Università degli studi del Piemonte Orientale
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Department of Pharmaceutical Sciences

Ph.D. in Chemistry & Biology
XXXI cycle a.y. 2015-2018

Novel molecular and pharmacological regulators of Neural Stem Cells in physiological and disease mechanisms

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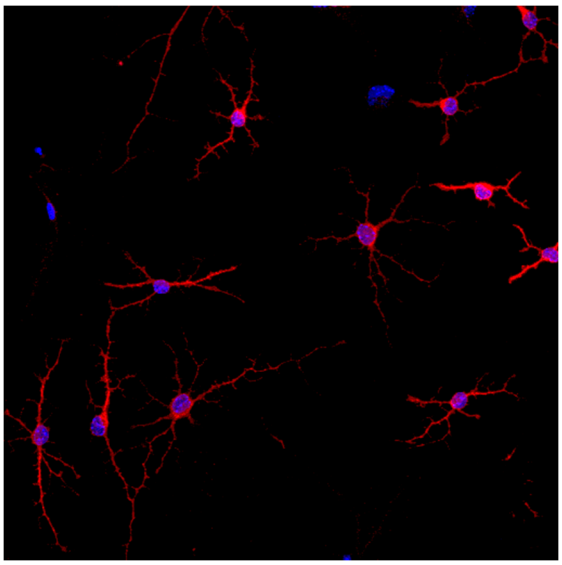

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SUMMARY

CHAPTER 1.

INTRODUZIONE

STEM CELLS: DEFINITION AND PROPERTIES

Stem Cells (SC) are defined as unspecialized cells characterized by an extensive self-renewal ability, while retaining their capacity to differentiate into different cell types.

The self-renewal capacity is maintained by an asymmetric cell division called "bivalent mitosis" a process in which stem cells can generate two daughter cells:

- one similar to the starting cell, to maintain the pool of stem cells in the tissue;
- another one with the ability to mature progressively towards phenotypically and functionally specialized cells.

SC are able to divide even in a symmetrical way generating two stem cells or two differentiated cells (Cai et al., 2004). These processes depend on the specific stimuli of the microenvironment where SC are located.

According to their differentiation potential, SC are divided in:

- Totipotent Stem Cells (TSC), cells that are capable to give rise to embryonic and extra-embryonic tissue. The zygote, originating from the fusion of the male gamete and the female gamete, is a typical example of TSC (Seydoux and Braun, 2006).
- Pluripotent Stem Cells reside in the internal mass of the blastocyst and are in an early embryonic differentiation stage in which the three embryonic sheets start to form: ectoderm, mesoderm and endoderm. These cells are able to differentiate in all tissues belonging to these three germinal lineages but cannot support the full development of an organism (Hadjantonakis and Papaioannou, 2001; Chou and Yabuuchi, 2011). Typical pluripotent cells are Embryonic Stem Cells (ESC).
- Multipotent Stem Cells are able to generate a limited number of somatic cells, restricted to a single embryo layer. Adult stem cells belong to this category (Flickinger, 1999).

- Unipotent Stem Cells can maturate into a single cell type of the belonging tissue and they ensure tissue repairing and maintenance. *E.g.* Unipotent luminar stem cells and spermatogonian stem/progenitor cells (Elias et al., 2017; Liu et al., 2016).

INDUCED PLURIPOTENT STEM CELLS

Induced pluripotent stem cells: an innovative technology

Before the birth of IPSC (Induced Pluripotent Stem Cells) technology, primary neural cell cultures isolated from animal models and immortalized cell lines have been widely used to study human diseases mechanisms and therapeutic development; however these models showed many limitations. Indeed, human patologies are not always completely reflected in animal models because of species differences and artificially manipulated cells do not represent the best choice in disease modelling (Gordon et al., 2013). Human tissues and many primary cell types isolated from donors or patients could represent a more reliable model. However, the difficulty to obtain fresh human disease tissues represents the major limitation for the use of human samples.

The pluripotency of ESC could be an excellent resource to create all cell types *in vitro*; nevertheless, the use of human ESC is severely limited in the clinical and preclinical fields due to ethic problems about embryo destruction. With the introduction of IPSC manipulation, different cell types were generated *in vitro* starting from a reprogrammed somatic cell and overcoming many ethical problems regarding ESC using. IPSC-derived cells are innovative since they can give the oppurtunity to create a patient-specific and disease-specific model *in vitro* and accelerate the pathophysiologic understanding of different diseases.

Properties and characterizations of IPSC

IPSC derive from reprogramming of somatic cells with retroviral transfection of essential pluripotency genes (usually cMyc, Octamer-binding Transcription Factor 4 (OCT4), SRY-box 2 (SOX2) and Kruppel Like Factor 4 (KLF4) (Takahashi and Yamanaka, 2006)).
To generate IPSC, different cocktail of genes and different techniques were used improving the efficiency of the reprogramming methods (as shown in table 1). Furthermore, IPSC were obtained from cells belonging to different species such as monkey fibroblasts (Liu et al., 2008), pig fibroblasts (Wu et al., 2009), marmot fibroblast (Wu et al., 2010), rat liver progenitor cells (Li et al., 2009), rabbit hepatocytes and rabbit stomach cells (Honda et al., 2010). Many cells belonging to different human tissues have also been reprogrammed: endothelial cells of the umbilical cord vein, peripheral blood cells, endothelial cells, cord blood SC, adipose SC amniotic cells, neural SC, mesenchymal bone marrow SC, hepatocytes, astrocytes, keratinocytes and urine cells (Singh et al., 2015).

	Reprogramming	Factors	Cell type	Efficiency %	References
Integrating methods	Retroviral transduction	OSKM	Mouse fibroblast	0.001–1	Takahashi and Yamanaka, 2006a
		OSK + VPA	Neonatal	1	Huangfu et al., 2008a
	Lentiviral	OSKM	Human fibroblast	0.1–1	Yu et al., 2007
		OK + parnate + CHIR99021	Neonatal	0.02	Li et al., 2009
	Inducible lentiviral	OSKM	Human fibroblast	0.1–2	Maherali et al., 2008
Non-integrating methods	Sendai virus	OSKM	Human fibroblast	~0.1	Fusaki et al., 2009
	Adeno viral transduction	OSKM	Mouse fibroblast	~0.001	Stadtfeld et al., 2008a
	Plasmid DNA transfer	OSK	Fibroblast	0.00	Okita et al., 2008
	lox p lentivirus	OSKM	Fibroblast	0.1–1	Somers et al., 2010
	PiggyBAC	OSKM	Fibroblast	0.01	Woltjen et al., 2009
	Polyarginine tagged polypeptide	OSKM	Neonatal fibroblast	0.00	Kim et al., 2009b
	RNA modified synthetic mRNA	OSKM	Human fibroblast	4.40	Warren et al., 2010

Table1. IPSC reprogramming: different delivery methods for transfer and different combinations of transcription factors have variable efficiencies of reprogramming (Singh et al., 2015).

Human IPSC and human ESC are very similar in their morphology, proliferation rate and gene expression (including pluripotency markers and epigenetic status). Moreover, both IPSC and ESC can give rise to all the cell types belonging to ectoderm, mesoderm and endoderm. These cells are characterized by a high telomerase activity allowing unlimited replication without showing senescence phenomena (Jiang et al., 2002). The maintenance of self-renewal ability is also supported by the expression of genes encoding for transcriptional factors that guarantee pluripotency capacity (*e.g.* NANOG, OCT4 and SOX2) or for proteins implicated in replicative pathways such as c-MYC. Moreover, IPSC express even specific embryonic stage antigens such as SSEA-3 and SSEA-4 and surface markers such as TRA-1-60 and TRA-1-81 (Nichols et al., 1998; Carpenter et al., 2003; Chambers et al., 2003).

In vitro maintenance

IPSC are coltured in a medium contain Fibroblast Growth Factor 2 (FGF2) and they grow as colonies on feeder layer (usually mitotically inactivated fibroblasts), which provides extracellular supports (Unger et al. 2009; Llames et al., 2015).
The combination of feeder layer and growth factors allows IPSC to maintain pluripotency and self-renewal capacity (Thomson et al., 1998; Reubinoff et al., 2000; Takahashi et al., 2007). However, with modern technologies IPSC grow even on biological or synthetic substrates in factors-rich medium such as FGF2 and Transforming Growth Factor β3 (TGFβ3) (Ludwig et al., 2006; Hayashi et al., 2007; Higuchi et al. 2011; Kim et al., 2009; Villa-Diaz et al., 2010). The mainly used biological substrate derives from murine Engelbreth-Holm-Swarm sarcoma, such as Matrigel or Geltrex.
These substrates recreate a microenvironment that contains elements to favourite the IPSC maintenance such as soluble factors (bioactive molecules, cytokines, growth factors and nutrients), cell-cell interaction and cell-biomacromolecole (or biomaterial) interaction (Dellatore et al.,

2008). When removed from the feeder layer or the mentioned substrates and transferred in suspension, IPSC colonies start to differentiate by spontaneously forming Embryoid Bodies (EB). *In vitro* differentiation experiments demonstrated that EB are composed by a considerable variety of cell types morphologically distinguishable when transferred in adhesion, such as cardiomyocytes, showing rhythmic contraction, neuronal cells with long processes (Odorico et al., 2001) or retinal pigment epithelial cells with a dark colour (Foltz et al., 2018).

Application of IPSC in neuroscience

Many IPSC from patients affected by neurodegenerative, neuropsychiatric and neurodevelopmental diseases have been generated. These include patients with:

- neurodegenerative diseases: amyotrophic lateral sclerosis (Dimos et al., 2008), Huntington's (Liu et al., 2015), Alzheimer's (Ooi et al., 2013) and Parkinson's disease (Soldner et al., 2009);
- neurodevelopmental diseases: Down syndrome (Brigida and Siniscalco, 2016) or fragile-X syndrome (Vershkov and Benvenisty, 2017);
- neuropsychiatric diseases: schizophrenia (Balan et al., 2018), autism (Habela et al., 2016) and depression (Licinio and Wong, 2016);
- other diseases of nervous system: stroke (Liu, 2013), spinal cord injury (Teng et al., 2018) and brain tumors (Young et al., 2014).

Furthermore, IPSC can differentiate in functionally and regionally specialized neuronal cell types. As reported (Ghaffari et al., 2018), through addition of different exogenous stimuli, IPSC are able to differentiate directly in different neuronal subtypes such as cortical glutamatergic neurons (Shi et al., 2012), dopaminergic neurons (Sánchez-Danés et al., 2012), inhibitory GABA-ergic neurons (Yang et al., 2017) and cholinergic neurons (Dimos et al., 2008).

The differentiation of IPSC in glial cells could be an additional opportunity to study the role of astroglial cells in several brain diseases such as Parkinson's disease, Alzheimer's disease, Down syndrome, psychiatric disorders, multiple sclerosis, autism and glioblastoma. GFAP and S100B positive astrocytes could be obtained from IPSC after 180 days of differentiation (Krencik et al., 2011) while oligodendrocyte precursors could be obtained after longer differentiation time to allow maturation and generation of myelin sheaths (Hu et al., 2009). More recently, IPSC-derived microglia, called microglia-like macrophages, were generated *in vitro*. These cells did not show a microglial morphology, but express IBA1, CD45 and CD11b, typical microglial markers (Muffat et al., 2016).

Generation of IPSC-derived Neural Stem Cells

IPSC differentiation in Neural Stem Cells (NSC) is the main key to study *in vitro* physiologic and pathologic mechanisms involved in proliferation and differentiation processes.

IPSC are switched from the self-renewing condition to differentiation conditions to allow the three embryonic germ layers induction. Under this condition, exogenous small molecules or growth factors are added to enhance the neuroephitelial differentiation and to inhibit the extraembryionic or meso-endodermal differentiation (Chambers et al., 2009). These exogenous growth factors are dual SMAD inhibitors such as noggin, an inhibitor of BMP-SMAD1/5/8 pathway, and SB431542, an inhibitor of Activin/Nodal-SMAD2/3 pathway, as shown in figure 1.

The synergistic action of dual SMAD inhibition can promote rapid neural commitment:

- inhibition of Activin/Nodal-SMAD2/3 pathways decreases NANOG expression of IPSC and enhances ZEB2 expression, a SMAD-binding protein that limits the mesoderm-inducing effects.
- inhibition of Activin/NodalSMAD2/3 and BMP-SMAD1/5/8 induces the expression of a COUP-TFII (NR2F2), which is one of the earliest transcription

factors expressed during neural commitment (Tao and Zhang, 2016; Ozair et al., 2015).

Furthermore, activation of the WNT and sonic hedgehog exerts a precise dose-dependent effect to drive neuroephitelial differentiation in dorso-ventral or rostro-caudal identities (Kirkeby et al., 2012).

These cells can differentiate in neural-restricted lineages by using two different type of protocols, through EB formation or through adherent monolayer culture (Tang et al., 2017). Both methods promote the generation of neural tube-like rosettes positive for ZO1 and N-cadherin. Characterization of human IPSC-derived NSC demonstrated that these cells are positive for SOX2, SOX1, PAX6, NESTIN, Ki67 (nuclear protein which could be used as a marker for proliferative cells) and even CD133, a marker for multipotent stem cells (Meneghini et al., 2017; Palm et al., 2015).

Human NSC require a serum-free, growth factor-poor medium and several weeks to differentiate and to mature into neurons, astrocytes and oligodendrocytes. These IPSC-derived cells are characterized by specific proteins expression such as PSA-NCAM as neuroblast marker, MAP2 and TUJ1 as neuronal markers, and GFAP and S100B, as glial cell markers. To identify neuronal subtypes γ-aminobutyric acid, vesicular glutamate transporter and tyrosine hydroxylase can be used to detect respectively GABAergic neurons, glutamatergic neurons and dopaminergic neurons.

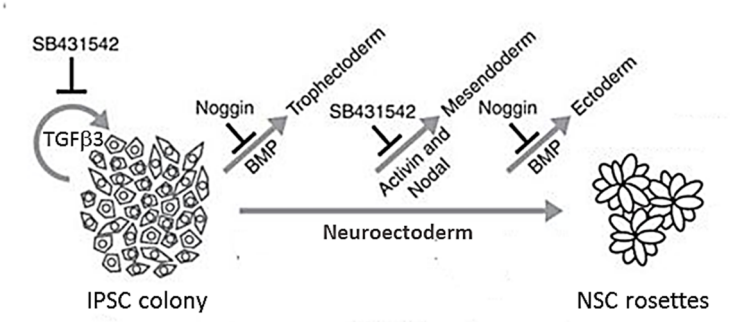

Figure 1. Schematic representation of IPSC differentiation in NSC with dual SMAD inhibitors: SB431542 inhibits the self-renewing capacity of IPSC by blocking of TGFβ3 and mesodermal differentiation, while Noggin inhibits trophectoderm and ectoderm differentiation (modified from Chambers el at., 2009).

ADULT STEM CELLS

Adult Stem Cells (aSC) are multipotent cells organized in small niches that guarantee a limited differentiation capacity. aSC have two principal functions: a functional role that consists in long-term tissue turnover (guaranteed mainly by asymmetric division), and after injury, they could switch from a functional state to a regenerative state to maintain the physiological tissue homeostasis (Wabik et al., 2015). The regeneration is due to enhanced proliferation rate in order to increase the SC population that they could differentiate into several specialized cells that participate directly in tissue regeneration (Wabik et al., 2015).

Typically, during aSC cell specialization, aSC generate an intermediate cell type, called precursor or progenitor. Progenitor cells are partially committed cells, but maintain their proliferative ability until they reach their completely differentiated state. Differentiated cells or somatic cells show mature phenotypes and are fully integrated into pre-existing circuits performing specialized functions (Clevers, 2015). Chemical modulation with small molecules and factors could improve the regeneration potency of aSC and this could be pharmacologically tagged to repair the dameged tissue (Qin et al., 2018). In such respect, study of aSC and their regulation mechanisms could be important to develop targeted therapies.

Recently, adult Neural Stem Cells (NSC) raised interest in the scientific community. NSC are multipotent stem cells that can generate neurons (neurogenesis) and glial cells (gliogenesis). During the foetal stage, NSC are involved in formation of the entire brain structure, but NSC were demonstrated to persist also in particular regions of adult mammalian brain (Altman and Das, 1965). These cells were named adult NSC or adult Neural Progenitor Cells since they were demonstrated to have limited differentiation and proliferation capacity *in vivo*, while *in vitro*, in presence of mitogens, they show long term proliferation capacity and neuronal/glial differentiation ability (Götz et al., 2015).

Adult Neural Stem Cells

In vitro analysis of NSC properties represents a very important approach to investigate the cellular and molecular mechanisms regulating neurogenesis and to find stem cell-based treatments to correct neurological disorders/injuries. There are different sources to obtain NSC by using current knowledge and technology: the direct isolation of primary cultures from tissues or through differentiation of pluripotent stem cells in NSC, as described above. For the murine model, the direct isolation of primary cells is the most used method. The first successful isolation of NSC was performed in rodents from Reynolds and Weiss in 1992 (Reynolds and Weiss, 1992). The isolated NSC are cultured in presence of growth factors and they can proliferate in suspension for long periods of time forming free-floating clusters called neurospheres (a characheteristic that contributes to prove the self-renewal capacity of NSC).
Characterization of these neurospheres revealed that cells are positive for two important neural stem cell markers, SOX2 and NESTIN. NESTIN is a type VI intermediate filament, which is expressed in NSC and in neuroblasts and is implicated in the radial growth of the axon. NSC *in vitro* can differentiate into astrocytes, oligodendrocyte precursors and neuroblasts/neurons.

ADULT NEUROGENESIS

The majority of neurons and glial cells are generated during the foetal period where they maturate and perform their specific function in the developed brain. In past years, it was firmly believed that new neurons could not be generated in adult life. This dogmatic view of an ever-immutable system has now changed as many studies demonstrated that new neuron generation occours in different brain regions from birth to adulthood (Eriksson et al., 1998). This continuous regeneration of new neurons in adult brain is called adult neurogenesis and is guaranteed by the

life-long persistence of adult NSC in neurogenic niches (Urbàn and Guillemot, 2015).

The neurogenic niches are a rich network of different elements that work in symphony to maintain stem cells after embryogenesis and for their progeny production in the adult brain. The other main components of neurogenic niches are different cell types, factors and extracellular matrix, which create an appropriate microenvironment with cell-cell interactions that balance NSC quiescence with proliferation or differentiation (Ma et al., 2009).

One of the main neurogenic niches, in the adult mammalian brain, is documented in the subgranular layer of the dentate gyrus (DG) of the hippocampus (Eriksson et al., 1998, Spalding et al., 2013). Adult hippocampal neurogenesis is a form of neural plasticity in the adult brain since it is important for the consolidation of new memory and for the emotional behaviour formation (Jessberger et al., 2009; Deng et al., 2010; Sahay et al., 2011; Toda et al., 2018).

Another well studied neurogenic niche is in the Subventricular Zone (SVZ) of the lateral ventricles. NSC in SVZ contribute to form new neurons, granule neurons or periglomerular inhibitory interneurons, which migrate to the olfactory bulb (Lois and Alvarez-Buylla, 1993; Doetsch et al., 1999). The continuous replacement of new neurons in the olfactory bulb may allow for adjustment of olfactory circuitry in response to odour experience and environmental changes (Alvarez-Buylla and García-Verdugo, 2002).

More recent reports demonstrated that adult neurogenesis also occurs in other brain regions in many animal models:

- the striatum of adult mice, monkeys and rabbits (Willaime-Morawek et al., 2006, Bédard et al., 2006; Luzzati et al., 2006);
- the amygdala of adult mice and monkeys (Bernier et al., 2002; Jhaveri et al., 2018);
- the spinal cord of adult mice (Sabelström et al., 2014);
- the cerebellum of adult mice (Lee et al., 2005);
- the hypothalamus of adult mice (Li et al., 2012; Kokoeva et al., 2015);

Many of these NSC niches were found also in human nervous system (Kempermann et al., 2018). Post-mortem tissue analysis using Ki-67, a marker of proliferative cells, and BrdU, a synthetic nucleoside that was incorporated in the new synthetized DNA (Eriksson et al., 1998), identified neurogenesis in the human hippocampal region. Furthermore, metabolic biomarkers were used in humans to localize hippocampal NSC (Manganas et al., 2007). Striatal neurogenesis was discovered by using histological and a carbon-14 dating approach in human post-mortem tissue (Ernst et al., 2014).

ADULT HIPPOCAMPAL NEUROGENESIS

Adult ippocampal neurogenesis (aHpNG) is characterized by complex processes that begin with NSC proliferation (Bonaguidi et al., 2011).
Subsequently, neural progenitors differentiate in excitatory dentate granule neurons. These neurons receive information from the entorhinal cortex (from perforant pathway) and converge their axons, called mossy fibers, in the CA3 area giving excitatory input to the pyramidal cells. From radial glia-like precursor cells, adult neurogenesis progresses over four phases to generate new granule cells and this process was estimated to take several weeks, as shown in figure 2 (Kempermann et al., 2015)

- Precursor cell phase: radial glial-like precursor cells expand with high proliferative activity and maturate in three main stages to create a pool of stem cells that is an important source from which progenies differentiate. These three progenitor stages are:
  Radial glia-like cells type 1 that are positive for GFAP, NESTIN and SOX2. These cells are quiescent and, under particular stimuli, are able to asymmetrically divide and generate a cell identical to the starting one and another cell with restricted neuronal lineage, called type 2 cell.
  Transient amplyfing progenitor cells type 2 express glial markers, such as GFAP, but they lost the typical morphology of radial cells. These cells express also Eomes (Tbr2), a transcription factor which appears to inhibit SRY-box 2 expression. This is a crucial point for the transition from stem

cells to more commitment cells called radial type 3 cells (Hodge et al., 2012).

Transient amplyfing progenitor cells type 3 start to express Doublecortin (DCX) and down-regulate the expression of NESTIN. They are characterized by reduced proliferative activity compared to that of type 1 and type 2 cells (Kempermann et al., 2004).

- In the **early survival phase**, type 3 cells enter in a quiescent state and start to send their axon to target the pyramidal cells in CA3 area forming appropriate synapses. At this stage, only a very small proportion of the newborn neurons survives and will be integrated into the dentate gyrus network (Sun et al., 2013; Kempermann et al., 2003).
- The establishment of functional connections of newborn neurons characterizes the **post-mitotic maturation phase** where axons, dendrites, spines and synapsis start to develop (Kempermann et al., 2015b).
- A **late survival phase**: after complete structural integration into the existing network, the newborn neurons become electrophysiological indistinguishable from their older neighbours and contribute to increase synaptic plasticity (van Praag et al., 2002; Ambrogini et al., 2004).

