrome (DS), a genetic condition due to triplication of Chromosome 21, are characterized by intellectual disability that worsens with age. Since impairment of neurogenesis and dendritic maturation are very likely key determinants of intellectual disability in DS, interventions targeted to these defects may translate into a behavioral benefit. While most of the neurogenesis enhancers tested so far in DS mouse models may pose some caveats due to possible side effects, substances naturally present in the human diet may be regarded as therapeutic tools with a high translational impact. Linoleic acid and oleic acid are major constituents of corn oil that positively affect neurogenesis and neuron maturation. Based on these premises, the goal of the current study was to establish whether treatment with corn oil improves hippocampal neurogenesis and hippocampus-dependent memory in the Ts65Dn model of DS. Four-month-old Ts65Dn and euploid mice were treated with saline or corn oil for 30 days. Evaluation of behavior at the end of treatment showed that Ts65Dn mice treated with corn oil underwent a large improvement in hippocampus-dependent learning and memory. Evaluation of neurogenesis and dendritogenesis showed that in treated Ts65Dn mice the number of new granule cells of the hippocampal dentate gyrus and their dendritic pattern became similar to those of euploid mice. In addition, treated Ts65Dn mice underwent an increase in body and brain weight. This study shows for the first time that fatty acids have a positive impact on the brain of the Ts65Dn mouse model of DS. These results suggest that a diet that is rich in fatty acids may exert beneficial effects on cognitive performance in individuals with DS without causing adverse effects.

Key Words: Down syndrome; intellectual disability; Ts65Dn model;

hippocampus; neurogenesis; dendrites; memory

INTRODUCTION

Triplication of Chromosome 21 causes Down syndrome (DS), a pathology characterized by brain hypotrophy and disability in several cognitive domains, including explicit memory. Since individuals with DS above 40 years of age are at high risk for the onset of Alzheimer's disease, intellectual disability may transform into dementia (Hartley et al., 2015). Widespread impairment of neural precursor proliferation starting from fetal life stages is a key hallmark of DS (Haydar and Reeves, 2012; Stagni et al., 2017a). Moreover, the DS brain is characterized by severe impairment in dendritic maturation (Bartesaghi et al., 2011; Benavides-Piccione et al., 2004). Both these defects are thought to be key determinants of cognitive impairment in DS. No effective pharmacotherapies currently exist for intellectual disability in individuals with DS. Ideally, therapies to improve brain development should be performed at early life stages. Yet, since neurons of the hippocampal dentate gyrus are continuously generated throughout life, interventions that are able to improve hippocampal neurogenesis may also have a positive impact on hippocampus-dependent learning and memory at adult life stages and could possibly delay the onset of Alzheimer's disease.

Among the various pharmacotherapies attempted in DS mouse models some of them proved to be effective in improving hippocampus-dependent learning and memory (Bartesaghi et al., 2011; Costa & Scott-McKean, 2013; Gardiner, 2015; Stagni et al., 2015). It should be noted, however, that some of the drugs used may also cause side effects, which diminishes their translational impact. For instance, lithium may impair renal function and inhibitors of $GABA_A$ receptors may have pro-convulsant effects. Ideally, the treatment of choice should be effective, safe and well tolerated. In this context, substances that are naturally present in the

human diet should be regarded as therapeutic tools with a potentially strong translational impact.

There is evidence that in addition to their role in metabolism, fatty acids can serve as signaling molecules by affecting intra- and extracellular receptor systems, either directly or after conversion to specific fatty acid derivatives (Georgiadi and Kersten, 2012). Poly- and/or mono-unsaturated fatty acids (PUFAs and MUFAs, respectively) have been implicated as critical nutritional factors for proper neural development and function (Gordon, 1997) and fatty acids appear to favor brain development and ameliorate cognitive functions in normal and diseased conditions (Hussain et al., 2013). Long chain poly-unsaturated fatty acids (LC-PUFAs), which make up 20% of the dry weight of the brain, are critical for healthy brain development and contribute to membrane structure and cytokine regulation. According to the position of the first double bond from the methyl end of the fatty acid chain, the most important PUFAs for humans can be divided into two families: n-6 (omega-6) and n-3 (omega-3) PUFAs. Linoleic acid (LA, 18:2n-6) is the parent fatty acid of omega-6 PUFAs, and produces principally arachidonic acid (AA, 20:4n-6), whereas α-linolenic acid (ALA, 18:3n-3) is the parent fatty acid of omega-3 PUFAs, and gives rise mainly to eicosapentaenoic acid (20:5n-3). LA and ALA must be supplied by food because they cannot be synthesized by the human body and for this reason they are called essential fatty acids. Essential fatty acids and their metabolites, especially n-6 PUFA-derived mediators, have 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; Maekawa et al., 2009; Sakayori et al., 2011; Tokuda et al., 2014). The observation that their deficiency alters neurogenesis (Coti Bertrand et al., 2006; Tang et al., 2016) highlights a possibly relevant role of n-6 PUFAs in the regulation of neurogenesis. The MUFA oleic acid (OA, C18:1n-9), which is the primary fatty acid in the white matter of the mammalian brain (O'Brien &

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), suggesting a role of OA in neuron maturation.

Corn oil, which is extracted from the germ of corn, contains a high percentage of both LA and OA. In view of the positive effects exerted by these fatty acids on neurogenesis and neuron maturation, the goal of the current study was to establish whether treatment with corn oil improves neurogenesis and neuron maturation in the hippocampal dentate gyrus of the Ts65Dn mouse model of DS and whether these effects are associated with a behavioral improvement.

MATERIALS AND METHODS

Mouse colony

In order to obtain Ts65Dn mice, B6EiC3Sn a/ATs(17<16>)65Dn females were mated with C57BL/6JEiJ x C3H/HeSnJ (B6EiC3Sn) F1 hybrid males provided by Jackson Laboratories (Bar Harbor, ME, USA). We used the first generation of this breeding. The genotyping of the animals was carried out as previously described (Reinholdt et al., 2011). The day of birth was designated postnatal day zero. The mice were kept in a room with a 12:12 h light/dark cycle and had free access to water and food. All efforts were made to minimize animal suffering and to keep the number of animals used to a minimum.

Experimental protocol

A total of 47 male mice aged 4 months were used. Mice were i.p. injected every other day for one month with i) saline (0.9% NaCl; n=17 euploid and n=8 Ts65Dn mice) or ii) corn oil (Sigma: C8267; 10 µl/g; n=12 euploid and n=10 Ts65Dn mice). At the end of treatment, mice were behaviorally tested with the Morris

Water Maze and Contextual Fear Conditioning tests. Because C3H/HeSnJ mice carry a recessive mutation that leads to retinal degeneration (*Rd*), animals were genotyped by standard PCR to screen out mice carrying this gene. Mice that did not carry a recessive mutation that leads to retinal degeneration entered the behavioral study. At the end of behavioral testing mice were killed, the brain was removed, fixed by immersion in PFA 4% and frozen. Mice injected with saline will be called hereafter control mice and mice injected with corn oil will be called treated mice.

Histological procedures

The frozen 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; 0.02% sodium azide; PBS1X to volume).

Immunohistochemistry

For doublecortin (DCX) immunohistochemistry, one out of six free-floating sections from the hippocampal formation (n=10 sections) were permealized with 0.4% Triton X-100 in KPBS and blocked for 2 h in 10% donkey serum in 0.4% Triton X-100 and KPBS. Sections were then incubated overnight at 4°C with a goat polyclonal anti-DCX antibody (Santa Cruz Biotechnology Cat# sc-8066, RRID: AB_2088494) diluted 1:100. Detection was performed with FITC-conjugated anti-goat secondary antibody (Abcam Cat# ab6881, RRID: AB_955236) diluted 1:200.

Measurements

Number of DCX-positive cells

Quantification of DCX-positive cells in the dentate gyrus was conducted in every 6th section using a fluorescence microscope (Nikon Eclipse TE 2000-S inverted

microscope; Nikon Corp., Kawasaki, Japan; objective: x 20, 0.50 NA; final magnification: x 200), equipped with a Nikon digital camera DS 2MBWc. DCX-positive cells were counted along the whole length of the granule cell layer and their number was expressed as number of cells for 100 µm of linear length.

Neuron sampling

Series of sections (n=10) across the dentate gyrus were used for reconstruction of the dendritic tree of DCX-positive neurons. DCX-positive neurons were sampled in the upper blade. Only neurons with branches extending beyond the outer one half of the molecular layer were selected. The total number of sampled neurons was 4-7 per animal.

Measurement of the dendritic tree

The dendritic tree of DCX-positive cells was traced as previously described (Guidi et al., 2013). 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. For each neuron, we evaluated total dendritic length, total number of branches, number of branches of each order and mean branch length.

Behavioral testing

Morris Water Maze (MWM)

The MWM test was used in order to examine hippocampus-dependent learning and memory. Mice were maintained in a room with reverse light/dark cycle. Animals were tested starting at 08.30-09.00am and before being tested were put in the behavior room for 1 h of habituation. Mice were trained in the MWM task to locate a hidden escape platform in a circular pool. The apparatus consisted of a large circular water tank (1.00 m diameter, 50 cm height) with a transparent round escape platform (10 cm^2). The pool was virtually divided into four equal quadrants identified as Northeast, Northwest, Southeast, and Southwest. The

pool was filled with tap water at a temperature of 20-22ºC up to 0.5 cm above the top of the platform and the water was made opaque with milk. The platform was placed in the pool in a fixed position (in the middle of the Southwest quadrant). The pool was placed in a large room with intra- (squares, triangles, circles and stars) and extra-maze visual cues. A video camera was placed above the center of the pool and connected to a videotracking system (ANY-maze Behavioral tracking software 5.0, Wheat Lane Wood Dale, IL, U.S.A.). The MWM test was organized as follows. Days 1-8: learning sessions; day 9: probe test. During the learning phase mice were subjected to 4 trials on day one and to two blocks of 4 trials separated by an interval of 45 minutes on days 2-8. Mice were released facing the wall of the pool randomly from the North, East, South, or West starting point and allowed to search for up to 60 s for the platform. If they reached the platform within this time they were left on the platform for 15 s, then they were returned to the home cage and tested again after 10 s. If mice did not reach the platform they were gently put on the platform, left there for 15 s and then returned to the home cage and tested again after 10 s. For the learning phase, we evaluated the latency to find the hidden platform, time in the periphery of the water tank (thigmotactic behavior), percentage of time in the periphery, path length, proximity to the platform, and swimming speed. Retention was assessed with one trial (probe trial), on the ninth day, 24 h after the last acquisition trial, using the same starting point for all mice. During this trial, the platform was removed from the tank. Mice were allowed to search for up to 60 s for the platform. For the probe trial, the latency of the first entrance in the trained platform zone, the frequency of entrances in the trained quadrant, the proximity to the trained platform position (Gallagher's test), the percentage of time spent at the periphery (thigmotaxis), the swimming speed and the percentage of time spent in each quadrant were evaluated. All experimental sessions were carried out between

8.30am and 5.00pm.

Contextual Fear Conditioning (CFC)

CFC was performed the day after the MWM. The test occurred in 30 x 24 x 21 cm operant chambers (Ugo Basile, Comerio VA, Italy). Each chamber was equipped with a stainless-steel rod floor through which a footshock could be administered, two stimulus lights, one house light, and a solenoid, all controlled by ANY-maze computer software (Behavioral tracking software 5.0, Wheat Lane Wood Dale, IL, U.S.A.). The chambers were located in a sound-isolated enclosure in the presence of red light. Mice were trained and tested on 2 consecutive days (Comery et al., 2005). The training procedure consisted of placing a subject in a chamber and allowing exploration for 2 min. An auditory cue [74 dB, 2000 Hz clicking via the solenoid; conditioned stimulus (CS)] was presented for 15 s. A 2 s footshock [0.6 mAmp; unconditioned stimulus (US)] was administered for the final 2 s of the CS. The entire procedure was repeated three times and mice were removed from the chamber 30 s later. Twenty hours after training, mice were returned to the same chamber in which training occurred (context), and freezing behavior was recorded by the experimenter using time sampling (10 s intervals). Freezing was defined as lack of movement except that required for respiration. At the end of the 5 min context test, mice were returned to their home cage. Approximately 1 h later, freezing was recorded in a novel environment and in response to the cue. The novel environment consisted of modifications including an opaque Plexiglas divider diagonally bisecting the chamber, a Plexiglas floor, and decreased illumination. Mice were placed in the novel environment, and time sampling was used to score freezing for 3 min. The auditory cue (CS) was then presented for 3 min, and freezing was again scored. All phases of the test were recorded and immobility was detected by using the video tracking and analysis software ANY-maze (Behavioral tracking software 5.0, Wheat Lane Wood Dale, IL, U.S.A.). Freezing scores for each subject were

expressed as a percentage for each portion of the test. Memory for the context (contextual memory) for each subject was obtained by subtracting the freezing percentage in the novel environment from that in the context.

Cultures of subventricular zone neural progenitor cells (NPCs)

Cells were isolated from the subventricular zone (SVZ) of newborn (age: 1-2 days) euploid and Ts65Dn mice. Briefly, brains were removed, SVZ regions were isolated and individually collected in ice-cold PIPES buffer pH 7.4. After centrifugation, tissue was digested for 10 min at 37°C using Trypsin/EDTA 0.25% (Life Technologies) aided by gentle mechanical dissociation. Cell suspension from each individual mouse was plated onto 25 cm^2 cell-culture flask (Thermo Fisher Scientific) and cultured as floating neurospheres in medium containing basic fibroblast growth factor (bFGF, 10 ng/ml; Peprotech) and epidermal growth factor (EGF, 20 ng/ml; Peprotech) using an established protocol (Meneghini et al., 2014). Primary (Passage 1, P1) neurospheres were dissociated using StemproAccutase (Life Technologies) after 7 days *in vitro* (DIV), thereafter neurospheres were passaged every 5 DIV. For proliferation studies, neurospheres (P3-P12) were dissociated in a single cell suspension and plated onto Nunclon™ Delta Surface 96-well plate (Thermo Fisher Scientific) at a density of 4×10^3 cells per well in DMEM/F-12 medium supplemented with B27, Glutamax™, heparin sodium salt (4 µg/ml; ACROS Organics), bFGF (10 ng/ml), and 100 U/100 µg/ml Penicillin/Streptomycin (Life Technologies). NPC cultures were treated for 96 h with LA-BSA complex (0.1-100 µM, Sigma-Aldrich), OA-BSA complex (0.1-100 µM, Sigma-Aldrich) and corresponding vehicle (BSA 3.3 mg/ml, Sigma-Aldrich). For antagonistic experiments, cells were treated with LA-BSA complex (100 µM) in presence of either GSK0660, a PPARβ/δ antagonist (0.1-10 µM, MCE), GW9662, a PPARγ antagonist (0.01-30 µM, MCE) and corresponding vehicle (DMSO 0.05 %- BSA 3.3 mg/ml). Lithium

chloride (LiCl, 2mM, Sigma-Aldrich) was used as positive control (Trazzi et al., 2014). Cell proliferation was quantified as relative luminescence units (RLU) values using CellTiter-Glo viability assay reagent (Promega) on a Victor^3-V plate reader (PerkinElmer).

Statistical analysis

Results are presented as mean ± standard error of the mean (SE). Data were analyzed with 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. Since the *in vitro* data were not normally distributed, statistical analysis was carried out using Kruskal-Wallis test followed by Mann–Whitney U test for comparisons between different doses of either LA or OA and vehicle, between LiCl and vehicle and different concentrations of the PPARβ/δ or the PPARγ antagonist and vehicle. A comparison between LA, OA, and LiCl was also carried out. Statistical analysis of the *in vivo* data was carried out using a two-way ANOVA with genotype (euploid, Ts65Dn) and treatment (saline, corn oil) as factors. *Post hoc* multiple comparisons were carried out using the Fisher Least Significant Difference (LSD) test. For the learning phase of MWM, statistical testing was performed using a three-way mixed ANOVA, with genotype and treatment as grouping factors and days as a repeated measure. For the probe test, save for the percentage of time spent in quadrants, we used a two-way ANOVA with genotype and treatment as factors followed by the Fisher LSD *post hoc* test. For the time spent in quadrants, we compared the percentage of time spent in the Northwest, Southeast, and Northeast quadrants with the percentage of time spent in the trained platform quadrant (Southwest) using a paired-sample t-test. Based on the "Box plot" tool available in SPSS Descriptive Statistics we excluded from each analysis the extremes, i.e. values that were larger than 3 times the IQ range [$x \geq$

Q3 + 3 * (IQ); x ≤ Q1 – 3 * (IQ)]. A probability level of $p \leq 0.05$ was considered to be statistically significant.

RESULTS

Effect of corn oil on body and brain weight

A two-way ANOVA on body weight before treatment showed no genotype x treatment interaction, a significant main effects of genotype [$F(1,43) = 12.25$, $p = 0.001$] but no main effect of treatment. Before treatment, there was no body weight difference between Ts65Dn mice destined to be treated with vehicle and those destined to be treated with corn oil (Fig. 1A). Both groups had a reduced body weight in comparison with euploid mice destined to be treated with vehicle (Fig. 1A). A two-way ANOVA on body weight after treatment showed a genotype x treatment interaction [$F(1,43) = 4.21$, $p = 0.046$], a main effects of genotype [$F(1,43) = 12.59$, $p = 0.001$] and a main effect of treatment [$F(1,43) = 4.27$, $p = 0.045$]. At the end of treatment, treated Ts65Dn mice had a body weight larger than control Ts65Dn mice and similar to that of control euploid mice (Fig. 1B). In euploid mice, treatment had no effect on body weight (Fig. 1B).

A two-way ANOVA on brain weight showed no genotype x treatment interaction and no main effect of genotype or treatment. Treatment with corn oil had no effect on the brain weight of either Ts65Dn or euploid mice in comparison with their control counterparts (Fig. 1C). Treated Ts65Dn mice, however, exhibited a significant increase in brain weight in comparison with treated euploid mice (Fig.1C).

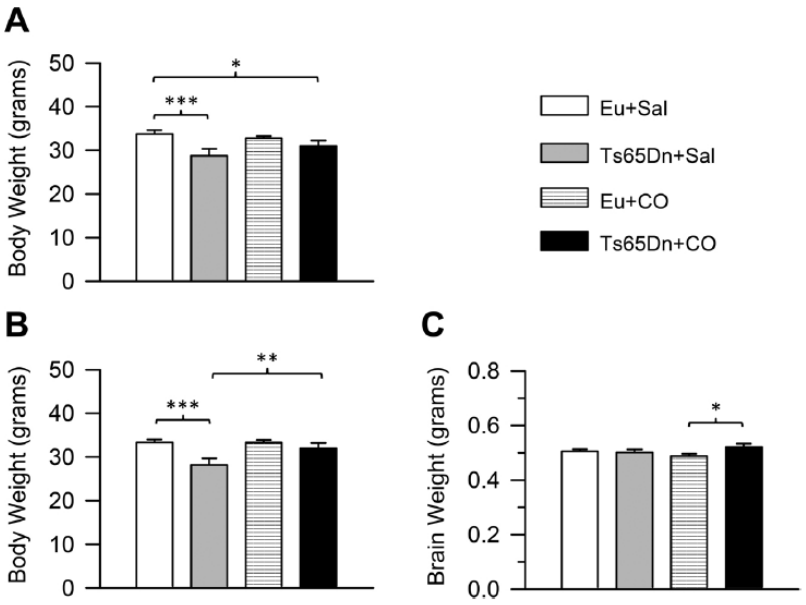

Fig. 1. Effect of treatment with corn oil on the body and brain weight.

A-C: Body weight before treatment (A) and body (B) and brain (C) weight after treatment (mean ± SE) in grams of control euploid mice (n=17), control Ts65Dn mice (n=8), treated euploid mice (n=12) and treated Ts65Dn mice (n=10). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Fisher LSD test after two-way ANOVA). Abbreviation: CO, corn oil; Eu, euploid; Sal, saline.

3.2. Effect of corn oil on learning and memory in Ts65Dn and euploid mice

MWM test is classically used in mouse models of DS to assess hippocampus-dependent learning and memory. For the learning phase, the following variables were evaluated: escape latency, time in periphery, percentage of time in periphery, path length, proximity. We additionally evaluated the swimming speed. We carried out a three-way mixed ANOVA for all variables followed by the *post hoc* Fisher LSD test. Results of ANOVA are reported hereafter and results of the *post hoc* test are summarized in Table 1.

A three-way mixed ANOVA on escape latency, with genotype and treatment as grouping factors and day as a repeated measure revealed no effect of genotype x

treatment x day. We found a genotype x day interaction [$F(7,301) = 5.64$, $p < 0.001$], a genotype x treatment interaction [$F(1,43) = 10.30$, $p = 0.003$], no treatment x day interaction, a main effect of genotype [$F(1,43) = 40.65$, $p < 0.001$], a main effect of treatment [$F(1,43) = 5.49$, $p = 0.024$], and a main effect of day [$F(7,301) = 45.62$, $p < 0.001$]. While control euploid mice exhibited a fast learning improvement with time, in control Ts65Dn mice the latency to reach the platform did not decrease as the test progressed, indicating poor learning capacity (Fig. 2A, Table 1). In contrast, Ts65Dn mice treated with corn oil showed a large learning improvement in comparison with their control counterparts, although their escape latency did not attain the values of control euploid mice (Fig. 2A, Table 1). In euploid mice treated with corn oil the latency was similar to that of control euploid mice (Fig. 2A; Table 1).