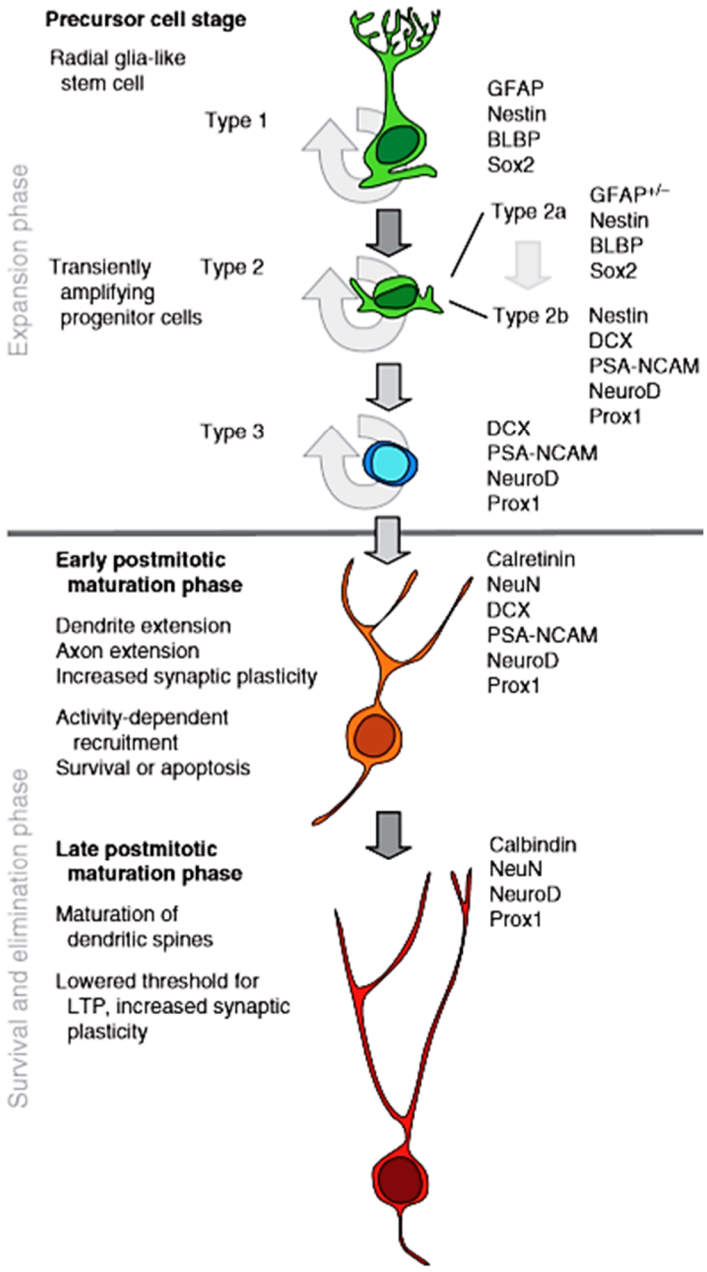

Figure 2. Schematic representation of adult hippocampal neurogenesis: developmental stages of adult hippocampal neuronal differentiation and the expression of stage specific markers (Kempermann et al., 2015b).

ROLE AND THE REGULATION OF ADULT HIPPOCAMPAL NEUROGENESIS

Hippocampus is a crucial structure implicated in the formation of emotional behaviour, cognitive functions, learning and spatial associative memory (Scoville and Milner, 1957; Oomen et al., 2014). Adult hippocampal neurogenesis confers an extra degree of neural plasticity in the DG and is important for the consolidation of new cognitive functions (Toda et al., 2018). Imparment in hippocampal neurogenesis is associated to alterated cognitive functions (Kempermann, 2002; Shohayeb et al., 2018).
Shors at al. demonstrate that the administration of low dose of the DNA methylating agent in rats, such as methylazoxymethanol (MAM), inhibits hippocampal NSC to complete the cell cycle with consequent impairment in hippocampus-mediated learning and memory functions (Shors et al., 2001). Moreover, recent studies demonstrated that mice, with significant numbers of new generated neurons, are able to learn faster in the Morris water maze (Merritt et al., 2015).

Regulation by endougenous factors

Adult hippocampal neurogenesis was demonstrated to be modulated by several extrinsic and intrinsic factors present in neural niche: ephrins, synapsins, growth factors, cell cycle regulators, neutrophines, paracrine signalling molecules, free radicals, transcriptional factors, neuropeptides, endogenous psychotropic systems and sex hormones. Furthermore, neurotransmitters solve an important role in the neurogenesis regulation. DG receives imputs from several brain regions where afferent terminals release different type of neurotransmitters that could modulate aHpNG process (Balu and Lucki, 2009; Shohayeb et al., 2018). The neurotransmitters involved are:

- Catecholamines in the DG are released by noradrenergic neurons that are localized massively in the locus coeruleus. A study in rats demonstrated that depletion of norepinephrine reduces the proliferation rate of NSC and

the derived neurons (Kulkarni et al., 2002) while a study in mice demonstrated that the reduced dopamine level is linked merely to an aHpNG alterations (Schlachetzki et al., 2016).

- Glutamatergic fibers originate from the entorhinal cortex and innervate the hippocampal DG via the perforant path. Depletion of glutamatergic-mediated neurons by inhibition of NMDA receptos was shown to increase neurogenesis in the adult hippocampus rats (Gould, 1994; Cameron et al., 1995).
- Cholinergic neurons reside in the septum and the nucleus basalis of Meynert and innervate different brain regions, mainly hippocampus. *In vivo* studies demonstrated that depletion of cholinergic pathway in rats do not affect the hippocampal proliferative cells rate (BrdU+ cells), but on the contrary reduces neurogenesis (Cooper-Kuhn et al., 2004).
- GABA levels is maintained by the activities of seven types of interneurons in the DG and these cells are involved in the modulation of newborn granule cell (Freund and Buzsaki, 1996) as well as in neurogenesis regulation (Sibbe and Kulik, 2017).
- Serotonergic neurons are localized in median and dorsal raphe nuclei and converge to multiple forebrain structures such as hippocampus (Oleskevich et al., 1991). The serotoninergic neurotransmission is suggested to be one of the central modulator of hippocampal neurogenesis, neuronal maturation and synaptogenesis (Djavadian et al., 2004; Daubert and Condron, 2010). This aspect will be deeply analysed below.

Regulation by environmental factors

Neurogenesis in the adult brain is influenced also by other extrinsic factors such as environmental variables. Adult hippocampal neurogenesis was found to correlate positively with voluntary physical activity that contributes to increase the survival and the number of newborn neurons (Van Praag et al, 1999). Environmental enrichment and running are shown

to positively modulate neurogenesis. Mice and rats living in enriched cages have a more efficient learning capacity compared to that of control rodents (Kempermann et al., 1997). These animals have also increased synaptogenesis (Hosseiny et al., 2015) and enhanced 5-HT1a receptor expression (Rasmuson et al., 1998). Moreover, learning and healthy diet consumption have a good effect on rodent neurogenesis (Lee et al., 2002). The same mechanisms are potentially translatable in humans, diet and exercise intervention could promote aHpNG and increasing cognitive function (Shors et al., 2014; Hueston et al., 2017).

Stress is another strong inhibitor of aHpNG in different mammalian species, including mice and humans (Mirescu and Gould, 2006). Stress reduces the survival of newborn neurons in the DG with consequences on behavioural functions such as reduction of learning and memory (Czéh et al., 2002; Westenbroek et al., 2004). Control mice show more neurogenesis compared to stressed mice: they use better spatially complex arrangements of food storage sites and have incresed learning and memory tasks compared to stressed mice (Garthe et al., 2016).

Moreover, aHpNG decreases drastically with age in rodents and also in human (Kempermann, 2015a; Manganas et al., 2007; Kempermann, 2015). Different studies carried out in rats suggested that with aging there is a reduction of proliferative and migrating cells into the granule cell layer and originating from the SGZ (Heine et al., 2004; McDonald and Wojtowicz, 2005).

Altered neurogenesis in disease and drug modulation

Interesting, aHpNG impairment is involved in different neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntington's disease. Moreover, an alteration of aHpNG occurs in several neuropsychiatric and neurodevelopmental diseases, such as major depression, bipolar disorder, schizophrenia, Down syndrome, Fragile X syndrome and Rett syndrome (Winner and Winkler, 2015).

Patients suffering from depression show impairment in cognitive flexibility, learning and memory (Austin et al., 2001; Fossati et al., 2002). These defects correlate with reduced hippocampal volume (Bremner et al., 2000) and neurogenesis (Sahay et al., 2007). Rats, mice and humans treated with antidepressants show enhanced aHpNG (Schmidt and Duman, 2007; Boldrini et al., 2009, Lee et al., 2013).
Lithium, a typical drug to treat bipolar disorder, is able to induce neuronal differentiation of NSC from the adult murine hippocampus (Bianchi et al., 2010; Guidi et al., 2017; Trazzi et al., 2014).
Furthermore, impaired hippocampal neurogenesis correlates with cognitive defects in mouse models of Alzheimer's disease. Some studies demonstrated that donepezil, a typical AD drugs, enhances the survival of newborn neurons in the DG (Kotani S et al., 2008) and improves spatial learning and memory deficits in adult rats.
Conversely, adult hippocampal neurogenesis is inhibited by addictive drugs (Xu et al., 2016) such as opiates (Bortolotto and Grilli, 2017), cocaine (Castilla-Ortega E et al., 2017) and alcohol (Morris et al., 2010). If these negative effects on hippocampal neurogenesis correlate with impairment of cognitive functions in addicted patients remains to be fully established.

ROLE OF SEROTONINERGIC SYSTEM IN HIPPOCAMPAL NEUROGENESIS

Serotonin, 5-hydroxytryptamine (5-HT), is a monoaminergic neurotransmitter. In the developing foetal brain, neurotransmitters like amino acids and monoamines, and in particular 5-HT, are expressed relatively early before they exert their functional role in the CNS maturation. In the human developing brain, the serotoninergic system is evident from 5^{th} week of gestation and increase rapidly through the 10^{th} week of gestation.
In adult brain, serotonergic terminals originate from the median and dorsal raphe nuclei of the brainstem and innervate multiple forebrain structures such as striatum, spinal cord, substantia nigra, cerebellum, nucleus accumbens and in the hippocampal formation, hilus, molecular and

granular layers of the dentate gyrus. In these regions, serotoninergic neurons synapses connect preferentially with interneurons of the stratum lacunosum-molecolare of the CA1 and CA3 regions.

5-HT plays a variety of roles in physiologic mechanisms including developmental, cardiovascular, gastrointestinal and endocrine functions. In the CNS, 5-HT is a regulatory neurotransmitter that modulates different brain functions like sleep, aggression, feeding, sex behaviour, thermoregulation and mood, depending on different target regions. In hippocampus, serotonin plays an important role in cognitive functions such as spatial learning and memory, and in stress regulation, including emotional behaviour and anxiety (Gould, 1999; Djavadian, 2004; Kraus et al., 2017).

Serotonin binds 15 specific receptors, grouped into 7 families, with distinct characteristics and expression patterns. These receptors have both excitatory and inhibitory action and many of them can regulate neurotransmitter release such as dopamine, acetylcholine, GABA, glutamate and noradrenaline.

Almost all these receptors are expressed in the dentate gyrus, on both stimulatory glutamatergic cells and inhibitory interneurons (Berumen et al., 2012). This evidence supports the possibility that 5-HT signalling may influence adult hippocampal neurogenesis and functions.

Many studies, in fact, demonstrated that administration of serotoninergic agonists results in increased cell proliferation and neurogenesis both in SVZ and in SGZ of the hippocampus. Following chronic or acute serotonin depletion there is a drastic decrease in the number of new generated neurons in the subventricular zone and dentate gyrus of rodents (Benninghoff et al., 2010; Ueda et al., 2005).

The 5-HT3 receptors are the only serotonin-gated ion channels and their activation in hippocampus is potentially involved in proneurogenic effects associated with exercise-induced hippocampal neurogenesis (Kondo et al., 2015).

The other six serotonin receptor families are G protein-coupled receptors (GPCRs):

- The 5-HT1 receptors are divided in subtypes 5-HT1a, 5-HT1b, 5-HT1d, 5-HT1e and 5-HT1f and are coupled to the Gi/o protein. Agonists for these receptor family decrease adenylyl cyclase activation and cAMP levels. Activation of 5-HT1 receptors increases cell membrane conductance for the potassium ions.
- The 5-HT4, 5-HT6 and 5-HT7 receptors are associated with Gs proteins and their activation supports the opposite action: increased cAMP level and decreased potassium conductance with consequent increase in neuronal excitability.
- The 5-HT2 receptors are classified in the subtypes 5-HT2a, 5-HT2b and 5-HT2c and are associated to the Gq proteins. Activation of these receptors induces phospholipase C (PLC) to catalyse the hydrolysis of PIP2 with consequent increase of inositol triphosphate (IP3), diacylglycerol (DAG) and intracellular calcium levels.
- The 5-HT5 receptors are divided in subtype 5-HT5a and 5-ht5b: as the 5-HT1 receptors, they are coupled to Gi/o proteins and their activation decreases cAMP levels.

The role of each serotonin receptor subtype is not yet fully elucidated in the aHpNG process. In the DG, neurons usually co-express different types of 5-HT receptors that could have similar or opposite effects on a specific function. Moreover, 5-HT receptors can combine each other forming homodimers or heterodimers so to contribute to further complexity in the 5-HT signalling. *E.g.*, heterodimers of 5-HT1a with 5HT7 receptors and 5-HT2A with mGlu2 receptors (metabotropic glutamate 2 receptors) have been shown to have characteristics that differ from their individual counterparts (Renner et al., 2012; Delille et al., 2013). Finally, the resulting effect of 5-HT on hippocampal neurogenesis depends also on local serotonin receptor concentration, on the ratio of different receptor subtypes in loco and on the density of 5-HT receptors in a specific cell population (Sahay et al., 2007; Alenina and Klempin, 2015; Dale et al., 2016).

Another important protein that binds 5-HT is the serotonin transporter (SERT or 5HTT). This transporter, localized on the presynaptic terminal

membrane, is able to reuptake 5-HT from the synaptic gap and to interrupt the action of the neurotransmitter, as shown in figure 3.

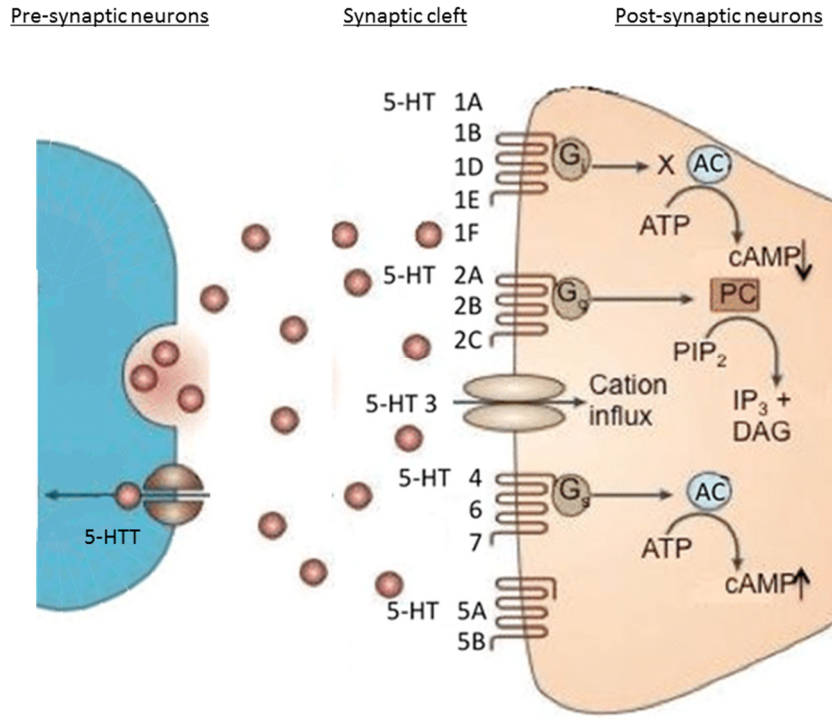

Figure 3. Schematic representation of 5-HT receptors and transpoters: 5-HT, released from pre-synaptic neurons in the synaptic cleft, are able to bind different post-synaptic 5-HT receptors that activates various signal cascades. 5-HTT is able to reuptake serotonin in the pre-synaptic neurons interrupting neurotransmitter effect. AC= adenylate cyclase, ATP= adenosine triphosphate, cAMP= cyclic adenosine monophosphate, PIP2 = phosphatidylinositol 4,5biphosphate, IP 3 = inositol triphosphate, DAG= diacylglycerol, PC= phospholipase C (adapted from Waider et al., 2012)

TRAZODONE

Trazodone (TZD), among antidepressants, belongs to SARIs drug class, since it is a serotonin receptor antagonist and reuptake inhibitor. It is used to treat psychiatric conditions including anxiety, insomnia, obsessive and compulsive disorder, post-traumatic stress disorder, substance use disorders, feeding and eating disorders, sexual dysfunction, behavioral disturbances associated with cognitive dysfunction, certain pain conditions

and rehabilitation after acute ischemic stroke (Lance et al., 1995; Roth et al., 2011; Khouzam, 2017). Recently, there has been an increasing interest in reconsidering trazodone as an effective antidepressant and as a drug with additional indications (Fagiolini et al., 2013).

Trazodone is a phenylpiperazine and a triazolopyridine derivative that is structurally uncorrelated to other major classes of antidepressants.

Trazodone is an antagonist of 5-HT2a and 5-HT2c receptors and has a partial agonism of serotonin 5-HT1a receptors. It inhibits serotonin reuptake by action on serotonin transporter. Furthermore, trazodone is an antagonist of α1- adrenergic receptors and, with lower affinity, of α2-adrenergic receptors. Lastly, TZD shows minimal anticholinergic effects. For its characteristics, TZD can be recognized as the first ever multimodal antidepressant.

TZD was demonstrated to have neuroprotective effects via the expression of mTOR, CREB and BDNF in neurons derived from human neural stem cells (Daniele et al., 2012). Electrofisiological studies in rat brain demonstrated that TZD is able to increase the serotoninergic neurotransmission (Ghanbari et al., 2010). Moreover, TZD was demonstrated to significantly improve cognitive performance in rat treated with the toxin 3-nitropropionic acid, which induces cognitive impairment, oxidative stress (glutathione) and mitochondrial dysfunction (Kumar et al., 2010). These studies suggest that TZD is involved in diffent mechanisms that are potentially correlated to the neurogenesis process.

DOWN SYNDROME, A NEURODEVELOPMENTAL DISORDER ASSOCIATED WITH NEURAL STEM CELL IMPAIRMENT

General aspects

Down syndrome (DS) is a neurodevelopmental disorder affecting 1 in every 787 live born babies each year (De Graaf et al., 2017a; De Graaf et al., 2017b; De Graaf et al., 2015). Among individuals affected by DS, in most cases (around 88% of DS patients), the disorder is caused by meiotic non-disjunction of the more likely maternal chromosome 21 resulting in a triplication of the entire chromosome 21. In some cases (around 4%), the

disorder is caused by robertsonian translocation of part or entire chromosome 21 to the long arm of the acrocentric chromosomes 14 or 22. Rarely (around 1%) DS presents as a mosaic conditions where only some cells have an extra copy of chromosome 21. These last cases are likely due to a non-disjunctional event occuring during the first stages of embryo formation. The phenotypic effects are very similar in all three DS forms, except for the severity of the disease: the mosaicism cases have a clinical phenotype milder than the typical full trisomy. Patients have reduced muscle tone that results in floppiness (hypotonia) and show typical craniofacial features that are geometrically well descripted (Cornejo et al., 2017). These facial abnormalities are microgenia (abnormally small chin), round face, slanting eye fissures with prominent ephicanthic folds, Brushfield spots in the iris, a flat facial profile, a flat nasal bridge, a protruding tongue, a shorter neck, a smaller nose and smaller ears than euploid individuals. Patients with DS are predisposed to a wide range of medical conditions. About 40-50% of newborns with DS present cardiac malformations, gastrointestinal defects, immune system anomalies, thyroid disorders, metabolic problems and increased frequency of leukaemia (Whooten et al., 2018; Hasle et al., 2016). However, with advances of medical technologies and increased access to medical care most of these medical issues have become treatable and life expectancy of DS patients has dramatically increased from 9 years old in 1929 to an average age of 60 years in the 2002 (Carfì et al., 2014). At present the most debilitating and unsolved problem of DS individuals is cognitive impairment.

Cognitive functions

Intellectual disabilities and mental retardation are invariably present in DS population, in a degree ranging from mild to severe. DS is the commonest identifiable cause of mental retardation (around 15-20% of the intellectually disabled population). Down syndrome children show memory profiles that are completely different from other genetic syndromes with

intellectual disabilities. DS childrens are characterized, in fact, by normal immediate visual-spatial short memory, but poor verbal working memory skills that worsen in the adolescence period (Conners et al., 2011; Edgin et al., 2010; Vicari and Carlesimo, 2006). Furthermore, cognitive functions degenerate with age due to several co-morbid factors such as sleep disruption, depression, sensory impairments, seizures, autism and other medical and psychiatric conditions. In the middle-late age, DS patients frequently develop dementia of the Alzheimer type. The increased risk of dementia results from the extra copy of the gene that codes for amyloid precursor protein (APP) which is strongly associated with the Alzheimer's disease development.

Anatomical and physiological defects

Mental disorders in DS patients derive from a combination of reduced neural development and functional alterations that appear in 4-5-months foetuses and drastically worsen in the last three months of gestation (Guihard-Costa et al., 2006). In the two to six months of gestation period, cortical neurons born in the proliferative ventricular zone migrate into the cortical plate where they assume a committed phenotype to form the specific layers of the cortex (Weitzdoerfer et al., 2001). Alteration in this period cold impact the final brain function. The late prenatal DS brain, in fact, shows delayed and disorganized cortical lamination with smaller and hypocellular hippocampal DG and hypomorphic cerebellum. Consequently, MRI and post-mortem studies of brain size, in children with DS between 10 and 20 years, show an approximate 17% decrease in volume, with selective loss in the hippocampus, cerebellum and frontal, temporal and occipital lobes. In adult age, brain size and weight is about 20% smaller than euploid brain and there are further reductions in the proportion between frontal lobe and temporal lobe volumes (Beacher et al., 2010; Anderson et al., 2013, Pinter et al., 2001). At the histological level, the cerebral cortex of adult brain shows a reduced number of pyramidal neurons and altered

granule cells distribution in the cortical layers II and IV. Ultra-structural studies show that trisomic neurons possess reduced dendritic arborisations, reduced synaptic density and length, and profound alterations in dendritic spine, which appear smaller and immature. Furthermore, there is an unbalance between the excitatory and inhibitory tone due to complex impairment of neurotransmitter receptor functions, but also of both excitatory and inhibitory neurons numbers. These defects have several consequences such aberrant maturation of neurons and defective synaptic transmission (Stagni et al., 2018). The cyto-architectonic alterations and their effect on the cognitive development in DS is not very well elucidated; however, some alterations may be associated with specific intellectual disabilities in DS. (Couzens et al., 2011; Tsao and Kindelberger, 2009). Deficits in memory consolidation may be consequent to temporal lobe and hippocampal dysfunction as well as to cerebellum and prefrontal cortex alterations (Pennington et al., 2003; Lott and Dierssen, 2010). It seems plausible, in fact, that cognitive dysfunctions in domains such as attention, executive control, language learning, spatial memory and emotional behaviours could be correlated with cerebellar–cortical–limbic circuitry impairment (Lott and Dierssen, 2010; Vicari, 2006).

THE MURINE MODEL OF DOWN SYNDROME

Over the last several years, very promising results have been obtained with a mouse model of DS, the Ts65Dn model. The murine chromosome 10, 16 and 17 show conserved synteny with human chromosome 21. The highest proportion of orthologues genes are on murine chromosome 16 (about 80%) and the remaining syntenic genes are on murine chromosome 10 (around 14% of syntenic gene) and murine chromosome 17 (around 6% of syntenic gene). In 1990 Davisson et al. created the first viable trisomy murine model, the Ts65Dn mouse, that is still the best-studied model and contains partial and segmental trisomy 16. The Ts65Dn mice have an extra chromosome composed by a region of murine chromosome 16

translocated onto a short segment of murine chromosome 17 and this correspond to trisomy of 104 genes orthologous to human chromosome 21 genes. In particular, Ts65Dn mice carry a triplication part between Mrpl39 and Znf295 genes that contain several studied genes like App, Sod1, Sim2 and Mx1, important in many human pathologic conditions. However, Ts65Dn mice have also three copies of 19 genes of murine chromosome 17 that are not syntenic and some their phenotypes might not be correlated to human DS (Gupta et al., 2016).