A three-way mixed ANOVA on the absolute time spent in the periphery zone (thigmotaxis), with genotype and treatment as grouping factors and day as a repeated measure revealed no effect of genotype x treatment x day. We found a genotype x day interaction [$F(7,301) = 4.27$, $p < 0.001$], a genotype x treatment interaction [$F(1,43) = 10.01$, $p = 0.003$], but no treatment x day interaction; a main effect of genotype [$F(1,43) = 21.35$, $p < 0.001$] was also found, as was a main effect of day [$F(7,301) = 60.27$, $p < 0.001$], while there was no main effect of treatment. A *post hoc* Fisher LSD test showed that, while control Ts65Dn mice spent more time in the periphery zone than control euploid mice, Ts65Dn mice treated with corn oil spent a similar time there as control euploid mice, save for days 4 and 7 (Fig. 2B, Table 1), suggesting an improvement in searching strategy. Euploid mice treated with corn oil showed no changes in thigmotaxis.

A three-way mixed ANOVA on the percentage of time spent in the periphery zone, with genotype and treatment as grouping factors and day as a repeated measure, revealed no effect of genotype x treatment x day. We found a genotype x day interaction [$F(7,301) = 2.17$, $p = 0.037$], while neither a treatment x day

interaction nor a genotype x treatment interaction were present. There was a main effect of genotype [$F(1,43) = 19.93$, $p < 0.001$], no main effect of treatment; but a main effect of day [$F(7,301) = 17.84$, $p < 0.001$]. A *post hoc* Fisher LSD test showed that the time that control Ts65Dn mice spent in the periphery zone, expressed as a percentage of the total latency, was larger than that of control euploid mice (Fig. 2C, Table 1). In treated Ts65Dn mice the percentage of time in thigmotaxis underwent a reduction and on days 5-8 it was significantly reduced in comparison with their control counterparts (Fig. 2C, Table 1), suggesting an improvement in spatial learning.

A three-way mixed ANOVA on path length, with genotype and treatment as grouping factors and day as a repeated measure revealed no effect of genotype x treatment x day. We found a genotype x day interaction [$F(7,301) = 8.90$, $p < 0.001$], while there was no treatment x day interaction, or genotype x treatment interaction. A main effect of genotype [$F(1,43) = 14.59$, $p < 0.001$] was observed, while no main effect of treatment emerged. A main effect of day [$F(7,301) = 47.16$, $p < 0.001$] was present. In all groups, the path length decreased from day 4 to day 8 (Fig. 2D). A *post hoc* Fisher LSD test showed no differences in the path length between control and treated Ts65Dn mice or between control and treated euploid mice (Table 1).

A three-way mixed ANOVA on proximity to the trained platform position (Gallagher's test; proximity), with genotype and treatment as grouping factors and day as a repeated measure revealed no effect of genotype x treatment x day. We found a genotype x day interaction [$F(7,301) = 3.78$, $p = 0.001$], a treatment x day interaction [$F(7,301) = 4.27$, $p < 0.001$], no genotype x treatment, a main effect of genotype [$F(1,43) = 18.85$, $p < 0.001$], no main effect of treatment, but a main effect of day [$F(7,301) = 23.58$, $p < 0.001$]. Fig. 2E shows that, while in control euploid mice the distance from the platform position decreased from day 1 to day 8, control Ts65Dn mice underwent no improvement. In contrast, treated

Ts65Dn mice underwent an improvement and on days 7-8 their distance from the platform was significantly reduced in comparison with their control counterparts; on days 5-8 their distance was similar to that of control euploid mice (Fig. 2E, Table 1).

A two-way ANOVA on the mean swimming speed during the 8 days of the learning phase, with genotype and treatment as grouping factors showed a genotype x treatment interaction [$F(1,43) = 9.43$, $p = 0.004$], a main effect of genotype [$F(1,43) = 4.96$, $p = 0.031$], and a main effect of treatment [$F(1,43) = 6.22$, $p = 0.017$]. A *post hoc* Fisher LSD test showed that in control Ts65Dn mice the swimming speed was reduced in comparison with control euploid mice. This difference disappeared in treated Ts65Dn mice (Fig. 2F).

In the probe test, we considered the following parameters as an index of spatial memory: i) latency to enter the trained platform zone (latency); ii) frequency of entrances into the trained quadrant (frequency); iii) proximity to the trained platform position (Gallagher's test; proximity); iv) percentage of time spent in the periphery zone (thigmotaxis); v) percentage of time spent in each quadrant. We additionally evaluated the swimming speed. A two-way ANOVA on the latency showed no genotype x treatment interaction, no main effect of genotype, and no main effect of treatment. A *post h*oc Fisher LSD test showed no significant differences between groups, although treated Ts65Dn mice underwent a latency reduction in comparison with their control counterparts (Fig. 3A). A two-way ANOVA on the frequency showed no genotype x treatment interaction, a main effect of genotype [$F(1,43) = 4.55$, $p = 0.039$] but no main effect of treatment. A *post hoc* Fisher LSD test showed that control Ts65Dn mice exhibited a reduced frequency of entrances in comparison with control euploid mice. In treated Ts65Dn mice there was an increase in the frequency that became similar to that of control euploid mice (Fig. 3B). A two-way ANOVA on the proximity showed no genotype x treatment interaction, while there was a main effect of genotype

[F(1,43) = 18.37, p < 0.001] and a main effect of treatment [F(1,43) = 11.45, p = 0.002]. A *post hoc* Fisher LSD test showed that control Ts65Dn mice swam at a greater distance from the trained platform zone in comparison with control euploid mice (Fig. 3C). Treated Ts65Dn mice swam closer to the trained platform zone and their performance became similar to that of control euploid mice (Fig. 3C).

A two-way ANOVA on the percentage of time spent in the periphery zone showed no genotype x treatment interaction, no main effect of genotype, and no main effect of treatment. A *post hoc* Fisher LSD test showed that in control Ts65Dn mice the percentage of time spent in the periphery zone was greater than that of control euploid mice (Fig. 3D). In treated Ts65Dn mice the percentage of time spent in the periphery zone was reduced in comparison with their control counterparts (Fig. 3D), suggesting restoration of the searching strategy.

A two-way ANOVA on the swimming speed showed no genotype x treatment interaction. A main effect of genotype [F(1,43) = 6.34, p = 0.016] was found, but there was no main effect of treatment. A *post hoc* Fisher LSD test showed that in control Ts65Dn mice swimming speed was reduced in comparison with control euploid mice. This difference disappeared in treated Ts65Dn mice (Fig. 3E).

A paired samples t-test showed that control Ts65Dn mice exhibited no differences in the time spent in the trained platform quadrant (SW) in comparison with the other quadrants (Fig. 3F). Treated Ts65Dn mice spent more time in the trained platform quadrant and in the Northwest (NW) quadrant, although these differences were not statistically significant. Control euploid mice spent marginally more time in the trained platform quadrant in comparison with the Northeast (NE) quadrant [t(16) = 2.07, p = 0.054] and the Southeast (SE) quadrant [t(16) = 2.06, p = 0.056] (Fig. 3F). Treated euploid mice spent significantly more time in the trained platform quadrant in comparison with the

SE [t(11) = 2.33, p = 0.040] and NE [t(11) = 3.38, p = 0.006] quadrants (Fig. 3F). The CFC paradigm is a test that allows for estimation of both hippocampus-independent (cued) and hippocampus-dependent (contextual) memory (McHugh et al., 2007). A two-way ANOVA with genotype and treatment as grouping factors on the performance in the old context showed an interaction between genotype and treatment [F(1,41) = 5.42, p = 0.025] and no main effect of either genotype or treatment. A *post hoc* Fisher LSD test showed that control Ts56Dn mice showed a lower freezing behavior in the trained environment (old context) compared to euploid control mice (Fig. 4A), although the difference was not significant (p = 0.075). In treated Ts65Dn mice the freezing behavior significantly increased in comparison with their control counterparts (Fig. 4A), indicating improvement of memory for the context. No effect of treatment was found in euploid mice (Fig. 4A). A two-way ANOVA with genotype and treatment as factors on the performance in the cued session showed no interaction between genotype and treatment, no main effect of treatment but a main effect of genotype [F(1,43) = 5.57, p = 0.023]. A *post hoc* Fisher LSD test showed no difference between groups save for treated Ts65Dn mice that had a reduced freezing in comparison with control euploid mice (Fig. 4B). Freezing in the cued session is expressed as difference between the percentage of freezing during the sound (cue) delivery and the spontaneous freezing in the new context. We considered of interest to establish possible differences between groups in the amount of freezing in the new context and during sound delivery, respectively. Fig. 4C shows that in the new context both treated euploid and Ts65Dn mice exhibited a higher freezing in comparison with their control counterparts,
although the difference was not statistically significant. During sound delivery, while control
Ts65Dn mice exhibited a lower freezing in comparison with euploid mice, this

difference disappeared in treated Ts65Dn mice (Fig. 4D).

Table 1. P values of the Fisher LSD test for the indicated variables.

		Escape Latency							
		D1	D2	D3	D4	D5	D6	D7	D8
Eu + Sal	Ts + Sal	.208	.004	< .001	< .001	< .001	< .001	< .001	< .001
	Eu + CO	.226	.976	.783	.568	.291	.809	.438	.575
	Ts + CO	.321	.159	.027	.007	.002	.032	.022	.028
Eu + CO	Ts + Sal	.853	.006	< .001	< .001	< .001	< .001	< .001	< .001
	Ts + CO	.883	.181	.065	.038	.039	.028	.136	.113
Ts + Sal	Ts + CO	.755	.123	.017	.002	.012	.001	.005	.001
		Time Periphery (sec)							
		D1	D2	D3	D4	D5	D6	D7	D8
Eu + Sal	Ts + Sal	.035	< .001	< .001	< .001	< .001	.002	< .001	< .001
	Eu + CO	.203	.426	.923	.197	.161	.654	.676	.796
	Ts + CO	.099	.019	.108	.027	.685	.660	.029	.437
Eu + CO	Ts + Sal	.331	.002	.001	.002	.014	.008	< .001	.001
	Ts + CO	.669	.125	.157	.336	.386	.988	.091	.619
Ts + Sal	Ts + CO	.580	.097	.031	.028	.002	.011	< .001	.007
		Time Periphery (%)							
		D1	D2	D3	D4	D5	D6	D7	D8
Eu + Sal	Ts + Sal	.040	< .001	.021	.002	.017	< .001	< .001	.012
	Eu + CO	.371	.694	.878	.631	.541	.604	.814	.483
	Ts + CO	.165	.001	.177	.009	.897	.035	.037	.810
Eu + CO	Ts + Sal	.221	.001	.022	.011	.075	.001	< .001	.004
	Ts + CO	.606	.005	.165	.040	.676	.125	.033	.401
Ts + Sal	Ts + CO	.472	.407	.320	.524	.038	.041	.004	.035
		Path Lenght							
		D1	D2	D3	D4	D5	D6	D7	D8
Eu + Sal	Ts + Sal	.014	.762	0.057	.016	< .001	.110	< .001	0.709
	Eu + CO	.323	.427	.832	.308	.460	.854	.992	.145
	Ts + CO	.666	.355	.121	.076	< .001	< .001	< .001	.037
Eu + CO	Ts + Sal	.002	.708	.105	.138	.004	.175	< .001	.121
	Ts + CO	.205	.870	.206	.436	< .001	< .001	< .001	.002
Ts + Sal	Ts + CO	.058	.612	.668	.461	.069	.038	.866	.150
		Proximity							
		D1	D2	D3	D4	D5	D6	D7	D8
Eu + Sal	Ts + Sal	.020	.002	.010	.002	.017	.001	< .001	< .001
	Eu + CO	.241	.014	.549	.553	.535	.416	.150	.686
	Ts + CO	.086	.011	.159	.022	.175	.052	.243	.120
Eu + CO	Ts + Sal	.203	.299	.004	.013	.077	< .001	< .001	< .001
	Ts + CO	.559	.834	.069	.099	.467	.013	.021	.073
Ts + Sal	Ts + CO	.480	.417	.227	.342	.287	.155	.011	.022

Abbreviations: D, day; Eu, euploid; CO, corn oil; Sal, saline; sec, seconds; Ts, Ts65Dn.

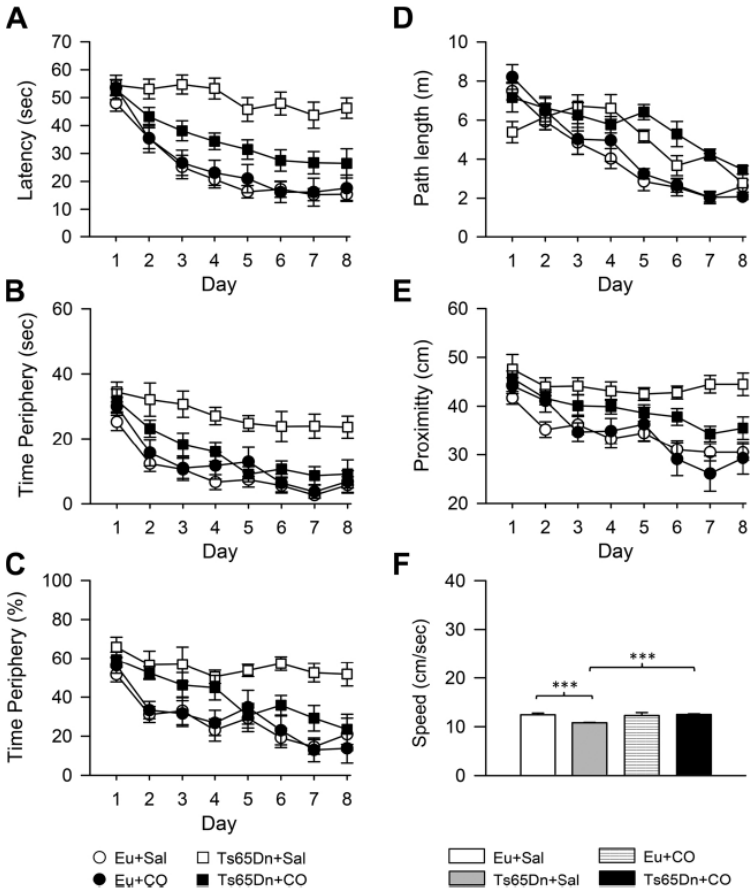

Fig. 2. Effect of treatment with corn oil on spatial learning in Ts65Dn and euploid mice.
Control euploid mice (n=17), control Ts65Dn mice (n=8), treated euploid mice (n=12) and treated Ts65Dn mice (n=10) were subjected to the MWM starting from the first day after treatment cessation (i.e. at 5 months of age). **A-E:** Learning phase of the MWM evaluated as latency to reach the platform (A), time spent at the periphery (thigmotaxis) (B), percentage of time spent at the periphery (C), path length (D), and proximity to the platform zone (E). **F:** Mean swimming speed of the four experimental groups obtained by averaging the speed of individual trials during the whole 8 day-period of the learning phase. Results of the *post hoc* Fisher LSD test are reported in Table 1. **B-D:** Values represent mean ± SE. Abbreviations: cm, centimeters; CO, Corn oil; Eu, euploid; m, meters; Sal, saline; sec, seconds.

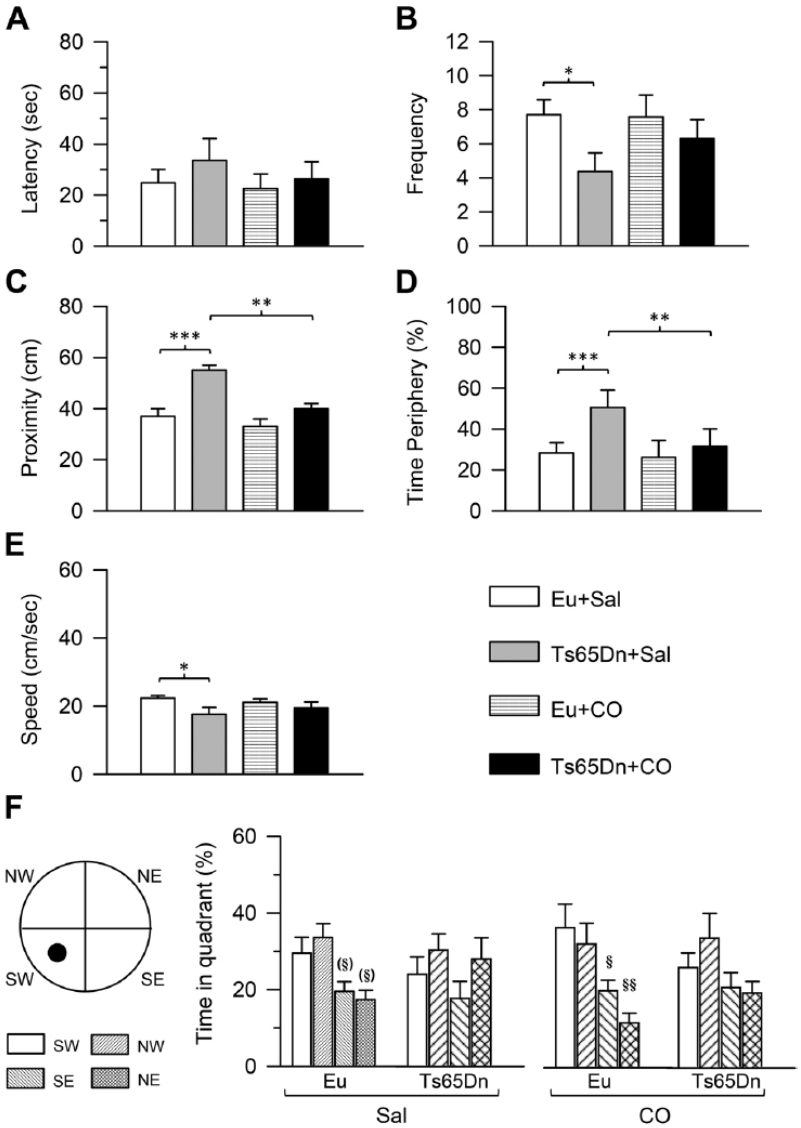

Fig. 3. Effect of treatment with corn oil on spatial memory in Ts65Dn and euploid mice.

Spatial memory was assessed in the probe test after spatial learning in control euploid mice (n=17), control Ts65Dn mice (n=8), treated euploid mice (n=12) and treated Ts65Dn mice (n=10). Mice are the same as in Fig. 2. In the probe test, memory was assessed as latency to reach the trained platform zone (**A**), number of crossings (frequency) over the trained platform quadrant (**B**), proximity to the trained platform zone (**C**), percentage of time spent at the periphery (**D**), percentage of time spent in quadrants (F). **E:** Swimming speed during the probe test. Values

represent mean ± SE. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Fisher LSD test after two-way ANOVA). The symbol § in (F) indicates a difference between each individual quadrant and the trained platform quadrant (see key on the left) for each experimental group. (§) $p < 0.06$; § $p < 0.05$; §§ $p < 0.01$ (two-sample paired t-test). Abbreviations: cm, centimeters; CO, Corn oil; Eu, euploid; NE, north-east; NW, north-west; Sal, saline; sec, seconds; SE, south-east, SW, south-west.

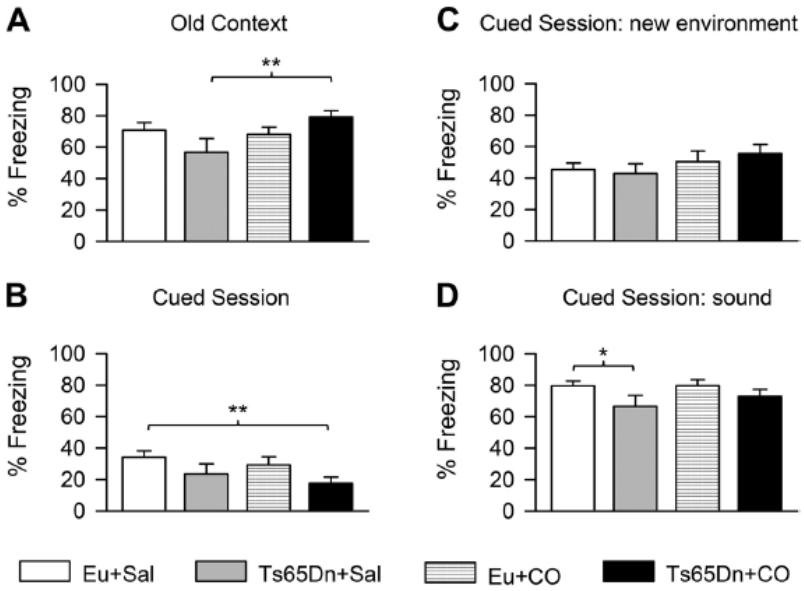

Fig. 4. Effect of treatment with corn oil on contextual fear conditioning in Ts65Dn and euploid mice.

Control euploid mice (n=17), control Ts65Dn mice (n=8), treated euploid mice (n=12) and treated Ts65Dn mice (n=10) were subjected to the CFC test. Based on exclusion criteria (see Methods) we excluded from the analysis of the old context session 1 control euploid mouse (yielding 16 mice) and 1 control Ts65Dn mouse (yielding 7 mice). **A-D:** Percentage of freezing in the old context (A), in the cued session (B), in the new environment of the cued session (C) and in the cued session during sound delivery (D). * $p < 0.05$; ** $p < 0.01$ (Fisher LSD test after two-way ANOVA). Abbreviations: CO, Corn oil; Eu, euploid; Sal, saline.

Effect of corn oil on hippocampal neurogenesis in Ts65Dn and euploid mice

Doublecortin (DCX) is a microtubule-associated phosphoprotein selectively located in the periphery of the soma with a pattern that overlaps microtubule distribution (Couillard-Despres et al., 2005). DCX is expressed in the cytoplasm of immature granule neurons during the period of neurite elongation (from one to four weeks after neuron birth), which allows evaluation of total number of new granule cells. In order to establish whether treatment with corn oil enhances neurogenesis in the hippocampal dentate gyrus, brain sections were subjected to immunohistochemistry for DCX. In agreement with the morphogenesis of the granule cell layer, DCX-positive cells were located in the innermost portion of the layer, close to the hilus (Fig. 5A). A two-way ANOVA on the number of DCX-positive cells showed no genotype x treatment interaction but a main effect of genotype [$F(1,14) = 29.39$, $p < 0.001$] and treatment [$F(1,14) = 10.30$, $p = 0.006$]. In agreement with previous evidence, control Ts65Dn mice had a reduced number of new granule cells in comparison with their euploid counterparts (Fig. 5A,B). In Ts65Dn mice treated with corn oil there was an increase in the number of new neurons in comparison with control Ts65Dn mice (Fig. 5A,B) that became similar to that of control euploid mice (Fig. 5A,B). In euploid mice treatment with corn oil did not affect the number of new granule cells (Fig. 5A,B). These results suggest that treatment with corn oil exerts a beneficial effect on hippocampal neurogenesis specifically in Ts65Dn mice.

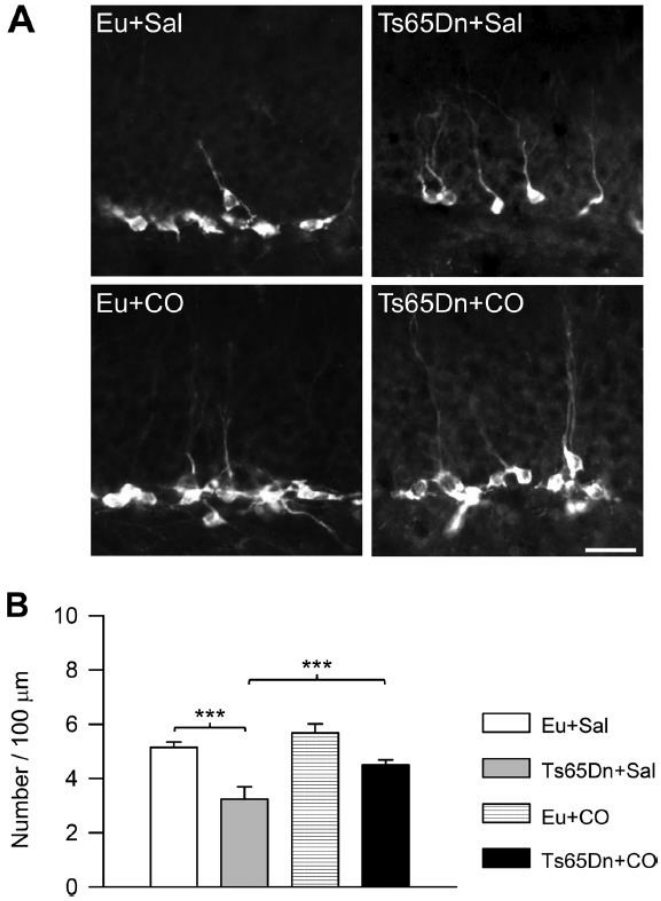

Fig. 5. Effect of treatment with corn oil on the number of new granule cells in the dentate gyrus.

The number of new granule cells was evaluated with DCX immunohistochemistry. **A:** Examples of sections processed for fluorescent immunostaining for DCX from the dentate gyrus of control euploid and Ts65Dn mice and euploid and Ts65Dn mice treated with corn oil. Calibration bar = 20 µm. **B:** Number of DCX-positive cells in the dentate gyrus of control euploid (n=5) and Ts65Dn (n=5) mice, corn oil treated euploid (n=4) and Ts65Dn (n=4) mice. Values represent mean ± SE. ** $p < 0.01$; *** $p < 0.001$ (Fisher LSD test after two-way ANOVA).. Abbreviations:

CO, Corn oil; Eu, euploid; Sal, saline.

Effect of corn oil on dendritic development in Ts65Dn and euploid mice

Dendritic morphology of newborn granule cells was analyzed in sections subjected to immunohistochemistry for DCX. Fig 6A shows examples of the dendritic pattern in each experimental group. It can be readily appreciated that the granule cells of control Ts65Dn mice had a poorly-branched dendritic tree and that treatment with corn oil increased its complexity.

A two-way ANOVA on the total length of the dendritic tree showed no genotype x treatment interaction, no main effect of genotype, but a main effect of treatment [$F(1,14) = 12.54$, $p = 0.003$]. A *post hoc* Fisher LSD test showed that in control Ts65Dn mice the dendritic length was reduced in comparison with control euploid mice and that treatment with corn oil fully restored total dendritic length (Fig. 6B). A two-way ANOVA on the total number of dendritic branches showed no genotype x treatment interaction and no main effect of genotype and treatment. A *post hoc* Fisher LSD test showed no differences between groups, although in treated Ts65Dn mice the total number of branches was marginally larger ($p = 0.06$) in comparison with their control counterparts (Fig. 6C). The analysis of the number of branches of each order showed no interaction between genotype x treatment and no main effect of genotype and treatment for all orders, save for order 5 that showed a main effect of treatment [$F(1,14) = 4.89$, $p = 0.044$]. A *post hoc* Fisher LSD test showed no differences between groups for orders 1-3 and 6-7 (Fig. 6E). However, control Ts65Dn mice had a reduced number of branches of order 5 in comparison with control euploid mice and treated Ts65Dn mice had a larger number of branches of order 4 and 5 in comparison with control Ts65Dn mice (Fig. 6E). Importantly, while control Ts65Dn mice lacked branches of orders 6 and 7, in treated Ts65Dn mice branches of orders 6 and 7 were also present (Fig. 6E). A two-way ANOVA on the mean

branch length showed no genotype x treatment interaction no main effect of genotype and no main effect of treatment. A *post hoc* Fisher LSD test showed no differences between groups (Fig. 6D).

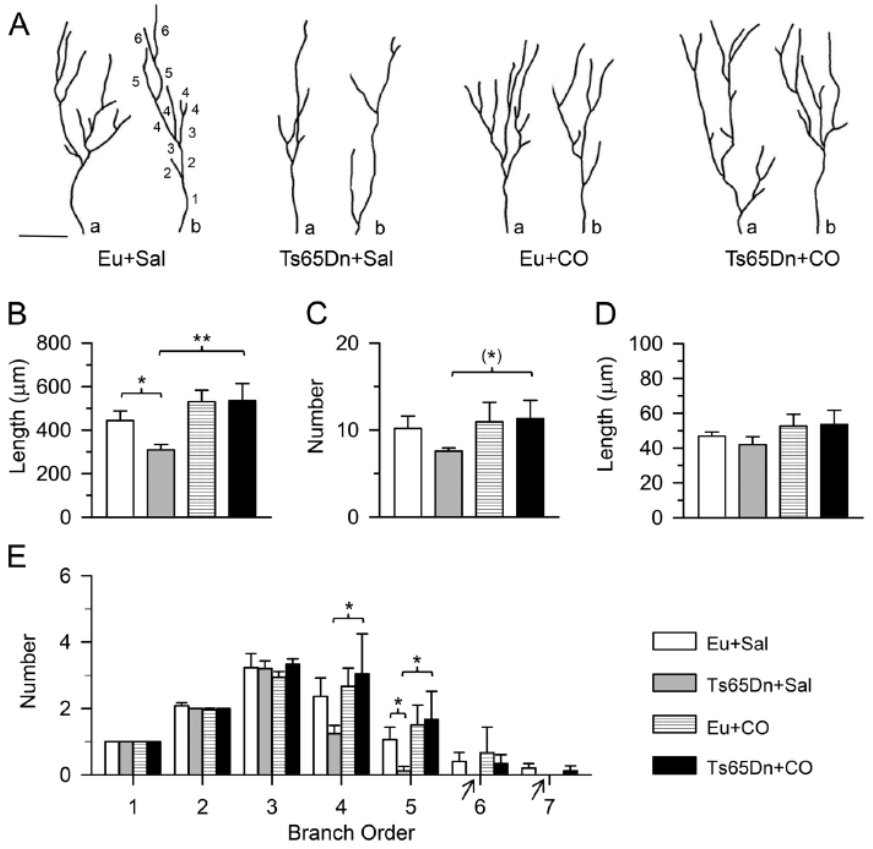

Fig. 6. Effect of corn oil on the dendritic size of newborn granule cells.

A: Two examples (a, b) of the reconstructed dendritic tree of DCX-positive granule cells from animals of each of the following experimental groups: control euploid (n=5) and Ts65Dn (n=5) mice and treated euploid (n=4) and Ts65Dn (n=4) mice. Numbers indicate the different dendritic orders. Calibration bar = 50 µm. **B-E:** Total dendritic length (B), mean number of dendritic segments (C), mean segment length (D) and mean number of branches of the different orders (E) in control euploid and Ts65Dn mice, and euploid and Ts65Dn mice treated with corn oil. The arrows in (E) indicate the absence of branches of order 6 and 7 in control Ts65Dn mice. Values in (B-E) represent mean ± ES. (*) $p < 0.06$; * $p < 0.05$; ** $p < 0.01$ (Fisher LSD test after two-

way ANOVA). Abbreviations: CO, Corn oil; Eu, euploid; Sal, saline.

Effect of linoleic acid and oleic acid on the proliferation rate of neural progenitor cells from Ts65Dn and euploid mice

Corn oil contains a high percentage (~54%) of PUFAs, mainly represented by LA (omega-6), and a high percentage (~28%) of the MUFA OA (omega-9). In order to investigate whether the most abundant PUFA and MUFA in corn oil could directly affect neurogenesis, we used cultures of neural progenitor cells (NPCs) from the subventricular zone of Ts65Dn mice. Cultures were exposed to different concentrations of LA or OA (from 0.1 to 100.0 µM). The Kruskal-Wallis test showed a significant effect of LA on proliferation rate [χ^2 (8) = 44.43, $p < 0.001$]. The Mann-Whitney test showed that in cultures exposed to LA there was a proliferation increase in comparison with cultures exposed to vehicle at concentrations of 60.0 µM (U = 0.001, p = 0.007), 80 µM (U = 0.001, p = 0.007), and 100 µM (U = 0.001, $p < 0.001$) (Fig. 7A). The Kruskal-Wallis test showed a significant effect of OA on proliferation rate [χ^2 (8) = 48.84, $p < 0.001$]. The Mann-Whitney test showed that in cultures exposed to OA there was a proliferation increase in comparison with cultures exposed to vehicle at concentrations of 80 µM (U = 0.001, p = 0.006), and 100 µM (U = 0.001, $p < 0.001$) (Fig. 7B).

A comparison of the pro-proliferative effect of different concentrations of LA showed that the effect of the concentration of 60 µM was lower in comparison with the concentration of 80 µM (U = 0.000, p = 0.050) and 100 µM (U = 6.000, p = 0.044), and that there were no differences between the concentrations of 80 µM and 100 µM (Fig. 7A). A comparison of the pro-proliferative effect of different concentrations of OA showed that the concentration of 80 µM had an effect that was lower in comparison with the concentration of 100 µM (U = 2.000, p = 0.011) (Fig. 7B). A comparison of the effects of LA and OA showed that the

proliferation increase caused by LA was larger than that caused OA (U = 68.000; p = 0.003) (Fig. 7C). While LA at a concentration of 100 µM caused a 45% increase in proliferation rate in comparison with cultures exposed to vehicle, OA at a concentration of 100 µM caused a 26 % increase only. This evidence indicates that LA is a more powerful neurogenesis enhancer in comparison with OA.

Since lithium has been shown to restore proliferation of NPCs of Ts65Dn mice *in vivo* (Bianchi et al., 2010; Contestabile et al., 2013) and *in vitro* (Trazzi et al., 2014), it seemed of interest to compare the pro-proliferative effect of lithium and those of LA/OA. We used a 2.0 mM concentration of LiCl based on previous evidence showing that this concentration restores neurogenesis in trisomic NPCs (Trazzi et al., 2014). As expected, LiCl increased the proliferation of trisomic cells (+ 34 %) compared to vehicle (Fig. 7C). Since LA and OA were effective at 100 µM, they appear to be more potent enhancers of NPCs proliferation than LiCl.

We exposed euploid cultures of NPCs from the subventricular zone of euploid mice to LA and OA in order to establish their effects on euploid cells. The Kruskal-Wallis test showed a significant effect of LA on proliferation rate [χ^2 (8) = 43.07, $p < 0.001$]. The Mann-Whitney test showed that in cultures exposed to LA there was a proliferation increase in comparison with cultures exposed to vehicle at the concentration of 100 µM (U = 0.000, $p < 0.001$) (Fig. 7D). The Kruskal-Wallis test showed a significant effect of OA on proliferation rate [χ^2 (8) = 40.71, $p < 0.001$]. The Mann-Whitney test showed that in cultures exposed to OA there was a proliferation increase in comparison with cultures exposed to vehicle at concentrations of 80 µM (U = 0.000, p = 0.001), and 100 µM (U = 8.000, $p < 0.001$) (Fig. 7E). In euploid cultures exposed to LiCl there was an

increase in proliferation (+ 41 %) compared to vehicle (Fig. 7F).

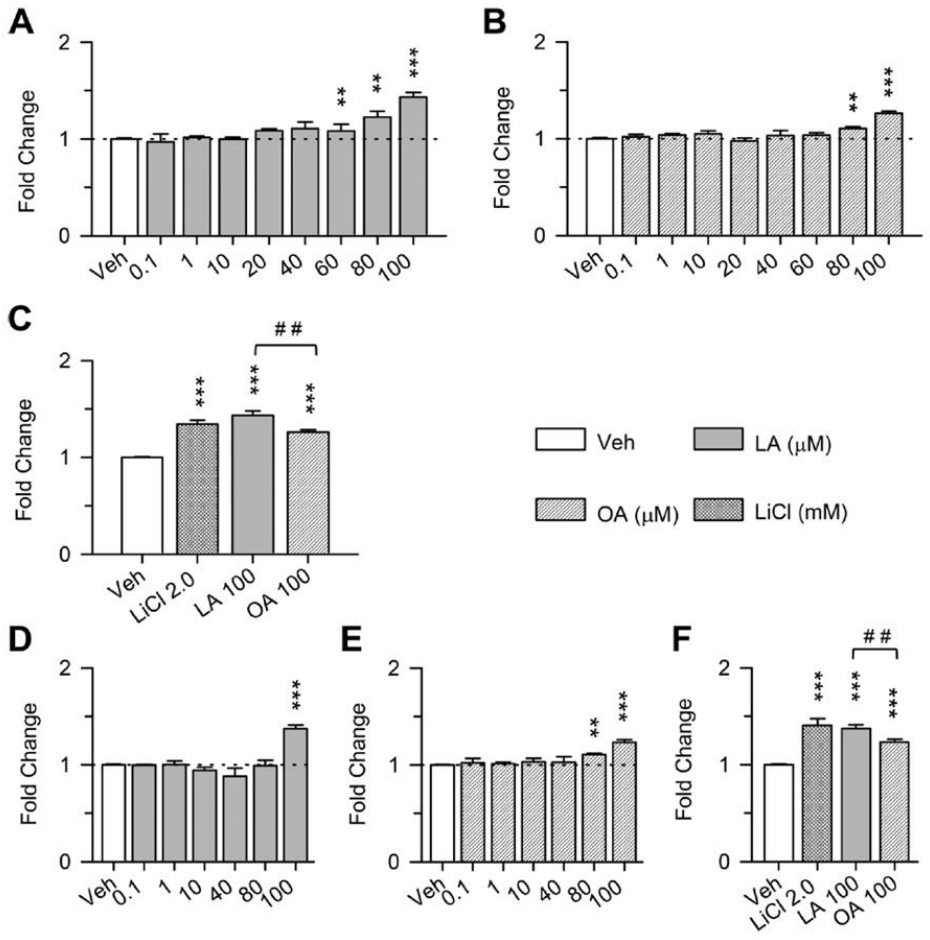

Fig. 7. Effect of treatment with oleic acid and linoleic acid on cultures of neural progenitor cells.

Neural progenitor cells from the subventricular zone of neonate (age: 1-2 days) Ts65Dn and euploid mice were maintained for 96 h in a vehicle composed of 10 ng/ml bFGF and BSA 3.3 mg/ml or in vehicle supplemented with either LA or OA, at the indicated concentrations (0.1-100 μM). Parallel cultures were maintained in a vehicle composed of 10 ng/ml bFGF and 0.05% DMSO or supplemented with LiCl 2.0 mM. **A-C:** A proliferation assay in Ts65Dn cultures showed a pro-proliferative effect of LA (A) and OA (B) in comparison with cultures exposed to vehicle and that the pro-proliferative effects of LA and OA were comparable to those exerted by LiCl (C). **D-F:** A proliferation assay in euploid cultures showed a pro-proliferative effect of LA

(D) and OA (E) in comparison with cultures exposed to vehicle and that the pro-proliferative effects of LA and OA were comparable to those exerted by LiCl (F). Data in A-C were obtained in pooled cultures from Ts65Dn mice (n=2). Data in D-F were obtained in pooled cultures from euploid mice (n=2). Values (mean ± SE) are expressed as fold change over the vehicle condition. ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney test after Kruskal-Wallis test). Abbreviations: LA, linoleic acid; LiCl, Lithium chloride; OA, oleic acid, Veh, vehicle.

The pro-proliferative effect of linoleic acid are blocked by PPARß/δ and PPARγ antagonists

The mechanisms of action of PUFAs on neurogenesis remain elusive (Dyall, 2015), although there are suggestions of a possible role of peroxisome-proliferator activated receptors (PPARs) (Bernal et al., 2015). PPARs are a group of transcription factors that represent the best recognized nuclear sensor system for fatty acids (Fidaleo et al., 2014; Georgiadi and Kersten, 2012). There are three isotypes of PPARs, named PPARα, PPARβ/δ and PPARγ (Fidaleo et al., 2014). Mouse NPCs express isotypes PPARβ/δ and PPARγ only (Bernal et al., 2015). In order to investigate whether the pro-proliferative effect exerted by the most abundant corn oil PUFA, LA, could be mediated by PPARs, trisomic cells were exposed to LA 100 µM in presence of either a PPAR ß/δ antagonist (GSK0660, 0.1-30 µM) or a PPARγ antagonist (GW9662, 0.01-10 µM). The Kruskal-Wallis test showed a significant effect of the PPAR ß/δ antagonist [χ^2 (5) = 19.86, p = 0.001] and the PPAR γ antagonist [χ^2 (4) = 33.57, $p < 0.001$] on proliferation. We found that both antagonists counteracted the LA-induced proliferative effects on trisomic NPCs (Fig. 8A,B). Exposure to the PPARβ/δ receptor antagonist GSK0660 caused a moderate reduction in the pro-proliferative effect of LA at all tested concentrations (0.1-30 µM), but did not completely abrogate its effect, even at the highest concentration (Fig. 8A). Exposure to the PPARγ receptor antagonist GW9662 at low concentrations (0.01 µM) did not reduce the pro-

proliferative effect of LA (Fig. 8B). However, exposure to higher concentrations (1, 3, and 10 µM) reduced and even abrogated the effect of LA (Fig. 8B). Taken together, these results suggested that, at least *in vitro*, PPARβ/ δ and PPAR γ contribute to the proliferative effects of LA on trisomic NPC.

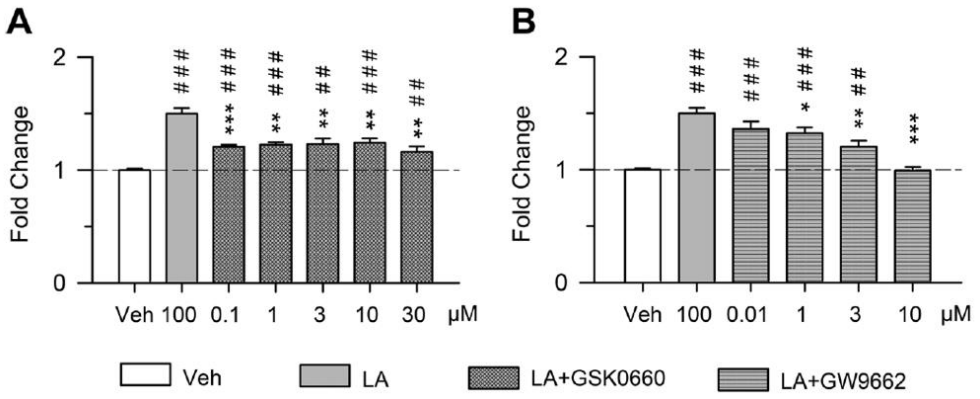

Fig. 8. Effect of PPAR β/ δ and PPAR γ antagonists on linoleic acid-mediated effects.