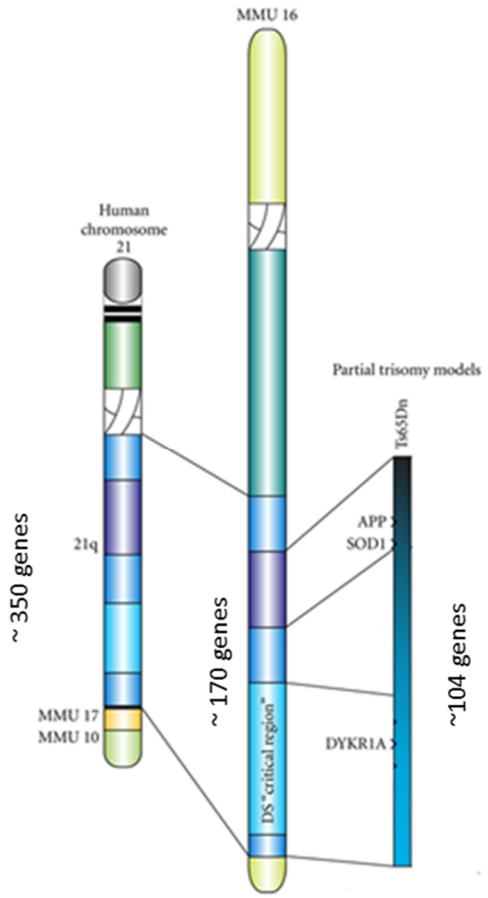

Figure 4. Schematic representation of the long-arm of human chromosome 21 comprised in the mouse chromosome 16, 17 and 10: Ts65Dn mice show triplication of approximately 104 of the 170 orthologous genes, but they include the most studied genes such as APP, SOD1, DSCR1 and DYRK1A (modified from Lockrow et al., 2012).

The Ts65Dn mice show several phenotypes and behavioural alterations that recapitulate the human counterpart, as shown in figure 5. As reported in a recent review (Stagni et al., 2018), they have several anatomical, biochemical and molecular alterations and, very importantly, they display cognitive alterations as in the human disease. Ts65Dn mice have altered cerebral architecture and reduced brain volume. Like DS patients, the size of the cerebellum and hippocampal granule cells layer are reduced very early and further decrease with age in Ts65Dn mice. Ts65Dn hippocampus shows reduced granule cell number in the DG associated with reduced dendritic spine density/shape and decreased synaptic structures across all postnatal ages (Benavides-Piccione et al., 2004).

Furthermore, the ratio between excitatory and inhibitory neurotrasmitters is altered in Ts65Dn mice due to increased inhibitory synapses and increased activity of GABAergic neurons, while the excitatory components are decreased (Créau, 2012). Many studies suggested that increased inhibitory interneurons in Ts65Dn forebrain is due to triplication of the genes Oligodendrocyte Transcription Factor 1 and 2 that are strongly involved in oligodendrogenesis and neurogenesis (Chakrabarti et al., 2010). The over-expression of inhibitory neruons and neurotrasmission was demonstrated to alter LTP and LTD in DG of trisomic mice, and these defects strongly correlate with learning and memory impairment (Kleschevnikov et al., 2004). Similarily to the human disease, in Ts65Dn mice there is also an unbalance between the astroglial and neuronal components, with a reduced number of neurons and increased number of astrocytes in multiple regions (Guidi et al., 2008; Stagni et al., 2018). Another similarity between the mouse model and the human disease is at the onset of Alzheimer's disease (AD). Starting from 12 months of age Ts65Dn mice show, in fact, an increased expression of APP protein in hippocampus and cortex and degeneration of basal forebrain cholinergic neurons. For this reason, Ts65Dn mice can be used also as a model of AD-like neurodegeneration.

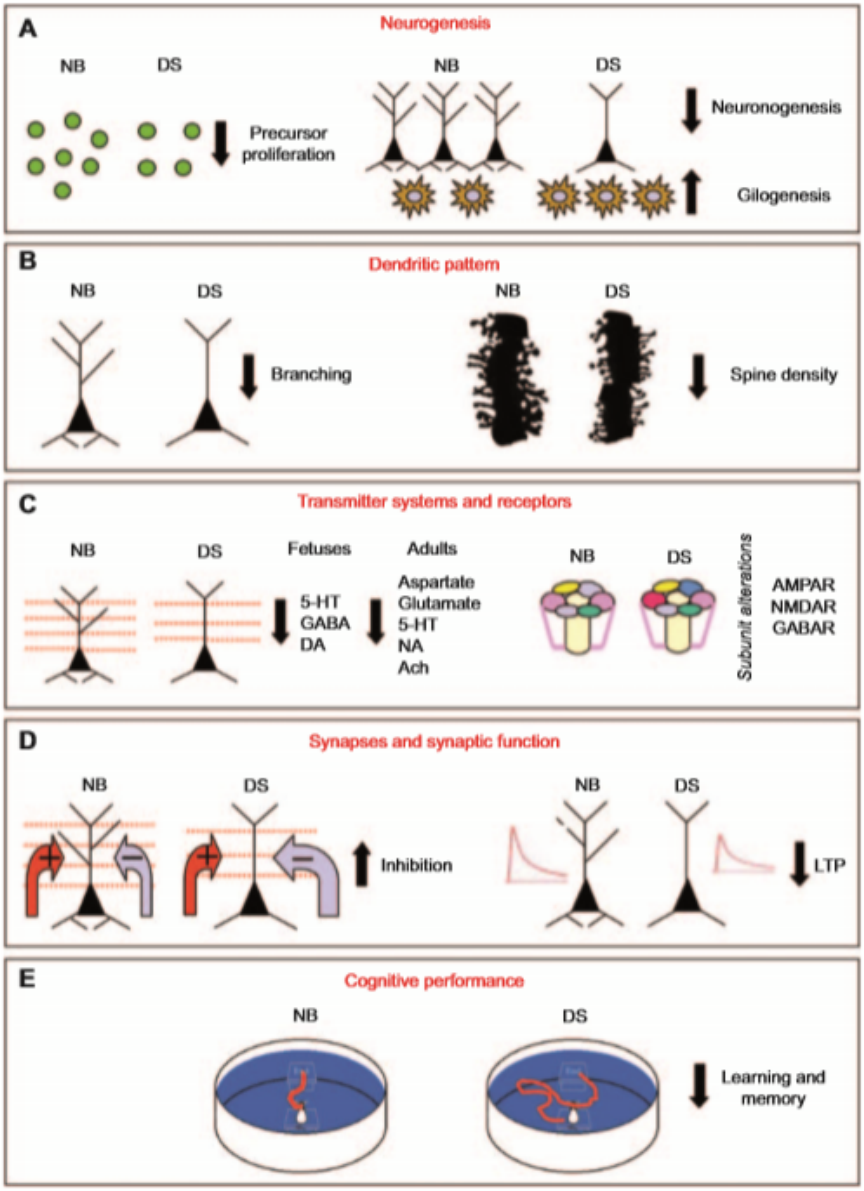

Figure 5. Schematic representation of brain abnormalities in Ts65Dn mice: impairment of NSC proliferation, reduced neuronogenesis and increased gliogenesis (A), dendritic atrophy and reduced density of dendritic spines (B), reduced levels of various neurotransmitter systems and receptor alterations (C), increased inhibition and impairment of long-term potentiation (D). The overall outcome is a severe impairment of cognitive functions, including hippocampus-dependent learning and memory (E) (from Bartesaghi et al., 2011).

NSC PROLIFERATION AND NEUROGENESIS DEFECT IN DOWN SYNDROME

Key deficiencies were demonstrated to occur during the NSC expansion and neurogenesis in the early foetal stage (Guidi et al., 2008). Stagni et al. show that the reduced proliferation rate and proneurogenic capacity in Ts65Dn mice and DS brain are likely due to common mechanisms (Stagni et al., 2018). Through immunohistological analysis of human foetus tissues for the cell cycle-associated marker such as Ki-67 (expressed in S+G2+M phases) and phospo-histone 3 (H3, mainly expressed in M phase), the NSC proliferative status was investigated in DS brain. In foetus during the first three months of gestation, cells expressing Ki-67 and H3 were drastically reduced in dentate gyrus, cortex and cerebellum in comparison with euploid NSC (Contestabile et al., 2007; Guidi et al., 2011). Studies in Ts65Dn mice confirmed the observations found in human brain. Histological analysis of Ts65Dn mice show that the numbers of $BrdU^+$ cells is reduced in the same areas of the human brain since prenatal stage (Contestabile et al., 2007).

It seems that reduced NSC proliferation rate is due to elongation of cell cycle and precocious exit from the cell cycle. In trisomic mice, cyclins and cyclin dependent kinases (CDK), that regulate cell cycle, are altered: 1. cyclin D1 is overexpressed causing prolongation of the G1 phase; 2. cyclin-dependent kinase inhibitor $p27^{KIP1}$ is increased inducing the cells to enter in G0 and premature neuronal differentiation; 3. p53 and $p21^{CIP1}$ (cyclin-dependent kinase inhibitor 1) are overexpressed impairing the transition from G1/G0 to S phase. It has been reported that triplication of the most studied genes such as Dirk1A, App, Rcan1 and Olig 1/2 has an active role in increasing activation and overexpression of cyclin D1, $P27^{KIP1}$, p53 and $p21^{CIP1}$ (Stagni et al, 2018).

In the same studies, defects in NSC proliferation were shown to result in a reduction of neurogenesis in the developing DS brain. Neurogenesis is highly compromised in favour of gliogenesis. Studies *in vivo* and *in vitro*,

with murine and human foetal NSC, demostrated that the newborn neurons significantly decrease in number, with paraller increased number of the astrocyte counterpart, in the hippocampus, parahippocampus gyrus and cerebellum (Guidi et al., 2008; Trazzi et al., 2014). This umbalance is due, at least in part, to triplication of genes that encode for several interferon receptors (such as IFNAR1, IFNAR2, IFNGR2) and IL10RB. The activation of interferon-receptors activates the JAK/STAT pathway, and in particular STAT3, that in turn activates transcription of GFAP and S100β, whose products are typical markers of astroglial cells (Ferrando-Miguel et al., 2003). Moreover, increased expression of APP and its intracellular domain portion (AICD) can repress Shh pathway through activation of PTCH1 expression. Consequently, the transcription factors GLI1 and GLI2, mediated by Shh pathway activation, are considerably reduced. GLI2 can induce neurogenesis through positive regulation of Mash1 and its downregulation affects neuronal differentiation (Trazzi et al., 2011).

TIMING OF POTENTIAL DRUG THERAPIES FOR DOWN SYNDROME

In the last 20 years, since the discovery that drugs are able to modulate neurogenesis and potentially restore cognitive functions, a growing interest had turned towards novel pharmacological approaches in DS. Experimental evidence further confirmed that there is the possibility to correct the DS NSC deficits. The target mechanisms of these studies involve different signalling pathways and molecules. The most important pharmacological approaches could be divided in 5 types, depending on targeted mechanisms:

A. Restoring of the physiological neurotransmission since in the DS brain there is an unbalance between reduced excitatory activity and increased inhibitory activity (Créau, 2012), both playing an important role in cognitive and neurodevelopment processes.

B. Prevention of neurodegeneration through antioxidant, neurotrophic molecules and free radical scavengers. Enhanced production of ROS, due to triplication of SOD1 gene (that plays a role in ROS scavenging) was

correlated with neurodegeneration and intellectual disabilities in DS (Perluigi and Butterfield, 2012). Treatments such as melatonin, vitamin E and oestrogens have shown some positive effects on learning and memory in Ts65Dn mice (Corrales et al., 2013; Lockrow et al., 2009; Granholm et al., 2002).

C. Recovery of disrupted downstream signalling pathways such as GSK3β signalling, whose dysregulation was suggested to be implicated in the neural impairment in DS (Trazzi et al., 2014). Inhibition of GSK3β by lithium reverted DS-associated defects not only at the cellular and anatomical levels, but ameliorated neurogenesis and cognitive functions in Ts65Dn mice (Stagni et al., 2013).

D. Correction of the protein dosage encoded by triplicated genes such as App and Dirk1A, which were demonstrated to be implicated in DS brain deficit. Several studies that blocked APP and DYRK1A downstream pathways using inhibitors such as DAPT (a γ-secretase inhibitor) and Epigallocatechin Gallate (EGCG, a Dirk1A kinase inhibitor) showed amelioration of behavioural functions in Ts65Dn mice (Netzer et al., 2010; Stagni et al., 2014).

E. Increasing neurogenesis by proneurogenic molecules such as P7C3 that was a well-know molecule to induce hippocampal neurogenesis in Ts65Dn mice (Latchney et al., 2015). Moreover, certain fatty acids demonstrated to have proneurogenic effects in Ts65Dn mice (Stagni et al., 2017).

More than half of these studies aimed to correct neurotransmission:

I. Inactivation of GABAergic neurotransmission by antagonizing GABAergic receptors. GABA neurotransmission is increased and is strongly correlated to cognitive and learning deficits in Ts65Dn mice. GABA antagonists or GABAA α5 negative allosteric modulators were demonstrated to ameliorate neurogenesis, LTP and to improve cognition in trisomic mice (Kleschevnikov et al., 2012; Martínez-Cué et al., 2013).

II. Restoring the cholinergic system that is degenerated in TS65dn mice (starting from 4-6 months and becoming evident at 10-12 months of age) and in DS individuals (Godrige et al, 1987; Chang and Gold, 2008). Reduction of the number of basal forebrain cholinergic neurons is correlated to

working memory/attention impairment. Drugs such as donezepil have been shown to partially restore learning and memory in Ts65Dn mice (Rueda et al., 2008).

III. Restoring noradrenergic neurotransmission that is largely affected in DS patients and in Ts65Dn mice. This impairment strongly correlates with cholinergic system degeneration, inflammation and cognitive decline in Ts65Dn. Restoration of the noradrenergic tone, using a norepinephrine precursor or β adrenergic receptor agonists, caused an improvement of cognitive functions in Ts65Dn mice (Salehi et al., 2009).

IV. Restoring the glutamatergic system: in DS patients and in Ts65Dn mice it has been demonstrated that glutamatergic neurotransmission and N-methyl-D-aspartate (NMDA) receptor signalling are altered and this might potentially contribute to behavioural disabilities. Mice trated with memantine, a moderate-affinity antagonist of NMDA receptors, show increased spatial learning capacity and restored electrophysiological abnormalities in trisomic mice (Lockrow et al., 2011).

V. Restoring the serotonergic system whose impairment in DS brain correlates to neurogenesis impairment and to declined cognitive functions (Whittle et al., 2007). Studies *in vivo* demonstrated that disruption of the serotoninergic tone in neonatal mice results in brain development dysfunctions, including generation of precursor cells, their migratory abilities and their differentiation in mature neurons and glial cells (Durig and Hornung, 2000). At the molecular level, the reason why the 5-HT pathway appears to be implicated in the DS may have also to do with S100β gene triplication since this gene product negatively regulates the outgrowth of serotonin terminals and reduces serotonin levels (Shapiro et al., 2010). In this respect, treatments that increase 5-HT availability may counteract the defects and even the mental retardation in DS individuals.

VI. Restoring other pathways such as histamine neurotransmission that was demonstrated, when deregulated, to contribute to cognitive impairment in DS (Kim et al., 2001).

Most of the studies listed above were performed in adult Ts65Dn mice and this is very relevant because it gives the demonstration that something

could be restore even in adult DS individuals. Among the studies done in adult Ts65Dn mice: 58% showed recovery of cognitive defects such as memory and learning, 19% only a partial improvement and 28% no effects. Unfortunately, very few studies concerned the neurogenesis process. Moreover, few studies took into consideration the duration of the treatment effects after treatments ending. Based on these considerations, some of the preclinical studies have built the rationale for studing in clinical trials the effects of drugs such as RG1662 (a GABAAα5 negative allosteric modulator), Memantine, donepezil and rivastagmine (acetylcholinesterase inhibitors), EGCG and vitamin E in adult and children affected by DS (Stagni et al., 2015).

An important critical aspect of the summarized preclinical studies performed in adult mice has to do with the fact that the overall brain development and function depends principally on events occurring during the early foetal stages. Moreover, after the birth neurogenesis is maintained in hippocampus until adulthood, while neurogenesis in the cerebellum occurs and ends in the first two post-natal weeks. For this reason, therapies tested in adult mice could modulate hippocampal neurogenesis with limited changes in the neural plasticity and with a partial recovery of the intellectual disabilities, while it may be too late to recover cerebellum-associated functions, such as attention and language capacity.
Consequently, therapies to improve neurogenesis defects should be started as soon as possible to allow the formation of appropriate neuronal connections. The two potential best time windows are likely neonatal and prenatal/perinatal treatments.
The most relevant studies in neonatal mice showed increased in hippocampal and cerebellum neurogenesis. *E.g. in vitro*, newborn Ts65Dn mice, treated with single injection of Shh agonist at birth (such as SAG-1), recovered cerebellar development and hippocampal LTP when they reached adult age (4 month-old), but with no effect on cerebellar-mediated cognitive functions (Das et al., 2013; Gutierrez-Castellanos et al., 2013). In another study, chronic treatment of fluoxetine in trisomic mice from

postnatal day 3 to day 15 (P3-P15) restored hippocampal neurogenesis and the number of granule cells in the neonatal mice. 1 and 4 months after treatment cessation, Ts65Dn mice maintained restored dendritic structure, spine density and cellular connectivity (Stagni et al., 2015). In addition, 7, 8-DHF, a natural small molecule, promoted neurogenesis and maturation of newborn neurons in neonatal Ts65Dn mice treated from P3 to P15 (Stagni et al., 2017). Furthermore, neonatal mice exposed to EGCG from P3-P15 fully restored hippocampal cellularity and neurogenesis (Stagni et al., 2016).

However, with the aim of fully correcting DS pathophysiology, prenatal stage seems to be the best treatment period and many studies demonstrated that drugs given to mothers had a larger impact to correct the whole brain functions. *E.g.* High concentration of choline in the diet of pregnant Ts65Dn mothers (beginning from embryonic day 1, or E1, to when pups reach P21) increased organization of cholinergic neurons in their progeny. When they reached 6 months-old age, mice showed increased cognitive functions as far as attention behaviour. The same diet, in combination with environmental enrichment, increased also hippocampal neurogenesis, spatial cognition and cholinergic neuron numbers in prenatally treated mice when they reached adulthood (Moon et al., 2010). The peptides NAPVSIPQ (that mimics the activity of the neuroprotective protein ADNP) and SALLRSIPA (that mimics the activity of neurotrofic factor ADNF) prevented neurodevelopmental delay, restored the altered subunit of GABA and improved learning in Ts65Dn mice treated in the prenatal period from E8-E12 (Vink et al., 2009). Prenatal treatment with tocoferol, the active form of vitamin E, increased cell density in DG and ameliorated learning and retention memory in Ts65Dn mice treated from E0 to adulthood P84 (Shichiri et al., 2011). Chronic administration of ECGC was able also to correct brain weight, thalamo-hypothalamic volume proportion and to completely restore the long-term memory (Guedj et al., 2009) in DYRK1A transgenic mice treated from gestation to adulthood (3 month-old).

Fluoxetine treatment in Ts65Dn mice from E10 to P2 (10 mg/kg for pregnant mother and 150 ug/g body weigth for pups) was able to restore proliferation and cellularity in all main brain regions of prenatally treated mice. When these mice reached adulthood (P43), they showed increased numbers of NSC in the SVZ and SGZ, corrected neuronal/astroglial cells balance, normalized number of granule cells, restored maturation of dendritic spines and improved cognitive function (Guidi et al., 2014).

Altogether, drug treatments in the prenatal phase showed a clear improvement in brain structures and functions to indicate that sooner treatment had more beneficial effects. However, in order to translate this protocol on human counterpart, it must be remembered that the human ontology is different from mice one. Mice development occurs mainly after birth, while in human nervous system maturation occurs during the prenatal stage and the main brain regions, except for SVZ, hippocampus and cerebellum, enter in a state of replicative quiescence after birth. This is an important critical issue to plan in the future any safe treatment in a human brain development phase. Since hippocampal and cerebellum neurogenesis in human begin from the 12th gestation week (Seress et al., 2011; ten Donkelaar et al., 2003) and in the same week it is possible to perform non-invasive prenatal testing to confirm DS, the remaining 28 weeks could potentially represent the optimal time window to treat foetus. Many studies are currently active with the final goal to develop new therapeutic drugs that have minimal side effects in mothers and unborn children, that are not teratogenic and that could pass through the placenta barrier and the blood brain barrier.

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CHAPTER 2.

OUTLINE OF THE THESIS

Neurogenesis is defined as the process of generating new neurons from neural stem cells (NSC). It occurs during embryogenesis to allow formation of the neuronal circuitries in the first stages of brain development. In recent years, alterations of this developmental process have been suggested to contribute to several neuropsychiatric disorders, including schizophrenia, and to neurodevelopmental disorders such as Down syndrome where brain abnormalities are associated with several impairments including cognitive defects.

Neurogenesis persists in discrete regions of adult mammalian brain. These regions, called neurogenic niches, include the subgranular zone of the dentate gyrus and in the subventricular zone in the lateral wall of the lateral ventricles (Eriksson et al., 1998, Spalding et al., 2013). Also in adulthood, neurogenesis relies on the presence of NSC, which have persisted in neurogenic niches. In these locations adult NSC are largely quiescent, but periodically they undergo proliferation and differentiation upon activation by extrinsic or intrinsic signals (Urbàn and Guillemot, 2015).

In the adult hippocampus, NSC and newborn neurons confer an extra degree of neural plasticity, which likely contributes to crucial brain functions. Although the physiological role of adult born neurons is not fully understood, increasing experimental evidence suggest that they are involved in key hippocampal-dependent functions, such as learning, memory and mood regulation (Kempermann, 2003; Shohayeb et al., 2018). In line with the idea that it is a peculiar form of neuroplasticity, adult neurogenesis is profoundly modulated by experiences and environmental conditions. Among positive modulators are environmental enrichment, physical exercise and learning (Van Praag et al, 1999; Lee et al., 2002). Negative modulators of adult hippocampal neurogenesis are aging, stress and social isolation (Kempermann, 2015; Czéh et al., 2002; Westenbroek et al., 2004; Bortolotto and Grilli, 2017a).

Deregulation of adult neurogenesis has been demonstrated in several neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, epilepsy, stroke, chronic pain, multiple sclerosis and in neuropsychiatric diseases such as major depression.

Major depression is one of the areas where adult hippocampal neurogenesis is extensively studied. Several preclinical studies indicate that chronic administration of antidepressant drugs results in enhanced neurogenesis in the adult rodent and human DG (Santarelli et al., 2003; Boldrini et al., 2009; Malberg et al., 2000), opening to the possibility that modulation of adult neurogenesis may contribute to the therapeutic effects of these drugs also in the clinical setting. More generally speaking, these studies once again suggest that adult hippocampal neurogenesis can be modulated pharmacologically and this may have therapeutic relevance in several undertreated disorders (Bortolotto and Grilli, 2017b; Bortolotto et al., 2014).