Neural progenitor cells from the subventricular zone of neonate (age: 1-2 days) Ts65Dn mice were maintained for 96 h either in a vehicle composed of 10 ng/ml bFGF and 0.05% DMSO or in vehicle supplemented with LA 100 µM alone or LA 100 µM plus either the PPARβ/ δ antagonist GSK0660 (**A**) or the PPARγ antagonist GW9662 (**B**) at the indicated concentrations (µM). Data were obtained in pooled cultures from Ts65Dn mice (n=3). Values (mean ± SE) are expressed as fold change over the vehicle condition. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. LA; ## $p < 0.01$; ### $p < 0.001$ vs. vehicle (Mann-Whitney test after Kruskal-Wallis test). Abbreviation: LA, linoleic acid; Veh, vehicle.

DISCUSSION

Corn oil positively impacts on hippocampal neurogenesis in Ts65Dn mice

While neurogenesis is a process that is largely accomplished before birth, in the hippocampal dentate gyrus neurogenesis persists throughout life in all examined mammals, including humans (Bayer, 1980; Eriksson et al., 1998; Guidi et al., 2011; Guidi et al., 2004; Kempermann & Gage, 2002; Ninkovic et al., 2007;

Spalding et al., 2013). Hippocampal neurogenesis has been shown to be severely impaired in fetuses with DS and in mouse models of DS at early life stages (Stagni et al., 2017a) and in adulthood (Belichenko & Kleschevnikov, 2011; Clark et al., 2006; Lorenzi and Reeves, 2006; Rueda et al., 2005). A number of studies has explored the effects of different drugs on hippocampal neurogenesis and hippocampus-dependent learning and memory in mouse models of DS (Gardiner, 2015; Stagni et al., 2015). In the current study, we found that treatment with a natural substance, corn oil, leads to restoration of neurogenesis in the hippocampus of adult Ts65Dn mice, which demonstrates for the first time that corn oil can enhance neurogenesis in DS.

While it is known that LA and OA increase cognitive performance (Jenkins et al., 2016; Moazedi et al., 2007), little evidence is available regarding the pro-neurogenic role of these fatty acids. By exploiting cultures of NPCs, we found that both LA and OA are able to increase the proliferation rate of NPCs, which suggests that both fatty acids of corn oil may potentially contribute to the neurogenesis increase observed in Ts65Dn mice treated with corn oil. The effects of LA, however, were larger than those of OA and had a magnitude that was comparable to that exerted by lithium, indicating that LA may act as a potent neurogenesis enhancer in the trisomic brain. Our *in vitro* results indicate that PPAR β/ δ and PPAR γ are involved in the direct effects of LA on NPCs suggesting that these receptors may contribute to the beneficial effects elicited by corn oil in trisomic mice. There is evidence that the triplicated gene Down syndrome critical region 2 (DSCR2) physically interacts with PPARβ in mammalian HEK293 cells and inhibits its ligand-induced transcriptional activity (Song et al., 2008). This suggests that this inhibition may contribute to impair neurogenesis in the DS brain and strengthens the conclusion that the positive effect of corn oil in Ts65Dn mice may be mediated by PPARs. Of course, other

mechanisms and contributors cannot be ruled out.

Corn oil positively impacts on dendritic development in Ts65Dn mice

In Ts65Dn mice, newborn granule cells had a reduced total dendritic length. This confirms previous evidence that dendritic hypotrophy starts at the initial stages of granule cells development (Guidi et al., 2013) and is in agreement with evidence of severe dendritic pathology in the Ts65Dn model of DS as well as in individuals with DS (Benavides-Piccione et al., 2004; Dang et al., 2014; Guidi et al., 2013). The reduction in total dendritic length was due to a reduction in the number of branches of intermediate order and a lack of high order branches, and not to a reduction in the mean branch length. In Ts65Dn mice treated with corn oil there was an increase in the number of branches of intermediate order and the *de novo* appearance of high order branches, with consequent restoration of total dendritic length. Neuron generation and dendritic maturation are severely compromised in DS. Thus, therapies to improve brain development should be aimed at restoring both of these processes. Current results show that corn oil restores the dendritic length of trisomic granule cells, indicating that the same treatment is able to restore not only the number of new granule neurons but also their "quality", in terms of correct maturation.

Corn oil positively impacts on hippocampus-dependent learning and memory in Ts65Dn mice

The deficits in hippocampus-dependent learning and memory in DS are attributable to reduced neurogenesis and synaptic alterations in the hippocampal formation, a region that is fundamental for declarative memory. We found that the restoration of neurogenesis and dendritic pattern of new granule neurons induced by treatment with corn oil in Ts65Dn mice was accompanied by a large improvement in hippocampus-dependent learning and memory as assessed with

the MWM test. During the learning phase, the reduction in the latency to reach the platform and in the time spent in the periphery showed that a large improvement had taken place; the proximity to the trained platform quadrant was similar to that of control euploid mice, indicating restoration of this parameter. In the probe test, the frequency of entrances in the trained platform quadrant, the proximity and the time spent in the periphery, became similar to that of control euploid mice, indicating restoration of these aspects of memory. It must be noted that control Ts65Dn mice had a slightly lower swimming speed in comparison with control euploid mice (-13%). It may be argued that the longer latency to reach the platform exhibited by control Ts65Dn mice in comparison with euploid mice during the learning phase of the MWM was due to their reduced speed, and that the latency reduction in treated Ts65Dn mice was due to the treatment-induced improvement in swimming speed and not to a learning improvement. However, on day 5 of the learning phase control Ts65Dn mice had a latency that was 3 times that of euploid mice, while their speed was only reduced by 13%, suggesting that the longer latency of Ts65Dn mice mainly reflects impairment of learning rather than a motor deficit. On day 5, the latency of treated Ts65Dn mice decreased by 40% in comparison with their control counterparts while swimming speed only increased by 15%, suggesting that the latency reduction was mainly due to an improvement in spatial learning and not in swimming speed. Moreover, the finding that treated Ts65Dn mice spent less time in the periphery and swam closer to the platform quadrant during the learning phase, and crossed the trained platform quadrant with increased frequency in the probe test, indicates an improvement in the searching strategy and, thus, in spatial learning.

Conflicting results are available regarding the swimming speed of Ts65Dn mice. While some reports show no swimming speed differences in comparison with euploid mice (Catuara-Solarz et al., 2015; Costa et al., 2010; Escorihuela et al., 1995; Faizi et al., 2011; Netzer et al., 2010; Stagni et al., 2017b), other studies

show that Ts65Dn mice have a reduced speed (Catuara-Solarz et al., 2016; Costa et al., 1999; Heinen et al., 2012). These discrepancies may be accounted for by differences in the sex and/or age of mice and/or by the notably higher degree of variability in swimming speed that characterizes Ts65Dn mice (Costa et al., 1999).

The CFC test showed that in treated Ts65Dn mice freezing in the old context increased in comparison with their control counterparts, suggesting improvement of context memory. During the cued session, however, treated Ts65Dn mice showed reduced freezing in comparison with control mice, which may suggest a poor association between the sound (cue) and the adverse stimulus (foot shock). Observation of Fig. 4D, however, shows that treated Ts65Dn mice demonstrated similar freezing to control euploid mice during sound delivery, suggesting no impairment in the retention of the association between sound and shock. On the other hand, treated Ts65Dn mice exhibited a higher (although not significant) level of freezing in the new context in comparison with control mice (Fig. 4C). Since freezing in the cued session is expressed as the difference between freezing during sound delivery and freezing in the new context, the reduced freezing of treated Ts65Dn in comparison with control mice during the cued session (Fig. 4B) may be due to a higher level of freezing in the new context rather than to an impairment in cue association learning.

Taken together, the current results are in agreement with a number of studies showing that Ts65Dn mice are impaired in learning and memory. In treated Ts65Dn mice, the parameters of the learning phase and probe test ameliorated in comparison with their control counterparts and some of the examined parameters became similar to those of control euploid mice. Moreover, the CFC test showed that treated Ts65Dn mice remembered the context to a similar extent as did control euploid mice. The finding that treatment with corn oil largely improved or even restored hippocampus-dependent learning and memory is consistent with

our finding that treatment restored hippocampal neurogenesis and dendritogenesis. The fact that treatment did not fully restore behavior may be related to the relatively low rate of neurogenesis at the examined age and, thus, to the relatively low number of new granule neurons added to the hippocampal circuits in comparison with pre-existing neurons.

Corn oil restores body weight in Ts65Dn mice

We found that after treatment with corn oil the body weight of Ts65Dn mice became similar to that of euploid mice. This effect is in agreement with evidence that in pre-term infants treated with PUFAs there is an improvement in growth and developmental scores (Fleith & Clandinin, 2005). To our knowledge, this is the first demonstration that it is possible to restore the reduced body weight of Ts65Dn mice through administration of corn oil. The effect of corn oil may be related to an improvement in metabolic processes exerted by fatty acids as well as to an increase in caloric intake. The finding that treatment increased the swimming speed of Ts65Dn mice suggests that corn oil may exert beneficial effects on the trophism of muscle cells.

We found no differences in the brain weight of five-month-old Ts65Dn mice compared to euploid mice, in contrast with our findings in younger mice (Stagni et al., 2017b). While treatment with corn oil had no effect in euploid mice, Ts65Dn mice underwent a small but significant brain weight increase. Since most of the brain neurons are born prenatally, the increase in brain weight may be related to an improvement in the composition of the neuronal membranes and/or growth of the neuronal processes.

Treatment with corn oil does not affect neurogenesis and cognitive performance in euploid mice

Unlike in Ts65Dn mice, treatment with corn oil did not increase proliferation and

dendritic maturation of granule cells in the hippocampal dentate gyrus of euploid mice and did not enhance hippocampus-dependent learning and memory. Yet, exposure to LA or OA was able to increase the proliferation rate of euploid NPCs *in vitro*, indicating that euploid NPCs are responsive to LA and OA similarly to trisomic NPCs. Taken together these results suggest that the lack of effects of corn oil *in vivo* in euploid mice may be due to a ceiling effect that prevents further enhancement of hippocampal development. This is consistent with previous observations that treatments that are effective in Ts65Dn mice may not have a similar efficacy in euploid mice (Corrales et al., 2014; Dang et al., 2014; Stagni et al., 2017b).

Conclusions

Nourishment with PUFAs and MUFAs has been shown to have a positive effect in various types of neurological disorders (Hussain et al., 2013). LA is the major PUFA, and OA is the major MUFA, of corn oil. Unlike OA, LA cannot be synthesized by the human body, so it must be exogenously supplied (Hussain et al., 2013). Arachidonic acid, which is a derivative of LA, has been shown to increase neurogenesis at postnatal stages when administered prenatally (Maekawa et al., 2009). Decreased levels of OA have been observed in the brain of Alzheimer's disease patients (Martin et al., 2010) and OA supplementation has been shown to inhibit the production of Aβ peptide and amyloid plaques (Amtul et al., 2011).

We found here that corn oil restores neurogenesis and improves hippocampus-dependent memory in adult Ts65Dn mice. Treatments that ameliorate hippocampal neurogenesis in adulthood may increase the "cognitive reserve" and postpone the onset of Alzheimer's-due dementia (Whalley et al., 2004). Although we administered corn oil intraperitoneally, our data may suggest that a diet based on an appropriate fatty acid intake may exert a benefit on cognitive performance

in individuals with DS. It is conceivable that treatment with fatty acids during the critical windows of neurogenesis (prenatal and early postnatal period) may have larger effects than those observed here and may possibly lead to a full behavioral rescue.

Various studies have explored the effects of a variety of pharmacological agents on hippocampus-dependent learning and memory in adult Ts65Dn mice (see (Gardiner, 2015)). Treatments i) targeted to transmitter/receptor systems, ii) employing neuroprotective agents, antioxidants, and free radical scavengers, iii) targeted to perturbed signaling pathways, iv) aimed at normalizing the expression of proteins coded by triplicated genes and v) that have used proneurogenic molecules demonstrated that it is possible to pharmacologically rescue or partially rescue behavioral deficits. While most of these studies show that it is possible to improve cognitive performance in a model of DS, some of the used therapies pose some caveats for human application in view of the nature of the used molecules that may potentially cause side effects. Corn oil, which is extracted from the germ of corn, contains a high percentage of fatty acids which are substances that are naturally present in the human diet. The current study shows that adult treatment with corn oil restores neurogenesis, dendritic development, and learning and memory in the Ts65Dn model of DS. Thus, supplementation of fatty acids may represent a promising and safe therapy for DS with a good translational potential in adulthood as well as at early life stages.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Chapter 7

Unpublished data

TSP-1/α2δ1 mediated signalling pathway in neural progenitor cells: potential relevance in Down syndrome pathophysiology

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INTRODUCTION

Down syndrome (DS) is a neurodevelopmental disorder caused by triplication of human chromosome 21. At present intellectual disability is the most disabling aspect of the pathology (Bartesaghi et al., 2011). In neonatal DS brain a decreased proliferation of neural progenitor cells (NPC) and a defective differentiation in their progeny are associated with a widespread neurogenesis impairment (Bianchi et al., 2010; Stagni et al., 2017; Vacca et al., 2019). These aspects are thought to be the main causes of DS intellectual disability (Stagni et al., 2018). DS phenotypic abnormalities are, at least in part, recapitulated in Ts65Dn mice, the most validated preclinical DS model (Bartesaghi et al., 2011; Herault et al., 2017). Exciting findings in animal models suggested that trisomy-linked brain abnormalities, including cognitive impairment, can be pharmacologically corrected by targeting, in early life stages, NPC (Guidi et al., 2014; Stagni et al., 2015, 2018, 2019a). Unfortunately, at present none of the drugs effective in animal models appear suitable for clinical application (Kazemi et al., 2016).

Recently in our laboratory, Cvijetic et al. identified novel signaling pathways that can affect NPC and their communication with astrocytes both in a cell-autonomous and non-cell autonomous manner (Cvijetic et al., 2017). To this regard, a piece of evidence hypothesized that cell-autonomous NPC dysfunctions can affect their communication with other cell types, including astrocytes, both in human DS cell models and in Ts65Dn mice (Chen et al., 2014; Mizuno et al., 2018). Indeed, DS astrocytes are not only more abundant, but exhibit functional alterations that can affect NPC and their progeny in a non-cell autonomous manner (Cresto et al., 2019; Mizuno et al., 2018). In particular, several studies showed that trisomic astrocytes secrete defective levels of thrombospondin-1 (Garcia et al., 2010; Torres et al., 2018) (TSP-1), a key astrocyte-derived signal involved in neurogenesis (Lu & Kipnis, 2010), synaptogenesis (Eroglu et al.,

2009) and spine formation (Risher et al., 2018). Despite these data, the role of TSP-1 in DS non-neuronal cells is largely unexplored.

All together these findings highlighted the need to investigate potential alterations of astrocyte-NPC communication in DS pathophysiology.

MATERIALS AND METHODS

Mouse colony

Ts65Dn mice were provided by Jackson Laboratories (Bar Harbor, ME, USA) and generated by mating B6EiC3Sn a/A-Ts(17^16)65Dn females with C57BL/6JEiJ x C3H/HeSnJ (B6EiC3Sn) F1 hybrid males (Reeves et al., 1995). Only first generation litters were used. Pups' genotyping was performed as previously described (Rheinold et al., 2011). Animals had *ad libitum* access to water and food in a room with a 12:12 h light/dark cycle. Experiments were performed in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) for the use of experimental animals and were approved by Italian Ministry of Public Health (1033/2015-PR and 47/2019).

Isolation and culture of neonatal neural progenitor cells

Cells were isolated from the subventricular zone (SVZ) and hippocampi (HP) of newborn (P1-2) euploid (EU) and Ts65Dn (trisomic, TS) mice, as previously described in Stagni et al. (Stagni et al., 2017; Stagni et al., 2019a). Briefly, SVZ and HP were removed and collected in ice-cold PIPES buffer. After that, tissues were digested with trypsin/EDTA 0.25 % (Life Technologies) and cell suspensions were seeded onto 25 cm^2 cell-culture flask (Thermo Fisher Scientific). Cells were cultured as free floating neurospheres in DMEM/F12 medium supplemented with B27 (Life Technologies), containing basic fibroblast growth factor (bFGF, 10 ng/ml; Peprotech) and human epidermal growth factor (hEGF, 20 ng/ml; Peprotech) using established protocols (Cvijetic et al., 2017;

Meneghini et al., 2014). Primary (Passage 1, P1) neurospheres were dissociated using Stempro Accutase (Life Technologies) after 7 days *in vitro* (DIV), thereafter single animals of the same genotype were pooled (pool of 3-6 pups) and neurospheres were then passaged every 5 DIV. For further *in vitro* studies cells from P3 to P12 were used, as described in Stagni et al. (Stagni et al., 2019a).

Neural progenitor cell proliferation

Proliferation was tested as previously described and data derived from 3-6 Ts65Dn (trisomic) and euploid pups pooled together (Giacomini et al., 2018; Stagni et al., 2017; Stagni et al., 2019b). Briefly, in order to evaluate cell proliferation trisomic (TS) and euploid (EU) SVZ NPC (P3-P12) were dissociated in a single cell suspension and plated onto Nunclon^{TM} Delta Surface 96-well plate (Thermo Fisher Scientific) at a density of 4×10^3 cells per well in DMEM/F-12 medium supplemented with B27, Glutamax^{TM} (2 mM, Life Technologies), heparin sodium salt (4 μg/ml; ACROS Organics), bFGF (10 ng/ml, Peprotech) and 100 U/100 μg/ml Penicillin/Streptomycin (Life Technologies) for 30 min, at 37°C. Vehicle (veh), thrombospondin-1 (TSP-1, 2000 ng/ml, Meridian Life Science) and pregabalin (PGB, 1 nM, Qventas) were then added to each well in triplicate. In parallel, hEGF (20 ng/ml, Peprotech) and LiCl (2 mM, Sigma-Aldrich) were used as positive controls as previously described (Bianchi et al., 2010; Stagni et al., 2017; Stagni et al., 2019b). Cell proliferation was quantified after 96 h incubation in a humidity chamber (to minimize evaporation) and quantified as relative luminescence units (RLU) values using a CellTiter-Glo kit (Promega) on a Victor^3-V plate reader (PerkinElmer) (Stagni et al., 2019b).

Neural progenitor cell differentiation

A phenotypic characterization was performed in order to evaluate NPC

differentiation ability in presence of differentiation medium (DMEM/F-12 medium, B27, Glutamax^{TM} and Penicillin/Streptomycin) or specific compounds following already validated protocols (Bortolotto et al., 2019; Cvijetic et al., 2017; Stagni et al., 2017). Briefly, neurospheres were dissociate into a single cell suspension and plated onto Nunc^{TM} LabTeK^{TM} chamber slides (Thermo Fisher Scientific) coated with laminin mouse protein (Thermo Fisher Scientific) at a density of $35x10^3$ cells per well in differentiation medium for 30 minutes. Cells were then exposed to vehicle (veh), LiCl (2 mM, Sigma-Aldrich), thrombospondin-1 (TSP-1, 2000 ng/ml, Meridian Life science), lipocalin-2 (LCN-2, 300 ng/ml, Cell Signaling), pregabalin (PGB, 1 nM, Qventas). For differentiation experiments in presence of selective inhibitors we used gabapentin (32 µM, Sigma-Aldrich); NSC23766, a selective Rac1 inhibitor (3 µM, Sigma-Aldrich); CK666, an Arp2/3 complex inhibitor (50 µM, MERCK) and the relative inactive control CK689 (50 µM, MERCK). In each experiment inhibitors were added 30 minutes before TSP-1 or PGB, as previously described (Valente et al. 2012). After 96 h cells were fixed for 20 min at room temperature using 4°C paraformaldehyde as previously described (Valente et al., 2012). Phenotypic characterization of NPC-derived cells was carried out by immunolocalization for MAP2 (rabbit polyclonal, 1:50,000; Abcam) and nestin (chicken monoclonal, 1:2,500; Neuromics). Secondary antibodies were used as follows: AlexaFluor555-conjugated goat anti rabbit (1:2,000; Molecular Probes), and AlexaFluor488-conjugated goat anti chicken (1:2,000; Molecular Probes). Nuclei were counterstained with 0.8 ng/ml Hoechst (Thermo Fisher Scientific) diluted in PBS. In each experiment, five fields/well (corresponding to about 150–200 cells/well) were counted with a 60X objective by a Leica DMIRB inverted fluorescence microscope. Immunoreactive cells were counted and their percentage over total viable cells was calculated. Apoptotic nuclei were calculated over total cells. All experiments were run in triplicate.

Protein isolation and western blot analysis

For protein isolation neural progenitor cells were disrupted in RIPA buffer (H_2O, 50 mM Tris-HCl pH 7.6, 150mM NaCl, 0.5 mM EDTA pH8, 1% (v/v) Triton X-100, 0.1% SDS, 10mM NaF, 1mM NaVO4, 1mM DTT and protease inhibitors, Sigma-Aldrich) as previously described (Cvijetic et al., 2017; Valente et al., 2012). Briefly, neurosphere homogenates were incubated on ice for 25 min. Then three serial incubations at -80°C (3 minutes) and 37°C (2 minutes) were performed. Lysates were centrifuged at 15,700 g for 10 min at 4°C and supernatants were collected. For protein isolation from day post-natal 1 (P 1) tissues, homogenates were disrupted in RIPA buffer by using a wheel at 4 °C as previously described (Denis-Donini et al., 2008). Briefly, tissue homogenates were incubated on ice for 60 min, then centrifuged at 15,700 g for 30 min at 4°C, then the supernatants were collected. Protein concentration was determined by Bradford assay (Sigma-Aldrich) and 25-30 μg of proteins were loaded for each sample. Protein separation was performed onto a 12% SDS-PAGE gel. Proteins were then transferred onto a nitrocellulose membrane. The membranes were blocked at RT in a 5% (wt/vol) milk in TBS-Tween20 0.01% solution for 1 hour. Immunoblots were carried out overnight at 4°C or 2h at RT in an antibody solution containing 5% (wt/vol) milk in TBS-T with the following antibodies: anti-Rac1 1:750 (mouse monoclonal, AB33186, Abcam), anti-Moesin 1:1,000 (rabbit monoclonal, AB52490, Abcam), anti-Dihydropyridine Receptor α2 subunit 1:500 (mouse monoclonal, D219, Sigma-Aldrich) and anti-βactin 1:1,000 (mouse monoclocal, Sigma-Aldrich). After washing, blots were incubated with a peroxidase-conjugated goat antimouse antibody (1:10,000, R&D systems) and a peroxidase-conjugated goat antirabbit antibody (1:10,000, R&D systems) for 60 minutes at RT and immunocomplexes were visualized by the Supersignal West Pico Chemiluminescent substrate (Thermo Scientific).

Densitometric analysis was performed using the Image Lab software system (Bio-Rad Laboratories) and each band was normalized to β-actin signal.

Primary astrocyte cultures and the astrocyte conditioned media

Primary mixed glial cultures were prepared from hippocampi of P1–2 Ts65Dn (trisomic, TS) and euploid (EU) pups. Cells were grown in DMEM high glucose, 10% FBS, 15 mM HEPES, 2 mM glutamine and 100 U/ml penicillin/streptomycin (Life Technologies) on 6 well plates coated with Poly-L-Lysine 0.04 mg/ml (Sigma Aldrich). When cells reached confluence (around 7 DIV), cells were detached using Trypsin/EDTA 0.25% (Life Technologies) and microglia was isolated from astrocytes as previously described (Rocchio et al., 2019; Tapella et al., 2019). Briefly, cells were labeled with antiCd11^{+} beads (Miltenyi Biotec) and passed through MS separation columns (Myltenyi Biotec) using the MACS separator (Myltenyi Biotec). The unlabeled cell fraction (astrocytes) was collected and plated onto Poly-L-Lysine coated wells (Sigma-Aldrich). When cell confluence was reached, cells were exposed to Neurobasal-A medium supplemented with B27, 2 mM L-glutamine and penicillin/streptomycin 100 U/ml (Life Technologies). After 48 h the astrocyte conditioned media (ACM) was collected as previously described (Cvijetic et al., 2017); astrocytes were detached, collected in ice-cold PBS and proteins were isolated and quantified, as described in the previous section.

Phenotypic characterization of astroglial cultures was performed by immunocytochemistry with antibodies against GFAP (mouse monoclonal, 1:600, Millipore) and CD11b (rat monoclonal; 1:150, Millipore) as described by Cvijetic et al. (Cvijetic et al., 2017).

TSP-1 measurements (ELISA)

Trombospondin-1 (TPS-1) levels were quantified in euploid (EU) and trisomic

(TS) astrocyte conditioned media (ACM) by ELISA following the vendor instructions (Mouse TSP-1 ELISA Kit, IK5156, Immunologial sciences). Briefly, standard, samples and control media were added to each well of a 96 well plate and incubated 90 minutes at 37°C. After that a biotin-detection antibody was added for 60 minutes at 37 °C. Wells were then washed and incubated with an HRP-Streptavidin (SABC) solution for 30 minutes at 37°C. A 3,3',5,5'-tetrametilbenzidina (TMB) substrate was then added for 7-15 minutes at 37°C, protected by light. The reaction was stopped and the absorbance was measured at 450 nm on a Victor^3-V plate reader (PerkinElmer). ELISA quantifications were normalized using the astrocytic protein content (µg), as previously described by Garcia et al. (Garcia et al., 2010)

Statistical analysis

Data were analyzed with GraphPad Prism 7.0®. The statistical analysis were performed using either a one-way ANOVA or a two-way ANOVA. Post hoc multiple comparisons were carried out using Tukey's test. In order to compare different proliferation and differentiation experiments a two way ANOVA followed by a Fisher's least significant difference (LSD) test was performed. For western blot and ELISA analyses a paired or unpaired student's t-test was performed. Results were considered statistically relevant with $p<0.05$.

RESULTS

Cell autonomous defects in trisomic neural progenitor cells

TS NPC *in vitro* are not only less proliferative (Stagni et al., 2019), but they show impaired neuronal phenotype acquisition when compared with EU NPC (Stagni et al., 2018). We seeded both EU/TS neonatal NPC under differentiative conditions in presence of vehicle or LiCl (2 mM). After 96 h, we evaluated the percentage of cells that were immunopositive for MAP2 (Microtubule Associated Protein 2, a marker of cells with a neuronal phenotype) and immunonegative for nestin (a marker of undifferentiated NPC; Bortolotto et al., 2019; Meneghini et al., 2010). When TS NPC were exposed to vehicle, we observed a statistically significant reduction in the percentage of $MAP2^{+}/nestin^{-}$ cells if compared to EU NPC (mean percentage decrease over vehicle-treated EU NPC: 32 % ; $p<0.05$ TS veh vs EU veh) (Fig. 1B). In the same experimental setting, LiCl increased the number $MAP2^{+}/nestin^{-}$ cells both in EU (mean percentage increase over vehicle-treated EU NPC: 59 % $p<0.001$ vs EU veh) and TS NPC (mean percentage increase over vehicle-treated TS NPC: 98 % ; $p<0.001$ vs TS veh), with no differences between genotypes ($p=0.1$). Altogether these results suggest that 2 mM LiCl corrects also the differentiative defects of TS and EU NPC.

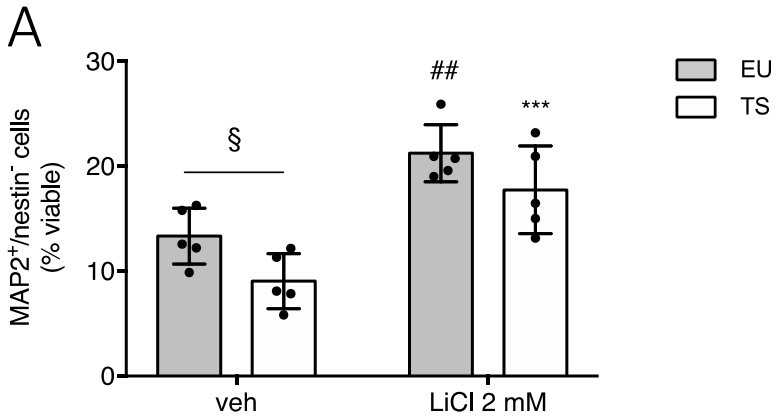

Figure 1. Neuronal differentiation in cultures of euploid (EU) and trisomic (TS) SVZ neural progenitor cells (NPC)

A. Cells are seeded in differentiation medium and experiments are performed on EU/TS NPC seeded in differentiating conditions in presence of vehicle (veh) or LiCl (2 mM). Data are expressed as percentage of $MAP2^+/nestin^-$ cells vs. total number of viable cells. Error bars represent the standard deviation of n=5 replicates, run in five different experiments and each dot represents the mean of three replicates. Results were considered statistically relevant with $p<0.05$ (## $p<0.01$; ### $p<0.001$ vs. vehicle-treated EU NPC; *$p<0.05$; *** $p<0.001$ vs. vehicle-treated TS NPC; Two way Anova, followed by Fisher LSD). § indicates a difference in neuronal differentiation rate between EU and TS genotypes (§ $p<0.05$ vs. vehicle-treated EU NPC; Two way Anova, followed by Fisher LSD test).

Non-cell autonomous defects of trisomic neural progenitor cells

In vivo NPC can communicate with several cell types, including astrocytes (Song et al., 2002). Astrocytes are secretory cells able to secrete a wide array of molecules that can affect progenitors in a non-cell autonomous way (Cvijetic et al., 2017). We isolated and cultured hippocampal (HP) astrocytes from P1-2 EU and TS pups, and we collected 48h-conditioned supernatant (astrocyte conditioned media, ACM). Thereafter, with the purpose of evaluating whether EU/TS ACM could affect neuronal differentiation (Fig. 2A) and apoptosis (Fig. 2B), we exposed both EU/TS NPC with EU/TS ACM. In all experiments ACM were compared to standard (STD) medium. After 24 h, we detected a decreased

percentage of $MAP2^+$/$nestin^-$ cells in STD-treated TS NPC, if compared to the corresponding EU NPC, confirming previous data that TS NPC produced less neurons than EU NPC (mean percentage of $MAP2^+$/$nestin^-$ cells ± S.D.: 6.1 ± 1 EU NPC and 2.9 ± 0.9 TS NPC with a mean percentage decrease: 52 % ; $p<0.001$ TS STD vs EU STD). EU ACM affected neuronal differentiation neither in EU nor in TS NPC. Conversely, TS ACM significantly reduced the percentage of $MAP2^+$/$nestin^-$ generated from EU NPC if compared to EU STD-treated cells (mean percentage of $MAP2^+$/$nestin^-$ cells ± S.D.: 6.1 ± 1 in STD-treated EU NPC and 3.7 ± 0.9 in TS ACM-treated EU NPC with a mean percentage decrease: 39 % ; $p<0.01$ vs EU STD), while did not affect TS NPC ($p>0.5$ TS ACM-treated TS NPC vs TS veh). Interestingly, we did not detect any difference between the two genotypes in presence of TS ACM ($p>0.05$), suggesting that TS ACM may potentially release signals (cytotoxic or antineurogenic) that negatively affect only EU NPC.

Under the same experimental setting, we evaluated apoptosis (Fig. 2B). We showed that, in STD-treated cells, no difference was reported between EU (mean percentage of apoptotic cells ± S.D: 21 ± 3.6) and TS NPC and progeny (mean percentage of apoptotic cells ± S.D: 17 ± 2.3), while both EU ACM and TS ACM significantly reduced the percentage of apoptotic cells both on EU and TS NPC and progeny. Based on these data, reduced neuronal differentiation in TS ACM-treated EU NPC cultures cannot be attributed to changes in cell survival.

In order to rule out a different time-dependent effect of EU/TS ACM on EU or TS NPC, we also exposed EU/TS SVZ-derived NPC to EU/TS ACM for 96 h. After 96 h, we obtained a result comparable to the one obtained at 24 h, with an exacerbated reduction of $MAP2^+$/$nestin^-$ cells only in SVZ-derived EU NPC in presence of TS ACM and, in parallel, a reduced apoptotic rate in TS ACM-treated SVZ-derived EU NPC. No effect of EU ACM was observed both in EU and TS SVZ-derived NPC *(data not shown).*

It is well known that astrocytes modulate NPC in a region-specific manner (Ma et al., 2009). Thus, EU/TS HP-derived NPC were exposed for 96 h to EU/TS HP-ACM or to STD medium and we evaluated the number of $MAP2^+/nestin^-$ cells over viable cells (Fig. 2C). Once again, as expected, we observed a reduced percentage of $MAP2^+/nestin^-$ cells in STD-treated TS HP-derived NPC if compared with STD-treated EU HP NPC (mean percentage decrease over STD-treated EU NPC: 32 % ; $p<0.05$ TS STD vs. EU STD). EU ACM significantly increased the percentage of $MAP2^+/nestin^-$ cells both in EU cells (mean percentage increase over vehicle-treated EU NPC: 60 %; $p<0.001$ EU ACM-treated EU NPC vs EU STD) and TS NPC (mean percentage increase over STD-treated TS NPC: + 107 %; $p<0.001$ EU ACM-treated TS NPC vs TS STD).

Conversely, TS ACM significantly reduced the number of $MAP2^+/nestin^-$ cells in EU NPC (mean percentage of $MAP2^+/nestin^-$ cells ± S. D.: 10.4 % ± 2.2 STD-treated EU NPC and 7 ± 1 % TS ACM-treated EU NPC, with a mean percentage decrease: 33 %; $p<0.05$ TS ACM-treated EU NPC vs EU veh), while did not affect the percentage of $MAP2^+/nestin^-$ TS cells. Surprisingly, TS ACM-treated EU NPC resulted significantly reduced if compared with TS ACM-treated TS NPC ($p<0.05$) meaning that TS ACM may contain molecules that affect neuronal differentiation of EU NPC in such a negative way that they differentiate less than TS ACM-treated TS NPC (Fig. 2C).

Regarding apoptosis (Fig. 2D), we observed no difference between EU and TS vehicle-treated cells, while both EU and TS ACM significantly reduced the number of apoptotic cells on EU ($p<0.001$) and TS NPC and progeny ($p<0.001$). Taken together, TS ACM, as EU ACM, reduces apoptosis in both EU and TS NPC and progeny, conversely, while EU ACM increases neuronal differentiation of both genotypes, TS ACM reduces neuronal differentiation selectively in EU NPC.

Recent evidence showed that astrocytes can affect NPC fate specification through the release of proneurogenic molecules such as thrombospondin-1 (TSP-1) (Clarke & Barres, 2013; Cvijetic et al., 2017). TSP-1 secretion is defective in human-derived and in murine trisomic astrocytes (Chen et al., 2014b; Garcia et al., 2010; Lu & Kipnis, 2010). We then evaluated, by ELISA, the levels of TSP-1 in EU and TS ACM. In line with literature data we detected reduced TSP-1 in TS ACM compared with EU ACM (mean percentage decrease: 59 % , $p<0.05$ vs EU ACM) (Fig. 2E).

Altogether these results demonstrated that: i) EU ACM positively affects neuronal differentiation of both EU and TS HP-derived NPC; ii) TS ACM, significantly reduces neuronal differentiation of EU NPC compared to STD medium; iii) TS ACM contains less TSP-1 compared to EU ACM; iiii) both EU and TS ACM reduced apoptotic rate of NPC and their progeny, regardless of their genotype.

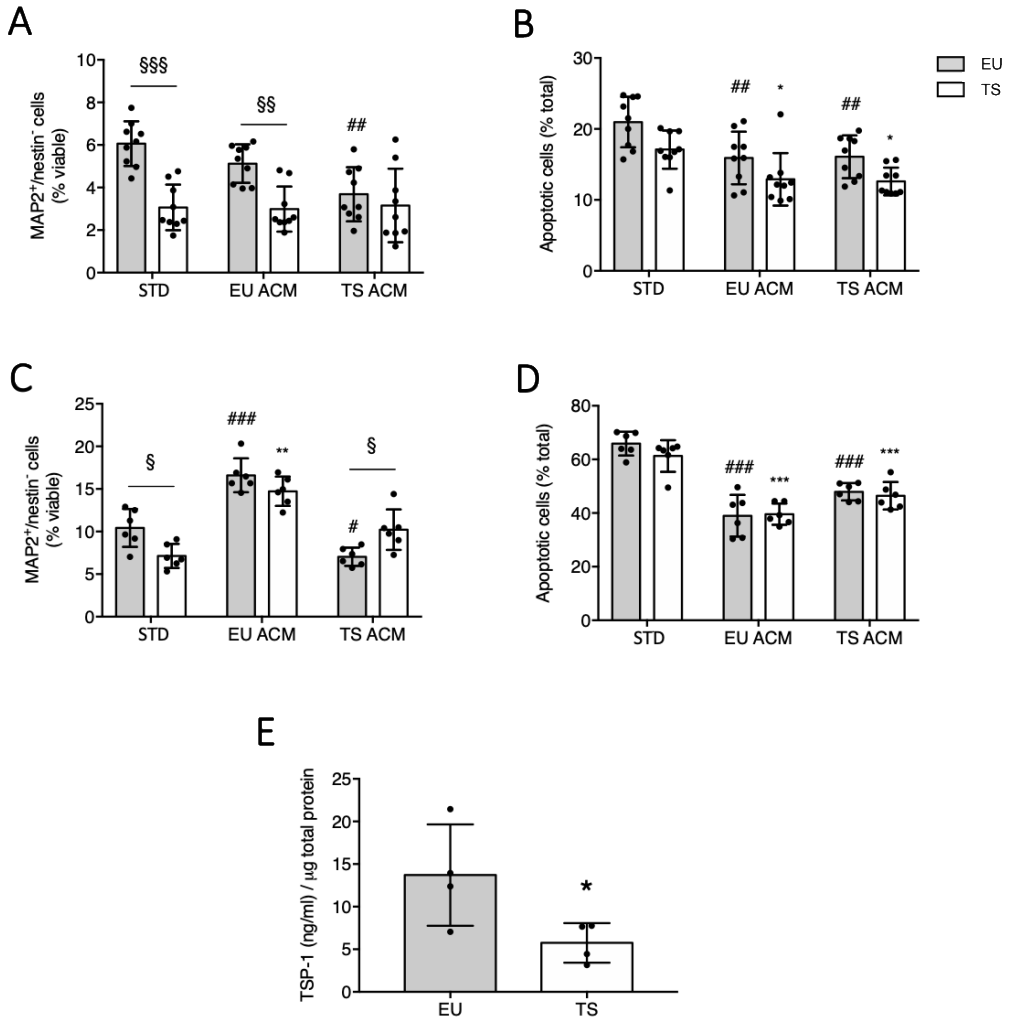

Figure 2. Effect of the astrocyte conditioned media (ACM) on euploid (EU) or trisomic (TS) neonatal neural progenitor cells (NPC)

Differentiation experiments were performed on euploid (EU, grey bar) and trisomic (TS, white bar) SVZ- (**A**-**B**) and HP- (**C**-**D**) derived NPC. **A.** EU/TS SVZ NPC are seeded in differentiating conditions in presence of vehicle (veh) or EU/TS ACM for 24 h. Data are expressed as percentage of $MAP2^+/nestin^-$ cells over viable cells. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate. **B.** EU/TS SVZ NPC are seeded in differentiating conditions in presence of vehicle (veh) or EU/TS ACM for 24 h. Data are expressed as percentage of apoptotic cells over total cells. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate. **C.** EU and TS hippocampal (HP) NPC are seeded in differentiating conditions

in presence of vehicle (veh) or EU/TS ACM for 96 h. Data are expressed as percentage of $MAP2^+/nestin^-$ cells vs the total number of viable cells. Error bars represent the standard deviation of n=6 replicates, run in two different experiments and each dot represents a single replicate. **D.** EU/TS HP are seeded in differentiating conditions in presence of vehicle (veh) or EU/TS ACM for 96 h. Data are expressed as percentage of apoptotic cells over total cells. Error bars represent the standard deviation of n=6 replicates, run in two different experiments and each dot represents a single replicate. Results are considered statistically relevant with $p<0.05$ (# $p<0.05$, ## $p<0.01$, ### $p<0.001$ vs. vehicle-treated EU NPC; ** $p<0.01$, *** $p<0.001$ vs. vehicle-treated TS NPC; Two way Anova, followed by Tukey's post hoc). The § indicate a difference in neuronal differentiation rate between EU and TS NPC (§ $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$; Two way Anova, followed by Tukey's post hoc vs. vehicle or EU/TS ACM **E.** ELISA quantification of TSP-1 detected in EU and TS ACM. Error bars represent the standard deviation of n=4 replicates, run in four different experiments and each dot represents the mean of a duplicate. The experiments were done using four different astrocyte preparations. Data are expressed as ng/ml over the total µg of astrocytic proteins and each dot is the mean of one ELISA experiment done in duplicate (* $p<0.05$ vs. EU ACM; student's t-test).

Thrombospondin-1 promotes *in vitro* neurogenesis in euploid, but not in trisomic NPC

In order to investigate whether TSP-1 differentially affected EU and TS NPC neuronal differentiation, we treated them in presence of a concentration range of TSP-1 (100-2000 ng/ml) or vehicle for 96 h (Fig. 3A-D). Compared to vehicle, TSP-1 100 ng/ml was ineffective in EU ($p=0.33$) and in TS NPC ($p=0.39$). Conversely, higher concentrations of the protein (250-500-2000 ng/ml) resulted in a concentration-dependent increase in the percentage of $MAP2^+/nestin^-$ cells, compared with vehicle, in EU NPC (mean percentage increase ± S.D over vehicle-treated EU NPC: 59 ± 22 , 60 ± 31, 57 ± 19 ; $p<0.001$ vs EU veh) (Fig. 3A). In the same experimental setting, LiCl (2 mM) increased the percentage of $MAP2^+/nestin^-$ EU cells (mean percentage increase ± S.D. over vehicle-treated EU NPC: 61 ± 20 SD ; $p<0.001$ vs EU veh).