In the last decade, the Laboratory of Neuroplasticity has contributed to the identification of novel molecular and pharmacological regulators of adult hippocampal neurogenesis of potential therapeutic relevance (Denis-Donini et al., 2008; Meneghini et al., 2013; Valente et al, 2015; Cvjietic et al, 2017). Through these activities our group has been able to demonstrate, for the first time, that additional drugs that are already utilized in the clinical setting are endowed with the ability to promote hippocampal neurogenesis *in vitro* and, more importantly, *in vivo* in animal models. Among others, we have proven this novel pharmacological activity for: i) the anticonvulsants, anxiolytics and analgesics $\alpha 2\delta$ ligands, namely pregabalin e gabapentin (Valente et al., 2012); ii) the analgesic drug and food supplement acetyl-L-carnitine (Cuccurazzu et al., 2013; Chiechio et al., 2017); iii) for the MOR-NRI drug tapentadol (Meneghini et al., Mol Pharmacology, 2014).

These accomplishments have allowed us to propose to the scientific community that the proneurogenic effects of these drugs may have therapeutic implications. Indeed our data provide the rationale of novel therapeutic indications for some drugs (for example as antidepressants and/or cognitive enhancers for acetyl-L-carnitine and $\alpha 2\delta$ ligands). On the other hand, they confirm the importance of increasing our current knowledge on drugs that are already in clinic to better understand their full

profile in terms of additional mechanisms of action and/or of potential side effects/tolerability issues (Bortolotto et al, 2014; Bortolotto and Grilli, 2017; Grilli; Grilli, 2017; Cuccurazzu et al., 2018).

My PhD activities were all focussed in such scientific context originally developed in the Laboratory of Neuroplasticity that is the concept that neural stem cells can be regarded as potential pharmacological targets.

One of my projects was aimed at a better understanding of the *in vitro* profile of the antidepressant drug trazodone and its potential effect on both murine and human NSC. Among many antidepressants trazodone (TZD) has a unique profile of interactions: it inhibits the 5-HT transporter and antagonizes 5-HT2a and 5-HT2c receptors. The synergistic action on serotonin transporter and receptors is proposed to correlate with its good antidepressant potency and tolerability. Surprisingly, very little it is known about the full pharmacological profile of TZD *in vitro* and no previous study has ever assessed its effects on NSC and its progeny. We were able to provide novel and interesting information about trazodone and its mode of action. Moreover, we also discovered novel and distinct functions of serotonin receptor subtypes in the modulation of neurogenesis, at least *in vitro.* The relevance of these studies will allow to reveal the mechanisms underlying neurogenesis and its regulation by the serotonergic system. In this way, it is possible to target specific pathways to obtain a therapeutic effect in disease where neurogenesis is compromised.

Furthermore, I gave some contribution in another laboratory project, which is focussed on Down syndrome. DS-associated brain abnormalities (atrophy, reduced neurogenesis and increased gliogenesis, aberrant synaptogenesis and spinogenesis, neurochemical alterations) have long been considered to be irreversible. However, recent experimental evidence suggested that early treatments of trisomic mice, in prenatal/perinatal stage, could restore the neurogenic defects and promote the correct consolidation of neural circuitry, together with an improvement of

cognitive functions (Bartesaghi et al., 2015; Stagni et al., 2015). The demonstration that neurodevelopment can be improved in DS mice provided proof of principle that intellectual disabilities in DS can be pharmacologically ameliorated. Most of the drugs which successed in animal models of DS are targeted on neural stem cells (Stagni et al., 2018). I was also involved in a project, which was aimed at identifying, among clinically approved drugs, novel molecules that may correct defective proliferation and neuronal differentiation of trisomic NSC derived from a murine model of Down syndrome, the Ts65Dn mice. These studies are relevant to develop in the future a potential therapy to treat mental disability in DS patients and improve their living conditions.

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CHAPTER 3.

Proneurogenic effects of trazodone in murine and human neural progenitor cells

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ABSTRACT

Several antidepressants increase adult hippocampal Neurogenesis (ahNG) in rodents, primates and, potentially, in humans. This effect may at least partially account for their therapeutic activity. The availability of antidepressants whose mechanism of action involves different neurotransmitter receptors represents an opportunity for increasing our knowledge on their distinctive peculiarities and for dissecting the contribution of receptor subtypes in ahNG modulation. The aim of this study was to evaluate, *in vitro*, the effects of the antidepressant trazodone (TZD) on ahNG by using primary cultures of adult hippocampal Neural Stem/Progenitor Cells (ahNSC) and human IPSC-derived NSC. We demonstrated that TZD enhances neuronal differentiation of murine as well as human NSC. TZD is a multimodal antidepressant, which binds with high affinity 5-HT_{2a}, α_1, 5-HT_{1a} and with lower affinity 5-HT_{2c} and 5-HTT. We demonstrated that TZD proneurogenic effects were mediated by 5-HT_{2a} antagonism both in murine and human NSC, and by 5-HT_{2c} antagonism in murine cells. Moreover NF-κB p50 nuclear translocation appeared to be required for TZD-mediated proneurogenic effects. Interestingly, TZD had no proneurogenic effects in 5-HT depleted ahNSC. The TDZ bell-shaped dose response curve suggested additional effects. However, in our model 5-HT_{1a} and α_1/α_2 receptors had no role on neurogenesis. Overall, our data also demonstrated that serotoninergic neurotransmission may exert both positive and negative effects on neuronal differentiation of ahNSC *in vitro*.

Keywords: antidepressant, neural stem/progenitor cells, trazodone, neurogenesis, serotonin, 5-HT_2

INTRODUCTION

Lifelong generation of new neurons takes place in the subgranular zone (SGZ) of the dentate gyrus (DG) from a pool of adult hippocampal Neural Stem/Progenitor Cells (ahNSC) (Bond et al., 2015). Newly generated neurons can integrate into preexisting neuronal networks and potentially contribute to specific functions such as hippocampal-dependent learning and memory, mood regulation, and stress response (Aimone et l., 2014). Extensive research efforts have contributed to the idea that adult hippocampal neurogenesis (ahNG) represents an important form of neural plasticity which is deregulated in several neuropsychiatric disorders, including major depressive disorder (MDD), and whose reduction is correlated with cognitive impairment and mood alterations (Kempermann and Kronenberng, 2003; Mirescu and Gould, 2006; Kempermann et al., 2008; Denis-Donini et al., 2008).

AhNG is susceptible to pharmacological modulation (Duman et al., 2001; Dranovsky and Hen, 2006; Zhao et al., 2008; Bortolotto et al., 2014; Xu et al., 2016). Several antidepressant drugs including tryciclic antidepressants (TCA), selective serotonin reuptake inhibitors (SSRI) and serotonin-norepinephrine reuptake inhibitors (SNRI), counteract stress-reduced ahNG in preclinical models of depressive-like behaviour (Pittenger and Duman, 2007; Eich and Petrik, 2012). Increased number of hippocampal neural progenitors was also reported in postmortem studies in MDD patients treated with antidepressants (Boldrini et al, 2009; Boldrini et al., 2012; Boldrine et al., 2014). Although still debated, ahNG may be required for some of the behavioral effects of antidepressants, at least in rodent models (Santarelli et al., 2003; David et al., 2009).

Trazodone (TDZ) is a triazolopyridine derivative that in clinical studies has demonstrated an antidepressant activity comparable to TCA, SSRI and SNRI (Fagiolini et al., 2012). TZD is structurally unrelated to other antidepressant classes and is characterized by a unique profile (Mittur, 2011). In the past the drug has been classified as a serotonin receptor antagonist and reuptake inhibitor (SARI) able to provide simultaneous inhibition of the

serotonin transporter (SERT), partial agonism of serotonin 5-HT_{1a} receptors, and antagonism of 5-HT_{2a} and 5-HT_{2c} receptors. With such a profile, the drug potentially avoids tolerability issues that are often associated with 5-HT_{2a} and 5-HT_{2c} receptor stimulation (*e.g.* insomnia, anxiety and sexual dysfunction) (Stahl, 2009). In addition to those properties, TDZ also exerts antagonistic activity against α_1- and α_2-adrenergic receptors (AR), with minimal anticholinergic effects (Stahl, 2009). In such respect, by taking advantage of a recently introduced concept, TZD can be recognized as the first ever multimodal antidepressant (Stahl et al., 2013).

In line with the accepted hypothesis that antidepressants may exert their therapeutic activity at least in part *via* positive effects on neurogenesis, modulation of ahNG have become part of the discovery activities of recently approved antidepressant drugs (Soumier et al., 2009; Guilloux et al., 2013). In addition, promotion of adult hippocampal neurogenesis is currently explored as a strategy for antidepressant drug screening (Pascual-Brazo et al., 2014; Cuccurazzu et al., 2013; Valente et al., 2012). Despite intense research efforts in this field, our current knowledge on the relative contribution of different receptors in the modulation of ahNG and in mediating the effects of antidepressant drugs is limited. Extensive literature data suggest a potential and complex contribution of receptors engaged by TZD, such as 5-HT_{1a}, 5-$HT_{2a/c}$, α_1, α_2, in the modulation of adult neurogenesis (Jha et al., 2008; Mori et al., 2014; Meneghini et al., 2014). How the unique pharmacological profile of TZD may affect adult neurogenesis is therefore of interest and could potentially increase our understanding of the ahNG modulation by neurotransmitters.

MATERIALS AND METHODS

Animals. Male C57BL/6 (C57BL/6, The Jackson Laboratories, US) mice were housed under light- and temperature-controlled conditions in high efficiency particulate air (HEPA)-filtered Thoren units (Thoren Caging System, Hazelton, PA). Mice were kept in number of 3-4/cage with *ad libitum* access to food and water at the animal facility of Piemonte Orientale University. Animal care and handling were carried out according to protocols approved by the European Community Directive and the local Institutional Animal Care and Use Committees (IACUC).

Drugs. All drugs concentrations were chosen based on binding affinity to their target receptors. The source of compounds was as follows: 5-Hydroxytryptamine hydrochloride (5-HT) trazodone hydrochloride (TZD), ketanserin (+) tartrate salt, DL-P-chlorophenylalanine (PCPA), phenylephrine hydrochloride (Phe), and fluoxetine hydrochloride were purchased from Sigma-Aldrich (Milan, Italy); 4F4PP oxalate, RS102221 hydrochloride, R-(+)-8-Hydroxy-DPAT hydrobromide (8-OH-DPAT), WAY161503 hydrochloride, TCB-2, NAD299 hydrochloride, idazoxan hydrochloride were purchased from Tocris (Bioscience, Bristol, UK).

Adult murine hippocampal NSC isolation and culture. Adult murine hippocampal NSC isolation was performed as previously described (Meneghini et al., 2014). Briefly, for each culture preparation three adult male mice (3-4 month-old) were euthanized by cervical dislocation, and their hippocampi dissected. Tissue was digested by a Papain Dissociation System (Worthington DBA, Lakewood, NJ). Cells were plated in NUNC EasyFlask 25 cm2 (Thermo Scientific, Waltham, MA) and cultured in serum-free complete proliferation medium [Neurobasal-A with B27 supplement, 2 mM glutamine (Gibco, Life Technologies, Monza, IT), 20 ng/ml recombinant human Epidermal Growth Factor (rhEGF), 10 ng/ml of recombinant human Fibroblast Growth Factor-2 (rhFGF-2, PeproTech, Rocky Hill, NJ) and 4 µg/ml heparin sodium salt (Sigma-Aldrich)]. Passage 1 (P1) neurospheres were

dissociated for the first time after ten days *in vitro* (DIV). From P2, dissociation was performed every five days and ahNSC were replated at a density of 12,000 cells/cm^2. NSC were used for proliferation and differentiation experiments from P5 to P30. Proliferating ahNSC were routinely tested for their undifferentiated state by expression of SOX2 (Sry-related HMG box transcription factor) and nestin (Type VI intermediate filament). For irreversible inhibition of tryptophane hydroxylase (TPH), cells were treated with 50 µM of PCPA or vehicle for 72 hours starting from the second day after dissociation. At day 5, cells were dissociated and plated for differentation.

Human iPSC-derived Neural Stem Cell culture. Human NSC derived from human induced pluripotent stem cells (IPSC) generated by genome footprint-free episomal reprogramming method were purchased from 101 Bio (Palo Alto, CA). Cells were cultured in DMEM/F12 complete medium supplemented with B27 and N2 (Gibco), GlutaMax (Gibco), 20 ng/ml rhEGF, 20 ng/ml rhFGF2, 2 µg/ml heparin and 100 U/100 µg/ml of Penicillin/Streptomycin. Every four days (80% confluence) hNSC were dissociated with StemPro Accutase (Thermo Fisher, Life Technologies) at 37°C, 5% CO2 for 4 min. Single cells were harvested by centrifugation at 1,000 g, counted and seeded onto GeltrexTM LDEV-Free hESC-qualified (Invitrogen, Life Technologies) coated dishes (3.5 cm, BD FalconTM) at a density of 52,000 cells/cm^2. Human NSC cultures were maintained at 37°C, 95% humidity, 5% CO2 and received daily medium changes. From P5 to P14 cells were used for differentiation.

Mouse ahNSC proliferation and differentiation. For evaluation of cell proliferation, dissociated NSC were seeded onto flat bottom 96-well plates (Falcon) at the density of 4,000 cells/well. Standard medium for basal conditions contained Neurobasal-A, B27, 2 mM glutamine, 10 ng/ml hbFGF, 4 µg/ml heparin and 100 U/100 µg/ml of Penicillin/Streptomycin. Cells were treated with indicated drug concentration or corresponding vehicle and incubated in a humidified atmosphere at 37°C with 5% CO2. Complete

proliferation medium with EGF (20 ng/ml) was included as positive control. After 72 hours, proliferation rates were determined using CellTiter-Glo luminescent cell viability assay (Promega), according to manufacturer's instructions. Values were normalized to standard medium values. For NSC differentiation, detailed procedure was previously described (Jha et al., 2006). Briefly, single NSC cells were plated onto laminin-coated Lab-Tek 8-well permanox chamber slides (Nunc) in differentiation medium containing Neurobasal-A, B27 and 2 mM glutamine, at a density of 43,750 cells/cm2. NSC were treated with indicated drug concentrations or corresponding vehicle for 24h. To investigate the involvement of different receptors in trazodone or serotonin effects, cells were treated for 30 min with selective agonists/antagonists, before addition of the drug. For inhibition of NF-κB p50 nuclear translocation, NSC were treated with 10 μg/ml SN-50 peptide (Calbiochem) for 30 min before addition of 5-HT or TZD.

Human NSC differentiation and drug treatment. NSC were cultured onto Geltrex coated Lab-Tek 8-well permanox chamber slides (Nunc) at a density of 25,000 cells/cm^2 in 200 μl of complete medium. Cells were allowed to attach for six hours, then medium was replaced by differentiation medium [DMEM/F12, B27 and N2 supplements, Glutamax, non-essential amino acids (NEAA, Sigma-Aldrich), 200 nM Ascorbic Acid (Stemcells technologies, FR) and 1,349 g/L of glucose (Sigma-Aldrich)] containing vehicle or drug treatment. Every four days medium was half changed with fresh differentiation medium.

Immunolocalization studies in differentiated murine ahNSC and human NSC. After differentiation, cells were fixed with 4% paraformaldehyde/4% saccharose solution (Sigma) and processed for immunocytochemistry as detailed elsewhere (Meneghini et al., 2014). Primary antibodies were as follows: anti-microtubule-associated protein-2 (MAP-2; rabbit polyclonal, 1:600; Millipore, Milan, IT), anti-nestin (Chicken monoclonal, 1:1500; Neuromics, DBA, IT), anti-Glial Fibrillary Acidic Protein (GFAP, mouse polyclonal, 1:600, Millipore), anti-chondroitin sulfate proteoglycan (NG2,

rabbit polyclonal, 1:500, Millipore), anti-S100 (rabbit polyclonal, 1:1000, Dako, DBA, IT). Secondary antibodies were as follows: Alexa Fluor 555–conjugated goat anti-rabbit (1:1400), Alexa Fluor 488-conjugated goat anti-chicken (1:1400), Alexa Fluor 555-conjugated goat anti-mouse (1:1,600), Alexa Fluor 488-conjugated goat anti-rabbit (1:1400) (all from Molecular Probes, Life Technologies). Nuclei were counterstained with 0.8 ng/ml Hoechst, 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. Positive cells for each marker were counted and their percentage was calculated over total viable cells.

Cytotoxicity Assay. To assess the potential cytotoxic effect of trazodone on ahNSC and their progeny, cells were plated for differentiation on laminin-coated flat bottom 96-well plates (Falcon) at the density of 43,750 cells/cm^2. Cells were treated with indicated trazodone concentration or vehicle for 24 hours. Cytoxicity was evaluated using CellToxTM Green Cytotoxicity Assay (Promega) according to manufacturer's instructions. Positive control for membrane damage was 0.8% Triton X-100 (Sigma) and four replicates for each condition were measured. Fluorescent signal was read by VICTOR Multilabel Plate Reader platform. The cytotoxicity results are expressed as Relative Fluorescent Unit (RFU). Apoptotic rate was determined as previously described (Meneghini et al., 2010).

mRNA extraction and Polimerase Chain Reaction (unpublished data). mRNA was extracted from cell pellets with TRIzol™ Reagent protocol (Invitrogen). mRNA was converted in cDNA trough reverse transcriptase reactions by using ImProm-II(TM) Reverse Transcription System (Promega). cDNA was amplified trough Polymerase Chain Reaction (PCR) by using GoTaq(R) G2 Flexi DNA Polymerase (Promega). The primers were chose to obtain transcripts for receptors 5-HT_{1a}, 5-HT_{2a}, 5-HT_{2c}, α1 adrenergic and α2 adrenergic, and the serotonin transporters (SERT), as shown in table 1. Finally, PCR products were then resolved by electrophoresis in a 1.2% agarose gel.

Primers for murine genes	
Receptors α1a (for)	5'-GGACAAGTCAGACTCAGAGCAAGTGACG-3'
Receptors α1a (rev)	5'-TATAGCCCAGGGCATGCTTGGAAGAC-3'
Receptors α1b (for)	5'-TTTCATGAGGACACCCTCAGCAGTACC-3'
Receptors α1b (rev)	5'-CTGCCGCTGTCATCCAGAGAGTCC-3'
Receptors α2a (for)	5'-CAAGATCAACGACCAGAAGT-3'
Receptors α2a (rev)	5'-GTGCGACGCTTGGCGATCT-3'
Receptors α2b (for)	5'-GCAGAGGTCTCGGAGCTAA-3'
Receptors α2b (rev)	5'-GCCTCTCCGACAGAAGATA-3'
Receptors 5-HT1a (for)	5'-ATCTCGCTCACTTGGCTCAT-3'
Receptors 5-HT1a (rev)	5'- CCACTACCTGGCTGACCATT -3'
Receptors 5-HT2a (for)	5'- ATAGCCGCTTCAACTCCAGA -3'
Receptors 5-HT2a (rev)	5'- ACGGCCATGATATTGGTGAT-3'
Receptors 5-HT2c (for)	5'- TGATTGGACTGAGGGACGAAAGCA -3'
Receptors 5-HT2c (rev)	5'- TTCCCACAAAGCACCGACAGGATA -3'
SERT (for)	5'-TCGCCCAGGACAACATCACCTGGAC-3'
SERT (rev)	5'-TATGTGATGAAAAGGAGGCTGG-3'
Primers for human genes	
Receptors 5-HT1a (for)	5'- GGCGGCAACACTACTGGTAT-3'
Receptors 5-HT1a (rev)	5'- AGCCAAGTGAGCGAGATGAG-3'
Receptors 5-HT2a (for)	5'-ACTCGCCGATGATAACTTTGTCCT-3'
Receptors 5-HT2a (rev)	5'- TGACGGCCATGATGTTTGTGAT-3
SERT (for)	5'-TCGCCCAGGACAACATCACCTGGAC-3'
SERT (rev)	5'-TATGTGATGAAAAGGAGGCTGG-3'

Table1. List of primers annealing the endogenous promoters of serotonin transporter gene and serotoninergic/adrenergic receptor genes.

LC-ESI-MS analysis. LC-ESI-MS analyses were carried out using a Thermo Finnigan LCQ Deca XP Plus system equipped with a quaternary pump, a Surveyor AS autosampler and a vacuum degasser (Thermo Finnigan, San Josè, CA). The liquid chromatography was performed on a Phenomenex Luna HILIC column (150 x 2 mm, 3 µm) with a Phenomenex Luna HILIC security guard column (4 mm x 2 mm), at 25 °C, at 200 µl/min flow rate, under isocratic elution. The mobile phase was composed by a 90:10 ratio of

acetonitrile and water (both with 0.2% v/v of formic acid). The injection volume was 5 µl and the run time was 20 min. The MS detection of serotonin was performed in positive ionization mode. Quantitation was carried out using single reaction monitoring (SRM) mode to monitor the transition m/z 177 > 160 (C.I.D. 22 eV). The principle instrument parameters were set as follows: ion spray voltage, 5.2 kV; source current, 80 µA; capillary temperature, 350°C.

Sample preparation for LC-ESI-MS analysis. Vehicle- or PCPA-treated ahNSC were differentiated in presence of vehicle, trazodone or fluoxetine. Differentiation media were collected after 24 hours and centrifuged for 10 min at 16,000 g. 140 µl of acetonitrile with 0.2% of formic acid were added to 60 µl of collected media. Proteins were precipitated by centrifugation for 10 min at 11,300 g. An aliquot of 180 µl of supernatant was evaporated to dryness in a rotational vacuum concentrator (for about 4h at 40 °C). The sample was reconstituted in 80 µl of water/acetonitrile with 0.2% of formic acid (3:7 vol/vol), before the injection. For quantification of serotonin in differentiation medium, a calibration curve was prepared in blank matrix (differentiation medium treated with acetonitrile with 0.2% of formic acid, 3:7 vol/vol) in the appropriate range.

Statistical analysis. All experiments were run in triplicates using different cell preparations and repeated at least three times. Data were expressed as mean ± S.D. and analyzed by one-way analysis of variance (ANOVA), followed by Tuckey's post-hoc test or by Student's t test. Statistical significance level was set for p values < 0.05

RESULTS AND DISCUSSION

Serotonin promotes neuronal differentiation of murine ahNSC.

For our studies, we used a well characterized *in vitro* model of murine ahNSC. In this experimental setting when ahNSC from the adult mouse hippocampus are grown in presence of growth factors they are phenotypically characterized by expression of nestin and SRY-related HMG-box gene 2 (SOX2), bonafide markers of undifferentiated neural progenitors, and by absence of markers of mature and immature neurons such as MAP-2, Tuj-1, and Doublecortin (Valente et al., 2012). Upon removal of growth factors and exposure to a serum-free defined medium, ahNSC stop dividing and differentiate onto laminin-coated chamber slides. By double immunolabeling for markers of neurons (MAP-2) and undifferentiated ahNSC (nestin), the appearance of new neurons that are MAP-2^+ and $nestin^-$ and newly generated neuroblasts (cells positive for both MAP-2 and nestin) can be evaluated and quantified as previously described (Cuccurazzu et al., 2013). Under these experimental conditions, within 24 h, ahNSC give rise to about 2% and 30% of neurons and neuroblasts, respectively (Figure 1a,b). We initially exposed ahNSC to increasing concentrations of the endogenous neurotransmitter 5-HT (0.0001-100 µM). As depicted in Figure 1, 5-HT is able to significantly increase the percentage of newly generated neurons and neuroblasts compared to vehicle-treated cells ($p < 0.001$, ANOVA, Figure 1a,b). Altogether these data confirm that in our *in vitro* model ahNSC differentiation is positively modulated by 5-HT levels, in line with previous reports (Alenina et al., 2015).

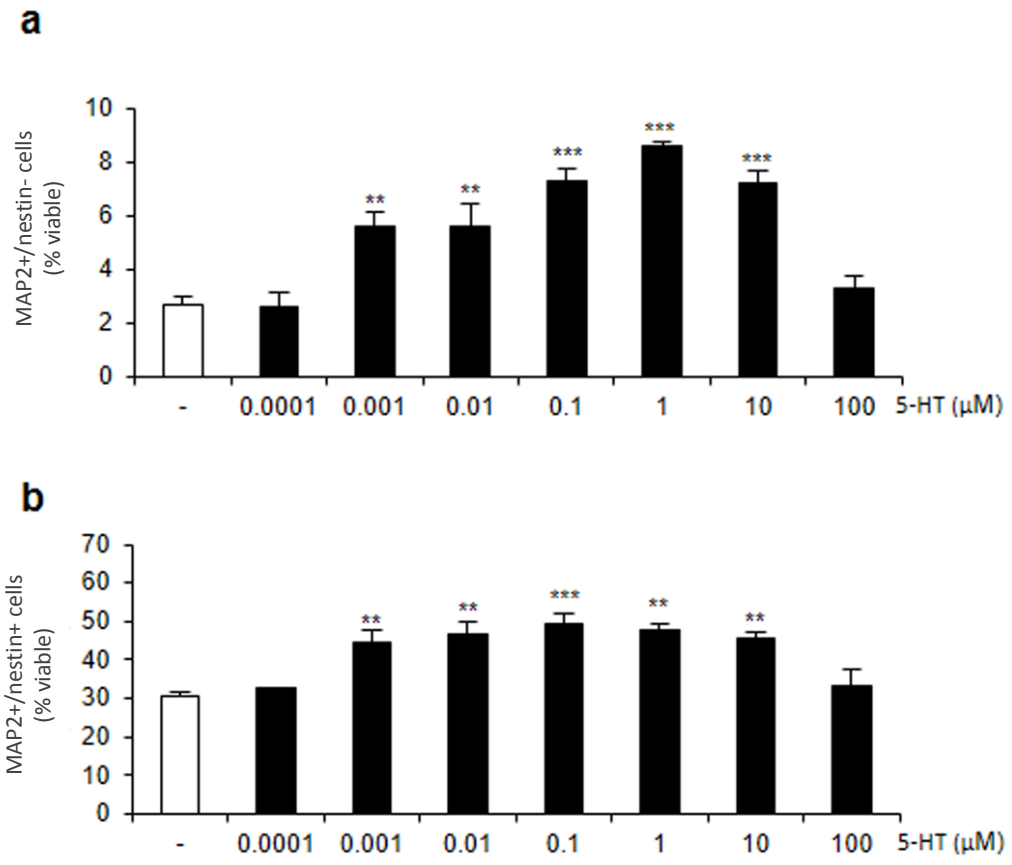


Fig. 1. 5-HT promotes neuronal differentiation of murine ahNSC *in vitro*. Adult hippocampal NSC were treated with 5-HT (0.0001-100 µM) or vehicle, for 24h. Effects on the percentage of MAP-2$^+$/nestin$^-$ (a) and MAP-2$^+$/nestin$^+$ (b) cells generated from ahNSC were evaluated. Data are expressed as mean ± S.D. ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells (One-way ANOVA, Tukey's post-hoc).

Murine ahNSC and human IPSC-derived NSC express serotoninergic and adrenergic receptors (unpublished data).

In order to characterize further murine and human NSC, we analysed the mRNA expression of different serotoninergic and adrenergic receptors. The transcript-based analysis of the two cell types demonstrated that murine ahNSC were characterized by distinct receptors expression such as 5-HT1a, 5-HT2a and 5-HT2c, and serotonin transporter (SERT). In parallel, we found that murine cells express even transcripts of α1 and α2 adrenergic receptors (as shown in figure 2a). On the other hand, human IPSC-derived NSC express 5-HT1a, 5-HT2a receptors and SERT. By this approach, we were able to define a unique expression signature of our model of murine and human NPC and it will be important to analyse the involvement of single receptors in the pro-neurogenic effect mediated by TZD (figura 2b).

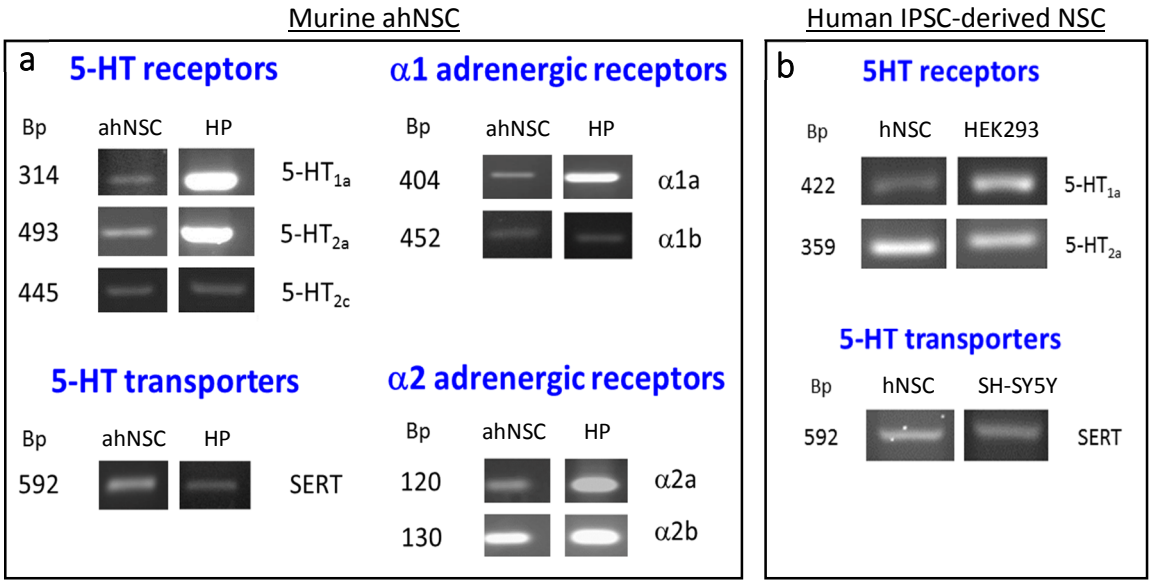


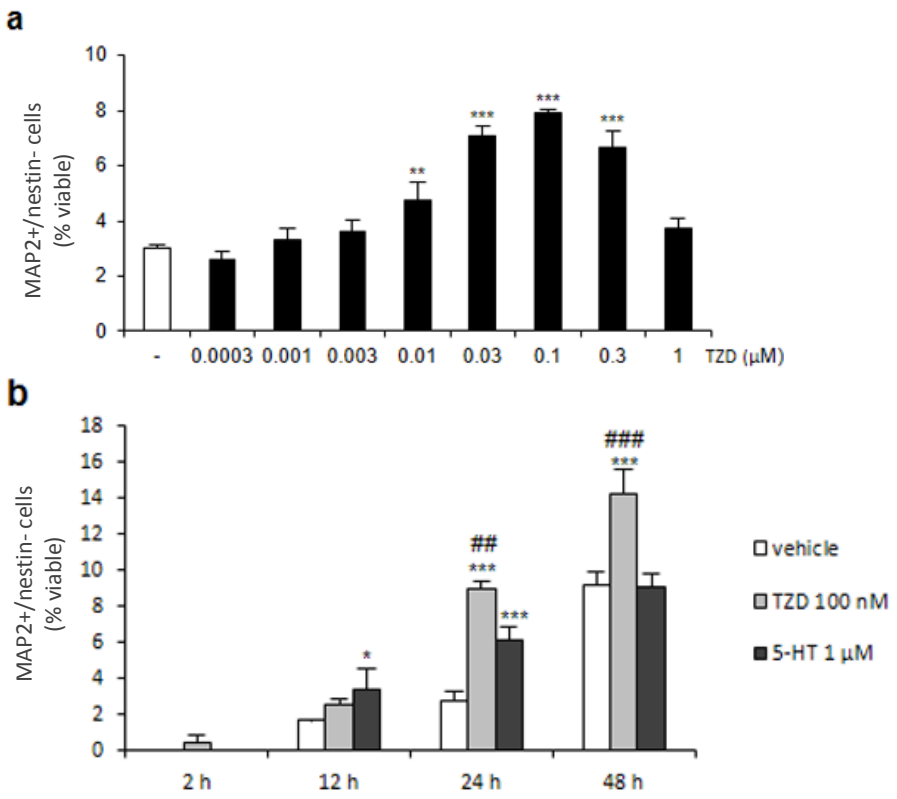
Figure 2. PCR analysis of 5-HT and adrenergic receptor subtypes in murine ahNSC and human IPSC-derived NSC. (a) Murine ahNSC express transcripts of receptors 5-HT1a, 5-HT2a, 5-HT2c, α1, α2 and SERT. (b) Human IPSC-derived NSC express transcripts of receptors 5-HT1a, 5-HT2a and SERT. As positive controls, murine hippocampal tissue (HP), HEK293 or SH-SY5Y were used.

TZD promotes neuronal but not glial differentiation of ahNSC *in vitro*.

Subsequently, ahNSC were differentiated in presence of a wide range of TZD concentrations (0.0003-1 μM) for 24 h. Such concentration range was chosen with the purpose of studying the potential involvement of all receptor subtypes bound, with high and low affinity, by TZD (Stahl, 2009). Compared to vehicle-treated cells, a significant increase in the percentage of MAP-2$^+$ (and nestin$^-$) cells was observed in presence of 0.01-0.3 μM TZD (0.1 μM TZD: $p < 0.001$ vs vehicle, ANOVA, Figure 3a), suggesting that the drug is able to promote neuronal differentiation of murine ahNSC in a concentration-dependent manner. Interestingly, at the highest concentration (1 μM) TZD was ineffective, suggesting that at that concentration the drug may activate mechanisms and/or receptors that potentially counteract those promoting neurogenesis at lower concentrations. Similar effects were obtained by the drug on the population of MAP-2$^+$/nestin$^+$ neuroblasts (*data not shown*). We also tested the effects of maximally effective concentrations of TZD (100 nM) and 5-HT (1 μM), in comparison with vehicle, as a function of time exposure (2-12-24-48 h). In presence of vehicle, the number of neurons (and neuroblasts, *data not shown*) progressively increased with time. When we analyzed the number of newly generated MAP-2$^+$/nestin$^-$ neurons, in comparison with vehicle, 5-HT promoted a significant increase at 12 and 24 h but not at 48 h (12 h: $p < 0.05$, 24h: $p < 0.001$, ANOVA, Figure 3b). Compared with 5-HT, TZD proneurogenic effects appeared slightly delayed but longer lasting and more pronounced, with a significantly higher number of neurons generated at 24 and 48 h (% of MAP-2$^+$/nestin$^-$ cells: 24h, 5-HT 6.1 ± 0.7, TZD 8.9 ± 0.4, $p < 0.01$ vs 5-HT-treated cells; 48h, 5-HT 9.1 ± 0.7, TZD 14.3 ± 1.3, $p < 0.001$ vs 5-HT treated cells, ANOVA, Figure 3b).

In absence of growth factors, ahNSC can also differentiate toward non-neuronal lineages. Interestingly, 24 h exposure of ahNSC to TZD (0.0003-1 μM) affected neither the percentage of newly generated GFAP$^+$ astrocytes (Figure 3c) nor the percentage of NG2$^+$ oligodendrocyte precursors (Figure 3d). In order to investigate whether TZD may affect survival of ahNSC and their progeny, we also analyzed apoptotic and necrotic rate in our cell

culture. As shown in Figure 3e, the percentage of apoptotic cells was not significantly different in TZD- versus vehicle-treated conditions. Similarly, no difference was observed in the number of necrotic cells, as established by disrupted membrane integrity in vehicle- vs TZD-treated ahNSC cultures (Figure 3f). Some antidepressants are also positive modulators of NSC proliferation (Boldrini et al., 2012). We tested the effect of TZD on the proliferation rate of ahNSC, using Epidermal Growth Factor (EGF) as a positive control. At all tested concentrations, 72 h-exposure to TZD had no effect on ahNSC proliferation rate (Figure3g). Overall, these data suggested that: i) TZD effects on ahNSC neuronal differentiation do not occur at the expense of NSC differentiation toward glial lineages; ii) the effect of TZD is merely differentiative in nature, since neither neuroprotective nor proliferative activity was elicited by the drug on ahNSC cultures.



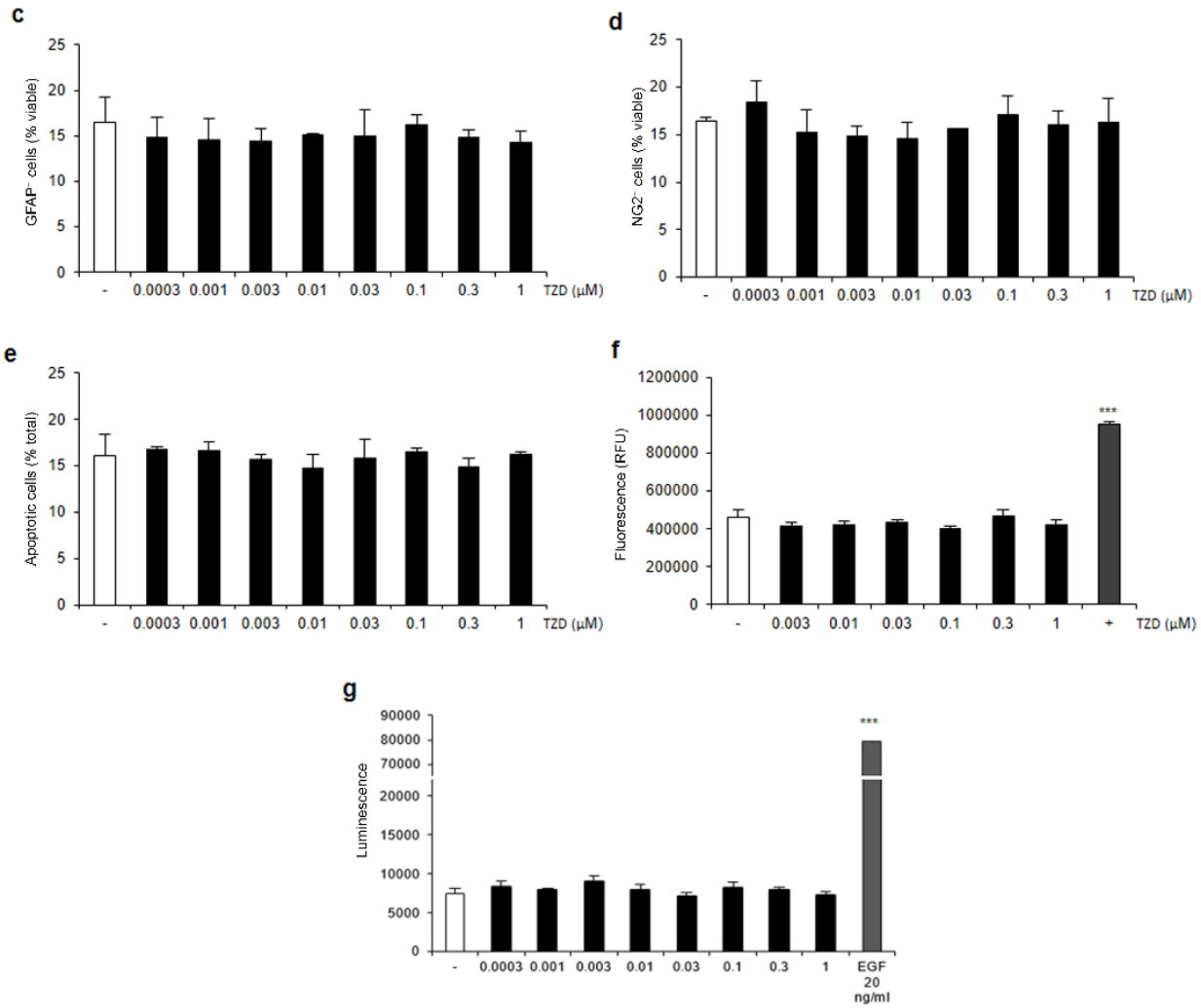


Fig. 3. Effects of TZD on murine ahNSC differentiation survival and proliferation. Adult hippocampal NSC were treated with TZD, at the indicated concentrations, or vehicle, for 24h. (a) TZD effects on the percentage of MAP-2^{+}/nestin^{-} cells generated from ahNSC. (b) Time course analysis of the proneurogenic effects of TZD and 5-HT. The percentage of MAP-2^{+} cells generated in presence of TZD (100 nM) and 5-HT (1 μM) was assessed after 2-12-24-48 h of treatment. Data are expressed as mean ± S.D. * $p < 0.05$, *** $p < 0.001$ vs vehicle-treated cells; ## $p < 0.01$, ### $p < 0.001$ vs 5-HT-treated cells (One-way ANOVA, Tukey's post-hoc). (c,d) Evaluation of TZD effect on the percentage of newly generated GFAP^{+} astrocytes (c) and NG2^{+} oligodendrocyte precursors (d). (e) Assessment of TZD effect on the apoptotic rate of ahNSC and their progeny. Data are expressed as the percentage of apototic cells over the total number of cells. (f) Evaluation of TZD cytotoxic effect. 0.8 % Triton X-100 was used as positive control (TX100). (g) Evaluation of TZD effect on ahNSC

proliferation. 20 ng/ml EGF was used as positive control. Data are expressed as mean ± S.D. *** $p < 0.001$ vs vehicle-treated cells (One-way ANOVA, Tukey's post-hoc).

The proneurogenic effects of TZD are mediated by 5-HT_{2a} antagonism.

We investigated the receptor subtypes involved in the proneurogenic effect of TZD. TZD binds with high affinity to 5-HT_{2a}, α_1, 5-HT_{1a} and with lower affinity to 5-HT2c, α_2 and 5-HTT (Mittur, 2011; Stahl, 2009). We first evaluated the involvement of 5-HT_{2a} receptors in TZD-mediated effect. When tested in ahNSC cultures, 10 nM TCB-2, a selective 5-HT_{2a} agonist [Ki $_{(5\text{-HT2a})}$: 0.73 nM], produced a rightward shift in TZD concentration-response curve, while the compound was inactive *per se* (Figure 4a). These data suggested that the proneurogenic effects elicited by TZD are mediated by its antagonistic activity at 5-HT_{2a} receptors. To further support the contribution of 5-HT_{2a} receptors in TZD proneurogenic effects, 1-30 nM 4F4PP, a 5-HT_{2a} selective antagonist, [Ki $_{(5\text{-HT2a})}$: 4 nM] and 0.3-100 nM ketanserin [a 5-$HT_{2a/c}$ antagonist, Ki $_{(5\text{-HT2a})}$: 3.6 nM, Ki $_{(5\text{-HT2c})}$: 200 nM], were both capable of promoting neuronal differentiation of ahNSC, in a concentration-dependent manner (ANOVA; Figure 4b,c). Interestingly, as shown for TZD, also for these drugs we observed an inverted U-shaped response curve.

At first sight the finding that antagonism at 5-HT_{2a} receptors may promote neurogenesis may be surprising, given the vast plethora of data supporting the view of 5-HT as an endogenous positive modulator of adult hippocampal neurogenesis (Brezun and Daszuta, 1999; Banasr et al., 2003, Brezun et al., 2000), in line with its proneurogenic effect in our experimental model. However, literature data on a role of 5-HT specific receptor subtypes in the regulation of neurogenesis also support the idea of distinct and even opposite effects on neurogenesis (Klempin et al., 2010). In particular, *in vivo* data suggested oppositional effects of serotonin receptors 5-HT_{1a} and 5-HT_2 in the regulation of adult hippocampal neurogenesis (Klempin et al., 2010). Unfortunately, in several reports these data appear contradictory, in part probably due to limited selectivity of

utilized agonists and antagonists. Klempin et al. (Klempin et al., 2010), by using cinanserin as a non-selective 5-HT_2 antagonist *in vivo*, demonstrated that it acutely produces an increase in cell proliferation in hippocampus. When the same authors tested the non-selective 5-HT_2 agonist α-methyl-5-HT, a net decrease in the number of $BrdU^+$ cells was found. Phenotypic analysis for 5-HT_2 agonist treatment revealed a net decrease in type 1/2a/2b cells after acute treatment and a significantly reduced number of newborn neurons after 7 days of treatment. Surprisingly, the same authors showed that *in vitro* blockade of 5-HT_2 receptors by cinanserin in NSC cultures produced a significant reduction in the number of βIII-tubulin positive cells, which represent newly generated neuroblasts (Klempin et al., 2010). Even more confusing, a similar result was obtained upon incubation with the non selective agonist α-methyl-5-HT. Kemplin's data are in contrast with those obtained by Banasr et al. (2004) who showed that the non selective 5-$HT_{2a/c}$ antagonist ketanserin produced a decrease in the number of BrdU-labeled cells whereas the 5-$HT_{2a/c}$ agonist DOI did not change cell proliferation. Similarly, Jha et al. (2008) demonstrated an enhanced adult hippocampal progenitor proliferation as a result of a sustained blockade of the 5-$HT_{2a/c}$ receptors by ketanserin treatment in mice. In our experimental setting we could confirm that TZD, but also a selective 5-HT_{2a} antagonist, promote neuronal differentiation of ahNSC and that TZD proneurogenic effects are counteracted in presence of a selective 5-HT_{2a} agonist. Based on these findings, herein we suggest that 5-HT_{2a} antagonism may have a significant role in neuronal differentiation of ahNSC, at least *in vitro,* and that TZD may act, at least in part, via this mechanism to promote neurogenesis. Recently, another novel pharmacological activity was described for TZD in a model of human neuronal cells, namely its neuroprotective effects against inflammatory insults (Daniele et al., 2015).

Interestingly, also this TZD-mediated effect involved antagonism at 5-HT_{2a} receptors, since it was counteracted by (R)-DOI (Daniele et al., 2015).