When TS NPC were exposed to TSP-1, we detected no increase in the percentage of $MAP2^+$/$nestin^-$ cells at any of the tested concentrations ($p>0.5$ TSP1-treated vs vehicle-treated TS cells) (Fig. 3B). As expected, TS NPC were responsive to LiCl (mean percentage increase ± S.D. over vehicle-treated TS NPC: 67 ± 50 ; $p<0.001$ vs TS veh) (Fig. 3A, B). Representative confocal microscopy images of EU $MAP2^+$ cells in presence of vehicle or 2000 ng/ml TSP-1 are shown in figure 3C, D.

Previous findings in our laboratory proposed that lipocalin-2 (LCN-2), another astrocyte-secreted factor, enhances neuronal differentiation in adult hippocampal NPC (ahNPC) (Cvijetic et al., 2017). We treated both EU/TS SVZ-derived NPC with LCN-2 (300 ng/ml. After 96 h LCN-2 significantly increased the number of $MAP2^+$/$nestin^-$ cells both in EU cells (mean percentage increase ± S.D. over vehicle-treated EU NPC: 69 ± 17; $p<0.001$ vs EU veh) and TS cells (mean percentage increase ± S.D. over vehicle-treated TS NPC: 53 ± 13 ; $p<0.001$ vs TS veh). In the same experimental condition, LiCl enhanced the number of $MAP2^+$/$nestin^-$ cells both in EU and TS NPC (mean percentage increase ± S.D. over vehicle: 66 ± 10 and 62 ± 29 in EU and TS NPC, respectively; $p<0.001$vs corresponding veh) (Fig. 3E, F).

Altogether these data demonstrate that TS NPC are selectively unresponsive to the proneurogenic effect of TSP-1, but not of another astrocyte-secreted molecule like LCN-2.

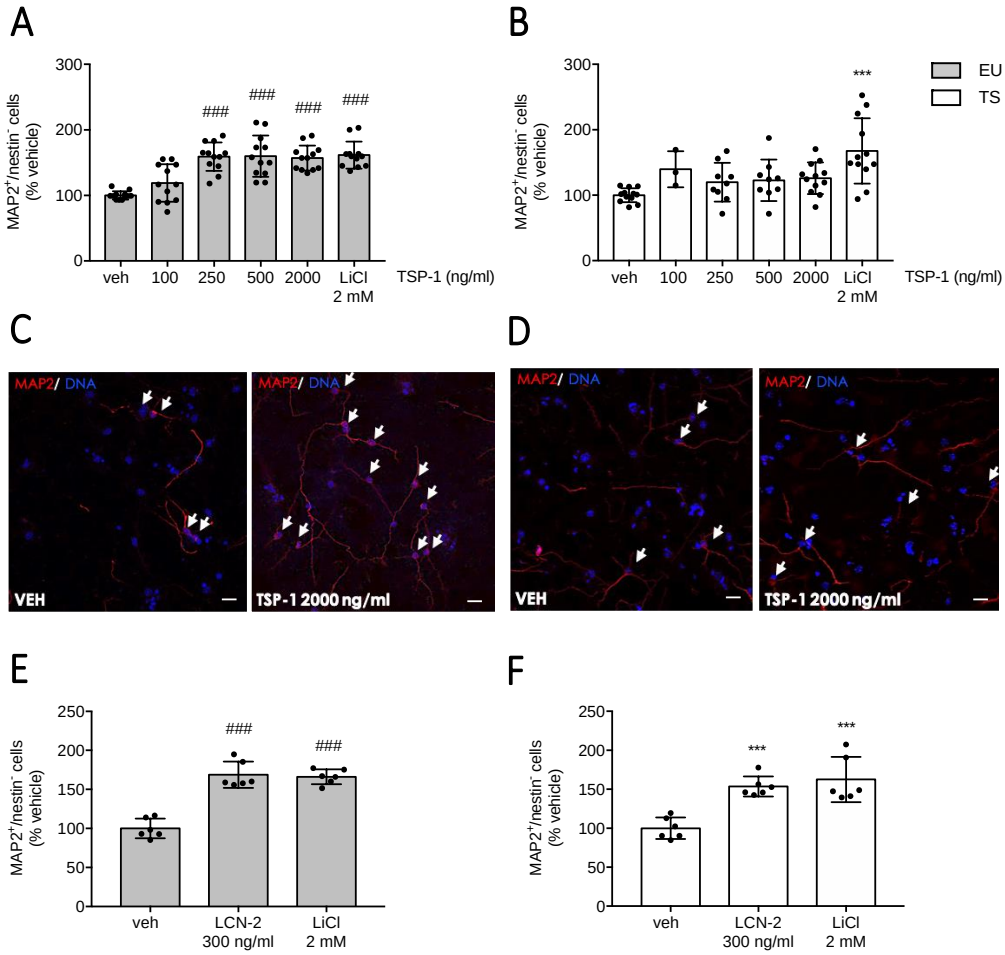

Figure 3. Effect of thrombospondin-1 (TSP-1) and lipocalin-2 (LCN-2) in neonatal euploid and trisomic SVZ neural progenitor cells

Differentiation experiments are performed on euploid (EU, grey bar) and trisomic (TS, white bar) SVZ-derived NPC. **A, B.** EU/TS NPC are seeded in differentiating conditions in presence of vehicle (veh), thrombospondin-1 (TSP-1, 100-2000 ng/ml) or lithium chloride (LiCl, 2 mM) for 96 h. Data are expressed as percentage of $MAP2^+$/$nestin^-$ cells normalized over vehicle–treated condition. For EU NPC, error bars represent the standard deviation of n=12 replicates, run in four different experiments and each dot represents a single replicate. For TS NPC, error bars represent the standard deviation of n=9 or n=3 replicates, run in three or one experiments. Each dot represents a single replicate. **C, D.** Representative confocal microscope images of $MAP2^+$ cells (red) in cultures of EU SVZ-derived NPC (C) and TS SVZ-derived NPC (D) in presence of vehicle (veh) or TSP-1 (2000 ng/ml). Nuclei are stained with DAPI (blue, DNA). White arrows

indicate positive cells. Scale bar=20 µm. **E, F.** EU/TS NPC are seeded in differentiating conditions in presence of vehicle (veh), lipocalin-2 (LCN-2, 300 ng/ml) or LiCl (2 mM) for 96 h. Data are expressed as percentage of $MAP2^+/nestin^-$ cells normalized over vehicle-treated condition. Error bars represent the standard deviation of n=6 replicates, run in two different experiments and each dot represents a single replicate for EU NPC. Results are considered statistically relevant with $p<0.05$ (### $p<0.001$ vs. vehicle-treated EU NPC; *** $p<0.001$ vs. vehicle-treated TS cells; One way Anova, followed by Tukey's post hoc).

The alpha2delta1 (α2δ1) subunit is responsible for TSP-1 mediated proneurogenic effects

TSP-1 binds several interactors (Resovi et al., 2014), including the α2δ1 subunit (Dolphin, 2013). Previous work in our laboratory showed that the anticonvulsant, anxiolytic and antihyperalgesic drug pregabalin (PGB), a known ligand of the α2δ1 subunit of voltage dependent calcium channels (Taylor et al., 2007), is able to increase adult hippocampal neurogenesis *in vitro* and *in vivo* and that these effects are mediated by α2δ1, expressed on the surface of ahNPC (Valente et al., 2012). Thus, in order to investigate whether PGB affected neuronal differentiation differentially in EU and TS NPC cultures, we treated cells with 1 nM concentration of the drug for 96 h (Valente et al., 2012). We observed that PGB increased the number of NPC-derived neurons in EU cultures (mean percentage increase ± S.D. over vehicle: 51 ± 18 ; $p<0.001$ vs EU veh) (Fig. 4A). Conversely, when TS NPC were exposed to the drug no effect was observed on the percentage of $MAP2^+/nestin^-$ cells (Fig. 4A). These data demonstrated TS NPC cultures were insensitive not only to TSP-1, but also to PGB proneurogenic effects. These findings suggested that the lack of TSP-1 and PGB effects in TS NPC may be linked to a defective α2δ1 signalling. In order to further investigate the molecular mechanisms underlying TSP-1 proneurogenic effect, we focused our efforts on EU NPC.

Gabapentin (GBP), another α2δ1 ligand, also binds the α2δ1 subunit (Valente et al., 2012). Past evidence showed that at high concentrations (32 μM) GBP antagonizes formation of excitatory synapses mediated by TSP1-α2δ1 interaction (Eroglu et al., 2009). Although TSP-1 synaptogenic and proneurogenic effects could be mediated by different mechanisms, we exposed EU NPC to the maximally effective concentration of TSP-1 (2000 ng/ml), in presence of vehicle or GBP (32 μM). As expected, we observed a statistically significant increase in the percentage of $MAP2^+/nestin^-$ cells in presence of TSP-1 (mean percentage increase ± S.D. over vehicle-treated EU NPC: 61 ± 39; $p<0.001$ vs EU veh) and this effect was completely counteracted in presence of GBP ($p<0.05$ PGB vs PGB+GBP). GBP alone had no effect on neuronal differentiation ($p>0.05$ if compared with vehicle-treated EU NPC). GBP counteracted also 1 nM PGB-induced increase of $MAP2^+/nestin^-$ (mean percentage increase over vehicle: 85 ± 19; $p<0.001$ PGB vs veh and 11 ± 22; $p<0.05$ PGB vs PGB+GBP). As expected, LiCl promoted an increase in the percentage of $MAP2^+/nestin^-$ cells (mean percentage increase ± S.D. over vehicle: 61 ± 47 ; $p<0.001$ vs veh) (Fig. 4C).

Taken together these data strongly suggested an involvement of the α2δ1 subunit in the proneurogenic effects mediated by PGB and TSP-1 in EU NPC.

Data in literature demonstrated that TSP-1 can affect not only neuronal differentiation, but also proliferation of adult NPC (Lu & Kipnis, 2010). In order to evaluate whether TSP-1 and PGB affected proliferation, we treated both EU and TS NPC with TSP-1 (2000 ng/ml) and PGB (1 nM). In our experimental conditions both drugs were ineffective in EU (Fig. 4D) and TS NPC (Fig. 4E).

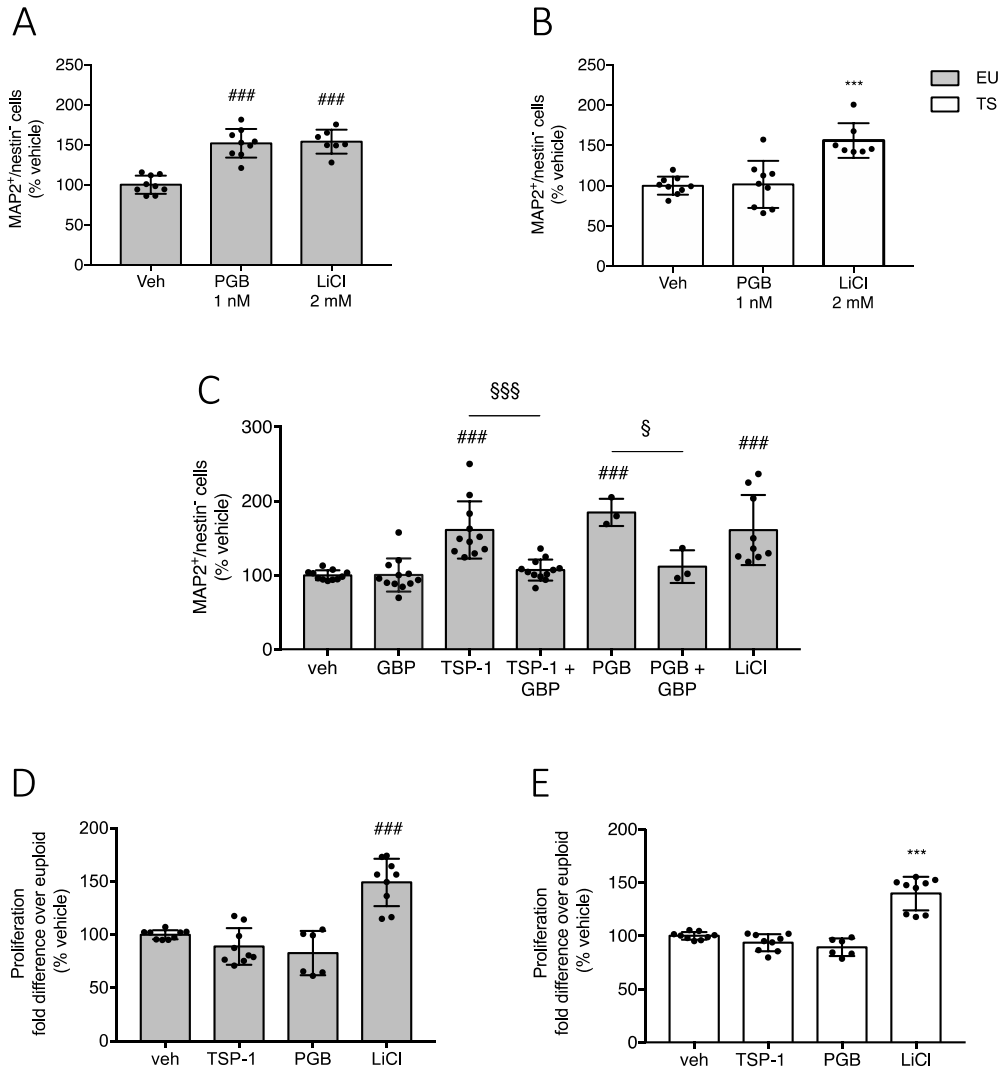

Figure 4. Effect of alpha2delta1 ligands in euploid neonatal SVZ neural progenitor cells

A-C. Differentiation experiments are performed on euploid (EU, grey bar) and trisomic (TS, white bar) SVZ-derived NPC. **A, B.** EU/TS NPC are seeded in differentiating conditions in presence of vehicle (veh), pregabalin (PGB, 1 nM) or lithium chloride (LiCl, 2 mM) for 96 h. Data are expressed as percentage of $MAP2^{+}/nestin^{-}$ cells normalized over vehicle. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate. **C.** EU NPC are seeded in differentiating conditions in presence of vehicle (veh), gabapentin (GBP, 32 μM), TSP-1 (2000 ng/ml), PGB (1 nM), LiCl (2 mM) or the cotreatments (TSP-1+GBP or PGB+GBP) for 96 h. Data are expressed as percentage of

$MAP2^+/nestin^-$ cells normalized over vehicle. Error bars represent the standard deviation of n=3, 9, 12 replicates, run in one, three, four different experiments and each dot represents a single replicate. **D, E.** EU/TS NPC are seeded in medium containing 10 ng/ml FGF and exposed to vehicle (veh), TSP-1(2000 ng/ml), PGB (1 nM) or LiCl (2 mM). Data are expressed as difference over vehicle-treated cells. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate for EU NPC. Results are considered statistically relevant with $p<0.05$ (### $p<0.001$ vs. vehicle-treated EU NPC; *** $p<0.001$ vs. vehicle-treated TS cells; One way Anova, followed by Tukey's post hoc). The § indicate a difference between the different conditions (§ $p<0.05$ PGB-treated vs. PGB+GBP, §§§ $p<0.001$, TSP1-treated cells vs. TSP1+GBP; One way Anova, followed by Tukey's post hoc).

α2δ1 expression levels are reduced in trisomic neural progenitor cells

We investigated whether α2δ1 expression levels was differently expressed in EU and TS SVZ-derived and HP-derived NPC. By western blotting we showed that α2δ1 was downregulated both in TS SVZ- and HP-derived NPC if compared with the corresponding EU NPC (mean percentage of reduction: 41 % and 32 %, respectively; $p<0.05$ TS vs EU) (Fig. 5A, B, E). Conversely, we observed no significative difference in α2δ1 expression levels in neonatal brain tissue at least at postnatal day 1, both in HP (Fig. 5C) and CX (Fig. 5D). These data suggested that the expression of α2δ1 may be selectively reduced in TS neural progenitor cells.

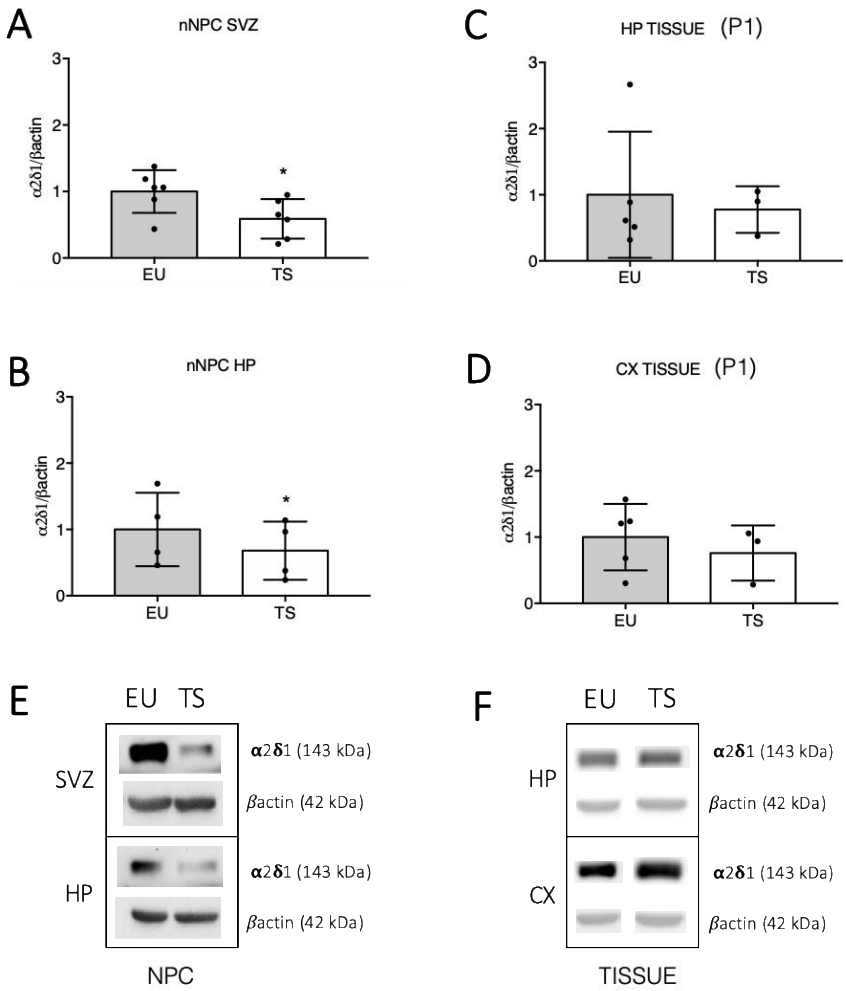

Figure 5. Western blot analyses to evaluate α2δ1 expression in neonatal SVZ and HP neural progenitor cells and in neonatal brain tissues

Western blot (WB) analyses are performed in neonatal EU (grey bar) and TS (white bar) NPC from the subventricular zone (SVZ) and hippocampi (HP). **A.** α2δ1 expression levels are examined in six different EU/TS SVZ-derived NPC preparations collected at different passages (4-10). α2δ1 protein levels are normalized over ß-actin and expressed as fold difference in comparison with EU NPC. Error bars represent the standard deviation of n=6 replicates, run in six different experiments and each dot represents a single NPC preparation. **B.** Expression levels of α2δ1 are examined in four different EU/TS HP NPC preparations collected at different passages (4-10). α2δ1 expression levels are normalized over ß-actin and expressed as fold difference in comparison with EU NPC. Error bars represent the standard deviation of n=4

replicates, run in four different experiments and each dot represents a single NPC preparation. **C.** Levels of α2δ1 were examined in hippocampi (HP) isolated from postnatal day 1 TS (n=3) and EU (n=5) pups. Error bars represent the standard deviation of n=5 (EU pups) and n=3 (TS pups), run in one experiment and each dot represents a single animal. **D.** Expression Levels of α2δ1 were examined in cortex (CX) isolated from postnatal day 1 TS (n=3) and EU (n=5) pups. Error bars represent the standard deviation of n=5 (EU pups) and n=3 (TS pups), run in one experiment and each dot represents a single animal. **E, F.** Representative immunoblot analysis of α2δ1 subunit (143 kDa) and ß-actin (42 kDa) in NPC (E) and neonatal tissues (F). Results were considered statistically relevant with $p<0.05$ and asterisks indicate a difference in comparison with EU NPC or EU tissue (* $p<0.05$, student's t-test).