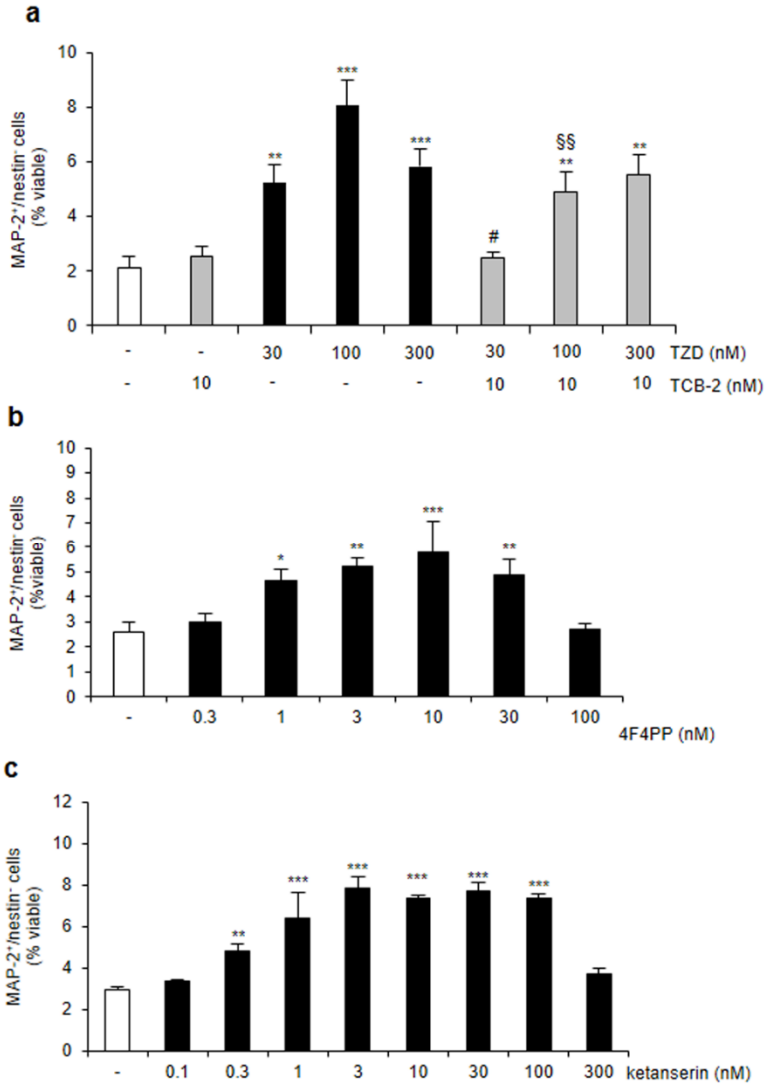


Fig. 4. TZD proneurogenic activity in murine ahNSC is mediated by 5-HT_{2a} antagonism. (a) Effects of the selective 5-HT_{2a} agonist TCB-2 (10 nM) on TZD-mediated increase of newly generated MAP-2^+ cells. (b) Effects of 4F4PP (0.3-100 nM), selective 5-HT_{2a} antagonist and (c) ketanserin (0.1-300 nM), a non-selective 5-HT_2 antagonist, on ahNSC differentiation toward the neuronal lineage. Data are expressed as mean ± S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells. # $p < 0.05$ vs 30 nM TZD-treated cells. §§ $p < 0.01$ vs 100 nM TZD-treated cells. (One-way ANOVA, Tukey's post-hoc).

5-HT_{2a}-mediated proneurogenic effects of TZD in human iPSC-derived NSC.

Very little is known about the effects elicited *in vitro* by antidepressant drugs in human NSC. We tested the proneurogenic activity of 5-HT on NSCs generated from a human iPSC line. Human iPSC-derived NSC were differentiated in absence of growth factors and in presence of vehicle or 5-HT (0.0001-100 μM) for 28 days. A significant increase in the percentage of MAP-2^+ cells was observed at all tested 5-HT concentrations (ANOVA, Figure 5a). In the same experimental setting we evaluated the effect of TZD. As shown in figure 5b, similarly to 5-HT, TZD significantly increased the percentage of newly generated MAP-2^+ cells (0.1 μM TZD: $p < 0.01$, ANOVA, Figure 5b-d). As demonstrated in murine cells, TZD produced no significant effect on human NSC differentiation toward the astroglial lineage, as measured by GFAP (Figure 5e) and S100B immunostaining (Figure 5f). Based on the results obtained on murine ahNSC, we then tested the effect of TZD (1-10 nM) in presence of the selective 5-HT_{2a} receptor agonist TCB-2 (10 nM). TCB-2 completely counteracted the effect of TZD (Figure 5g), suggesting that, as in murine ahNSC, also in human NSC TZD promotes neuronal differentiation via 5-HT_{2a} antagonism. To further confirm this hypothesis, the selective 5-HT_{2a} antagonist 4F4PP (3-30 nM) significantly increased the percentage of MAP-2^+ cells generated *in vitro*, when compared to vehicle-treated cells (ANOVA, Figure 5h).

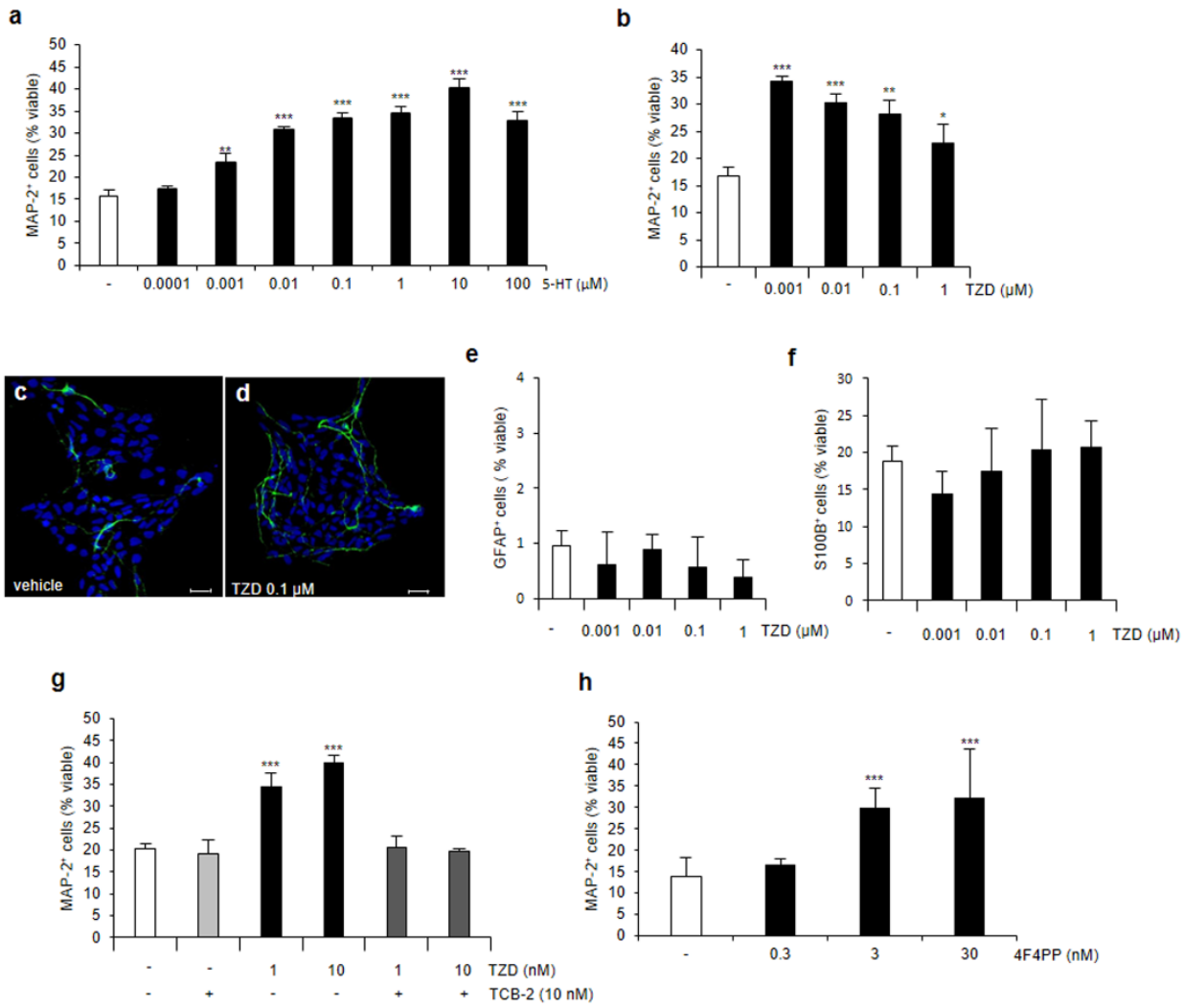


Fig. 5. Neuronal differentiation of human iPSC-derived NSC is increased in presence of 5-HT and of TZD: drug activity is mediated by 5-HT$_{2a}$ antagonism. (a) Effect of 28 day-long 5-HT treatment (0.0001-100 µM) on the percentage of MAP-2$^+$ cells generated from human NSC, compared to vehicle. (b) Effect of 28 day-long TZD treatment (0.001-1 µM) on the percentage of MAP-2$^+$ cells generated from human NSC, compared to vehicle. (c-d) Representative confocal microscopy images for MAP-2 immunolabelling (green) of human NSC in presence of vehicle (c) and TZD 100 nM (d). Nuclei are counterstained with Hoechst (blue). Magnification= X40, Scale bar: 20 µM. Effect of TZD treatment on the differentiation of human NSC toward astroglial lineages (e-f), as indicated by the percentages of GFAP (e)

and S100B (f) immunopositive cells. (g) Effect of the selective 5-HT_{2a} agonist TCB-2 (10 nM) on TZD-mediated effects. (h) Evaluation of the percentage of *in vitro* generated MAP-2^+ cells in presence of the selective 5-HT_{2a} antagonist 4F4PP (0.3-30 nM). Data are expressed as mean ± S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells (One-way ANOVA, Tukey post hoc).

Evaluation of the involvement of other receptor subtypes in TZD-mediated proneurogenic effects: a potential role for 5-HT_{2c} receptors.

In light of the multimodal mechanism of action of TZD we further expanded our investigation. TZD indeed binds with high affinity also α_1 and 5-HT_{1a} receptors (Stahl, 2009). Previous data in our laboratory suggested that α_1 antagonists like doxazosin could promote *in vitro* neuronal differentiation of murine ahNSC (Meneghini et al., 2014). However, when the α_1 agonist phenylephrine hydrocloride (30-100 nM) was tested in presence of TZD, it did not counteract its proneurogenic effects in ahNSC cultures (Figure 6a). Although with low affinity, TZD also exerts antagonistic activity at α_2 receptors (Stahl, 2009). Blockade of this receptor subtype has been demonstrated to accelerate the proneurogenic effects of chronic antidepressant treatment (Yanpallewar et al., 2010). Based on these observations and on a previous report of a noradrenergic tone in our cellular model (Meneghini et al., 2014) we then tested idazoxan (0.001-1 μM), an α_2 antagonist [$Ki_{(\alpha 2a)}$: 10 nM; $Ki_{(\alpha 2b)}$: 100 nM]. In our experimental setting idazoxan was devoid of proneurogenic effects on ahNSC (Figure 6b). Altogether these data strongly suggested the lack of involvement of both α_1 and α_2 receptors in TZD proneurogenic activity *in vitro*.

A vast array of information suggested that 5-HT_{1a} may be the predominant receptor for antidepressant activity and, more recently, also for serotonin-mediated regulation of hippocampal neurogenesis *in vivo* (Gardier et al., 1996; Cryan and Leonard, 2000; Thomas and Hagan, 2004). Although our cellular model expressed 5-HT_{1a} receptor mRNA, when murine ahNSC were exposed to the 5-HT_{1a} agonist 8-OH-DPAT [$Ki_{(5\text{-}HT1a)}$: 1.78 nM; 0.1-10 nM] we did not observe any proneurogenic effect (Figure 6c), suggesting that 5-

HT_{1a} receptors may not be involved in neuronal differentiation elicited by TZD and even by 5-HT. To further corroborate this finding, the selective 5-HT_{1a} antagonist NAD299 [Ki $_{(5\text{-}HT1a)}$: 0.6 nM; 30 nM] had no effect on the proneurogenic response elicited by 100 nM TZD (Figure 6d). NAD299 alone had no effect on ahNSC neuronal differentiation (Figure 6d). Of note, also human NSC expressed 5-HT_{1a} mRNA but, as in murine ahNSC, we could not observe any effect of NAD299 on TZD-mediated effects on these cells (data not shown). The lack of involvement of 5-HT_{1a} receptors in TZD-mediated neuronal differentiation of both murine and human NSC is in agreement with previously published data suggesting that 5-HT_{1a} receptor subtype is not involved in modulation of ahNSC differentiation but rather in their proliferation. In line with our results, two 5-HT_{1a} agonists, 8-OH-DPAT and ipsapirone, failed to regulate neuronal differentiation of NSC *in vitro* (Klempin et al., 2010). Conversely, *in vivo* blockade of 5-HT_{1a} receptors significantly reduced the number of proliferating ahNSC in rat dentate gyrus (Radley and Jacobs, 2002). Moreover, after serotonin depletion by para-chlorophenylalanine (PCPA), an irreversible inhibitor of the synthetizing enzyme TPH2, treatment with 8-OH-DPAT could rescue the number of proliferative ahNSC $BrdU^+$ in the dentate gyrus (Banasr et al., 2003). Similarly, *in vitro*, PCPA treatment and a 5-HT_{1a}R antagonist hampered proliferation, but not differentiation of ahNPSC (Benninghoff et al., 2010). Again, NSC proliferation was restored after treatment with 5-HT or a 5-HT_{1a} receptor agonist (Benninghoff et al., 2010). Interestingly, in a different model of human neuronal cells where TZD elicited neuroprotective effects against inflammatory insults, the antidepressant drug activated ERK phosphorylation and BDNF gene transcription, and this effect appeared to be mediated by activation of 5-HT_{1a} receptors (Daniele et al., 2015).

TZD binds, although with low affinity, also 5-HT_{2c} receptors. When tested in murine ahNSC cultures, the selective 5-HT_{2c} agonist WAY161503 [Ki $_{(5\text{-}HT2c)}$: 4 nM] produced a rightward shift in TZD concentration- response curve (Figure 6e). WAY was inactive *per se* (Figure 6e). These data suggested that the proneurogenic effects elicited by TZD are also mediated by its antagonistic activity at 5-HT_{2c} receptors. To confirm our observation we

differentiated murine ahNSC in presence of 0.3-300 nM RS102221, a 5-HT_{2c} selective antagonist [$Ki_{(5\text{-}HT2c)}$: 10 nM]. RS102221 significantly increased the percentage of MAP-2^+ cells (10 nM RS102221: $p < 0.001$, ANOVA, Figure 6f). These *in vitro* results are in line with the observation that the antidepressant drug agomelatine can promote hippocampal neurogenesis both via melatonergic agonistic activity and 5-HT_{2c} antagonism in rodents (Soumier et al., 2009). Altogether these data suggest: i) the lack of involvement of 5-HT_{1a} and α_1/α_2 receptors in TZD proneurogenic activity *in vitro;* ii) a possible involvement of 5-HT_{2c} antagonism in TZD effect, at least in murine cells. Indeed, we failed to demonstrate 5-HT_{2c} mRNA expression in human iPSC-derived NSC (*data not shown*).

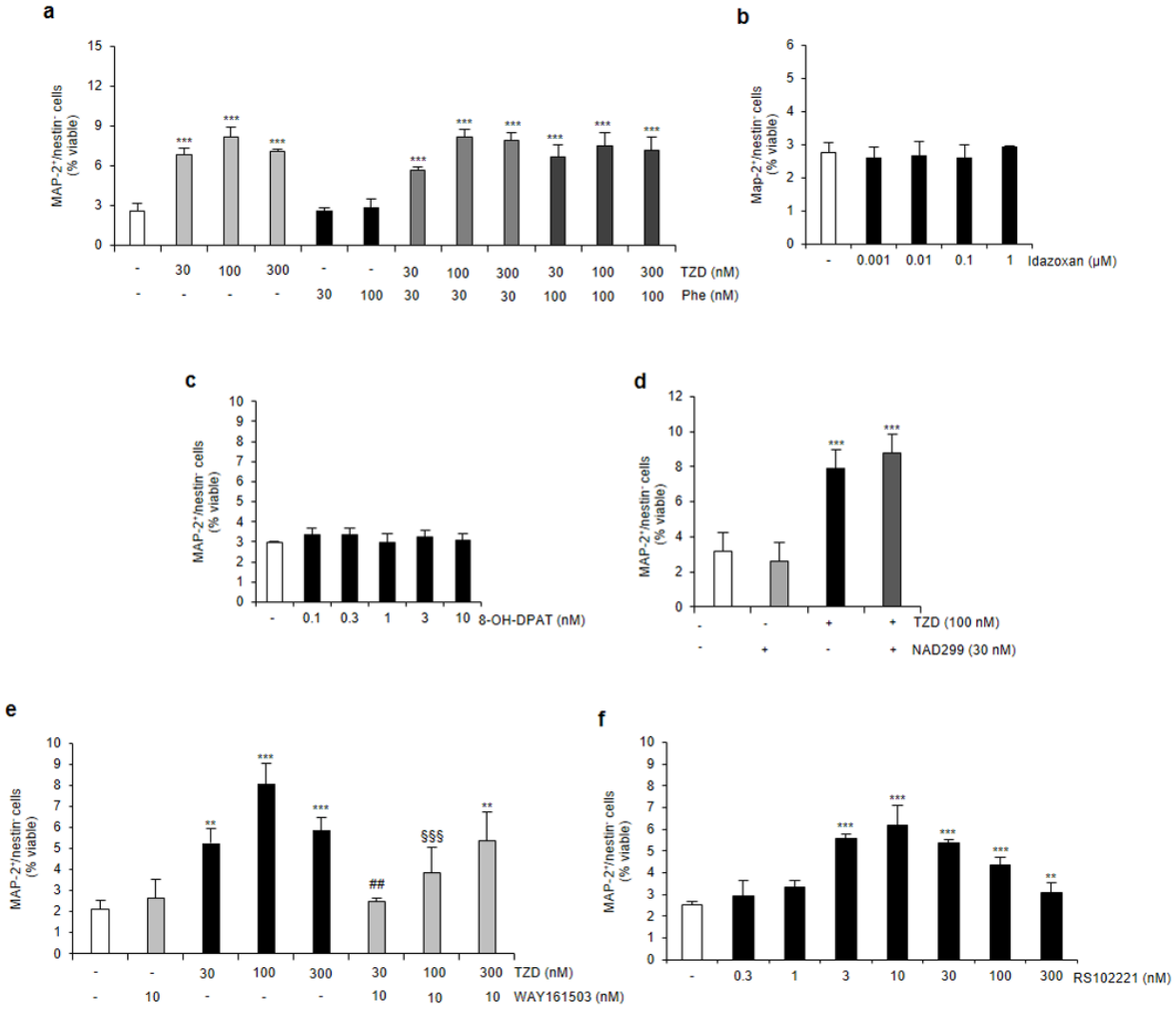


Fig. 6. 5-HT$_{2c}$, but not 5-HT$_{1a}$ and α_1/α_2 adrenergic receptors, contribute to the proneurogenic effects of TZD in murine ahNSC. (a) Effect of the α_1 agonist phenylephrine hydrochloride (Phe) on TZD proneurogenic effects. (b) Effect of the α_2 antagonist idazoxan (0.001-1 μM) on neuronal differentiation of ahNSC. (c) Effect of the 5-HT$_{1a}$ agonist 8-OH-DPAT (0.1-10 nM) on neuronal differentiation of ahNSC. (d) Evaluation of the effect of pretreatment of ahNSC with the selective 5-HT$_{1a}$ antagonist, NAD299 (30 nM), on TZD-mediated proneurogenic effects. (e) Effect of the selective 5-HT$_{2c}$ agonist WAY161503 (10 nM) on TZD-mediated increase of newly generated MAP-2$^+$ cells. **(f)** Evaluation of the percentage of newly generated neurons in presence of the 5-HT$_{2c}$ antagonist RS102221 (0.3-300 nM). Data are expressed as mean ± S.D. ** $p < 0.01$, *** $p < 0.001$ vs vehicle-

treated cells, ## $p < 0.01$ vs 30 nM TZD-treated cells. §§§ $p < 0.001$ vs 100 nM TZD-treated cells (One-way ANOVA, Tukey's post-hoc).

The *in vitro* proneurogenic effects of TZD require 5-HT synthesis by murine ahNSC.

The fact that TZD exerted its effects via 5-$HT_{2a/c}$ antagonism supported the idea of an endogenous serotoninergic tone in our *in vitro* model and also raised the question of serotonin source both under basal conditions and in response to TZD. We measured, by LC-ESI-MS, extracellular 5-HT concentration in our murine cultures. Values were, on average, 0.86 ± 0.19 ng/ml (Figure 7a), corresponding to 4.9 ± 1.1 nM. Interestingly, murine and human ahNSC not only express several 5-HT receptors, but also 5-HTT (Klempin et al., 2010; Benninghoff et al., 2010). TZD is a low affinity 5-HTT blocker (Stahl, 2009) and this drug activity can result in an increased availability of extracellular 5-HT. Interestingly, a slight, although not statistically significant, increase in 5-HT extracellular concentration was observed in presence of a proneurogenic concentration of 100 nM TZD compared to vehicle (TZD: 1.25 ± 0.20 ng/ml; vehicle: 0.86 ± 0.19 ng/ml, $p = 0.07$, Figure 7a). Under the same experimental conditions the SSRI antidepressant fluoxetine (0.003-3 μM) promotes neuronal differentiation of murine ahNSC in a concentration-dependent manner ($p < 0.001$, ANOVA, Figure 7b). The proneurogenic effect of fluoxetine correlated with a significant increase in 5-HT extracellular concentration, compared with vehicle-treated conditions (1 μM FLX: 1.76 ± 0.1 ng/ml, $p < 0.01$ vs vehicle, *t* test), suggesting that our cellular model indeed does express functional 5-HTT (Figure 7a). Undifferentiated ahNSC also express both isoforms of tryptophane-hydroxylase, TPH1 and TPH2 (Benninghoff et al., 2010). We performed a three-day treatment of proliferating ahNSC with 50 μM PCPA so to irreversibly inhibit TPH and deplete ahNSC of endogenous serotonin (Benninghoff et al., 2010). As expected, extracellular 5-HT levels became undetectable by LC-ESI-MS in culture medium of PCPA- compared to vehicle-treated cells. Although literature data report that PCPA treatment significantly decreases proliferation of ahNSC both *in vitro* and *in vivo*

(Benninghoff et al., 2010; Jha et al., 2006), in our hands blockade of 5-HT synthesis caused a small but not statistically significant reduction (about -23 %) in the number of recovered PCPA-treated NSC compared to vehicle-treated cells, suggesting a more limited effect of 5-HT depletion on ahNSC proliferation. Similarly, PCPA treatment did not affect the survival of ahNSC (% dead cells/total cells: 12.9 ± 2.5 and 16.2 ± 2.9 in vehicle- and PCPA-treated cells, respectively, $p > 0.05$, *t* test). After PCPA/vehicle treatment ahNSC were washed and plated, at the same cellular density, under conditions promoting neuronal differentiation either in presence of TZD (30-300 nM) or the corresponding vehicle. The percentage of newly generated MAP-2^+ cells was then determined in vehicle- and PCPA-treated cells. Under basal conditions we observed a statistically significant reduction in the percentage of MAP-2^+ cells in PCPA-treated cells compared to vehicle-treated cells (Figure 7c; - 59.1 %, $p < 0.01$, *t* test). This effect cannot be ascribed to a reduction in cell viability since we did not observe significant differences in the apoptotic/necrotic rate of ahNSC and their progeny under the same experimental conditions (*data not shown*). Even more interestingly, 30-300 nM TZD promoted neuronal differentiation in vehicle-treated, but not in PCPA-treated ahNSC (Figure 7c). As a control, exogenously added 5-HT (1 μM) promoted neurogenesis in both vehicle- and PCPA-treated ahNSC ($p < 0.001$ vs vehicle, ANOVA Figure 7c), suggesting that PCPA-treated cells were not affected in their ability to respond to proneurogenic signals. Of note, although not significantly different, the percent of increase of MAP-2^+ cells over basal differentiating conditions was higher in PCPA-treated compared to vehicle-treated ahNSC (+260.9 ± 83.9 and +127.2 ± 32.3 % in PCPA- and vehicle-treated cells, respectively, $p = 0.062$, *t* test; Figure 7c). Altogether, these data strongly support the finding that the effects of TZD on neuronal differentiation of ahNSC are mediated by drug antagonism at 5-HT_2 receptors, in presence of a serotoninergic tone which, in our experimental setting, is not significantly affected by the antidepressant drug.

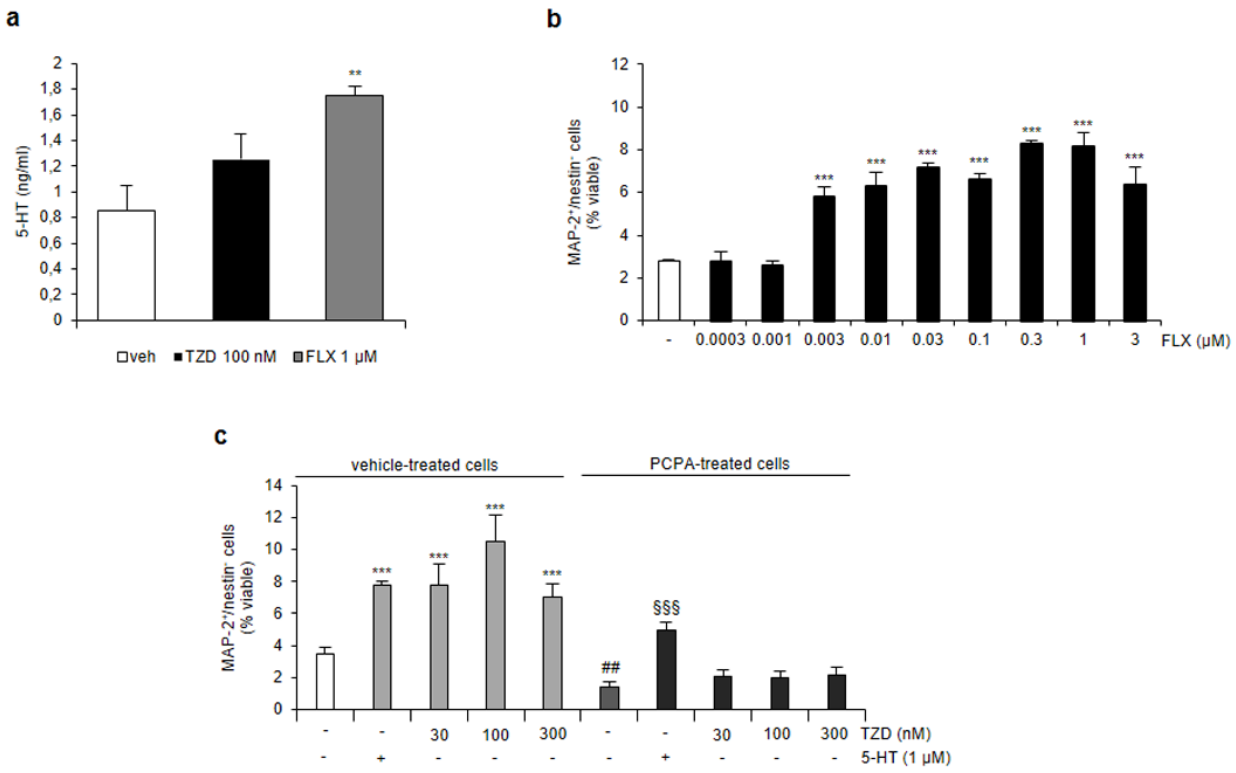


Fig. 7. TZD proneurogenic activity requires synthesis of endogenous 5-HT by murine ahNSC. (a) LC-ESI-MS analysis of extracellular 5-HT content in our *in vitro* model under basal conditions and after treatment with 1 µM fluoxetine (FLX) and 100 nM TZD. (b) Effect of fluoxetine (0.0003-3 µM) on the percentage of MAP-2$^+$ cells generated from ahNSC. Data are expressed as mean ± S.D. *** $p < 0.001$ vs vehicle-treted cells. (One-way ANOVA, Tukey's post-hoc). (c) Effect of irreversible inhibition of tryptophan hydroxylase by para-chlorophenylalanine (PCPA, 50 µM, 3 day-treatment of proliferating ahNSC) on the proneurogenic activity by TZD. Evaluation of exogenous 5-HT (1 µM) treatment on neuronal differentiation of both PCPA-treated and vehicle-treated ahNSC. Data are expressed as mean ± S.D. *** $p < 0.001$ vs basal condition in vehicle-treated cells; §§§ $p < 0.001$ vs basal condition in PCPA-treated cells (One-way ANOVA, Tukey's post-hoc), ## $p < 0.01$ vs basal condition in vehicle-treated cells (Student's *t* test).

Nuclear translocation of NF-κB p50 is required for the proneurogenic effects elicited by TZD .

Based on the current results our working hypothesis is that 5-HT exerts proneurogenic and antineurogenic effects by interacting with different serotonergic receptor subtypes. While the 5-HT receptors responsible for the serotonin proneurogenic effect in the present model remain to be clarified, we investigated which downstream signaling pathways could play a role in the TZD effect mediated by 5-HT2_a and 5-HT2_c receptors subtypes. Previous data from our laboratory established the relevance of NF-κB proteins, and in particular of the p50 subunit, in the *in vitro* and *in vivo* proneurogenic effect of several drugs (Cuccurazzu et l., 2013; Valente et al., 2012) as well as of neural mediators (Meneghini et al., 2010; Meneghini et al., 2013; Cvijetic et al., 2017). Based on these data we tested the involvement of NF-κB p50 in TZD-mediated proneurogenic effects on ahNSC. Interestingly, co-treatment of ahNSC with SN50 (10 µg/ml), an inhibitor of NF-κB p50 nuclear translocation, completely counteracted the proneurogenic effects of 1-10-100 nM TZD (Figure 8a).

However, unlike TZD, in same experimental setting the enhancement of the proneurogenic effect obtained by adding external 5-HT did not require NF-κB p50 nuclear translocation (Figure 8b). These results would need additional studies to further characterize the signaling pathways that operate downstream the different 5-HT receptor subtypes and their balance in producing the proneurogenic effects of 5-HT and trazodone.

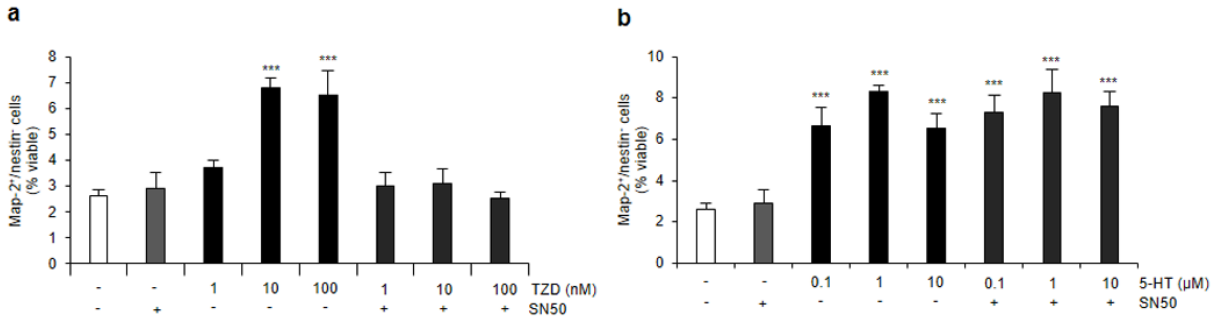


Fig. 8. NF-κB p50 nuclear translocation is required for TZD, but not for 5-HT proneurogeic effect. (a) Effect of TZD after inhibition of NF-κB p50 nuclear translocation. Data are expressed as mean ± S.D. *** $p < 0.001$ vs vehicle treated cells. (b) 5-HT proneurogenic activity in presence of NF-κB p50 nuclear translocation inhibition. Data are expressed as mean ± S.D. *** $p < 0.001$ vs vehicle treated cells (One-way ANOVA, Tukey's post-hoc).

CONCLUSIONS

This *in vitro* study suggests, for the first time, a new pharmacological activity of TZD, namely its ability to promote neuronal differentiation of murine and human NSC. An extensive pharmacological characterization of receptors involved in TZD proneurogenic activity in murine cells revealed that 5-$HT_{2a/c}$ antagonism is mainly responsible for that effect. Interestingly, also in IPSC-derived human NSC the antidepressant drug promotes neuronal differentiation via blockade of 5-HT_{2a} receptors. This piece of information is quite important since most *in vitro* studies evaluating the activity of clinically relevant drugs on neurogenesis have been performed in rodent cells. Moreover in our hands, unlike the SSRI fluoxetine, the proneurogenic effects of TZD did not appear to correlate with an increase in 5-HT extracellular concentration, as a result of 5-HTT blockade. Finally, our data add further complexity to the role played by serotoninergic neurotransmission in the regulation of adult hippocampal neurogenesis. Previous *in vivo* data suggested oppositional effects of serotonin receptors 5-HT_{1a} and 5-HT_2 in the regulation of adult hippocampal neurogenesis (Klempin et al., 2010). Although obtained *in vitro*, our data also support the concept that serotonin, acting on different receptor subtypes, may promote but also counteract, via 5-HT_2 receptors, neuronal differentiation of adult NSC. Despite the widespread view of 5-HT as an endogenous positive modulator of hippocampal neurogenesis it is not surprising that homeostasis within this process may be reached also by oppositive effects via different receptor subtypes. In our experimental setting we could not demonstrate that 5-HT_{1a} receptors may play a role in the positive modulation of ahNG neither in murine and human cells, so at this stage we cannot point to the receptor subtype which mediates the *in vitro* proneurogenic effects of 5-HT in these cells. Based on literature reports, 5-HT_4 is known to promote neurogenesis both *in vitro* and *in vivo* (Lucas et al., 2007; Samuels et al., 2016), but mRNA for that receptor subtype was not expressed in our cellular model (*data not shown*). The other receptor subtype which has been proposed to play a role in neurogenesis is the

ionotropic 5-HT_3, although conflicting results have been obtained since both activation and blockade of that receptor have been proposed to promote neuronal differentiation of ahNSC (Kondo et al., 2015; Bétry et al., 2015). Again, mRNA for this receptor subtype was not detected in our model (*data not shown*).

Interestingly, at least in murine cells, in our experimental setting the time course of TZD pro-neurogenic effects was also different compared to that of 5-HT, again pointing to differences in the overall effects on neurogenesis on molecules that act via distinct receptor subtypes. The different time course profile of TZD and 5-HT could be also potentially explained by changes in receptor subtype expression by ahNSC and their progeny over time *in vitro*. Such changes could even be triggered differently by TZD or 5-HT during *in vitro* cell maturation stages. For these reasons, future studies will need to further explore the differential contribution of distinct 5-HT receptors on NSC, their progeny and their overall impact on adult hippocampal neurogenesis.

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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 Stem/Progenitor Cells (NSC) 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 seems important 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/EMA-approved drugs, we found that the immunosuppressant cyclosporine A (CSA) restored proliferation, acquisition of a neuronal phenotype, and maturation of NSC 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 NSC proliferation in the SVZ and in the subgranular zone of the hippocampal dentate gyrus, 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, a key determinant of proliferation impairment. Results show that neonatal treatment with CSA restores the whole triad of defects of the trisomic brain. In DS CSA treatment 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 stem/progenitor cells (NSC); 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 NSC 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 NSC 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 and 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 NSC 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

Animals. 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 Communities Council Directive of 24 November 1986 (86/609/EEC) for the use of experimental animals and were approved by Italian Ministry of Public Health. In this study, all efforts were made to minimize animal suffering and to keep the number of animals used to a minimum.

IN VITRO EXPERIMENTS

Isolation and Culture of neonatal SVZ NSC. 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 cm2 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 NSC (P3-P12) pooled from at least 3-5 pups were dissociated in a single cell suspension and plated onto NunclonTM Delta Surface 96-well plate (Thermo Fisher Scientific) at a density of 4×103 cells per well in DMEM/F-12 medium supplemented with B27, GlutamaxTM (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 NSC 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 Victor3-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).

NSC 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 4×103 cells per well in DMEM/F-12 medium supplemented with B27, GlutamaxTM, 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 10µM. 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 Victor3-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 35×103 per well in differentiation medium (DMEM-F12 supplemented with B27, 2 mM Glutamax and 100 U/100 mg/ml penicillin/streptomycin). NSC 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 NSC-derived cells was carried out by immunolocalization for MAP2 (rabbit polyclonal, 1:50000; Abcam) and Nestin (chicken monoclonal, 1:2500; Neuromics). Secondary antibodies were as follows: AlexaFluor555-conjugated goat anti rabbit (1:1400; MolecularProbes), and AlexaFluor488-conjugated goat anti chicken (1:1400; Molecular Probes). In

additional experiments, in which NSC 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 and Morshead, 2016). Based on this evidence, we treated euploid and Ts65Dn mice with CSA (MedChem Express, 15 mg/kg/day in vehicle; subcutaneos 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 sacrified. 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 TM 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 TM Kit) and stored at room temperature in the dark for 2 weeks. Then, brains were transferred into Solution C (FD Rapid Golgi Stain TM 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).

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 6th section using a modified unbiased stereology protocol that has previously been reported to successfully quantify BrdU labeling (Malberg et al., 2000, Kempermann and Gage, 2002, Tozuka et al., 2005). 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.

RESULTS

Identification and characterization of CSA effects in trisomic NSC phenotypic assays

NSC 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 NSC (% 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 NSC (Fig. 1B). By using a cytotoxicity assay, we could exclude that CSA-mediated effect on the number of NSC 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 NSC displayed a proliferation rate

similar to untreated euploid NSC.

In addition to proliferation impairment, trisomic NSC 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 NSC 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 NSC); 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 NSC 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 NSC at the expense of their commitment toward an astrocytic phenotype.

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 NSC exposed to different concentrations of CSA. A one-way ANOVA on the percentage of NSC 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 NSC.

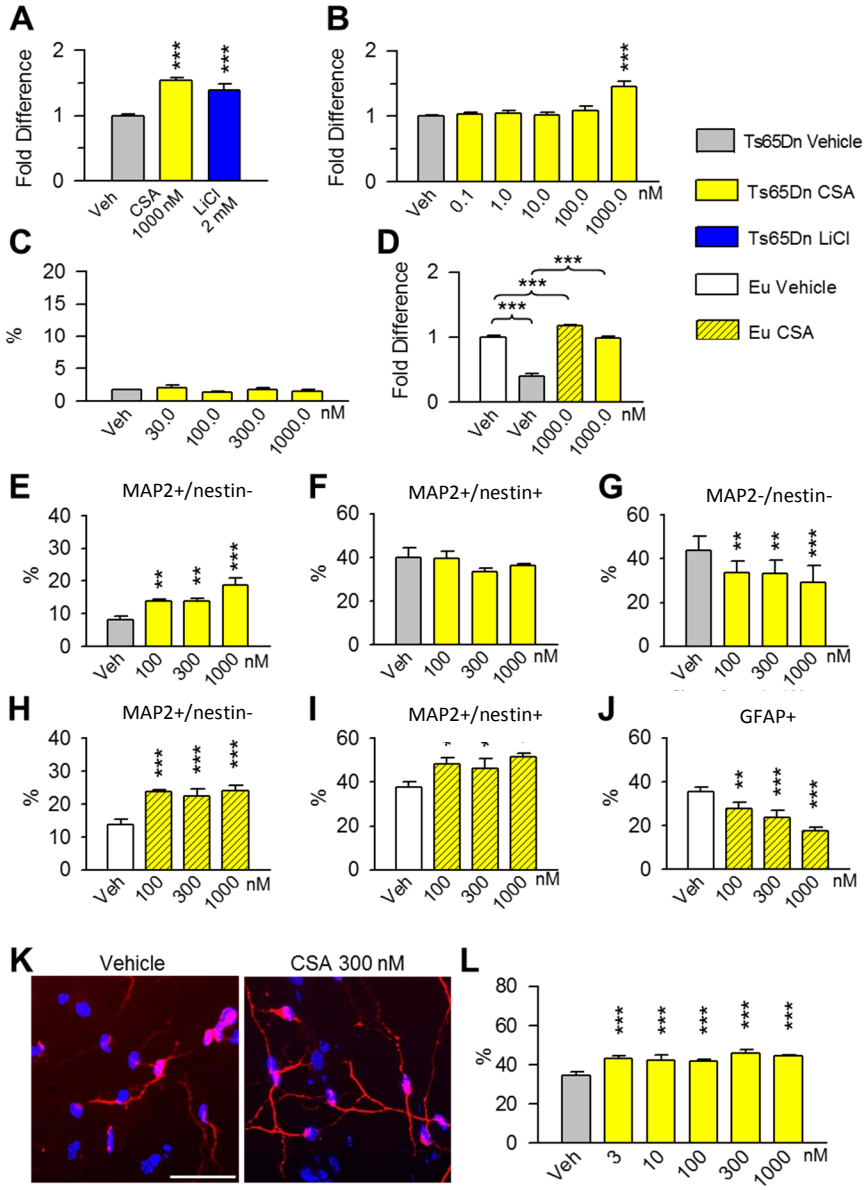

Fig. 1. Effect of CSA on proliferation, differentiation and maturation of NSC derived from the SVZ. A,B: Effect of CSA 1000 nM or LiCl 2.0 mM (A) and of different concentrations of CSA (B) on the proliferation rate (evaluated as relative luminescence units, RLU; see Methods) of NSC of Ts65Dn mice at 96 h in culture. Data are expressed as fold change in comparison with NSC 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 NSC exposed to vehicle or to CSA 1000 nM for 72 h. Data

are expressed as fold change in comparison with euploid NSC 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 NSC 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 NSC 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 300 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: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ (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 and 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 NSC 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 and 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 NSC 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 NSC in both the dentate gyrus and SVZ of Ts65Dn mice.

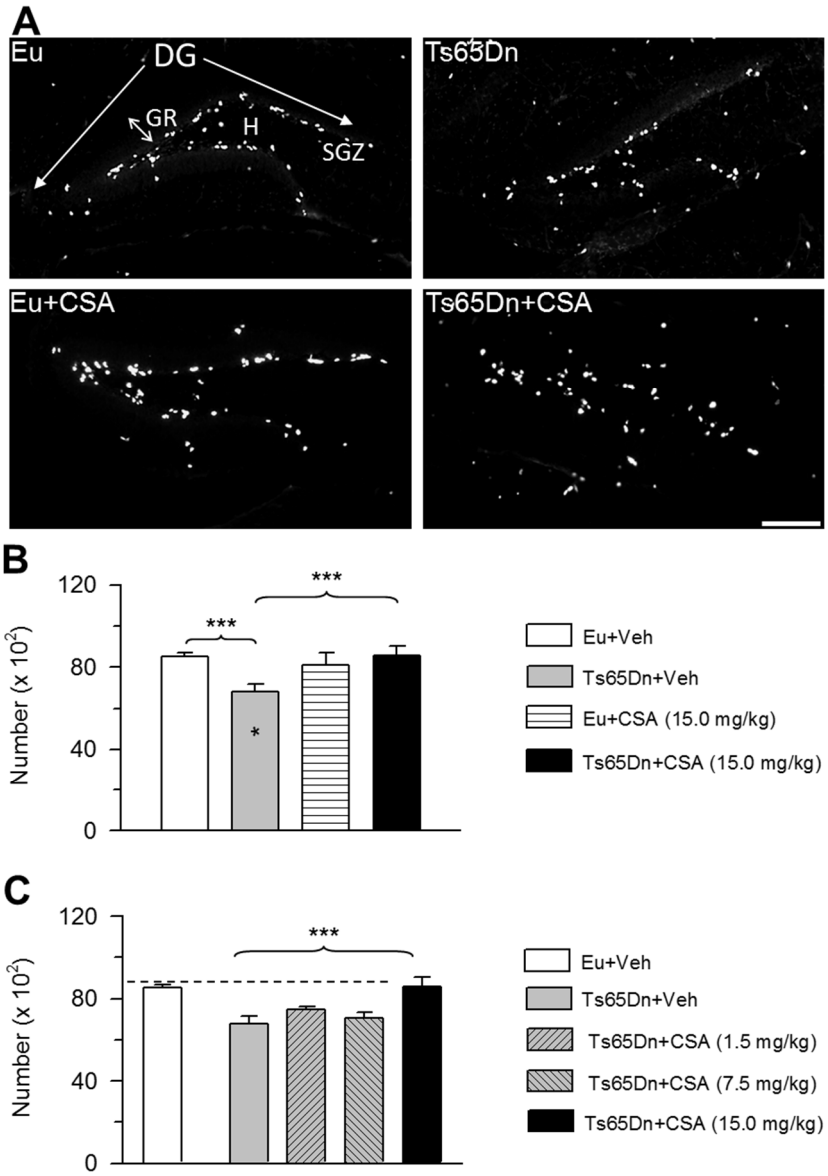

Fig. 2. Effects of neonatal treatment with CSA on the size of the population of cells in the S-phase of the cell cycle in the dentate gyrus of Ts65Dn and euploid mice. A: Representative images of sections immunostained for BrdU from the dentate gyrus of untreated euploid and Ts65Dn mice, and of euploid and Ts65Dn mice that were treated daily with 15 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 ≤ 0.05; *** p ≤ 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.

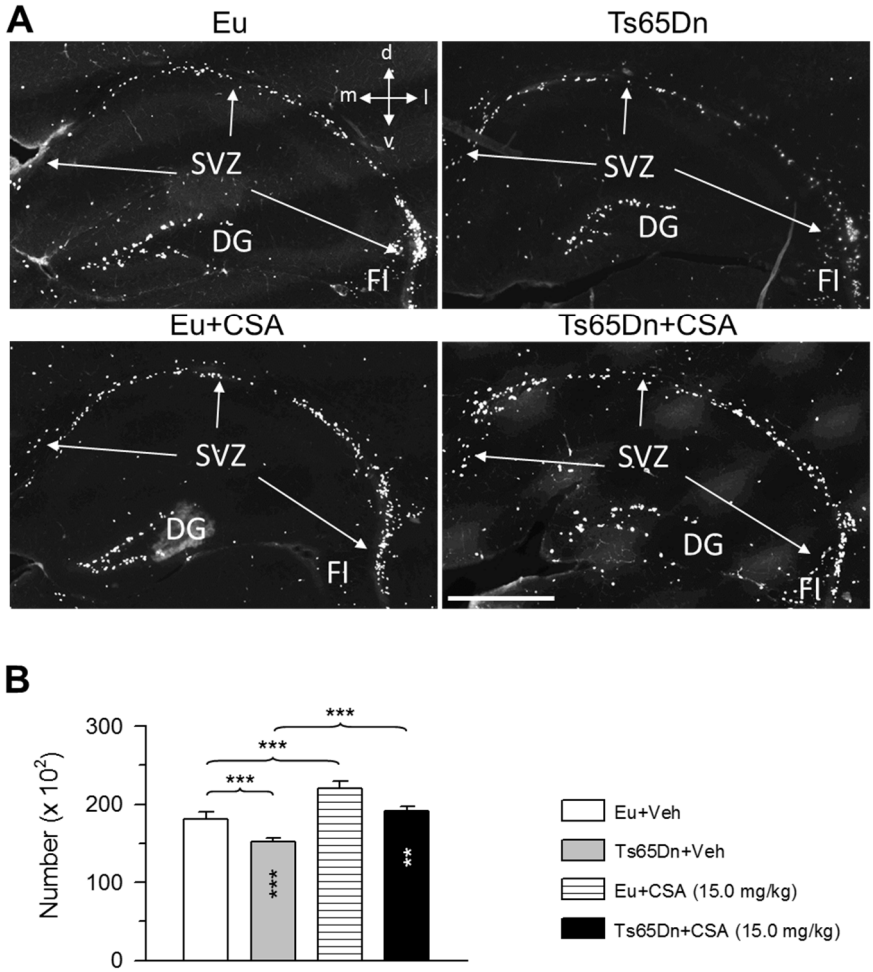

Fig. 3. Effects of neonatal treatment with CSA on the size of the population of cells in the S-phase of the cell cycle in the SVZ zone of Ts65Dn and euploid mice. A: Representative images of sections immunostained for BrdU from the SVZ of untreated euploid and Ts65Dn mice, and of euploid and Ts65Dn mice 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≤0.01; *** p ≤ 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 and Bayer, 1975). Thus, in view of the treatment-induced increase in the proliferation potency of NSC 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.