Rac1 activation is required for the proneurogenic effect of TSP-1 in EU NPC

TSP-1 controls the rearrangement of the actin cytoskeleton that plays a pivotal role in synapse formation and dendritic morphology, in particular by activating Rho GTPases (Risher & Eroglu, 2012). Recent evidence suggested that the small Rho GTPase Rac1 (Ras-related C3 botulinum toxin substrate 1) is a key component of the signaling pathway initiated by TSP1-α2δ1 association at least in the formation of new synapses (Risher et al., 2018). No information is currently available on the possibility that Rac1 activation may also be involved in the proneurogenic effects of TSP-1. Thus, in order to investigate the role of Rac1 in the proneurogenic effect mediated by TSP1-α2δ1 interaction, we exposed EU NPC to TSP-1 (2000 ng/ml) or PGB (1 nM), in presence of NSC23766 (3 µM), a selective Rac1 inhibitor or vehicle. NSC23766 alone had no effect on neuronal differentiation of EU NPC ($p>0.05$ N vs veh). As expected, TSP-1 significantly increased the percentage of $MAP2^{+}$/$nestin^{-}$ cells (mean percentage increase over vehicle-treated EU NPC: 96 ± 40 ; $p<0.001$ vs veh), and this effect was completely prevented by co-treatment with Rac1 inhibitor (mean percentage increase ± S.D. over vehicle-treated EU NPC: 24 ± 28 ; $p<0.001$ TSP-1 vs TSP-1+N) (Fig. 6A). In parallel, we observed that the Rac1 inhibitor completely

counteracted PGB-induced increase of $MAP2^+$/$nestin^-$ cells ($p<0.01$ PGB vs PGB+N) (Fig. 6B).

These data suggested that Rac1 activation may be potentially involved in the proneurogenic effect elicited by TSP-1 and PGB in EU NPC. In order to evaluate Rac1 expression levels in EU and TS SVZ- and HP-derived NPC we performed a western blot analysis. No difference in Rac1 expression levels were observed in SVZ (n=6; p=0.7) (Fig. 6C) and HP NPC of both genotypes (n=4; p=0.2) (Fig. 6D). Similar results were obtained in neonatal EU/TS HP and CX tissues (*data not shown).* These data exclude the possibility that TSP-1 and PGB are ineffective in TS NPC due to reduced expression levels of Rac1.

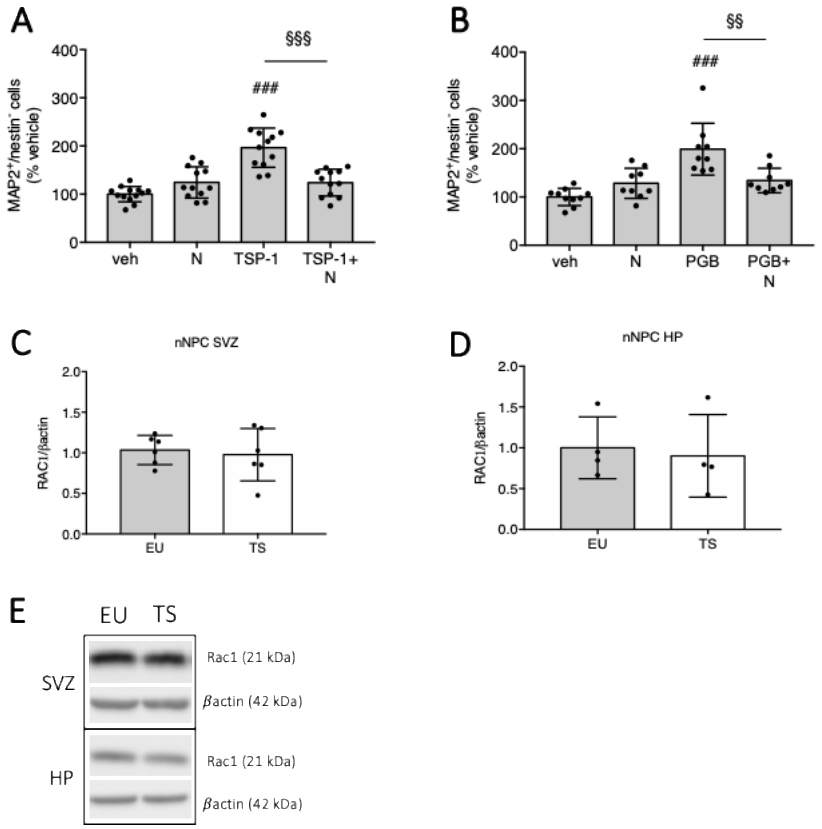

Figure 6. Effect of Rac1 inhibition on the proneurogenic effects of TSP-1 and PGB in EU NPC.

A, B. Differentiation experiments are performed in euploid (EU, grey bar) SVZ NPC. **A.** EU NPC

are seeded in differentiating conditions in presence of vehicle (veh, 0.05% DMSO), Rac1 selective inhibitor NSC23766 (N, 3 µM), TSP-1 (2000 ng/ml) or NSC23766 (N) + TSP-1 for 96 h. Data are expressed as percentage of $MAP2^+/nestin^-$ cells over vehicle. Error bars represent the standard deviation of n=12 replicates, run in four different experiments and each dot represents a single replicate. **B.** EU NPC are seeded in differentiation condition in presence of vehicle (veh, 0.05% DMSO), NSC23766 (N, 3 µM), PGB (1 nM) or NSC23766 (N) + PGB for 96 h. Data are expressed as percentage of $MAP2^+/nestin^-$ cells over vehicle. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate. **C.** Expression Levels of Rac1 were examined in six different EU/TS SVZ-derived NPC and **D.** in four different EU/TS HP-derived NPC preparations collected at different passages. Expression levels are normalized over ß-actin. Error bars represent the standard deviation of n=6 (EU/TS SVZ-derived NPC) or n=4 (EU/TS HP-derived NPC) replicates, run in one different experiment and each dot represents a single NPC preparation. **E.** Representative immunoblot showing immunoreactivity for Rac1 (21 kDa) and ß-actin (42 kDa) in SVZ NPC (up) and HP NPC (down). Results are considered statistically relevant with $p<0.05$ (# $p<0.05$; ### $p<0.001$ vs. vehicle-treated EU NPC; One way Anova, followed by Tukey's post hoc). The § indicate a difference between conditions (§§ $p<0.01$ vs. PGB-treated EU NPC, §§§ $p<0.001$, vs. TSP1-treated EU NPC; One way Anova, followed by Tukey's post hoc).

ARP2/3 complex activation is required for TSP-1 and PGB proneurogenic effects

Previous evidence demonstrated a downregulation in DS fetal brain of the actin-related protein complex 2/3 (ARP 2/3) (Weitzdoerfer et al., 2002). This complex is involved in the formation of neuronal cytoskeleton and importantly ARP2/3 could be activated downstream to Rac1 (Chen et al., 2017). Thereby, with the purpose to investigate whether ARP 2/3 complex was involved in the signaling activated by TSP1-α2δ1 interaction, we treated EU NPC with TSP-1 (2000 ng/ml), in presence of CK666, a selective inhibitor of the ARP2-3 complex (50 µM), CK689, its inactive control (50 µM) (Ilatovskaya et al., 2013) or the corresponding vehicle under differentiative conditions. When EU NPC were exposed to CK666 alone no signifcant effect was observed, compared to vehicle.

As expected, TSP-1 significantly promoted EU NPC neuronal differentiation (mean percentage increase ± S.D. over vehicle-treated EU NPC: 71 ± 32 ; $p<0.001$ vs veh) and such effect was counteracted by CK666 co-treatment ($p<0.05$ TSP-1+CK666 vs TSP-1) (Fig.7A). Under the same experimental setting, the inactive control CK689 had no effect alone and did not affect TSP1 proneurogenic effect on EU NPC (mean percentage increase ± S.D. over vehicle-treated NPC: 64 ± 28 ; $p<0.001$ TSP-1+CK689 vs veh or CK689) (Fig 7B).
Under the same conditions, CK666 also prevented the PGB-induced increase in the number of NPC-derived neurons ($p<0.001$ PGB+CK666 vs PGB). CK689 was devoid of effects (mean percentage increase ± over vehicle-treated NPC: 72 ± 23; $p<0.001$ PGB+CK689 vs PGB or CK689) (Fig. 7 C, D). Altogether these results suggested that ARP2-3 complex inhibition prevents the proneurogenic effect of TSP-1 and PGB and that AR2-3 complex activation lies downstream of $\alpha2\delta1$ engagement by these ligands.

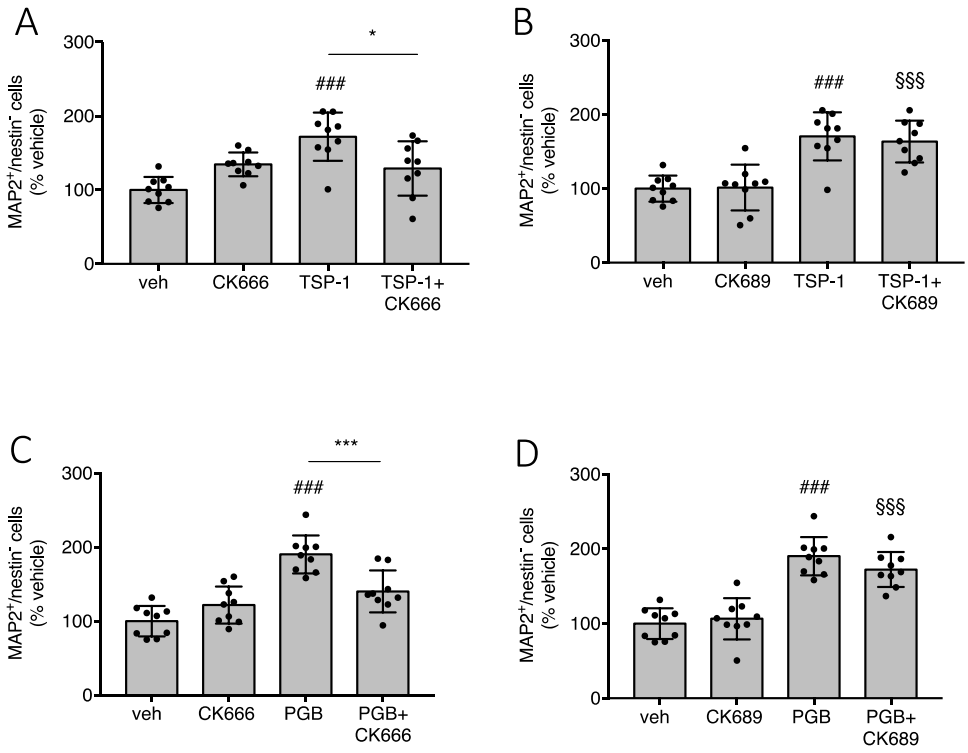

Figure 7. Effect of ARP2-3 complex inhibition on TSP-1 and PGB proneurogenic effects on EU NPC.

A. Differentiation experiments are performed on EU SVZ-derived NPC. **A.** EU NPC are seeded in differentiating conditions in presence of vehicle (veh, 0.05% DMSO), ARP2/3 complex inhibitor CK666 (50 µM), TSP-1 (2000 ng/ml) or CK666 + TSP-1 for 96 h. Data are expressed as percentage of $MAP2^+/nestin^-$ cells normalized over vehicle. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate. **B.** EU NPC are seeded in differentiating conditions in presence of vehicle (veh, 0.05% DMSO), the inactive control CK689 (50 µM), TSP-1 (2000 ng/ml) or CK689 +TSP-1 for 96 h. Data are expressed as percentage of $MAP2^+/nestin^-$ cells normalized over vehicle. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate. **C.** EU NPC are seeded in differentiating conditions in presence of vehicle (veh, 0.05% DMSO), CK666 (50 µM), PGB (1 nM) or CK666 + PGB for 96 h. Data are expressed as percentage of $MAP2^+/nestin^-$ normalized over vehicle. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate. **D.** EU NPC are seeded in differentiating conditions in presence of vehicle (veh,

0.05% DMSO), CK689 (50 µM), PGB (1 nM) and CK689 + PGB for 96 h. Data are expressed as percentage of $MAP2^+/nestin^-$ normalized over vehicle. Error bars represent the standard deviation of n=9 replicates, run in three different experiments and each dot represents a single replicate. Results are considered statistically relevant with $p<0.05$ (### $p<0.001$ vs. vehicle-treated EU NPC; §§§ $p<0.001$ vs. CK689-treated EU NPC; One way Anova, followed by Tukey's post hoc). Asterisks indicate a difference between conditions (* $p<0.05$, *** $p<0.001$ vs. TSP-1 or PGB; One way Anova, followed by Tukey's post hoc).

DISCUSSION

In this chapter I have presented some unpublished and still preliminary results. Based on the data obtained, we confirmed, once again, that neonatal trisomic NPC showed *in vitro* phenotypic defects, including reduced NPC proliferation *(data not shown)* and a decreased neuronal differentiation, if compared with euploid cells. We also confirmed that these cell-autonomous defects could be corrected by lithium chloride, a well-established neurogenesis enhancer both *in vivo* (Bianchi et al., 2010; Contestabile et al., 2013) and *in vitro* (Stagni et al., 2019b).

Since recent evidence of astrocytic alterations in DS (Chen et al., 2013), we decided to focus our attention on the possibility that dysfunctional communication between NPC and astrocytes could also contribute to DS pathophysiology. Using astrocyte conditioned media (ACM) collected from euploid and trisomic hippocampal astrocytes, we observed that trisomic ACM negatively affected neuronal differentiation in euploid NPC. These results were confirmed both in SVZ-derived NPC and in HP-derived NPC. Despite Chen et al. showed that human trisomic astrocytes negatively affect neuronal differentiation of human derived trisomic NPC (Chen et al., 2013; Cresto et al., 2019), in our study we did not observe negative effects of trisomic ACM on trisomic NPC.

Under the same experimental conditions, we observed a proneurogenic effect of euploid ACM on euploid hippocampal NPC, as previously shown (Cvijetic et al., 2017; Song et al., 2002) and on trisomic hippocampal NPC in agreement with previous studies (Chen et al., 2014; Garcia et al., 2010). Not surprisingly, EU ACM had no proneurogenic effect in SVZ-derived NPC, regardless of their genotype, likely for the hippocampal source of the astrocyte used for ACM collection (Decimo et al., 2012; Ma et al., 2009). On the other hand, since trisomic ACM reduced neuronal differentiation both in SVZ and HP derived EU NPC, this may suggest that antineurogenic molecules released by trisomic hippocampal astrocytes exert their negative effects regardless of NPC origin in terms of neurogenic niche.

In parallel, a reduction in the apoptotic rate was observed in EU and TS ACM-treated EU and TS NPC cultures, compared to STD medium. Altogether these data suggested that: i) as previously shown (Cvijetic et al., 2017), ACM elicit prosurvival effects on NPC and their progeny, regardless of their genotype; ii) that the negative effect of trisomic ACM on neuronal differentiation of euploid cells could be due to the presence of antineurogenic molecules. At present we have no evidence on the nature of such astrocyte-derived molecule(s). In the future studies should be undertaken to unravel secretome composition of trisomic astrocytes.

During this project we rather concentrated our attention on a matricellular protein, thrombospondin-1 (TSP-1), a molecule whose secretion has been demonstrated to be defective in murine and human trisomic astrocytes (Garcia et al., 2010; Torres et al., 2018). We confirmed that also in our hands, TS ACM contained less TSP-1 compared to EU ACM.

TSP-1 is a key astrocytic signal involved in neurogenesis (Cvijetic et al., 2017; Lu & Kipnis, 2010), widely expressed during brain development (Meiniel et al., 2003). For the first time we showed that trisomic NPC do not respond to the proneurogenic effect of TSP-1 and also of pregabalin, a clinically relevant drug known to bind the α2δ1 subunit (Dolphin, 2013; Valente et al., 2012). Since TSP-1 binds α2δ1 as well and our group demonstrated that the subunit is expressed by NPC and mediates both TSP-1 and PGB proneurogenic effects (Valente et al., 2012; Cvijetic et al., 2017), we hypothesize that defective α2δ1 signalling in TS NPC. α2δ1 is a regulatory subunit of neuronal voltage-dependent calcium channels (VGCC, Dolphin, 2013), but several lines of research have proposed that some of its ligand-mediated effects can be elicited in a VGCC-independent manner (Eroglu et al., 2009). Also in our *in vitro* model, preliminary experiments done in the lab showed that TSP-1 and PGB did not activate a calcium response in EU and TS NPC *(data not shown)*, supporting the hypothesis that in this cell type α2δ1 can be engaged by its ligands in a VGCC independent way.

Investigating the molecular mechanisms underlying the proneurogenic effect of TSP-1, we showed that: i) another α2δ1 ligand, gabapentin (GBP, 32 μM) was able to block the proneurogenic effect of both TSP-1 and PGB in EU NPC (Eroglu et al., 2009; Risher et al., 2018); ii) α2δ1 expression was downregulated in trisomic compared to EU NPC. Taken together these data support the idea that in euploid NPC the proneurogenic effect of TSP-1 is mediated by the interaction with α2δ1 and that reduced expression of α2δ1 in trisomic cells may, at least in part, contribute to their lack of proneurogenic response to TSP-1 and PGB. Since TS NPC are insensitive to such ligands we hypothesized that additional defects in α2δ1 signalling may occur in these cells.

In principle, defects in α2δ1 signalling in TS NPC may lie in disrupted association of α2δ1 with its interactors (Risher & Eroglu, 2012) and/or impaired

activation of downstream signaling. We showed that pharmacological inhibition of Rac1, a Rho GTPase protein downstream α2δ1 subunit, counteracted the proneurogenic effect of TSP-1 and PGB in euploid NPC. Our data are consistent with other studies that showed that prosinaptogenic effects of TSP-1 are mediated by the activation of Rac1 (Risher et al., 2018). Rac1 is a small GTPase existing in two forms: an inactive form (GDP-bound) and an active form (GTP-bound) (Tejada-Simon, 2015). The relationship between Rac1 inactivated and activated states is important for proper interaction of Rac1 with other targets downstream the signaling pathway. Moreover, Rac1 activated moves from the cytosol to the membrane where it could interact with several proteins (Tejada-Simon, 2015), including α2δ1 (Risher et al., 2018).

There is evidence showing that Rac1 is upstream of the actin-related protein complex 2/3 (ARP 2-3) complex activation (Chen et al., 2017). ARP-2/3 complex is also important for brain development and studies showed that it is downregulated in DS fetal brain (Weitzdoerfer et al., 2002). Here we showed that pharmacological inhibition of ARP 2/3 counteracted the proneurogenic effect mediated by TSP-1 and PGB.

Based on reported evidence, altogether we demonstrated that in EU NPC TSP-1 and PGB proneurogenic effects are mediated by interaction with α2δ1, and that this effect requires activation of Rac1 and of ARP2/3, once activated ARP2/3 binds pre-existing actin filaments in order to allow the new filaments to grow on the old ones and form a functional actin cytoskeleton (Ilatovskaya et al., 2013). Impaired actin filament assembling leads to neuronal abnormalities (Griesi-Oliveira et al., 2018).

At present we have no direct and solid evidence of dysfunctional rac1 signaling in trisomic NPC and their progeny. Two distinct hypothesis could be raised: i) differential expression levels of Rac1 protein in TS compared to EU NPC; ii)

impaired Rac1 activation in TS NPC. Here we demonstrated no difference in Rac1 expression levels in NPC of both genotypes. In very preliminary experiments *(data not shown)*, we observed activation of Rac1 in presence of TSP-1 in EU but not in TS NPC. Although further experiments are needed to dissect Rac1 activity in basal conditions and in presence of both TSP-1 and PGB, both in EU and TS NPC, at the current stage of knowledge we hypothesize impaired activition of Rac1 in response to α2δ1 ligands in TS NPC.

Rac1 is involved in several brain functions including learning and memory and dendritic spine development, features of several neurodevelopmental disorders including Down syndrome (Désiré et al., 2005; Kikuchi et al., 2019; Risher et al., 2018; Torres et al., 2018). Furthermore, several mutations in Rac1 have been reported in individuals with intellectual disability (Zamboni et al., 2018). These mutations can contribute to hypoactivity of the pathway downstream Rac1 (Zamboni et al., 2018). In preliminary experiments we also demonstrated that moesin, another protein involved in rac1 signaling (Ivetic & Ridley, 2004), is downregulated in trisomic NPC. Althogh these data are in line with the observation that moesin is downregulated in DS fetal brain (Lubec et al., 2001), this is the first demonstration of such alteration in TS NPC.

Here we showed that not only trisomic astrocytes secrete less TSP-1 than euploid one, but that trisomic NPC are not responsive to TSP-1 and other α2δ1 ligands like PGB, suggesting an impaired machinery downstream α2δ1 in TS NPC. Taken together these data raise future questions whether upregulation of the α2δ1 subunit and/or pharmacological correction in the TSP1-1/α2δ1/rac1/arp2-3 signaling could correct phenotypic alterations in trisomic NPC and their progeny.