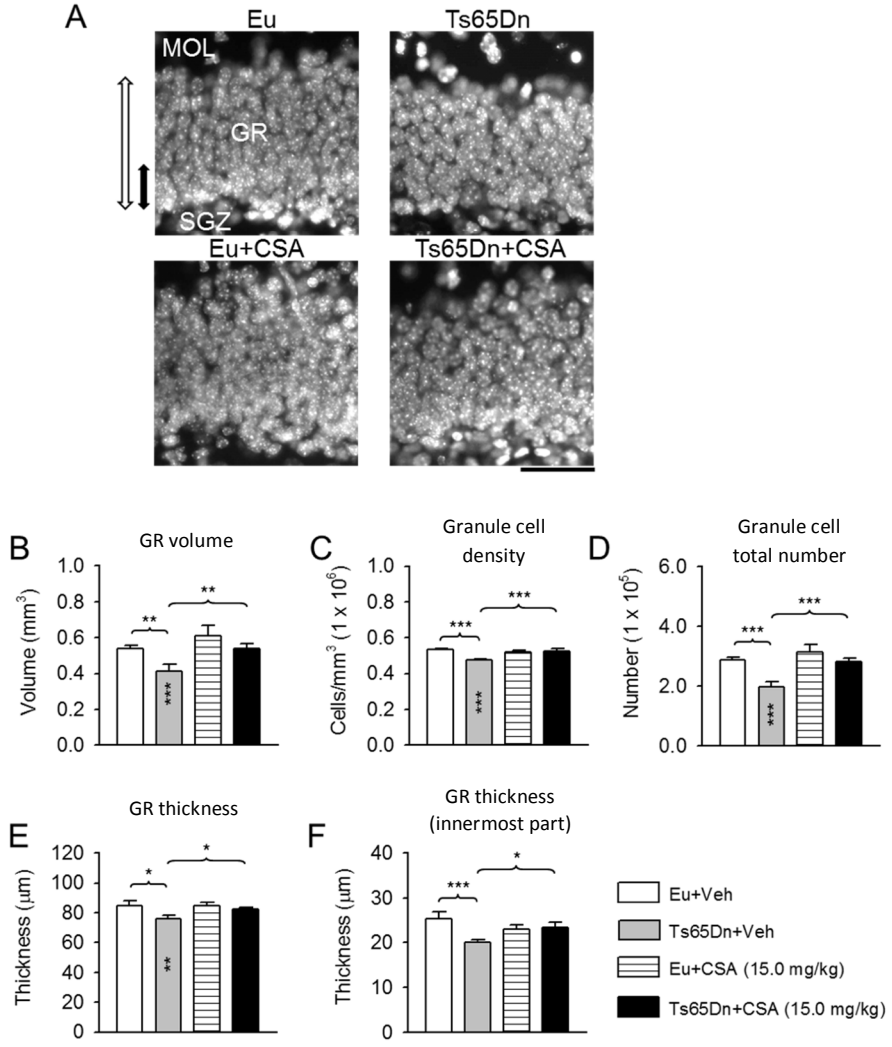

Fig. 4. Effects of neonatal treatment with CSA on the stereology of the granule cell layer of Ts65Dn and euploid mice. A: Representative images of Hoechst-stained sections showing the granule cell layer of an animal from each experimental group. The double-headed white arrow indicates the thickness of 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≤ 0.05; ** p ≤ 0.01; *** p ≤ 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).

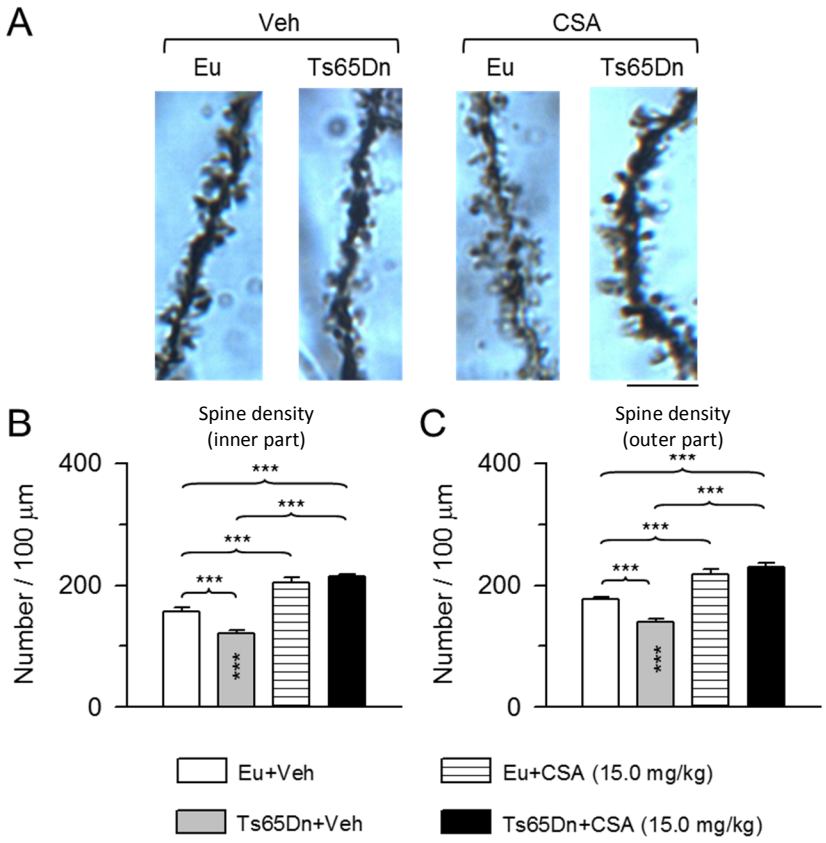

Fig. 5. Effects of neonatal treatment with CSA on dendritic spine density in the dentate gyrus of Ts65Dn and euploid mice. A: Photomicrographs of Golgi-stained granule cell dendrites showing spines on distal dendritic branches in an animal from each experimental group. Calibration bar=5 µm. B,C: Spine 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).

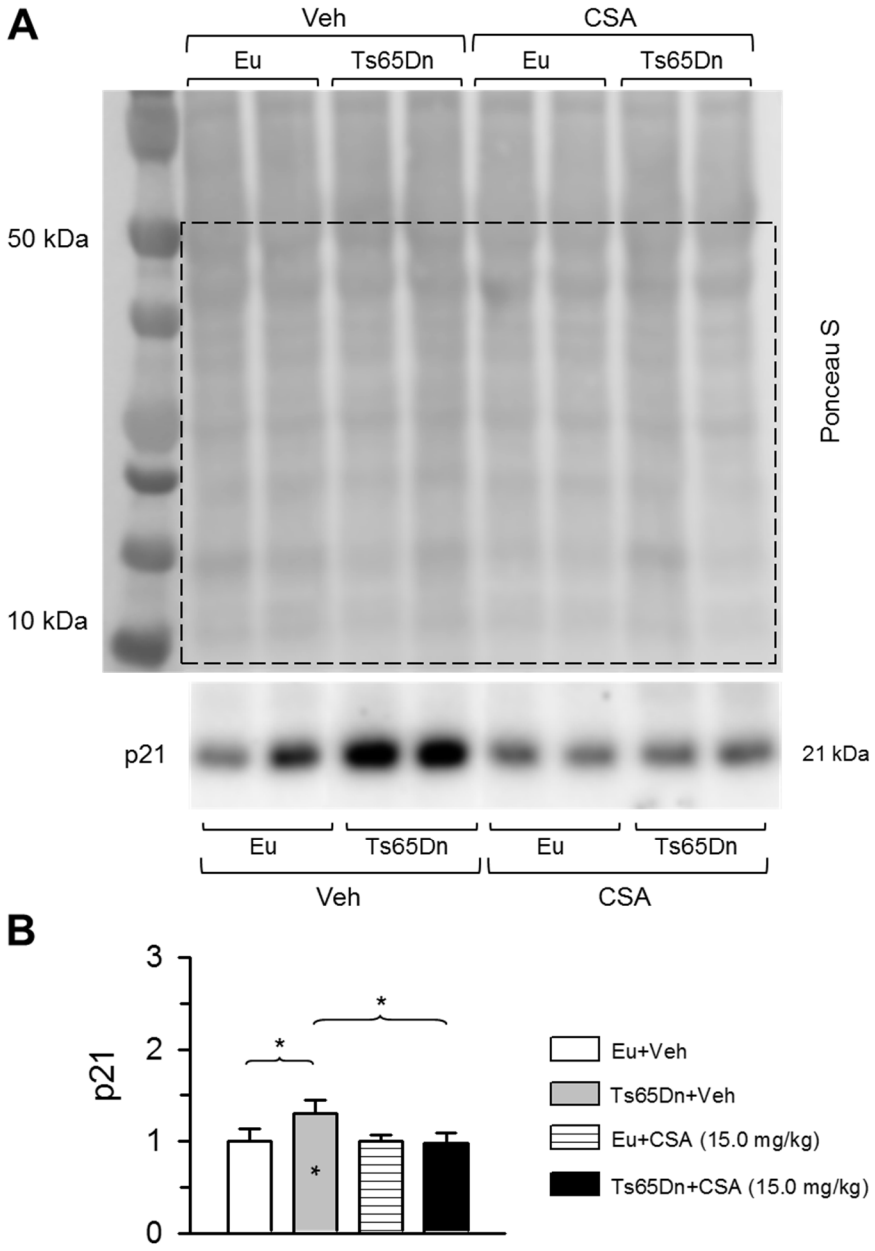

Fig. 6. Effects of neonatal treatment with CSA on p21 protein levels in the hippocampal formation of Ts65Dn and euploid mice. A: Representative western blot showing immunoreactivity for p21 and Ponceau S-stained gel. Protein levels of p21 were normalized to all proteins with molecular weight between 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.

	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, based on the criteria explained in the Statistics section. The *p* value in the column on the right refers to the comparison between untreated and treated mice of the same genotype (Fisher's LSD test after two-way ANOVA).

DISCUSSION

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

By exploiting cultures of NSC from the SVZ, we found that CSA i) restores the reduced proliferation rate that characterizes trisomic NSC; ii) rescues the aberrant differentiation program of trisomic NSC, because it increases the number of cells that differentiate into neurons and, concomitantly, reduces the number of cells that differentiate into astrocytes; iii) increases the development of neuritic processes. The pro-proliferative and pro-neurogenic effects of CSA in trisomic NSC 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 and Morshead, 2016) at the same concentrations as those used here. It has been shown that CSA increases neurite outgrowth of cultured dorsal root ganglion cells with an EC_{50} of 50 nM (see (Hamilton and Steiner, 1998)). This is in line with the current findings that CSA fosters neurite outgrowth of trisomic NSC 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 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 NSC proliferation suggests a threshold for the pro-neurogenic effects of CSA.

The NSC of the SGZ give origin to granule neurons and astrocytes destined to the dentate gyrus. In agreement with the pro-*neurogenic* 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 NSC 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 NSC 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 perforant pathway, which takes its origin from the medial and lateral divisions of the entorhinal cortex (Amaral and 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 NSC 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 and 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 and 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 Ts65Dn mouse brain (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 and 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 PERSPERCTIVES

DS is characterized by impairment of NSC proliferation, acquisition of a neuronal phenotype, and dendritic development. An obvious question regards the possibility of pharmacologically restoring this whole triad of deficits. 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 deficit is attributable to the alteration of specific pathways. Based on the complexity of these alterations, it may be necessary to use drug combinations to fully correct brain development. Importantly, the current study shows that treatment with a single drug, CSA, administered for a short but critical time window, can restore the entire triad of deficits of the trisomic brain.

In view of its extensive effects in the murine model, CSA may be potentially effective in DS patients. However, caution must be exercised because CSA is an immunosuppressant drug and its clinical use is limited by side effects that include nephrotoxicity, neurotoxicity and hepatotoxicity (Matsuda and Koyasu, 2000, Bartynski et al., 2001). 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 tolerated treatment in individuals with DS, the possibility of side effects must be considered. To this regard, it is noteworthy that we did not observed apparent effects in 13 day-long CSA treatment on mice health status, as evidenced by no change in body weight of both genotypes. Conversely, in treated mice we found a small (4-5%) reduction in overall brain weight, which is in line with similar evidences obtained in rats (Setkowicz and Kadulski, 2007). The causes of this brain weight reduction remain to be elucidated. Recent evidences show that 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 largely tied to its calcineurin-mediated mechanism of action. It must be remarked that various studies have shown that the immunosuppressive and neurotrophic actions of immunosuppressants can be dissociated and that the neurotrophic properties of immunosuppressant drugs are not mechanistically linked to their immunosuppressive actions but operate by separate pathways (see (Hamilton and 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 CSA NIM811 mimics the pro-survival effects of CSA on NSC *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 positive effects on brain development as those elicited by CSA, in absence of

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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CHAPTER 5.

DISCUSSION AND CONCLUSIONS

Neurogenesis, the process by which new neurons are formed, occurs during brain development, contributing to the formation of the main neuronal circuitries. Neurogenesis is guaranteed by the presence of Neural Stem Cells (NSC) that, in addition to proliferation and maintenance of the pool of stem cells, are able to generate new functional neurons.
Alterations in brain development period could lead to the formation of an abnormal brain with altered anatomical structures, loss of physiological cellular functions and consequent deficit in cognitive abilities. A typical example is the Down Syndrome (DS), a pathology characterized by brain reduced size and volume, hypocellularity (in particular in the hippocampus), altered neurotransmission functions, and decreased synaptic density associated to reduced learning and memory capacity (Bartesaghi et al., 2011).

NSC also persist in the adult brain in specific regions, such as in subventricular zone and in the subgranular zone of hippocampus where they continue to produce new neurons (Eriksson et al., 1998, Spalding et al., 2013). The hippocampal newborn neurons contribute to neural plasticity and it was demonstrated that in the murine and human hippocampus, they are involved in the acquisition of behavioural abilities (Kempermann, 2002; Shohayeb et al., 2018). Alterations in this process, could lead to deficits in hippocampus-mediated learning and memory (Garthe et al., 2016).

In the several brain-related diseases, such as Alzheimer's and Parkinson's diseases, epilepsy, stroke, chronic pain, multiple sclerosis and in neuropsychiatric diseases (such as major depression), a reduced neurogenesis was shown. Besides pathologies, also many behavioural factors can positively and negatively modulate neurogenesis such as stress, diet, physical activities, enriched environment (Van Praag et al, 1999; Lee et al., 2002; Kempermann, 2015; Czéh et al., 2002; Westenbroek et al.,

2004; Bortolotto and Grilli, 2017a). Surprisingly, depressed patients treated with antidepressants show a neurogenesis improvement that contributes to cognitive recovery (Santarelli et al., 2003; Boldrini et al., 2009). These data suggest that a pharmacological recovery is possible, not only in depressed patients, but also in other diseases where neurogenesis is impaired (Bortolotto and Grilli, 2017b; Bortolotto et al., 2014).
In our laboratory, we previously demonstrated that some anticonvulsants, anxiolytics and analgesics are able to promote neurogenesis (Valente et al., 2012; Cuccurazzu et al., 2013; Chiechio et al., 2017; Meneghini et al., 2013). Therefore, given the vastness of existing treatments in clinic, the chance that other drugs, in addition to their clinical effect, could solve an additional function as modulators of neurogenesis is incredibly high and still uninvestigated. This research approach is called drug-repurposing strategy.

In such respect, during the three years of my PhD program, I contributed to enrich this knowledge in this field in two ways:

1) *Investigation of trazodone (TZD) pharmacological profile to elucidate its role in neurogenesis.*

We demonstrated that TZD has a pro-neurogenic effect on murine adult hippocampal and human IPSC-derived NSC. This effect is mediated by antagonism of receptors 5-HT2a and 5-HT2c, as shown *in vitro* experiments. Since we found that TZD produces its proneurogenic effect by antagonizing 5-HT2a and 5-HT2c receptors, we proposed that there is an endogenous serotoninergic tone, that acts on the 5-HT receptors, leading to an autocrine anti-neurogenic effect. In fact, we detected the presence of 5-HT in murine NSC culture, indicating that not only neurons, but also NSC are able to synthetize and release 5HT in the extracellular environment.
Since serotonin are able to activate all serotoninergic receptors it is very interesting that both serotonin and TZD have a resulting proneurogenic effect on murine NSC despite their contradictory action on 5-HT2a and 5-HT2c receptors. Experimental evidence show that NSC express different

serotonin receptors and their activation is able to modulate positively neurogenesis (Banars et al., 2004; Nandam et al., 2007; Kondo et al., 2015; Samuels et al., 2016). Lucas et al. demonstrated in vivo that activation of receptors 5-HT4 was able to increase the number of newborn neurons in the dentate gyrus (Lucas et al., 2007). Kondo et al., shown that 5-HT3 receptors activation was essential in exercise-induced hippocampal neurogenesis and antidepressant effects (Kondo et al., 2015). *In vivo* studies in rodents demonstrated even that activation of 5-HT1b and 5-HT6 had antidepressant effects and the potential role in neurogenesis induction (Yohn et al., 2017). We suggest that, the effect of serotonin was due to the synergic effect obtained by all serotonin receptor activation. Furthermore, by blocking SERT, TZD increase extracellular level of serotonin that contributing in the same way to increase neurogenesis.

We shown that the proneurogenic effect of TZD was not mediated by receptors 5-HT1a, α1 and α2 as demonstrated by the non-effects of selective agonists/antagonists. We suggested that the serotonin receptors 5-HT1a was involved in the proliferation mechanism of adult hippocampal NSC, in accordance with previously publications where selective agonists were able to increase their proliferation rate both *in vivo* and *in vitro* (Klempin et al., 2010; Benninghoff et al., 2010; Radley and Jacobs, 2002).
Regarding the adrenergic receptors, we demonstrated that they were involved in the neurogenesis process since inhibition of receptors α1 and activation of receptors α2 were both able to induce neuronal differentiation of adult hippocampal NSC (Meneghini et al., 2014). As expected, the proneurogenic effect of TZD was not mediated by α1 and α2 receptors since the α1 agonist was not able to counteract the proneurogenic effect of TZD and the α2 antagonist was not able to increase the percentage of MAP2 positive cells.

From these studies, serotonin receptors contribute in different way to the proneurogenic effect once activated. However, to fully understand the complex system of serotonin signalling in our *in vitro* model of NSC, it will

be essential to study also the expression of other 5-HT receptors and their singular contribution in the neurogenesis mechanisms.

To further investigate TZD dependent downstream signalling, we evaluated the involvement of NF-κB. Our group previously demonstrated that NF-κB subunit p50 is implicated in drug induced neurogenesis (Valente et al., 2012; Cuccurazzu et al., 2013) Interestingly inhibition of p50 translocation in the nucleus, prevented TZD-mediated proneurogenic effect. However, in presence of exogenous serotonin, cells responded to TDZ. These data need to be further clarified, especially regarding signalling pathways, since NF-κB seems to be involved, but in the presence of exogenous serotonin, other mechanisms appears to be activated.

Through this project, we contributed to reveal novel potential mechanisms underlying the proneurogenic effect of antidepressants such as TZD. In particular, many neurogenesis studies have been carried out on rodents and in our laboratory, we studied murine adult hippocampal NSC since many years. However, there are few data concerning the human counterpart. During my PhD, I validated an *in vitro* method to study NSC derived from human IPSC. These cells, unlike murine one, need an enriched condition to maintain their proliferative state and a very long time to differentiate into progeny. Consequently, I investigated the effect of TZD and the involved receptors in the neurogenesis on human cells. As obtained in murine model, TZD had the same proneurogenic effect mediated at least by 5-HT2a in human IPSC-derived NSC, confirming further our hypothesis. Furthermore, Daniele et al. shown that trazodone has a neuroprotective effect in human NSC and this effect was mediated by the antagonism of the 5-HT2a receptors. These data further suggested the involvement of the receptors 5-HT2a antagonism in the NSC regulatory mechanisms (Daniele et al., 2015).

2) *Identification of a new drug that could recover DS deficit at anatomical and cellular levels in neonatal Ts65Dn mice.*

By our screening, we demonstrated that cyclosporine (CSA) has a pro-proliferative and a pro-neurogenic effect in neonatal trisomic mice both *in vitro* and *in vivo*. In fact, we found that CSA *in vitro* restores cell proliferation, with no effect on cell survival, and increases neuronal differentiation at the expenses of astroglial differentiation. *In vivo*, in collaboration with Bartesaghi's groups, we demonstrated that CSA restored the number and the volume of newborn granule cells in SVZ and DG, increased spine density and reduced the overexpression of the cyclin-dependent kinase inhibitor p21 in trisomic mice.

With this study, we contributed to identify CSA, a known immunosuppressant, as a treatment able to correct some neural deficits in trisomic mice *in vivo*. Experimental evidences are in agreement with our hypothesis since in vitro and in vivo CSA could improve neurogenesis (Chow and Morshead, 2016) and it seems trough calcineurin-independent pathways (Sachewsky et al., 2014). Moreover, since we have previously shown that some treatments could contribute to restore cognitive impairment in trisomic mice (Stagni et al., 2017; Giacomini et al., 2018), it will be important to study the effect of CSA also on cognitive functions and understand whether this effect will be maintained after cessation of treatment. This would be a very relevant result since, given the potentiality of CSA, we could think of a possible transferability of this therapy in DS patients.

However, since TS65Dn mice show triplication of only a subset of DS associated genes, studies in human cells are highly needed. It was demonstrated that the neuronal differentiation and the proliferation capacity were severally altered in human IPSC-derived NSC of DS patients to confirm that the pathological aspect was maintained *in vitro* (Hibaoui et al., 2014). Lu et al. suggested that human IPSC-derived NSC expressed high

level of amyloid precursor protein *in vitro* to indicate even the correlation of Alzheimer disease (Lu et al., 2013). Furthermore, Hibaoui et al. shown that neurogenesis impairment of IPSC-derived NSC could be pharmacologically rescued using DYRK1A inhibition (Hibaoui et al., 2014). These data proposed that drugs could restore neural defects even in human model. In such respect, in our laboratory we have available human IPSC derived from DS patients (Weick et al., 2013). During my PhD, I tried to add more information besides the murine data. In particular, I was trying to differentiate human IPSC to obtain an expandable and pure population of NSC from healthy and DS affected patients. This resource could be relevant to validate further the proneurogenic hits discovered in murine cells, such as cyclosporine A, and investigate the downstream signalling involved.

In conclusion, these studies demonstrated that drug repurposing could be an effective strategy for translational research aimed at treating brain diseases. In fact, both TZD e CSA have been discovered to have an "off-labelled" action: the modulation of neurogenesis of neonatal and adult murine NSC. The finding of these drugs could be proposed in the future to treating diseases where neurogenesis is compromised, such as in neurodegenerative (depression, ect.) or neurodevelopmental disease (Down syndrome, ect.), and to greatly increase the therapeutic potential existing right now.

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CHAPTER 6.

List of Publications

Proneurogenic Effects of Trazodone in Murine and Human Neural Progenitor Cells.

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

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