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Chapter 8

Final discussion

Down syndrome (DS) is the most common genetic cause of intellectual disability caused by the triplication of chromosome 21, with a frequency of one in 700 births worldwide. Although individuals with DS have various medical problems, intellectual disability (ID) is the most disabling aspect of the pathology. Indeed, DS brain develops differently from an euploid child and is characterized by hypocellularity, reduced neurogenesis and increased astrogliogenesis (Bartesaghi et al., 2011; Stagni et al., 2018). In the past years studies hypothesized that DS brain abnormalities might be caused by a widespread reduction in neural progenitor cell proliferation, registered both in DS fetuses (Guidi et al., 2008; Contestabile et al., 2007) and in Ts65Dn pups (Bianchi et al., 2010; Guidi et al., 2014; Stagni et al., 2016), the most validated preclinical model to study DS (Guidi et al., 2008; Stagni et al., 2018)

A revolutionary study in the field showed for the first time that a pharmacological treatment in the perinatal period (prenatal and neonatal) could correct NPC defective proliferation and differentiation and, importantly, rescue cognitive impairment in adulthood (Stagni et al., 2015).

Thus, the perinatal period seems to be the therapeutic window in which correct DS brain neurodevelopmental alterations. Indeed, at present part of the drug discovery in DS is mainly focused in early pharmaceutical interventions (Hart et al., 2017). However, several drugs efficacious in the animal model such as lithium chloride (LiCl) appears not suitable for clinical application mainly due to unfavorable tolerability and/or safety issues (Kazemi et al., 2016).

Based on these premises, during my PhD we targeted neural progenitor cell alterations in neonatal NPC *(in vitro)* and in Ts65Dn pups *(in vivo)* using different pharmacological approaches in order to identify drugs able to correct phenotypic alterations of NPC (defective proliferation and neuronal

differentiation) and identify new molecular mechanisms altered in NPC, which may contribute to the dysfunctional crosstalk with other cell types, including astrocytes.

During the first part of my PhD we mainly focused on phenotypic alterations of neural progenitor cells, aimed at the identification of novel potential drugs to treat DS brain abnormalities, profiting from a drug repurposing approach. This methodology allows to save time and money in the preclinical part and dramatically shorten the gap between drug discovery and availability to the patient (Clout et al., 2019; Reaume, 2011).

In particular, the first activity in which I was involved consisted in the set up of a miniaturize, simple, reproducible and ready to use *in vitro* phenotypic assay, based on the reduced proliferation of trisomic NPC. We used the well established neurogenesis enhancer LiCl (2 mM) (Bianchi et al., 2010, Contestabile et al., 2013) as positive control in all the experiments and as threshold value during the screening activities. We screened 1890 already clinical approved drugs for their ability to promote trisomic NPC proliferation and this effort resulted in the identification of 30 potential hits more potent than LiCl, belonged mainly to three pharmacological classes: the glucocorticoids, the β2 adrenergic agonists/antiasthmatic drugs and the immunosuppressant drugs.

Between the potential hits one of the most surprising ones were the glucocorticoids (GC). Despite the majority of the studies showed a negative effect of glucocorticoids on neurogenesis both *in vitro* (*i.e.* dexamethasone 1 µM) (Sundberg et al., 2006) and *in vivo* (*i.e.* dexamethasone 0.5 mg/kg/day from P4 to P7) (Kanagawa et al., 2006), in our *in vitro* cell cultures we observed a pro-proliferative effect of glucocorticoids (GC) both in trisomic and euploid NPC. At least one other group showed that several GC (0.005 – 50 µM) increased

proliferation of NPC derived from human iPSCs in a dose dependent manner (Ninomiya et al., 2014), consistent with our data.

The mechanism of action of GC is not still well elucidated. There is evidence that GC can regulate cell proliferation (both positively or negatively) binding the glucocorticoid receptors (GR), with transcriptional (Nürnberg et al., 2018) or non-transcriptional effects (Hapgood, Avenant & Moliki, 2016), or as agonists of the Smoothened (Smo) receptor in the Sonic Hedgehog pathway (Shh) (Vicario et al., 2019; Wang et al., 2010). In all these cases their binding may activate signaling pathways know to be altered in DS (*i.e.* Akt/PI3K; Shh) (Pelleri et al., 2016; Perluigi et al., 2014; Trazzi et al., 2013). However, we observed positive effect of GC both in trisomic and euploid NPC, leaving us clueless about the mechanisms behind these results. In the future we will better elucidate GC mechanism of action and evaluate whether GC affect neuronal differentiation of trisomic NPC.

During the screening activities, we identified also several β2 adrenergic agonists, antiasthmatic drugs widely used in clinic (Molimard et al., 1998). These data are consistent with previous findings that suggested β2 adrenergic receptors as targets to improve cognitive performances and synaptic plasticity in 4-6-month-old Ts65Dn mice (formoterol 2 mg/kg, 4 h before cognitive tests) (Dang et al., 2014). Here, we suggest that NPC, that express β2 receptors (Bortolotto et al., 2019), are specific target of β2 agonist drugs.

Importantly, we observed also an effect on neuronal differentiation of TS NPC after treatment with β2 agonists *(data not shown)*. These results are in agreement with previous studies in the laboratory that demonstrated that salmeterol and formoterol promoted neurogenesis *in vitro* (0.1-10 nM), in adult hippocampal NPC, and *in vivo*, in wild type mice (10 µg/kg) (Bortolotto et al., 2019). Based on these results and our *in vitro* screening data, a currently ongoing study at the University of Bologna (in the Laboratory of Prof. Bartesaghi) is evaluating the

effects of subchronic administration of β2AR agonists that pass the blood brain barrier in neonatal TS and EU pups with very promising preliminary results (*data not shown*).

Another interesting and surprising hit identified during our screening campaign was the immunosuppressant cyclosporine A (CSA). In the above findings, we described the phenotypic characterization of NPC *in vitro*, the part in which I personally contributed, and *in vivo* in Ts65Dn and euploid pups. I*n vitro* we demonstrate that CSA: i) restored the reduced proliferation rate of TS NPC, without affecting cell death, ii) increased TS NPC neurogenesis and concomitantly reduced astrogliogenesis and iii) enhanced the development of neuritic processes. These data are consistent with previous studies *in vitro* (1 µM, the same concentration we used) (Chow & Morshead, 2016; Hunt & Morshead, 2010) and *in vivo* in wild type mice (Steiner et al., 1997; Sachewsky et al., 2014). Importantly, *in vivo* we demonstrated that a neonatal treatment with CSA (15 mg/kg/day; P3-P15) significantly i) increased the number of proliferating NPC in the SGZ and SVZ of Ts65Dn pups, ii) increased neuronal density of the dentate gyrus and iii) largely increased the spine density in granule cells of Ts65Dn-treated mice.

Worthy of attention is the possibility that a single drug, CSA, administered in an early critical time window, is able to correct the main defects of DS brain. In the future, it may be important to evaluate the long-term effects of CSA treatment on cognitive performances of treated-Ts65Dn mice. At present, since the extensive effects of CSA in the murine model, CSA may be a potentially effective drug in DS. Taken together these data are important since showed that we were able to set up a reproducible and sensitive trisomic NPC-based phenotypic assay and proved once again the concept that NPC may represent pharmacological targets not only *in vitro*, but *in vivo*, in DS.

Unfortunately, CSA toxicity is likely to prevent its use in the DS clinical setting, but since the remarkable effects of the drug in the murine model, we believe it will be very important in the near future to unravel its underlying mechanism(s) of action.

In such case, the phenotypic approach we used may contribute to unravel the mechanism(s) of action of the identified hits, which may be different from the one(s) involved in the primary action of approved drugs. Indeed, we showed that CSA decreased the expression levels of p21 in trisomic hippocampi. P21 is a cyclin-dependent kinase inhibitor that regulates the transition between G1 and S phase during the cell cycle (Stagni et al., 2018). P21 is overexpressed in DS brain and this overexpression may negatively affect TS NPC proliferation (Chen et al., 2013; Stagni et al., 2018). Based on these data and previous literature evidence (Steiner et al., 1997; Sachewsky et al., 2014), in the present study we hypothesize that CSA may mediate its proneurogenic effect *in vitro* and *in vivo* not as calcineurin inhibitor, but through other mechanisms, such as blocking the activity of p38. P38 is a mitogen-activated protein kinase, MAPK (Matsuda & Koyasu, 2000), overexpressed in DS brain, able to increase p53 and p21 levels (Tramutola et al., 2016), thus the inhibition of p38 by CSA may result in reduced p21 levels. In line with this hypothesis, *in vivo,* studies showed that the inhibition of p38 can improve spine growth and density (Fernandez et al., 2012), data consistent with the large increased in spine density observed in CSA treated-Ts65Dn mice.

Based on our results and on previous evidence, in the future we will further investigate the mechanism of action of CSA *in vitro*, how it affects neurogenesis and astrogliogenesis (showed reduced in TS NPC for the first time in our cell model). The CSA dependent decrease in astrogliogenesis may be potentially linked to an inhibition of the Janus kinase-signal transducer and activator (JAK-STAT), a pathway known to be upregulated in DS that contributes to the dysregulated phenotype acquisition of trisomic NPC (Lee et al., 2016; Stagni et

al., 2018).

The above findings showed a drug repurposing strategy associated with a phenotypic-based approach, without an *a priori* known target. To our knowledge, these studies represent the first attempt to identify therapies in DS based on murine neonatal trisomic NPC using such a strategy.

However, in the past, preclinical research in Down syndrome was mainly focused on a target-based approach based on the investigation of DS brain defects as starting point to find the rational basis to devise therapies that could correct DS brain developmental defects. Based on this evidence, in parallel to the first project, we took advantage of our *in vitro* cell model in order to dissect the effect of specific treatments on NPC, already under testing by our colleagues *in vivo* (Prof. Bartesaghi, UNIBO) in Ts65Dn and euploid mice (Croston, 2017).

We tested a flavone derivative, 7,8-dihydroxyflavone (7,8-DHF), agonist of the tropomyosin-related kinase B (TrkB) receptor of the brain-derived neurotrophic factor (BDNF). This compound was chosen based on the evidence that BDNF levels are reduced in hippocampus (Guedj et al., 2009) and cerebral cortex of DS fetuses (Toiber et al., 2010) and that perinatal treatment with fluoxetine restores BDNF levels in Ts65Dn mice (Stagni et al., 2013).

We showed that *in vivo* neonatal treatment with 7,8-DHF (5 mg/kg/day, P3-P15) i) increased the number of NPC in the dentate gyrus, ii) restored the number of granule cells and iii) increased dendritic spine density in Ts65Dn pups. Importantly, an administration of 7,8-DHF from P3 to adolescence P45 improved memory and learning tasks in treated Ts65Dn mice. The timing of 7,8-DHF administration is really critical since a recent study proved that 4-month-old mice treated with 7,8-DHF (5 mg/kg/day) does not show any improvement in learning and memory (Giacomini et al., 2019).

In vitro, we showed that 7,8-DHF strongly increased neuronal differentiation and neuronal maturation of trisomic NPC and progeny but did not affect NPC proliferation.

This work contributes in the field confirming again that NPC are a pharmacological target and the time of drug administration is really critical.

Here, we observed the first discrepancy between *in vitro* and *in vivo* data. Indeed, *in vitro* 7,8-DHF did not increase TS NPC proliferation (compared with *in vivo*), while strongly impacted on trisomic neuronal differentiation both in NPC derived from SVZ and hippocampi. We hypothesize that 7,8-DHF pro-proliferative effect observed *in vivo* may be mediated by non-cell autonomous effects regulated by other elements present in the neurogenic niche, that may be target of 7,8-DHF, including astrocytes (Cvijetic et al., 2017). Indeed, a recent study showed that BDNF receptors, the tyrosine kinase receptor B (TrkB), are present on astrocytes, especially the truncated form TrkB.T1 (Holt et al., 2019). However, other studies showed that stimulation of astrocytes with BDNF can increase the production of nitric oxide species and favor the activation of detrimental pathways (Colombo & Farina, 2016). Since the increased neuronal-astroglial ratio in DS brain (Contestabile et al., 2013) and the role of BDNF-TrkB system in the NPC phenotype acquisition in DS brain (Stagni et al., 2013; Li et al., 2008), in the future we may study whether 7,8-DHF impact on the secretome of trisomic astrocytes *in vitro* and the effect of this astrocyte conditioned media on neonatal NPC. These data may help to further dissect the effects of 7,8-DHF in a more complex system composed by different cell types (NPC and astrocytes).

Again in the overview of a target-based approach, taking advantage of our *in vitro* cell model, we collaborated on another study aimed to investigate two mono-unsaturated fatty acids, linoleic (LA) and oleic acid (OA), main components of corn oil. OA is a major constituent of membrane phospholipids

and is highly concentrated in myelin (Song et al., 2019), while LA is an essential fatty acid that cannot be synthetize in the human body (Kaur et al., 2014) and that can positively impact on NPC (Kang et al., 2014). Based on this evidence and on the cytoprotective and neurotrophic effect of mono-unsaturated fatty acids (Song et al., 2019) we tested OA and LA *in vitro* in both TS and EU NPC.

We observed a strongly increase in TS and EU NPC proliferation, in agreement with previous observations obtained *in vitro* using oleic acid (100 µM, as the concentration we used) (Belal et al., 2018). Then, we focused our attention on LA and for the first time in this cell model we showed that the pro-proliferative effect of LA was promoted through the binding of ß/δ and γ peroxisome-proliferator activated receptors (PPARs), the two main isoforms expressed in brain and involved in NPC proliferation and differentiation (Cimini et al., 2007). These data are important because the use of natural diet components is regarded as a therapeutic tool with a potential translational impact, especially to prevent cognitive decline linked to age, that manifests early in young DS adults (Vacca et al., 2019; Wang et al., 2013). Thus, in collaboration with Prof. Bartesaghi, we tested corn oil *in vivo* in 4-month old Ts65Dn mice. We showed that corn oil (10 µl/g, 1 month treatment) 1) increased neurogenesis, 2) dendritic development and in parallel 3) increased learning and memory, exclusively in Ts65Dn mice.

As for 7,8-DHF we observed a discrepancy between *in vitro* and *in vivo* data: OA and LA both promoted proliferation in EU and TS NPC, whereas corn oil effect *in vivo* was selectively observed in Ts65Dn mice. These data may suggest or a different sensitivity of neonatal NPC versus adult NPC or, *in vivo*, other cell types may contribute to the effect mediated by corn oil in Ts65Dn mice.

Recent literature data demonstrated that astrocytes expressed fatty acid transporters and that these cells are also able to release polyunsaturated fatty acids (Barber & Raben, 2019; Bernoud et al., 1998).

Based on these data, in the future we may better elucidate the effect of mono-unsaturated fatty acids on the secretome of trisomic astrocytes and then the effect of this media on TS NPC. Furthermore, we need to deeper investigate LA and OA mechanism of action on TS NPC proliferation and differentiation.
Taken together, we showed that fatty acids positively impact on Ts65Dn brain. At least another group showed that administration of LA to pregnant rat mothers (1% LA with the diet from pregnancy to lactation) positively impacted in offspring cognitive performances (Queiroz et al., 2019), suggesting that treatment of pregnant Ts65Dn dams and offspring may be evaluated in the future.

The above studies showed that: i) clinically approved drugs are able to correct phenotypic alterations of NPC, ii) in parallel NPC phenotype may be affected also by other cell types present *in vivo*, such as astrocytes, in a non-cell autonomous manner.
In the work I was involved during the last months of my PhD we tested the effect of TS/EU astrocyte-conditioned media (ACM) on TS /EU NPC and we suggested that: i) as previously shown (Cvijetic et al., 2017), ACM elicit prosurvival effects on NPC and their progeny, regardless of their genotype; ii) that the negative effect of trisomic ACM on neuronal differentiation of euploid cells could be due to the presence of antineurogenic molecules. At present we have no evidence on the nature of such astrocyte-derived molecule(s). In the future studies should be undertaken to unravel secretome composition of trisomic astrocytes.
Together these data highlighted the importance to investigate the crosstalk between NPC and astrocytes in DS. This study can disclose new potential pharmacological targets.

In such a perspective during the last years of my PhD we rather concentrated our attention on a matricellular protein, thrombospondin-1 (TSP-1), a molecule

whose secretion has been demonstrated to be defective in murine and human trisomic astrocytes (Garcia et al., 2010; Torres et al., 2018). We confirmed that also in our hands, TS ACM contained less TSP-1 compared to EU ACM. For the first time, we showed that neither TSP-1 nor the clinically approved drug pregabalin (PGB) (Valente et al., 2012) were able to affect TS NPC neuronal differentiation. Consistently with previous observations, we showed that TSP-1 and PGB exerted their proneurogenic effect via the α2δ1 subunit (Eroglu et al., 2009; Risher et al., 2018). We detected reduced expression of α2δ1 in TS NPC, data consistent with previous findings in our lab in adult hippocampal NPC lacking of the transcription factor subunit NF-κB p50 (ahNPC p50 KO) (Cvijetic et al., 2017). Importantly, the cited study showed that ahNPC p50 KO are also defective in their response to proneurogenic signals (Cvijetic et al., 2017).

We proved that the pharmacological inhibition of Rac1, a protein downstream α2δ1 blocked the proneurogenic effect of TSP-1 and PGB in EU NPC. These data are consistent with findings obtained by Rishler et al. demonstrating that the prosinaptogenic effect of TSP-1 is mediated by activation of the Rho GTPase Rac1 (Risher et al., 2018). Data showed that Rac1 is upstream ARP-2/3 (Chen et al., 2017), a complex involved in the actin cytoskeleton, important for brain development and downregulated in DS fetal brain (Weitzdoerfer, Fountoulakis & Lubec, 2002). We showed that a pharmacological inhibtion of the ARP2/3 complex blocks the proneurogenic effect of TSP-1 and PGB.

No difference in expression levels of Rac1 was observed between genotypes. A preliminary experiment in the lab showed that Rac1 is activated by TSP-1 in EU NPC with a very rapid kinetic. Based on these results we hypothesize that TSP-1 activates Rac1 in EU cells, while TS NPC may be defective in Rac1 activity. These data will be further investigated in the future.

In general, based on the data presented, we suggest an important role played by the activation of the Rho GTPase signaling via TSP-1/α2δ1 association in

euploid cells that mediate TSP-1 proneurogenic effect. This consideration prompts us to hypothesize an impaired TSP-1/α2δ1 association and a defective Rho GTPase signaling in TS NPC.

In the future, several points need to be better dissected. First, we need to evaluate if the silencing of α2δ1 in EU NPC may result in defective properties, similar to TS NPC. Moreover, if after silencing we will observe that TSP-1 and PGB effects are abolished, this should increase our confidence on the involvement of the α2δ1 subunit in the defective neuronal differentiation of TS NPC. Subsequently, it is important to further dissect the RhoGTPase pathway in TS NPC that we hypothesize to be impaired. Thus, all together these data raised up the question whether an upregulation of the α2δ1 subunit or a pharmacological correction in the RhoGTPase signaling could restore a normal phenotype in trisomic cells, highlighting new potential pharmacological targets in trisomic NPC.

In conclusion, with this PhD thesis we confirmed that is possible to pharmacologically restore the altered phenotype (proliferation and differentiation) of trisomic NPC in early life stages, not only *in vitro*, but also in Ts65Dn pups. We showed two different strategies of correction: using a phenotypic-based assay associated to a drug repurposing strategy or a target-based approach.

In parallel, we increased our knowledge on the molecular mechanisms underlying the key astrocytic signal TSP-1. We highlighted two main problems: on one hand trisomic astrocytes secrete less TSP-1 (non-cell autonomous defects), on the other hand TS NPC fail in the reception of the signal, maybe due to defects in the TSP-1 receptor and, in general, of the downstream signaling pathway. Furthermore, we showed that *in vitro* NPC may be affected by astrocytes: trisomic astrocytes may release antineurogenic molecules for euploid NPC, but trisomic astrocytes may also be target of drugs and may contribute to the positive

effects observed *in vivo*. Thus with this thesis, as for already previous evidence (Chen et al., 2013), we hypothesize a role of the crosstalk between astrocytes and NPC in DS that needs to be better elucidated.

Taken together this thesis demonstrates that NPC are important novel pharmacological target and the investigation of the molecular mechanisms underlying trisomic neural progenitor cell defects may help to disclose new pharmacological targets in early life stages of DS.

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List of peer reviewed publications

1st November 2016-1st November 2019

"Neonatal treatment with cyclosporine A restores neurogenesis and spinogenesis in the Ts65Dn model of Down syndrome"
Stagni F.*, Salvalai M.E.*, Giacomini A., Emili M., Uguagliati B., Xia E, Grilli M., Bartesaghi R., Guidi S., Neurobiol Dis, 2019, 129:44-45

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"Treatment with corn oil improves neurogenesis and cognitive performance in the Ts65Dn mouse model of Down syndrome"
Giacomini A., Stagni F., Emili E., Guidi S., Salvalai M.E., Grilli M., Vidal-Sanchez V., Martinez-Cué C., Bartesaghi R.. Brain Research Bullettin, 2018,140, 378-391.

"A flavonoid agonist of the TrkB receptor for BDNF improves hippocampal neurogenesis and hippocampus-dependent memory in the Ts65Dn mouse model of DS"
Stagni F., Giacomini A., Guidi S., Emili M., Uguagliati B., Salvalai M.E., Bortolotto V., Grilli M., Rimondini R., Bartesaghi R. Experimental Neurology sept 2017, 298, 79-96.

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